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Étude de la pathogenèse de l'infection et de l'inflammation causées par des souches de *Streptococcus suis* de différentes origines

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RÉSUMÉ

Streptococcus suis est l'un des plus importants pathogènes bactériens du porc, causant des pertes économiques substantielles à l'industrie porcine. De plus, c'est un agent zoonotique représentant de sérieux risques pour la santé humaine. Cette bactérie cause diverses pathologies, dont la méningite, la mort subite et le choc septique sont les plus fréquentes. Ces pathologies sont la conséquence d'une inflammation exacerbée caractéristique de l'infection systémique et du système nerveux central (SNC).

Des différents systèmes de classification, le sérotypage et le « multilocus sequence typing » sont les plus utilisés pour *S. suis*. Bien qu'il existe 35 sérotypes, la quasi-totalité des études a utilisé des souches "classiques" européennes et/ou asiatiques appartenant aux types alléliques (STs) 1 ou 7 du sérotype 2, et ce en dépit du fait que *S. suis* est une bactérie hautement hétérogène génétiquement et phénotypiquement. Nos connaissances sont donc basées presque exclusivement sur celles-ci, mais ont été extrapolées à l'ensemble des souches de *S. suis*. Par conséquent, il y a un manque critique d'information sur le rôle des composants de *S. suis* de différentes origines dans la virulence et la pathogenèse, de même que sur la réponse inflammatoire induite. Ainsi, l'objectif de cette thèse était de mieux comprendre la pathogenèse de l'infection et l'inflammation causées par *S. suis* à l'aide de souches différentes et d'évaluer l'impact de ces différences sur celles-ci.

Dans un premier temps, nous avons démontré que les composants de surface, dont la capsule polysaccharidique, l'antigène I/II et les acides lipotéichoïques, ainsi que leurs rôles et leurs propriétés, peuvent différer selon l'origine de la souche (le sérotype, le ST ou le lieu géographique) en raison de leur bagage génétique et de leurs caractéristiques phénotypiques. De plus, le choix de la souche et du modèle expérimental peut créer un biais important dans les études de virulence et de pathogenèse et affecter les conclusions qui en découlent.

D'autre part, nous avons démontré que les cellules de l'hôte du compartiment systémique reconnaissent des composants conservés de *S. suis* à l'aide de la voie des « Toll-like receptors » et de la signalisation MyD88-dépendante en aval, menant à l'induction d'une réponse inflammatoire, à laquelle participe les monocytes et les neutrophiles sanguins. Toutefois, l'amplitude de cette réponse est modulée par le bagage génétique et les

caractéristiques phénotypiques de la souche. Bien que bénéfique pour l'hôte, l'inflammation induite doit être contrôlée, car trop peu d'inflammation permet à la bactérie de se répliquer, tandis qu'une inflammation exacerbée cause des dommages tissulaires, évolutions qui peuvent toutes deux être mortelles pour l'hôte.

Enfin, le SNC est très sensible à l'infection par *S. suis* et répond rapidement et agressivement à sa présence par l'entremise d'une réponse inflammatoire exacerbée localisée, qui est, plus souvent qu'autrement, néfaste pour l'hôte. Cependant, la reconnaissance de *S. suis* dans le SNC ne nécessite pas la signalisation MyD88-dépendante. De plus, l'infiltration des monocytes et des neutrophiles est une conséquence de l'inflammation induite, ce qui indique que ce sont les cellules résidentes qui en sont responsables, bien que les leptoméninges n'y participent peu.

Mots-clés : *Streptococcus suis*, sérotype, type allélique, facteur de virulence, infection systémique, infection du système nerveux central, réponse inflammatoire, choc septique, méningite, modèle murin

ABSTRACT

Streptococcus suis is one of the most important porcine bacterial pathogens and is responsible for substantial economic losses to the swine industry. Moreover, it is also a zoonotic agent representing serious risks to human health. This bacterium causes a variety of pathologies, of which meningitis, sudden death, and septic shock are the most frequent, and which are the consequence of an exacerbated inflammation that characterizes the systemic and central nervous system (CNS) infections.

Of the different classification systems, serotyping and multilocus sequence typing are the two most commonly used for *S. suis*. Though 35 serotypes exist, nearly all studies have used "classical" European and/or Asian serotype 2 sequence type (ST) 1 or 7 strains, regardless of the fact that *S. suis* is a highly heterogenous bacterium, both genetically and phenotypically. As such, our knowledge is based almost exclusively on these strains, yet it has been extrapolated to the entire *S. suis* population. This demonstrates the clear lack of information on the role of *S. suis* components from different origins in its virulence and pathogenesis, as well as in the induced inflammatory response. Consequently, the main objective of this thesis was to better understand the pathogenesis of the infection and the inflammation caused by *S. suis* using different strains and to evaluate the impact of these differences on pathogenesis and inflammation.

Firstly, we demonstrated that surface components, including the capsular polysaccharide, antigen I/II, and lipoteichoic acids, as well as their roles and properties, may vary according to the origin of the strain used (serotype, ST, geographical isolation) due to differences in genetic background and phenotypic characteristics. Furthermore, the choice of strain and experimental model can introduce an important bias in virulence and pathogenesis studies that can affect the conclusions reached.

Alongside, we demonstrated that systemic compartment host cells recognize conserved components of *S. suis* via the Toll-like receptor pathway and downstream MyD88-dependent signaling, which leads to the induction of an inflammatory response in which blood monocytes and neutrophils participate. However, the magnitude of this response is modulated by the genetic background and phenotypic characteristics of the strain. Though beneficial for the host, the induced inflammation must be controlled, as too little inflammation allows the bacteria to

replicate, while exacerbated inflammation causes tissue and organ damage. Indeed, these two outcomes may result in host death.

Finally, the CNS is extremely sensitive to *S. suis* infection and quickly and aggressively responds to its presence via an exacerbated inflammatory response that will remain localized and that is, more often than not, detrimental to the host. However, recognition of *S. suis* in the CNS does not necessarily require MyD88-dependent signaling. Moreover, infiltration of blood monocytes and neutrophils is a consequence of the induced inflammation, which indicates that resident cells are responsible for this response, though the leptomeninges do not appear to greatly participate therein.

Keywords: *Streptococcus suis*, serotype, sequence type, virulence factor, systemic infection, central nervous system infection, inflammatory response, septic shock, meningitis, mouse model

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LISTE DES ABRÉVIATIONS

ADN : Acide désoxyribonucléique

AgI/II : Antigène I/II

AIM2 : « Absent in melanoma 2 »

APC : Cellule présentatrice d'antigène

ARN : Acide ribonucléique

BBB : Barrière hématoencéphalique

BCSFB : Barrière hémato-liquide céphalorachidien

BMEC : Cellules endothéliales microvasculaires du cerveau

CC : Complexe clonal

CCL : « C-C motif chemokine ligand »

CMH-II : Complexe majeur d'histocompatibilité-II

CPEC : Cellules épithéliales du plexus choroïdien

CPS : Capsule polysaccharidique

CXCL : « C-X-C motif chemokine ligand »

DC : Cellule dendritique

DPPIV : Dipeptidyl peptidase IV

EF : Facteur extracellulaire

G-CSF : « Granulocyte colony-stimulating factor »

Gal : Galactose

GalNAC : *N*-acétylgalactosamine

GAS : Streptocoque du groupe A

GBS : Streptocoque du groupe B

Glc : Glucose

Glc-ol : Glucitol

GlcNAC : *N*-acétyleglucosamine

GM-CSF : « Granulocyte-macrophage colony-stimulating factor »

ICE : Élément intégratif et conjugatif

IFN : Interféron

IFNAR : « IFN-α/β receptor »

Ig : Immunoglobuline

IL : Interleukine

IRAK : « IL-1 receptor-associated kinase »

IRF : Facteur régulateur de l’interféron

LCR : Liquide céphalorachidien

Lgt : Pré-prolipoprotéine diacylglycéryle transférase

LTA : Acide lipotéichoïque

MAPK : Protéine kinase associée aux mitogènes

MEC : Matrice extracellulaire

MLST : « Multilocus sequence typing »

MRP : « Muramidase-released protein »

MyD88 : « Myeloid differentiation primary response 88 »

NET : « Neutrophil extracellular trap »

Neu5Ac : Acide sialique

NF-κB : Facteur nucléaire-κB

NK : Natural Killer

NLR : « NOD-like receptor »

NLRP : « NLR pyrin domain »

NOD: « Nucleotide-binding oligomerization domain »

P : Phosphate

PAMP : Motif moléculaire associé aux pathogènes

PRR : Récepteur de reconnaissance des motifs moléculaires

Rha : Rhamnose

RIP2 : « Receptor-interacting protein kinase 2 »

SAS : Espace sous-arachnoïdien

SNC : Système nerveux central

ST : Type allélique

STAT : « Signal transducer and activator of transcription »

STSLS : Syndrome apparenté au choc toxique streptococcique

TIR : Toll/interleukine-1

TLR : « Toll-like receptor »

TNF : Facteur de nécrose tumorale

TRAF : « TNF receptor-associated factor »

TRIF : « TIR-domain-containing adapter-inducing IFN-β »

UFC : Unité formatrice de colonies

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I. INTRODUCTION

Streptococcus suis est une bactérie à Gram positif et l'un des plus importants pathogènes du porc, causant des pertes économiques substantielles à l'industrie porcine. De plus, c'est un agent zoonotique représentant de sérieux risques pour la santé humaine, particulièrement en Asie du Sud-Est [1]. *S. suis* cause une multitude de pathologies chez les porcelets, dont la méningite, la mort subite et l'arthrite. Chez l'humain, *S. suis* est principalement responsable de méningites et de chocs septiques. En effet, ces pathologies sont la conséquence d'une inflammation exacerbée, qui est la caractéristique principale des infections à *S. suis* [1, 2].

La classification la plus utilisée pour caractériser *S. suis* est celle du sérotypage. À ce jour, 35 sérotypes ont été décrits, basés sur l'antigénicité de leur capsule polysaccharidique (CPS) [2]. De ces sérotypes, le sérotype 2 est le plus fréquemment isolé de cas cliniques porcins et humains, et le plus virulent. Toutefois, le sérotype 14 est en émergence en Asie du Sud-Est, où il présente un risque pour la santé humaine. Finalement, le sérotype 9 est prévalent en Europe, où il est la plus grande cause de cas porcins en Allemagne, en Espagne et aux Pays-Bas [3]. De plus, sa prévalence a augmenté au Canada et en Chine dans les dernières années [4, 5].

En parallèle, l'utilisation du « multilocus sequence typing » a permis la classification des souches de *S. suis* en types alléliques (STs) en se basant sur l'analyse de gènes conservés [6]. Cette technique a surtout été utilisée pour le sérotype 2 [3]. La grande majorité des souches virulentes européennes et asiatiques dites "classiques" appartiennent au ST1, tandis que les souches ST7 ont été responsables des éclosions humaines de 1998 et 2005 en Chine. Enfin, les souches ST25 et ST28, considérées comme moins virulentes, sont principalement retrouvées en Amérique du Nord, mais aussi en Thaïlande et au Japon, respectivement [3, 7].

La pathogenèse de *S. suis* est complexe et est basée sur les études du sérotype 2 [8]. Chez le porc, *S. suis* colonise les voies respiratoires supérieures, où il peut persister pendant plusieurs années. Toutefois, les souches virulentes peuvent envahir les muqueuses afin d'atteindre la circulation sanguine [9]. De manière similaire, l'humain se contamine suite au contact de lésions cutanées avec des porcs infectés ou leurs dérivés, ou suite à l'ingestion d'aliments peu ou pas cuits [1]. Une fois dans la circulation sanguine, *S. suis* résiste à l'effet bactéricide des leucocytes, ce qui lui permet de se répliquer, puis de se disséminer. La présence bactérienne active les cellules de l'immunité innée, menant à la production d'une

réponse inflammatoire qui, lorsqu'exacerbée, peut causer la mort de l'hôte [10]. Si l'individu survit à cette infection systémique, la bactérie peut atteindre le système nerveux central (SNC), dans lequel elle va activer les cellules résidentes. Celles-ci vont répondre par l'entremise d'une réponse inflammatoire qui est responsable de l'infiltration de leucocytes circulants périphériques. Enfin, son exacerbation va mener au développement de méningites [10].

Cependant, la quasi-totalité des études portant sur la pathogenèse de *S. suis* et sur ses facteurs de virulence a été réalisée avec des souches ST1 ou ST7 de sérotype 2 [8, 10]. En effet, très peu d'information est disponible sur d'autres STs du sérotype 2, de même que sur d'autres sérotypes, et ce, malgré leur importance. Bien que plusieurs composants de *S. suis* ont été suggérés comme étant des facteurs impliqués dans sa pathogenèse et sa virulence, la notion de ce qu'est un facteur de virulence critique pour cette bactérie est de plus en plus sujette à controverse [8]. En effet, les méthodes expérimentales utilisées diffèrent grandement entre laboratoires. Toutefois, aucune étude n'a abordé cette problématique à l'aide de données scientifiques.

Des différents facteurs de virulence proposés, la CPS est considérée comme critique pour la virulence de *S. suis*, car elle participe à une multitude de fonctions, dont à la résistance à la phagocytose et à l'effet bactéricide et elle masque les composants de surface responsables de l'activation du système immunitaire [10]. De plus, les acides lipotéichoïques (LTA) sont des composantes majeures de la paroi des bactéries à Gram positif [11]. Malgré cela, leurs structures et leurs propriétés immunostimulatrices restent inconnues. Enfin, les antigènes I/II (AgI/II) sont des protéines multimodales impliquées dans plusieurs fonctions chez les streptocoques [12]. Cependant, bien que présents chez *S. suis*, leurs fonctions et leur rôle dans l'infection n'ont pas été étudiés.

La reconnaissance de *S. suis* par les cellules immunitaires de l'hôte s'effectue à l'aide de récepteurs de surface ou intracellulaires. Parmi ceux-ci, les « Toll-like receptors » (TLRs) sont les plus importants [13]. Leur activation initie une cascade de signalisation incluant le recrutement de protéines adaptatrices, dont le « myeloid differentiation primary response 88 » (MyD88), qui est central à cette voie [13]. La cascade de signalisation mène à la production d'une réponse inflammatoire composée de divers médiateurs pro-inflammatoires, dont les interférons (IFN) de type I [14]. Bien que traditionnellement associés aux infections virales, ces

derniers pourraient aussi participer dans les infections par les bactéries extracellulaires [15-17].

Parmi les acteurs de la réponse immunitaire, les cellules du système immunitaire sont responsables de la réponse inflammatoire induite par *S. suis* [10]. Dans la circulation sanguine, les neutrophiles et les monocytes sont parmi les cellules de l'immunité innée les plus importantes. En plus d'être des phagocytes professionnels, ils participent à la production de médiateurs inflammatoires [18, 19]. De plus, ils infiltrent massivement le SNC lors de la méningite causée par *S. suis* [20-22]. Malgré leur importance avérée, leur rôle lors des infections à *S. suis* reste inconnu. Comme pour le compartiment systémique, le SNC est constitué de différents types cellulaires participant non seulement à maintenir son intégrité, mais aussi à sa défense [23]. Les leptoméninges sont une fine couche de cellules entourant le SNC le protégeant des microorganismes envahissants, tandis que les astrocytes sont retrouvés à travers l'entièreté du SNC [24, 25]. Bien que ces cellules soient parmi les premières à rencontrer *S. suis* dans le SNC et que *S. suis* cause une méningite, c'est-à-dire une inflammation des leptoméninges, leur rôle dans le cadre de sa pathogenèse et de son infection est mal compris.

Ainsi, l'**hypothèse générale** de cette thèse est que la pathogenèse de l'infection systémique et du SNC par *S. suis*, de même que la réponse inflammatoire induite, dépendent du bagage génétique et des caractéristiques phénotypiques des souches étudiées. En effet, les composants bactériens, ainsi que l'inflammation induite par la bactérie, varient selon l'origine de la souche, incluant le sérotype, le ST et le lieu géographique, entre autres.

L'**objectif général** de cette thèse est de mieux comprendre la pathogenèse de l'infection et de l'inflammation causées par *S. suis* à l'aide de souches différentes et d'évaluer l'impact de ces différences sur celles-ci.

Les **objectifs spécifiques** sont :

1. Étudier le rôle de différents composants de *S. suis* dans sa pathogenèse, notamment dans ses interactions avec l'hôte

2. Étudier l'impact de l'origine des souches et du choix des modèles expérimentaux sur les études de virulence et de pathogenèse de *S. suis*

3. Caractériser l'infection et l'inflammation causées par différents *S. suis* au niveau systémique et du SNC

À travers cette thèse, nous démontrons que la présence, le rôle et les propriétés des composants de *S. suis* dans sa pathogenèse et sa virulence peuvent varier selon le bagage génétique et les caractéristiques phénotypiques, ce qui rend la généralisation des résultats obtenus imprudente. De plus, le choix des modèles expérimentaux utilisés peut influencer les conclusions tirées. D'autre part, bien que la reconnaissance de *S. suis* par l'immunité innée s'effectue de manière similaire via des motifs conservés et cause une réponse inflammatoire exacerbée caractéristique de ce pathogène, le bagage génétique et les caractéristiques phénotypiques de la souche module cette réponse, tant au niveau systémique qu'au niveau du SNC, ce qui peut déterminer si l'hôte succombe ou non à l'infection. Les connaissances découlant de ces travaux fourniront de nouvelles avenues pour le développement de stratégies préventives et thérapeutiques face à ce pathogène, de même que des outils de diagnostic pour mieux le contrôler.

II. REVUE DE LITTÉRATURE

1. *Streptococcus suis*

1.1. Caractéristiques générales

S. suis est une bactérie encapsulée à Gram positif en forme de coque, fréquemment retrouvée sous forme isolée ou en paire, et plus rarement en courtes chaînes, d'un diamètre inférieur à 2 µm [1]. Il est un anaérobie facultatif non-motile et forme des colonies mucoïdes gris-blanc α-hémolytiques sur gélose supplémentée de sang de mouton [26].

1.2. *Streptococcus suis* chez le porc

S. suis est une bactérie commensale des voies respiratoires supérieures des porcs, et plus particulièrement des cavités nasales et des amygdales, mais est également retrouvé dans les tractus génitaux et gastro-intestinaux [2, 27]. En effet, 100% des porcs sont naturellement colonisés par au moins un sérotype de *S. suis*, bien que la présence de plusieurs sérotypes est fréquente chez le même animal [28]. Toutefois, bien que le porc soit un porteur asymptomatique de *S. suis*, cette bactérie est la cause d'infections graves entraînant des pertes économiques importantes pour l'industrie porcine, et ce mondialement [1, 3]. Cependant, bien que différentes souches appartenant à un seul ou plusieurs sérotypes peuvent être présentes au sein d'un troupeau fermé, une seule souche est normalement responsable de la majorité des cas cliniques [28].

1.2.1. Transmission et facteurs de risques

S. suis peut se transmettre de manière verticale et/ou horizontale. La transmission verticale se fait durant la mise bas, lorsque les porcelets sont en contact direct avec les bactéries présentes dans les sécrétions vaginales [2]. De plus, la bactérie se transmet de manière horizontale par des contacts entre les porcelets ou avec la truie [2]. À ce jour, différents facteurs de risque ont été identifiés comme pouvant influencer le développement des infections à *S. suis* à la ferme [2]. Ainsi, certaines pratiques d'élevage, dont des fluctuations excessives de température, l'humidité relative élevée, la mauvaise ventilation, l'entassement, l'écart d'âge entre les porcelets à l'intérieur d'une même pièce, ainsi que le stress lié au transport et à la vaccination constituent des facteurs augmentant la susceptibilité des animaux à l'infection [2]. De plus, la faiblesse du statut immunitaire du troupeau et la présence d'autres pathogènes, tels que le virus du syndrome reproducteur et respiratoire porcin et les mycoplasmes, peuvent augmenter les risques d'une infection à *S. suis* [29, 30].

1.2.2. Signes cliniques et pathologies associées

S. suis peut causer une variété d'infections conduisant à des pathologies de gravités variables chez le porc : la mort subite, la méningite, l'arthrite et l'endocardite sont les plus fréquentes [31, 32]. La plupart des manifestations cliniques surviennent entre cinq et dix semaines d'âge, étant donné que les porcelets sont plus susceptibles à développer une infection suite au sevrage en raison des différents stress qu'ils subissent à cette occasion (séparation de la mère, fin de l'immunité passive, changements liés à leur environnement et à leur diète, etc.) [33].

Un des premiers signes observés chez les animaux malades à *S. suis* est une hyperthermie qui sera, dans la plupart des cas, accompagnée d'une importante perte de poids et de l'apparition de signes de dépression [2]. La bactérie peut également être retrouvée dans la circulation sanguine où elle pourra induire le sepsis si l'animal n'est pas traité rapidement [2]. Différents signes cliniques neurologiques peuvent être observés chez les animaux atteints de méningites, parmi lesquels il est possible de retrouver l'ataxie, l'opisthotonos, les convulsions et le nystagmus [2]. Pour leur part, les animaux atteints d'autres pathologies, tels que des endocardites, qui sont souvent accompagnées de sepsis, présenteront plutôt des signes de dyspnée respiratoire, de cyanose et de dépression [2]. Les lésions attribuées à *S. suis* sont habituellement caractérisées par des infiltrations neutrophiliques (inflammation suppurative), parfois accompagnées de dépôt de fibrine et sont globalement les mêmes pour tous les sérotypes, du moins sur le plan macroscopique [22].

1.2.3. Traitements et prévention

À ce jour, les antibiotiques restent le premier choix pour traiter les infections à *S. suis* [34]. Toutefois, le choix de l'antibiotique doit se faire selon la sensibilité de la souche responsable de l'infection, tout en tenant compte des résistances rapportées dans la filière porcine suite à la surutilisation des antibiotiques [34, 35]. Néanmoins, l'ampicilline, le ceftiofur, la tiamuline, le trimethoprime et les sulfonamides restent de bons choix pour le traitement de la maladie [2]. Il est important de garder en tête que *S. suis* ne sera pas complètement éliminé de l'animal [34]. D'autre part, lorsque les animaux présentent des signes de méningites, l'administration d'un anti-inflammatoire en combinaison avec l'antibiotique choisi pourrait améliorer la réponse de l'animal à son traitement [2].

Afin de prévenir le développement de maladies causées par *S. suis*, la vaccination est la mesure la plus recommandée. Toutefois, il existe très peu d'information concernant son efficacité et peu de fermes au Canada utilisent un vaccin [36]. De plus, le plus grand problème auquel font face les études de vaccination est la faible immunogénicité de *S. suis* [36]. Non seulement la CPS est peu immunogène, mais elle masque les composants sous-capsulaires qui le sont [37, 38]. De plus, *S. suis* a développé plusieurs mécanismes pour déjouer les différentes étapes de la réponse adaptative. En effet, nous avons récemment démontré que *S. suis*, en partie par l'entremise de sa CPS, interfère dans la présentation antigénique par les cellules dendritiques (DCs), que ce soit en retardant/compromettant l'expression du complexe majeur d'histocompatibilité de classe II (CMH-II) ou en interférant dans la production d'interleukine (IL)-12p70, impliquée dans la différenciation des lymphocytes T $CD4^+$ (**Annexes – Article I**). Ceci a pour conséquence une activation sous-optimale des lymphocytes T, des lymphocytes B et de la réponse adaptative globalement [36, 38, 39].

Néanmoins, différents types de vaccins expérimentaux ont été testés au cours des dernières années [36]. Les bactérines sont généralement le premier choix et sont présentement les seuls vaccins utilisés sur le terrain [36]. Elles sont constituées de la bactérie entière inactivée soit à la chaleur ou au formol [36]. Néanmoins, leur efficacité reste controversée [2, 36]. De plus, les bactérines autogènes sont conçues à partir de la souche causant la maladie dans l'élevage, limitant ainsi la possibilité de les utiliser dans d'autres fermes [36].

Outre les bactérines, l'approche vaccinale étudiée est l'utilisation de composants de la bactérie, en particulier la CPS ou des protéines. En effet, la CPS de *S. suis* sérotype 2 pourrait être un excellent candidat vaccinal, tel que démontré récemment par l'entremise d'un vaccin glycoconjugué CPS-toxoïde tétanique [40, 41]. Un avantage majeur d'un vaccin ciblant la CPS est que les animaux pourront être protégés contre toutes les souches faisant partie d'un même sérotype [36, 41]. Toutefois, comme la CPS est faiblement immunogène, la conjugaison avec des protéines porteuses est nécessaire, ce qui rend ce type de vaccin présentement trop dispendieux pour une utilisation sur le terrain [36]. De plus, comme la protection est spécifique au sérotype en question, plusieurs CPSs purifiées seront nécessaires dans un même vaccin afin de cibler plusieurs sérotypes [36]. Pour leur part, les vaccins sous-unitaires, vaccins composés d'un ou de plusieurs composants (antigènes) du pathogène, sont encore au stade expérimental pour *S. suis* [36]. Dans ce type de vaccin, l'antigène choisi doit idéalement être

présent chez plusieurs souches et/ou sérotypes de *S. suis* afin de conférer une protection universelle contre le pathogène [36]. Bien qu'un grand nombre de protéines ont été étudiées, ces vaccins ne sont pas encore commercialement disponibles [36].

1.3. Infections zoonotiques causées par *Streptococcus suis*

En plus d'être un important pathogène du porc, *S. suis* est aussi un agent de zoonose principalement responsable de méningites et de chocs septiques chez l'humain [31]. Depuis le premier cas d'infection humaine rapporté au Danemark en 1968, plus de 1 600 cas ont été décrits à ce jour (**Annexes – Article II**). En effet, le nombre de cas rapportés a augmenté de manière exponentielle dans les dernières années, particulièrement dans les pays d'Asie, où deux éclosions mortelles ont eu lieu en Chine en 1998 et en 2005 [3]. En Amérique du Nord et en Europe, les infections humaines à *S. suis* sont considérées comme des maladies professionnelles, c'est-à-dire que les travailleurs de l'industrie porcine, les éleveurs et les vétérinaires sont les plus à risque de développer la maladie suite au contact d'un animal infecté avec des lésions cutanées [3, 31]. Cependant, en Asie, et plus particulièrement au Vietnam et en Thaïlande, la consommation d'aliments crus ou peu cuits dérivés du porc est une cause importante de la propagation de *S. suis* [3, 31]. Toutefois, la rareté de l'infection suggère l'implication concourante d'autres facteurs tels l'immunosuppression, le diabète, l'alcoolisme, l'asplénie et le cancer [42].

Le nombre croissant d'infections humaines à *S. suis* a grandement augmenté l'intérêt pour ce pathogène, particulièrement à la suite de l'éclosion de 2005 [31]. Non seulement le nombre d'articles scientifiques publiés à son sujet a significativement augmenté depuis le début des années 2000, mais les conséquences pour la santé publique ont amélioré la surveillance et les méthodes diagnostiques en Asie [3, 31]. Toutefois, la prévalence en Asie reste beaucoup plus élevée que celle en Europe et en Amérique du Nord. De plus, les taux de mortalité varient de moins de 3% dans la plupart des pays occidentaux à 26% dans certains pays asiatiques [3].

1.3.1. Signes cliniques et pathologies associées

Chez l'humain, *S. suis* cause une infection systémique à la suite d'une période d'incubation variant de quelques heures à deux jours [31]. Comme chez le porc, la méningite est la manifestation clinique la plus fréquente, mais le développement de sepsis et de choc septique est aussi très commun [3]. De manière particulière, la souche responsable des éclosions en

Chine a causé des centaines de cas du syndrome apparenté au choc toxique streptococcique (STSLS) [3]. En plus d'une fièvre élevée, ce syndrome est caractérisé par des frissonnements, des maux de tête, des vomissements, des douleurs abdominales, et par des signes cliniques d'hypotension, de tachycardie, de dysfonction hépatique, de coagulation intravasculaire disséminée, de défaillance rénale aiguë et de syndrome de détresse respiratoire aiguë [43]. Alors que la mort suit souvent le choc septique, la séquelle la plus fréquente suite à la méningite est une perte auditive [43, 44].

1.3.2. Traitements et prévention

Lors de la méningite, une antibiothérapie agressive est habituellement recommandée [31]. Toutefois, l'utilisation d'antibiotiques a un effet limité chez l'humain lors du sepsis et du choc septique [31]. Comme il n'existe présentement aucun vaccin contre *S. suis*, la prévention de la transmission aux humains dépend du contrôle de la maladie chez le porc [31]. Des mesures de santé publique accompagnées de campagnes de sensibilisation concernant les mesures d'hygiène à adopter permettront de réduire le nombre d'infections humaines [31].

1.4. Typage

Deux approches de typages sont couramment utilisées pour *S. suis*, soit le typage sérologique, qui différencie les souches selon l'antigénicité de leur CPS, et les tests par réactions en chaîne par polymérase multiplexe [3].

1.4.1. Tests sérologiques et sérotypes décrits

La majorité des laboratoires de diagnostic utilisent le test sérologique de coagglutination afin de déterminer le sérotype des souches de *S. suis* [3]. Ce test utilise la protéine A exprimée à la surface de *Staphylococcus aureus* afin de lier la portion Fc d'une immunoglobuline (Ig) de classe G, laissant sa portion Fab libre de lier son antigène [45]. Ainsi, un sérum de lapin immunisé avec la souche de référence d'un sérotype permettra l'agglutination des cellules de *S. aureus* potentialisées en présence de leur antigène, soit la CPS exprimée par une souche de *S. suis* appartenant au même sérotype (utilisation de la bactérie entière) [45].

À ce jour, 35 sérotypes différents de *S. suis* ont été caractérisés en fonction de l'antigénicité de la CPS exprimée, soit les sérotypes 1 à 34 et le sérotype 1/2 [46]. Bien que cette méthode soit relativement fiable, le sérotypage comporte certaines limites, dont la présence de réactions

croisées et l'existence de souches non-typables ou auto-agglutinantes [3, 47-50]. De plus, les avancées en génétique et en biologie moléculaire ont permis de démontrer que les sérotypes 20, 22, 26, 32, 33 et 34 appartiennent à une espèce autre que *S. suis* [51-53]. Ainsi, les sérotypes 20, 22 et 26 appartiendraient à *Streptococcus parasuis*, le sérotype 33 à *Streptococcus ruminantium* et les sérotypes 32 et 34 à *Streptococcus orisratti* [46].

1.4.2. Typage moléculaire

Afin de contourner les limites des tests sérologiques, des tests moléculaires basés sur l'amplification de gènes spécifiques au sérotype ont été développés [46]. Le gène codant pour la polymérase impliquée dans la synthèse de la CPS est ciblé, puisque celui-ci est sérotype-spécifique, à l'exception des sérotypes 1 et 14 et les sérotypes 1/2 et 2, qui ne peuvent pas être différenciés à l'aide de cette méthode [54, 55]. Toutefois, certaines souches non-sérotypables demeurent non-typables par ces techniques d'identification moléculaires et appartiennent probablement à de nouveaux sérotypes. En effet, des études récentes ayant séquencé le gène codant pour la polymérase impliquée dans la synthèse de la CPS de souches non-sérotypables ont identifié au moins dix-sept nouveaux loci capsulaires [50, 56]. Ainsi, chaque locus se distingue par la présence d'un gène codant pour une polymérase spécifique et pourrait conséquemment représenter de nouveaux sérotypes. Cependant, près de 40% des isolats identifiés comme appartenant à un nouveau NCL sont non encapsulés à cause de mutations dans leur locus capsulaire [50, 56].

1.4.3. Typage allélique par « multilocus sequence typing »

Le typage moléculaire allélique ou « mulitlocus sequence typing » (MLST) est une technique permettant la caractérisation de souches au sein d'une espèce bactérienne en se basant sur la séquence interne de sept gènes hautement conservés (gènes de ménage) [57]. Pour *S. suis*, le MLST se base sur des variations dans les gènes *aroA* (gène codant la 5-enolpyruvylshikimate 3-phosphate syntétase), *cpn60* (gène codant une chaperonne de 60 kDa), *dpr* (gène codant une protéine putative de résistance au peroxyde), *gki* (gène codant une glucose kinase), *mutS* (gène codant une enzyme de réparation des erreurs dans l'acide désoxyribonucléique [ADN]), *recA* (gène codant un facteur de recombinaison homologue) et *thrA* (gène codant une aspartokinase/homosérine déshydrogénase). Chaque séquence interne des gènes, appelée allèle, est associée à un numéro afin de créer un profil composé des sept allèles des gènes cibles. Ce profil est ensuite associé à un ST précis [6].

Cette technique ne considère pas l'ensemble des polymorphismes nucléotidiques de la séquence, mais cible plutôt quelques variations précises au sein de la séquence [57]. Elle permet donc d'avoir une vue d'ensemble de l'évolution de la bactérie et de mieux comprendre l'évolution phylogénétique de *S. suis* [6]. Cette méthode a suscité beaucoup d'intérêt au cours des dernières années et est fréquemment utilisée depuis la disponibilité des technologies de séquençage, ainsi que de l'accessibilité de la banque d'allèles et de profils en ligne [3]. Néanmoins, le MLST comporte certains points faibles. Entre autres, l'utilisation de gènes de ménage peut masquer des différences génotypiques, et par conséquent phénotypiques, importantes entre les souches. L'acquisition récente d'ADN exogène de phages, d'îlots génomiques, d'éléments intégratifs et conjugatifs ou des phénomènes de recombinaison homologue pourraient ainsi passer inaperçus [57].

À ce jour, 1 096 différents STs de *S. suis* ont été rapportés. Cependant, plusieurs d'entre eux sont étroitement reliés, suggérant un ancêtre commun (**Figure 1**). Au moment d'amorcer cette thèse, il avait été suggéré qu'une association entre la virulence d'une souche et le ST pourrait exister, du moins pour le sérotype 2 [7]. En effet, le complexe clonal (CC) 1, auquel appartient le ST1 (prédominant en Europe, en Asie et en Amérique du Sud) et le ST7 (présent en Chine et responsable des éclosions humaines de 1998 et 2005) est fortement associé aux cas d'infections sévères caractérisées par le sepsis, la méningite ou l'arthrite [3]. Au contraire, le ST25 et le ST28, fréquemment retrouvés en Amérique du Nord, sont associés avec une virulence intermédiaire et faible, respectivement [7]. De plus, certains STs sont associés avec la présence de gènes codant pour certains marqueurs de virulence comme la suilysine (*sly*), la « muraminidase-released protein » (MRP) (*mrp*) et le facteur extracellulaire (EF) (*epf*) [7]. Les souches appartenant au ST1 et au ST7 possèdent les marqueurs de virulence *sly, mrp* et *epf* alors que les ST25 et ST28 sont négatifs pour *sly* et *epf*, avec la présence ou l'absence de *mrp* permettant de distinguer ces deux STs [7]. Curieusement, les souches d'un même sérotype peuvent être regroupées en plusieurs STs, de même qu'un ST particulier peut être composé de souches de sérotypes différents [3]. Malgré l'intérêt suscité, très peu d'études ont exploré l'association entre ST et virulence, ni l'association du ST et la pathogenèse de *S. suis* et la réponse inflammatoire induite.

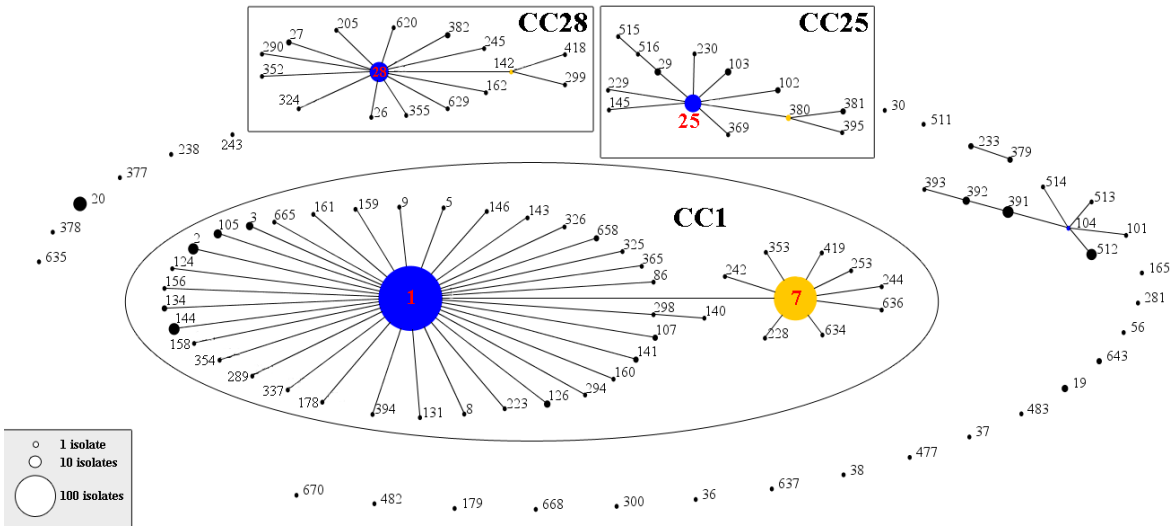

Figure 1. Vue d'ensemble de la population de *Streptococcus suis* par l'entremise des différents types alléliques. La banque de données entière du *S. suis* MLST Database est illustrée à l'aide d'eBURST. Chaque point représente un ST individuel, avec sa taille représentant le nombre d'isolats. Les points reliés représentent des complexes clonaux (CC), notamment les CC1, CC25 et CC28 qui sont les plus importants mondialement. Les fondateurs primaires et secondaires de ces CCs sont identifiés en bleu et jaune, respectivement. Les STs sont indiqués en noir, à l'exception des STs les plus importants (ST1, ST7, ST25 et ST28), en rouge. Les isolats appartenant au même ST possèdent les mêmes allèles pour les sept gènes utilisés, tandis que les isolats appartenant au même CC partagent six ces sept gènes [58].

1.5. Épidémiologie

1.5.1. Chez le porc

Chez le porc, les infections causées par *S. suis* ont été rapportées partout dans le monde [3]. Bien que le sérotype 2 soit le plus prévalent mondialement, son importance varie grandement. En effet, le sérotype 2 prédomine dans la plupart des pays européens et asiatiques, tandis qu'en Amérique du Nord, la prévalence du sérotype 2 est similaire à celle du sérotype 3, tous deux d'environ 20% [3, 59]. Curieusement, le sérotype 2 est plus isolé que le sérotype 3 au Canada, alors qu'aux États-Unis le sérotype 3 est davantage isolé [59]. En effet, les sérotypes les plus fréquemment isolés au Canada en ordre décroissant sont le 2, 3, 4, 1/2 et 7, bien que plus de 30% des souches soient non-typables [48]. Toutefois, non seulement la prévalence du sérotype 9 a augmenté dans les dernières années au Canada et en Chine, mais le sérotype 9 est le plus important en Allemagne, en Espagne et aux Pays-Bas [3, 4, 60].

Quant à la distribution des STs (**Figure 2**) , le ST1 est le plus isolé d'infections porcines à l'échelle mondiale, à l'exception de l'Amérique du Nord [3]. En Amérique du Nord, ce sont les ST25 et ST28 qui prédominent, à pourcentage presque égal, réunissant 95% des isolats [7]. Le ST28 est aussi fréquemment retrouvé au Japon. D'autre part, le ST7 est endémique en Chine. Finalement le ST20, aussi associé à des infections humaines, a été isolé de plusieurs cas de sérotype 2 aux Pays-Bas, tandis que les infections causées par le sérotype 9 dans ce même pays sont associées au ST16 [3].

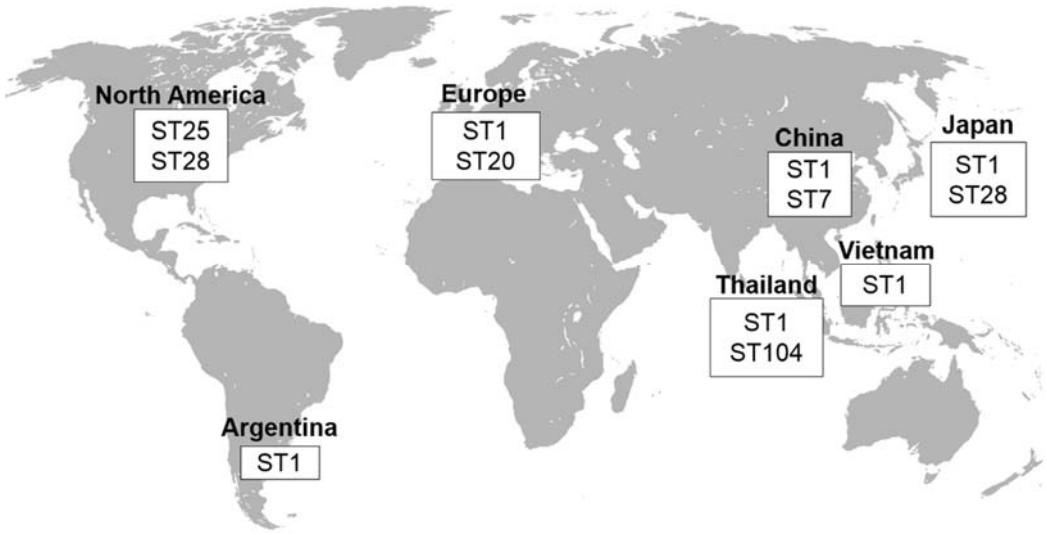

Figure 2. Distribution mondiale des plus importants types alléliques (STs) de *Streptococcus suis* sérotype 2 isolés de cas cliniques porcins et humains. Tiré de l'Annexes – Article II.

1.5.2. Chez l'humain

Tel que mentionné à la section 1.3., depuis le premier cas humain d'infection à *S. suis* en 1968, plus de 1 600 cas ont été rapportés à ce jour dans la plupart des pays élevant des porcs [3]. Le nombre de cas a augmenté de manière exponentielle dans les dernières années, particulièrement dans les pays d'Asie du Sud-Est, où plusieurs centaines de cas sont rapportés annuellement [3]. Ceci pourrait s'expliquer en partie par la surveillance accrue et l'amélioration des méthodes diagnostiques dans ces pays, mais aussi par des raisons de santé publique et d'hygiène [31]. En effet, le système de production de type cours arrière, dans lequel les porcs et les humains vivent ensembles côte à côte, est très commun en Asie du Sud-Est, contrairement à l'Occident [1]. De plus, la consommation de porc peu ou pas cuit, dont le sang,

est une pratique commune [1]. Ainsi, l'ensemble de ces pratiques, en combinaison avec le nombre élevé de cas humains à *S. suis*, a forcé les gouvernements de ces pays à mettre en place des systèmes de surveillance et d'investir dans le développement et la mise en place de méthodes diagnostiques, tant chez le porc que chez l'humain, afin de mieux combattre l'infection [1].

S. suis a été identifié comme la première cause de méningite chez les adultes au Viêtnam, la deuxième en Thaïlande et la troisième cause la plus fréquente de méningite bactérienne acquise dans la communauté à Hong Kong, où elles se présentent souvent sous forme de cas groupés ou d'éclosions [3]. En revanche, seuls des cas sporadiques ont été rapportés au Canada et aux États-Unis [3]. Cette différence pourrait s'expliquer par des erreurs de diagnostic, mais possiblement aussi par une plus faible prévalence des souches virulentes pour les humains en Amérique du Nord [31]. De plus, l'hypothèse a été émise que les souches nord-américaines sont moins virulentes que celles retrouvées en Europe et en Asie et qu'elles ont un potentiel de transmission réduit, malgré des méthodes d'élevage similaires (du moins entre l'Amérique du Nord et l'Europe) [31]. Toutefois, la virulence des souches nord-américaines a été très peu étudiée et l'impact de l'origine géographique à l'intérieur d'un même sérotype reste inconnu.

Le sérotype 2 est responsable de plus de 95% des cas humains mondialement, dont les éclosions en Chine qui ont suscité un intérêt marqué [3]. Durant l'éclosion de 2005, plus de 215 cas humains ont été déclarés dans la province de Sichuan, dont 39 sont décédés suite à l'infection [3]. Le taux de mortalité élevé est une des caractéristiques inquiétantes de cette éclosion [61]. En plus du sérotype 2, le sérotype 14 a émergé en Asie du Sud-Est dans les dernières années, particulièrement en Thaïlande, où il a causé plusieurs dizaines de cas humains [3]. Finalement, des cas humains causés par les sérotypes 5, 9, 16, 21, 24 et 31 ont également été rapportés (**Annexes – Article III**) [62-66].

De manière similaire au porc, le ST1 prédomine en Europe, en Asie et en Amérique du Sud (Argentine) (**Annexes – Article IV**) [3]. Au contraire, des souches appartenant au ST7 n'ont été retrouvées qu'en Chine et ce sont elles qui ont été responsables des éclosions mortelles de 1998 et 2005 [3]. Pour leur part, les souches appartenant au ST20 sont retrouvées particulièrement aux Pays-Bas, comme chez le porc [67]. Pour ce qui est de l'Amérique du

Nord, ce sont principalement des souches appartenant aux ST25 (également retrouvées en Thaïlande) et aux ST28 (également retrouvées au Japon) qui sont les plus souvent isolées de cas cliniques [3]. Finalement, des souches de sérotype 2 appartenant aux ST101 et ST104 et des souches de sérotype 14 appartenant au ST105 sont endémiques en Thaïlande (**Figure 2**) [3, 68, 69].

1.6. Pathogenèse de l'infection et réponse inflammatoire

Afin de causer son infection, *S. suis* doit traverser plusieurs barrières physiques et immunitaires [10]. En effet, la bactérie doit coloniser, puis envahir l'épithélium, afin d'atteindre la circulation sanguine. Une fois dans le sang, la bactérie doit résister à l'effet bactéricide des leucocytes, afin de pouvoir se multiplier, persister et se disséminer. Ceci lui permet donc de causer une infection systémique caractérisée par le sepsis suite à l'induction d'une production exacerbée de médiateurs pro-inflammatoires pouvant éventuellement mener au choc septique. Si l'hôte survit à l'infection systémique, *S. suis* peut franchir l'une des deux barrières séparant le SNC du sang, soit la barrière hématoencéphalique (BBB) ou la barrière hémato-liquide céphalorachidien (BCSFB). Une fois dans le SNC, *S. suis* cause une infection locale caractérisée par une réponse inflammatoire exacerbée, menant au développement de la méningite accompagnée ou non d'une encéphalite (méningoencéphalite) [10].

1.6.1. Voies d'entrée chez l'hôte et colonisation des amygdales chez le porc

La voie d'entrée la plus commune chez le porc est les voies respiratoires supérieures, où la bactérie colonise les amygdales palatines ou pharyngées [70, 71]. Les amygdales sont décrites comme étant un des sites primaires d'invasion bactérienne, suivi des nodules lymphatiques pharyngés [70]. En effet, des antigènes de *S. suis* y ont été identifiés et des bactéries entières ont fréquemment été observées dans les cryptes luménales et dans l'épithélium des zones subépithéliales immédiates [70].

Toutefois, le tractus gastro-intestinal ne peut pas être exclu comme site d'infection secondaire, puisque les intestins des porcelets sont rapidement colonisés lors du sevrage [9]. Tel que mentionné précédemment, les voies d'entrée principales chez l'humain sont soit au niveau de lésions cutanées suite au contact avec des porcs malades ou avec leurs dérivés, ou au niveau intestinal suite à l'ingestion de produits contaminés crus ou peu cuits [9]. À ce jour, l'entrée par les voies respiratoires n'a jamais été démontrée chez l'humain [9].

1.6.2. Adhésion et invasion de l'épithélium

Les mécanismes par lesquels *S. suis* parvient à franchir l'épithélium demeurent encore peu connus à ce jour [9]. En effet, *S. suis* adhère et envahit faiblement les cellules épithéliales [10]. De plus, la production de biofilm par *S. suis*, afin de survivre et coloniser les amygdales, demeure controversée [9]. Toutefois, l'adhésion de *S. suis* aux cellules épithéliales dépend de différentes adhésines, qui sont partiellement masquées par la présence de la CPS [9]. En effet, plus de vingt facteurs ont été décrits comme participant à l'adhésion et à l'invasion des cellules épithéliales [10, 72-87]. Ainsi, il a été proposé que *S. suis* peut réguler à la baisse l'expression de sa CPS durant les premières étapes de l'infection, afin de favoriser les interactions avec ces cellules [9]. En effet, seules les souches non-encapsulées ont la capacité d'envahir les cellules épithéliales [10]. De plus, la suilysine (une toxine produite par certaines souches de *S. suis*) pourrait aussi participer dans la perturbation de la surface épithéliale avec son activité cytotoxique [88]. Une fois la barrière épithéliale traversée, *S. suis* peut ensuite interagir avec la matrice extracellulaire (MEC) [10]. Plus de quarante protéines ont été décrites comme ayant un rôle dans la liaison à la MEC, que ce soit à la fibronectine, au fibrinogène, à la laminine ou encore au collagène [8]. L'adhésion de *S. suis* aux protéines de la MEC pourrait faciliter les interactions avec les cellules épithéliales en plus de l'invasion du tissu sous-jacent [9].

1.6.3. Survie dans le sang et dissémination systémique

Une fois l'épithélium franchi, *S. suis* se retrouve dans la circulation sanguine. Dans le sang, *S. suis* circule par l'intermédiaire de facteurs encore inconnus, soit librement ou lié à la surface des monocytes, selon l'hypothèse du cheval de Troie modifié [10, 88, 89]. *S. suis* possède plusieurs facteurs lui permettant de résister à la phagocytose et à l'effet bactéricide des neutrophiles et des monocytes sanguins [10]. À ce stade, la CPS est un facteur critique permettant à *S. suis* de persister dans le sang [10, 90, 91]. Contrairement aux étapes d'adhésion et d'invasion des surfaces épithéliales, la présence de la CPS est nécessaire pour la survie bactérienne, comme démontré par l'utilisation de souches non-encapsulées. En effet, celles-ci, contrairement à leur souche-mère, sont rapidement éliminées de la circulation sanguine [10, 92, 93]. Ainsi, il a été suggéré que *S. suis* peut réguler à la hausse l'expression de sa CPS durant cette étape [10]. Plusieurs autres facteurs sont également impliqués dans la tolérance au stress oxydatif [10]. De plus, la suilysine, agissant en synergie avec des composants de la paroi cellulaire de *S. suis,* cause la libération d'hémoglobine des érythrocytes, contribuant à l'augmentation des niveaux de médiateurs pro-inflammatoires [94].

Finalement, SspA, une protéase de type subtilisine, est un inducteur de médiateurs pro-inflammatoires des macrophages [95]. Ensemble, ces mécanismes permettent à *S. suis* de se multiplier, de persister et de se disséminer, causant ainsi une bactériémie pouvant mener au sepsis [10].

1.6.4. Activation de la réponse inflammatoire systémique, sepsis et choc septique

Bien que l'activation du système immunitaire soit protectrice pour l'hôte lors d'une infection bactérienne, le sepsis suivi ou non d'un choc septique peut se développer suite à une régulation déséquilibrée de cette réponse [96]. La libération excessive de médiateurs pro-inflammatoires peut être dommageable pour l'hôte, car cela perturbe l'homéostasie vasculaire, ce qui peut conduire à une dysfonction multiorganes [96]. En effet, la persistance de *S. suis* dans le sang et sa dissémination dans différents organes internes (particulièrement le foie, la rate, le cœur et les reins), cause une activation rapide et déséquilibrée des cellules de la réponse immunitaire innée (les neutrophiles, les monocytes et les cellules Natural Killer [NK] dans le sang, et les macrophages et les cellules dendritiques dans les organes) et l'induction massive de médiateurs pro-inflammatoires menant directement au développement du sepsis puis, dans la majorité des cas, au choc septique [10, 97-100]. De plus, certaines souches virulentes, dont celles responsables des éclosions en Chine, caractérisées par des cas de STSLS, manipulent à leur avantage le système immunitaire de l'hôte [101]. Ainsi, *S. suis* induit non seulement une production élevée de médiateurs pro-inflammatoires par les cellules porcines, murines et humaines *in vitro* [10], mais également des niveaux systémiques élevés de médiateurs pro-inflammatoires *in vivo* durant le sepsis et le choc septique chez la souris, le porc et l'humain, dont le facteur de nécrose tumoral (TNF), l'IL-6, l'IL-12p70, IFN-γ, le « C-C motif ligand » (CCL) 2, CCL3, CCL5 et le « C-X-C motif ligand » (CXCL) 1 [21, 102-104]. De plus, *S. suis* induit l'expression de molécules d'adhésion impliquées dans l'inflammation, provoquant ainsi le recrutement massif de leucocytes et l'amplification de la réponse inflammatoire [10]. L'activation cellulaire par *S. suis* implique des récepteurs de reconnaissance et des motifs moléculaires, notamment le CD14 et le TLR2, mais d'autres TLRs pourraient aussi intervenir [10]. Des études, à l'aide de mutants non-encapsulés, ont démontré le rôle des composants de la paroi bactérienne dans l'induction de la réponse inflammatoire : bien que ces composants impliqués soient peu caractérisés, les lipoprotéines à la surface de la bactérie sont fort probablement impliquées [10, 105, 106]. Toutefois, il est important de

garder en tête que bien que l'inflammation exacerbée induite par *S. suis* soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes.

1.6.5. Voies d'entrée dans le système nerveux central

Si l'hôte survit à l'infection systémique causée par *S. suis*, il est susceptible de développer une infection du SNC, lorsque la bactérie atteint celui-ci. Pour ce faire, la bactérie doit traverser la BBB ou la BCSFB (**Figure 3**). Toutefois, les mécanismes utilisés par *S. suis* pour envahir le SNC restent nébuleux à ce jour [10].

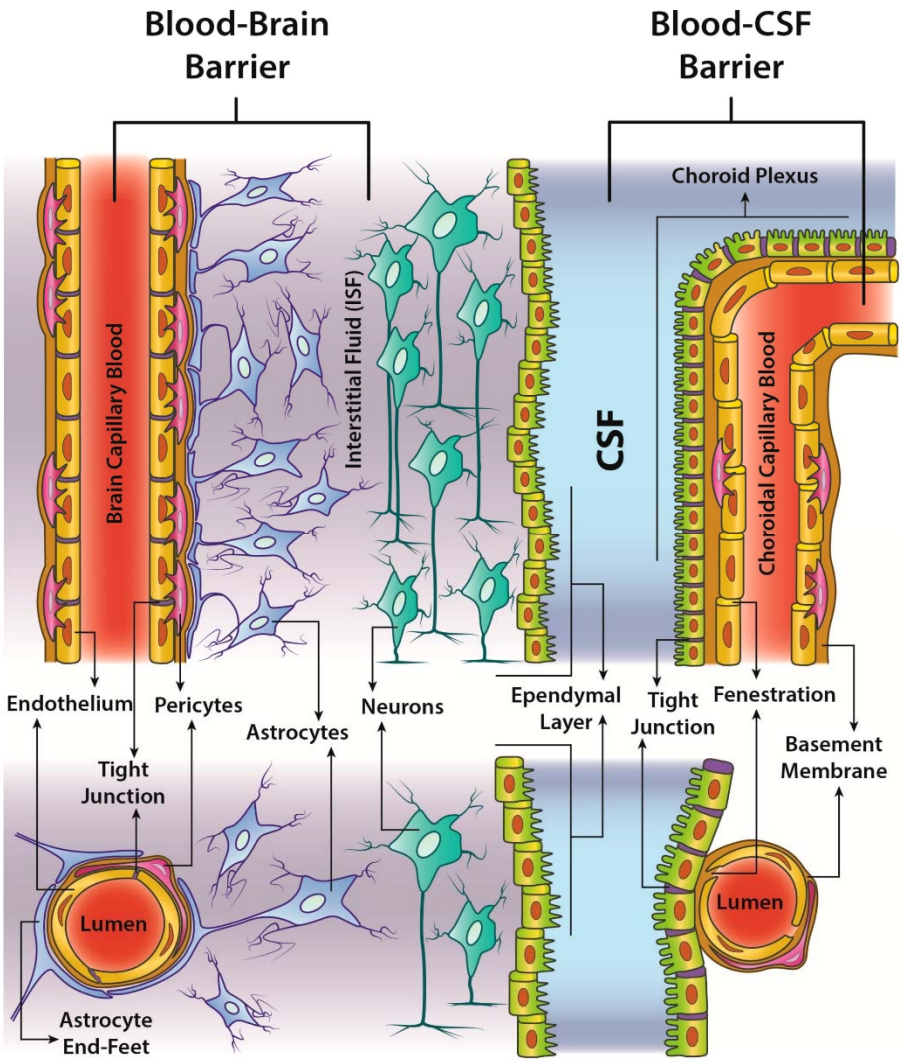

Figure 3. **Les barrières séparant le sang du système nerveux central.** Il existe deux barrières principales séparant le sang du système nerveux central, soit la barrière

hématoencéphalique (BBB; à gauche) et la barrière sang-liquide céphalorachidien (BCSFB; à droite). La BBB est formée de cellules endothéliales microvasculaires recouvrant les vaisseaux sanguins et des pieds astrocytaires. Au contraire, la BCSFB est composée des cellules épithéliales du plexus choroïdien qui produisent et sécrètent le liquide céphalorachidien [107].

1.6.5.1. La barrière hématoencéphalique

La BBB est une barrière structurelle et fonctionnelle formée par les cellules endothéliales microvasculaires du cerveau (BMEC). Les BMECs possèdent plusieurs caractéristiques, dont la présence de jonctions serrées, une résistance électrique élevée et un faible taux de pinocytose [108, 109]. Ces microvaisseaux sont recouverts de péricytes et des pieds astrocytaires [110]. La BBB fonctionne comme barrière hautement résistante aux macromolécules circulantes, protégeant ainsi le SNC des microorganismes et des toxines circulant dans le sang. Une perturbation de la BBB est une caractéristique de la pathophysiologie de la méningite bactérienne [108, 111]. Ainsi, différents facteurs impliqués dans la virulence de *S. suis* ont été caractérisés comme participant dans l'adhésion et l'invasion des BMECs porcines [10]. Contrairement aux cellules épithéliales, *S. suis* peut envahir les BMECs porcines et y survivre de manière intracellulaire jusqu'à 7 heures, ce qui pourrait être un élément crucial pour traverser la BBB et favoriser le développement de la méningite [112]. Une fois de plus, la suilysine participe à l'invasion des BMECs par son activité hémolytique [10]. Plus récemment, il a été démontré que l'énolase de *S. suis* possède la capacité d'augmenter la perméabilité de la BBB [113]. De plus, certaines composantes du sérum peuvent également participer aux interactions entre *S. suis* et les BMECs porcines [10]. Il a aussi été rapporté que *S. suis* peut induire la libération d'acide arachidonique, précurseur des prostaglandines, des les BMECs via l'activité de sa suilysine, mécanisme qui faciliterait la capacité des bactéries à pénétrer dans le SNC et à moduler l'inflammation locale en augmentant la perméabilité vasculaire de la BBB et la production d'oxyde nitrique des BMECs [114]. *S. suis* peut également moduler l'expression de molécules d'adhésion sur les monocytes humains et les cellules endothéliales, ayant comme résultante une augmentation de l'adhésion [115, 116]. D'autres études ont montré que *S. suis* est capable d'induire la libération de cytokines pro-inflammatoires et de chimiokines par les BMECs humaines et porcines [21, 117].

1.6.5.2. La barrière hémato-liquide céphalorachidien

Le plexus choroïdien est composé d'un épithélium (cellules épithéliales du plexus choroïdien [CPEC]) qui produit et sécrète le liquide céphalorachidien (LCR) dans les ventricules du

cerveau [118]. Comme avec la BBB, la BCSFB est constituée de jonctions serrées inhibant la diffusion paracellulaire de molécules hydrosolubles [109]. Bien que la BBB représente une plus grande surface d'invasion, des études anatamopathologiques ont démontré que le plexus choroïdien des porcs est perturbé suite à l'infection par *S. suis*, incluant la présence de cellules immunitaires dans les ventricules [119]. En effet, *S. suis* peut diminuer la résistance transépithéliale des CPECs [89, 120]. De plus, *S. suis* possède la capacité d'adhérer aux CPECs et de traverser la BCSFB du côté basolatéral (sang) pour se rendre au côté latéral (LCR) via l'endocytose [121]. *S. suis* peut aussi induire la mort des CPECs, en partie due à la présence de la suilysine [122]. D'autre part, l'étude du transcriptome de CPECs infectées par *S. suis* a démontré que les gènes impliqués dans la réponse inflammatoire et dans la production de médiateurs inflammatoires sont régulés à la hausse [120, 123]. Ensemble, ces interactions pourraient permettre à *S. suis* d'atteindre le LCR. Une fois dans le LCR, *S. suis* est non seulement libre d'atteindre le parenchyme cérébral, mais aussi de s'y multiplier, étant donné la présence abondante de nutriments et l'absence générale d'Igs, de complément et de leucocytes [124].

1.6.6. Activation de la réponse inflammatoire du système nerveux central et développement de la méningite

Comment mentionné ci-dessus, il existe deux voies d'entrées principales permettent à *S. suis* d'atteindre le SNC, soit la BBB et le BCSFB (**Figure 3**). Si *S. suis* traverse la BBB, la bactérie se retrouve directement dans le parenchyme cérébral où elle fait face aux cellules résidentes [111]. Parmi ces cellules, la microglie et les astrocytes sont des cellules de l'immunité innée [25]. Au contraire, une fois dans le LCR (après avoir traversé la BCSFB), *S. suis* doit interagir avec les cellules des méninges (plus particulièrement celles de la pie-mère), qui devront être envahies, avant de pouvoir atteindre le parenchyme cérébral sous-jacent [108]. Toutefois, bien que l'infection du SNC causée par *S. suis* se caractérise par la méningite, c'est-à-dire une inflammation des méninges, aucune étude n'avait déterminé le rôle que joue les méninges dans la pathogenèse de l'infection, ni leurs interactions avec *S. suis*, lorsque cette thèse a été amorcée.

Les dommages neurologiques et la mort neuronale associés à la méningite induite par *S. suis* sont la conséquence d'une reconnaissance de composants bactériens par les cellules immunitaires de l'hôte [10, 22]. En effet, la présence de *S. suis* dans le SNC augmente

l'expression transcriptionnelle de TLRs (TLR2 et TLR3, mais pas TLR4), du récepteur CD14 et de médiateurs pro-inflammatoires (IL-1β et CCL2) et ce, dans la plupart des régions du cerveau, incluant la BBB, le plexus choroïdien et les méninges [21]. La microglie, et moindrement les astrocytes, ont été démontrés comme les sources principales de ces médiateurs [21]. Bien que les composants bactériens causant cette activation soient peu connus, des études avec un mutant non-encapsulé ont démontré une augmentation de la production de médiateurs inflammatoires lors de l'infection de microglies et d'astrocytes murins, suggérant, comme lors de l'infection systémique, un rôle des composants sous-capsulaires de *S. suis* [125, 126]. Plus récemment, une étude de co-culture avec des astrocytes et des cellules de la microglie murins a rapporté que des facteurs sécrétés par les astrocytes augmentent la production d'oxyde nitrique par la microglie suite à l'infection par *S. suis* [127]. Comme pour le compartiment systémique, il est important de garder en tête que bien que l'inflammation exacerbée induite par *S. suis* dans le SNC soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes. Toutefois, contrairement à l'infection systémique, les connaissances des mécanismes et du rôle des différentes cellules de l'hôte lors de l'infection du SNC causée par *S. suis* sont très limitées.

1.7. Composants de la surface bactérienne et facteurs de virulence

Une panoplie de facteurs de virulence de *S. suis* favorisent sa colonisation de l'hôte et son infection menant au développement de la maladie clinique [9, 10]. En effet, il y a eu un nombre croissant de publications portant sur l'étude des facteurs de *S. suis*, avec plusieurs d'entre eux ayant été décrits comme "critiques" pour sa virulence [8]. Toutefois, les méthodes expérimentales employées pour les étudier varient énormément entre laboratoires, autant pour les études *in vitro* que pour les études *in vivo,* et sont, dans plusieurs cas, inappropriées et/ou inadéquates [8]. Ainsi, il existe un manque de rigueur scientifique en ce qui concerne ce qu'est un facteur de virulence pour *S. suis* et comment le déterminer [8]. De plus, la complexité de ce microorganisme rend difficile sa catégorisation basée seulement sur la présence ou l'absence de facteurs de virulence décrits ou proposés. Ainsi, lorsque cette thèse a débuté, ce manque de rigueur scientifique, et donc de répétabilité des résultats entre laboratoires, même si présent, n'avait jamais été évalué.

1.7.1. La capsule polysaccharidique

La CPS est une couche structurée de polysaccharides que certaines bactéries sont capables de synthétiser et d'exporter à leur surface. La CPS de *S. suis* est l'un des facteurs de virulence les plus importants, selon les études réalisées avec le sérotype 2 [10, 92, 128]. De plus, la classification de *S. suis* en différents sérotypes est basée sur l'antigénicité des CPSs due à des différences de composition et de structure [46].

1.7.1.1. Composition et structure

À ce jour, seules les structures de la CPS des sérotypes 1, 1/2, 2, 3, 9, 14 et 18 ont été décrites [49, 129-132]. Dans le cadre de cette thèse, nous nous intéresserons plus particulièrement aux sérotypes 1, 1/2, 2, 9 et 14 comme ils sont parmi les plus importants pour les infections chez le porc et chez l'humain mondialement.

1.7.1.1.1 Compsition générale

La CPS du sérotype 2 est composée de galactose (Gal), de glucose (Glc), de *N*-acétylglucosamine (GlcNAc), de rhamnose (Rha) et d'acide sialique (Neu5Ac), tandis que celle du sérotype 1/2 possède un *N*-acétylgalactosamine (GalNAc) à la place du galactose dans la chaîne latérale portant l'acide sialique (**Figure 4**) [49, 130]. Il a été suggéré que la chaîne latérale et l'acide sialique constituent les principaux épitopes reconnus par les anticorps dirigés contre la CPS des sérotypes 1/2 et 2 [93]. Quant à elle, la CPS du sérotype 14 est semblable à celle du sérotype 2, mais ne contient pas de rhamnose [129]. Comme pour les sérotypes 1/2 et 2, la seule différence entre les CPSs des sérotypes 1 et 14 est la présence du *N*-acétylgalactosamine chez le sérotype 1 à la place du galactose chez le sérotype 14 (**Figure 4**) [49]. Chez la CPS du sérotype 1, l'épitope principale semble être également la chaîne latérale, tandis qu'elle ne semble pas être un épitope majeur chez le sérotype 14 [49]. De plus, l'acide sialique joue un rôle très limité chez les sérotypes 1 et 14 dans la reconnaissance par les anticorps [49, 93]. Finalement, la CPS du sérotype 9 est composée de glucitol (Glc-ol), de phosphate (P), de galactose, de rhamnose et d'un sucre 4-céto labile (2-acetamido-2,6-dideoxy-b-D-xylo-hexopyranos-4-ulose) (**Figure 4**) [131]. Toutefois, la CPS du sérotype 9 ne contient pas d'acide sialique [131].

Sérotype 1

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc
1
↓
3
[6)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

Sérotype 14

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc
1
↓
3
[6)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

Sérotype 2

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc α-D-Gal*p*
1 1
↓ ↓
3 3
[4)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

Sérotype 1/2

α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc α-D-Gal*p*
1 1
↓ ↓
3 3
[4)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→]$_n$

Sérotype 9

α-D-Gal*p*
1
↓
2
[3)-α-L-Rha*p*-(1→3)-Glc-ol-(6→*P*→3)-β-D-Gal*p*-(1→3)-β-D-6d*xyl*Hex*p*NAc-4-ulo-(1→]$_n$

Figure 4. Structure de la capsule polysaccharidique des sérotypes 1, 14, 2, 1/2 et 9 de *Streptococcus suis*. L'acide sialique (α2,6) est représenté en rouge. La seule différence entre les structures des sérotypes 1 et 14 et entre les sérotypes 1/2 et 2 est la présence de *N*-acétylgalactosamine (en vert) chez les sérotypes 1 et 1/2 à la place de galactose chez les sérotypes 14 et 2 (en bleu) [49, 129-131].

1.7.1.1.2 L'acide sialique

Le terme acide sialique réfère à une famille d'acide de sucres composés de neuf carbones, se retrouvant en position terminale de glycoconjugués exposés à la surface de plusieurs cellules eucaryotes, dont le plus abondant est l'acide *N*-acétylneuraminique [133]. Chez les eucaryotes, la présence de ce sucre en position terminale des glycoconjugués interfère dans l'activation du complément et de plusieurs fonctions immunitaires [133]. Ainsi, les pathogènes bactériens qui ont développé des mécanismes pour incorporer l'acide sialique à leurs composants de surface possèdent un avantage particulier dans la résistance au système immunitaire de l'hôte, mais

également dans l'interaction avec différents récepteurs cellulaires [133]. Ainsi, la sialylation de la CPS du streptocoque du groupe B (GBS) protège contre la phagocytose en inhibant l'opsonisation de la bactérie et augmente la résistance à l'activation du complément par la voie alternative [134]. De plus, sa présence inhibe les interactions avec la Siglec-9 et l'activation des neutrophiles humains [135]. Ainsi, la sialylation de la CPS semble jouer un rôle important dans la virulence de GBS.

Malheureusement, aucun mutant exprimant une CPS sans acide sialique n'est disponible chez *S. suis*, ce qui complique les études sur le rôle exact de l'acide sialique dans les interactions avec les cellules de l'hôte et dans la pathogenèse de l'infection. En effet, la délétion d'un des gènes impliqués dans la synthèse de l'acide sialique cause une perte de la CPS chez *S. suis* [93]. Ainsi, le rôle de l'acide sialique dans la virulence de *S. suis* reste ambigu. Toutefois, il ne semble pas être un déterminant crucial de la virulence de *S. suis* sérotype 2, tel que démontré à l'aide de souches traitées de manière enzymatique avec la sialidase (permettant de cliver l'acide sialique) [136].

1.7.1.2. Le locus capsulaire

Selon les connaissances actuelles, les gènes présents dans le locus codant pour la CPS sont transcrits sous un même acide ribonucléique (ARN) polycistronique et régulé sous un seul et même promoteur situé en amont du gène *cpsA* [54]. La taille des loci de la CPS varie entre 15 274 et 40 198 paires de bases en fonction du sérotype [54]. Les gènes codant pour la synthèse, l'exportation et la polymérisation de la CPS se retrouvent généralement entre les gènes *orfZ* et *aroA*, tandis que les gènes de régulation de la synthèse de la CPS sont les gènes *cpsA*, *cpsB*, *cpsC* et *cpsD*. [54] De plus, les gènes codant pour des polymérases, flippases et différentes glycosyltransférases sont également retrouvés dans l'ensemble des sérotypes [54]. Finalement, une caractéristique importante de certains sérotypes est la présence de gènes associés à la synthèse et au transfert (sialyltransférase) de l'acide sialique, soit les gènes *neuA* à *neuD* (synthèse) et *cpsN* (sialyltransférase), présents chez les sérotypes 1, 1/2, 2, 6, 13, 14, 16 et 27 [54].

À l'exception des sérotypes 1, 1/2, 2 et 14, les sérotypes de *S. suis* possèdent un gène codant pour une polymérase sérotype-spécifique [54]. Pour les sérotypes 1, 1/2, 2 et 14, aucune polymérase, glycosyltransférase ou flippase sérotype-spécifique n'a été trouvée [54]. Tel que

décrit ci-dessus, les chaînes latérales semblent être des épitopes majeurs chez ces sérotypes, à l'exception du sérotype 14, et seraient responsables des réactions croisées observées [49]. En fait, les différences d'antigénicités observées entre les sérotypes 1 et 14 et 1/2 et 2 découlent d'un seul polymorphisme nucléotidique dans le gène *cpsK* codant pour la glycosyltransférase (**Annexes – Article V**) [137].

1.7.1.3. Mutants de la capsule polysaccharidique

Les premiers mutants de la CPS chez *S. suis* ont été obtenus par l'insertion de transposons [138]. Une seule insertion était suffisante afin d'inhiber la synthèse de la CPS, ce qui a eu pour conséquence sa perte et un phénotype non-encapsulé [138]. De plus, ces travaux ont démontré que l'absence de CPS modifie l'hydrophobicité à la surface bactérienne, diminue la résistance à la phagocytose par les phagocytes murins et porcins et favorise l'élimination rapide de la bactérie du sang [138].

Depuis, plusieurs mutants de la CPS ont été obtenus, mais tous chez le sérotype 2. En effet, les rôles de la protéine régulatrice CpsB [92], de la transférase initiale CpsE [92], différentes glycosyltransférases (CpsF, CpsG, CpsJ et CpsL) [139, 140] ainsi que de NeuB et NeuC, enzymes impliquées dans la synthèse de l'acide sialique [93, 141], ont été décrits, et ce avec des souches européennes de ST1 ou les souches ST7 responsables des éclosions humaines en Chine. Peu importe la délétion, un phénotype non encapsulé a été obtenu. Plus récemment, un mutant de la CPS a été obtenu chez une souche de sérotype 14 (CpsB) (**Annexes – Article VI**) [142]. Toutefois, malgré l'importance des autres sérotypes de *S. suis*, aucun mutant d'un sérotype autre que les sérotypes 2 et 14 n'a été étudié.

1.7.1.4. Propriétés et fonctions

Le rôle de la CPS dans la virulence de *S. suis* sérotype 2 a été extensivement étudié dans les dernières années et plusieurs fonctions ont été attribuées à sa présence lors de l'infection, dont la modulation de l'adhésion et de l'invasion, un rôle anti-phagocytaire et un rôle immunomodulateur [10]. Toutefois, ces études ont toutes été effectuées avec des souches européennes de ST1 ou de ST7 (Chine) [92, 93, 128, 138], bien que les souches nord-américaines (ST25 et ST28) diffèrent grandement génétiquement [7]. Une étude récente avec une souche de sérotype 14 a démontré que la présence de sa CPS est importante pour sa pathogenèse (**Annexes – Article VI**) [142]. Comme mentionné aux sections précédentes, les

CPS des sérotypes 2 et 14 sont relativement similaires en termes de structure et de composition et contiennent toutes deux de l'acide sialique [54, 129, 130]. Ainsi, très peu d'information est disponible sur le rôle de la CPS chez d'autres sérotypes plus divergents et dont la CPS ne contient pas d'acide sialique.

1.7.1.4.1. Rôle dans la modulation de l'adhésion et de l'invasion

La CPS de *S. suis* sérotype 2 a été décrite comme pouvant moduler l'adhésion et l'invasion aux cellules de l'hôte en interférant avec l'action des adhésines [10]. En effet, l'absence de la CPS favorise l'adhésion aux cellules épithéliales et endothéliales et favorise également l'invasion des cellules épithéliales du larynx humain et des BMECs porcines [112, 141, 143, 144]. Ainsi, étant donné que l'adhésion aux cellules figure parmi les premières interactions hôte-pathogène et qu'elle est cruciale pour la colonisation, l'expression de la CPS pourrait nuire à celle-ci [9]. Comme mentionné précédemment, *S. suis* pourrait moduler à la baisse l'expression de sa CPS afin de favoriser le contact avec ses adhésines à la surface cellulaire lors de la colonisation [9].

1.7.1.4.2. Propriétés anti-phagocytaires

La CPS est en premier lieu un facteur anti-phagocytaire qui permet à *S. suis* sérotypes 2 et 14 d'éviter les premières lignes de défense immunitaire de l'hôte, c'est-à-dire les cellules phagocytaires de la réponse immunitaire innée, dont les macrophages, les cellules dendritiques et les neutrophiles [10, 142]. En effet, la CPS du sérotype 2 inhibe spécifiquement la transduction des signaux intracellulaires nécessaires à la phagocytose chez les macrophages [98]. De plus, lorsque *S. suis* est en contact avec les macrophages, sa CPS déstabilise les microdomaines lipidiques afin d'empêcher sa reconnaissance [145]. La présence de CPS interfère également dans la phagocytose et le « killing » par les cellules dendritiques murines et porcines et par les neutrophiles porcins [90, 91, 139, 146]. Cette propriété que confère la présence de la CPS, du moins aux sérotypes 2 et 14, est cruciale pour leur virulence. En son absence, *S. suis* est incapable de résister au « killing » par les leucocytes sanguins et est rapidement éliminé de la circulation sanguine [92, 93]. De plus, la présence de la CPS du sérotype 2 interfère dans le dépôt du complément à la surface bactérienne, empêchant l'opsonophagocytose [90, 91, 139].

Bien que les études sur le rôle de la CPS se soient concentrées sur le sérotype 2, et moindrement sur le sérotype 14, une étude *in vitro* a démontré que le sérotype 1 est moins internalisé par les cellules dendritiques humaines dérivées de monocytes que les sérotypes 2, 7 et 14, tandis que les sérotypes 4 et 9 sont plus internalisés que ceux-ci [147]. Ainsi, il est possible que la composition de la CPS puisse influencer les propriétés anti-phagocytaires de celle-ci. Cependant, d'autres facteurs tels que l'épaisseur de la CPS et le bagage génétique pourraient également influencer les propriétés anti-phagocytaires des souches à l'étude [147].

1.7.1.4.3. Immunomodulation

La CPS participe également à la modulation de la réponse inflammatoire [10]. En effet, la présence de CPS permet de masquer la surface bactérienne, incluant les composés sous-capsulaires ayant des propriétés immunostimulatrices [10]. Des tests *in vitro* avec différents types cellulaires, dont les DCs et les macrophages, ont permis de démontrer qu'un mutant non-encapsulé de *S. suis* sérotype 2 induit davantage l'expression de cytokines pro-inflammatoires, telles que le TNF, IL-6, l'IL-12p70 et le CXCL1 [99, 139, 148]. De plus, la CPS des sérotypes 2 et 14 induit elle-même la production des chimiokines CCL2 et CCL3 des DCs murines, des macrophages et des BMECs humaines [37, 148, 149]. Par exemple, CCL2 favorise l'infiltration de monocytes et déstabilise l'intégrité de la BBB, ce qui pourrait permettre à *S. suis* d'infiltrer le SNC [10, 148].

1.7.2. La paroi cellulaire bactérienne et ses modifications

Chez les bactéries à Gram positif, la paroi cellulaire est composée d'une épaisse couche de peptidoglycane qui contient des LTAs, ancrés aux têtes des membranes lipidiques [150]. Ensemble, ces polymères comptent pour 60% de la masse de la paroi des bactéries à Gram positif [150]. De plus, une multitude de protéines sont présentes à la surface bactérienne, attachées soit à des ancres lipidiques insérées dans la membrane, directement au peptidoglycane ou même aux LTAs [150].

1.7.2.1. Les acides lipotéichoïques

Les LTAs sont des molécules amphiphiles retrouvées dans la membrane de la plupart des bactéries à Gram positif [151]. Historiquement, ils ont été associés au développement du sepsis et du choc septique causé par les bactéries à Gram positif, puisqu'ils activent les leucocytes et stimulent la production exacerbée de médiateurs pro-inflammatoires, suite à leur

reconnaissance par le TLR2 [152, 153]. Toutefois, de plus en plus d'études suggèrent que ce sont les lipoprotéines de la surface bactérienne et non les LTAs qui seraient reconnues et responsables de cette activation inflammatoire [154, 155]. Lorsque cette thèse a débuté, il n'y avait aucune information disponible concernant la structure des LTAs de *S. suis* ou leurs propriétés immunostimulatrices.

1.7.2.1.1. Composition et structure

Les LTAs sont des polymères de polyphosphoglycérol, la substitution d'une D-alanine ou d'un résidu glycosyl les rendant fonctionnels. Ils sont ancrés dans la membrane bactérienne par un motif diacylglycérol terminal [152, 156]. Basés sur l'architecture des unités répétitives, les LTAs sont classés en cinq types (**Figure 6**) [11]. Le type I contient du polyglycérol phosphate, les types II et III un complexe glycosyl-glycérol-phosphate, le type IV du glycosyl-ribitol-phosphate et le type V du glycosyl-phosphate [11]. Ainsi, les LTAs de type II à V sont considérés comme étant plus complexes [11]. Les LTAs de type I sont les plus communs et sont retrouvés entre autres chez *S. aureus*, chez *Bacillus subtilis* et chez *Listeria monocytogenes*, tandis que *Streptococcus pneumoniae* contient du LTA de type IV [11]. Il est important de mentionner que la plupart des bactéries ne contiennent normalement qu'un seul type de LTA.

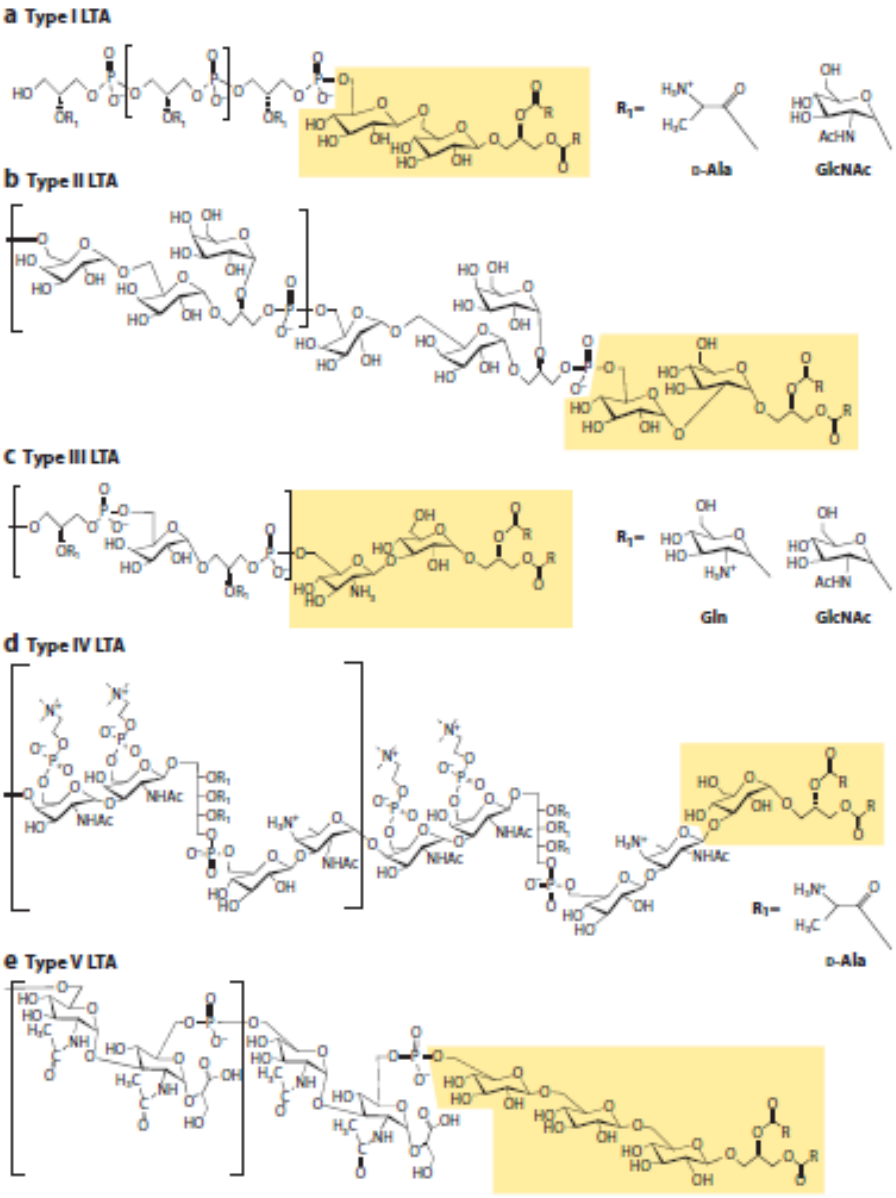

Figure 5. Schématisation des différentes classes d'acides lipotéichoïques des bactéries à Gram positif. Les ancres glycolipidiques sont indiquées en orange. Tiré de [11].

1.7.2.1.2. La D-alanylation des acides lipotéichoïques

La D-alanylation du LTA, codé par le gène *dltA,* permet aux bactéries à Gram positif de moduler leur charge de surface, de réguler la liaison de ligands et de contrôler les propriétés électromécaniques de la paroi cellulaire [157]. La D-alanylation du LTA de *S. suis* est importante pour la virulence de la bactérie, car elle confère une résistance à l'action des peptides antimicrobiens cationiques, une résistance à l'effet bactéricide des neutrophiles

porcins et des cellules dendritiques murines et interfère dans le dépôt du complément à la surface bactérienne [139, 158]. De plus, la D-alanylation du LTA augmente l'adhésion aux, et l'invasion des BMECs porcines [158, 159]. L'ensemble de ces rôles contribue partiellement à la virulence de *S. suis* étant donné qu'un mutant isogénique de la D-alanylation du LTA est moins virulent lors de l'infection systémique chez la souris et le porc [158].

1.7.2.2. La *N*-désacétylation du peptidoglycane

Comme mentionné plus haut, la composante principale de la paroi cellulaire des bactéries à Gram positif est le peptidoglycane, qui procure une résistance au stress osmotique, entre autres [152, 156]. Ce polymère est composé d'une longue chaîne alternante de deux dérivés de sucres, le *N*-acétylglucosamine et l'acide *N*-acétylmuramique, hautement réticulée par des sous-unités peptidiques y formant des ponts [152, 160]. Parmi les différentes espèces bactériennes, la structure des chaînes de sucres est hautement conservée tandis que la composition des sous-unités peptidiques varie [156]. Il a été suggéré que le peptidoglycane agit en synergie avec le LTA pour déclencher la réponse inflammatoire de l'hôte et causer le sepsis [153]. Par contre, l'hôte tire avantage de cette structure plutôt invariable, et reconnaît les bactéries à l'aide des protéines de domaines de liaison des « nucleotide-binding oligomerization domain » (NOD) 1 et 2, qui reconnaissent les muropeptides relâchés, lors de la reconnaissance de la paroi cellulaire [161]. Afin, certaines bactéries ont développé des mécanismes spécifiques permettant de modifier la structure de leur peptidoglycane, dont la *N*-désacétylation, ce qui déjoue les défenses de l'hôte [156].

Il a été démontré que *S. suis* sérotype 2 est capable de *N*-désacétylation du peptidoglycane grâce au gène *pdgA* [162]. *In vitro*, l'expression du gène *pdgA* est augmentée suite à l'interaction de *S. suis* avec les neutrophiles, ce qui est aussi le cas *in vivo* lors d'infections à l'aide d'un modèle murin [162]. De plus, la virulence d'un mutant isogénique pour *pgdA* est hautement atténuée lors d'infections expérimentales, tant chez la souris, que chez le porc [162]. Finalement, la *N*-désacétylation du peptidoglycane augmente la persistance de *S. suis* dans le sang, en permettant de mieux résister à l'effet bactéricide des neutrophiles porcins et à l'effet bactéricide complément-dépendante des cellules dendritiques murines [139, 162].

1.7.2.3. Les lipoprotéines

Présentes au niveau de l'enveloppe cellulaire, les lipoprotéines sont une classe importante de protéines membranaires amphiphiles constituées à la fois d'une portion protéique hydrophile et d'une portion lipidique hydrophobe [163, 164]. Cette dernière interagit avec les phospholipides de la paroi bactérienne et assure l'ancrage des lipoprotéines au niveau de la membrane [165]. Elles constituent une famille relativement abondante de protéines représentant environ 2% ou plus du protéome des bactéries à Gram positif et sont impliquées dans un éventail de fonctions importantes pour la bactérie, que ce soit pour sa survie, sa reproduction ou même sa virulence [163, 164]. Au moins 45 gènes codant pour des lipoprotéines putatives ont été identifiés dans le génome de la souche de référence de *S. suis* sérotype 2 P1/7.

1.7.2.3.1. Biosynthèse et maturation

Les lipoprotéines partagent une similarité structurelle résultant d'un processus de maturation unique et conservé [150]. En effet, elles sont initialement traduites dans le cytoplasme sous forme de pré-prolipoprotéines immatures. La majorité de celles-ci vont ensuite être exportées sous forme de polypeptides linéaires [164, 166]. Une fois exportées, les pré-prolipoprotéines vont être redirigées vers la machinerie de biosynthèse des lipoprotéines à l'aide d'un second signal [163]. Il s'agit d'une séquence fortement conservée de quatre acides aminés appelée lipobox [163]. Parmi ces acides aminés, seule la cystéine est invariable et obligatoirement présente, car elle constitue le site à partir duquel toutes les futures modifications structurales auront lieu [163].

Chez la plupart des bactéries à Gram positif, dont *S. suis*, la maturation des lipoprotéines se fait en deux étapes. Dans un premier temps, les pré-prolipoprotéines subissent une modification par la pré-prolipoprotéine diacylglycéryle transférase (Lgt) intégrée dans la membrane plasmique [167, 168]. Cet enzyme catalyse l'attachement covalent d'un fragment diacylglycérol au niveau de la liaison sulfhydrile de la cystéine présente à l'intérieur du lipobox. Une fois cette étape de diacylation terminée, les pré-prolipoprotéines deviennent des prolipoprotéines ancrées à la membrane cytoplasmique. Cette modification lipidique est importante pour le maintien des lipoprotéines au niveau de la membrane plasmique [165]. À la suite de l'ajout du fragment diacyle par la Lgt, la prolipoprotéine signal peptidase de type II est

responsable du clivage du signal peptidique au niveau de la cystéine conservée. Celle-ci devient alors la nouvelle extrémité N-terminale de la lipoprotéine [166, 167].

1.7.2.3.2. Rôles et fonctions

Comme mentionné, les lipoprotéines sont impliquées dans une panoplie de fonctions chez la bactérie. Parmi celles-ci, il y a la réplication et la survie bactérienne via le maintien de la stabilité de l'enveloppe bactérienne [164], le transport de substrats et de nutriments (en fait, le groupe fonctionnel le plus abondant des lipoprotéines est celui des protéines liant de substrats à haute affinité) [164], la résistance aux antibiotiques [169] et la sécrétion et le repliement des protéines [170, 171]. Toutefois, le rôle d'environ 30% des lipoprotéines reste inconnu à ce jour [150].

De plus, les lipoprotéines sont connues pour jouer un rôle important dans la virulence des bactéries en participant à l'adhésion aux et à l'invasion des cellules de l'hôte, à la persistance et à la dissémination bactérienne et à l'initiation des processus inflammatoires [166]. En effet, étant très conservées, les lipoprotéines sont reconnues par des récepteurs de l'immunité innée, dont le TLR2 en association avec soit le TLR1 ou le TLR6 pour les lipoprotéines triacylées et diacylées, respectivement. Ainsi, des souches déficientes pour la Lgt de plusieurs pathogènes tels que *S. aureus*, GBS et *S. pneumoniae,* présentent une diminution de l'induction de la production de cytokines [163, 172-175]. En accord, l'absence de la Lgt de *S. suis* sérotype 2 réduit l'expression des médiateurs pro-inflammatoires IL-1β et CXCL8 produits par les cellules mononuclées porcines. Finalement, il a été démontré que l'activation de l'hétérodimère TLR2/6 par les lipoprotéines varie d'un sérotype à l'autre, avec une activation plus forte par le sérotype 9, que par le sérotype 2 [105]. Toutefois, à l'exception de ces deux études, le rôle des lipoprotéines de *S. suis* et leurs propriétés immunostimulatrices sont peu connus.

1.7.3. Autres protéines de surface ancrées à la paroi bactérienne

1.7.3.1. L'autolysine

L'autolysine est une protéine permettant la dégradation du peptidoglycane bactérien et la lyse cellulaire lors de conditions non-favorables à la survie [176]. De plus, les autolysines sont impliquées dans diverses fonctions biologiques, dont la division et la séparation cellulaire et le renouvellement de la paroi cellulaire [176]. Chez *S. suis*, la présence de l'autolysine confère des propriétés bactériolytiques et des activités de liaison à la fibronectine [177]. De plus, elle est impliquée dans la formation de biofilm par *S. suis*, dans l'adhésion aux cellules épithéliales

humaines HEp-2 et dans la virulence, telle qu'évaluée à l'aide d'un modèle d'infection chez le poisson zèbre [177].

1.7.3.2. La dipeptidyl peptidase IV

La dipeptidyl peptidase IV (DPPIV) est une protéase de 70 kDa qui peut être retrouvée sécrétée et/ou associée à la surface cellulaire de *S. suis* [178]. Son activité spécifique permet le clivage des protéines après les résidus X-proline et X-alanine du domaine N-terminal des polypeptides [178]. Ceci est très important, étant donné que plusieurs cytokines ont un résidu proline en deuxième position de leur chaîne polypeptidique [178]. Ainsi, la DDPIV de *S. suis* peut moduler la réponse de l'hôte lors de l'infection [178]. De plus, l'élimination des dipeptides du domaine N-terminal peut générer des peptides biologiquement actifs [178]. Il a donc été suggéré que la DPPIV pourrait contribuer à la dérégulation de la réponse inflammatoire lors de la méningite, à la destruction tissulaire et à la dissémination systémique de la bactérie [178]. Finalement, la DPPIV peut interagir avec la fibronectine humaine et jouerait un rôle important dans la virulence (grandement atténuée chez *S. suis* sérotype 2 lors de l'inactivation de la DPPIV) [179].

1.7.3.3. La protéine Zmp

Initialement décrite comme une protéase clivant les IgAs chez une souche de *S. suis* ST7 (responsable de l'éclosion humaine de 2005 en Chine) [180], la protéine Zmp est une zinc métalloprotéase apparentée à la protéine ZmpC de *S. pneumoniae* [181]. Bien que ZmpC possède plusieurs activités, dont l'activation de la matrice métalloprotéase-9, la libération des ectodomaines de la glycoprotéine de liaison à la P-sélectine, de la mucine 16 et du syndécane-1 [181-185], il a été démontré que Zmp clive partiellement les ectodomaines de ces deux dernières cibles seulement. Toutefois, aucune activité IgA protéase n'a pu être démontrée. De plus, Zmp n'est pas un facteur critique pour la colonisation des voies respiratoires ou pour la virulence de *S. suis* sérotype 2 dans des modèles murins et porcins d'infection (**Annexes – Article VII**). Ainsi, cette protéine est un exemple de la controverse entourant les facteurs de virulence de ce pathogène, quelle est la définition d'un facteur de virulence critique et la répétabilité des résultats obtenus entre laboratoires.

1.7.4. Les marqueurs de virulence classiques

1.7.4.1. La suilysine

S. suis sécrète de très nombreux composés, dont une toxine hémolytique appelée suilysine. Avec une taille de 54 kDa, elle appartient au groupe de toxines cytolytiques liant le cholestérol de la membrane cellulaire eucaryote [186]. Cette toxine n'est cependant pas retrouvée chez tous les sérotypes de *S. suis*. La suilysine est structurellement et fonctionnellement similaire à la pneumolysine de *S. pneumoniae*, mais cette dernière n'est pas sécrétée contrairement à la toxine de *S. suis*. La suilysine se rapproche également de la streptolysine O du streptocoque du groupe A (GAS), la listeriolysine de *L. monocytogenes* et la perfringolysine *de Clostridium perfringens* [187]. Ces toxines, de même que la suilysine, sont caractérisées par une perte d'activité et un regain de celle-ci, suite à l'oxydation et à la réduction, respectivement. De plus, elles sont inhibées par de faibles quantités de cholestérol et sont responsables de la formation de pores transmembranaires [188, 189].

Le rôle de la suilysine dans la pathogenèse de l'infection causée par *S. suis* a été investigué, mais reste tout de même nébuleux [190]. La suilysine est toxique pour les cellules endothéliales et épithéliales de même que pour les neutrophiles, les monocytes et les macrophages [90, 97, 144, 191, 192]. De plus, la sulysine induit la production de médiateurs pro-inflammatoires par les BMECs humaines et porcines, les cellules mononuclées du sang porcin, les macrophages alvéolaires porcins et les cellules dendritiques murines [112, 117, 139, 149, 193, 194]. Elle induit également une régulation à la hausse des molécules adhésives des monocytes humains et est impliquée dans la sécrétion de l'acide arachidonique, précurseur des prostaglandines, par les cellules endothéliales humaines [114, 115]. Finalement, la suilysine contribue à la résistance contre l'opsonophagocytose par les cellules dendritiques murines [139].

In vivo, le rôle de cette toxine reste controversé. En effet, aucune mortalité n'a été observée chez des souris infectées avec le surnageant d'une culture d'une souche sécrétant de la suilysine [195]. Toutefois, un mutant déficient pour la suilysine est avirulent dans un modèle murin, mais sa virulence n'est que légèrement réduite lors de l'infection systémique chez le porc [195]. Enfin, lors d'une étude de challenge chez le porc, la souche mutante a induit une maladie similaire à celle de la souche-mère [194]. Ainsi, il est difficile de considérer cette toxine en tant que facteur de virulence critique en raison des différences entre hôtes.

1.7.4.2. Le facteur extracellulaire et la « muraminidase-released protein »

Ces deux protéines ont été parmi les premiers facteurs de virulence putatifs décrits pour *S. suis* sérotype 2 [196]. La MRP, est une protéine de 136 kDa ancrée dans la paroi cellulaire par un motif LPXTG, mais qui peut être relâchée dans le surnageant lors de la croissance bactérienne [32, 196]. Au contraire, le EF, codé par le gène *epf*, est une protéine de 110 kDa présente seulement dans le surnageant des cultures bactériennes [32, 196]. Les souches de *S. suis* sérotype 2 de phénotype MRP+EF+ sont associées à des infections plus sévères chez le porc que les souches MRP-EF- [197, 198]. Toutefois, les souches de *S. suis* sérotype 2 nord-américaines de phénotype MRP-EF- sont généralement plus virulentes dans des modèles d'infection expérimentale chez le porc et chez la souris que les souches MRP+EF- [7, 199]. De plus, lorsque des mutants isogéniques ont été inoculés chez des porcelets, leur virulence était égale à celle des souches-mères [200]. Une étude récente a démontré que parmi les souches nord-américaines de *S. suis* sérotype 2, les phénotypes les plus communs sont MRP-EF-SLY- à 44% et MRP+EF-SLY- à 51%, correspondant aux ST25 et ST28, respectivement [7]. Cependant, étant donné que les rôles spécifiques de la MRP et EF dans la pathogenèse de *S. suis* n'ont pas été clarifiés (aucune différence de virulence en leur absence), ces protéines ne devraient être considérées que des marqueurs associés à la virulence [7].

1.7.5. Autres facteurs

En plus des facteurs décrits plus haut, plusieurs centaines d'autres facteurs de virulence confirmés ou putatifs ont été décrits pour *S. suis*, en particulier pour le sérotype 2 [8-10]. Le **Tableau I** ci-dessous répertorie plusieurs d'entre eux, mais n'est pas exhaustif.

Tableau 1. Liste de certains facteurs de virulence proposés ou confirmés impliqués dans la pathogenèse de l'infection causée par *S. suis*

Facteur	Fonction	Virulence du mutant	Référence
	Régulation		
1910KH/RR	Système de régulation à deux composantes	Atténuée (porc)	[201]
AdcR	Régulation de l'acquisition du zinc	Atténuée (souris)	[202]
ArcD	Arginine-ornithine anti-porteur (système arginine déiminase)	Mutant non testé	[203]
ArgR	Régulateur transcriptionnel d'arcBCD (système arginine déiminase)	Mutant non testé	[204]
Ccpa	Régulateur transcriptionnel du « carbon catabolite repressor »	Atténuée (souris)	[205, 206]
CiaRH	Système de régulation à deux composantes	Atténuée (souris)	[207]
CodY	Protéine régulatrice	Atténuée (souris)	[208]
CovR	Protéine régulatrice	Augmentée (porc)	[209]
Fur	Régulation de l'acquisition du fer	Atténuée (souris)	[202]
IhK/Irr	Système de régulation à deux composantes	Atténuée (souris)	[210]
NadR	Régulateur transcriptionnel	Atténuée (porc)	[211]
NisK/NisR	Système de régulation à deux composantes	Atténuée (souris)	[80]
RevS	Protéine régulatrice	Atténuée (souris)	[212]
RevSC21	Protéine régulatrice	Atténuée (porc)	[213]
Rgg	Régulateur transcriptionnel	Atténuée (porc)	[214]
Rss04	Petit ARN (régulateur transcriptionnel)	Atténuée (souris)	[79]
SalK/SalR	Système de régulation à deux composantes	Avirulent (porc)	[215]
Stk	Sérine/thréonine kinase	Atténuée (porc/souris)	[86]
Tran	Régulateur transcriptionnel	Atténuée (poisson zèbre)	[216]
TreR	Régulateur transcriptionnel	Atténuée (porc)	[211]
	Facteurs exposés à la surface et protéines sécrétées		
6-phosphogluconate-dehydrogenase	Adhésion aux cellules épithéliales	Mutant non disponible	[217]
Abpb	Arginine peptidase	Atténuée (souris)	[75]
ApuA	Pullulanase	Mutant non testé	[218]
Atl	Autolysine	Atténuée (poisson zèbre)	[177]
BgaC	Béta-galactosidase	Identique (souris)	[219]
Cbp40	Adhésion au collagène de type I	Atténuée (poisson zèbre)	[220]
Collagenase	Dégradation du collagène	Atténuée (porc)	[211]
Collagenase-like protéase	Adhésion/invasion de la BBB	Mutant non disponible	[221]
Endo-β-N-acétylglucosaminidase	Dégradation des oligosaccharides de l'hôte	Atténuée (porc)	[211]
Enolase	Adhésion à la fibronectine, au plasminogène et au collagène	Mutant non disponible	[222-225]
Fbps	Adhésion à la fibronectine	Atténuée (porc)	[226, 227]
Fhb	Protéine liant le facteur H humain	Atténuée (porc)	[228]

Fhbp	Protéine liant le facteur H humain	Identique (souris)	[229]
GAPDH	Adhésion aux cellules épithéliales	Mutant non disponible	[230, 231]
Hhly3	Hémolysine-III	Atténuée (poisson zèbre)	[232]
Htps	Histidine triade protéine	Mutant non disponible	[233]
IdeSsuis	IgM protéase	Identique (porc)	[234, 235]
IgA1	IgA protéase	Atténuée (porc)	[180]
Igde	IgG protéase	Mutant non disponible	[236, 237]
Mac	IgM (porcin) protéase	Identique (porc, souris, poisson zèbre)	[238]
Pilus (srtBCD Pilus)	Adhésion aux cellules épithéliales	Atténuée (poisson zèbre)	[78]
Pilus srtE	Adhésine potentielle	Mutant non disponible	[239]
Pilus srtF	Adhésine potentielle	Identique (souris)	[240]
Pilus srtG	Adhésine potentielle	Mutant non disponible	[239, 241]
Facteur d'opacification du sérum	Opacification du sérum	Atténuée (porc)	[242]
SrtA	Sortase	Identique (souris)/ Atténuée (porc)	[243, 244]
Ssa	Adhésion à la fibronectine	Atténuée (souris)	[245]
SsnA	DNase	Atténuée (souris)	[246]
SspA	Protéase	Atténuée (porc/souris)	[247-249]
SsPep	Protéine extracellulaire	Atténuée (porc)	[250]
SadP	Adhésion au galactosyl-α1–4-galactose	Mutant non disponible	[251]
Résistance			
Dpr	Résistance à la toxicité (fer)	Mutant non disponible	[252]
Nox	Tolérance stress oxydatif	Atténuée (porc/souris)	[253]
PerR	Protéine de la famille 'Fur'	Atténuée (souris)	[254]
SodA	Résistance à la toxicité (superoxyde dismutase)	Mutant non disponible	[255]
Spx1	Tolérance au stress	Atténuée (souris)	[253, 256]
Spx2	Tolérance au stress	Atténuée (souris)	[253, 256]
Système arginine déiminase	Résistance à l'acidité	Mutant non testé	[257, 258]
Tig	Tolérance au stress thermique, oxydatif et acide	Avirulent (souris)	[259]
Zur	Résistance à la toxicité (zinc)	Mutant non testé	[260]
Facteurs impliqués dans le métabolisme et transport			
3-kétoacyl-ACP réductase	Adhésion à la fibronectine	Mutant non disponible	[225]
Abpb	Adhésion à l'amylase	Atténue (souris)	[75]
Adénylosuccinate synthétase	Adhésion à la fibronectine	Mutant non disponible	[225]
Cdd	Cytidine déaminase	Atténuée (porc)	[211]
Chaperonine GroEL	Adhésion à la fibronectine	Mutant non disponible	[225]
Protéine de la famille DHH	Adhésion/invasion de la BBB	Mutant non disponible	[221]
DivIVA	Adhésion aux cellules épithéliales	Mutant non disponible	[261]
DNA-directed polymerase subunit RNA beta	Adhésion/invasion de la BBB	Mutant non disponible	[221]
DnaJ	Adhésion aux cellules épithéliales	Mutant non disponible	[262]
DnaK	Adhésion aux cellules épithéliales	Mutant non disponible	[261]

EF-Tu	Adhésion aux cellules épithéliales	Mutant non disponible	[263]
FeoB	Transporteur (fer)	Atténué (souris)	[264]
Fhs	Formate-tetrahydrofolate ligase	Atténuée (porc/souris)	[265]
Fructose biphosphate aldolase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[225]
GdpP	c-di-AMP phosphodiestérase	Atténué (souris)	[73]
GlnA	Glutamine synthétase	Atténué (souris)	[266]
Glutamate déhydrogénase	Adhésion à la fibronectine	Mutant non disponible	[225, 267]
GtfA	Sucrose phosphorylase	Atténuée (porc)	[211]
GuaA	GMP synthétase	Identique (porc)	[211]
HtpsC	Adhésion aux cellules épithéliales	Atténue (souris)	[76]
IMPDH	Inosine 5-monophosphate déshydrogénase	Atténue (souris)/ Avirulent (porc)	[268]
Lactate déshydrogénase	Adhésion aux cellules épithéliales	Mutant non disponible	[263]
LuxS	Quorum sensing	Atténué (poisson zèbre)	[269-271]
ManN	Transport spécifique du mannose	Atténuée (porc)	[211]
MsmK	ATPase du transporteur de type ABC	Atténuée (souris)	[272]
O-acétylhomosérine sulfhydrylase	Adhésion/invasion de la BBB	Mutant non disponible	[221]
Oligopeptide-binding protein OppA precursor	Adhésion à la fibronectine et au collagène	Mutant non disponible	[225]
Perméase	Transporteur de type ABC (acide aminé)	Atténuée (porc)	[211]
Phosphoglycérate mutase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[225]
Phospholipase C	Modulation de la production d'acide arachidonique	Mutant non disponible	[114]
Phosphopantothenoyl cystéine décarboxylase	Adhésion/invasion de la BBB	Mutant non disponible	[221]
Potentielle glycogène-phosphorylase	Adhésion/invasion de la BBB	Mutant non disponible	[221]
PurA	Adénylosuccinate synthétase	Atténuée (porc)	[211]
PurD	Phosphoribosylamine-glycine ligase	Atténuée (porc)	[211]
Pyruvate déshydrogénase composant E1, sous-unité alpha	Adhésion à la fibronectine	Mutant non disponible	[225]
Pyruvate kinase	Adhésion à la fibronectine et au collagène	Mutant non disponible	[225]
ScrB	Sucrose-6-phosphate hydrolase	Atténuée (porc)	[211]
ScrR	Répresseur de l'opéron du sucrose	Atténuée (porc)	[211]
Signal transduction histidine kinase	Adhésion/invasion de la BBB	Mutant non disponible	[221]
SsTGase	Glutamine-glutamyltransférase	Atténuée (porc)	[273]
Stp	Sérine/thréonine phosphatase	Atténuée (souris)	[5]
Trag	Protéine impliquée dans le transfert d'ADN	Atténué (poisson zèbre)	[274]
Translation elongation factor G	Adhésion à la fibronectine et au collagène	Mutant non disponible	[225]
TroA	Acquisition du manganèse	Avirulent (souris)	[275]

	Autres		
Hp0197	Inconnu/antigène de surface	Atténuée (souris)	[276-278]
Sao	Inconnu/antigène de surface	Identique (souris)	[279]
VirA	Inconnu	Atténuée (lapin)	[280]

1.7.6. L'antigène I/II – Nouveau facteur de virulence?

Les AgI/II sont des composantes immunostimulatrices et des protéines multimodales retrouvées à la surface bactérienne et impliquées dans diverses fonctions [12]. Ces protéines sont présentes chez plusieurs streptocoques pathogènes, dont *Streptococcus mutans*, *Streptococcus gordonii*, GAS et GBS [12]. Leur séquence primaire est composée de sept régions, soit (1) le peptide signal, (2) le domaine N-terminal, (3) une région riche en alanine, (4) une région variable, (5) une région riche en proline, (6) le domaine C-terminal et (7) le domaine d'ancrage à la paroi cellulaire (**Figure 6**) [12].

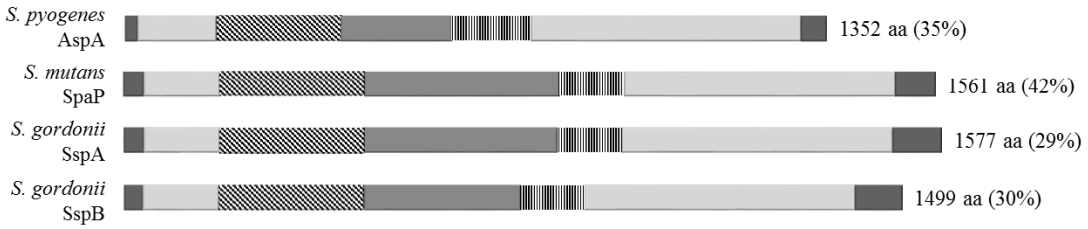

Figure 6. Caractéristiques structurales des antigènes I/II des différents streptocoques. De gauche à droite : le signal peptide, le domaine N-terminal, la région riche en alanine, la région variable, la région riche en proline, le domaine C-terminal et le domaine d'ancrage à la paroi cellulaire. Le taille en acides aminés (aa) est indiquée. Tiré de l'Article IV.

Plusieurs études ont démontré que les AgI/II participent à la persistance et à la dissémination des bactéries dans les voies respiratoires et dans la cavité orale de l'hôte [12]. Par leurs fonctions, ils permettent l'auto-agrégation bactérienne, l'agrégation aux composantes solubles et immobilisées aux surfaces, dont des composants de la MEC tels que le collagène, le fibrinogène et la fibronectine plasmatique, la formation de biofilm, la résistance au stress acide et l'adhésion aux cellules de l'hôte [12]. De plus l'AgI/II du GAS (AspA) est un facteur anti-phagocytaire protégeant la bactérie contre l'effet bactéricide des macrophages et des neutrophiles [281]. Une autre de leurs caractéristiques est leur affinité de liaison aux glycoprotéines salivaires, en particulier la glycoprotéine 340, qui est présente en grandes quantités dans la salive des mammifères sous forme immobilisée ou en solution. De plus, la

glycoprotéine 340 est présente sur toutes les surfaces mucosales, incluant les muqueuses nasales et intestinales [12]. Enfin, il a été rapporté que les AgI/II de *S. gordonii* (SspA et SspB) induisent la production de médiateurs pro-inflammatoires par les cellules épithéliales respiratoires et les DCs [282].

Bien que des gènes des sérotypes 2 et 9 de *S. suis* ayant une homologie à ceux codant pour l'AgI/II chez d'autres streptocoques, aient été trouvés, leur rôle dans la colonisation de l'hôte et dans la pathogenèse de l'infection, pas plus que leurs fonctions, n'avaient encore été étudiés lorsque cette thèse a débuté.

1.7.7. Facteurs de virulence – Sujet de controverse important

Dans les dernières années, le nombre de publications portant sur *S. suis* et s'intéressant à l'étude des facteurs de virulence a augmenté exponentiellement [8]. De plus, plusieurs de ces facteurs se sont avérés être critiques pour la virulence de la bactérie [8]. En effet, malgré les différences importantes entre les souches de *S. suis* (nombre de sérotypes et STs élevés, entre autres), au moins 37 gènes différents ont été rapportés comme facteurs de virulence critiques, en raison de l'avirulence du mutant de délétion [8]. Ainsi, il semblerait que *S. suis*, bien qu'un des plus importants pathogènes du porc, soit très sensible et peu adapté aux changements. Toutefois, la définition de ce qu'est un facteur de virulence reste ouverte à interprétation, puisqu'aucun barème n'a été établi. Entre autres, l'état clinique de l'hôte duquel la souche a été isolée, le modèle d'infection expérimentale *in vivo* utilisé et les études *in vitro* effectuées vont tous affecter cette définition [8]. De plus, le choix de la souche, en raison de son bagage génétique et de ses caractéristiques phénotypiques, pourrait aussi influencer le résultat [8]. Ainsi, la recherche portant sur *S. suis* fait face à une problématique importante depuis plusieurs années, qui malgré cela, n'avait jamais été adressée à l'aide d'une approche expérimentale lorsque cette thèse a débuté.

1.8. Modèles d'infection expérimentale

Le développement de modèles d'infection expérimentale a été nécessaire, afin d'étudier l'infection causée par *S. suis* et de mieux comprendre la pathogenèse de cette bactérie. Bien que nécessaire pour comprendre les mécanismes des interactions entre *S. suis* et son hôte, l'utilisation de lignées cellulaires et de cellules primaires isolées directement de l'hôte a plusieurs limites et ne permet pas d'avoir une vue d'ensemble de l'infection. D'autre part,

même si le porc est l'hôte naturel de *S. suis*, différents organismes, tel que le poisson zèbre, la souris et le cochon d'Inde ont été utilisés comme modèles d'infection expérimentale, afin de démontrer l'implication d'un facteur précis dans la virulence et d'étudier les étapes de la pathogenèse de *S. suis* [8, 283, 284]. Chacun de ces modèles présente ses avantages et désavantages (**Figure 7**), que ce soit au niveau du coût de la mise en oeuvre, de la reproductibilité des résultats ou encore de la reproductibilité des signes cliniques associés à la maladie et la transposition des résultats aux hôtes naturels (porc et humain) [8]. C'est pour cela que la majorité des études ont été réalisées chez le porc ou chez la souris.

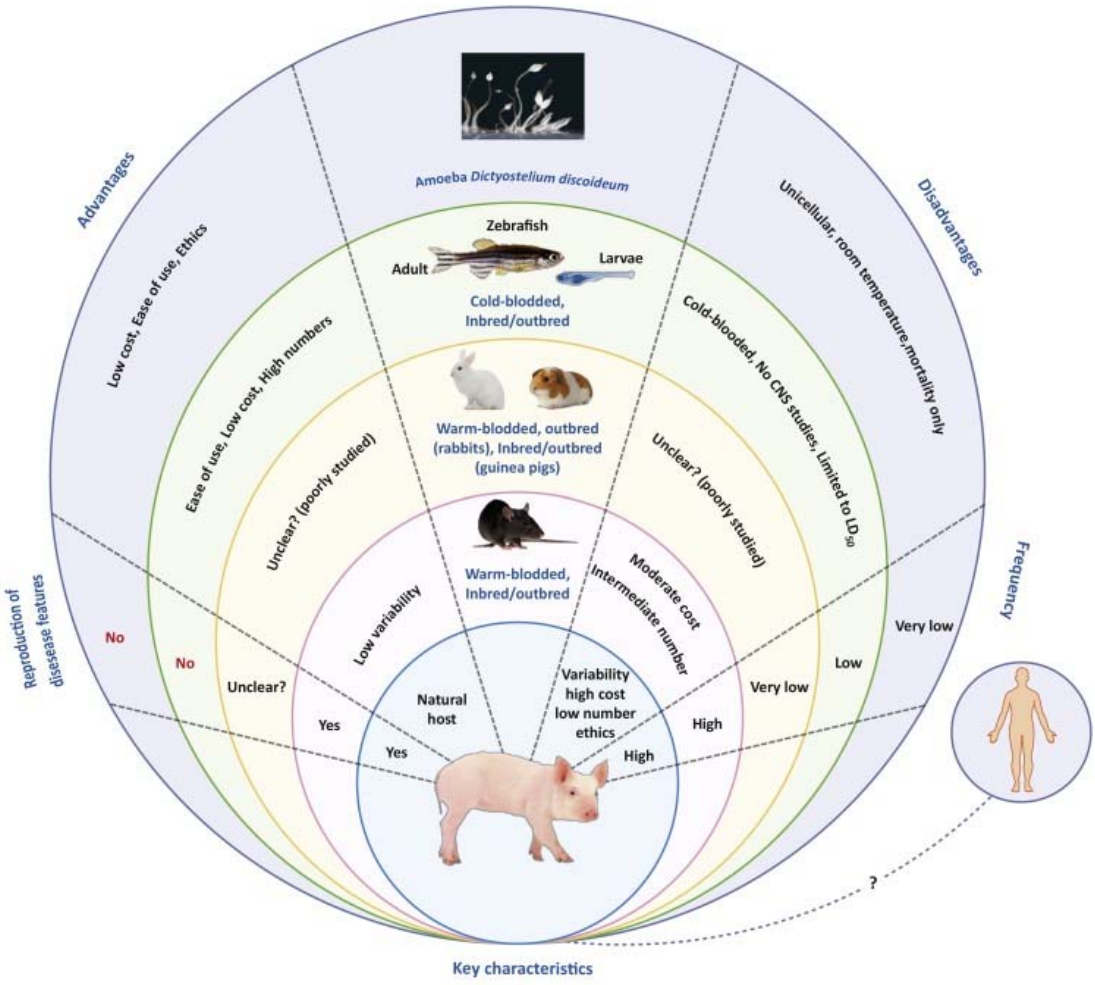

Figure 7. Résumé des avantages, des désavantages et des caractéristiques clés des modèles animaux décrits pour l'étude de *Streptococcus suis*. Tirée de [8].

1.8.1. Le porc

Bien que l'hôte naturel de *S. suis* soit le porc, le développement d'un modèle d'infection expérimentale chez le porc n'est pas nécessairement pratique, en raison de la taille des animaux, de leur variabilité génétique, du coût associé et que peu d'animaleries sont adaptées pour la réalisation d'études avec un grand nombre de porcs [285]. De plus, tel que mentionné précédemment, 100% des porcs sont naturellement colonisés par au moins un sérotype de *S. suis*, les rendant beaucoup moins susceptibles aux infections expérimentales, sans oublier que l'âge des animaux a un effet important sur cette susceptibilité [2]. Afin de surmonter ce problème, des porcs nés de césariennes et privés de colostrum ont été obtenus [8]. Puisque ces porcs sont exempts de *S. suis* et qu'ils n'ont pas d'anticorps maternels, ils sont beaucoup plus susceptibles à l'infection par *S. suis* [8]. Toutefois, leur rareté et leur coût les rendent inaccessibles pour une utilisation de routine. De plus, il est important de se rappeler que ces porcs constituent un modèle "artificiel", duquel il peut rester difficile d'extrapoler les résultats obtenus à ceux issus d'une même expérience chez des porcs conventionnels [8]. Bien que certains de ces désavantages, dont la variabilité génétique et la colonisation naturelle, peuvent être perçus comme des avantages, ils restent des contraintes et des variables pouvant compliquer la répétabilité des études expérimentales et des résultats en découlant.

D'autre part, la voie intranasale est la voie d'entrée "normale" de *S. suis* chez le porc. Cependant, l'inoculation par cette voie donne des résultats mitigés variant d'une expérience à l'autre au sein d'un même laboratoire et entre différents laboratoires [8]. Une irritation préalable des muqueuses respiratoires avec de l'acide acétique, ou une pré-infection avec *Bordetella bronchiseptica,* peuvent tout deux augmenter la reproductibilité des résultats et est également nécessaire afin d'observer des signes cliniques de la maladie [8]. Ainsi, l'inoculation par voie intrapéritonéale ou intraveineuse est fréquemment utilisée afin d'augmenter l'efficacité de l'infection, mais ces voies d'entrée contournent l'étape de colonisation des voies respiratoires supérieures et l'invasion de l'épithélium, pouvant ainsi biaiser les conclusions obtenues [8]. En effet, les interactions entre *S. suis* et l'hôte dans les voies respiratoires supérieures, même si mal comprises, pourraient influencer l'initiation et le développement des réponses immunitaires et inflammatoires appropriées et affecter la physiologie bactérienne, dont l'expression différentielle de facteurs et composants pouvant être impliqués dans sa virulence [9].

1.8.2. La souris

La souris, en raison de sa petite taille, de sa reproduction rapide et du nombre d'outils disponibles, est une alternative intéressante au porc [8]. De plus, les souris sont susceptibles à l'infection causée par *S. suis* [285]. Plusieurs études ont utilisé des lignées de souris « outbred » (exogames), dont les CD-1 sont les plus communes [8]. L'utilisation de ces souris offre une diversité phénotypique et génétique qui peut être importante, afin d'imiter les variations naturelles lors d'une réponse à une infection dans la population normale (porc ou humain) [8]. Par contre, de plus en plus d'études se sont tournées vers l'utilisation de souris « inbred » (endogames), dont les C57BL/6 et les BALB/c sont les plus populaires, étant donné que la variabilité est plus limitée entre les individus [8]. De plus, une multitude de souris endogames dites knockout, chez lesquels l'inactivation de gènes spécifiques permet l'identification de la participation de différents facteurs dans la réponse de l'hôte, ont été développées dans les dernières années [8]. Malheureusement, l'inoculation par voie intranasale chez la souris ne donne pas des résultats très convaincants, rendant les études de colonisation pratiquement impossible [8], diminuant par la même occasion le nombre d'études ayant utilisé cette voie d'infection. Ainsi, comme chez le porc, l'inoculation par voie intrapéritonéale ou intraveineuse est privilégiée [8].

Au cours des années, notre laboratoire a développé plusieurs modèles d'infection de *S. suis* chez la souris, mais tous avec le sérotype 2. Des modèles d'infections hématogènes chez les souris CD-1 (exogames) et C57BL/6 (endogames), dans lesquels *S. suis* est inoculé par voie intrapéritonéale, permettent d'étudier la réponse inflammatoire lors du sepsis et du choc septique et lors de l'infection du SNC [102, 286]. Lors de la phase systémique de la maladie, les signes cliniques observés sont le poil ébouriffé, les yeux gonflés, l'œdème oculaire, la prostration, la dépression et la léthargie en raison de sepsis et/ou de choc septique pendant les premières 48 h à 72 h de l'infection [102, 286]. Chez les souris ayant survécu au sepsis et/ou au choc septique, des signes cliniques de méningite peuvent par la suite être observés, normalement entre quatre à dix jours post-infection [286]. En fait, la méningite est souvent accompagnée d'une encéphalite (méningoencéphalite), caractérisée, entre autres, par des signes neurologiques, dont l'hyperexcitation, l'opisthotonos, la désorientation spatiale, une démarche circulaire avec la tête penchée, le pédalage, les mouvements cloniques et une excitation soudaine suivie de décubitus [286]. Ainsi, l'inoculation par voie intrapéritonéale

permet d'induire la méningite tout en causant un traumatisme physique minime sans anesthésie et permet à *S. suis* de causer une bactériémie avant d'atteindre le SNC [286].

Puisque ce ne sont pas toutes les souris infectées par voie intrapéritoneale qui développent de la méningite et que le temps requis au développement de la maladie varie grandement entres les animaux, un modèle d'infection par voie intracisternale a été développé, afin de mieux étudier l'infection du SNC [21, 287]. Ainsi, les bactéries sont inoculées directement dans la *cisterna magna* du cerveau (ouverture dans l'espace sous-arachnoïdien entre la membrane arachnoïde et la pie-mère des méninges), donc directement dans le LCR, en passant par l'ouverture entre l'os occipital et la vertèbre d'Atlas [287]. Cette méthode cause de la méningite/méningoencéphalite chez 100% des souris infectées en moins de 24 h lors de l'utilisation d'une dose standard (1 x 10^5 unités formatrices de colonies [UFC]) [287]. Bien qu'elle n'ait été utilisée que dans le cadre d'une seule étude lorsque cette thèse a débuté, cette voie d'inoculation a beaucoup de potentiel, afin de permettre de mieux comprendre l'infection causée par *S. suis* au SNC.

2. La réponse immunitaire innée et l'inflammation

Lorsque confronté à un agent infectieux, l'hôte dispose de deux principaux mécanismes de défense pour l'éliminer, soit la réponse immunitaire innée et la réponse immunitaire adaptative. Le système immunitaire inné représente la première ligne de défense contre les pathogènes, jouant un rôle critique dans la reconnaissance des microorganismes et le déclenchement de la réponse inflammatoire [13, 288, 289]. Ce système est constitué, à la fois, de barrières physiques et chimiques, dont l'épiderme, les muqueuses et les sécrétions antimicrobiennes, ainsi que de plusieurs types cellulaires tels que les monocytes, les macrophages, les DCs et les neutrophiles, qui reconnaissent les pathogènes et permettent la mise en place d'une réponse rapide et relativement non spécifique [290]. Cette réponse est induite par la reconnaissance de motifs moléculaires associés aux pathogènes (PAMPs). Les PAMPs sont des structures microbiennes conservées qui seront reconnues par des récepteurs de reconnaissance des motifs moléculaires (PRRs). Ces récepteurs sont exprimés à la surface cellulaire, dans les compartiments intracellulaires, ou sécrétés dans la circulation sanguine [289]. Ils contribuent à plusieurs fonctions, dont à l'opsonisation, à l'activation de la cascade du complément, à la phagocytose, ainsi qu'à l'activation des voies de signalisation pro-inflammatoires menant à la sécrétion de médiateurs [290].

2.1. Les phagocytes professionnels

2.1.1. Les monocytes

Appartenant au groupe des cellules mononuclées, les monocytes sont, après les neutrophiles, les cellules d'origine myéloïde les plus nombreuses en circulation sanguine [291]. Ils se développent à partir des cellules hématopoïétiques de la moelle osseuse et subissent plusieurs étapes de maturation avant de rejoindre la circulation périphérique [292]. La demi-vie d'un monocyte circulant a été estimée à 3 jours chez l'humain et à 1 jour chez la souris, mais des études récentes ont remis ceci en question [292, 293]. Historiquement, les monocytes ont été considérés comme des réservoirs de précurseurs myéloïdes qui permettent le réapprovisionnement constant des populations de macrophages et de DCs résidant au niveau des tissus lors de conditions homéostatiques [18, 292]. De plus, les monocytes migrent rapidement vers le site concerné à la suite d'une infection et se différencient soit en macrophages ou en DCs, afin de mettre en place la réponse immunitaire innée et adaptative [293]. Cependant, des études récentes ont mis en évidence que les rôles des monocytes

seraient beaucoup plus complexes que de servir uniquement de source pour les macrophages et les DCs tissulaires [291]. En effet, les monocytes sont des cellules matures et fonctionnelles, dès qu'elles entrent en circulation sanguine et peuvent y jouer des rôles [18]. Cette complexification est d'ailleurs liée, en partie, à l'hétérogénéité et la plasticité des monocytes.

Bien que les études antérieures aient supposé que les monocytes soient une population homogène, des études plus récentes ont démontré que ces cellules sont en fait composées de différentes sous-populations, selon les marqueurs exprimés à leur surface [18, 293]. En effet, deux sous-types principaux ont été répertoriés, tant chez l'humain que chez le porc et la souris [18]. Chez l'humain et chez le porc, ces populations se distinguent par l'expression du CD14 et du CD16 [18]. Les monocytes qualifiés de classiques ou inflammatoires possèdent un fort niveau d'expression de CD14, mais un faible niveau de CD16 ($CD14^{high}CD16^{low}$) [294]. Au contraire, les monocytes non classiques ou patrouilleurs, aussi appelés résidents, expriment moins fortement le CD14, mais beaucoup plus le CD16 ($CD14^{low}CD16^{high}$) [294]. Bien que les monocytes expriment les récepteurs CD11b et CD115 chez la souris, les monocytes inflammatoires se caractérisent par une forte expression des marqueurs Ly6C et CCR2, mais une faible expression de CX_3CR1 ($Ly6C^{high}CCR2^{high}CX_3CR1^{low}$), tandis que les monocytes patrouilleurs expriment faiblement le Ly6C et CCR2, mais fortement le CX_3CR1 ($Ly6C^{low}CCR2^{low}CX_3CR1^{high}$) [294].

En plus d'être différenciés par les niveaux de récepteurs exprimés, ces deux sous-populations de monocytes présentent des propriétés fonctionnelles différentes. En effet, comme leur nom l'indique, les monocytes inflammatoires participent directement à la réponse inflammatoire de deux différentes manières. D'une part, ils peuvent migrer vers le site d'infection et y sécréter à la fois des médiateurs pro-inflammatoires, dont le TNF et l'IL-6, et des dérivés réactifs à l'oxygène [18, 295]. Des études réalisées chez la souris ont démontré que les monocytes inflammatoires deviennent les phagocytes mononuclés dominants des tissus lors de l'inflammation, en particulier des macrophages inflammatoires et/ou des DCs productrices de TNF et d'oxyde nitrique [291, 293]. D'autre part, ils peuvent également acquérir la capacité de présenter des antigènes et de migrer vers les organes lymphoïdes afin d'initier la réponse adaptative [296]. En revanche, le rôle des monocytes patrouilleurs reste incertain. Des études récentes semblent indiquer que cette sous-population reste dans la circulation sanguine et est impliquée dans la surveillance des dommages tissulaires et leur réparation lors de

l'homéostasie [297], alors que d'autres soutiennent leur contribution dans la réponse anti-inflammatoire [298]. Bien que le rôle des monocytes inflammatoires ait été démontré lors d'infections bactériennes systémiques et du CNS, le rôle des monocytes patrouilleurs, lors de celles-ci, reste nébuleux [299-301].

Une caractéristique importante des monocytes inflammatoires chez la souris est qu'ils nécessitent la présence du récepteur CCR2 (récepteur liant la chimiokine pro-inflammatoire CCL2), afin de sortir de la moelle osseuse et d'entrer en circulation sanguine. Ainsi, en absence du CCR2, les monocytes inflammatoires sont trappés dans la moelle osseuse [302]. De plus, leur migration vers les tissus infectés ou endommagés dépend de la chimiokine CCL2 [303]. Quant à eux, les monocytes patrouilleurs nécessitent le facteur de transcription nucléaire Nr4a1 pour assurer leur différenciation et leur survie [304]. Cependant, il semble que ces deux sous-populations ne sont pas si différentes l'une de l'autre, en ce qui a trait à leur origine. En effet, les monocytes inflammatoires, dont la demi-vie est estimée à 2 jours, seraient en fait les précurseurs des monocytes patrouilleurs dotés d'une demi-vie plus longue de 11 jours [305]. Autrement dit, pour allonger leur durée de vie, les monocytes inflammatoires seraient capables de se différencier en monocytes patrouilleurs, une fois l'inflammation terminée, de telle sorte de disposer d'un nombre suffisant de monocytes patrouilleurs pour surveiller avec efficacité l'intégrité de l'organisme [293, 305]. Ainsi, les monocytes sont des cellules beaucoup plus complexes que ce qui a été suggéré dans le passé.

2.1.2. Les macrophages

Tout comme les monocytes, les macrophages contribuent à la fois à l'homéostasie et à l'évolution des maladies [306]. Il s'agit de larges phagocytes professionnels dotés d'une durée de vie de plusieurs mois, dont l'une des fonctions principales est d'éliminer les débris cellulaires et corps étrangers présents. Pour ce faire, ils sont distribués dans les tissus à travers le corps, afin de surveiller les invasions microbiennes ou l'accumulation de particules étrangères. Chaque tissu possède sa propre population spécifique de macrophages dérivée du développement embryonnaire. Bien que des études antérieures aient suggéré que ces populations sont renouvelées grâce aux monocytes, des études plus récentes indiquent qu'ils seraient capables de se régénérer, de sorte à maintenir leur population en conditions homéostatiques [306]. Par contre, lors d'infections ou suite à une blessure, les monocytes sortent de la circulation sanguine par diapédèse, afin de se rendre au niveau des tissus

infectés, dans lesquels ils se différencient en macrophages [307]. Ces derniers ingèrent alors les pathogènes et/ou déchets cellulaires qui se retrouvent piégés dans leur phagosome. De plus, ils sont capables de récupérer des peptides digérés, afin d'effectuer la présentation antigénique nécessaire au déclenchement de la réponse adaptative [288].

Les macrophages interviennent également dans la réponse inflammatoire en sécrétant des médiateurs pro-inflammatoires nécessaires non seulement au recrutement d'autres leucocytes au site d'infection, mais aussi à l'activation des cellules [293]. Une fois activés, ces macrophages adoptent des phénotypes contexte-dépendant qui vont soit promouvoir ou inhiber la défense immunitaire et les réponses inflammatoires de l'hôte [307]. C'est pour cette raison que les macrophages ne sont pas divisés en sous-populations stables et discrètes, mais plutôt en plusieurs phénotypes activés. En effet, il a été démontré qu'ils seraient en mesure de changer d'un phénotype fonctionnel à un autre en réponse à des signaux du microenvironnement [308]. On peut néanmoins différencier deux groupes principaux de macrophages désignés M1 et M2. Les macrophages activés de manière classique ou M1 (inflammatoires) sont activés par les PAMPs ou l'IFN-γ et ils ont des fonctions pro-inflammatoires, bactéricides et phagocytaires [307]. Au contraire, les macrophages M2 (anti-inflammatoires), aussi appelés macrophages activés alternativement, sont stimulés par l'IL-4 ou l'IL-13 et sont impliqués dans des processus constructifs tels que la réparation des tissus [307]. Ils permettent de maintenir les tissus en bonne santé en éliminant les cellules mortes et les matériaux toxiques [307]. De plus, ils seraient aussi impliqués dans l'atténuation de la réponse inflammatoire excessive en sécrétant la cytokine anti-inflammatoire IL-10, afin de protéger l'intégrité des tissus et d'assurer le retour homéostatique à la suite d'une infection ou d'une blessure [288, 307]. Ainsi, les macrophages sont parmi les principaux effecteurs cellulaires de l'immunité innée et sont impliqués à la fois dans les processus pro- et anti-inflammatoires. Ils assurent également l'homéostasie des tissus, supportent leur développement et réparent les dommages présents [288].

2.1.3. Les cellules dendritiques

Décrites pour la première fois au début des années 1970, les DCs sont caractérisées par une morphologie étoilée ou dendritique [309]. Les DCs sont dérivées de la moelle osseuse et retrouvées dans tous les tissus lymphoïdes et la plupart des tissus non-lymphoïdes [310]. Bien qu'originellement considérées comme un groupe de cellules homogènes, les DCs peuvent être

classées en différentes sous-populations, selon leur stade de différenciation, leur phénotype et leurs fonctions [311]. Autrement dit, les DCs sont plutôt définies par leurs propriétés fonctionnelles et par une combinaison de marqueurs cellulaires, dont une forte expression du CD11c et du CMH-II [310]. Ainsi, quatre principaux sous-types ont été décrits chez la souris : (1) les DCs conventionnelles, qui prédominent à l'état d'équilibre et sont spécialisées dans l'apprêtement et la présentation antigénique aux lymphocytes T $CD4^+$; (2) les DCs plasmacytoïdes qui sont responsables de la sécrétion d'importantes quantités d'IFN de type I; (3) les cellules de Langerhans qui résident dans la peau et migrent aux nœuds lymphatiques pour présenter l'antigène et (4) les DCs dérivées des monocytes qui sont induites en réponse à l'inflammation et pourraient potentiellement constituer un réservoir de cellules présentatrices d'antigènes (APC) utiles en situation d'urgence [312].

Tout comme les autres phagocytes, les DCs participent activement à une multitude de fonctions immunologiques, afin d'assurer l'homéostasie de l'organisme. En effet, en conditions stables, les DCs immatures circulent librement dans le corps. Elles migrent ensuite vers les tissus pour échantillonner l'environnement à la recherche d'antigènes étrangers [311]. En absence d'inflammation ou de pathogène, les DCs présentent de grandes capacités phagocytaires et expriment de faibles niveaux de CMH-II et des molécules de co-stimulation (CD80, CD86 et CD40). En revanche, suite à la reconnaissance d'un signal de danger, les DCs subissent plusieurs changements physiologiques, afin d'effectuer leur fonction principale d'APC [313]. En effet, tout comme les macrophages, les DCs sont capables de phagocyter, mais plutôt que d'effectuer une destruction complète, elles ne dégradent les protéines que de manière partielle, afin de préserver des courtes séquences peptidiques [313]. Ces dernières sont ensuite présentées sur les molécules du CMH-II à la surface des cellules. Durant ce processus de maturation, les DCs produisent aussi une grande quantité de médiateurs inflammatoires qui vont permettre la différenciation et la polarisation des lymphocytes T $CD4^+$ [311]. Ainsi les DCs jouent un rôle important dans la défense de l'hôte face aux pathogènes et elles sont le lien critique entre la réponse innée et la réponse adaptative [314]. Finalement, étant des cellules de l'immunité innée, les DCs sont une source importante de médiateurs pro-inflammatoires impliqués dans le déclenchement et le maintien de l'inflammation [293].

2.1.4. Les neutrophiles

Contrairement aux autres cellules présentées ci-dessus, non seulement les neutrophiles ne sont pas des APCs professionnelles, mais ils appartiennent plutôt à la famille des leucocytes polymorphonucléés. En effet, ces cellules sont caractérisées par un noyau divisé en plusieurs lobes et par la présence de différentes classes de granules intracytoplasmiques porteuses d'enzymes et autres agents potentiellement toxiques impliqués dans la défense de l'hôte [315]. Chez la souris, les neutrophiles matures expriment fortement les récepteurs CD11b et Ly6G à leur surface [316]. Les neutrophiles proviennent des cellules hématopoïétiques de la moelle osseuse différenciées en présence du facteur de croissance «granulocyte colony-stimulating factor » (G-CSF), puis se rendent, une fois matures, dans la circulation sanguine, dans laquelle ils participent à la surveillance contre tout corps étranger ou signe d'une réponse inflammatoire [315]. En présence d'une infection, les cellules endothéliales présentes au niveau du site produisent des molécules d'adhésion permettant le roulement des neutrophiles le long de l'endothélium. Les neutrophiles peuvent ensuite se fixer à ces cellules, afin de les traverser, ce qui leur permet d'effectuer la diapédèse et de pénétrer à l'intérieur des tissus infectés [317].

Comme avec les autres phagocytes professionnels, des études récentes ont démontré que les neutrophiles sont une population hétérogène. Les deux sous-types principaux se distinguent par leur densité lors de l'utilisation de gradients de Ficoll-Paque. Bien que la population classique soit lourde (neutrophiles de forte densité), une certaine proportion d'entre eux sont plus légers (neutrophiles de faible densité) [318]. Toutefois, les fonctions différentielles de ces deux sous-types, qui pourraient représenter des stades de maturation différents [318], sont peu connues à ce jour.

Les neutrophiles sont attirés par chimiotaxie au niveau du site d'infection par plusieurs chimiokines de type ELR^+ CXC, telles que le CXCL1 et le CXCL2 (qui partagent le même récepteur, CXCR2) et le CXCL8, principale chimiokine pour les neutrophiles [317]. Une fois en présence des agents infectieux, les neutrophiles ont recours à une panoplie de fonctions différentes pour se débarrasser des pathogènes. La plus commune est la phagocytose : les neutrophiles ingèrent les débris ou pathogènes et les détruisent via l'action de protéases ou de produits antimicrobiens [319]. La phagocytose est grandement facilitée par l'opsonisation, qu'elle soit réalisée par le complément ou par les Igs [319]. Une fois le pathogène détruit, les neutrophiles subissent l'apoptose avant d'être éliminés par les macrophages. Ce mécanisme

programmé de mort cellulaire permet d'éviter une présence trop longue de ces cellules dans l'organisme. En effet, les neutrophiles sont dotés d'une durée de vie de quelques heures en circulation en raison des produits antimicrobiens qu'ils transportent à l'intérieur de leurs granules [317]. Toutefois, il a été suggéré que les neutrophiles peuvent avoir une durée de vie tissulaire plus longue afin de favoriser leurs fonctions immunologiques de phagocytose et de killing [320]

En plus de leur fonction de phagocytose, les neutrophiles sont capables de produire des dérivés réactifs à l'oxygène, dont l'oxyde nitrique, l'anion superoxyde et le peroxyde d'hydrogène, dans le but d'éliminer les microorganismes à l'intérieur et à l'extérieur de la cellule [317]. Lors d'un processus appelé dégranulation, ils libèrent également le contenu de leurs granules [317]. Enfin, les neutrophiles sont capables de libérer des « neutrophil extracellular traps » (NET) constitués de fibres composées de chromatines et de sérine protéases qui attrapent et tuent les microorganismes extracellulaires [315]. En plus de leurs propriétés antimicrobiennes, ces pièges peuvent également servir de barrières physiques pour empêcher la dispersion des pathogènes [321]. En tant qu'un des premiers types cellulaires présents au niveau du site d'infection, les neutrophiles participent également à la mise en place de la réponse inflammatoire en produisant plusieurs médiateurs, dont l'IL-1β, l'IL-6, IL-12, l'IL-18, le CCL2, le CCL3, le CXCL1, le CXCL2, le CXCL8 et le G-CSF, entre autres [317, 319, 322]. Ainsi, les neutrophiles sont des effecteurs cellulaires clés de la réponse immunitaire innée [315], puisqu'ils possèdent un arsenal de mécanismes antimicrobiens, dont le but ultime est d'éliminer tout pathogène présent dans l'organisme.

2.2. Le système du complément

Le système du complément est destiné à maintenir l'homéostasie, à reconnaître et à éliminer les cellules endommagées ou modifiées ainsi que les pathogènes. Il est composé de 30 à 50 protéines qui circulent dans le plasma ou qui sont présentes à la surface de cellules. De façon générale, les composants sont groupés en précurseurs inactifs, en effecteurs suite à leur activation, en régulateurs et en inhibiteurs [323]. L'activation du complément provoque une boucle d'amplification permettant la formation de peptides qui améliorent les fonctions effectrices du complément. Les molécules régulatrices veillent à ce que la cascade soit correctement activée et contrôlée et que les actions des peptides effecteurs se produisent au bon moment et au bon emplacement [324]. Ainsi, le complément est activé via trois voies

principales : (1) la voie alternative, initiée par contact avec la surface des microorganismes; (2) la voie classique, induite par des Ig liées à l'antigène et (3) la voie des lectines, induite par des structures d'hydrates de carbone spécifiques [323].

2.3. Les récepteurs et les voies de signalisation associées

Afin de répondre rapidement à la présence d'un microorganisme ou à un dommage cellulaire, les cellules immunitaires présentent à la surface de leurs membranes cellulaires et dans leurs compartiments cytoplasmiques plusieurs familles de PRRs impliquées dans la reconnaissance des PAMPs et responsables de l'initiation des processus inflammatoires [13]. À ce jour, plusieurs familles de récepteurs PRRs ont été décrites [325]. Les TLRs et les « NOD-like receptors » (NLRs) seront discutés dans le cadre de cette thèse comme ils sont importants pour la reconnaissance bactérienne et qu'ils ont été les mieux étudiés à ce jour.

2.3.1. Les « Toll-like receptors » (TLRs)

Les TLRs sont la famille de PRRs la plus étudiée à ce jour [326, 327]. Structurellement, ce sont des protéines transmembranaires de type I caractérisées par trois types de domaines différents : (1) un domaine extracellulaire contenant des motifs de répétitions riches en leucines, qui est responsable de la liaison au ligand, (2) un domaine transmembranaire et (3) un domaine intracellulaire d'homologie au récepteur cytoplasmique de signalisation Toll/interleukine-1 (TIR) requis pour la transduction du signal en aval [326]. Une fois le PAMP lié au TLR, le récepteur s'oligomérise afin de déclencher une cascade de signalisation conduisant à la production de médiateurs inflammatoires, dont des cytokines et des chimiokines [328].

À ce jour, dix et douze TLRs fonctionnels ont été identifiés chez l'humain et la souris, respectivement [325]. Parmi ceux-ci, seuls les TLR1 à TLR9 sont conservés chez les deux espèces. Le TLR10 murin n'est pas fonctionnel dû à l'insertion d'un rétrovirus, tandis que les TLR11, TLR12 et TLR13 ont été perdus dans le génome humain [329]. Les TLRs sont principalement divisés en deux sous-groupes selon leur localisation cellulaire. Les TLR1, TLR2, TLR4, TLR5, TLR6 et TLR10 sont exprimés à la surface des cellules et reconnaissent essentiellement des composants de la membrane microbienne, tels que les lipides, les lipoprotéines et les protéines [325, 329]. De plus, l'activation de ces TLRs induit principalement la production de cytokines et chimiokines inflammatoires. D'autre part, les TLR3, TLR7, TLR8,

TLR9 TLR11, TLR12 et TLR13 se retrouvent au niveau des membranes des endosomes [329]. Ces TLRs reconnaissent plutôt des acides nucléiques et induisent majoritairement la production des IFNs de type I [325, 329]. Toutefois, il est important de mentionner que les TLRs peuvent aussi reconnaître certains composants endogènes de l'hôte servant de signaux de danger appelés motifs moléculaires associés aux dommages cellulaires [330].

Le **tableau II** ci-dessous résume les principales molécules identifiées comme ligand respectif de chaque TLR. Ces derniers sont exprimés non seulement par des cellules immunitaires, mais aussi par d'autres types cellulaires, comme les cellules endothéliales, les cellules épithéliales et les fibroblastes [331]. Chaque type cellulaire possède son propre profil d'expression de TLRs. Ainsi, le fait de disposer de plusieurs TLRs permet à l'hôte de stimuler plusieurs types cellulaires de sorte à augmenter l'intensité de la réponse inflammatoire et à éviter une invasion du système immunitaire par un agent infectieux [329].

Tableau 2. Liste des « Toll-like receptors » chez l'humain et chez la souris et leurs ligands [155, 327].

« Toll-Like Receptor »	Localisation	Ligands
TLR1	Membrane plasmique	Lipoprotéines triacylées
TLR2	Membrane plasmique	Acides lipotéichoïques Lipoprotéines
TLR3	Membrane endolysosomale	Poly(I:C) ARN double brin
TLR4	Membrane plasmique	Lipopolysaccharide
TLR5	Membrane plasmique	Flagelline
TLR6	Membrane plasmique	Lipoprotéines diacylées
TLR7/TLR8	Membrane endolysosomale	ARN simple brin
TLR9	Membrane endolysosomale	ADN CpG
TLR10	Membrane plasmique	Inconnu
TLR11 (souris)	Membrane endolysosomale	Composantes des bactéries uropathogènes
TLR12 (souris)	Membrane endolysosomale	Inconnu
TLR13 (souris)	Membrane endolysosomale	Inconnu

2.3.1.1. Signalisation par les TLRs

Suite à la reconnaissance de PAMPs, les TLRs activent différentes voies de signalisation intracellulaire permettant d'induire une réponse inflammatoire [325]. Ces voies de signalisation débutent par le recrutement de différentes protéines adaptatrices présentes à l'intérieur de la cellule, dont les plus importantes sont le MyD88 et le « TIR-domain-containing adapter-inducing IFN-β » (TRIF) [325, 326]. Leur recrutement mène donc à l'initiation de deux voies principales de signalisation, soit la voie MyD88-dépendante et la voie TRIF-dépendante [328]. En effet, a l'exception du TLR3, tous les TLRs nécessitent l'implication de la protéine MyD88 pour initier leur signalisation [327]. N'ayant pas recours à la protéine adaptatrice MyD88, le TLR3 emprunte une voie de signalisation MyD88-indépendante pour déclencher la réponse

inflammatoire, impliquant la protéine adaptatrice TRIF [155]. De plus, la signalisation TRIF-dépendante peut également être utilisée par le TLR4 lorsque celui-ci est endocyté, suite à l'expression du CD14 [326]. Bien que les cascades de signalisation intracellulaires varient entre ces deux voies, elles mènent toutes deux à la production de médiateurs pro-inflammatoires [327]. En effet, la voie MyD88-dépendante entraîne principalement la production de cytokines et chimiokines pro-inflammatoires, tandis que la voie TRIF-dépendante induit la production des IFNs de type I [327]. La signalisation par ces deux voies implique une multitude de protéines adapatatrices et est schématisée à la **Figure 8**.

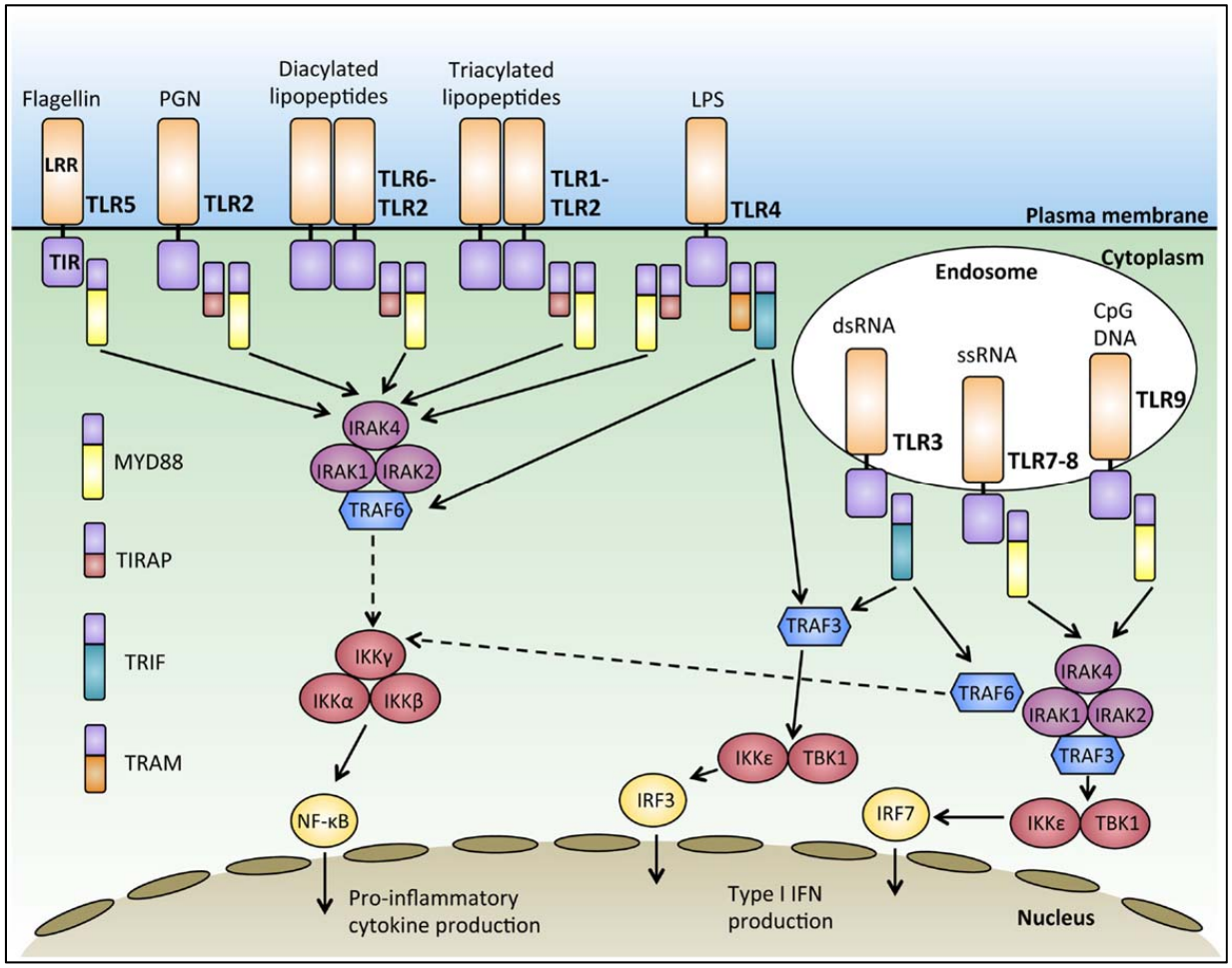

Figure 8. La signalisation MyD88-dépendante et TRIF-dépandente des « Toll-like receptors » (TLRs). La signalisation MyD88-dépendante débute par l'association de la protéine adaptatrice MyD88 au domaine cytoplasmique TIR des TLRs activés. Cependant, les TLR1/2, TLR2/6 et TLR4 ont également besoin de la protéine adaptatrice TIRAP, qui agit de pont de liaison entre le domaine TIR de ces TLRs et la protéine MyD88. Une fois liée, MyD88

s’associe avec « IL-1 receptor-asssociated kinase » (IRAK) 4. Le complexe MyD88/IRAK4 stimule l’autophosphorylation d’IRAK4 et le recrutement de deux autres membres de la famille, soit IRAK1 et IRAK2. L’activation des IRAKs permet de mobiliser le déplacement de « TNF receptor-associated factor » (TRAF) 6, avec qui elles forment un complexe qui entraîne la libération du facteur nucléaire-κB (NF-κB). Cette activation aura pour conséquence le déclenchement de la réponse inflammatoire en favorisant la transcription des gènes codant pour les cytokines et chimiokines pro-inflammatoires. Quant à la voie TRIF-dépendante, celle-ci est utilisée par le TLR3 et le TLR4, quoique ce dernier a besoin d’une deuxième protéine adaptatrice, TRAM, qui agit de pont de liaison entre TRIF et le domaine TIR du TLR4. Une fois le complexe formé, les protéines TRAF6 et TRAF3 s’y lient, ce qui entraîne la phosphorylation du facteur de transcription des IFN (IRF) 3 ou 7, et enclenche l’activation de plusieurs gènes inductibles codant pour les IFNs de type I. Tiré de [332]

2.3.2. Les « nucleotide-binding oligomerization domain-like receptors »

Les NLRs ont été découverts peu de temps après les TLRs et jouent un rôle important dans la surveillance au niveau du cytosol [13]. Ils ont une architecture typique en trois parties, avec un domaine de liaison nucléotidique central conservé nommé « central nucleotide-binding domain » [333]. Après la reconnaissance du ligand, la région C-terminale des protéines NLRs initie leur activation, tandis que le domaine effecteur N-terminal, spécifiant la fonction du NLR, est moins conservé [333]. En effet, les NLRs peuvent abriter un domaine pyrine ou un domaine de recrutement dépendant de l’activation de la caspase, entre autres [333].

À ce jour, la famille des NLRs inclut 22 et 34 gènes chez l’humain et la souris, respectivement. Chacun de ces gènes joue un rôle différent dans la reconnaissance des pathogènes, dans l'homéostasie et dans l'apoptose [334]. Les membres de la famille NLR peuvent être divisés en deux groupes : (1) les récepteurs NOD1 et NOD2, activant divers facteurs de transcription/activation, tels que NF-κB, les IRFs et les MAPKs et (2) des NLRs tels que « NLR pyrin domain » 3 (NLRP3) et NLRP1, régulant l'assemblage d'un complexe multiprotéique nommé l’inflammasome, ce qui conduit à l'activation de la pro-caspase-1 et à la maturation des cytokines de la famille de l'IL-1 [333]. Plus particulièrement, les récepteurs NOD1 et NOD2 reconnaissent l’acide glutamyl-méso-diaminopimélique et le muramyl dipeptide présent dans le peptidoglycane des bactéries à Gram négatif et à Gram positif, respectivement [333].

2.3.3. Voies des facteurs régulateurs de l'interféron

Les IRFs sont une famille de facteurs de transcription composée de neuf membres régulant l'expression des IFN de type I [335]. Toutefois, depuis les premières études, il a été démontré que les IRFs sont aussi impliqués dans une panoplie de fonctions biologiques, dont le contrôle du développement des cellules hématopoïétiques et de la réponse adaptative [335]. Ils contiennent un domaine de liaison à l'ADN conservé de moins de 120 acides aminés, qui reconnaît une séquence consensus de l'ADN appelée « IFN-stimulated response element » [335]. En plus d'être constitutivement exprimés, certains IRFs peuvent aussi être induits, afin d'amplifier leur effet. C'est notamment le cas de l'IRF1 (induit par l'IFN-γ), l'IRF5 (induit par les IFNs de type I et par l'activation de TLRs) et l'IRF7 (induit par les IFNs de type I) [335].

Tel que mentionné précédemment, les IRFs sont activés par différentes voies de signalisation. L'activation de la voie TRIF-dépendante mène à la phosphorylation, la dimérisation, puis la translocation de l'IRF3, tandis que la voie MyD88-dépendente active les IRF1, IRF5 et IRF7 [335]. Ces quatre IRFs sont des régulateurs positifs de la transcription des gènes codant pour les IFNs de type I [335]. Par contre, il est important de noter que leur distribution varie d'un type cellulaire à l'autre et entre les tissus [336]. En effet, les macrophages expriment surtout l'IRF3, tandis que les DCs expriment plutôt les IRF1, IRF5 et IRF7 [337].

Traditionnellement associés aux infections virales en raison du rôle que jouent les IFNs de type I dans le contrôle de ces infections, les IRFs ont été démontrés comme étant impliqués dans diverses pathologies, dont les maladies métaboliques, cardiovasculaires et neurologiques, entre autres [336]. Plus récemment, ils ont été rapportés comme participant dans la production d'IFNs de type I lors d'infections bactériennes. En effet, les IRF1 et IRF7, mais pas l'IRF3, sont impliqués lors d'infections systémiques par GBS [338]. Au contraire, l'IRF3 est important pour la production d'IFN-β induit par GAS et *S. pneumoniae* pour les macrophages, tandis que ce sont les IRF1 et IRF5 pour les DCs [16, 17]. Bien que les IRFs jouent un rôle important dans l'infection causée par des streptocoques pathogènes, aucune étude n'a évalué leur rôle lors de l'infection à *S. suis*.

2.4. Les médiateurs pro-inflammatoires

La réponse inflammatoire est composée de molécules produites par l'hôte, dont les cytokines, qui sont des glycoprotéines solubles de faible poids moléculaire régulant tant la réponse

immune innée qu'adaptative [339]. Les cytokines sont produites par plusieurs types cellulaires et ont des effets pléiotropiques, agissant sur différentes cellules cibles [339]. À faible concentration, les cytokines ont des effets paracrines tandis qu'à des plus fortes concentrations, elles ont des effets endocrines et agissent systémiquement [339].

2.4.1. Le facteur de nécrose tumorale

Le TNF est une cytokine de 25 kDa qui joue un rôle déterminant dans l'inflammation [340]. Il est produit par les monocytes/macrophages, les neutrophiles, les cellules gliales du cerveau, les cellules Kupffer du foie, les mastocytes, les cellules NK et les lymphocytes B et T [341]. Les facteurs stimulant le TNF incluent le lipopolysaccharide, des composants de la paroi bactérienne et les produits de l'activation du complément [341]. Le TNF interagit avec au moins deux récepteurs membranaires, soit le TNF-R1 et TNF-R2, qui sont tous deux exprimés par la plupart des types cellulaires, à l'exception des érythrocytes, en plus d'être présents sous forme soluble dans le plasma [341]. Le TNF-R1 est principalement responsable de l'induction de la réponse inflammatoire via la voie de NF-κB, mais peut aussi induire l'apoptose à la suite de son association avec les radeaux lipidiques [342]. Au contraire, le TNF-R2 est moins bien défini et semble impliqué comme supporteur dans les fonctions médiées par le TNF-R1 [342].

2.4.2. L'interleukine-1

L'IL-1 est un médiateur central à la réponse inflammatoire et est principalement produit par les monocytes et les macrophages activés, mais aussi par les DCs, les neutrophiles et une panoplie d'autres types cellulaires [342]. L'IL-1 est composée de deux protéines, soit IL-1α et IL-1β, qui sont les produits de gènes distincts [342]. Leur récepteur partagé, l'IL-1R, est exprimé à la surface de pratiquement toutes les cellules de l'hôte [342]. Une des caractéristiques uniques de l'IL-1 est qu'il existe un antagoniste naturel, l'IL-1Ra, qui est exprimé par les monocytes et neutrophiles [342]. Comme l'IL-1α et l'IL-1β lient le même récepteur, il n'y a pas de différence dans leur activité biologique [342]. Par contre, l'IL-1α est associée à la cellule et peut agir de facteur transcriptionnel, tandis que l'IL-1β est sécrétée et joue un rôle dans l'inflammation systémique [340].

Une particularité de l'IL-1β est son processus de maturation complexe [343]. En effet, sa production nécessite deux étapes. Dans un premier temps, l'activation de PRRs, dont les TLRs, mène à la production de la proIL-1β, qui doit par la suite être clivée, afin de pouvoir

effectuer ses fonctions biologiques [344]. Cette deuxième étape implique plusieurs protéines, dont la caspase-1 et les inflammasomes. Les inflammasomes sont des complexes multiprotéiques composés d'une molécule senseur spécifique à chaque inflammasome et d'une protéine adaptatrice commune qui lie le senseur à la caspase-1 [343]. Bien que plusieurs inflammasomes ont été décrits, les mieux caractérisés sont NLRP3, NLRP1 et le « absent in melanoma 2 » (AIM2) [345]. Leur assemblage suite à divers stimuli mène au clivage de la pro-caspase-1 qui peut, par la suite, cliver la proIL-1β afin de la rendre mature [343].

2.4.3. L'interleukine-6

Plusieurs cellules peuvent sécréter l'IL-6 qui est une protéine de 26 kDa [342]. Elle est multifonctionnelle et joue un rôle central dans les mécanismes de défense de l'hôte, la régulation des réponses immunes innées et adaptatives [342, 346]. Les activités biologiques de l'IL-6 chevauchent partiellement celles de l'IL-1β [342, 346]. De plus, l'IL-6 stimule la synthèse de protéines hépatiques, lors de la phase aiguë, et agit comme un pyrogène endogène [342, 346]. Ainsi, l'IL-6 est décrit comme étant une alarmine qui est relâchée lors de dommages tissulaires, afin de protéger l'hôte contre les réactions inflammatoires [342, 346].

2.4.3. L'interleukine-12

L'IL-12 est un hétérodimère codé par deux gènes différents, soit l'IL-12A (p35) et l'IL-12B (p40). À la suite de la synthèse protéique, l'hétérodimère actif p70 et un homodimère de p40 sont formés. Au contraire des autres cytokines décrites dans cette section, la production d'IL-12 n'est limitée qu'à quelques types cellulaires, dont les DCs et les macrophages [347]. Cette interleukine est un lien important entre l'immunité innée et l'immunité adaptative, puisqu'elle est un facteur stimulant la différenciation et la polarisation des lymphocytes T $CD4^+$ [347]. De plus, elle stimule la production d'IFN-γ et de TNF par les cellules NK et les lymphocytes T et promeut l'activité cytotoxique des cellules NK et des lymphocytes T cytotoxiques $CD8^+$ [347].

2.4.4. Les interférons de type I

2.4.4.1. Caractéristiques générales

Les IFNs de type I sont une famille de cytokines ayant plusieurs fonctions, dont la modulation de la réponse immunitaire innée [348]. Cette famille est composée de plusieurs membres, parmi lesquels les deux membres les mieux décrits et les plus exprimés sont l'IFN-α, qui comporte 16 isoformes, et l'IFN-β, qui sont ensembles communément appelés IFN-α/β [349].

Bien que grandement décrits pour leurs rôles lors d'infections virales, les IFNs de type I ont plus récemment été décrits comme participant dans la réponse immunitaire face aux bactéries intracellulaires et, de manière plus surprenante, aux bactéries extracellulaires [349]. Ainsi, la conséquence de la production des IFNs de type I lors d'infections est hautement dépendante du contexte de celles-ci.

Même si la plupart des types cellulaires peuvent produire de l'IFN-β, la production d'IFN-α est beaucoup plus restreinte, étant donné qu'elle est limitée aux cellules hématopoïétiques et aux DCs plasmacytoïdes [348]. Leur production est la conséquence d'une activation des PRRs, dont les TLRs et NLRs, et plus particulièrement par les PRRs retrouvés dans la membrane des endosomes et dans le cytosol [348]. Tel que mentionné précédemment, la reconnaissance de motifs microbiens par les PRRs peut mener à l'activation des IRFs et leur translocation au noyau où ils vont participer dans la transcription des IFNs de type I [335]. Une fois produit, les IFN-α/β vont lier leur récepteur commun appelé IFNAR. Ce récepteur se retrouve à la surface de plusieurs types cellulaires différents et est composé de deux chaînes (IFNAR1 et IFNAR2) [348]. La liaison des IFN-α/β à IFNAR enclenche une cascade de signalisation débutant par l'activation de la kinase Janus 1 et de la tyrosine kinase 2. La phosphorylation d'IFNAR par ces kinases mène au recrutement des protéines « signal transducer and activator of transcription » (STAT), qui sont par la suite phosphorylées. Leur phosphorylation permet leur dimérisation et leur translocation au noyau où ils vont participer à la transcription d'une panoplie de gènes, dont ceux codant pour des médiateurs pro-inflammatoires [349].

2.4.4.2. Rôle lors des infections bactériennes

Bien que les IFNs de type I aient été étudiés dans le contexte des infections virales depuis plusieurs années, cette section se concentrera sur leurs rôles lors des infections bactériennes, et plus précisément celles par les bactéries extracellulaires. Il est important de noter que les IFNs de type I ont été beaucoup mieux étudiés dans le cadre des infections par les bactéries intracellulaires, en particulier *L. monocytogenes*, *Salmonella enterica* et *Mycobacterium tuberculosis* [349].

Depuis environ dix ans, il a été rapporté que des bactéries extracellulaires, dont des streptocoques, induisent la production d'IFNs de type I par les DCs et les macrophages et que l'IFN-α/β peut moduler la réponse de l'hôte lors de l'infection et le développement de la maladie

clinique [349]. La reconnaissance de l'ARN, et moindrement de l'ADN de GBS par les TLR7 et TLR9, respectivement, mène à la production d'IFNs de type I par les DCs [338]. Par contre, pour GAS, bien que la signalisation dépendante de MyD88 soit nécessaire pour la production d'IFN-β par les macrophages et les DCs, elle ne nécessite pas le TLR7 [17]. Finalement, la production d'IFN-β induite par *S. pneumoniae* ne dépend ni de MyD88, ni de TRIF, nécessitant plutôt des PRRs cytosoliques, tout en étant tout de même activée par l'ADN de la bactérie [350]. Ainsi, même si les streptocoques semblent tous induire la production d'IFNs de type I, et ce majoritairement via leurs acides nucléiques, les mécanismes cellulaires impliqués dépendent du pathogène.

Lors des infections bactériennes *in vivo*, les IFNs de type I peuvent jouer un rôle, tant bénéfique que néfaste. En effet, un rôle bénéfique a été observé lors des infections par GAS, GBS et *S. pneumoniae* [15, 17, 350]. Ceci peut s'expliquer par une croissance bactérienne non-contrôlée, en raison d'une réponse inflammatoire sous-optimale, qui est requise pour l'élimination bactérienne dans le sang et les organes internes. Cependant, une induction forte de la réponse des IFNs de type I est considérée comme étant un facteur clé, qui permet à *S. pneumoniae* de causer une infection systémique par un mécanisme encore inconnu [351]. Ainsi, comme pour les mécanismes *in vitro*, le rôle que jouent les IFNs de type I lors des infections par les streptocoques dépend de la bactérie en question.

Récemment, il a été rapporté que *S. suis* induit l'expression d'IFN-β par les splénocytes lors de l'infection systémique chez un modèle expérimental murin [352]. De manière surprenante, son expression était inverse à la virulence de la souche testée, c'est-à-dire qu'une souche ST25 nord-américaine de virulence intermédiaire a induit une expression plus élevée d'IFN-β qu'une souche ST1 européenne virulente, tandis que la souche ST7 responsable de l'éclosion humaine de 2005 en Chine a induit les plus faibles niveaux [352]. Malgré cela, les sources cellulaires, les mécanismes impliqués et son rôle dans le cadre de l'infection à *S. suis* n'ont jamais été étudiés.

2.4.5. L'interféron de type II

L'IFN-γ, aussi appelé interféron de type II, est une cytokine importante, tant pour l'immunité innée, que pour l'immunité adaptative. Elle est principalement produite par les cellules NK et les lymphocytes T $CD4^+$ et T $CD8^+$ [353]. L'IFN-γ promeut l'activité des cellules NK, l'activation

et l'augmentation de la présentation antigénique et l'activité lysosomale des macrophages, en plus de réguler à la hausse la production de TNF et d'IL-12, entre autres. [353, 354]. De plus, l'IFN-γ est la cytokine principale définissant les lymphocytes T $CD4^+$ de profil Th1 [353].

2.4.6. Les chimiokines

Les chimiokines, qui sont de petites protéines de 8 à 10 kDa, sont regroupées en deux familles importantes, basées sur leur structure. En effet, les chimiokines C-C contiennent deux résidus cystéines adjacents et ont la capacité d'attirer et d'activer principalement les cellules mononucléées. Pour leur part, les chimiokines C-X-C sont caractérisées par le fait que leurs deux premiers résidus cystéines sont séparés par un seul acide aminé, en plus d'être typiquement chimiotactiques pour les neutrophiles [355].

2.4.6.1. Les chimiokines de motif C-C

2.4.6.1.1. CCL2

La chimiokine CCL2 est principalement produite par les monocytes inflammatoires, bien que d'autres types cellulaires puissent aussi la produire [356, 357]. CCL2 est critique pour la mobilisation des monocytes inflammatoires qui sont parmi les seules cellules à exprimer son récepteur CCR2 [355]. De manière similaire à l'IL-1β et à l'IL-6, CCL2 élicite le métabolisme oxydatif, l'expression d'intégrines β_2, le relâchement d'enzymes lysosomales et l'induction de la production de différentes cytokines [356, 357].

2.4.6.1.2. CCL3

CCL3 est produit par plusieurs types cellulaires, dont les monocytes/macrophages, les mastocytes, les DCs, les fibroblastes et les lymphocytes T. Cette chimiokine est impliquée dans l'inflammation accrue et dans le recrutement des monocytes, des lymphocytes B et T et des cellules NK, mais aussi des neutrophiles [358, 359]. De plus, CCL3 lie le récepteur CCR5, qu'il partage avec les chimiokines CCL4 et CCL5 [359].

2.4.6.2. Les chimiokines de motif C-X-C

2.4.6.2.1. CXCL1 et CXCL2

Les séquences d'acides aminés des chimiokines CXCL1 et CXCL2 partagent 90% d'homologie et elles sont toutes les deux capables de lier le récepteur CXCR2 [360]. Elles sont produites

par plusieurs types cellulaires, dont les monocytes, les macrophages, les DCs, les neutrophiles et les lymphocytes T, et partagent le même rôle principal, soit la chimiotaxie des neutrophiles [360].

2.4.6.2.2. CXCL8

CXCL8 est produit par une grande variété de cellules, dont les cellules endothéliales, les monocytes, les macrophages, les DCs et les neutrophiles, et lie les récepteurs CXCR1 et CXCR2 [356]. Elle joue un rôle important dans la chimiotaxie des neutrophiles [356]. De plus, CXCL8 induit des changements morphologiques chez les neutrophiles et la libération d'enzymes lysosomales par ceux-ci [356]. Il promeut aussi l'adhésion des neutrophiles à l'endothélium en augmentant l'expression des intégrines β_2 et en régulant la migration transendothéliale de ces cellules [356]. Toutefois, il est important de noter que cette chimiokine n'est pas produite chez la souris et qu'aucun homologue n'y a été identifié à ce jour [356].

2.5. Rôle de la réponse immunitaire innée lors de l'infection à *Streptococcus suis*

Au cours des années, le rôle des différentes cellules de l'immunité innée dans les interactions entre *S. suis* et son hôte a été décrit *in vitro*, mais presque exclusivement pour le sérotype 2 [10]. Toutefois, aucune étude ne s'est penchée sur le rôle de ces cellules, incluant les monocytes, les macrophages, les DCs et les neutrophiles *in vivo*. Ainsi, l'information disponible sur le modèle animal est très limitée. De plus, comme pour la plupart des pathogène, il est important de garder en tête que bien que l'inflammation exacerbée induite par *S. suis* soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes.

2.5.1. Rôle des monocytes

Bien que les monocytes soient un composant essentiel des leucocytes sanguins et qu'ils infiltrent les tissus lors d'infections bactériennes, très peu d'études se sont attardées à leurs rôles lors de l'infection à *S. suis*. En fait, la plupart des études ayant utilisé des monocytes ont été réalisées afin de pouvoir les différencier par la suite, que ce soit en macrophages à l'aide de l'IFN-γ [100, 148] ou en DCs à l'aide du facteur de croissance « granulocyte-macrophage colony-stimualting factor » (GM-CSF) et d'IL-4 [361]. Toutefois, il a été démontré que la lignée de monocytes humains THP-1 sécrète d'importantes quantités de TNF, de CCL2 et de CXCL8, à la suite de l'infection par une souche ST1 de *S. suis* sérotype 2 [100]. De plus, *S. suis* induit

l'expression d'IL-12p40, d'IL-23p19, de CCL5 et de CCL20 par des monocytes porcins isolés du sang périphérique [361]. Comme ces monocytes ont été isolés à l'aide d'une sélection positive ciblant le CD14, ils correspondent à la sous-population de monocytes classiques [361]. Finalement, bien que les monocytes porcins isolés du sang possèdent une certaine capacité à phagocyter *S. suis*, cette fonction est moindre que celle des DCs dérivées de monocytes et que celle des neutrophiles porcins [90, 91, 361]. Toutefois, la présence de la CPS interfère dans l'internalisation de *S. suis* par les monocytes porcins [91]. Ainsi, il y a un manque d'information important concernant le rôle et les fonctions des monocytes lors de l'infection à *S. suis.*

2.5.2. Rôle des macrophages

S. suis utilise plusieurs stratégies afin de déjouer les macrophages, qui sont les cellules phagocytaires par excellence [10]. Parmi celles-ci, la CPS joue un rôle central. En effet, la présence de CPS à la surface de *S. suis* déstabilise les microdomaines lipidiques à la surface des macrophages, inhibant ainsi la phagocytose et empêchant la reconnaissance lactosylcéramide-dépendante de la bactérie [145]. De plus, la présence de la CPS module les voies de signalisations intracellulaires impliquées dans la phagocytose par les macrophages murins [98]. En plus d'inhiber la phagocytose de *S. suis* par les macrophages, la présence de sa CPS peut augmenter la survie intracellulaire des bactéries dans les macrophages péritonéaux murins et porcins [362].

Comme mentionné plus haut, une hypothèse voudrait que plutôt que de participer à l'élimination de *S. suis*, les macrophages pourraient, au contraire, servir de véhicules de transport pour les bactéries extracellulaires, favorisant ainsi leur dissémination dans l'hôte. En effet, *S. suis* sérotype 2 adhère fortement aux macrophages murins, sans pour autant être ingéré par les cellules [97]. Son adhésion à la surface des cellules serait possible, au moins en partie, par l'acide sialique de sa CPS [97]. De plus, en interagissant avec les macrophages, *S. suis* induit la libération de plusieurs médiateurs inflammatoires, dont le TNF, l'IL-1β, l'IL-6, le CCL2 et le CXCL8 de manière phagocytose-indépendante [99, 148]. Chez les macrophages alvéolaires porcins, la production de médiateurs pro-inflammatoires induits par *S. suis* implique le facteur de transcription NF-κB et les MAPK [363]. La libération de médiateurs pro-inflammatoires est un mécanisme de défense utilisé par l'hôte, afin de mettre en place une

réponse inflammatoire et recruter d'autres cellules au site d'infection, dans le but d'éliminer la bactérie [88, 364].

2.5.3. Rôle des cellules dendritiques

Tout comme les macrophages, les DCs sont responsables de la phagocytose des agents microbiens présents dans l'organisme. Toutefois, plutôt que de détruire complètement les pathogènes, elles ne dégradent leurs protéines que de façon partielle, de telle sorte à les présenter à leur surface cellulaire, leur permettant ainsi de jouer leur rôle principal d'APC [313]. Une fois ces antigènes capturés, les DCs subissent un processus de maturation complexe marqué par l'augmentation de l'expression du CMH-II et des molécules de co-stimulation et par la libération de cytokines [314]. Le rôle anti-phagocytaire de la CPS de *S. suis,* contre les DCs murines, porcines et humaines, a également été confirmé [139, 147, 314]. Cependant, il a été démontré que la CPS de *S. suis* n'est pas suffisante pour permettre à la bactérie de résister à l'opsonophagocytose et à l'effet bactéricide en conditions opsonisantes par les DCs murines [139]. En effet, des modifications de la paroi cellulaire bactérienne, ainsi que la production de suilysine, interfèrent dans le dépôt du complément à la surface de *S. suis*, limitant par conséquent sa reconnaissance par les récepteurs du complément et son élimination par phagocytose [139]. En revanche, une fois phagocytée par les DCs porcines ou humaines, la survie intracellulaire de la bactérie n'est pas affectée par la présence de sa CPS [147, 314].

De plus, la présence de CPS interfère dans le processus de présentation antigénique, en affectant l'expression du CMH-II et des molécules de co-stimulation à la surface des DCs murines, porcines et humaines, en plus d'affecter la production de cytokines, dont l'IL-12p70 (**Annexes – Article I**) [146, 147]. Elle masque partiellement les composés de la paroi cellulaire de *S. suis* reconnus par les DCs et responsables de leur activation [139]. Ainsi, en altérant l'activation et la maturation des DCs, *S. suis* diminue leur processus de présentation d'antigènes, ainsi que l'activation subséquente des lymphocytes T. Ceci pourrait expliquer la faible activation des lymphocytes T $CD4^+$ et la faible réponse mémoire humorale observées chez la souris et le porc [365, 366]. Il est à noter que *S. suis* modifie la production des ratios des cytokines IL-10/IL-12p70 et IL-10/TNF vers un profil de réponse anti-inflammatoire [147]. De plus, l'interaction de *S. suis* avec les DCs entraînent la libération de divers médiateurs pro-inflammatoires, dont le TNF, l'IL-1β, l'IL-6, l'IL-12p70, l'IL-15, IL-23p19, le CCL2, le CCL3, le CXCL1, le CXCL9 et le CXCL10 [147, 314, 361, 367]. Autrement dit, par le biais de ces

mécanismes, *S. suis* empêche la mise en place, par l'hôte, d'une réponse immunitaire adaptative efficace et assure sa persistance [365]. Globablement, l'effet net de l'activation des DCs par *S. suis* est une réponse pro-inflammatoire, qui est toutefois, sous-optimale.

2.5.4. Rôle des neutrophiles

Comme pour les monocytes, le rôle des neutrophiles lors de l'infection à *S. suis* a été peu décrit. Un intérêt pour ces cellules et leurs interactions avec *S. suis* a été suscité dans les dernières années, mais l'information disponible reste toujours limitée. Toutefois, les neutrophiles sont les phagocytes ayant la capacité la plus élevée pour internaliser et tuer *S. suis*, même en présence de sa CPS [91]. De plus, la suilysine interfère dans la capacité des neutrophiles à phagocyter *S. suis*, mais le rôle de cette toxine, dans le dépôt du complément à la surface bactérienne qui pourrait favoriser l'opsonophagocytose, reste controversé [90, 91]. Il a aussi été rapporté que la suilysine est toxique pour les neutrophiles et induit leur mort [90]. Finalement, *S. suis* produit une DNase (SsnA) qui contribue à la dégradation des NETs et à l'évasion immunitaire [368, 369]. De manière surprenante, malgré l'importance des neutrophiles dans les infections bactériennes, aucune étude n'a évalué la capacité des neutrophiles à produire des médiateurs pro-inflammatoires lors de l'infection par *S. suis.*

2.5.5. Rôle des « Toll-like receptors »

Puisque les TLRs sont la famille de PRRs la mieux étudiée et qu'ils sont exprimés sur toutes les cellules de l'hôte, plusieurs études se sont intéressées à leurs rôles dans la reconnaissance de *S. suis* et le déclenchement de la réponse inflammatoire [10]. L'implication de la voie des TLRs a été évaluée via l'utilisation de cellules déficientes pour la protéine adaptatrice MyD88. Comme mentionné précédemment, cette protéine est utilisée par tous les TLRs, à l'exception du TLR3, pour déclencher la cascade de signalisation et l'induction des médiateurs inflammatoires [329]. En son absence, la production de médiateurs pro-inflammatoires par les macrophages et les DCs murins est pratiquement abrogée à la suite de l'infection par *S. suis* sérotype 2 [148, 367]. De plus, *S. suis* module sa reconnaissance par les TLRs par l'entremise de sa CPS. En effet, en absence de celle-ci, *S. suis* n'est plus en mesure de masquer les composants de sa paroi cellulaire qui interagissent, de ce fait, avec différents TLRs pour induire la production de médiateurs pro-inflammatoires [148]. Cependant, la CPS des sérotypes 2 et 14 contribue également à la production des chimiokines CCL2 et CCL3 par les macrophages et les DCs murins de façon MyD88-indépendante pour CCL2 et TLR2- et

MyD88-dépendante pour CCL3 [37, 148]. Autrement dit, hormis les TLRs, d'autres familles de récepteurs pourraient être impliquées dans la reconnaissance de *S. suis* [370]. Néanmoins, les TLRs restent la famille de PRRs la plus probablement impliquée dans la reconnaissance de cette bactérie.

S. suis dispose d'un arsenal de composés non seulement exposés à sa surface, mais également sécrétés, qui peuvent interagir avec les TLRs extracellulaires des cellules de l'hôte. Parmi ces composés sécrétés, il a été proposé que la suilysine peut être reconnue par le TLR4 [331]. En effet, la suilysine de *S. suis* induit une forte libération de TNF par des macrophages murins et humains [331].

Cependant, la majorité des études indique que le TLR2 serait le récepteur principal impliqué dans la reconnaissance de *S. suis* [370]. En effet, la stimulation de monocytes humains par *S. suis* entraîne une régulation à la hausse de l'expression du TLR2 et non du TLR4 [148]. De même, la production de TNF, de l'IL-1β, de l'IL-6, de CCL2 et de CXCL8 est réduite lorsque les monocytes sont préalablement traités avec des anticorps monoclonaux neutralisants dirigés contre le TLR2, mais pas contre le TLR4 [148]. Enfin, des macrophages et DCs murins déficients pour le TLR2 induisent moins de médiateurs pro-inflammatoires en réponse à *S. suis* [148, 367]. Malgré ces études ciblées sur le TLR2, il est important de mentionner que celui-ci ne peut initier les cascades de signalisation et la production de médiateur inflammatoire sans former un hétérodimère, que ce soit avec le TLR1 ou le TLR6 [105]. Il s'agit également d'un récepteur versatile en raison de sa capacité à reconnaître une variété de composantes microbiennes, tel que mentionné précédemment. Cependant, il semble de plus en plus probable que les lipoprotéines soient le ligand privilégié du TLR2 et que les différences subtiles entre les formes triacylées et diacylées de celles-ci soient distinguées par la dimérisation du TLR2 avec soit le TLR1 ou le TLR6, respectivement [105]. En effet, les lipoprotéines de *S. suis*, qui sont retrouvées sous forme diacylées, activent le TLR2/6, mais pas le TLR1/2 [105]. La régulation à la hausse de l'expression des TLR2 et TLR6 par les DCs porcines, après stimulation avec *S. suis*, soutient davantage l'implication unique du complexe TLR2/6 dans la reconnaissance du pathogène [314]. De même, le blocage par anticorps du TLR6 entraîne une diminution de la production du TNF par les cellules mononuclées du sang périphérique [371].

Bien que le TLR2 en association avec le TLR6 joue un rôle important dans la réponse inflammatoire causée par *S. suis*, la réduction importante, mais non complète de la production de médiateurs inflammatoires, suggère l'implication d'autres TLRs MyD88-dépendant dans sa reconnaissance [148]. Étant un pathogène extracellulaire, la reconnaissance des TLRs endosomaux, lors de l'infection à *S. suis*, a été très peu étudiée. Plus récemment, il a été démontré que l'ADN de *S. suis* stimule la libération d'IL-6 et de TNF par les cellules mononuclées du sang périphérique de manière dose-dépendante, et ce via le TLR9 [371]. De plus, lors de l'activation par *S. suis* de DCs murines déficientes à la fois pour le TLR9 et le TLR2, une différence significative de l'expression de l'IL-12p70 et de CXCL10 a été observée. Ces résultats suggèrent que le TLR9 pourrait agir en coopération avec le TLR2 ou de manière redondante dans l'activation des DCs par *S. suis* [367].

Toutefois, il est important de noter que le rôle des TLRs lors de l'infection à *S. suis* n'a été que très peu étudié *in vivo*. En effet, il n'y a qu'une seule étude portant sur le rôle du TLR2, et ce lors de l'infection systémique par *S. suis* sérotype 2 [370]. Il a été démontré que le TLR2 participe à l'exacerbation de la réponse inflammatoire induite par une souche ST1 virulente (européenne), mais pas par la souche ST7 hautement virulente et responsable de l'éclosion humaine de 2005 en Chine [370]. Ainsi, d'autres études seront nécessaires afin de mieux comprendre le rôle de la voie des TLRs lors de l'infection à *S. suis.*

2.5.6. Rôle des « nucleotide-binding oligomerization domain-like receptors »

Si l'information du rôle des TLRs lors de l'infection à *S. suis* est limitée, celle du rôle des NLRs est pratiquement inexistante. En effet, une seule étude a évalué le rôle des NLRs, et plus principalement celui de NOD2, dans le cadre de l'infection à *S. suis* sérotype 2. Les résultats ont démontré que NOD2 est partiellement impliqué dans la production d'IL-23p19 et de CXCL1 par les DCs murines [367]. Toutefois, la production d'IL-23-p19 NOD2-dépendante est indépendante du TLR2, et ce malgré le fait que le TLR2 est également impliqué dans la production d'IL-23p19 par les DCs. Ceci suggère donc un rôle non-redondant de ces deux récepteurs dans la production de cette cytokine [367].

3. Le système nerveux central et son immunité innée

Historiquement, le SNC a été considéré comme étant un site immunoprivilégié, en raison de l'absence relative de drainage lymphatique, du nombre limité de phagocytes et des APCs et des barrières qui le séparent du sang [372]. Entre autres, celui-ci est entouré de plusieurs couches de tissus protecteurs appelées les méninges. Malgré cela, le SNC possède son propre système immunitaire inné qui s'active en réponse à des défis immuns cérébraux et systémiques [372]. D'une part, l'activation du système immunitaire inné et la production de médiateurs pro-inflammatoires contrôlent l'infection et favorisent la réparation du SNC. D'autre part, ils peuvent aussi causer des pathologies, dont la méningite [372]. Comme les tissus périphériques, le SNC contient des phagocytes mononucléés, dans ce cas dérivés de précurseurs embryonnaires du sac vitellin capables de s'auto-renouveler, dont la microglie du parenchyme et les macrophages périvasculaires, des méninges et du plexus choroïdien [372]. De plus, les astrocytes, qui constituent la plus grande population de cellules gliales (cellules formant l'environnement des neurones) du SNC, sont une composante importante de l'immunité innée du SNC [25].

3.1. Les méninges et la membrane gliale limitante superficielle

Les méninges regroupent les trois couches de tissus enveloppant le SNC et le protégeant de dommages physiologiques dus aux traumatismes crâniens [373]. Elles sont constituées des pachyméninges correspondant à la dure-mère et des leptoméninges, soient la membrane arachnoïdienne et la pie-mère sous-jacente (**Figure 9**) [374]. La dure-mère est composée d'une épaisse couche de fibres de collagène et couvre l'intérieur du crâne, tandis que les leptoméninges sont deux minces couches de cellules interconnectées par des trabécules [374]. La membrane arachnoïdienne forme une couche translucide et imperméable qui contient l'espace sous-arachnoïdien (SAS), dans lequel se retrouve le LCR [374]. Quant à elle, la pie-mère recouvre directement la surface du cerveau et la membrane gliale limitante superficielle se retrouve directement en dessous. La membrane gliale limitante superficielle est la première couche du cerveau et est composée des pieds astrocytaires associés à la lame basale du parenchyme entourant le cerveau [374]. Ainsi, elle est la couche externe du tissu nerveux et prévient la migration des neurones et des cellules gliales vers les méninges. De plus, elle régule le mouvement des petites molécules et des cellules dans le parenchyme du cerveau [374].

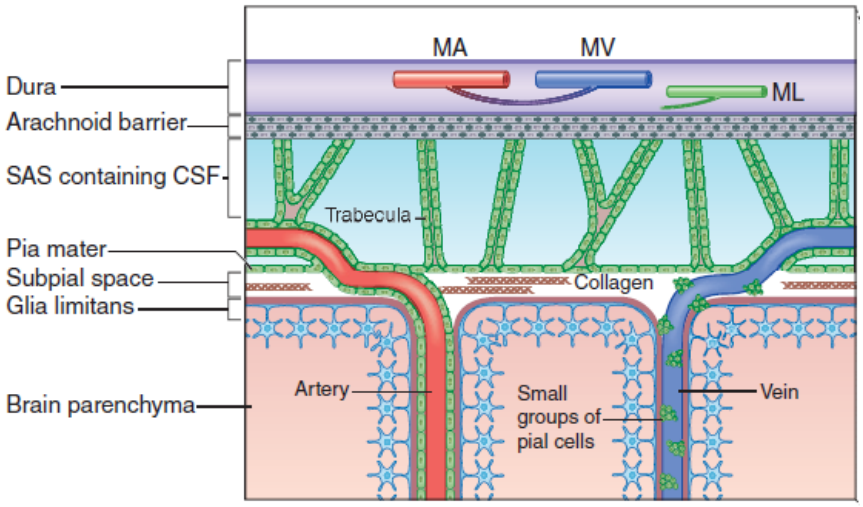

Figure 9. Les barrières à la surface du système nerveux central. La dure-mère contient des artères (MA), de veines (MV) et plusieurs vaisseaux lymphatiques (ML). Directement en dessous de la dure-mère se retrouve la membrane arachnoïdienne qui forme l'extérieur de la barrière hémato-liquide céphalorachidien de l'espace sous-arachnoïde (SAS). Le SAS contient le liquide céphalorachidien (LCR) et est traversé par des trabécules joignant la pie-mère. Finalement, la membrane gliale limitante superficielle forme une barrière à la surface du parenchyme et autour des vaisseaux sanguins [23].

Outre un rôle protecteur, les méninges participent activement à l'homéostasie du SNC en modulant les fonctions corticales et celles des cellules souches, entre autres. De plus, elles sont une source importante de plusieurs facteurs trophiques, dont le facteur de croissance des fibroblastes, le facteur de croissance de type insuline, l'acide rétinoïque et le CXCL12 [374].

Comme les leptoméninges sont la porte qu'utilisent les pathogènes se retrouvant dans le LCR pour entrer dans le cerveau, elles sont impliquées dans la réponse inflammatoire locale [24]. En fait, la méningite bactérienne se caractérise par une inflammation suppurative de la membrane arachnoïdienne et de la pie-mère [375]. Ces cellules expriment plusieurs PRRs, dont le TLR2 et le TLR4, et produisent des niveaux élevés d'IL-6, de GM-CSF, de CCL2, de CCL5 et de CXCL8 à la suite de l'infection par *Neisseria meningitidis* et d'IL-1β, d'IL-15 et de CXCL2 à la suite de l'infection par *S. pneumoniae*, mais pas de CXCL8 [299, 376, 377]. De plus, *N. meningitidis*, *Escherichia coli* K1, *S. pneumoniae* et *Haemophilus influenzae* type b adhèrent tous aux leptoméninges, mais seul *E. coli* peut les envahir [378]. Toutefois, bien qu'une importante inflammation suppurative des leptoméninges soit observée lors de la méningite causée par *S. suis,* leur rôle reste inconnu.

3.2. Les cellules de l'immunité innée

3.2.1. La microglie

Le terme microglie fait référence aux macrophages retrouvés à travers le parenchyme du SNC. Bien que la microglie partage beaucoup de caractéristiques en commun avec les macrophages retrouvés dans le compartiment systémique, les cellules microgliales ne proviennent pas des cellules hématopoïétiques de la moelle osseuse, mais plutôt des cellules précurseurs embryonnaires du sac vitellin [379]. De plus, contrairement à ce qui avait été suggéré dans le passé, les cellules de la microglie ont une très longue durée de vie et s'autorenouvellent, ne nécessitant pas l'apport de monocytes sanguins [379]. Leur développement et leur maintien dépendent de l'activation continuelle du récepteur CD115 (récepteur du « macrophage colony-stimulating factor ») [380]. En soit, la microglie compte pour 10% des cellules du SNC et est présente dans toutes les régions de la matière blanche et grise [380].

Une des caractéristiques importantes de la microglie est son activation rapide lors d'infection ou d'atteintes physiques au SNC. En effet, l'activation de la microglie précède celle des autres types cellulaires du SNC [25]. Dans leur état non-activé, les cellules de la microglie ont une morphologie dite ramifiée typique [25]. Tout comme pour les macrophages systémiques, la microglie exprime de manière constitutive le CMH-II à sa surface, en plus du récepteur CD11b [380]. Les cellules microgliales ramifiées ont la capacité de changer dramatiquement leur morphologie en réponse à différents stimuli externes, dont la présence microbienne. Alors, elles rétractent leurs ramifications afin d'acquérir une forme ronde appelée amiboïde [25]. De plus, elles régulent rapidement à la hausse un grand nombre de récepteurs et sécrètent plusieurs médiateurs impliqués dans la défense du SNC, mais aussi dans son dommage [381].

Les cellules de la microglie expriment plusieurs PRRs, dont un grand éventail de TLRs, incluant les TLR1 à TLR9, avec une expression plus faible du TLR9 [25]. Lorsqu'activées via les TLRs, elles peuvent produire une panoplie de médiateurs pro-inflammatoires, dont le TNF, l'IL-1, IL-6, IL-12 et l'IL-18, en plus de plusieurs chimiokines telles CCL2, CCL3, CCL5, CXCL1 et CXCL2, et des espèces réactives de l'oxygène [382]. De plus cette activation mène à l'expression de molécules de co-stimulation et du CMH-II, ce qui les rendent fonctionnellement capables de présenter des antigènes aux lymphocytes T $CD4^+$ [383].

Les cellules de la microglie expriment aussi NOD2, qui une fois activé par *N. meningitidis* et par *Borrelia burgdorgeri* mène à l'activation de la « receptor-interacting protein kinase 2 » (RIP2) et à la production d'une réponse inflammatoire [384]. L'activation de NOD2 dans les cellules microgliales participe aussi à l'astrogliose (augmentation du nombre d'astrocytes suite à leur multiplication) et à la démyélination induites par ces deux pathogènes bactériens [385].

3.2.2. Les astrocytes

Les astrocytes sont l'autre population importante de cellules gliales dans le SNC. En effet, les astrocytes couvrent environ 10% du cortex. Ces cellules portent bien leur nom, ayant une morphologie en forme d'étoile [386]. Bien que les astrocytes aient été considérés comme jouant des rôles structurels, de support et dans l'approvisionnement de nutriments au tissu nerveux, ils sont responsables d'équilibrer les niveaux de potassium dans le SNC et d'effectuer l'ajustement de l'équilibre aqueux, entre autres [25]. Ces cellules sont une source remarquable de substances neuroactives, dont des facteurs de croissance et des neurostéroïdes, aussi appelés stéroïdes neuoactifs (stéroïdes altérant rapidement l'excitabilité des neurones) qui peuvent influencer le développement et la survie neuronale [387]. Plus récemment, les astrocytes ont été démontrés comme des contributeurs importants de la réponse inflammatoire du SNC lors d'infections ou de dommages tissulaires [388]. En effet, leur production de TNF et d'IL-1β augmente radicalement lors d'états neuropathologiques et contribue à la dégradation de la BBB et au recrutement de leucocytes périphériques [388].

De manière similaire à la microglie, les astrocytes expriment certains TLRs, dont les TLR2 à TLR5 et TLR9 [389]. De plus, ils expriment NOD2, qui tout comme pour la microglie, mène à la production d'une réponse inflammatoire à la suite de l'infection par *N. meningitidis* et par *B. burgdorgeri* [384]. L'activation des TLRs ou NLRs des astrocytes participe à la production de plusieurs médiateurs pro-inflammatoires, dont l'IL-1β, l'IL-6, le CCL2 et le CXCL1 [25].

3.2.3. Les macrophages périvasculaires, des méninges et du plexus choroïdien

Les macrophages périvasculaires, des méninges et du plexus choroïdien sont, au contraire de la microglie, les macrophages des tissus non-parenchymaux [390]. Les macrophages périvasculaires se retrouvent dans l'espace périvasculaire, entre les vaisseaux sanguins cérébraux et les pieds astrocytaires, tandis que ceux des méninges et du plexus choroïdien se

retrouvent dans les tissus eux-mêmes (**Figure 10**) [390]. Tout comme la microglie, ces macrophages résidents sont dérivés de cellules précurseures embryonnaires du sac vitellin et ont une durée de vie très longue [379] Les macrophages périvasculaires et des méninges s'autorenouvellent, tandis que ceux du plexus choroïdien nécessitent l'apport de monocytes classiques/inflammatoires sanguins [379]. Ensemble, ils jouent des rôles importants dans le maintien de l'homéostasie en échantillonnant les débris locaux, en phagocytant les cellules mortes et en communiquant avec les cellules environnantes [390]. De plus, l'expression du MHC-II à leur surface leur permet d'effectuer la présentation antigénique [379]. Comme ces différents macrophages se retrouvent près de la BBB ou de la BCSFB, ils participent à l'immunosurveillance locale et sont parmi les premières cellules immunitaires rencontrées par les pathogènes dans le SNC. Ainsi, ils produisent une panoplie de médiateurs pro-inflammatoires, dont l'IL-1β, l'IL-6, le CCL2, le CXCL1 et le CXCL2 [379, 390]. En fait, les macrophages périvasculaires et des méninges jouent un rôle bénéfique lors de l'infection à *S. pneumoniae* en participant à la réponse inflammatoire requise pour éliminer le pathogène du SNC [391].

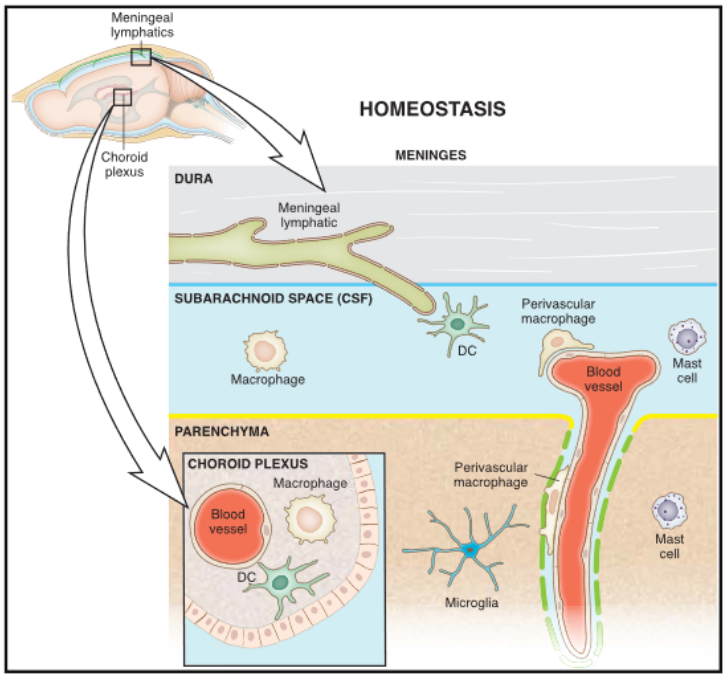

Figure 10. Localisation des différentes populations de macrophages retrouvés dans les tissus non-parenchymaux dans le système nerveux central. Ces cellules sont situées à des positions stratégiques en bordure du parenchyme cérébral, près des vaisseaux sanguins cérébraux, dans les méninges et dans le plexus choroïdien [390].

3.3. Activation de la réponse inflammatoire dans le système nerveux central lors de l'infection à *Streptococcus suis*

Bien que *S. suis* cause une importante infection du SNC qui est responsable du développement de la méningite, les cellules impliquées sont peu connues. En effet, le nombre d'études portant sur cette infection était très limité au début de cette thèse. Il est à noter que les interactions des cellules résidentes du SNC et des cellules infiltrantes avec d'autres bactéries causant des méningites l'humaines (adhésion, invasion, phagocytose et réponse inflammatoire, mort cellulaire, etc.) peuvent différer selon le pathogène, probablement en raison de composants bactériens variés, ce qui justifie la nécessité de les étudier lors des infections à *S. suis* [299, 392-394].

3.3.1. Rôle des cellules résidentes et infiltrantes

Le rôle des cellules constituant les barrières du cerveau, soit les BMECs de la BBB et les CPECs de la BCSFB, dans la réponse inflammatoire a été peu étudié. Il apparaît cependant que les BMECs porcines et humaines produisent de l'IL-6 et du CXCL8 à la suite de l'infection par *S. suis* sérotype 2 [117, 149]. Cette réponse est due à la reconnaissance de composants de surface partiellement masqués par la présence de la CPS [117, 149]. De plus, la suilysine est, elle aussi, impliquée dans cette induction [117, 149]. Les BMECs humaines peuvent aussi sécréter du CCL2, ce qui n'a jamais été évalué avec les BMECs porcines [149]. Des analyses transcriptomiques des CPECs infectées avec *S. suis in vitro* ont démontré une régulation à la hausse de l'expression d'une panoplie de médiateurs pro-inflammatoires, dont le TNF, l'IL-1α, l'IL-1β, l'IL-6, le CCL2, le CCL4, le CCL5, le CXCL2, le CXCL5, le CXCL8 et les facteurs de croissance M-CSF et G-CSF [123]. De plus, la sécrétion de quantités élevées d'IL-6 et de CXCL8 a aussi été mesurée [123]. Ainsi, les cellules constituant la BBB et la BCSFB sont activées par *S. suis* et participent directement à la réponse inflammatoire induite. L'augmentation de l'expression de CCL2 dans le plexus choroïdien et les vaisseaux sanguins des cerveaux de souris infectées par *S. suis*, et d'IL-1β dans le plexus choroïdien, soutiennent ces résultats [286].

Quant aux cellules gliales, il a été démontré que la lignée de cellules microgliales BV-2 sécrète des niveaux élevés de TNF, d'IL-6 et de CCL2 à la suite de l'infection de *S. suis*, en plus d'une production marquée d'oxyde nitrique [125, 395]. Comme l'absence de CPS augmente leur production, cela indique que ce sont des composants de surface qui en sont responsables

[125, 395]. De plus, les astrocytes peuvent produire de l'IL-6, du CCL2 et du CXCL1 [126]. Comme pour la microglie, cette production par les astrocytes est la conséquence de la reconnaissance de composants de surface, mais la suilysine semble aussi y jouer un rôle. Finalement, il a été démontré, plus récemment, à l'aide de co-cultures de microglie et d'astrocytes, que les astrocytes sécrètent des composés solubles induisant la production d'oxyde nitrique par la microglie, lorsque celle-ci est infectée avec *S. suis* [127]. Cela indique qu'il y a une communication importante entre les différentes cellules du SNC lors de l'infection par ce pathogène.

Enfin, bien que les monocytes et les neutrophiles infiltrent massivement le SNC, lors de la méningite causée par *S. suis* [20, 22, 286], leur rôle, comme pour le compartiment systémique, reste inconnu dans celui-ci. En fait, à l'exception de la microglie, des astrocytes, des BMECs et des CPECs, le rôle d'aucun autre type cellulaire n'a été déterminé lors de l'infection du SNC causée par *S. suis*. Comme le milieu dans lequel se retrouve une cellule et les signaux reçus peuvent grandement influencer sa réponse, sa polarisation, son rôle et ses fonctions [396-398], le rôle des monocytes et neutrophiles infiltrants pourraient différer du compartiment systémique.

3.3.2. Rôle des « Toll-like receptors »

Comme pour le compartiment systémique, les travaux portant sur le rôle des TLRs dans le SNC étaient limités à deux études au début de cette thèse. Suite à l'infection par *S. suis*, une augmentation de l'expression transcriptionnelle du TLR2 a été observée dans la plupart des régions du cerveau, avec une expression plus marquée au niveau du corps calleux, de l'hippocampe et du cortex [286]. Cette expression du TLR2 a été associée aux astrocytes. De plus, une augmentation de l'expression du TLR3, mais pas du TLR4, a aussi été rapportée. Comme mentionné précédemment, le TLR3 ne dépend pas de la protéine adaptatrice MyD88, recrutant plutôt TRIF. Comme le TLR3 reconnaît l'ARN double brin, typiquement retrouvé chez les virus, cela indique que *S. suis* l'active par un ligand non identifié à ce jour [286]. Cependant, on ne peut affirmer, sans aucun doute possible, que le TLR3 est bel et bien impliqué dans la reconnaissance de *S. suis*. L'activation de la transcription de son ARN messager pourrait être le fruit d'un effet indirect. La seule autre étude portant sur le rôle des TLRs a démontré que le TLR2 est partiellement impliqué dans la production de TNF et de CCL2 par les astrocytes murins primaires. Ainsi, à l'exception de ces deux études, aucune autre information n'est

disponible concernant non seulement la voie des TLRs, mais aussi d'autres voies de signalisation, dans le SNC à la suite de l'infection à *S. suis*.

3.3.3. Réponse pro-inflammatoire locale

Malheureusement, outre les résultats présentés aux sections précédentes, très peu d'information supplémentaire est disponible sur la réponse inflammatoire locale qu'induit *S. suis in vivo*. En effet, si l'augmentation de l'expression d'IL-1β et de CCL2 dans la plupart des régions du cerveau, lors de l'infection, indique le développement d'une réponse pro-inflammatoire [286]. L'expression faible ou variable de TNF et d'IL-6 [286] contraste avec ce qui est observé pour d'autres pathogènes bactériens, dont *S. pneumoniae* [399, 400]. De plus, comme mentionné plus haut, l'expression de ces médiateurs ne se traduit pas nécessairement par leur production, particulièrement pour l'IL-1β, qui nécessite une étape de maturation supplémentaire. Ainsi, d'autres études sont primordiales, afin de mieux comprendre la réponse induite.

Basé sur cette recension de la littérature, l'**hypothèse générale** de cette thèse est que la pathogenèse de l'infection systémique et du SNC par *S. suis*, de même que la réponse inflammatoire induite, dépendent du bagage génétique et des caractéristiques phénotypiques des souches étudiées. En effet, les composants bactériens, ainsi que l'inflammation induite par la bactérie, varient selon l'origine de la souche (le sérotype, le ST, le lieu géographique, etc.).

L'**objectif général** de cette thèse est de mieux comprendre la pathogenèse de l'infection et de l'inflammation causées par *S. suis* à l'aide de souches différentes et d'évaluer l'impact de ces différences sur celles-ci.

Les **objectifs spécifiques** sont :

1. Étudier le rôle de différents composants de *S. suis* dans sa pathogenèse, notamment dans ses interactions avec l'hôte

2. Étudier l'impact de l'origine des souches et du choix des modèles expérimentaux sur les études de virulence et de pathogenèse de *S. suis*

3. Caractériser l'infection et l'inflammation causées par différents *S. suis* au niveau systémique et du SNC

III. MÉTHODOLOGIE ET RÉSULTATS

OBJECTIF 1 – ÉTUDIER LE RÔLE DE DIFFÉRENTS COMPOSANTS DE *STREPTOCOCCUS SUIS* DANS SA PATHOGENÈSE, NOTAMMENT DANS SES INTERACTIONS AVEC L'HÔTE

ARTICLE I

Interactions of *Streptococcus suis* serotype 9 with host cells and role of the capsular polysaccharide: Comparison with serotypes 2 and 14

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Manuscrit en préparation pour soumission à *PLoS One*

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur du manuscrit. J'ai été responsable de la conception de l'étude et des approches méthodologiques, j'ai participé activement aux expériences *in vitro* impliquant les cellules et j'ai effectué les expériences *in vivo*. J'ai aussi effectué l'analyse des résultats, l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci.

Mise en contexte

Des différents composants qu'expriment *S. suis*, la CPS est un facteur critique à sa virulence et au développement de la maladie clinique. En effet, des études précédentes ont démontré qu'en absence de CPS, *S. suis* est rapidement éliminé de la circulation par les leucocytes sanguins. De plus, sa présence interfère dans la capacité de *S. suis* à adhérer aux et d'envahir les cellules épithéliales et masque les composants de surface responsables de l'activation des cellules de l'hôte. Toutefois, ces propriétés et fonctions ont été démontrées avec des souches de sérotype 2, puis extrapolées aux autres sérotypes de *S. suis*. En effet, il n'y a qu'une seule publication ayant étudié un autre sérotype, soit le serotype 14 (annexe – article VI). Il est important de noter que la la CPS du sérotype 14 est très similaires à celle du sérotype 2 (structure et composition), dont la présence d'acide sialique. De plus, bien que les sérotypes 2 et 14 sont très importants mondialement, le sérotype 9 prédomine dans plusieurs pays européens et sa prévalence a grandement augmenté au cours des dernières années chez le porc au Canada et en Chine. Néanmoins, le rôle de la CPS du sérotype 9 en tant que facteur de virulence n'avait pas été évalué auparavant, et ce malgré une structure et une composition distincte.

Abstract

Streptococcus suis is an important porcine bacterial pathogen and a zoonotic agent responsible for sudden death, septic shock, and meningitis, of which serotype 2 is the most widespread, with serotype 14 causing human infections in South-Est Asia. Current knowledge of the *S. suis* pathogenesis and virulence is almost exclusively based on these two serotypes. Though serotype 9 is most commonly associated with porcine infections in Spain, the Netherlands, and Germany, very little information is currently available regarding the pathogenesis of this serotype. Of its different factors, the capsular polysaccharide (CPS) is required for *S. suis* virulence as it promotes resistance to phagocytosis and killing and masks surface components responsible for host cell activation. However, these roles have been described for serotypes 2 and 14, whose CPSs are structurally and compositionally similar. Consequently, we evaluated herein the interactions of serotype 9 with host cells and the role of its CPS, which greatly differs from those of serotypes 2 and 14. Results demonstrated that serotype 9 adhesion to but not invasion of respiratory epithelial cells was greater than that of serotypes 2 and 14. Furthermore serotype 9 was more internalized by macrophages but equally resistant to whole blood killing. Though recognition of serotypes 2, 9, and 14 by DCs required MyD88-dependent signaling, *in vitro* pro-inflammatory mediator production induced by serotype 9 was much lower than that by the other two serotypes. *In vivo*, however, serotype 9 causes an exacerbated inflammatory response responsible for host death during its systemic infection, suggesting a role of other innate immune cells. Meanwhile, presence of the serotype 9 CPS differentially modulated interactions with host cells in comparison to serotypes 2 and 14 by masking surface components less efficiently. However, its CPS remains a critical virulence factor required for bacterial survival in blood and development of clinical disease regardless of its unique composition and structure.

Introduction

Streptococcus suis is an important encapsulated bacterial pathogen of young piglets and a zoonotic agent causing a variety of diseases including sudden death (pigs), septic shock (humans), and meningitis (both species) [1]. Classification is based on serotyping defined by the antigenicity of the capsular polysaccharide (CPS) or its genes [1]. Of the thirty-five described serotypes, serotype 2 is the most widespread and virulent, being responsible for the majority of porcine and human cases of infection [2]. Alongside, serotype 14 is an emerging threat to human health in South-Est Asia [2]. Yet, our current understanding of the *S. suis* pathogenesis and virulence is almost exclusively based on serotype 2 [5, 6]. As such, our knowledge of other serotypes remains very limited. Alongside serotypes 2 and 14, serotype 9 has emerged in Europe in recent years and is presently responsible for the greatest number of porcine cases of *S. suis* infection in Spain, the Netherlands, and Germany [2]. Furthermore, its prevalence in China [7] and Canada [8] has significantly increased in recent years in pigs, and the first human case was recently reported in Thailand [9]. Nevertheless, very few studies have addressed the interactions of this serotype with host cells [10].

Though a variety of virulence factors have been described for *S. suis*, its CPS is one of the few truly critical factors required for its pathogenesis and is implicated in a multitude of functions [5, 6], which include, for example, resistance to phagocytosis and killing by innate immune cells [11-16] and masking of surface components responsible for host cell activation [15-17]. Indeed, presence of CPS interferes with recognition of *S. suis* by Toll-like receptor (TLRs), a family of evolutionarily conserved membrane-associated innate immune receptors that mainly signal via myeloid differentiation primary response 88 (MyD88) [17, 18]. Moreover, studies using experimental animal infection models have demonstrated that the CPS is required for survival of *S. suis* in blood [16, 19, 20]. Alongside, it was recently demonstrated that *S. suis* can modulate the presence of its CPS within the host [21], a mechanism that could participate in host cell adhesion and invasion since these functions are hampered by its presence [5].

However, these roles have been described for serotype 2 and, more recently, for serotype 14 [3, 14, 16, 20]. Moreover, the presence of CPS was also reported to confer anti-phagocytic properties to serotypes 1 and 1/2 [22]. Though certain structural and composition differences exist between the CPSs of these four serotypes, they are minimal: the serotype 14 and 1 and serotype 2 and 1/2 CPSs, respectively, differ by the substitution of a galactose to a *N*-

acetylgalactosamine resulting from a single nucleotide polymorphism in the glycosyltransferase CpsK [22-25]. In fact, switching CPS expression between serotypes 1 and 14 or between serotypes 1/2 and 2 had limited impact on virulence due to similar anti-phagocytic properties [22]. Furthermore, the CPSs of these four serotypes are characterized by presence of a sialic acid (*N*-acetylneuraminic acid) sidechain [23-25]. Sialic acid is commonly present in host cells and confers important properties to the cell surface [26]. Though different pathogens have evolved to express sialic acid at their surface, *S. suis* is one of only two sialylated Gram-positive bacteria, the other being Group B *Streptococcus* (GBS) [26]. Importantly, presence of sialic acid in GBS is associated with modulation of immune cell activation [27, 28]. Unfortunately, it has not yet been possible to evaluate the role of sialic acid in *S. suis* pathogenesis since deletion of sialyltransferase or sialic acid synthesis genes results in complete non-encapsulation, while mutations blocking its assembly are lethal due to an accumulation of intracellular sialic acid [16, 19, 29, 30]. As such, knowledge regarding the role of *S. suis* CPS in absence of sialic acid remains unknown. Interestingly, the composition and structure of the serotype 9 CPS was recently described and differs greatly from that of serotypes 2 and 14 [31]. While it includes presence of glucitol, phosphate, and a labile 4-keto sugar, it does not contain sialic acid [31].

Consequently, given the lack of information regarding the serotype 9 pathogenesis, its interactions with host cells and the role of its CPS were evaluated in comparison to the well-characterized serotypes 2 and 14. Results demonstrated that serotype 9 adhesion to, but not invasion of, respiratory epithelial cells was greater than that of serotypes 2 and 14. Furthermore serotype 9 was more highly internalized by macrophages but equally resistant to whole blood killing. While recognition of serotypes, 2, 9, and 14 by DCs required MyD88-dependent signaling, pro-inflammatory mediator production induced by serotype 9 was much lower that induced by serotypes 2 and 14. Presence of the serotype 9 CPS differentially modulated interactions with host cells in comparison to serotypes 2 and 14 by masking surface components less efficiently. However, its presence was critical for the development of clinical disease as it confers anti-phagocytic properties required for resisting killing by blood leukocytes.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (rech-1570).

Bacterial strains and growth conditions

The highly encapsulated wild-type *S. suis* serotype 2, 9, and 14 strains and their non-encapsulated isogenic mutants used in this study are listed in **Table 1**. As previously described, the serotype 9 1135776 strain was isolated from a diseased pig in Canada and belongs to sequence type 788 [32]. *S. suis* strains were cultured in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada). For *in vitro* cell culture assays, bacteria were prepared as previously described [15, 33] and resuspended in cell culture medium. For experimental infections, early stationary phase bacteria were washed twice in phosphate-buffered saline, pH 7.4, and resuspended in THB [34-36]. Bacterial cultures were appropriately diluted and plated on THB agar (THA) to accurately determine bacterial concentrations. The *Escherichia coli* strain and different plasmids used in this study are also listed in **Table 1**. When needed, antibiotics (Sigma-Aldrich, Oakville, ON, Canada) were added to the media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/mL; for *E. coli*, kanamycin and spectinomycin at 50 µg/mL and ampicillin at 100 µg/mL.

DNA manipulations

Genomic DNA was extracted from the *S. suis* serotype 9 1135776 strain using InstaGene Matrix solution (BioRad Laboratories, Hercules, CA, USA). Mini-preparations of recombinant plasmids were carried out using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Restriction enzymes and DNA-modifying enzymes (Fisher Scientific, Ottawa, ON, Canada) were used according to the manufacturer's recommendations. Oligonucleotide primers (**Table 2**) were obtained from Integrated DNA Technologies (Coralville, IA, USA) and PCRs carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or the Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using an ABI 310 Automated DNA

Sequencer and ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Construction of the serotype 9 non-encapsulated isogenic mutant

The serotype 2 and 14 non-encapsulated isogenic mutants P1/7Δ*cpsF* and DAN13730Δ*cpsB* were previously constructed and characterized in our laboratory [3, 15]. Precise in-frame deletion of *cpsG* gene from strain 1135776 was constructed using splicing-by-overlap-extension PCRs as previously described [37, 38]. Overlapping PCR products were cloned into pCR2.1 (Invitrogen, Burlington, ON, Canada), extracted with EcoRI, recloned into the thermosensitive *E. coli–S. suis* shuttle plasmid pSET4s, and digested with the same enzyme, giving rise to the knockout vector p4Δ*cpsG*. Electroporation of the serotype 9 wild-type strain 1135776 and procedures for isolation of the mutants were previously described [39]. Allelic replacement was confirmed by PCR and DNA sequencing analyses. Amplification products were purified with the QIAgen PCR Purification Kit (Qiagen) and sequenced as described above. mRNA expression of upstream and downstream genes flanking the *cpsG* gene in the mutant strain was confirmed by RT-PCR, validating in-frame gene deletion (data not shown). Growth of both mutants was similar to that of the wild-type strain (data not shown).

Bacterial surface hydrophobicity assay

Relative surface hydrophobicity of the *S. suis* wild-type strains and non-encapsulated mutants was determined by measuring adsorption to *n*-hexadecane as previously described [16].

Transmission electron microscopy

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Transmission electron microscopy was carried out as previously described [33, 40]. Briefly, bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer, pH 7.3 (Canemco & Marivac, Canton de Gore, QC). The CPS was stabilized using specific antibodies as previously described [40]. Anti-*S. suis* serotype 9 rabbit serum, produced as previously described [41], was used to gently resuspend bacteria. Next, cells were immobilized in 4% agar in 0.1 M cacodylate buffer, pH 7.3. Pre-fixation was performed by adding 0.1 M cacodylate buffer containing 0.5% glutaraldehyde, and 0.15% ruthenium red for 30 min. Fixation was performed for 2 h at room temperature with 0.1 M cacodylate buffer containing 5% glutaraldehyde and 0.05% ruthenium red. Post-fixation was carried out with 2% osmium tetroxide in water overnight

at 4°C. Samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in an increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections were post-stained with uranyl acetate and lead citrate and examined using a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

NPTr epithelial cell culture and adhesion and invasion assays

The porcine tracheal epithelial NPTr cell line (provided by M. Tonelli, Istituto Zooprofilattico Sperimentale, Italy) was used and cultured until confluence as previously described [42]. Cells were infected with 1 x 10^6 CFU/well (multiplicity of infection [MOI] = 10) of the different *S. suis* strains and incubated for 2 h or 4 h at 37 °C in 5% CO_2. The adhesion assay, which quantifies total cell-associated bacteria (surface-adherent and intracellular bacteria), and invasion assay (using the antibiotic protection assay) were performed as previously described [42].

J774A.1 macrophage culture and phagocytosis assays

The J774A.1 murine macrophage cell line (ATCC TIB-67; Rockville, MD, USA) was maintained in Dulbecco's Modified Eagle's Medium (Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Gibco) and grown at 37 °C with 5% CO_2. Confluent cell cultures were scraped, seeded at 1 x 10^5 cells/mL, and incubated for 3 h at 37 °C with 5% CO_2 to allow cell adhesion. Cells were infected by adding 1 x 10^7 CFU/mL of bacterial suspension in complete culture medium (MOI = 100), incubated for 1 h or 2 h at 37 °C with 5% CO_2, and phagocytosis assays performed as previously described using the antibiotic protection assay [34].

Whole blood bactericidal (killing) assay

Blood was collected from six- to ten-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA) and mixed with sodium heparin (Sigma-Aldrich). Whole blood containing 9 x 10^6 leukocytes/mL on average was transferred to a microtube containing 9 x 10^6 CFU/mL of the different *S. suis* strains (MOI = 1) and incubated for 4 h, mixing every 20 min. Assay conditions were chosen based on the kinetics of *S. suis* killing by murine blood [34]. After incubation, cells were lysed and appropriate dilutions plated on THA to determine viable bacterial counts. Resistance to bacterial killing by blood leukocytes was compared to incubation in plasma alone (obtained by centrifuging whole blood at 1 800 x *g* for 10 min at 4 °C). The

percentage of bacteria killed was determined using the following formula: 1 - (bacteria in blood / bacteria in plasma) / 100%.

Generation of bone marrow-derived dendritic cells and activation

The femur and tibia from C57BL/6, MyD88$^{-/-}$ (B6.129P2(SJL)-*MyD88*$^{tm1.Defr}$/J), TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmKir}/J), and TLR4$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps-del}$/JthJ) mice on C57BL/6J background (Jackson Research Laboratories, Bar Harbor, ME, USA) were used to generate bone marrow-derived DCs, as previously described [15]. Briefly, bone marrow cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (Gibco) and complemented with 20% granulocyte-macrophage colony-stimulating factor from mouse-transfected Ag8653 cells [43]. Cell purity was confirmed to be at least 85% CD11c^{+} by flow cytometry as previously described [15]. Prior to infection, cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different strains *S. suis* strains (1 x 10^6 CFU/mL; initial MOI = 1). Conditions used were based on those previously published [15, 18]. Supernatants were collected 16 h following infection with *S. suis*, time at which secreted cytokine levels were maximal in the absence of *S. suis*-induced DC cytotoxicity as determined by lactate dehydrogenase release (data not shown) [15, 18]. Non-infected cells served as negative controls. Secreted levels of tumor necrosis factor (TNF), interleukin (IL)-6, C-C motif chemokine ligand (CCL) 2, CCL3, and C-X-C motif chemokine ligand (CXCL) 1 were quantified by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations.

S. suis *virulence mouse model of infection*

A CD-1 mouse model of infection was used [19, 35]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering through the use of humane endpoints, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. Thirty 6-week-old female CD-1 mice (Charles River Laboratories) were used for these experiments (15 mice per strain). Mice were inoculated with 1 x 10^7 CFU via the intraperitoneal route and health and behavior monitored at least thrice daily until 72 h post-infection and twice thereafter until the end of the experiment (10 days post-infection) for the development of clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, prostration, and lethargy. Blood samples were

collected from the caudal vein of surviving mice 12 h and 24 h post-infection and plated as previously described [34].

Statistical analyses

Normality of data was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test) or non-parametric tests (Mann-Whitney rank sum test), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare survival between wild-type-infected mice and those infected with the non-encapsulated strains. Each *in vitro* test was repeated in at least three independent experiments. $p < 0.05$ was considered as statistically significant.

Results

Deletion of the Streptococcus suis *serotype 9 cpsG gene causes a non-encapsulation phenotype*

Previous studies have demonstrated that deletion of various CPS biosynthesis genes from serotypes 2 and 14 results in a non-encapsulated phenotype [3, 15, 16, 19, 20, 44]. As such, an isogenic mutant in which the *cpsG* gene was deleted from the North American serotype 9 1135776 strain was constructed and compared with serotypes 2 and 14. Surface hydrophobicity (an indicator of encapsulation) of serotype 9 was low (less than 5%) and comparable to that of serotype 2 and 14 (**Fig. 1**). Meanwhile, deletion of *cpsG* gene significantly increased surface hydrophobicity ($p = 0.02$), with similar values to those obtained with the serotype 2 and 14 non-encapsulated mutants ($p = 0.02$) (**Fig. 1**). This confirms that deletion of *S*. *suis* CPS biosynthesis genes results in high surface hydrophobicity regardless of serotype.

Encapsulation levels of the serotype 9 wild-type strain and Δ*cpsG* mutant were then confirmed by transmission electron microscopy following antibody stabilization. The wild-type strain possessed a layer of CPS at its surface, indicative of being well-encapsulated (**Fig. 2A**). This is similar to the serotype 2 strain wild-type P1/7 strain and serotype 14 wild-type DAN13730 strain used herein [3, 19, 33]. Meanwhile, the serotype 9 mutant clearly lacked presence of CPS (**Fig. 2B**), as previously reported for the serotype 2 and 14 non-encapsulated mutants [3, 19]. Consequently, these results confirm that deletion of CPS biosynthesis genes from serotype 9 results in non-encapsulation.

Presence of capsular polysaccharide partially or significantly modulates* Streptococcus suis *serotype 9 adhesion to and invasion of respiratory epithelial cells, respectively

The serotype 2 CPS has been described to mask bacterial surface adhesins involved in the initial interactions with host cells, including adhesion to and invasion of epithelial cells [5]. Using NPTr porcine tracheal epithelial cells, the adhesion and invasion capacities of the different wild-type and non-encapsulated mutant strains were evaluated. After 2 h of incubation, the serotype 9 wild-type strain adhered to epithelial cells, with adhesion levels increasing by 4 h (**Fig. 3A-B**). However, adhesion of serotype 9 was significantly greater than that of serotypes 2 and 14 regardless of incubation time ($p = 0.012$), which were similar (**Fig. 3A-B**). Meanwhile, although non-encapsulation significantly increased adhesion of serotypes 2 and 14 regardless of incubation time ($p = 0.002$), that of serotype 9 slightly modulated the adhesion to epithelial cells, but only at 2 h incubation ($p = 0.027$) (**Fig. 3A-B**). Consequently, presence of the serotype 9 CPS hardly masks surface adhesins.

Following adhesion, *S. suis* may invade cells. Serotype 9 invaded little epithelial cells, even after 4 h of incubation, which was similar to serotypes 2 and 14 (**Fig. 3C-D**), indicating that higher adhesion is not necessarily followed by increased bacterial internalization. On the other hand, invasion by the non-encapsulated serotype 9 mutant was significantly greater than that of the wild-type strain at both 2 h and 4 h ($p = 0.008$) (**Fig. 3C-D**). Not only were similar differences also reported for the non-encapsulated serotype 2 and 14 mutants ($p = 0.008$), but levels of internalized bacteria were comparable between non-encapsulated mutants (**Fig. 3C-D**).

Presence of capsular polysaccharide confers anti-phagocytic properties to* Streptococcus suis *from macrophages regardless of serotype

Phagocytosis studies with *S. suis* serotype 9 were carried out with J774A.1 murine macrophages, as previously reported for serotypes 2 and 14 [3, 14, 34]. Serotype 9 was internalized after 1 h of incubation, with intracellular bacteria increasing with time (**Fig. 4A-B**). By contrast, the serotypes 2 and 14 were not internalized (1 h) or significantly less internalized (2 h) by macrophages than serotype 9 ($p = 0.008$) (**Fig. 4A-B**). Of the different properties attributed to the presence of the *S. suis* serotype 2 and 14 CPS, resistance to phagocytosis is amongst the most important [3, 14, 19]. Indeed, results were confirmed in the present study

(p = 0.002) (**Fig. 4A-B**). Results also showed that non-encapsulation of serotype 9 significantly increased phagocytosis by macrophages (p = 0.01), with levels of intracellular bacteria remaining stable (**Fig. 4A-B**). Taken together, these results demonstrate that though the *S. suis* CPS confers anti-phagocytic properties regardless of the serotype, the serotype 9 CPS protects less efficiently against phagocytosis than those of serotypes 2 and 14.

Presence of capsular polysaccharide is required for* Streptococcus suis *whole blood bactericidal resistance regardless of serotype

Results showed that the serotype 9 wild-type strain was as resistant to whole blood bacterial killing as serotypes 2 and 14 **(Fig. 5)**. Confirming previous data [3, 16, 19, 34], results also showed that the CPS is required for survival and persistence of *S. suis* serotypes 2 and 14 in blood (**Fig. 5**). In addition, the serotype 9 non-encapsulated mutant was significantly less resistant to killing (p = 0.0002) than its wild-type strain, with 30% of bacteria killed after 4 h (**Fig. 5**). Although the serotypes 9 and 14 non-encapsulated mutants were comparably killed, they were both significantly more resistant than the serotype 2 non-encapsulated mutant (p = 0.02) (**Fig. 5**).

Modulation of dendritic cell pro-inflammatory mediator production by presence of* Streptococcus suis *capsular polysaccharide and recognition by the Toll-like receptor pathway are comparable between serotypes

Bone marrow-derived DCs were used as an innate immune cell model given that DCs play a critical rôle during *S. suis* pathogenesis and that their response to *S. suis* serotype 2 has been well-characterized [15, 16, 18, 33, 45]. Surprisingly, and with the exception of TNF, serotype 9 strain induced lower levels of the different pro-inflammatory mediators evaluated, especially of CCL2 and CXCL1, when compared to serotypes 2 and 14 (p = 0.006) (**Fig. 6**). Meanwhile, levels induced by serotypes 2 and 14 were comparable (**Fig. 6**).

Alongside phagocytosis resistance, the serotype 2 CPS was been well-described to interfere in recognition of *S. suis* by innate immune cells by masking immunostimulatory bacterial surface components [5], a property not yet investigated for serotypes 9 and 14. Lack of CPS significantly increased production of TNF, IL-6, and CXCL1 (p = 0.01), but not of CCL2 nor CCL3 by serotype 9, with similar results for serotypes 2 and 14 (p = 0.001) (**Fig. 6**). Consequently, the inflammatory response induced by serotype 9 from DCs is markedly lower

than that by serotypes 2 and 14, with presence of its CPS modulating this production somewhat less.

S. suis-induced inflammation results from its recognition via different conserved pathways, of which the TLR pathway has been best described for serotype 2 [5]. Moreover, since recognition of *S. suis* serotype 2 mainly occurs at the host cell surface, TLR2 is the main receptor involved [17, 18]. In addition, TLR4 has been suggested to recognize the pore-forming toxin suilysin [46], produced by the serotype 2 and 14 strains used herein, but not by the serotype 9 strain [32, 47]. Importantly, recruitment of the adaptor protein MyD88 is central to the TLR pathway [48]. As such, the role of the TLR pathway in the recognition of *S. suis* by DCs was investigated. Absence of MyD88 lead to a near complete abrogation of pro-inflammatory mediator production induced by serotypes 9, 2, and 14 (p = 0.0002) (**Fig. 7**). Moreover, production of these mediators as induced by serotype 9 was partially dependent on TLR2, with a 50% to 60% decrease in its absence (p = 0.02), which was similar to what was observed with serotypes 2 and 14 (p = 0.01) (**Fig. 7**). By contrast, recognition of serotype 9, as with serotypes 2 and 14, was TLR4-independent (**Fig. 7**). Taken together, these results demonstrate that pro-inflammatory mediator production induced by *S. suis* serotype 9 from DCs is MyD88-dependent and partially requires TLR2 but not TLR4.

Presence of capsular polysaccharide is required for virulence of* Streptococcus suis *serotype 9 and development of systemic disease in a mouse model of infection

To evaluate the role of the *S. suis* serotype 9 CPS in virulence and development of clinical disease, CD-1 mice, commonly used for serotype 2 virulence studies [16, 19, 35], were infected with the serotype 9 wild-type and mutant strain by intraperitoneal inoculation. Wild-type strain-infected mice rapidly developed clinical signs of systemic disease characteristic of sepsis and septic shock with 100% of mice succumbing to infection within 48 h (**Fig. 8A**). By contrast, none of the mice infected with the non-encapsulated mutant succumbed to disease (p = 0.0002) (**Fig. 8A**). In fact, these mice only developed transient signs of infection such as rough coat hair following inoculation of bacteria. Viability of inocula was verified prior to and after infection with no differences between (data not shown).

To better explain differences in virulence, blood bacterial burden was evaluated 12 h and 24 h post-infection. All mice infected with the wild-type strain presented elevated blood bacterial

burdens that averaged 5 x 10^8 CFU/mL (**Fig. 8B-C**). In fact, levels were comparable to those obtained upon euthanasia of mice suffering from septic shock (2 x 10^9 CFU/mL). On the other hand, almost no bacterial burden was detected in the blood of mice infected with the non-encapsulated mutants, levels of which were not only significantly lower than those of mice infected with the wild-type strain (p = 0.0002), but almost undetectable (**Fig. 8B-C**).

Discussion

Colonization (establishment of the pathogen at the portal of entry in the absence of infection) is amongst the first steps of the *S. suis* pathogenesis, with CPS expression playing an important role therein for serotype 2 [49-51]. The serotype 9 strain adhered more to tracheal epithelial cells than serotypes 2 and 14 and, differently from the latter two, its CPS hardly inhibited these interactions. This would not be due to a lower expression of CPS in the serotype 9 strain since all three serotypes were similarly well-encapsulated. Indeed, these results suggest that the serotype 9 surface adhesins may remain at least partially exposed in the presence of its CPS or that serotype 9 possesses different adhesins. Using these same strains, it was previously reported that serotype 9 adheres more to extracellular matrix components (collagen I, fibrinogen, and fibronectin) and salivary agglutinin proteins than serotype 2 [52]. In addition, though serotypes 2 and 9 both express the adhesin SadP, its amino acid sequence greatly differs between them, which might translate in functional or affinity differences [10, 53]. Although serotype 9 also adhered more to porcine intestinal epithelial cells than serotype 2, adhesion by a non-encapsulated serotype 9 mutant was greater than that of its wild-type strain, by contrast with serotype 2 [10]. Furthermore, its was demonstrated, at least for serotype 9, that adhesion significantly varies depending on genotype [10]. While previous results with intestinal cells were obtained using a highly virulent sequence type 16 European strain, the North American serotype 9 strain used herein and belonging to sequence type 788, is probably less virulent as indicated by the absence of classical virulence markers [32]. Though encapsulation inhibited serotype 9 invasion of tracheal epithelial cells by rendering the bacterial factors involved inaccessible, this effect was comparable to that of serotypes 2 and 14. This indicates that the factors involved in serotype 9 adhesion and invasion probably differ. Interestingly, invasion of intestinal epithelial cells was greater by non-encapsulated serotype 2 than serotype 9 [10], suggesting that different adhesins may be involved in bacterial-host interactions in both the respiratory and intestinal tracts.

It has been reported that the CPS is a critical anti-phagocytic and virulence factor for *S. suis* [5, 54]. However, these conclusions are based on serotype 2 and, more recently, on serotype 14 studies [3]. Herein, serotype 9 was more susceptible to phagocytosis than serotypes 2 and 14, with similar results for the latter two. It has been previously demonstrated that serotype 9 is also more internalized by human monocyte-derived DCs than serotypes 2 and 14 [55]. Despite these observations, the absence of CPS significantly increased phagocytosis levels of *S. suis* serotype 9, confirming a certain anti-phagocytic role as in serotypes 2 and 14. It can be hypothesized that the composition of the serotypes 2 and 14 CPSs, which are similar, including presence of a sialic acid sidechain, may play a role in such increased resistance to phagocytosis [23, 24]. Indeed, the composition and structure of the CPS of serotype 9 greatly differs from these two serotypes, as it contains glucitol, phosphate, and a labile 4-keto sugar, all of which are absent from the serotype 2 and 14 CPSs but does not contain sialic acid [31]. This last component has been suggested to reduce complement-dependent phagocytosis [56, 57]. Also, and similarly to what may happen during the interactions with epithelial cells, the serotype 9 CPS might not mask the surface components responsible for activation of phagocytic mechanisms as efficiently as serotypes 2 and 14, or these might be differentially expressed and/or exposed.

Although clear differences were observed with the phagocytosis assay, encapsulated serotypes 2, 9 and 14 were all equally resistant to killing by whole blood, with the CPS playing a significant role in blood survival. However, neutrophils and monocytes are the main phagocytes in blood, with little to no macrophages being present [58]. Moreover, it was reported that *in vitro* phagocytosis assays do not necessarily correlate with blood killing and virulence *in vivo* [34]. In this study, the protective role of the CPS was confirmed *in vivo* since non-encapsulation of serotype 9 resulted in near complete elimination from the bloodstream after 24 h of infection. In fact, in absence of its CPS, serotype 9 was unable to persist, disseminate systemically, and cause disease, being avirulent. This indicates that components and structure of the CPS (as well as the presence or absence of sialic acid) do not influence the capacity of the CPS to protect *S. suis*.

Levels of pro-inflammatory mediators induced by serotype 9 from DCs were, with the exception of TNF, markedly lower than those induced by serotypes 2 and 14. In addition, the absence of CPS from serotype 9 also increased the release of pro-inflammatory mediators. However, this

effect was less notable than for serotypes 2 and 14, suggesting that even when present, CPS only partially masks the lipoproteins and other immunostimulatory surface components involved in DC activation. Since serotype 9 lipoproteins were suggested to possess greater immunostimulatory properties than those of serotype 2 [55, 59], these results were somewhat unexpected. Although we do not have a clear explanation, these differences might be due to host cell origin or geographical origin of the strain used, since the North American serotype 9 strain used herein does not produce, amongst other things, suilysin [32], which has also been described to induce pro-inflammatory mediators [15, 46]. Though this serotype 9 strain induced lower pro-inflammatory mediator production from DCs than serotypes 2 and 14, its immunostimulatory potential remains notable since 100% of mice infected with the wild-type strain developed septic shock within 48 h of infection. This indicates that probably other cell types contribute to serotype 9-induced systemic inflammation, including monocytes, neutrophils and Natural Killer cells, which are important sources of plasma pro-inflammatory mediators during bacterial infection [60]. However, it is impossible to exclude that induced bone marrow derived-DCs have the exact same properties and/or phenotypes as their "natural" counterparts. Finally, although differences in inflammatory mediator production were observed, receptors involved in recognition of serotypes 2, 9, and 14 are similar. Indeed, the importance of TLR pathway, and mainly TLR2, was comparable for the three serotypes.

In conclusion, the interactions between *S. suis* serotype 9 and host cells contrast with those of serotypes 2 and 14 due, at least in part, to differential modulation by its CPS. Indeed, even when present, the serotype 9 CPS does not mask surface adhesins and prevents phagocytosis as efficiently as those of serotype 2 and 14. However, the serotype 9 CPS remains a critical virulence factor required for bacterial survival in blood and development of clinical disease regardless of its unique composition and structure, including absence of sialic acid. Though serotype 9 induces lower production of pro-inflammatory mediators from DCs, it causes an exacerbated inflammatory response responsible for host death during its systemic infection *in vivo*, suggesting a role of other innate immune cells. Meanwhile, recognition of *S. suis* requires MyD88-dependent signaling and mostly TLR2, regardless of the serotype, indicating that evolutionarily conserved bacteria components are responsible for initial host cell recognition. Future studies using additional strains will help better understand and confirm these results.

Acknowledgements

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Tables

Table 1. List of strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
Streptococcus suis		
P1/7	Virulent serotype 2 (S2) strain isolated from a case of pig meningitis in the United Kingdom	[401]
P1/7Δ*cpsF*	Non-encapsulated isogenic mutant derived from P1/7; in frame deletion of *cpsF* gene	[139]
DAN13730	Virulent serotype 14 (S14) strain isolated from a human case in the Netherlands	[402]
DAN13730Δ*cpsB*	Non-encapsulated isogenic mutant derived from DAN13730; in frame deletion of *cpsB* gene	[142]
1135776	Virulent serotype 9 (S9) strain isolated from a diseased pig in Canada	[72]
1135776Δ*cpsG*	Non-encapsulated isogenic mutant derived from 1135776; in frame deletion of *cpsG* gene	This study
Escherichia coli		
TOP10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
Plasmids		
pCR2.1	Ap^r, Km^r, pUC *ori*, *lac*ZΔM15	Invitrogen
pSET4s	Spc^r, pUC *ori*, thermosensitive pG+host3 *ori*, *lac*ZΔM15	[403]
p4Δ*cpsG*	pSET-4s carrying the construct for *cpsG* allelic replacement	This study

Table 2. List of oligonucleotide primers used in this study.

Name	Sequence (5' – 3')
cpsG-1	GGTAAGATTGAGTTGGTCC
cpsG-2	CTGATTGAGTGGCCCATCCTC
cpsG-3	GCAAACATTGATGAAACACT
cpsG-4	ATGAGATATGATGGCAAGCC
cpsG-5A	GGTGGACCTGTTACGGTTACC
cpsG-5B	GCGCGAATTCGTCTTGGATATGGGCGAGCCAG
cpsG-6	TCCATAAATGAGTTTTTCCCTAAGAAACTC
cpsG-7	GAGTTTCTTAGGGAAAAACTCATTTATGGA
cpsG-8A	CCCATCACGTCAAATAATGTC
cpsG-8B	CGCGGAATTCATCATCGTCATCCTTCATTGC

Figures

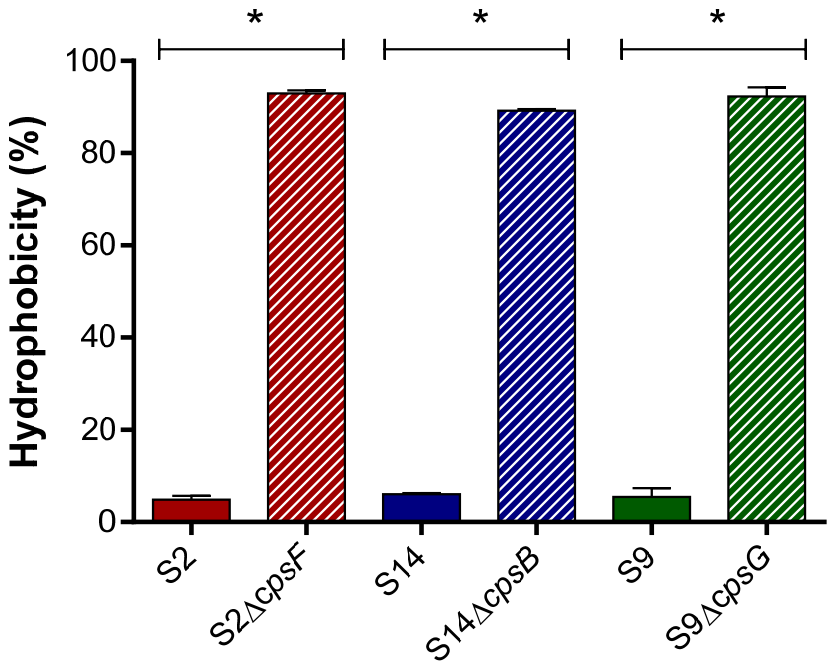

Figure 1. Absence of *Streptococcus suis* capsular polysaccharide is associated with increased surface hydrophobicity. Surface hydrophobicity of the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type and mutant strains was determined using *n*-hexadecane. Data represent the mean ± SEM (n = 3). * ($p < 0.05$) indicates a significant difference between wild-type and mutant strains.

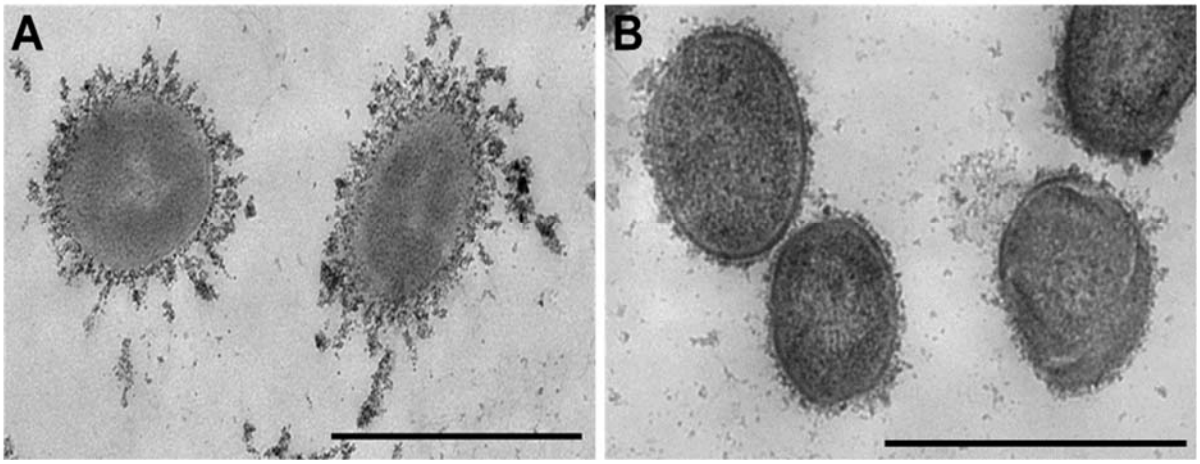

Figure 2. Deletion of *Streptococcus suis* serotype 9 *cpsG* gene results in non-encapsulation. Transmission electron microscopy following stabilization with anti-*S. suis* serotype 9 rabbit serum of the serotype 9 (S9) wild-type (**A**) and mutant strain (**B**). Black bars = 1 µm.

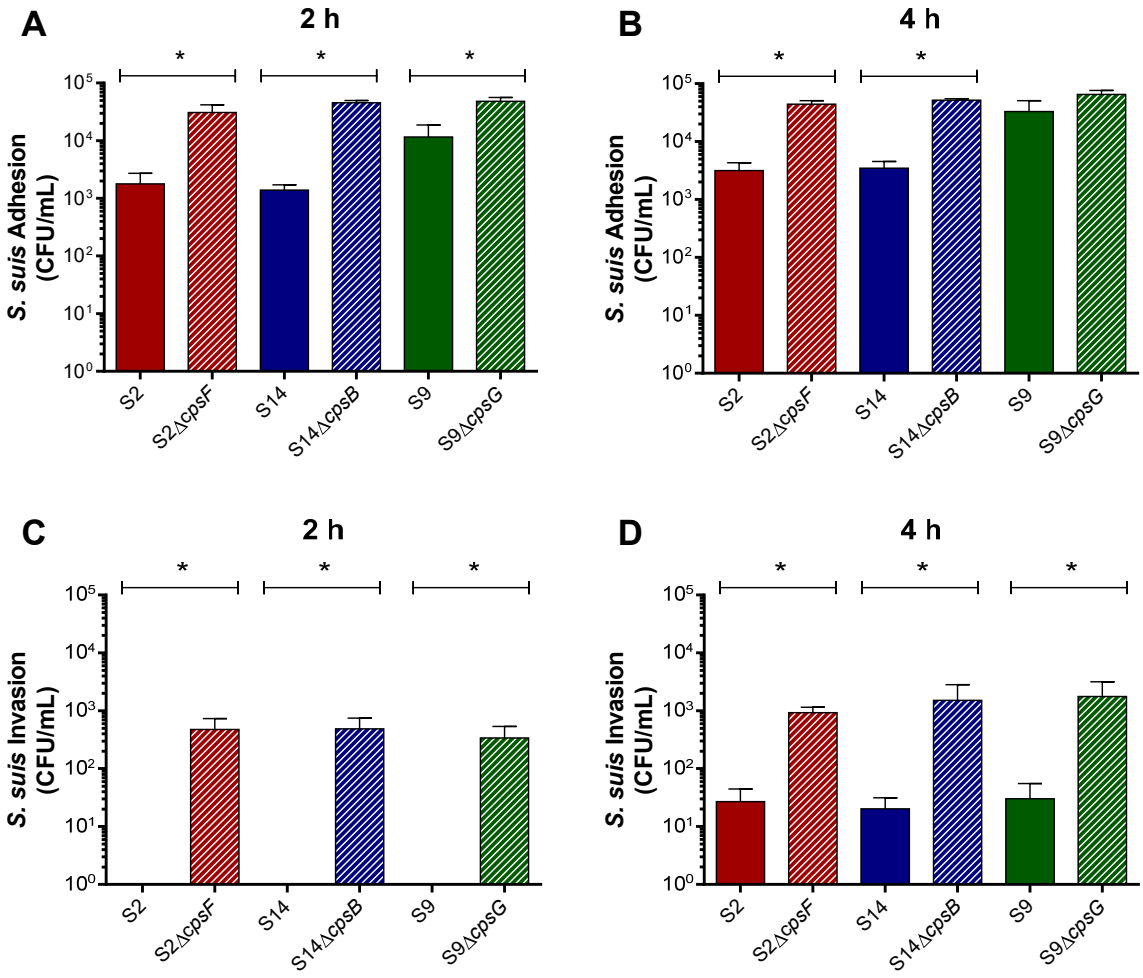

Figure 3. Presence of capsular polysaccharide partially or significantly modulates *Streptococcus suis* serotype 9 adhesion to and invasion of respiratory epithelial cells, respectively. Adhesion (**A** & **B**) and invasion (**C** & **D**) of the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type and mutant strains to NPTr porcine tracheal epithelial cells after 2 h (**A** & **C**) or 4 h (**B** & **D**) of incubation. Data represent the mean ± SEM (n = 4). * ($p < 0.05$) indicates a significant difference between wild-type and mutant strains.

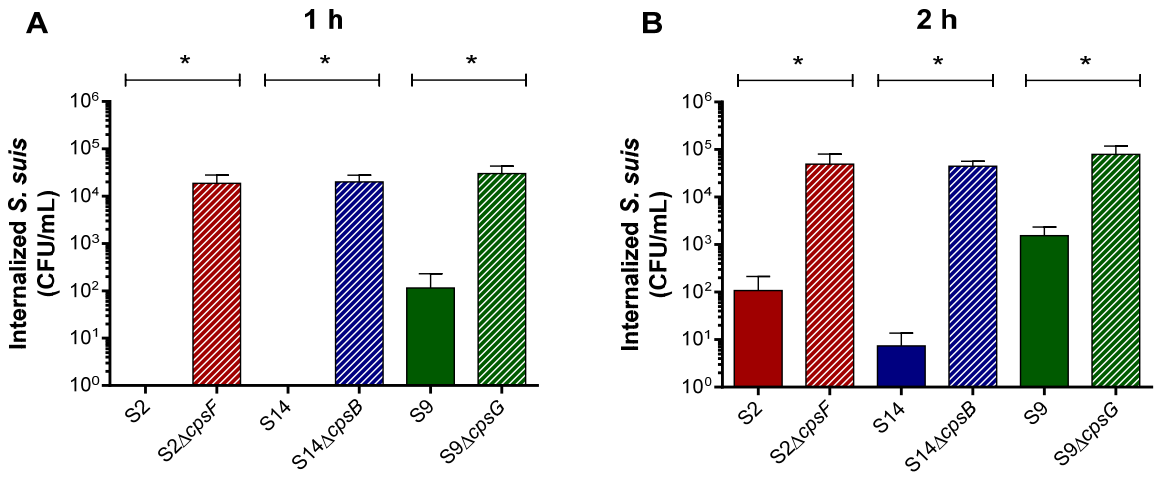

Figure 4. Presence of *Streptococcus suis* capsular polysaccharide confers anti-phagocytic properties towards macrophages regardless of serotype. Internalization of the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type and mutant strains by J774A.1 murine macrophages after 1 h (**A**) or 2 h (**B**) of incubation. Data represent the mean ± SEM (n = 4). * ($p < 0.05$) indicates a significant difference between wild-type and mutant strains.

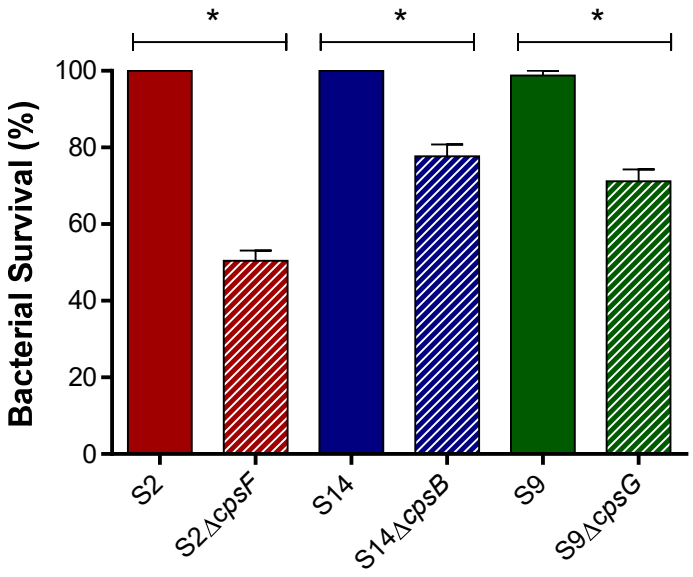

Figure 5. Presence of capsular polysaccharide is required for *Streptococcus suis* whole blood bactericidal resistance regardless of serotype. Capacity of the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type and mutant strains to resist the bactericidal effect of murine whole blood after 4 h of incubation. Percentage of bacterial survival was calculated in comparison to bacteria in plasma alone. Data represent the mean ± SEM (n = 3). * ($p < 0.05$) indicates a significant difference between wild-type and mutant strains.

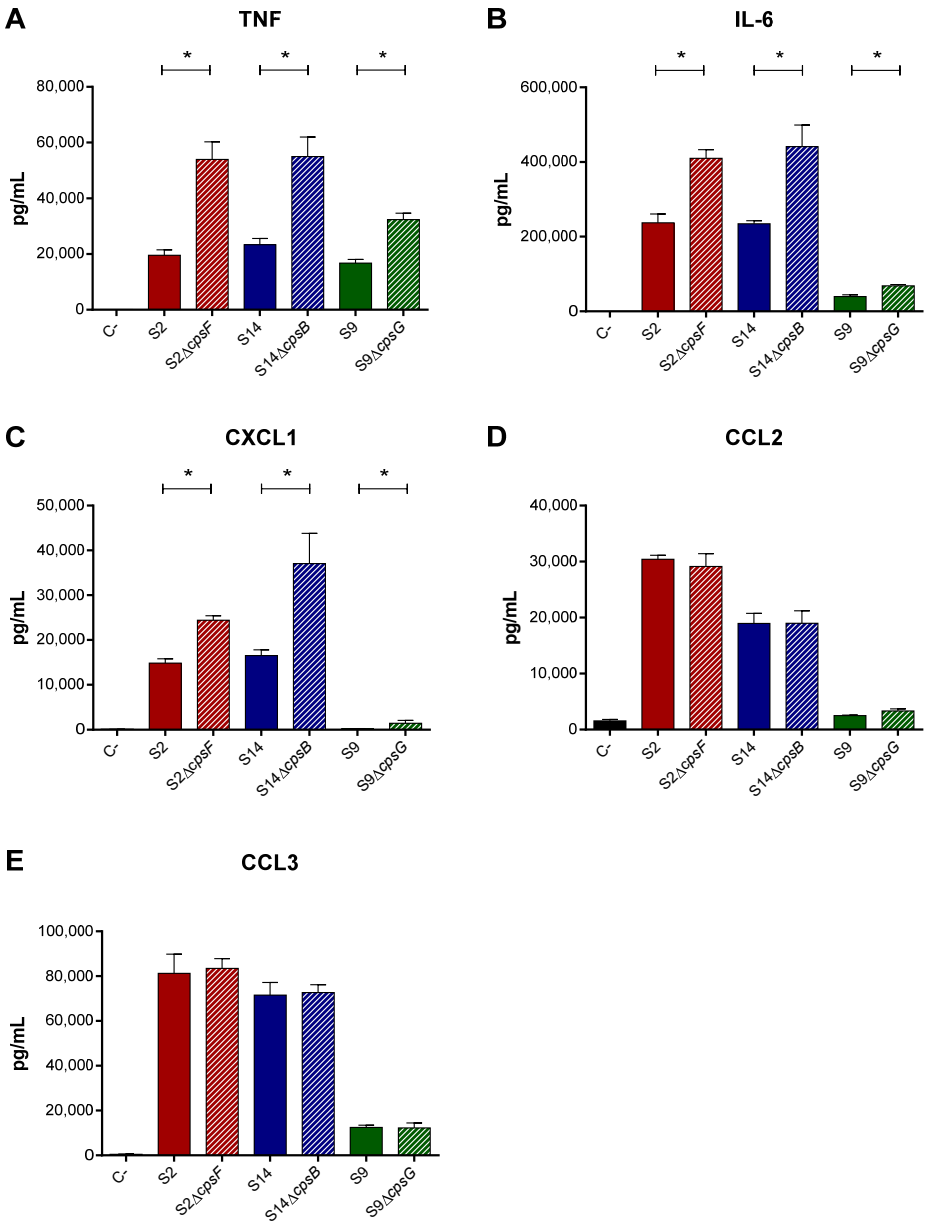

Figure 6. Presence of capsular polysaccharide modulates *Streptococcus suis*-induced pro-inflammatory mediator production by dendritic cells (DCs) regardless of serotype. Pro-inflammatory mediator production by DCs following infection with the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type and mutant strains after 16 h of incubation, as measured by ELISA. Production of TNF (**A**), IL-6 (**B**), CXCL1 (**C**), CCL2 (**D**), and CCL3 (**E**). Data represent the mean ± SEM (n = 4). C- denotes cells in medium alone. * ($p < 0.05$) indicates a significant difference between wild-type and mutant strains.

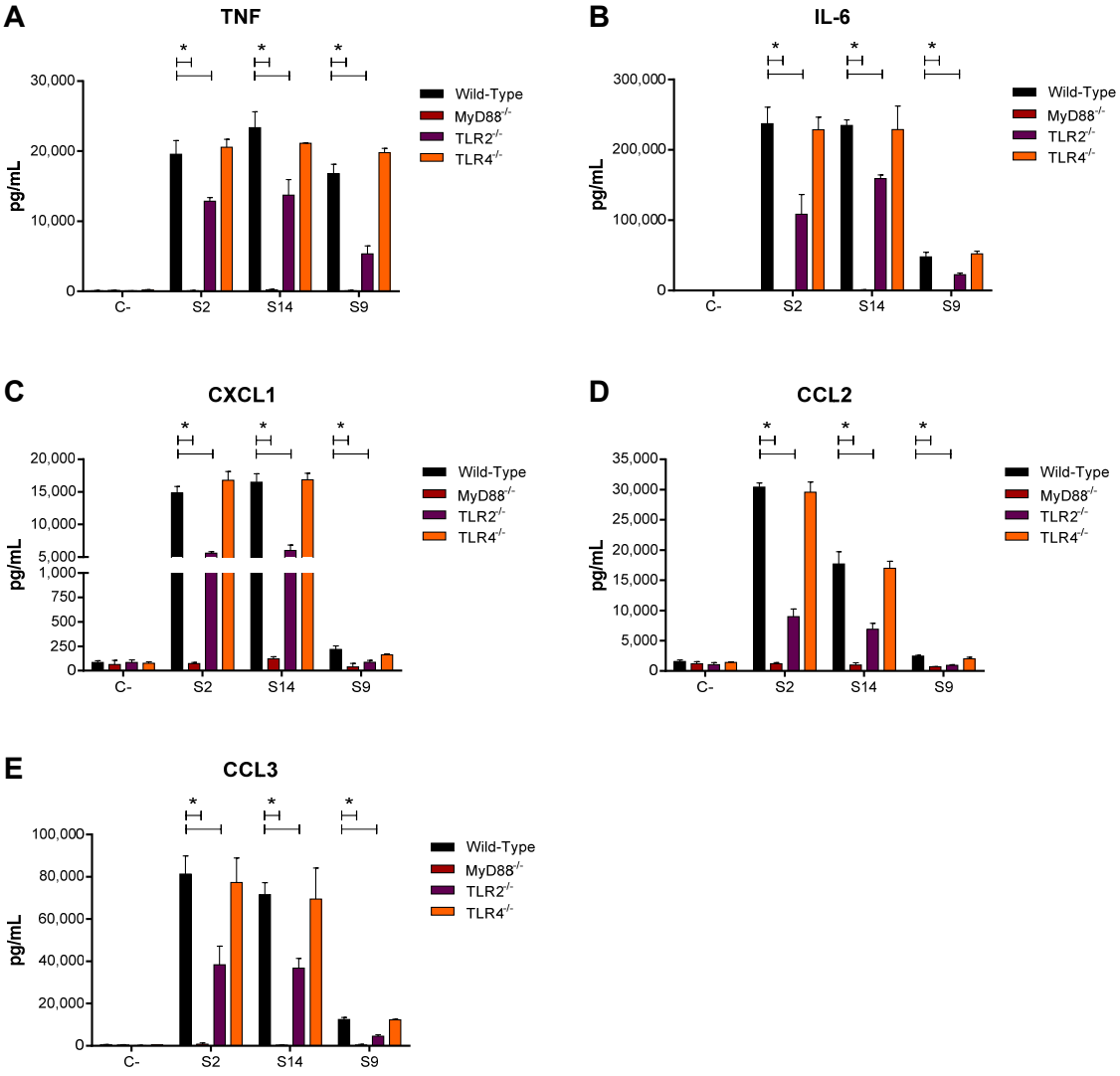

Figure 7. MyD88-dependent Toll-like receptor (TLR) signaling is required for *Streptococcus suis*-induced pro-inflammatory mediator production by dendritic cells (DCs) regardless of serotype. Pro-inflammatory mediator production by wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs following infection with the *S. suis* serotype 2 (S2), 9 (S9) or 14 (S14) wild-type strains after 16 h of incubation, as measured by ELISA. Production of TNF (**A**), IL-6 (**B**), CXCL1 (**C**), CCL2 (**D**), and CCL3 (**E**). Data represent the mean ± SEM (n = 4). C- denotes cells in medium alone. * ($p < 0.05$) indicates a significant difference between wild-type and knockout cells.

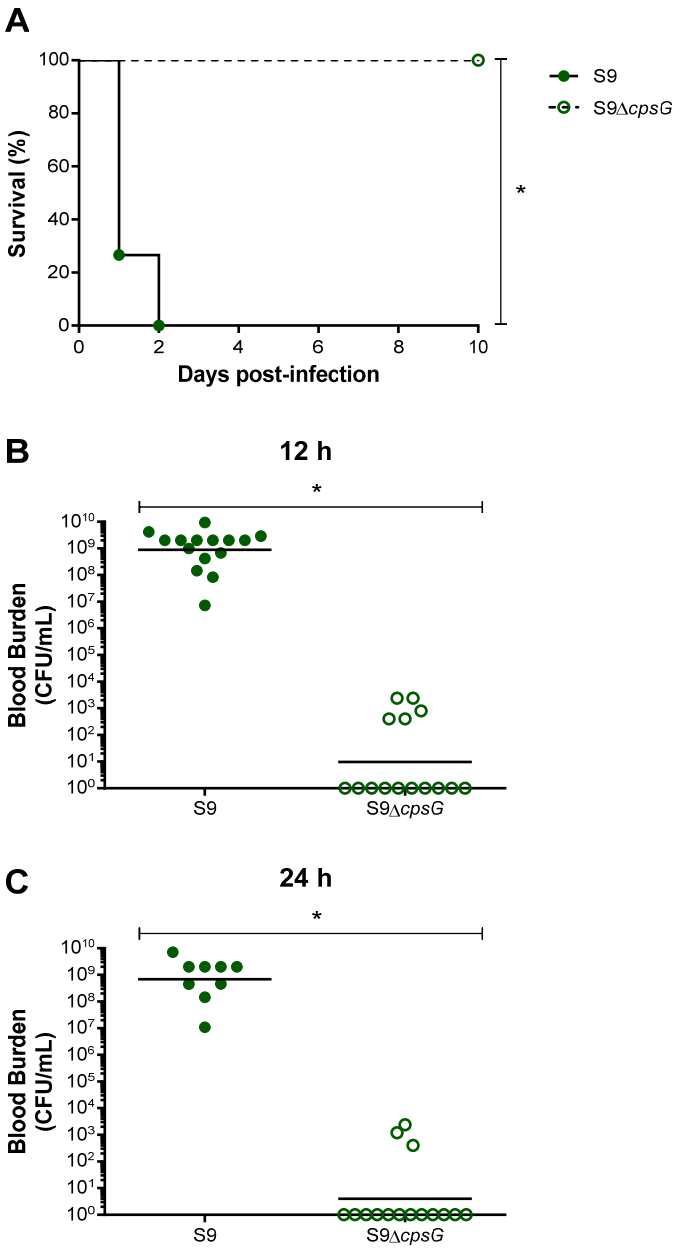

Figure 8. Capsular polysaccharide is required for *Streptococcus suis* serotype 9 virulence and persistence in blood in a mouse model of infection. Survival (**A**) and blood bacterial burden 12 h (**B**) and 24 h post-infection (**C**) of CD-1 mice following intraperitoneal inoculation of the *S. suis* serotype 9 wild-type (S9) or mutant (S9Δ*cpsG*) strain. Data represent survival curves (A) or geometric mean (B & C) (n = 15). * ($p < 0.05$) indicates a significant difference between survival or blood bacterial burden of mice infected with wild-type and mutant strain.

ARTICLE II

Role of the *Streptococcus suis* serotype 2 capsular polysaccharide in the interactions with dendritic cells is strain-dependent but remains critical for virulence

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai été responsable de la conception de l'étude et des approches méthodologiques, j'ai participé activement aux expériences *in vitro* impliquant les cellules, aux ELISA et à la RT-qPCR et j'ai effectué les expériences *in vivo*. J'ai effectué l'analyse des résultats, l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci. J'ai aussi effectué les corrections lors des étapes de révision pour publication.

Mise en contexte

Les résultats obtenus dans l'article I ont démontré que la présence de la CPS confère à *S. suis* d'importantes propriétés et participe grandement à sa virulence, et ce pour les sérotypes 2, 9 et 14. Toutefois, cette étude et les études précédentes ont utilisé soit des souches de référence ou des souches dites "classiques". En effet, toutes les études portant sur le rôle et les fonctions de la CPS du sérotype 2 ont été effectuées avec des souches appartenant au ST1 et/ou ST7 d'origines européennes ou asiatiques. Toutefois, les souches nord-américaines diffèrent grandement des souches européennes et asiatiques, autant sur le plan génétique que phénotypique. Entre autres, 95% de souches nord-américaines appartiennent au ST25 ou au ST28 et ont été suggérées d'avoir une virulence moins grande que celle des souches ST1 et ST7. Il est donc important de savoir si le rôle, les propriétés et les fonctions attribués à la présence de la CPS de souches de sérotype 2 ST1 et ST7 s'appliquent aussi aux souches de sérotype 2 nord-américaines.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and zoonotic agent responsible for sudden death, septic shock, and meningitis. However, serotype 2 strains are heterogeneous, composed of a multitude of sequence types (STs) whose distribution greatly varies worldwide. Of the virulence factors presently described for *S. suis*, the capsular polysaccharide (CPS) is a critical factor implicated in a multitude of functions, including in impairment of the phagocytic capacity and innate immune cell activation by masking underlying bacterial components. However, these roles have been described using Eurasian ST1 and ST7 strains, which greatly differ from North American ST25 strains. Consequently, the capacity of the CPS to mask surface antigens and putative virulence factors in non-Eurasian strains remains unknown. Herein, the role of the *S. suis* serotype 2 CPS of a prototype intermediate virulent North American ST25 strain, in comparison with that of a virulent European ST1 strain, with regards to interactions with dendritic cells, as well as virulence during the systemic phase of infection, was evaluated. Results demonstrated that the CPS remains critical for virulence and development of clinical disease regardless of strain background, due to its requirement for survival in blood. However, its role in the interactions with dendritic cells is strain-dependent. Consequently, certain key characteristics associated with the CPS are not necessarily applicable to all *S. suis* serotype 2 strains. This indicates that though certain factors may be important for *S. suis* serotype 2 virulence, strain background could be as determining, reiterating the need of using strains from varying backgrounds in order to better characterize the *S. suis* pathogenesis.

Introduction

Streptococcus suis is an important bacterial pathogen of young piglets and a zoonotic agent causing sudden death (pigs), septic shock (humans), and meningitis (both species) [1]. Classification is based on serotyping defined by the capsular polysaccharide (CPS) present or its genes [1]. Worldwide, serotype 2 is the most widespread and virulent, responsible for the majority of porcine and human cases of infection [2]. However, an extensive heterogeneity exists within serotype 2 strains, both genotypically and phenotypically, which can be grouped into a multitude of sequence types (STs), as determined by multilocus sequence typing, and whose distribution greatly varies [2]. Furthermore, experimental mouse and pig models of infection have been used to determine virulence of the most important STs (ST1, ST7, and ST25) [3-7]. While the ST7 strain responsible for the Chinese human outbreaks is highly virulent, European ST1 strains are virulent; by contrast, ST25 strains, typical in North America, are of intermediate virulence [3, 8]. Albeit these important differences, studies on the pathogenesis of the infection have almost exclusively used Eurasian strains, even though ST25 strains account for nearly 50% of serotype 2 isolates recovered from diseased pigs in North America [9].

Though a variety of virulence factors have been described for *S. suis*, the CPS, which is a critical factor whose composition and structure is identical for all serotype 2 strains, is implicated in a multitude of functions [10, 11]. Of these, resistance to phagocytosis by innate immune cells [12-16] and masking surface components responsible for host cell activation [16, 17] are the most important. Studies using experimental animal infection models have demonstrated that the CPS is required for survival in blood [18, 19]. Alongside, it was recently demonstrated that *S. suis* can modulate presence of its CPS within the host [20]. However, these roles have been described using Eurasian ST1 and ST7 strains, which greatly differ from their North American counterparts.

The innate immune host response is composed of numerous cell types of which phagocytes play a central role in the *S. suis* pathogenesis [10, 11]. Of these, dendritic cells (DCs) are key players involved in phagocytosis and cytokine production [21]. Indeed, DCs have been previously described as playing an important role in the *S. suis* pathogenesis by contributing to the exacerbated inflammatory host response [16, 22]. Nevertheless, the interactions between DCs and *S. suis* serotype 2 have been mostly studied using Eurasian strains.

Though the CPS is antigenically identical for all *S. suis* serotype 2 strains, its capacity to mask surface antigens and putative virulence factors in non-Eurasian strains remains unknown. Consequently, the objective of this study was to evaluate the role of the *S. suis* serotype 2 CPS of a prototype intermediate virulent North American ST25 strain, in comparison with that of a virulent European ST1 strain, with regards to interactions with DCs, as well as virulence during the systemic phase of infection. Results obtained demonstrate that while the CPS is required for survival in blood and remains critical for virulence and development of clinical disease regardless of strain origin, its role in the interactions with DCs (resistance to phagocytosis and interference of cytokine production) is strain-dependent.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (permit number rech-1570).

Bacterial strains and growth conditions

The well-characterized and highly encapsulated wild-type *S. suis* serotype 2 strains and their non-encapsulated isogenic mutants used in this study are listed in Table 1. *S. suis* strains were cultured in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON). For *in vitro* cell culture assays, bacteria were prepared as previously described [16] and resuspended in cell culture medium. For experimental infections, early stationary phase bacteria were washed twice in phosphate-buffered saline pH 7.4 and resuspended in THB [4, 23, 24]. Bacterial cultures were appropriately diluted and plated on THB agar (THA) to accurately determine bacterial concentrations. The *Escherichia coli* strain and different plasmids used in this study are also listed in Table 1. When needed, antibiotics (Sigma-Aldrich, Oakville, ON) were added to the media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/mL; for *E. coli*, kanamycin and spectinomycin at 50 µg/mL and ampicillin at 100 µg/mL.

DNA manipulations

As previously described [28], *S. suis* genomic DNA was extracted using InstaGene Matrix solution (BioRad Laboratories, Hercules, CA). Mini-preparations of recombinant plasmids were

carried out using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Restriction enzymes and DNA-modifying enzymes (Fisher Scientific, Ottawa, ON) were used according to the manufacturer's recommendations. Oligonucleotide primers (Table 2) were obtained from Integrated DNA Technologies (Coralville, IA) and PCRs carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON) or the Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using an ABI 310 Automated DNA Sequencer and ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA).

*Construction of the 89-1591 non-encapsulated isogenic mutants 89-1591Δ***cpsF** *and 89-1591Δ***neuC**

The P1/7 non-encapsulated isogenic mutants P1/7Δ*cpsF* and P1/7Δ*neuC* were constructed and characterized elsewhere [16, 18]. In this study, the DNA genome sequence of the *S. suis* wild-type strain 89-1591 was used for construction of its non-encapsulated isogenic mutants. Precise in-frame deletions of *cpsF* or *neuC* genes were constructed using splicing-by-overlap-extension PCRs as previously described [28, 29]. Overlapping PCR products were cloned into pCR2.1 (Invitrogen, Burlington, ON), extracted with EcoRI, recloned into the thermosensitive *E. coli*–*S. suis* shuttle plasmid pSET4s, and digested with the same enzyme, giving rise to the knockout vector p4Δ*cpsF* or p4Δ*neuC*. Electroporation of the wild-type strain 89-1591 and procedures for isolation of the mutants were previously described [27]. Allelic replacement was confirmed by PCR and DNA sequencing analyses. Amplification products were purified with the QIAgen PCR Purification Kit (Qiagen) and sequenced as described above. Growth of both mutants was similar to that of the wild-type strain (data not shown).

Bacterial surface hydrophobicity assay

Relative surface hydrophobicity of the *S. suis* wild-type strains and non-encapsulated mutants was determined by measuring adsorption to *n*-hexadecane as previously described [30].

Transmission electron microscopy

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Transmission electron microscopy was carried out as previously described [31, 32]. Briefly, bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer pH 7.3 (Canemco & Marivac, Canton de Gore, QC) containing 2.5% glutaraldehyde and 0.05% ruthenium red.

Ferritin was then added to a final concentration of 1 mg/mL and incubated for 30 min at room temperature. Cells were then immobilized in 3% agar in 0.1 M cacodylate buffer pH 7.3, washed five times in cacodylate buffer containing 0.05% ruthenium red, and fixed in 2% osmium tetroxide for 2 h at room temperature. Afterwards, samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in an increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were post-stained with uranyl acetate and lead citrate and examined using a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

Generation of bone marrow-derived dendritic cells

The femur and tibia from eight C57BL/6 mice (Jackson Research Laboratories, Bar Harbour, ME) were used to generate bone marrow-derived DCs, as previously described [16]. Briefly, hematopoietic bone marrow stem cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (Gibco, Burlington, ON). Complete medium was complemented with 20% granulocyte macrophage-colony stimulating factor from mouse-transfected Ag8653 cells [33]. Cell purity was confirmed to be at least 85% $CD11c^+$ by flow cytometry as previously described [16]. Prior to infection, cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different strains of *S. suis* serotype 2 listed in Table 1 (1 x 10^6 CFU/mL; initial multiplicity of infection [MOI] = 1). Conditions used were based on those previously published [16, 22].

Internalization assay

Cells were infected with the different *S. suis* strains and phagocytosis was left to proceed for different times (0.5 to 4 h) at 37°C with 5% CO_2. MOI and assay conditions were chosen based on previous studies regarding the kinetics of *S. suis* phagocytosis by DCs [16]. After incubation, penicillin G (5 mg/mL; Sigma-Aldrich) and gentamicin (100 mg/mL; Gibco) were added to the wells for 1 h to kill extracellular bacteria. Supernatant controls were taken in every test to confirm that extracellular bacteria were efficiently killed by the antibiotics. After antibiotic treatment, cells were washed three times and water added to lyse the cells. The number of CFU recovered per well was determined by plating viable intracellular bacteria on THA.

Cytokine measurement

Supernatants were collected 16 h following infection with *S. suis*, time at which secreted cytokine levels are maximal in the absence of *S. suis*-induced DC cytotoxicity [16, 22]. Non-infected cells served as negative controls. Secreted levels of tumor necrosis factor (TNF), interleukin (IL)-6, IL-12p70, and C-X-C motif chemokine ligand (CXCL) 1 were quantified by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN) according to the manufacturer's recommendations.

Determination of interferon-β mRNA expression by quantitative RT-PCR

Cell mRNA was extracted 6 h post-infection (p.i) according to the manufacturer's instructions (Qiagen) and cDNA generated using the Quantitect cDNA Synthesis Kit (Qiagen) as previously described [32]. Incubation time was chosen based on maximal expression of interferon (IFN)-β by DCs following *S. suis* infection [32]. Real-time qPCR was performed on the CFX-96 Touch Rapid Thermal Cycler System (Bio-Rad) using 250 nM of primers (Integrated DNA technologies) and the SsoFast Evagreen Supermix Kit (Bio-Rad). Cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 sec and 57°C for 5 sec. Melting curves were generated after each run to confirm presence of a single PCR product. The sequences of primers used in this study are shown in Table 2 and were verified to have reaction efficiencies between 90 % and 110 %. The reference genes *Atp5b* and *Gapdh*, determined to be the most stably expressed using the algorithm geNorm, were used to normalize data. Fold changes in gene expression were calculated using the quantification cycle threshold (Cq) method using the CFX software manager v.3.0 (Bio-Rad). Samples from mock-infected cells served as calibrators. IFN-β was measured by RT-qPCR in order to compare with published results [32].

Whole blood bactericidal (killing) assay

Blood was collected from twelve six- to ten-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA) and mixed with sodium heparin (Sigma-Aldrich). Leukocytes (9×10^6 cells/mL on average) were transferred to a microtube containing 9×10^6 CFU/mL of the different *S. suis* strains (MOI = 1) and incubated for 4 h, mixing every 20 min. Assay conditions were chosen based on the kinetics of *S. suis* killing by murine blood [4]. After incubation, cells were lysed by vortexing and vigorous pipetting and appropriate dilutions plated on THA to determine viable bacterial counts. Resistance to bacterial killing by blood leukocytes

was compared to incubation of the different bacterial strains in plasma only (obtained by centrifuging whole blood at 1 800 x *g* for 10 min at 4°C). The percentage of bacteria killed was determined using the following formula: 1 - (bacteria in blood / bacteria in plasma) / 100%.

S. suis *virulence mouse model of infection*

A well-standardized CD-1 mouse model of infection was used [18, 23]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering through the use of humane endpoints, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. No additional considerations or housing conditions were required. All staff members received the required animal handling training as administered by the University of Montreal Animal Welfare Committee. Forty-five six-week-old female CD-1 mice (Charles River Laboratories) were used for these experiments (15 mice per strain evaluated). Mice were inoculated with 5 x 10^7 CFU via the intraperitoneal route and health and behavior monitored at least thrice daily until 72 h p.i. and twice thereafter until the end of the experiment (10 days p.i.) for the development of clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, and lethargy. Mice were also monitored for the development of clinical signs of meningitis. Clinical scores were determined according to the grid approved by the University of Montreal Animal Welfare Committee (S1 Appendix) and required actions undertaken. Mice were immediately euthanized upon reaching endpoint criteria using CO_2 followed by cervical dislocation. No mice died before meeting endpoint criteria and all surviving mice were euthanized as described above at the end of the experiment (10 days p.i.). Blood samples were collected from the caudal vein of surviving mice 24 h p.i. and plated as previously described [4].

Statistical analyses

Normality of data was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test) or non-parametric tests (Mann-Whitney rank sum test), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare survival between wild-type-infected mice and those infected with the non-encapsulated strains. Each *in vitro* test was repeated in at least three independent experiments. $p < 0.05$ was considered as statistically significant.

Results

Deletion of cpsF *or* neuC *genes involved in* S. suis *serotype 2 capsular polysaccharide biosynthesis results in non-encapsulation of a ST25 strain*

Though deletion of various CPS biosynthesis genes, including those for the sialic acid side-chain, was previously described to result in absence of CPS in Eurasian ST1 and ST7 strains [16, 18, 19, 34], the effect of *cpsF* and *neuC* gene deletion on CPS expression in a ST25 strain was unknown. Surface hydrophobicity (an indicator of encapsulation) was similar between wild-type strains P1/7 (ST1) and 89-1591 (ST25) (Fig 1). Deletion of *cpsF* or *neuC* gene significantly increased surface hydrophobicity of all mutants ($p < 0.001$), which averaged 95% (Fig 1). These results confirm that deletion of *S. suis* serotype 2 CPS biosynthesis genes results in high surface hydrophobicity, regardless of strain background.

Encapsulation of the ST25 strain 89-1591 and its *cpsF*- and *neuC*-deficient mutants was then evaluated by transmission electronic microscopy following labelling with polycationic ferritin, which binds negatively charged structures such as the *S. suis* serotype 2 CPS [31]. In accordance with previously published micrographs [26, 32], wild-type strain 89-1591 is well-encapsulated, presenting a thick layer of CPS at its surface as evidenced by the polycationic ferritin labelling (Fig 2A). This is similar to results obtained with the European ST1 strain P1/7 [18]. On the other hand, 89-1591 mutants deficient for either *cpsF* (Fig 2B) or *neuC* (Fig 2C) gene clearly lack polycationic ferritin marker at their surface, indicating a lack of CPS. Consequently, these results confirm that deletion of *S. suis* serotype 2 CPS biosynthesis genes results in non-encapsulation of mutant strains, regardless of strain background.

Role of capsular polysaccharide in resistance of S. suis *serotype 2 to phagocytosis by dendritic cells is strain-dependent*

Resistance to internalization by phagocytes is an important property conferred to *S. suis* serotype 2 by the presence of its CPS [22]. To evaluate this function, DCs from C57BL/6 mice were used. This breed of mice has been largely used in the literature [16, 22, 32] since it is inbred, which greatly reduces variability between individuals and the number of animals required. Moreover, results obtained using DCs from C57BL/6 mice have been demonstrated to be representative of porcine and human DCs [35, 36]. As previously described, the ST1 strain P1/7 was little internalized by DCs (less than 10 CFU/mL), with internalization levels remaining constant over time (Fig 3). By contrast, non-encapsulated mutants P1/7Δ*cpsF* and

P1/7Δ*neuC* were significantly more internalized ($p < 0.05$), with levels of internalized bacteria increasing with time (Fig 3). Meanwhile, the ST25 strain 89-1591 was significantly more internalized than the ST1 wild-type strain P1/7 ($p < 0.01$), and this regardless of the incubation time (Fig 3). Surprisingly, no differences were observed between internalization levels of the ST25 strain 89-1591 and its non-encapsulated mutants, 89-1591Δ*cpsF* and 89-1591Δ*neuC*, by murine DCs (Fig 3). Lack of difference between the ST25 strain 89-1591 and its non-encapsulated mutants was not due to low or non-encapsulation of the wild-type strain in cell culture medium as determined by hydrophobicity assay following infection of DCs (data not shown).

Interference of dendritic cell cytokine production by presence of the* S. suis *serotype 2 capsular polysaccharide is strain-dependent

Presence of the *S. suis* serotype 2 CPS has also been associated with interference of cytokine production by myeloid cells, including DCs [16, 17]. While the ST1 strain P1/7 and the ST25 strain 89-1591 both induced a variety of cytokines following infection of murine DCs (TNF, ^{IL6}, IL-12p70, and CXCL1), only levels of IL-6 and CXCL1 induced by P1/7 (ST1) were significantly higher than those induced by 89-1591 (ST25) ($p < 0.01$) (Fig 4). As previously reported [16], absence of CPS from strain P1/7 (ST1) significantly increased production of TNF, IL-6, ^{IL12p70}, and CXCL1 ($p < 0.001$) by DCs (Fig 4A-D). In contrast, absence of CPS did not affect production of these mediators by DCs following infection with the ST25 strain 89-1591 (Fig 4A-D). Once again, lack of difference between the ST25 strain 89-1591 and its non-encapsulated mutants was not due to low or non-encapsulation of the wild-type strain in cell culture medium as determined by hydrophobicity assay following infection of DCs (data not shown).

We recently demonstrated a mechanism complementary to surface-associated receptor activation whereby internalization of *S. suis* can lead to induction of IFN-β by DCs [32]. Since internalization levels of the 89-1591 non-encapsulated mutants were similar to those of the wild-type strain, it was hypothesized that IFN-β expression might be similar between strain 89-1591 and its non-encapsulated mutants. As previously described, the ST25 strain 89-1591 induced significantly higher levels of IFN-β expression from DCs than did the ST1 strain P1/7 6 h p.i. ($p < 0.001$) (Fig 4E) [32]. Both non-encapsulated mutants of the ST1 strain P1/7 induced

significantly higher levels of IFN-β expression than did the wild-type strain ($p < 0.001$) (Fig 4E). By contrast, IFN-β expression was similar between 89-1591 and its two mutants (Fig 4E**)**.

Capsular polysaccharide is required for S. suis *serotype 2 whole blood bactericidal resistance regardless of strain background*

While presence of the North American ST25 CPS only minimally modulated interactions with murine DCs *in vitro*, its role in a context more reflective of the *in vivo* situation remained unknown. Consequently, the role of the presence of the ST25 strain CPS in resisting the bactericidal effect of murine whole blood was evaluated. As previously reported [4], the ST1 strain P1/7 completely resisted killing by whole blood, while the ST25 strain 89-1591 was somewhat less resistant (Fig 5). The non-encapsulated mutants of strain P1/7 were significantly less resistant to the bactericidal effect of murine whole blood than their wild-type strain ($p < 0.001$), with nearly 60% of bacteria killed after 4 h of incubation (Fig 5). Similarly, non-encapsulation of strain 89-1591 resulted in significantly greater killing by whole blood ($p < 0.001$), with levels of killed bacteria similar to those of P1/7 non-encapsulated mutants (Fig 5).

Capsular polysaccharide is required for virulence of the S. suis *serotype 2 ST25 strain 89-1591 in a mouse model of infection*

To evaluate the role of the North American ST25 CPS in virulence and development of clinical disease, a well-developed CD-1 mouse model of infection was used in which bacteria were inoculated via the intraperitoneal route [9, 23, 37-39]. As previously reported, the ST25 wild-type strain 89-1591 induced disease in mice, with 100% of mice succumbing within six days of infection (Fig 6A) [4, 9]. While some mice succumbed to septic shock, most mice developed clinical signs of meningitis, which is a characteristic of this strain. By contrast, none of the mice infected with either of its non-encapsulated mutants (89-1591Δ*cpsF* or 89-1591Δ*neuC*) succumbed to disease ($p < 0.001$) (Fig 6A). In fact, these mice only developed transient signs of infection such as rough hair coat following inoculation of bacteria. Viability of the inoculum was verified prior to and after infection with no differences between (data not shown).

In order to explain these differences in virulence, blood bacterial burden was evaluated 24 h p.i., time at which only bacteria capable of persisting are detected. All mice infected with the wild-type strain 89-1591 presented blood bacterial burdens that averaged 5×10^5 CFU/mL

(Fig 6B). On the other hand, almost no bacterial burden was detected in blood of mice infected with either of the non-encapsulated mutants, levels of which were significantly lower than those of mice infected with the wild-type strain ($p < 0.001$) (Fig 6B).

Discussion

S. suis serotype 2 is an important porcine bacterial pathogen and zoonotic agent responsible for sudden death, septic shock, and meningitis [1]. Of its different virulence factors, the CPS is considered one of the only "truly" critical virulence factors, being associated with anti-phagocytic properties, protection against bacterial killing, and a capacity to interfere with cytokine production [10]. However, previous studies have used Eurasian strains (virulent ST1 and ST7), which greatly differ from their North American counterparts, including intermediate virulent ST25 strains [10].

Biosynthesis of the *S. suis* serotype 2 CPS involves a variety of genes encoded by the *cps* locus of which mutants deficient for *cpsB*, *cpsE, cpsF*, *cpsG*, *cpsJ*, *cpsL*, *neuB*, and *neuC* have been created, all using Eurasian ST1 strains or the Chinese ST7 strain [16, 18, 19, 34, 40]. In accordance with these studies, deletion of *cpsF* or *neuC* genes also caused non-encapsulation of the ST25 strain 89-1591, which suggests that deletion of a gene from the serotype 2 *cps* locus probably causes non-encapsulation regardless of strain background. A similar phenomenon was observed for *S. suis* serotype 14, whereby deletion of the *cpsB* or *neuC* genes caused a loss of CPS [41]. Based on current knowledge obtained with the serotypes 2 and 14 only, it may be hypothesized that tampering of the *S. suis cps* locus results in non-encapsulation independently of serotype. However, given the structural similarities between serotypes 2 and 14, including presence of sialic acid, the use of more divergent serotypes will be required to confirm this hypothesis [42-44].

Previous studies with Eurasian strains have demonstrated that serotype 2 CPS is a critical anti-phagocytic factor that protects against internalization by DCs (as well as other phagocytes) of murine, porcine, and human origin [13, 16, 35, 36, 45]. However, this property was not observed herein using a ST25 strain: as such, it would be interesting to confirm using other cell types. It is important to note that composition and structure are responsible for serotype determination [42, 46], and that all serotype 2 strains have the same CPS antigenically, which indicates a highly similar structure and composition. In addition, the *cps* locus is identical in all serotype 2

strains sequenced to date, regardless of sequence type [47]. Moreover, thickness of CPS was determined to be similar between strains P1/7 and 89-1591 by electron microscopy [32]. As such, though results are currently unavailable, it can be hypothesized that differences in internalization might be due to differential exposition and/or composition of the underlying surface components. Unfortunately, identity of all sub-capsular *S. suis* serotype 2 components remains unknown, and future studies will be necessary to both confirm this hypothesis and identify differences between strains. Houde *et al*. demonstrated that presence of serotype 2 CPS destabilizes host cell lipid microdomains, thus preventing lactosylceramide accumulation at the cell membrane, which blocks *S. suis* phagocytosis and lactosylceramide-dependent recognition of *S. suis* [48]. Although lactosylceramide-dependent recognition of *S. suis* appears to be important for internalization, the bacterial component recognized remains unknown, though it is supposed to be located at the bacterial surface [48]. Since the ST25 strain 89-1591 was more internalized by DCs, regardless of it being well-encapsulated, it may be hypothesized that expression, surface distribution, and/or exposition/masking of the yet unknown *S. suis* component recognized by lactosylceramide, might vary between the two strains.

This differential role of the CPS regarding its anti-phagocytic properties suggests a potential paradigm shift. In fact, certain misconceptions regarding the *S. suis* CPS are commonplace: while CPS protects by masking surface components, many of which are important immune activators, it is not impenetrable, rather being analogous to a net. Indeed, the correct term regarding the serotype 2 CPS structure is random coil, indicating that it is constantly shifting over the entirety of the bacterium, being fluid rather than static [42]. Moreover, *S. suis* serotype 2 possesses the ability to modulate presence of its CPS, whose thickness might depend on the environment faced [20]. Finally, data suggest that unlike Group B *Streptococcus*, the *S. suis* serotype 2 CPS is not firmly attached to its surface, as it is easily recovered following autoclave treatment [42]. Consequently, though the CPS surrounds *S. suis* and protects it from recognition by phagocytes, it does not necessarily mask all surface components simultaneously.

While phagocytosis assays are important to better understand the interactions between *S. suis* and phagocytic cells such as DCs, they do not necessary represent the complex reality of the host. Moreover, clearance of *S. suis* by phagocytes depends not only on its internalization, but most importantly on its killing, which *S. suis* attempts to thwart in order to survive and induce

disease [10]. In fact, the anti-phagocytic role of the CPS has long been associated with survival of the pathogen in blood via resistance to bacterial killing [10]. Unlike results obtained with DCs alone, presence of CPS was required for resisting the bactericidal effect of murine blood regardless of strain background. Differently from phagocytosis assays, whole blood is composed of a variety of immune cell types, of which neutrophils and monocytes predominate; while dendritic cells are important phagocytes, they are more commonly distributed throughout tissues rather than in blood [21, 49]. Furthermore, the phagocytic and killing capacity of immune cells differs: while DCs were reported to internalize *S. suis* more efficiently than monocytes, it was also demonstrated that neutrophils, which constitute the most important subset of blood phagocytes, are the most efficient at killing *S. suis* [13, 45]. Differences between results obtained using the phagocytosis assay with DCs alone and whole blood bactericidal assay confirm the lack of correlation between resistance to phagocytosis by a single cell type and resistance to killing in a complex model reflective of the systemic compartment [4]. Though phagocytosis assays are crucial for dissecting the cell-pathogen interactions, conclusions should be carefully interpreted and may not always be extrapolated to the more complex systems required to better comprehend the interplay *in vivo*.

Recognition of *S. suis* by various cell receptors results in production of cytokines, which participate in the inflammatory response required to clear the pathogen [22, 23]. Interference of cytokine production by myeloid cells is thus an important tactic used by *S. suis* to avoid detection by host cells, and in which presence of the serotype 2 CPS participates. While absence of CPS from strain P1/7 (ST1) resulted in increased cytokine production due to recognition of surface components by host receptors, as previously reported with other myeloid cells [16, 17, 35, 36, 50, 51], the lack of differences between the well-encapsulated ST25 wild-type strain 89-1591 and its non-encapsulated mutants is novel. Interestingly, the ST1 strain P1/7 induced higher levels of IL-6 and CXCL1, but not of TNF and IL-12p70, than the ST25 strain 89-1591. This result, while unexpected, is difficult to explain based on current knowledge since very little studies have used North American ST25 strains. It was previously demonstrated that plasma levels of TNF, IL-6, and CXCL1, but not IL-12p70, are higher during systemic infection in mice infected with strain P1/7 than those infected with strain 89-1591 [4]. *In vitro*, however, a collection of North American and European strains induced similar mRNA levels of TNF, IL-6, and IL-8 from porcine whole blood [52], while another virulent European serotype 2 ST1 strain (reference strain 735) induced higher levels of IL-6, but not IL-8, from human brain

microvascular endothelial cells [53]. Once again, these differences in cytokine production might be due to differential activation resulting from varying exposition and/or composition of underlying immunostimulatory surface components. Finally, given that these cytokines were generally more induced by strain P1/7 than 89-1591 *in vivo*, but not necessarily with DCs, indicates that other cell types are probably involved in inflammatory mediator production *in vivo*, including monocytes, neutrophils, and/or Natural Killer cells. Together though, the current lack of knowledge justifies the need in studying strains from different genetic backgrounds other than ST1 and ST7, but also with other immune cell types, in order to better understand the complex pathogen that is *S. suis*.

Since *S. suis* is a classical extracellular pathogen, activation of surface-associated receptors, including Toll-like receptors (TLR), is crucial for cytokine production [17, 22]. As such, the differential role of the serotype 2 CPS between strains P1/7 and 89-1591 might be due to differential exposition and/or composition of surface components. Though identity of the surface components involved is unknown, potential candidates might include lipoproteins, which are immunostimulatory proteins whose composition is highly variable between bacterial species and possibly even between strains [54]. Alongside phagocytosis-independent surface-associated cell receptor-dependent cytokine production, we recently demonstrated a new complementary mechanism whereby, following phagocytosis, the *S. suis* nucleic acids activate the endosomal TLR7 and TLR9, leading to production of IFN-β [32]. Differential results in IFN-β induction by strain P1/7 and its mutants, in contrast to strain 89-1591, illustrate how production of internalization-dependent cytokines correlates with phagocytosis levels.

The contrasting interactions between DCs and the *S. suis* serotype 2 strains P1/7 and 89-1591 *in vitro* suggested potentially differential roles of their CPS in virulence. Absence of CPS was previously reported to result in avirulence, regardless of the *cps* gene mutated in Eurasian ST1 or ST7 strains [18, 19, 34]. In accordance, yet in contrast to interactions with DCs alone, non-encapsulation of the ST25 strain also resulted in avirulence, due to an inability of the mutants to persist in the bloodstream. This inability is in accordance with whole blood killing results, confirming that this test is a good correlate of virulence [4].

Interestingly, while lack of serotype 2 CPS resulted in similar or increased cytokine production *in vitro* depending on the strain, these non-encapsulated strains are avirulent *in vivo*. Though

S. suis-induced exacerbated inflammation is a hallmark of disease [10], the lack of resistance of non-encapsulated mutants to the bactericidal effect of whole blood results in their rapid clearance and overall reduced inflammatory activation of host cells *in vivo*. By contrast, resistance of well-encapsulated *S. suis* serotype 2 strains to killing by blood leukocytes favors their replication, persistence, stimulation, and activation of host cells, resulting in an exacerbated inflammation responsible for clinical disease and host death [4, 23].

In conclusion, this study is the first to demonstrate that while the *S. suis* serotype 2 CPS remains critical for persistence in blood, development of clinical disease, and overall virulence, its role in the interactions with immune cells is strain-specific. In fact, the CPS has been classically associated with anti-phagocytic properties and masking of bacterial surface components. However, these properties are not necessarily applicable to all *S. suis* strains. This shift in paradigm suggests that while certain factors may be important for virulence of *S. suis* serotype 2, strain background could be as determining [55]. As such, further studies using additional ST25 strains as well as strains from other STs and of varying virulence, will be necessary to confirm these results as being strain-specific, ST-specific or other. Importantly, this study reiterates that the use of strains from varying backgrounds is required in order to better characterize the *S. suis* pathogenesis and that generalized conclusions should avoided.

Acknowledgements

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Supporting Information (Available at the PLoS One Website)

S1 Appendix. Evaluation of clinical signs and scoring following intraperitoneal injection of *Streptococcus suis* serotype 2 in mice.

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Tables

Table 1. List of strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
Streptococcus suis		
P1/7	Virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	[25]
P1/7Δ*cpsF*	Non-encapsulated isogenic mutant derived from P1/7; in frame deletion of *cpsF* gene	[16]
P1/7Δ*neuC*	Non-encapsulated isogenic mutant derived from P1/7; in frame deletion of *neuC* gene	[18]
89-1591	Intermediate virulent North American ST25 strain isolated from a case of pig sepsis in Canada	[26]
89-1591Δ*cpsF*	Non-encapsulated isogenic mutant derived from 89-1591; in frame deletion of *cpsF* gene	This study
89-1591Δ*neuC*	Non-encapsulated isogenic mutant derived from 89-1591; in frame deletion of *neuC* gene	This study
Escherichia coli		
TOP10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
Plasmids		
pCR2.1	Ap^r, Km^r, pUC *ori*, *lac*ZΔM15	Invitrogen
pSET4s	Spc^r, pUC *ori*, thermosensitive pG+host3 *ori*, *lac*ZΔM15	[27]
p4Δ*cpsF*	pSET-4s carrying the construct for *cpsF* allelic replacement	This study
p4Δ*neuC*	pSET-4s carrying the construct for *neuC* allelic replacement	This study

Table 2. List of oligonucleotide primers used in this study.

Name	Sequence (5' – 3')
PCR	
cpsF-1	CCA GCA AAG TAT GGT GGT TTC G
cpsF-2	GCG CAC CAA CTT CTC TTA ATG C
cpsF-3	CTT AGT CAC TCC GAA CTC ACC G
cpsF-4	CCA CGC CAG ATT CAA TGA GC
cpsF-5	AGA CGG TCA TGA ATG GCT ACG
cpsF-6	GAG GGA GGT GTA GAC TTC TGC TCC AGC ATG
cpsF-7	CAT GCT GGA GCA GAA GTC TAC ACC TCC CTC
cpsF-8	CAT CAG AAT GAT GCC AAA CAG G
neuC-1	TGC CCG TTT ATA AGA TTC CAT C
neuC-2	TGA GTT GCT CTG TCA AGG TC
neuC-3	TGA TTG AAG TGC CCT CAT TAC
neuC-4	TAA ACC TTT TGA TCC TGA CCG
neuC-5	TGA AAA GCA CTT TAC TCT GGA C
neuC-6	CCT TGT AAA GCA GAA TCA GGT TGA TGC ATG GCT GTC ACT AC
neuC-7	GTA GTG ACA GCC ATG CAT CAA CCT GAT TCT GCT TTA CAA GG
neuC-8	ATG TTC CAC AAT GGC ACC C
Quantitative real-time PCR	
Atp5b	F: ACC AGC CCA CCC TAG CCA CC
	R: TGC AGG GGC AGG GTC AGT CA
Gapdh	F: CCC GTA GAC AAA ATG GTG AAG
	R: GAC TGT GCC GTT GAA TTT G
Ifnb	F: CCC AGT GCT GGA GCC ATT GT
	R: CCC TAT GGA GAT GAC GGA GA

Figures

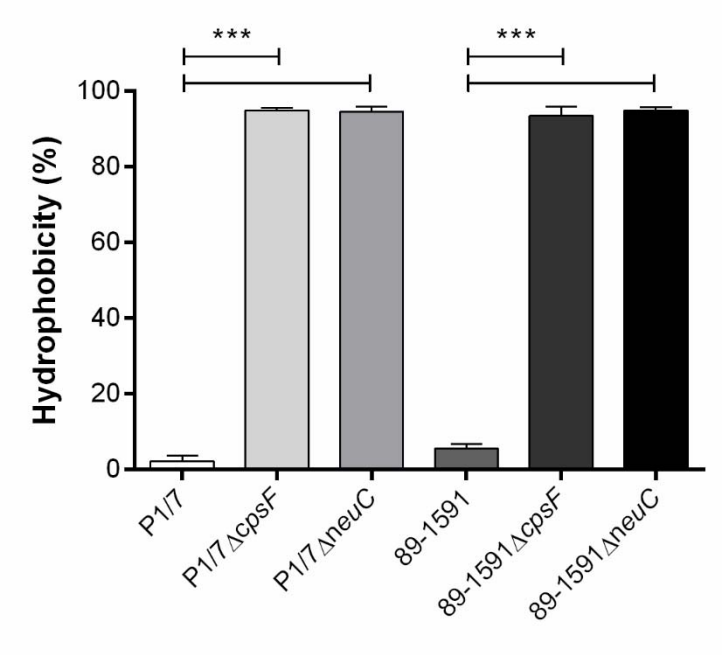

Figure 1. Absence of capsular polysaccharide is associated with increased surface hydrophobicity of *S. suis* serotype 2. Hydrophobicity of wild-type and mutant strains was determined using *n*-hexadecane. Data represent the mean ± SEM from three independent experiments. *** ($p < 0.001$) indicates a significant difference between wild-type strains (P1/7 or 89-1591) and their non-encapsulated mutants (Δ*cpsF* and Δ*neuC*).

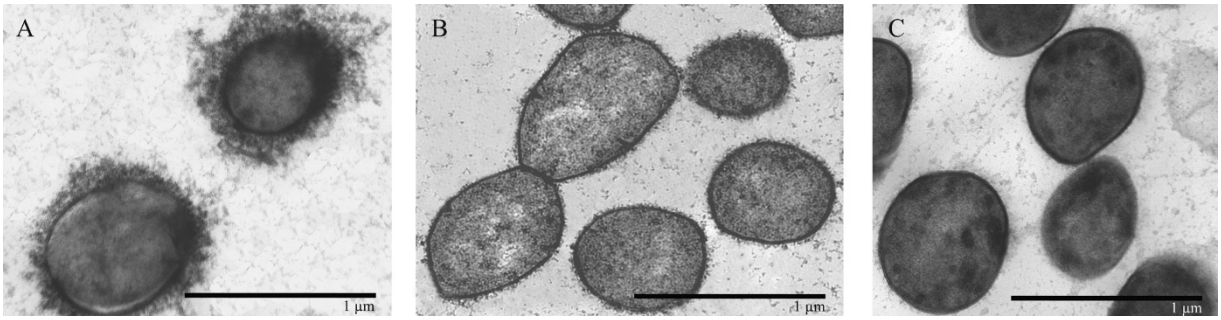

Figure 2. Deletion of genes involved in *S. suis* serotype 2 capsular polysaccharide biosynthesis (*cpsF* and *neuC*) results in non-encapsulation of the ST25 strain 89-1591. Transmission electron microscopy following labelling with polycationic ferritin of the ST25 wild-type strain 89-1591 (A) or its isogenic mutants 89-1591Δ*cpsF* (B) and 89-1591Δ*neuC* (C). Black bars = 1 µm.

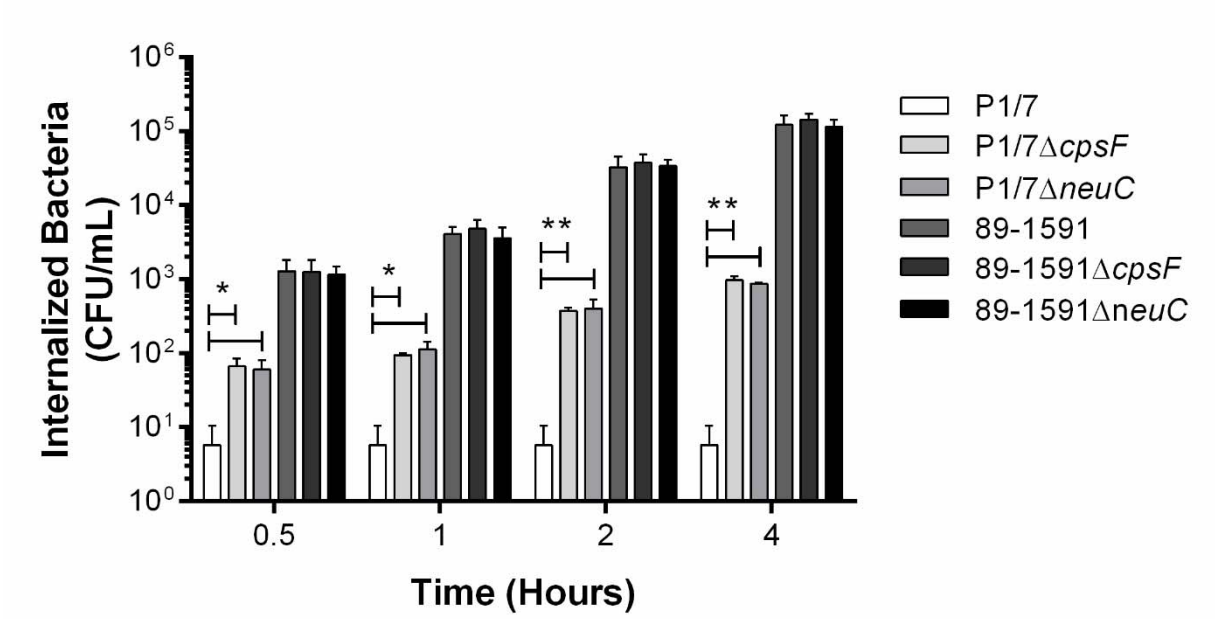

Figure 3. Strain-dependent role of the capsular polysaccharide in *S. suis* serotype 2 resistance to phagocytosis by dendritic cells (DCs). Internalization kinetics (0.5 to 4 h) of wild-type and non-encapsulated mutant strains by DCs. Data represent the mean ± SEM from four independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between P1/7 and P1/7Δ*cpsF* or P1/7Δ*neuC*.

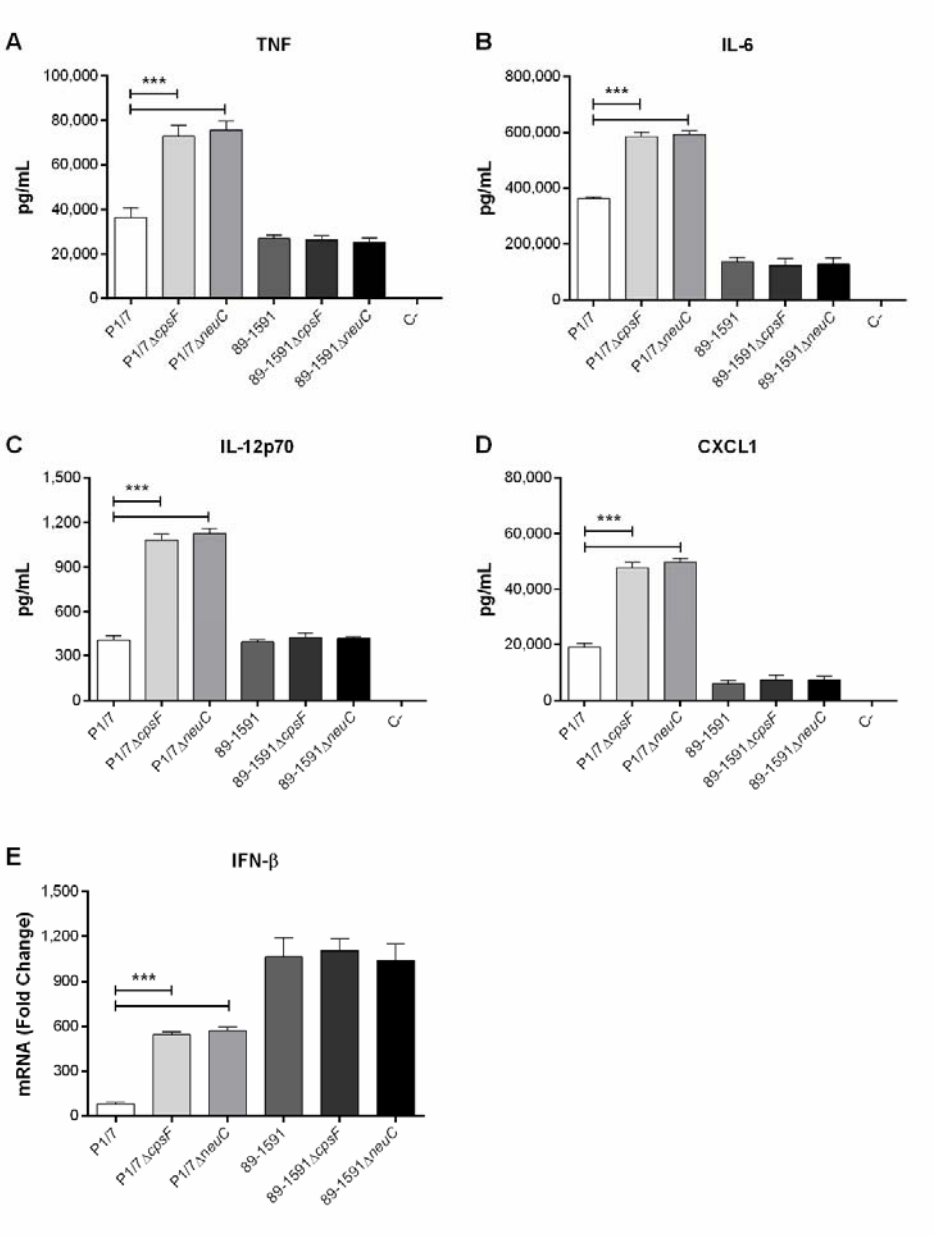

Figure 4. Strain-dependent rôle of capsular polysaccharide in interference of *S. suis* serotype 2-induced cytokine production by dendritic cells (DCs). Cytokine production by DCs following infection with wild-type and non-encapsulated mutant strains after 16 h of incubation, as measured by ELISA, with the exception of IFN-β, after 6 h of incubation, by RT-qPCR. Production of TNF (A), IL-6 (B), IL-12p70 (C), CXCL1 (D), and IFN-β (E). Data represent the mean ± SEM from four independent experiments. C- denotes the negative control (cells in medium alone). *** ($p < 0.001$) indicates a significant difference between P1/7 and P1/7Δ*cpsF* or P1/7Δ*neuC*.

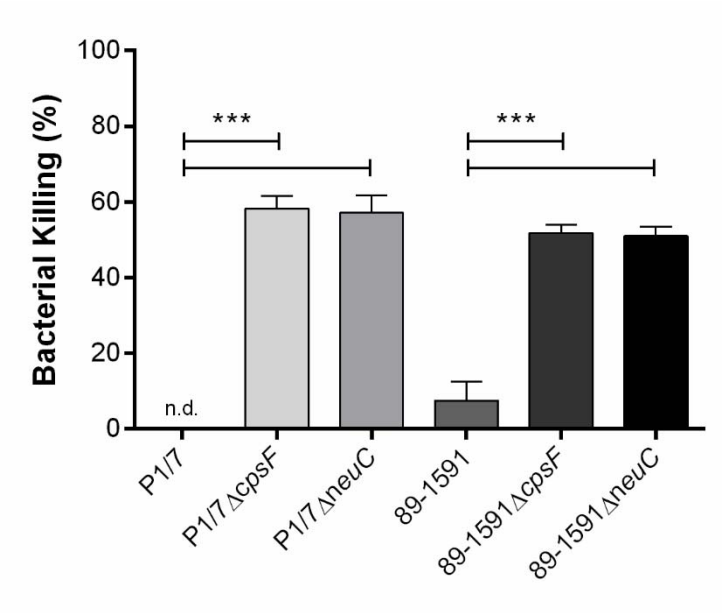

Figure 5. The *S. suis* serotype 2 capsular polysaccharide is required for resisting the bactericidal effect of whole blood regardless of strain background. Capacity of wild-type strains and non-encapsulated mutants to resist the bactericidal effect of murine whole blood after 4 h of incubation. Percentage of bacterial killing was calculated in comparison to bacteria in plasma alone. Data represent the mean ± SEM from four independent experiments. n.d. denotes not detected. *** ($p < 0.001$) indicates a significant difference between wild-type strains (P1/7 or 89-1591) and their mutants (Δ*cpsF* or Δ*neuC*).

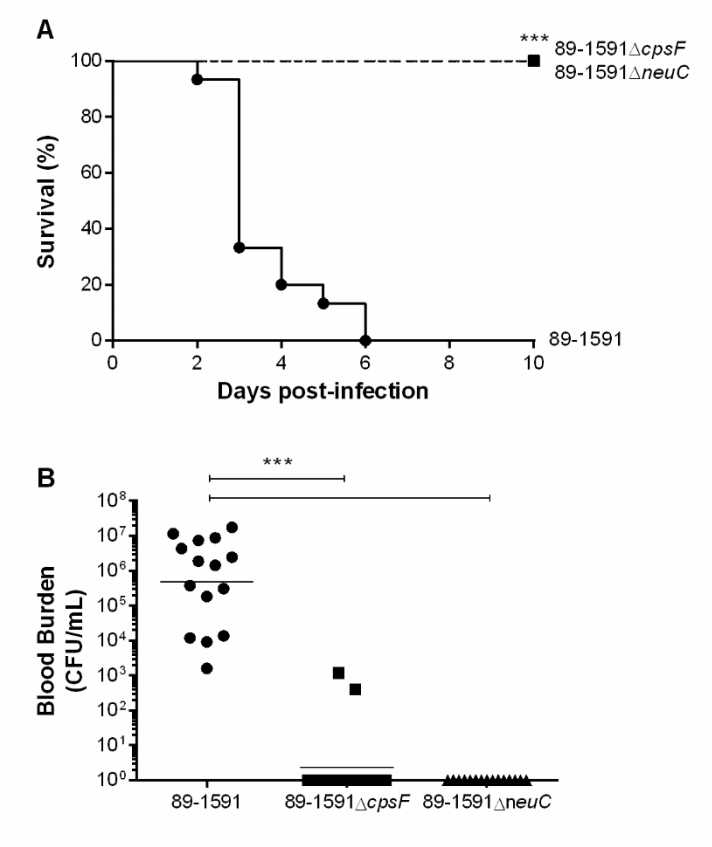

Figure 6. Capsular polysaccharide is required for virulence of the *S. suis* serotype 2 ST25 strain 89-1591 in a mouse model of infection and persistence in blood. Survival (A) and blood bacterial burden 24 h post-infection (B) of CD-1 mice following intraperitoneal inoculation of 5×10^7 CFU of wild-type strain 89-1591 or its non-encapsulated mutants 89-1591Δ*cpsF* and 89-1591Δ*neuC*. Data represent the survival curves (A) or geometric mean (B) of 15 mice/strain. *** ($p < 0.001$) indicates a significant difference between survival or blood bacterial burden of mice infected with wild-type strain 89-1591 and those infected with non-encapsulated mutants (89-1591Δ*cpsF* and 89-1591Δ*neuC*).

ARTICLE III

Recovery of *Streptococcus suis* serotype 2 capsule *in vivo*

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé à la conception de l'étude et des approches méthodologiques et j'ai effectué les dot-ELISA et les expériences *in vivo*. J'ai participé à l'analyse des résultats, à l'écriture de la première version du manuscrit et à la révision de celui-ci. J'ai aussi participé aux corrections lors des étapes de révision pour publication.

Mise en contexte

La présence de CPS est associée à plusieurs fonctions et rôles chez *S. suis* et est critique pour sa virulence (article I et article II). Toutefois, sa présence interfère aussi dans l'adhésion aux et l'invasion des cellules épithéliales, entre autres. En effet, seul un mutant dépourvu de CPS est capable de les envahir. Au contraire, la présence de la CPS est requise une fois dans la circulation afin de résister à l'effet bactéricide des leucocytes sanguins. Il a donc été suggéré, et ce depuis plusieurs années maintenant, que *S. suis* pourrait moduler la présence, ou du moins l'épaisseur de sa CPS, selon le milieu dans lequel il se retrouve. Ainsi, les pressions sélectives de l'environnement auquel il fait face pourraient influencer l'expression et/ou l'épaisseur de la CPS à sa surface. Toutefois, aucune étude n'avait évalué cette hypothèse de manière expérimentale.

Abstract

Many *Streptococcus suis* isolated from porcine endocarditis in slaughterhouses have lost their capsule and are considered avirulent. However, we retrieved capsule- and virulence-recovered *S. suis* after in vivo passages of a non-encapsulated strain in mice, suggesting that non-encapsulated *S. suis* are still potentially hazardous for individuals in the swine industry.

Introduction

Streptococcus suis is a major pathogen that causes severe economic losses to the swine industry. Moreover, it is an important zoonotic agent that causes severe diseases to people in close contact with diseased pigs or their products [1]. In Japan, *S. suis* has been frequently isolated from pigs with endocarditis in slaughterhouses, and most of the isolates were expected to be sequence types (STs), which are potentially hazardous to humans [2]. Interestingly, many isolates from porcine endocarditis lost their capsule, and all the non-encapsulated isolates analyzed had mutations in the capsular polysaccharide synthesis (*cps*) genes [3-4]. The capsule of *S. suis* is an important virulence factor [1]. Although loss of the capsule gives *S. suis* some benefit in causing endocarditis by enhancing the ability of bacterial cells to adhere to porcine and human platelets, a major virulence determinant for infective endocarditis [3], non-encapsulated *S. suis* are generally considered to be avirulent [5]. However, it is unknown whether or not non-encapsulated *S. suis* lurking in porcine endocarditis pose a threat to people working in the swine industry.

The Study

To investigate whether or not non-encapsulated *S. suis* can restore the ability to express the capsule and become virulent again, we repeated in vitro or in vivo passages of non-encapsulated *S. suis* and attempted to retrieve capsule-recovered strains.

For the in vitro passages, 29 *S. suis* strains isolated from pigs with endocarditis were used. They had the *cps* gene cluster of serotype 2 but had lost their capsule due to mutations in the *cps* genes (Table I). We subcultured them twice in liquid media and separated the cells according to the buoyant density by Percoll density gradient centrifugation (Technical Appendices). Because encapsulated cells show lower density than non-encapsulated cells [6-7], capsular expression of *S. suis* cells with low density was investigated by coagglutination tests using serotype 2 antiserum (Technical Appendices). The retrieved *S. suis* was also used for the next subcultures. We repeated four cycles of this experiment (in total eight subcultures); however, no encapsulated *S. suis* was obtained from any of the strains tested.

Although these results suggested that mutations in *cps* genes are not repaired easily, the conditions faced by *S. suis* in vivo could influence capsular expression. To investigate this, we

selected strain NL119 as a representative. NL119 is an ST1 strain, one of the types hazardous to humans, but has lost the capsule due to a point mutation that occurred at nucleotide position 490 (T490C, Cys164Arg) of a glycosyltransferase gene (*cps2F*) (Table I and Figure 1A) [4]. As described in the Technical Appendices, groups of five mice were inoculated with 5×10^8 CFU of NL119. Bacteria persistent in mice were retrieved 36 h post-infection (p.i.) from the blood, in which capsular expression works favorably for survival. Capsular expression of the retrieved NL119 was investigated by coagglutination tests, and the colony giving the strongest reaction within 30 sec was used for the subsequent in vivo passage.

The coagglutination test of the parental strain NL119 showed a negative result as expected. Similarly, NL119 after the first and second passages (NL119 P1 and P2, respectively) gave weak reactions, comparable to those of the parental strain, suggesting poor encapsulation. Meanwhile, NL119 after the third and fourth passages (NL119 P3 and P4, respectively) gave strong reactions, suggesting recovery of the capsule. To confirm this, we further analyzed formalin-killed bacteria by dot-ELISA using monoclonal antibody Z3, which reacts with the sialic acid moiety of the serotype 2 capsule, and an anti-*S. suis* serotype 2 serum adsorbed with parental strain NL119 in order to select CPS-specific antibodies (Technical Appendices) [8]. In accordance with the coagglutination test, NL119 P1 and P2 gave weak reactions similar to those of NL119, while strong signals were detected in NL119 P3 and P4 with both the monoclonal antibody and serum (Figure 1B and 1C). Because NL119 P1-P4 were also ST1 as determined by multilocus sequence typing, these results suggest that NL119 had recovered the capsule during passages in animals.

To find mutations which had contributed to the capsule recovery, we sequenced the *cps2F* gene of NL119 P1-P4. Although the cytosine residue at nucleotide position 490 was not changed compared with the parental strain, we found a further missense mutation at nucleotide position 491 (G491C, Arg164Pro) of the *cps2F* gene in NL119 P3 and P4 (Figure 1A). To investigate whether this mutation was involved in the capsule recovery, we cloned *cps2F* of NL119 P4 into a gene expression vector pMX1 [9] and introduced it into the parental strain NL119 (Technical Appendices). A coagglutination test using serotype 2 antiserum showed positive reactions in all transformants tested, demonstrating that the further missense mutation restored the function of *cps2F*, resulting in capsule recovery of NL119 P3 and P4, although it is unknown how the Cps2F function was recovered by the amino acid substitution. It is unclear

at present whether successful isolation of the capsule-recovered strains resulted from particular conditions in vivo that promote additional *cps* mutations or selection of encapsulated cells, which were already present as a sub-population in the original non-encapsulated NL119 population, by host immunity including phagocytosis. However, because NL119 was a non-encapsulated strain well-isolated by repeated passages in vitro, and no encapsulated sub-population was retrieved in vitro by the selection using Percoll density gradient centrifugation, the capsule-recovered *S. suis* is more likely to have been generated in vivo.

In order to evaluate if the capsule-recovered *S. suis* also recovered its virulence, mice were infected with either NL119 or NL119 P4 (Technical Appendices). A significant difference was observed in the mortality ($p<0.05$), with 50% mortality in the NL119 P4-infected mice 14 days p.i., compared with 0% for the non-encapsulated NL119 (Figure 2A). Recovery of the capsule also significantly increased its survival in the blood at 24 h p.i. ($p<0.05$). All surviving NL119 P4-infected mice, except one, presented significant blood bacterial titers (5×10^3 CFU/mL or higher) with a geometric mean of 1×10^4 CFU/mL. In contrast, except for one mouse, all mice infected with NL119 presented blood bacterial titers lower than 10^4 CFU/mL, with a geometric mean of 1×10^2 CFU/mL (Figure 2B).

Conclusions

Although capsule loss may contribute to *S. suis* infection by enhancing bacterial adherence to host cells and biofilm formation [3, 10-12], capsule loss makes *S. suis* cells susceptible to phagocytosis; therefore, the virulence of non-encapsulated mutants was attenuated when evaluated in animal models [5]. In accordance with previous studies, non-encapsulated NL119 was avirulent. However, NL119 P4, which recovered its capsule in vivo, also recovered virulence. Because various mutations in *cps* genes including large deletions and insertions cause capsule loss in *S. suis* [3-4], not all mutations will be repaired like NL119. However, our results demonstrated the presence of a non-encapsulated mutant, which can recover the capsule and virulence in vivo. Although further investigations using a variety of naturally occurring and laboratory-derived mutants will be needed for comprehensive understanding of the biological significance and mechanisms of this phenomenon, as suggested from this example, some of the non-encapsulated *S. suis* may cause severe diseases to the next hosts by recovering the capsule i.e., some non-encapsulated *S. suis* lurking in pigs with endocarditis are still potentially hazardous to people handling such pigs and their products.

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Technical appendices are available at the Emerging Infectious Diseases website

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Tables

Table I. Non-encapsulated *S. suis* strains used for passages

Strains	Affected gene(s)	Types of mutations	Affected nucleotide(s) (Affected amino acid)	Ref.
NL100	*cps2F*	Nonsense	T696G (Tyr232TERM)	4
NL119	*cps2F*	Missense	T490C (Cys164Arg)	4
NL122	*cps2F*	Missense	G52A (Gly18Ser)	4
NL126	*cps2F*	Frameshift by insertion	TCCG	4
NL132	*cps2E*	Missense	G1199A (Arg400Lys)	4
	cps2H	Frameshift by deletion	TA	4
NL143	*cps2F*	Missense	G493T (Asp165Tyr)	4
	cps2K	Insertion	AATCATTGG	4
	cps2R	Missense	G496A (Gly166Arg)	4
NL146	*cps2F*	Nonsense	T482A (Leu162TERM)	4
NL171	*cps2E*	Insertion	IS element: 1,619 bp	3
NL174	*cps2H*	Frameshift by deletion	A	4
NL175	*cps2H*	Frameshift by deletion	A	4
NL184	*cps2E*	Insertion	IS element: 1,115 bp	3
NL194	*cps2E*	Insertion	IS element: 1,416 bp	3
NL208	*cps2E*	Frameshift by deletion	TAAG	4
NL219	*cps2E*	Frameshift by deletion	TAAG	4
NL225	*cps2F*	Frameshift by insertion	CCAAA	4
NL230	*cps2F*	Frameshift by insertion	A	4
NL240	*cps2E*	Nonsense	C1189T (Gln397TERM)	4
NL245	*cps2E*	Frameshift by insertion	T	4
NL249	*cps2E*	Frameshift by insertion	AGCA	4
NL255	*cps2E*	Insertion	IS element: 1,619 bp	3
NL257	*cps2E*	Frameshift by insertion	ATCT	4
NL266	*cps2E*	Frameshift by deletion	A	4
NL278	*cps2F*	Missense	T259C (Ser87Pro)	4

NL295	*cps2F*	Missense	T492G (Cys164Trp)	4
NL303	*cps2F*	Deletion	81 bp	4
NL322	*cps2B*	Missense	G469A (Asp157Asn)	4
	cps2G	Deletion	50 bp	4
NL328	*cps2F*	Frameshift by deletion	AG	4
NL342	*cps2E*	Frameshift by deletion	TAAG	4
NL345	*cps2H*	Deletion	23 bp	4
	cps2N	Missense	C706T (Pro236Ser)	4

Figures

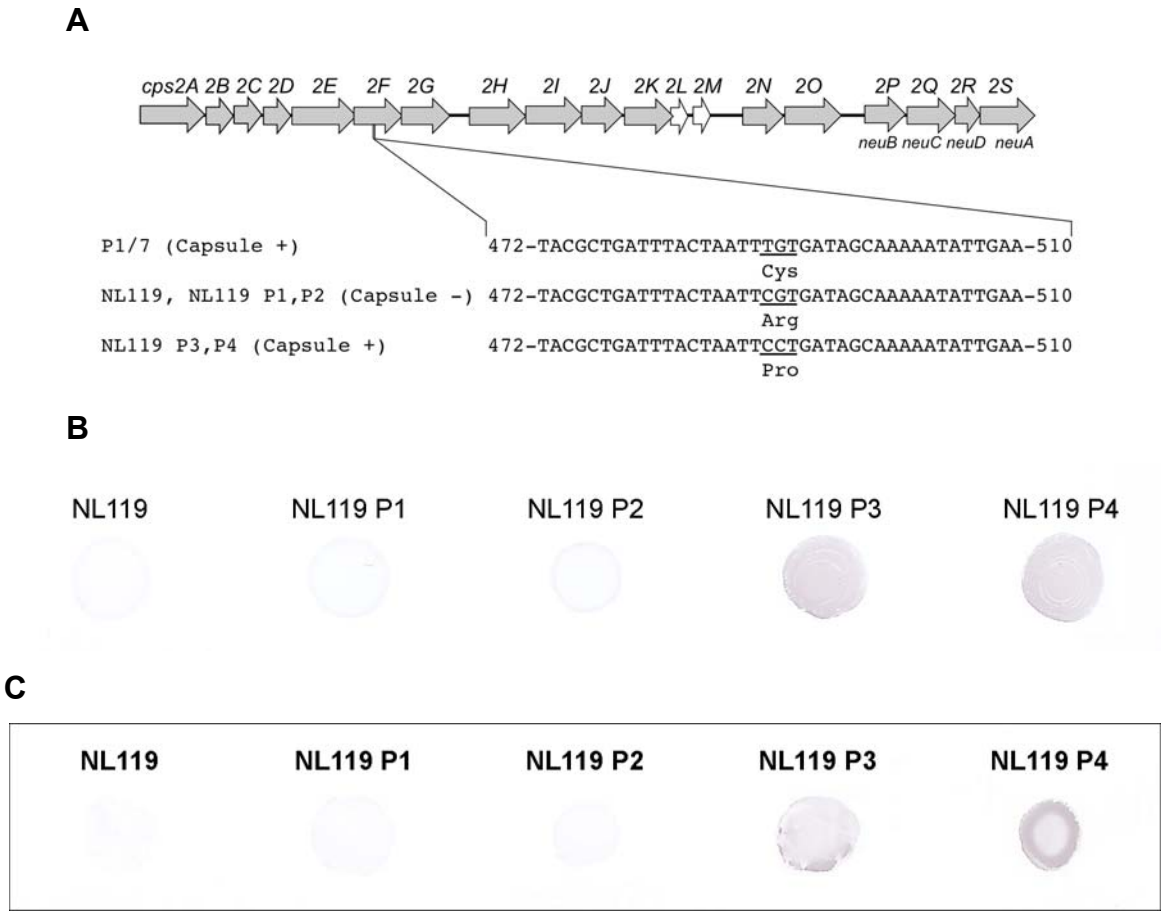

Figure 1. Capsule recovery of NL119 *in vivo*. (A) The genetic organization of the *S. suis* serotype 2 capsular polysaccharide synthesis (*cps*) gene cluster and mutations observed in NL119 and strains retrieved from NL119-infected mice after each in vivo passage (NL119 P1-P4) (DDBJ/EMBL/GenBank accession nos.: LC147077, LC147078, LC147079, LC147080 and LC077855, respectively). The gray arrows represent genes putatively involved in capsule synthesis. The open arrows indicate genes with unknown functions. The numbers indicate nucleotide positions in *cps2F*. NL119 lost the ability to synthesize the capsule due to a missense mutation at nucleotide position 490 (T490C, Cys164Arg) of *cps2F* (*4*). As shown in Figure 1B and C, NL119 P1 and P2, retrieved from mice after the first and second in vivo passages, respectively, were still non-encapsulated, and their *cps2F* sequences were identical to that of NL119. In NL119 P3 and P4, retrieved after the third and fourth passages, respectively, a further missense mutation at nucleotide position 491 (G491C, Arg164Pro) of the *cps2F* genes restored the function of the genes, resulting in capsule recovery of the strains. B) and C) Dot-ELISA of NL119 and strains retrieved from NL119-infected mice after each in vivo passage (NL119 P1-P4) using monoclonal antibody Z3 (B) and polyclonal anti-*S. suis* serotype 2 serum adsorbed with NL119 (C). Monoclonal antibody Z3 specifically recognizes the sialic acid moiety of the *S. suis* serotype 2 capsule.

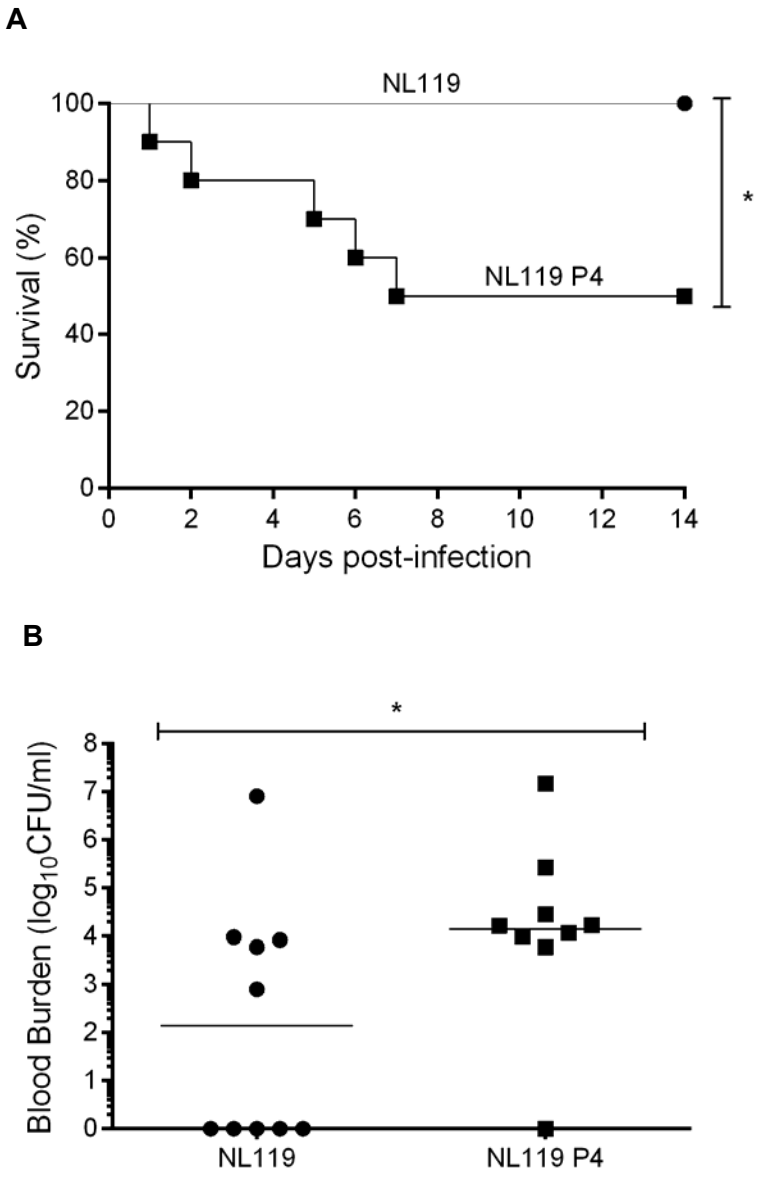

Figure 2. Virulence of non-encapsulated NL119 and capsule-recovered NL119 P4 in mice. (A) Survival of C57BL/6 mice (n= 10 mice per strain; until 14 days p.i.) inoculated intraperitoneally with 5×10^7 CFU of either NL119 or NL119 P4. (B) Blood bacterial burden at 24 h p.i. Data of individual mice are presented as $\log_{10}$ CFU/mL with the geometric mean. Asterisks indicate a significant difference between NL119 and NL119 P4 ($p<0.05$).

ARTICLE IV

Serotype-specific role of antigen I/II in the initial steps of the pathogenesis of the infection caused by *Streptococcus suis*

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-premier auteur de l'article. J'ai participé à la conception des approches méthodologiques et aux expériences *in vitro* et *in vivo*. J'ai été impliqué dans la révision du manuscrit et j'ai aussi effectué les corrections lors des étapes de révision pour publication.

Mise en contexte

Après la CPS, nous nous sommes intéressés à l'AgI/II comme composant de surface de *S. suis* Les AgI/II sont des composants immunostimulateurs et des protéines multimodales retrouvés à la surface bactérienne et impliqués dans diverses fonctions. Non seulement sont-ils présents chez plusieurs espèces de streptocoques (GAS, GBS, *S. mutans* et *S. gordonii*, etc.), mais ils sont impliqués dans leur colonisation de l'hôte, leur persistance et leur dissémination. Très récemment, nous avons démontré la présence de gènes ayant une homologie à ceux codant pour les AgI/II d'autres streptocoques chez des souches de sérotypes 2 et 9 de *S. suis*. Toutefois, leurs rôles, leurs fonctions et leurs propriétés dans la colonisation de l'hôte, qui est la première étape de la pathogenèse de l'infection, restent inconnus. L'AgI/II pourrait donc correspondre à un nouveau facteur important pour la virulence de *S. suis* et une cible thérapeutique intéressante le cas advenant.

Abstract

Streptococcus suis is one of the most important post-weaning porcine bacterial pathogens worldwide. The serotypes 2 and 9 are often considered the most virulent and prevalent serotypes involved in swine infections, especially in Europe. However, knowledge of the bacterial factors involved in the first steps of the pathogenesis of the infection remains scarce. In several pathogenic streptococci, expression of multimodal adhesion proteins known as antigen I/II (AgI/II) have been linked with persistence in the upper respiratory tract and the oral cavity, as well as with bacterial dissemination. Herein, we report expression of these immunostimulatory factors by *S. suis* serotype 2 and 9 strains and that AgI/II-encoding genes are carried by integrative and conjugative elements. Using mutagenesis and different in vitro assays, we demonstrate that the contribution of AgI/II to the virulence of the serotype 2 strain used herein appears to be modest. In contrast, data demonstrate that the serotype 9 AgI/II participates in self-aggregation, induces salivary glycoprotein 340-related aggregation, contributes to biofilm formation and increased strain resistance to low pH, as well as in bacterial adhesion to extracellular matrix proteins and epithelial cells. Moreover, the use of a porcine infection model revealed that AgI/II contributes to colonization of the upper respiratory tract of pigs. Taken together, these findings suggest that surface exposed AgI/II likely play a key role in the first steps of the pathogenesis of the *S. suis* serotype 9 infection.

Introduction

Streptococcus suis is one of the most important post-weaning bacterial pathogens of pigs and a major economic problem for the porcine industry [1]. Septicemia with sudden death, meningitis, arthritis, and endocarditis are the most frequent clinical signs caused by *S. suis* in pigs [2]. *S. suis* is also a zoonotic agent responsible for numerous human cases of meningitis, septicemia, and streptococcal toxic shock-like syndrome [2]. In Western countries, human *S. suis* infections mostly occur in individuals directly or indirectly linked with the porcine industry. In contrast, the general population is at risk of *S. suis* disease in certain Asian countries where this pathogen has been shown to be an important cause of adult meningitis [3]. Serotype 2 is, globally, considered the most virulent serotype and the one most frequently isolated from both porcine and human infections [4]. The use of multilocus sequence typing has revealed that serotype 2 strains belonging to certain sequence types (STs) are more virulent than others. ST1 strains (virulent) predominate in most Eurasian countries, whereas ST25 and ST28 strains (intermediate and low virulence, respectively) are mainly present in North America [4]. Meanwhile, highly virulent ST7 strains, responsible for at least two important human outbreaks in China, have only been reported in that country [5]. The serotype 9 has recently emerged in certain European countries, such as Spain, the Netherlands, and Germany [4]. Yet, very few studies have addressed the presence of virulence factors in this serotype, and putative virulence factors described for serotype 2 strains may not always be present in serotype 9 strains [6]. Moreover, the first *S. suis* serotype 9 human case of infection was reported in 2015 [7].

The early steps of the pathogenesis of the *S. suis* infection are not well understood [1,8]. Currently, the most accepted hypothesis is that virulent strains reach the bloodstream after breaching the mucosal epithelium of either the upper respiratory or the gastrointestinal tracts of pigs [1]. Similarly, infection of humans occurs via skin wounds or at the intestinal interface following ingestion of raw or undercooked infected meat [1]. However, the precise mechanisms and virulence factors involved remain unknown. Of note, the upper respiratory tract of pigs, particularly the tonsils and nasal cavities, are important reservoirs of *S. suis* [1]. Furthermore, *S. suis* has also been shown to be present in nearly half of the submaxillary lymph node samples of clinically healthy pigs [9]. Bacterial loads in saliva swab and tonsillar brush samples are similar, indicating that *S. suis* is indeed a natural inhabitant of the oral cavity [10].

Antigens I/II (AgI/II) have been extensively described in oral as well as invasive pathogenic streptococci, including *Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* [11]. AgI/II are immunostimulatory components and multimodal adhesion proteins implicated in host upper respiratory tract and oral cavity persistence and dissemination [11]. Affinity of AgI/II-like proteins for binding salivary glycoproteins, especially the glycoprotein (gp) 340 (also called DMBT1 protein) is a common feature of this protein family [12]. Large quantities of gp340 are present in the saliva of mammals in either a surface-immobilized-form or fluid phase-form. It is also present at all mucosal surfaces, including the nasal and intestinal cavities [13,14]. Interestingly, it has been shown that *S. suis* is able to adhere to gp340 and that this protein aggregates certain strains of *S. suis* [15]. However, the strains tested did not express AgI/II when using a heterologous monospecific antibody [15].

In this study, using in silico analyses, genes with homology to those coding for AgI/II were identified in *S. suis* serotype 2 and 9 strains. Using isogenic mutants deficient for the expression and production of AgI/II in both serotype 2 (S2Δ*agI/II*) and serotype 9 (S9Δ*agI/II*), the role of this protein in different aspects of the pathogenesis of the infection caused by *S. suis* was evaluated. We report for the first time that these proteins play a limited or important role in the pathogenesis of the infection caused by *S. suis* serotype 2 and 9, respectively.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. The virulent serotype 2 ST7 strain SC84, responsible for the 2005 human outbreak in China [5], and the serotype 9 strain 1135776 (isolated from a diseased pig in Canada) were used herein as models to study the role of Ag I/II in the pathogenesis of the infection caused by *S. suis*. Twenty-five additional *S. suis* serotype 9 strains recovered from diseased pigs were also used to evaluate the prevalence of *agI/II* genes by PCR (**Additional File 1**). Seventeen of these strains originated from Canada, 3 from Brazil, 1 from Denmark (reference strain), and 4 from Germany. A strain isolated from a human case of infection was also included [7]. The *S. mutans* strain Ingbritt was

used as a tool for collection of porcine salivary agglutinins (pSAGs) whereas the *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA), MC1061 [16], and BL21(DE3) (Invitrogen) strains were used for DNA manipulations and/or AgI/II protein production. The different *Streptococcus* and *E. coli* strains were grown at 37 °C in Todd Hewitt (THB) under static conditions or in Luria-Bertoni broth (Becton Dickinson, Franklin Lakes, NJ, USA) with shaking, respectively. Antibiotics (Sigma-Aldrich, St-Louis, MO, USA), where needed, were used at the following concentrations for *S. suis* and *E. coli*: spectinomycin at 500 µg/mL and 50 µg/mL and erythromycin at 5 µg/mL and 200 µg/mL, respectively. Ampicillin was also used at a concentration of 50 µg/mL for *E. coli*.

Bioinformatics analyses

In silico analyses of AgI/II-coding DNA sequences (CDS) in *S. suis* genomes were performed using BLASTN (expected threshold < 10^{-3}) as previously described [17]. The *S. suis* nucleotide collection nr/nt database available in GenBank (taxid 1307) was queried for *S. suis* genomes. Alongside, a bank of *S. suis* serotype 2 North American ST25 and ST28 strains isolated from diseased pigs whose genomes were previously published [18,19] were also queried. Moreover, BLASTN was used to detect homologies with genes coding for AgI/II or orthologues that have already been described in other bacterial species: *S. mutans* SpaP (accession number NC_004350.2), *S. gordonii* SspA and SspB (accession number CP000725.1), *S. pyogenes* (accession number NC_007296.1), *S. agalactiae* (accession number AAJP01000002.1), and *Enterococcus faecalis* (accession number AY855841.2). Examination of CDS carriage by putative integrative and conjugative elements (ICEs) was conducted using the ICEberg database [20], followed by BLASTN using the *S. suis* serotype 2 SC84 (accession number GCA_000026725.1) and serotype 9 D12 (accession number GCA_000231905.1) genomes as queries. Protein domains were analyzed using the NCBI conserved domain database with the help of the BatchCD tool [21]. Cell wall anchored domains were predicted using CW-PRED [22], while transmembrane domains and signal peptide cleavage sites were detected using the TMHMM [23] and the SignalP [24] tools, respectively. The Expasy bioinformatics resource portal was used to determine the theoretical protein molecular weight [25].

DNA manipulations

Chromosomal *S. suis* DNA was prepared using standard methods [26] or InstaGene matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Plasmid DNA

preparations and purification of PCR amplicons were performed using the QIAprep Spin Miniprep Kit and the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. Oligonucleotide primers (listed in **Additional File 2**) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Primers were designed from the available *S. suis* serotype 2 (strain SC84) and serotype 9 (strain D12) genomes. DNA ligations and transformation of competent *E. coli* were performed as previously described [27]. Sequencing reactions were carried out using an ABI 3730xl Automated DNA Sequencer and the ABI PRISM Dye Terminator Cycle Version 3.1 (Applied Biosystems, Foster City, CA, USA) and analyses of sequences performed using the BioEdit© software and/or BLASTN.

Generation of the isogenic* agI/II-*deficient mutants and complemented strains

For precise in-frame deletions of the *agI/II* genes in the *S. suis* serotype 2 strain SC84 and serotype 9 strain 1135776, regions upstream and downstream of the genes were amplified and fused by overlap-extension PCR. The amplification products were subcloned into vector pCR2.1 (Invitrogen), excised using HindIII (Promega, Madison, WI, USA), and cloned into the thermosensitive gene replacement vector pSET4s as previously described [27]. The resulting serotype 2 and serotype 9 pSET4S-*agI/II* vectors were introduced into recipient serotype 2 and 9 strains, respectively. Allelic replacement and absence of AgI/II expression in resulting serotype 2 and serotype 9 *agI/II*-deficient mutants were confirmed by sequencing and Western blot, respectively.

The pOri23 plasmid [28], which carries a gene conferring resistance to erythromycin, was used for complementation assays. A DNA fragment composed of the full sequence of the *agI/II* genes, as well as their putative endogenous promotors and terminators was cloned into pOri23 using the EcoRI and PstI restriction enzymes (two constructs, one for the serotype 2 *agI/II* and another for the serotype 9 *agI/II*). Since the serotype 9 strain used is highly resistant to erythromycin (data not shown), and several reports have described increased resistance to this antimicrobial among serotype 2 strains [29,30], a spectinomycin resistance cassette derived from pSET4s was introduced into the pOri23-S2*agI/II* and pOri23-S9*agI/II* plasmids. Following subcloning steps using *E. coli* MC1061, the generated pOri23$_{spc}$-S2*agI/II* and pOri23$_{spc}$-S9*agI/II* plasmids were then introduced into the S2Δ*agI/II* and S9Δ*agI/II* strains to generate the complemented S2CΔ*agI/II* and S9CΔ*agI/II* strains, respectively.

Cloning, expression, and purification of the His-tagged recombinant AgI/II protein and production of polyclonal mono-specific antibodies

A 4430 bp fragment of the serotype 2 *agI/II* gene, excluding the sequences coding for the cell wall anchorage and the LPXTG domains, was cloned into the pET151 expression vector (Invitrogen) according to the manufacturer's instructions (**Figure 1**). Protein synthesis was induced using 0.5 mM of isopropyl β-D-1-thiogalactopyranoside and cells lysed using lysozyme (Sigma-Aldrich) and sonication. The resulting recombinant His-tagged AgI/II, henceforth rAgI/II, was purified by affinity chromatography using the His-Bind Resin Chromatography Kit (Novagen, Madison, WI, USA,) according to manufacturer's instructions. Protein purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis following dialysis. Protein concentration was determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Rabbits were inoculated with the purified rAgI/II to produce a mono-specific polyclonal serum as previously described [31]. This serum was then used to verify presence of the protein in wild-type, isogenic *agI/II*-deficient mutants, and complemented strains by Western blot as previously described [32].

Cell surface hydrophobicity

The relative surface hydrophobicity of the *S. suis* wild-type strains and *agI/II*-deficient mutants was determined by measuring their adsorption to n-hexadecane as previously described [33]. A serotype 2 non-encapsulated mutant strain showing a high percentage of hydrophobicity was used as a positive control [33].

In vitro *pathogenesis assays*

Self-aggregation and biofilm assays

For the self-aggregation assay, overnight cultures of *S. suis* were washed twice with phosphate-buffered saline (PBS), pH 7.3, and re-suspended in THB to obtain an optical density (OD) at 600 nm of 0.05. Samples were incubated at 37 °C for 24 h under static conditions and self-aggregation quantified as previously described [34]. Biofilm formation capacity was determined as previously described [35] in the absence or presence of 2 mg/mL of porcine fibrinogen (Sigma-Aldrich).

S. suis *aggregation to soluble porcine salivary agglutinins*

Saliva was obtained from pigs as previously described [36] with a few modifications. Briefly, cotton ropes were suspended for 30 min to allow a total of 80 growing pigs from a high health status herd with no recent history of endemic *S. suis* disease to chew. No clinical signs of disease were present during collection. Whole saliva was decanted and impurities eliminated by centrifugation at 8000 × *g* for 20 min at 4 °C. pSAGs were then purified from clarified saliva as previously described for human salivary agglutinins using *S. mutans* [37]. The pSAGs were dialyzed in PBS and the concentration determined using the Pierce BCA Protein Assay Kit. Bacterial aggregation was quantified every 20 min for 1 h in the absence or presence of pSAGs [37].

Evaluation of S. suis *adhesion to extracellular matrix proteins, porcine salivary agglutinins, and the gp340-derived SRCRP2 peptide by ELISA*

Bacterial cultures were produced as previously described [38]. Formaldehyde-killed bacteria were washed using either PBS-T (PBS containing 0.05% Tween-20) for experiments involving extracellular matrix proteins (ECM), or TBS-T (10 mM Tris-HCl, 150 mM NaCl, pH 7.5 containing 0.1% Tween-20) supplemented with 1 mM $CaCl_2$, for experiments involving pSAGs and the gp340-derived SRCRP2 peptide [39]. Maxisorp flat-bottom microtiter plates (NUNC, Rochester, NY, USA) were coated with 12.5 µg/mL of human plasma fibronectin (Sigma-Aldrich), 15 µg/mL of human type I collagen (Corning, Corning, NY, USA), 1 mg/mL of porcine fibrinogen or 50 µg/mL of pSAGs, all diluted in carbonate coating buffer (0.1 M, pH 9.6), or with 200 µg/mL of the SRCRP2 peptide (Bio Basic Canada Inc., Markham, ON, Canada) diluted in water, overnight at 4 °C. After washing with PBS-T or TBS-T and blocking with non-fat dry milk, bacterial suspensions equivalent to 1 × 10^8 CFU/mL were added to the plates and incubated at 37 °C for 2 h. Subsequent steps were undertaken as previously described [38] using serotype 2 or 9 specific rabbit antisera and the OD at 450 nm determined.

Acid stress killing assay

The ability of *S. suis* to withstand acid challenge was determined as previously described with some modifications [39]. Briefly, *S. suis* strains were grown in THB, washed twice with PBS, and adjusted to a concentration of 1 × 10^8 CFU/mL. Cells were then resuspended in 0.1 M glycine buffer adjusted to either pH 3.0 or 5.0 and incubated at 37 °C. Surviving bacteria were

accurately determined using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA, USA).

Cell adhesion and invasion assays

The newborn porcine tracheal epithelial cell line (NPTr) was cultured until confluent as previously described [40]. Cells were infected with *S. suis* as previously described with minor modifications [41]. Briefly, PBS-washed NPTr cells were incubated at 37 °C with 5% CO_2 and infected with *S. suis* at a multiplicity of infection of 10. After 2 h of incubation, wells were washed with PBS to remove non-associated bacteria. For adhesion assays, cells were lysed with 1 mL of cold water, while the invasion assay was performed using the antibiotic protection method as previously described [40], and associated or intracellular bacteria enumerated as described above.

Intranasal colonization in a porcine model of infection

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and the Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal, which approved the protocols and procedures used herein (permit number RECH-1570). Four-week old pigs (providing from the same high health status herd mentioned above) were used. The 10 pigs were randomly separated into two rooms upon arrival and their nasal cavities, saliva, and tonsils swabbed to confirm absence of serotype 9. The *S. suis* serotype 9 wild-type strain 1135776 and *agl/ll*-deficient mutant were cultured as previously described [42] to obtain a final concentration of 2×10^9 CFU/mL. Intranasal infections were carried out as previously described with some modifications [43]. Pigs were inoculated with 1 mL of 2% acetic acid per nostril 1 h prior to infection with 1 mL per nostril of either the wild-type or the S9Δ*agl/ll* mutant strain.

Nasal cavities were swabbed using sterile cotton-tipped applicators. Swabs were placed in sterile tubes containing PBS supplemented with 0.1% bovine serum albumin and immediately cultured. Serial dilutions of swab samples (10^0 to 10^{-6}) were plated on Colombia agar supplemented with 5% defibrinated sheep blood (Cedarlane, Burlington, ON, Canada), *Streptococcus* selective reagent SR0126 (Oxoid, Hampshire, UK), and selected antibiotics to which the serotype 9 strain is resistant at the concentrations used (50 µg/mL spectinomycin,

5 µg/mL erythromycin, 0.2 µg/mL penicillin G, and 1 µg/mL tetracycline). After incubation for 24 h at 37 °C with 5 % CO_2, plates containing 30 to 300 colonies were selected. Suspected alpha-hemolytic colonies were enumerated and 10 *S. suis*-like colonies per plate were sub-cultured and tested by coagglutination assay using anti-*S. suis* serotype 9 rabbit serum as previously described [44]. Three weeks post-infection, pigs were euthanized and tonsils recovered. Tonsil samples were processed as previously described [45] and *S. suis* serotype 9 carriage evaluated as described above.

Statistical analyses

At least three independent biological replicates were performed for each experiment and results expressed as mean ± standard error of the mean (SEM). Raw data were analyzed using the non-parametric statistical Mann Whitney test. Statistical differences are defined as being greater than $p < 0.05$.

Results

Prevalence and molecular characteristics of the S. suis *AgI/II*

Bioinformatics analyses using the *S. suis* (taxid 1307) genome database available in GenBank revealed the presence of genes coding for AgI/II-like proteins in the genomes of serotype 2 strains, including the ST7 strain SC84, ST1 strain BM407, ST25 strain 89-1591, and in a bank of North American *S. suis* serotype 2 ST25 and ST28 strain genomes [18,19]. However, they were absent from the genome of the reference ST1 strain P1/7. The gene was also present in the genome of the serotype 9 strain D12. Given the low number of published *S. suis* serotype 9 genomes, PCR analyses were undertaken using field strains, which confirmed the presence of the gene in the 25 strains tested (**Additional File 1**), including strains from Canada, Germany, and Brazil, as well as in the reference *S. suis* serotype 9 strain from Denmark and a human isolate from Thailand. *S. suis* serotype 2 and 9 genes coding for AgI/II share approximately 95% of nucleotide identity. In addition, the promotors share 92% of nucleotide identity with the -35 and -10 boxes and the ribosome binding site for *agI/II* genes being present in all available genomes. Moreover, the terminators of *agI/II* genes are conserved in all strains (100% of nucleotide identity). The percentage of identity between the AgI/II proteins of serotypes 2 and 9 is 95 %, being both highly similar. Alignment of the amino acid sequence of both proteins is presented in **Additional File 3**. Bioinformatics analyses revealed that the *S. suis* AgI/II has a theoretical molecular weight of 180 kDa, which is slightly larger than that of

other described AgI/II, probably due to the SspB-like isopeptide-forming domain being repeated thrice in the C-terminal part of the *S. suis* AgI/II (**Figure 1**) [11]. The *S. suis* AgI/II shares between 29% and 42% of protein sequence identity with other streptococcal AgI/II, such as AspA (*S. pyogenes*), SpaP (*S. mutans*), SspA (*S. gordonii*), and SspB (*S. gordonii*) (**Figure 1**). Alongside, the *S. suis* AgI/II also shares 32% of protein identity with the aggregation substance PrgB (also called Asc10) of *E. faecalis* [46]. The *S. suis* AgI/II has similar characteristic domains to those described in oral streptococci (**Figure 1**) [11].

Further bioinformatics investigations, including the use of the ICEberg database, revealed that the gene encoding for the AgI/II protein in the serotype 2 strain SC84 is carried by the 89 K integrative and conjugative element (ICE) (89 Kbp) [47], while that of the serotype 2 ST1 strain BM407 is carried by two putative ICEs annotated as ICE*Ssu*(BM407)1 and ICE*Ssu*(BM407)2) (75 Kbp and 80 Kbp, respectively). Moreover, the gene coding for AgI/II in the serotype 9 strain D12 is also carried by an element sharing 95% of nucleotide identity with the whole sequence of ICE*Ssu*(BM407)1. Altogether, these analyses suggest that the *S. suis* AgI/II are mainly carried by ICEs.

Confirmation of AgI/II-deficient mutants in both *S. suis* serotypes 2 and 9

Production of AgI/II by the serotype 2 and 9 strains SC84 and 1135776, respectively, was confirmed by immunoblotting using mono-specific antisera produced with the recombinant protein, rAgI/II (**Figure 2**). The proteins had a molecular weight of approximately 180 kDa, as predicted by bioinformatics analyses. Deletion of the *agI/II* gene resulted in absence of detectable signal while complementation of the mutant strains restored detection with a band at the expected molecular weight (**Figure 2**). Growth of the S2Δ*agI/II* and S9Δ*agI/II* mutants as well as that of the complemented strains was similar to their respective wild-type strains (data not shown).

It was previously described that AgI/II positively impacts surface hydrophobicity of oral streptococci. However, we did not observe significant differences in hydrophobicity between the *S. suis* serotype 2 or 9 wild-type strains and their AgI/II-deficient mutants (S2Δ*agI/II* and S9Δ*agI/II*) (**Additional File 4**). Interestingly, the serotype 2 wild-type strain was significantly more hydrophobic than that of serotype 9 ($p < 0.05$).

In vitro *pathogenesis assays*

Serotype-dependent role of the* S. suis *AgI/II in self-aggregation and biofilm formation

S. suis serotype 2 self-aggregation was not modified by the absence of AgI/II (**Figure 3A**). However, deletion of AgI/II significantly reduced self-aggregation of *S. suis* serotype 9 by 80% ($p < 0.01$) (**Figure 3A**). On the other hand, self-aggregation was completely restored when using the complemented S9CΔ*agI/II* strain (**Figure 3A**). Thus, the serotype 9 AgI/II, but not that of serotype 2, is involved in bacterial self-aggregation.

The role of AgI/II in biofilm formation was evaluated for both serotype 2 and 9 in the presence of porcine fibrinogen. The capacity of the serotype 2 strain to form biofilm was relatively low, and no difference was observed in the absence of AgI/II (**Figure 3B**). On the other hand, the serotype 9 wild-type strain showed a significantly greater capacity to form biofilm than the wild-type serotype 2 strain in the presence of porcine fibrinogen ($p < 0.01$). Furthermore, the serotype 9 AgI/II was significantly involved in this bacterial function ($p < 0.001$) (**Figure 3B**). The capacity to form biofilm was restored in the complemented S9CΔ*agI/II* strain (**Figure 3B**). Minimal biofilm formation was observed in the absence of porcine fibrinogen for both the serotype 2 and 9 strains (**Additional File 5**). Consequently, the serotype 9 AgI/II, but not that of serotype 2, plays an important role in the capacity to form biofilm.

The* S. suis *AgI/II increases both porcine salivary agglutinin induced-aggregation and adhesion to salivary agglutinins

Salivary agglutinins are major receptors of streptococcal AgI/II [12]. Thus, we investigated the interactions of the *S. suis* serotype 2 and 9 AgI/II with fluid-phase (miming the conditions in saliva) and surface-immobilized (miming mucosa such as in the oral cavity) pSAGs. pSAGs collected from pig saliva was obtained at a concentration of 50 µg/mL, which is similar to that usually obtained for human salivary agglutinins [37].

Results showed a significantly more rapid and greater aggregation of both *S. suis* serotype 2 or serotype 9 strains in the presence of pSAGs ($p < 0.05$) (**Figure 4**). Moreover, this fluid-phase pSAG-induced aggregation significantly increased with time ($p < 0.05$) (**Figure 4**). However, the pSAG-mediated aggregation induced by the serotype 9 strain was significantly higher than that induced by the serotype 2 strain, but only after 60 min of incubation ($p < 0.05$) (**Figure 4**).

AgI/II-deficiency significantly reduced fluid phase pSAG-induced aggregation for both serotypes ($p < 0.05$) (**Figures 5A and B**), and complementation of AgI/II-deficient mutants restored fluid phase pSAG-induced aggregation ($p < 0.01$) (**Figures 5A and B**).

The adhesion of *S. suis* to surface-immobilized pSAGs was then evaluated using ELISA. Since background obtained with crude pSAGs was very elevated (data not shown), the gp340-derived peptide SRCRP2, described as the major binding sequence for AgI/II [37], was used. Results showed that deletion of the *S. suis* serotype 2 *agI/II* had no effect on adhesion to SRCRP2 (**Figure 5C**), while that of serotype 9 significantly reduced adhesion to SRCRP2 ($p < 0.05$), but only at a concentration of 200 µg/mL (**Figure 5D**). As expected, complementation of the *S. suis* serotype 9 AgI/II-deficient mutant restored adhesion to SRCRP2 (**Figure 5D**).

Taken together, these results demonstrate that AgI/II promotes pSAG-induced aggregation when in fluid phase for both serotypes, and adhesion to the gp340-derived peptide SRCRP2 at a high concentration for serotype 9 only.

The* S. suis *AgI/II confers protection to acid stress

Once swallowed, *S. suis* will reach the stomach, in which it must overcome hostile environmental conditions such as low pH. We thus investigated the role of AgI/II and aggregation in resistance to low pH. Acid stress killing assays revealed that the *S. suis* serotype 2 AgI/II was not involved in acid resistance at pH 3 (**Figure 6A**) nor at pH 5 (**Figure 6C**). On the other hand, results showed that the S9Δ*agI/II* mutant strain survived significantly less than its wild-type strain ($p < 0.05$) at both pH 3 (**Figure 6B**) and pH 5 (**Figure 6D**). Thus, AgI/II confers partial protection to *S. suis* serotype 9, but not to serotype 2, against acidic environments.

The* S. suis *serotype 9 AgI/II contributes to adhesion to extracellular matrix proteins and to porcine epithelial cells

AgI/II was previously described in other streptococci as binding ECM proteins and contributing to adhesion to and invasion of epithelial cells. Our results showed that while the serotype 2 AgI/II was not involved in adhesion to collagen I, that of the serotype 9 played a significant role ($p < 0.01$) (**Figures 7A and B**). In accordance, complementation of the S9Δ*agI/II* mutant restored the wild-type phenotype (**Figure 7B**). Moreover, as previously described with other

serotype 2 strains [38], the serotype 2 wild-type strain used in this study (SC84) did not bind porcine fibrinogen (**Figure 7C**). On the other hand, the serotype 9 wild-type strain did bind to porcine fibrinogen, with absence of AgI/II significantly reducing this ability ($p < 0.05$) (**Figure 7D**). Once again, complementation of the S9Δ*agI/II* mutant strain restored this adhesion capacity (**Figure 7D**). Finally, the deletion of the *S. suis* serotype 9 *agI/II* gene and, to a lesser extent, that of the serotype 2, significantly decreased adhesion to plasma fibronectin ($p < 0.05$) (**Figures 7E and F**). Consequently, these results demonstrate the importance of AgI/II as a multimodal adhesin for *S. suis* serotype 9 while only playing a minor rôle for serotype 2.

The role of AgI/II in adhesion to and invasion of porcine tracheal epithelial cells was subsequently investigated. Interestingly, the serotype 9 wild-type strain adhered significantly more to epithelial cells than did the serotype 2 ($p < 0.05$) (**Figure 8**). Adhesion assays revealed a significant decrease in adhesion to epithelial cells in the absence of AgI/II for the serotype 9 ($p < 0.05$), equivalent to 30% of wild-type strain adhesion, with complementation restoring adhesion (**Figure 8**). On the other hand, no differences were observed between the *S. suis* serotype 2 wild-type strain and its AgI/II-deficient mutant (**Figure 8**). Low levels of epithelial cell invasion were observed for both serotypes, with no role of AgI/II being evident (data not shown). Taken together, these results reveal that AgI/II is implicated in adhesion to host proteins and epithelial cells for serotype 9 and, to a lesser extent, for serotype 2.

Role of AgI/II in colonization of the oral and nasal cavities of pigs

Given that *in vitro* results demonstrated an important role of AgI/II for *S. suis* serotype 9, we next evaluated the contribution of this protein in colonization using a porcine infection model. Animals were divided into two groups and infected with either the serotype 9 wild-type strain or the AgI/II-deficient mutant by intranasal inoculation. Evaluation of serotype 9 colonization revealed that the number of wild-type strain recovered from the nasal cavities significantly increased over time until day 12 post-infection (pi) ($p < 0.05$), whereas the number of S9Δ*agI/II* remained stable throughout the experiment (**Figure 9A**). Moreover, AgI/II-deficient mutants were recovered in significantly lower numbers from the nasal cavities of pigs on days 5, 8, and 12 pi ($p < 0.05$) (**Figure 9A**). Although the number of serotype 9 wild-type strain and AgI/II-deficient mutant in the nasal cavities of pigs was similar 21 days pi (**Figure 9A**), AgI/II-deficiency resulted in significantly reduced colonization of tonsils ($p < 0.05$) (**Figure 9B**).

Together, these results strongly suggest that the serotype 9 AgI/II contributes to colonization of the porcine respiratory tract.

Discussion

AgI/II proteins have been extensively described in oral pathogenic streptococci as multimodal adhesion proteins and immunostimulatory components implicated in host upper respiratory tract and oral cavity persistence and dissemination [11]. In addition, it has been shown that AgI/II proteins potentially play multiple roles in *Streptococcus* adherence, colonization, and microbial community development [11]. These proteins have also been described in pyogenic streptococci, such as *S. pyogenes* and *S. agalactiae,* but they have never been identified in *Streptococcus pneumoniae* [11]. An initial goal of this study was to determine whether *S. suis* possesses these putative virulence factors. We showed that most of the *S. suis* serotype 2 available genomes, including from different STs, possess genes encoding AgI/II. Interestingly, the gene was absent from the ST1 strain P1/7, which is commonly used as a reference for investigation of virulence [6]. We also identified AgI/II-encoding genes in the genome of the Chinese serotype 9 strain D12, in the serotype 9 reference strain 22083, as well as in a collection of 25 serotype 9 field strains (added herein given the limited number of serotype 9 genomes available), alongside a human isolate, tested by PCR.

It is widely recognized that mobile genetic elements such as insertion sequences, transposons, bacteriophages, plasmids, and genomic islands are key drivers of genomic evolution and bacterial adaptation. Among them, ICEs are chromosomal genetic elements that play an important role in horizontal gene transfer [48]. In both *S. pyogenes* and *S. agalactiae*, AgI/II are encoded by genes carried by ICEs, which can spread not only to other *S. pyogenes* and *S. agalactiae* strains, but also to other streptococci [49,50]. Meanwhile, different ICEs have been described in *S. suis* [51], of which the 89 K ICE carried by the *S. suis* serotype 2 strain SC84 has been suggested to be responsible, at least in part, for the higher virulence of this strain [52]. Interestingly, results obtained in this study showed that the *S. suis agl/II* genes are mainly carried by ICEs. As such, it may be suggested that acquisition of AgI/II by *S. suis* occurred via horizontal transfer following acquisition of ICEs.

Persistence of *S. suis* in the oral cavity may contribute to the pathogenesis of the infection. Our data showed that AgI/II plays an important rôle in self-aggregation for *S. suis* serotype 9. This role was even more important in the presence of salivary glycoproteins, such as gp340. It has been previously shown that human salivary gp340 was able to aggregate an untypeable, a serotype 1, and a serotype 2 *S. suis* strain [15]. However, these strains were negative for the expression of AgI/II as evaluated by immunoblot using a polyclonal antibody raised against the *S. mutans* proteins [15]. In the present study, we showed that purified soluble pSAGs increase the ability of *S. suis* to aggregate and that AgI/II played an important role in such interactions for serotype 9 and, to a lesser extent, serotype 2. Fluid-phase and surface-immobilized gp340 expose different binding properties and, consequently, differentially recognize adhesive phenotypes of diverse bacterial species. Herein, we showed that AgI/II also played a role in the *S. suis* serotype 9 adhesion to the surface-immobilized gp340-derived peptide SRCRP2. Similarly, the AgI/II from *S. suis* serotype 9 also played an important role in biofilm formation.

The relationship between the saliva-dependent aggregation, attachment to salivary glycoproteins, and biofilm formation in the oral cavity and pathogenesis of the infection caused by pathogenic streptococci is not very clear. On the one hand, aggregation (clumping) may presumably allow “bacterial clearance” from the oral cavity via swallowing [53]. It is usually accepted that the main route of infection for pigs is through the respiratory tract. However, more recently, the oral route (as clearly described in humans) has also been suggested as a portal of entry in pigs [54]. Although a recent report showed that disease could not be induced in an experimental infection by the oral route in post-weaned animals [55], a role of early colonization of the intestine of pre-weaned piglets followed by direct invasion through intestinal epithelial cells in animals under post-weaned stress could not be completely ruled out [1]. In the present study, an increased susceptibility to low pH (usually found in the stomach) was observed for *S. suis* serotype 9 in the absence of the *agI/II* gene. As such, it may be hypothesized that AgI/II induces bacterial self-mediated and salivary agglutinin-mediated aggregation and biofilm formation for serotype 9, which would increase, at certain moments, the swallowing of large amounts of bacteria. AgI/II would subsequently increase bacterial protection against the low pH of the stomach, thus allowing colonization of the intestine. However, this hypothesis remains to be confirmed.

It has been proposed that adhesion to epithelial cells is one of the most important initial steps of the pathogenesis of the infection caused by *S. suis* [1]. Similarly to other pathogens, *S. suis* is also able to bind ECM components, which have been suggested to be implicated as cell receptors [1]. At least 28 different *S. suis* components have been described to be involved in such interactions so far [1,6]. In the present study, it was clearly shown that the AgI/II plays an important role in the adhesion of *S. suis* serotype 9 to collagen I, fibrinogen, and fibronectin. In the case of serotype 2, this protein plays a minimal role in adhesion to fibronectin and none to collagen I. As previously described, the serotype 2 strain was unable to bind fibrinogen [38]. The lack of binding to the latter may also explain differences observed in biofilm formation (in the presence of this protein) between serotype 2 and serotype 9 strains and the important role played by the serotype 9 AgI/II.

The implication of AgI/II in the adhesion to epithelial cells was further evaluated using porcine tracheal epithelial cells as a model [40]. Firstly, it was interesting to note that the serotype 9 wild-type strain presented higher adhesion levels than the serotype 2 strain, a fact that has been previously reported with other porcine cells [54]. A role was attributed to AgI/II in the adhesion of serotype 9 since a significant reduction of adhesion to these cells was observed using the S9Δ*agI/II* mutant. This reduction of adhesion could be explained by a reduction in the interactions with ECM components (as described above) or through a direct effect of the AgI/II as an adhesin. In fact, this protein has been described to be directly involved in epithelial cell adhesion and invasion by *S. gordonii* through β1 integrin recognition [56]. Using a different mechanism, this protein was also involved in adhesion/invasion of *S. pyogenes* to these cells [56].

Previous studies showed that the *S. pyogenes* AgI/II is implicated in upper respiratory tract colonization [57]. Since results showed that AgI/II plays important roles in vitro for serotype 9, its implication in colonization of the upper respiratory tract was investigated in pigs. As previously described, pigs infected by the serotype 9 wild-type strain and its isogenic S9Δ*agI/II* mutant via the intranasal route did not develop clinical signs of infection [43]. However, a slight, yet significantly lower colonization of the upper respiratory tract by the mutant strain, and, subsequently at the tonsillar level, was observed, suggesting that this protein may collaborate

in bacterial colonization during the first steps of the infection. However, additional studies should be carried out to confirm this hypothesis.

In conclusion, the presence of AgI/II is herein reported for the first time in *S. suis*. This protein appears to play important or limited roles during the first steps of the pathogenesis of the infection caused by serotypes 9 and 2, respectively. Since the gene and protein sequences are highly similar between both serotypes, the observed differences are more difficult to explain than anticipated, and several hypotheses may be proposed. Firstly, a particular motif specific to the gene coding for the serotype 9 AgI/II might be responsible for the phenotypic differences highlighted in this study. Secondly, the *S. suis* serotype 2 and 9 *agI/II* genes are both carried by ICEs, which vary, creating differing genetic contexts and, consequently, differential gene regulation. Thirdly, critical *S. suis* virulence factors still remain poorly known [6]; the lack of a dominant role of the serotype 2 AgI/II observed herein might also be due to compensation by other virulence factors that result in bacterial redundancy [6]. Further studies are presently underway to explore these avenues. Overall, AgI/II may contribute to the colonization of the upper respiratory tract of pigs and could represent important surface bacterial components implicated in the first steps of the pathogenesis of the infection caused by *S. suis*.

List of Abbreviations

AgI/II: antigen I/II; BCA: bicinchoninic acid; CDS: coding DNA sequence; ECM: extracellular matrix protein; gp340: glycoprotein 340; ICE: integrative and conjugative element; NPTr: newborn porcine tracheal epithelial cell; OD: optical density; PBS: phosphate-buffered saline; pi: post-infection; pSAG: porcine salivary agglutinin; SEM: standard error of the mean; ST: sequence type; THB: Todd Hewitt broth.

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Supporting Information (Available at the Veterinary Research Website)

Additional File 1. List of *S. suis* serotype 9 strains used in this study and their characteristics.

Additional File 2. List of primers used in this study.

Additional File 3. S. suis serotype 2 (S2) and serotype 9 (S9) AgI/ II amino acid sequence alignment.

Additional File 4. Percent hydrophobicity of the *S. suis* serotype 2 (S2) and serotype 9 (S9) wild-type and *agI/II*-deficient mutant strains.

Additional File 5. Biofilm formation by the *S. suis* serotype 2 (S2) and serotype 9 (S9) wild-type and *agI/II*-deficient mutant strains in the absence of porcine fibrinogen.

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Tables

Table 1. Strains and plasmids used in the study

Strain or plasmid	Characteristics	Reference
Streptococcus suis		
SC84	Serotype 2 strain isolated from a patient with streptococcal toxic shock-like syndrome in China	[47]
1135776	Serotype 9 strain isolated from pig following sudden death in Canada	This study
S2Δ*agl/rl*	SC84-derived strain carrying an in-frame deletion of the *agl/rl* gene	This study
S9Δ*agl/rl*	1135776-derived strain carrying an in-frame deletion of the *agl/rl* gene	This study
S2CΔ*agl/rl*	SC84-derived strain carrying pOri23-S2*agl/rl*	This study
S9CΔ*agl/rl*	1135776-derived strain carrying pOri23-S9*agl/rl*	This study
Escherichia coli		
TOP10	Host for pCR2.1 and pSET4s derivatives	Invitrogen
MC1061	Host for pOri23 derivatives	[16]
BL21(DE3)	Host for pET151 derivatives	Invitrogen
Plasmids		
pET151	Ap^r, pBR322 *ori*, T7 promotor	Invitrogen
pCR2.1	Ap^r, Km^r, pUC *ori*, *lac*ZΔM15	Invitrogen
pSET4s	Spc^r, pUC *ori*, thermosensitive pG+host3 *ori*, *lac*ZΔM15	[37]
pOri23	Erm^r, ColE1 ori, *P23*	[28]
pET151-S2*agl/rl*	pET151 carrying the S2 *agl/rl* gene	This study
pSET4s-S2*agl/rl*	pSET4s carrying regions upstream and downstream of the S2 *agl/rl* gene	This study
pSET4s-S9*agl/rl*	pSET4s carrying regions upstream and downstream of the S9 *agl/rl* gene	This study
$pOri23_{spc}$-S2*agl/rl*	pOri23 carrying the S2 *agl/rl* gene as well as its promotor and terminator	This study
$pOri23_{spc}$-S9*agl/rl*	pOri23 carrying the S9 *agl/rl* gene as well as its promotor and terminator	This study

Figures

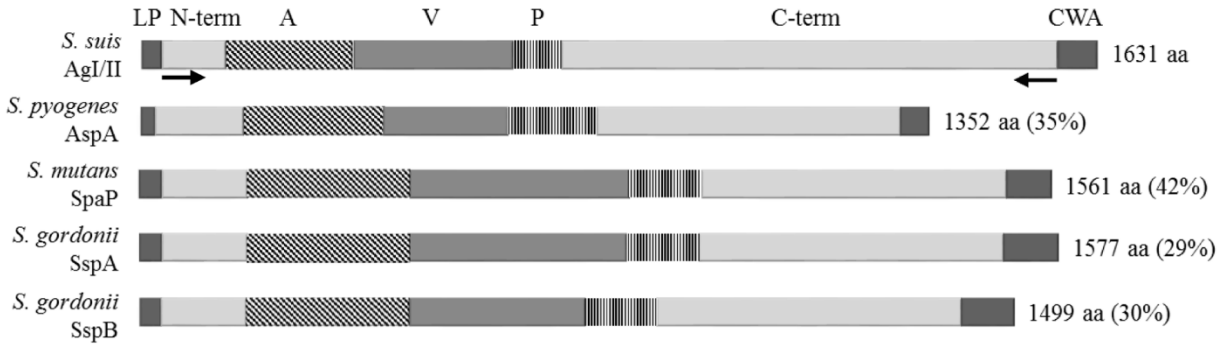

Figure 1. Characteristics of AgI/II proteins present in different streptococci. The leader peptide signal (LP), N-terminal domain (N-term), alanine-rich region (A), variable region (V), proline-rich region (P), and C-terminal domain, and the cell wall anchorage domain (CWA) containing the LPXTG domain are illustrated for the *S. suis* AgI/II, *S. pyogenes* AspA, *S. mutans* SpaP, and *S. gordonii* SspA and SspB. Amino acid (aa) size and percentage of *S. suis* AgI/II protein identity are also indicated. Black arrows indicate the location of primers pET151_S2*agI/II*Δ*CWA*_F and pET151_S2*agI/II*_Δ*LPXTG*_R, which were used to produce the His-tagged recombinant AgI/II protein, rAgI/II.

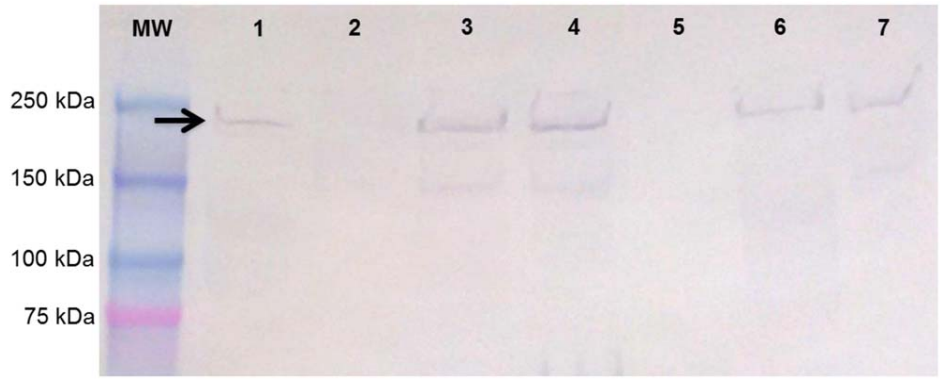

Figure 2. The AgI/II protein is expressed in the *S. suis* serotype 2 and 9 wild-type strains but is absent in S2Δ*agI/II* and S9Δ*agI/II* mutant strains. Western blot using cell wall extracts from *S. suis* serotype 2 (wells 1 to 3) and serotype 9 (wells 4 to 6): serotype 2 wild-type strain SC84 (well 1) and serotype 9 wild-type strain 1135776 (well 4); mutant strains S2Δ*agI/II* (well 2) and S9Δ*agI/II* (well 5); and complemented strains S2CΔ*agI/II* (well 3) and S9CΔ*agI/II* (well 6). Expected bands at approximately 180 kDa, shown by the black arrow, were observed for the serotype 2 and 9 wild-type and complemented strains, similar to that obtained with the purified AgI/II protein, rAgI/II (well 7), used as a positive control. MW: molecular weight marker.

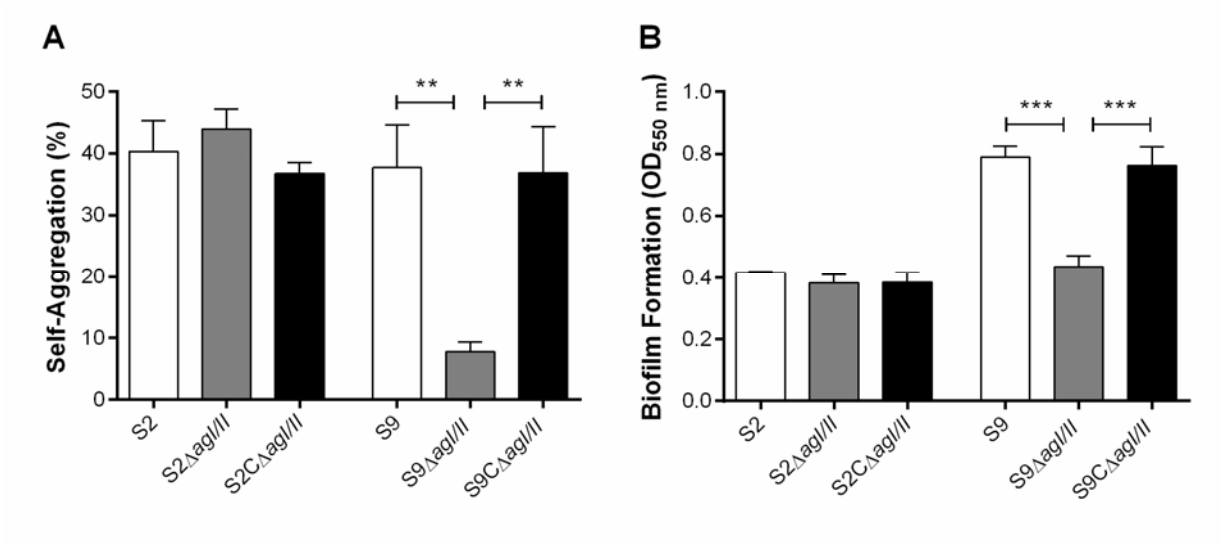

Figure 3. The *S. suis* serotype 9 (S9) AgI/II, but not that of the serotype 2 (S2), is implicated in bacterial self-aggregation and biofilm formation. The role of the *S. suis* AgI/II was evaluated with regards to cell-to-cell aggregation in fluid phase (A) and biofilm formation capacity in the presence of porcine fibrinogen (B) after 24 h of incubation at 37 °C. Data represent the mean ± SEM from at least three independent experiments. ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agI/II*) and *agI/II*-deficient mutant (S9Δ*agI/II*).

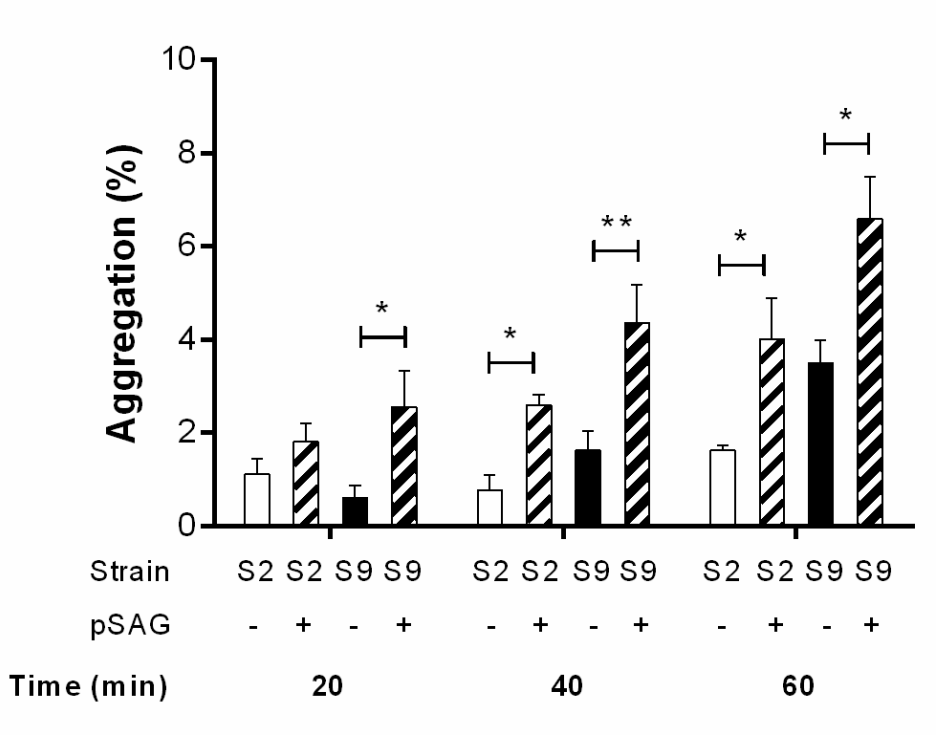

Figure 4. Porcine salivary agglutinins (pSAGs) aggregate *S. suis* serotype 2 (S2) and serotype 9 (S9). Evaluation of the fluid phase aggregation of the wild-type *S. suis* S2 and S9 strains in the absence (−) or presence (+) of pSAGs. Aggregation in the absence of pSAGs reflects self-aggregation only. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference of *S. suis* S2 or S9 aggregation in the absence and presence of pSAGs.

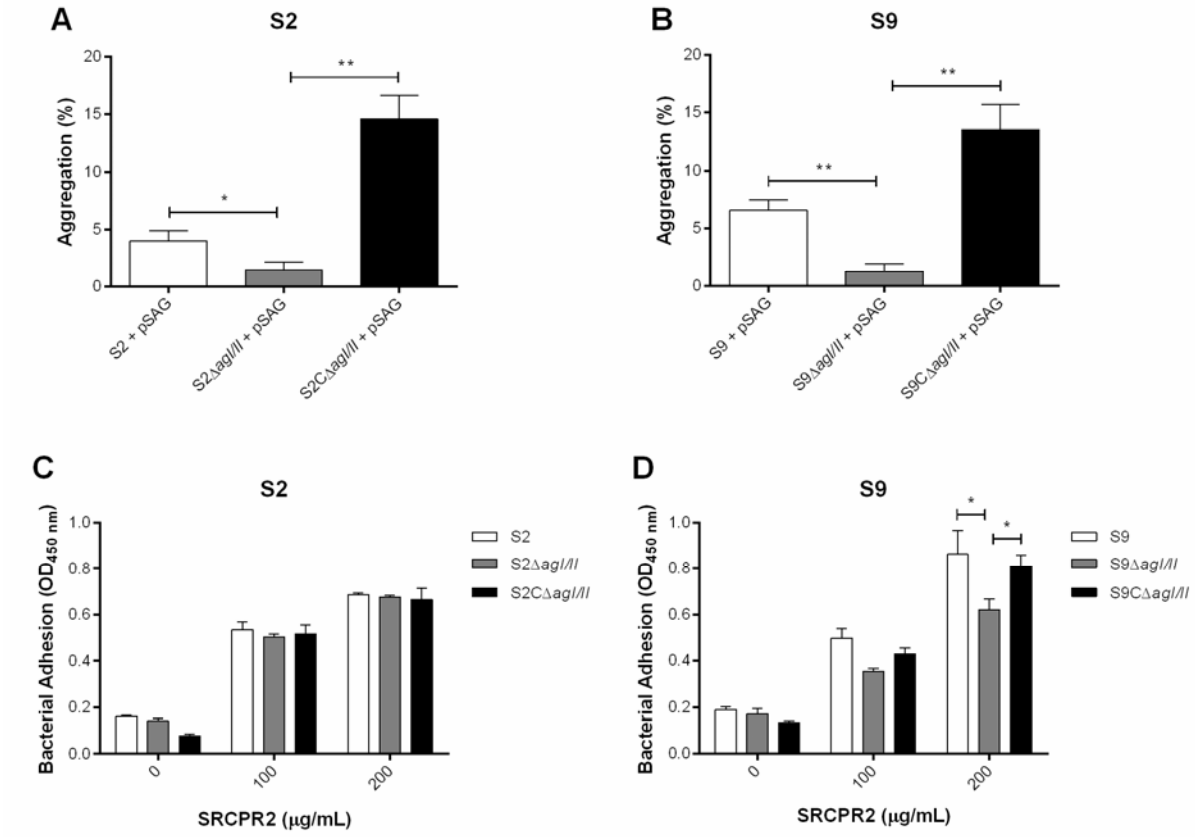

Figure 5. The *S. suis* serotype 2 (S2) and serotype 9 (S9) AgI/II are involved in adhesion to fluid phase porcine salivary agglutinins (pSAGs), but only for S9 with surface-immobilized pSAGs. Evaluation of the fluid phase aggregation of S2 (A) and S9 (B) strains to pSAGs or to surface-immobilized gp340-derived peptide SRCRP2 by S2 (C) and S9 (D), the latter being measured by ELISA. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between the *S. suis* S2 or S9 wild-type or complemented strain (S2CΔ*agl/II* or S9CΔ*agl/II*) and the *agl/II*-deficient mutants (S2Δ*agl/II* or S9Δ*agl/II*).

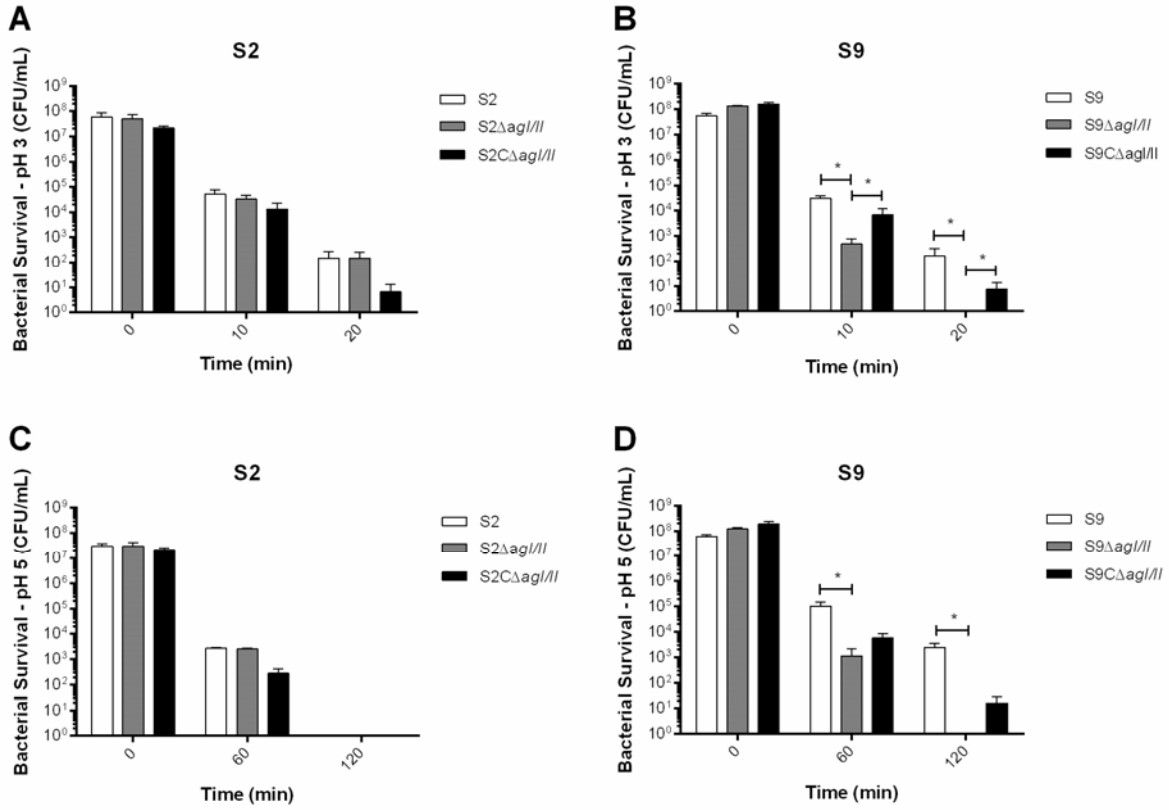

Figure 6. The *S. suis* serotype 9 (S9) AgI/II, but not that of serotype 2 (S2), is involved in protection against acid stress. Effect of acid stress on *S. suis* S2 and S9 viability, determined at pH 3 (A and B) and pH 5 (C and D). Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agl/II*) and *agl/II*-deficient mutant (S9Δ*agl/II*).

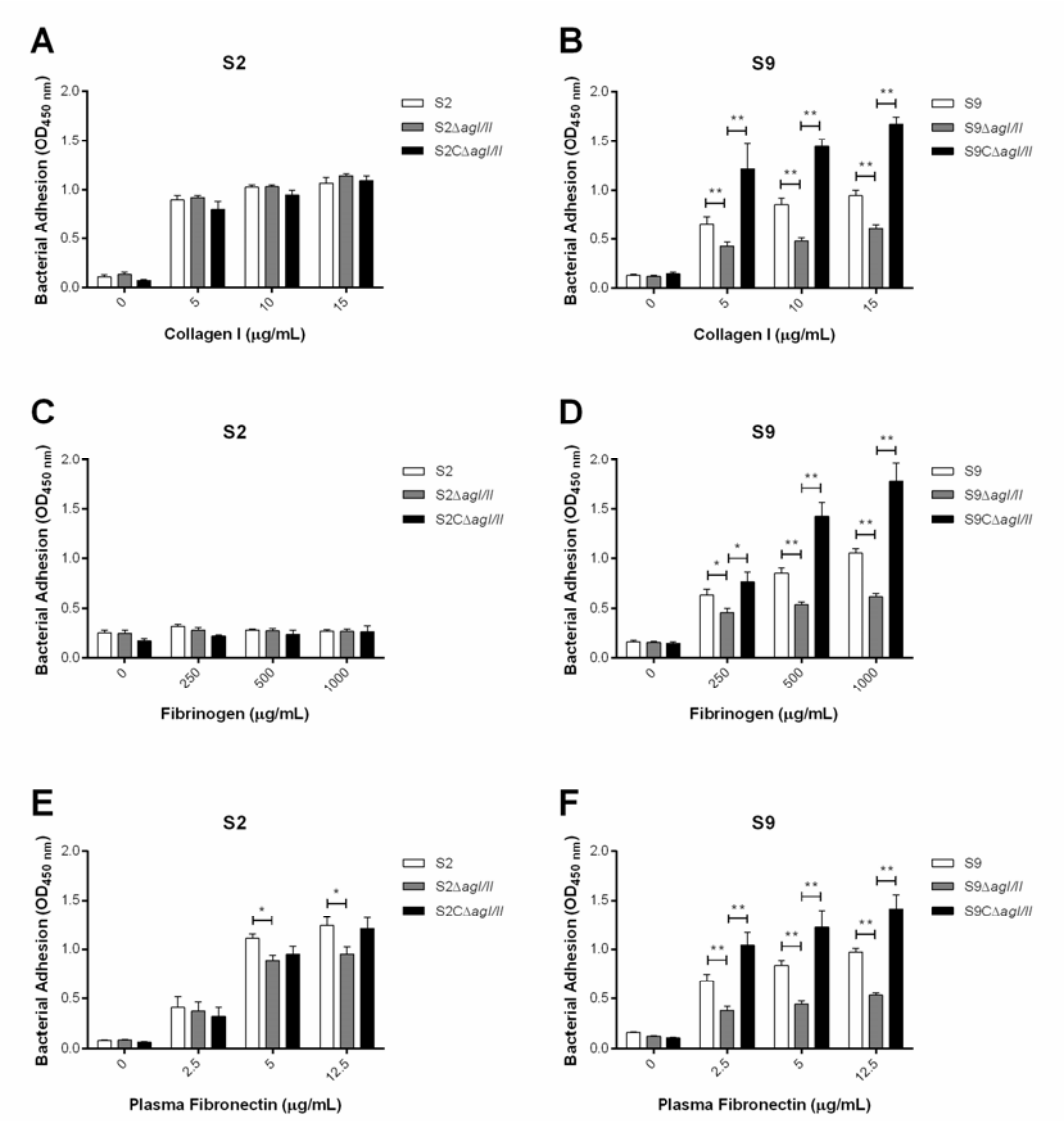

Figure 7. The *S. suis* serotype 9 (S9) Agl/Rl and, to a lesser extent, that of serotype 2 (S2), are bacterial adhesins for extracellular matrix proteins. Adhesion of the *S. suis* S2 and S9 strains to different concentrations of collagen I (A and B), fibrinogen (C and D), and plasma fibronectin (E and F) as evaluated by ELISA. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between the wild-type or complemented strain (CΔ*agl/rl*) and the *agl/rl*-deficient mutant (Δ*agl/rl*).

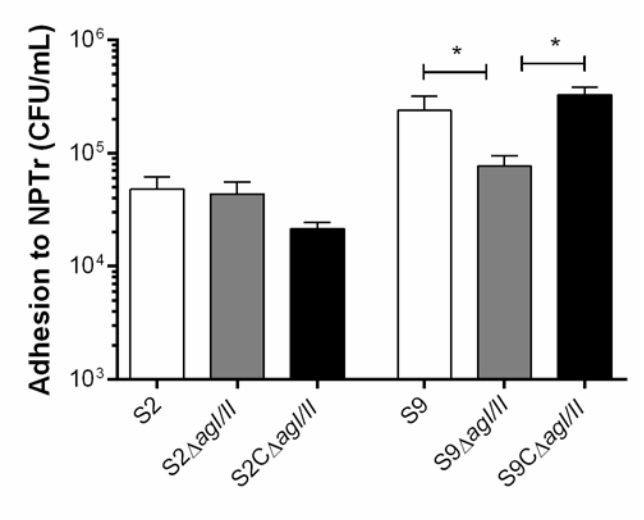

Figure 8. The *S. suis* serotype 9 (S9) AgI/II, but not that of the serotype 2 (S2), is involved in adhesion to porcine tracheal epithelial cells. Adhesion of the *S. suis* S2 and S9 strains to NPTr cells after 2 h of incubation with a multiplicity of infection of 10. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between the *S. suis* S9 wild-type or complemented strain (S9CΔ*agI/II*) and the *agI/II*-deficient mutant (S9Δ*agI/II*).

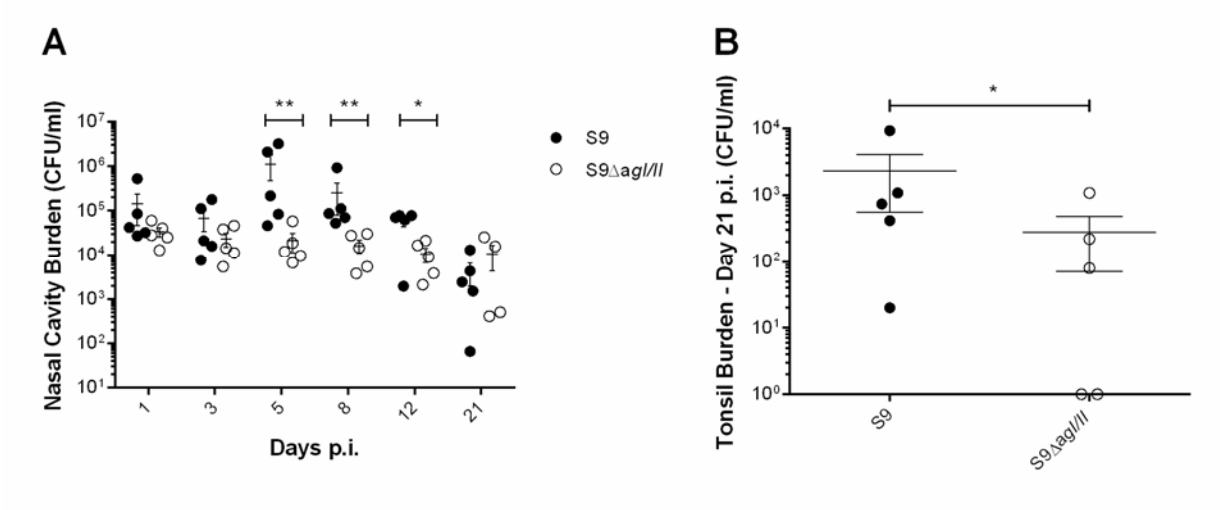

Figure 9. The *S. suis* serotype 9 (S9) AgI/II is implicated in colonization of the porcine respiratory tract. An intranasal porcine model of infection was used to determine the implication of the *S. suis* S9 AgI/II in colonization of the nasal cavity (A) and tonsils 21 days post-infection (B). Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between presence of the *S. suis* S9 wild-type strain and the *agI/II*-deficient mutant (S9Δ*agI/II*).

ARTICLE V

Antigen I/II participates in the interactions of *Streptococcus suis* serotype 9 with phagocytes and the development of systemic disease

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Soumis à *Frontiers in Cellular and Infectious Microbiology.* **Septembre 2018**

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur du manuscrit. J'ai participé à la conception de l'étude et des approches méthodologiques et aux expériences *in vitro* impliquant les cellules et aux ELISA. J'ai aussi effectué les expériences *in vivo*, l'analyse des résultats, l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci.

Mise en contexte

Nous avons démontré dans le cadre de l'article IV que l'AgI/II du sérotype 9 participe dans plusieurs fonctions importantes requises pour la colonisation de l'hôte, dont l'autoagrégation, la formation de biofilm, la résistance aux pH acides et l'adhésion aux protéines de la matrice extracellulaire et aux cellules épithéliales. Toutefois, son rôle dans les étapes subséquentes de sa pathogenèse, dont dans l'infection systémique, reste inconnu. Bien qu'il y ait très peu d'information de disponible sur le rôle des AgI/II des streptocoques dans d'autres compartiments que les voies respiratoires supérieures et la cavité buccale, cela ne veut pas dire qu'ils n'y sont pas impliqués. En effet, l'AgI/II de GAS, appelé AspA, est un facteur anti-phagocytaire protégeant la bactérie contre l'effet bactéricide des macrophages et des neutrophiles, mais n'est pas nécessaire à sa virulence. Nous avons donc voulu évaluer le rôle de l'AgI/II du sérotype 9 de *S. suis* lors de l'infection systémique et dans les interactions avec différents phagocytes.

Abstract

Streptococcus suis is an important porcine bacterial pathogen and a zoonotic agent causing a variety of pathologies including sudden death, septic shock, and meningitis. Though serotype 2 is the most studied serotype due to its presence worldwide, serotype 9 is responsible for the greatest number of porcine cases in Spain, the Netherlands, and Germany. Regardless of its increasing importance, very few studies have investigated *S. suis* serotype 9 virulence factors and pathogenesis. Antigens I/II (AgI/II) are multimodal adhesion proteins implicated in host respiratory tract and oral cavity persistence of various pathogenic human streptococci. It was recently demonstrated that AgI/II is involved in various bacterial functions for serotype 9, participating in the initial steps of the pathogenesis of the infection. However, its contribution to systemic infection remains unknown. As such, we evaluated herein the role of *S. suis* serotype 9 AgI/II in the interactions with phagocytes and the development of systemic disease in a mouse model of infection. Results demonstrated that presence of AgI/II is required for the development of clinical systemic disease by promoting bacterial survival in blood and tissues due to its anti-phagocytic properties, as shown with macrophages and dendritic cells. Furthermore, AgI/II also participates in cell activation and pro-inflammatory mediator production, which contribute to the exacerbated systemic inflammation responsible for host death. Taken together, AgI/II is a critical virulence factor required not only for the initial steps of the *S. suis* serotype 9 pathogenesis, but also for virulence during systemic infection and development of disease. In fact, this is the first study to describe a role of an AgI/II family member in systemic bacterial disease.

Introduction

Streptococcus suis is a bacterial pathogen of post-weaning piglets responsible for important economic losses, with sudden death, meningitis, and arthritis being the most frequent clinical manifestations [1]. Moreover, *S. suis* is a zoonotic agent responsible for meningitis and septic shock in humans [2]. Of the different described serotypes, serotype 2 is the most widespread and virulent [3]. In recent years, however, serotype 9 has emerged in Europe and is presently responsible for the greatest number of porcine cases of *S. suis* infection in Spain, the Netherlands, and Germany [3]. Furthermore, its prevalence in China [4] and Canada [5] has significantly increased, and the first human case of *S. suis* serotype 9 infection was reported in 2015 [6]. Regardless of its increasing importance, very few studies have investigated serotype 9 virulence factors and pathogenesis. Consequently, our understanding of the *S. suis* pathogenesis is based on serotype 2 studies [2, 7, 8].

Following colonization of the upper respiratory tract or tonsils of pigs, virulent strains may reach the bloodstream after breaching the mucosal epithelium [7]. Similarly, infection in humans occurs via skin wounds or at the intestinal interface following ingestion of raw or undercooked infected pork products [7]. In the bloodstream, *S. suis* resists killing by phagocytes, which allows bacterial multiplication resulting in bacteremia, organ dissemination, and development of systemic infection [8]. Moreover, *S. suis* activates blood and tissue-resident innate immune cells, including macrophages and dendritic cells (DCs), which participate in the massive release of pro-inflammatory mediators. This response results in exacerbated inflammation responsible for sepsis leading to sudden death in pigs and septic shock in humans [1]. If untreated, *S. suis*-induced systemic inflammation may end in host death [1]. However, the precise mechanisms and virulence factors involved are poorly understood.

Antigens I/II (AgI/II) have been extensively described in oral and invasive pathogenic streptococci, including *Streptococcus mutans*, *Streptococcus gordonii*, Group A *Streptococcus* (GAS; *Streptococcus pyogenes*), and Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) [9]. These multimodal adhesion proteins are implicated in host respiratory tract and oral cavity persistence and in dissemination [9]. Importantly, they have been described to possess a wide variety of functions ranging from self-aggregation, aggregation to soluble and surface-immobilized components, biofilm formation, and cell adhesion [9]. We recently demonstrated that not only is AgI/II present in *S. suis* serotypes 2 and 9, but that its role is serotype-specific

[10]. Indeed, while AgI/II is involved in above mentioned functions for serotype 9, very little functions are associated with its presence in serotype 2 [10]. As such, AgI/II participates in the initial steps of the *S. suis* serotype 9 pathogenesis, more specifically in colonization, persistence, and carriage in the upper respiratory tract and tonsils of pigs [10].

It must be noted that studies on the role of streptococcal AgI/II have been generally limited to respiratory tract colonization and persistence. In fact, little information is available regarding other functions and/or environments. Yet, the *S. suis* serotype 9 AgI/II might also be involved elsewhere, as evidenced by GAS AgI/II protein (termed AspA), that protects bacteria against phagocytosis and killing by macrophages and neutrophils [11]. In fact, such functions could be important for the *S. suis* pathogenesis since resistance to phagocytosis and killing are required for bacterial survival and dissemination and consequent development of systemic disease [8].

Consequently, we evaluated the role of the *S. suis* serotype 9 AgI/II in the interactions with phagocytes and the development of systemic disease in a mouse model of infection. Results demonstrated that AgI/II promotes bacterial survival in blood and tissues due to its anti-phagocytic properties. Furthermore, its presence also participates in innate immune cell activation and pro-inflammatory mediator production, which contribute to the exacerbated systemic inflammation and host death.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (permit number Rech-1570).

Bacterial strains and growth conditions

The well-encapsulated wild-type *S. suis* serotype 9 1135776 strain, isolated from a diseased pig in Canada and belonging to sequence type 788 [12], and its previously constructed isogenic AgI/II-deficient mutant (Δ*agI/II*) and complemented strain (CΔ*agI/II*) [10], were used. Strains were cultured in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada). For *in vitro* cell culture and whole blood bactericidal assays, bacteria were prepared as previously

described [13] and resuspended in cell culture medium. For experimental infections, early stationary phase bacteria were washed twice in phosphate-buffered saline, pH 7.4 (PBS), and resuspended in THB [14-16]. Bacterial cultures were appropriately diluted and plated on THB agar (THA) to accurately determine bacterial concentrations. For the complemented strain, spectinomycin (Sigma-Aldrich, Oakville, ON, Canada) was added at a concentration of 500 µg/mL.

S. suis *serotype 9 systemic infection mouse virulence models*

Six-week-old male and female C57BL/6 mice (Jackson Research Laboratories, Bar Harbor, ME, USA) were used throughout this study. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [14, 16]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. After standardization trials with the wild-type strain to determine the ideal dose, *S. suis* strains were inoculated at a final dose of 1 x 10^7 colony forming units (CFU) to groups of 10 mice by either the intraperitoneal or intravenous (caudal vein) route for survival and blood bacterial burden. Mice were monitored at least three times daily until 72 h post-infection and twice thereafter until 14 days post-infection. Blood bacterial burden of surviving mice was assessed 24 h post-infection by collecting 5 µL of blood from the caudal vein, appropriately diluting, and plating on THA as described above. Blood bacterial burden was also measured upon euthanasia.

Measurement of plasma (systemic) pro-inflammatory mediators

In addition, eight mice per group were intraperitoneally mock-infected (THB) or infected with 1 x 10^7 CFU of the *S. suis* strains and blood collected 12 h post-infection by intracardiac puncture following euthanasia and anti-coagulated with EDTA (Sigma-Aldrich) as previously described [14, 17]. Plasma supernatants were collected following centrifugation at 10 000 x *g* for 10 min at 4 ºC and stored at -80 ºC. Plasmatic concentrations of interleukin (IL)-6, IL-12p70, interferon (IFN)-γ, C-C motif chemokine ligand (CCL) 2, CCL3, CCL4, C-X-C motif chemokine ligand (CXCL) 1, and CXCL2 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Acquisition

was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Peritoneal macrophage isolation

Resident peritoneal macrophages were isolated from C57BL/6 mice as previously described [18] with some modifications. Cells were recovered by washing the peritoneal cavity with cold PBS without prior elicitation, pooled, and resuspended in Dulbecco's Modified Eagle's Medium (Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and plated at 1 x 10^5 cells/mL. Peritoneal macrophages were allowed to adhere for 1 h at 37 °C with 5% CO_2 and then washed twice with warm PBS to remove non-adherent cells prior to infection.

Whole blood bactericidal (killing) assay

Blood was collected from six- to ten-week-old C57BL/6 mice and mixed with sodium heparin (Sigma-Aldrich). Leukocytes (9 x 10^6 cells/mL on average) were transferred to a microtube containing 9 x 10^6 CFU/mL of the *S. suis* strains (multiplicity of infection [MOI]=1) and incubated for 4 h, mixing every 20 min. Assay conditions were chosen based on the kinetics of *S. suis* killing by murine blood [14]. After incubation, cells were lysed by vortexing and appropriate dilutions plated on THA to determine viable bacterial counts. Resistance to bacterial killing by blood leukocytes was compared to incubation of the different strains in plasma only (obtained by centrifuging whole blood at 1 800 x *g* for 10 min at 4 °C). Percentage of bacterial survival was determined using the following formula: (bacteria in blood/bacteria in plasma) / 100%.

Generation of bone marrow-derived dendritic cells

The femur and tibia of C57BL/6 mice were used to generate bone marrow-derived DCs, as previously described [13]. Briefly, hematopoietic bone marrow stem cells were cultured in RPMI-1640 medium (Gibco) supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), and 50 µM 2-mercaptoethanol (Gibco). Complete medium was complemented with 20% granulocyte-macrophage colony-stimulating factor from mouse-transfected Ag8653 cells [19]. Prior to infection, cells were plated at 1 x 10^6 cells/mL. Cell purity was determined to be at least 85% $CD11c^+$ by flow cytometry.

Internalization and intracellular survival assays

Bacteria were pre-opsonized with either 20% complete or heat-inactivated normal C57BL/6 mouse serum in PBS for 30 min at 37 °C with shaking as previously described [13]. Cells were infected with *S. suis* strains at optimal conditions in culture medium (1 x 10^7 CFU for peritoneal macrophages and 1 x 10^8 CFU for DCs [MOI=100]) and phagocytosis was left to proceed for 1 h at 37 °C with 5% CO_2. After incubation, penicillin G (5 mg/mL; Sigma-Aldrich) and gentamicin (100 mg/mL; Gibco) were directly added to the wells for 1 h to kill extracellular bacteria. Supernatant controls were taken in every test to confirm that extracellular bacteria were efficiently killed by the antibiotics. After antibiotic treatment, cells were washed three times and lysed using water. The number of CFU recovered was determined by plating viable intracellular bacteria on THA. Intracellular survival assays were performed as described for phagocytosis, except that after 1 h of infection, cell monolayers were washed twice with PBS and antibiotic-containing medium was added to infected cells for up to 3 h post-infection. At different times (0, 1, 2, and 3 h), infected monolayers were washed three times, lysed with water, and the number of CFU was determined. Intracellular survival was expressed as the percentage of the initial number of viable bacteria 1 h post-infection.

Dendritic cell activation and pro-inflammatory mediator measurement

DCs were stimulated with the pre-opsonized *S. suis* strains (as described above) in culture medium (1 x 10^6 CFU/mL; initial MOI=1). Supernatants were collected 16 h following infection with *S. suis*, time at which secreted cytokine levels were maximal in the absence of *S. suis*-induced cytotoxicity as confirmed by measurement of release lactate dehydrogenase using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) (data not shown). Non-infected cells served as negative controls. Secreted levels of tumor necrosis factor (TNF), IL-1β, IL-6, and CCL3 were quantified by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations.

Statistical analyses

Normality of data was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test) or non-parametric tests (Mann-Whitney rank sum test) were performed to evaluate statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare

mouse survival. Each test was repeated in at least three independent experiments. $p < 0.01$ was considered as statistically significant.

Results

Antigen I/II is required for Streptococcus suis *serotype 9 virulence and development of systemic disease*

Unlike with *S. suis* serotype 2 [15, 16, 20, 21], experimental swine models of serotype 9 infection are very limited, which has greatly hindered pathogenesis studies. Using C57BL/6 mice, which are routinely used as a model for serotype 2 virulence studies [14, 16, 17], the role of AgI/II in *S. suis* serotype 9 systemic infection was evaluated following intraperitoneal inoculation. Mice infected with the wild-type strain developed clinical signs of systemic infection characterized by rough hair coat, swollen eyes, prostration, depression, and lethargy. These animals succumbed to infection within 3 days, with 60% of mortality 24 h post-infection (**Fig. 1A**). By contrast, only 10% of mice infected with the Δ*agI/II* mutant succumbed to infection after 14 days ($p < 0.001$) (**Fig. 1A**), and animals only presented transient signs of systemic infection (rough coat hair and swollen eyes) in the first 24 h of infection. Meanwhile, mice infected with the complemented strain, CΔ*agI/II*, presented similar clinical signs to those of mice infected with the wild-type strain, with 100% of mice succumbing to infection within the same time frame (**Fig. 1A**). These results were confirmed in two subsequent infections (data not shown).

Since *S. suis* serotype 2 systemic infection is associated with persistent bacteremia [14], blood bacterial burden of mice infected with the serotype 9 wild-type, Δ*agI/II* or CΔ*agI/II* strain was evaluated. Twenty-four hours following infection, mice infected with the wild-type strain presented elevated blood bacterial burdens averaging 1×10^8 CFU/mL (**Fig 1B**). On the other hand, mice infected with the Δ*agI/II* mutant did not present measurable bacteremia 24 h post-infection ($p < 0.001$), with the exception of a single individual who eventually succumbed to infection 5 days post-infection (**Fig. 1B**). Finally, and in accordance with survival, mice infected with the complemented strain, CΔ*agI/II*, presented similar blood bacterial burdens to those infected with the wild-type strain (**Fig. 1B**).

Exacerbated inflammation is a hallmark of the *S. suis* serotype 2-induced systemic infection and is responsible for host death due to sepsis and/or septic shock. In accordance, plasmatic

levels of the different pro-inflammatory mediators evaluated (IL-6, IL-12p70, IFN-γ, CCL2, CCL3, CCL4, CXCL1, and CXCL2) were elevated in mice infected with the wild-type and CΔ*agI/II* strains (**Fig. 2**). Meanwhile, not only were plasmatic levels of these mediators significantly lower in mice infected with the Δ*agI/II* mutant ($p < 0.001$), but they were similar to those of mock-infected mice (**Fig. 2**).

Antigen I/II promotes Streptococcus suis *serotype 9 resistance to phagocytosis by resident peritoneal macrophages*

The compromised blood presence/persistence of *S. suis* serotype 9 in absence of AgI/II following intraperitoneal inoculation suggested that AgI/II might be implicated in the interactions with peritoneal macrophages, which are the first innate immune cells encountered following intraperitoneal inoculation. Consequently, the role of *S. suis* serotype 9 AgI/II in resistance to phagocytosis by resident peritoneal macrophages was evaluated by infecting cells with bacteria pre-opsonized with either 20% complete or heat-inactivated normal mouse serum, used to evaluate the role of complement. After 1 h of incubation, 1 x 10^5 CFU of the wild-type strain pre-opsonized with complete serum were internalized, representing 1% of inoculum (**Fig. 3A**). Meanwhile the Δ*agI/II* mutant was significantly more internalized ($p < 0.01$), with approximately 10% of bacteria recovered (**Fig. 3A**). On the other hand, complementation (CΔ*agI/II*) restored wild-type phenotype (**Fig. 3A**). Interestingly, pre-opsonization with heat-inactivated mouse serum significantly reduced phagocytosis of all strains by approximately ten-fold ($p < 0.01$) (**Fig. 3A**), although the deficient mutant was still more internalized than controls (**Fig. 3A**).

Unlike with phagocytosis, AgI/II did not modulate intracellular survival of *S. suis* serotype 9 pre-opsonized with either complete serum (**Fig. 3B**) or heat-inactivated serum (data not shown). Indeed, only 15% of initially internalized wild-type, Δ*agI/II* or CΔ*agI/II* bacteria were viable after 1 h, with less than 5% remaining after 2 h, and no viable bacteria recovered after 3 h of incubation (**Fig. 3B**). Taken together, these results suggest that while AgI/II confers anti-phagocytic properties promoting resistance of *S. suis* serotype 9 to resident peritoneal macrophages, it does not promote intracellular bacterial survival, nor does it interfere with complement deposition.

Antigen I/II promotes* Streptococcus suis *serotype 9 resistance to killing by whole blood

From the peritoneal cavity, non-internalized bacteria will rapidly reach the bloodstream, where resistance to killing by leukocytes is required for persistence and systemic dissemination. Consequently, the role of AgI/II in resistance of *S. suis* serotype 9 to killing by mouse whole blood was evaluated. The wild-type strain was almost completely resistant to killing by blood, with 99% of survival after 2 h (results not shown) and 4 h of incubation (**Fig. 4**). By contrast, the Δ*agI/II* mutant was significantly less resistant to killing ($p < 0.001$), with 80% of survival (**Fig. 4**). Meanwhile, complementation (CΔ*agI/II*) restored wild-type phenotype (**Fig. 4**).

Antigen I/II modulates the interactions of* Streptococcus suis *serotype 9 with dendritic cells: Role in phagocytosis resistance, intracellular survival, and pro-inflammatory mediator induction

As mentioned, persistent bacteremia results in systemic dissemination of *S. suis*, with elevated bacterial burdens recovered in various internal organ following infection with serotype 2 [15]. In these organs, *S. suis* will encounter different resident innate immune cells, including DCs. As such, the role of AgI/II in the interactions with DCs was evaluated. The *S. suis* serotype 9 wild-type strain pre-opsonized with complete serum was little internalized by DCs after 1 h of incubation, with 1 x 10^5 CFU recovered, corresponding to 0.1% of inoculum (**Fig. 5A**). However, the Δ*agI/II* mutant was significantly more internalized ($p < 0.01$), with 1 x 10^6 CFU recovered, equivalent to 1% of inoculum, while complementation (CΔ*agI/II*) restored wild-type levels (**Fig. 5A**). Similarly to what was observed with macrophages, pre-opsonization with heat-inactivated serum significantly reduced DC phagocytosis of all strains by ten-fold ($p < 0.01$) (**Fig. 5A**). Once again, however, the Δ*agI/II* mutant was significantly more internalized by DCs ($p < 0.01$) (**Fig. 5A**).

Survival of internalized wild-type bacteria was initially elevated, with 60% of viability after 1 h (**Fig. 5B**). However, viability quickly decreased thereafter, with 15% and 0% after 2 h and 3 h, respectively (**Fig. 5B**). By contrast, intracellular survival of the Δ*agI/II* mutant was significantly reduced ($p < 0.01$), with only 30% after 1 h, before reaching 0% after 2 h (**Fig. 5B**). Importantly, complementation (CΔ*agI/II*) restored wild-type phenotype (**Fig. 5B**). Similar results were also obtained using heat-inactivated of serum (data not shown).

Recognition of *S. suis* by DCs not only activates phagocytic mechanisms involved in bacterial clearance, but it also induces pro-inflammatory mediator production [13]. The *S. suis* serotype 9 wild-type strain induced important levels of TNF, IL-1β, IL-6, and CCL3 from DCs (**Fig. 6**). Pro-inflammatory mediator production by DCs was significantly reduced following infection with the Δ*agI/II* mutant by 30% to 50% depending on the mediator ($p < 0.01$) (**Fig. 6**). Meanwhile, complementation (CΔ*agI/II*) restored wild-type phenotype (**Fig. 6**). Taken together, these results demonstrate that presence of AgI/II modulates the interactions of *S. suis* serotype 9 with DCs.

Intravenous inoculation of* Streptococcus suis *serotype 9 confirms the role of antigen I/II in virulence and development of systemic disease

To confirm that the route of infection does not influence results on the role of the AgI/II, mice were also infected by intravenous injection. Inoculation of the *S. suis* serotype 9 wild-type strain resulted in development of systemic clinical disease similar to following intraperitoneal inoculation, with 100% of mice succumbing to infection within 2 days (**Fig. 7A**). Meanwhile, mice infected with the Δ*agI/II* mutant succumbed significantly less to infection ($p < 0.01$), with 40% of mortality after 14 days (**Fig. 7A**). Though clinical signs were less severe in Δ*agI/II*-infected mice, most mice presented some signs of systemic infection such as rough coat hair, swollen eyes, prostration, and depression, which persisted for at least 48 h (data not shown). By contrast, the complemented strain, CΔ*agI/II,* caused similar mortality to the wild-type strain (**Fig. 7A**).

In accordance with mortality, blood bacterial burden of mice infected with the wild-type strain was elevated 24 h post-infection, averaging 1 x 10^8 CFU/mL, while that of mice infected with the Δ*agI/II* mutant was significantly lower ($p < 0.001$) (**Fig. 7B**). Unlike following intraperitoneal inoculation, however, mice infected with the Δ*agI/II* mutant presented detectable blood bacterial burdens, which averaged 1 x 10^3 CFU/mL (**Fig. 1B**). Finally, blood bacterial burden of mice infected with the complemented strain was similar to that of wild-type strain-infected mice (**Fig. 7B**).

Discussion

S. suis serotype 9 has emerged as one of the most frequently isolated serotypes in recent years, yet little information is available regarding its pathogenesis. Though we recently

demonstrated that AgI/II is required for the initial steps of the *S. suis* serotype 9 pathogenesis [10], its role in systemic infection, including interactions with phagocytes, remained unknown.

Unfortunately, researchers are faced with a major setback when studying serotypes other than serotype 2, which is the lack of well-standardized experimental animal models of infection [22]. In the case of serotype 9, it has been shown that reproducibility of the disease is more difficult than with serotype 2 (Beineke *et al.* 2008). Though successful experimental infections with serotype 9 have been carried out in pigs, they have all, with the exception of one, used caesarean-derived colostrum-deprived animals, which are germ-free. This model is far from "conventional" pigs, which are colonized by *S. suis*, rendering them more resistant to infection (Dekker *et al.* 2012, Dekker *et al.* 2013, Dekker *et al.* 2017). Moreover, it is important to note that Canadian strains (including that used in the present study) present a lower virulence profile than European strains [12]. Indeed, we were previously unable to induce disease with this strain [10].

Due to these constraints, a C57BL/6 mouse model of *S. suis* serotype 9 systemic infection was developed in this study using the intraperitoneal and intravenous route of inoculation. Mice presented similar clinical signs of systemic disease characteristic of sepsis and septic shock to those previously described for serotype 2 [14, 16], and this using both inoculation routes. Furthermore, and similarly to serotype 2 [14, 16], serotype 9-induced systemic disease was characterized by elevated and persistent blood bacterial burden and a strong host systemic inflammatory response composed of a variety of pro-inflammatory mediators, resulting in host death. These commonalities between serotypes 2 and 9 regarding pathogenesis and host response suggest that *S. suis* possesses certain evolutionarily conserved components/motifs that are recognized via similar mechanisms. Yet, serotype-specific components such as virulence factors are probably responsible for differences observed in swine models between these two serotypes [12, 23]. As such, this infection model could be useful for future *S. suis* serotype 9 pathogenesis studies, especially given the limited availability of experimental porcine models of infection.

We demonstrated a critical role of AgI/II in systemic infection and development of clinical disease during *S. suis* serotype 9 infection. Interestingly, host survival following infection with the Δ*agI/II* mutant differed between routes of inoculation. Though different immune cells reside

in the peritoneal cavity, macrophages are the main innate immune cell type [24]. After intraperitoneal inoculation of the Δ*agI/II* mutant, local cells are probably responsible for partial bacterial elimination. In fact, the lack of AgI/II increased internalization by macrophages (with rapid intracellular killing), demonstrating that the *S. suis* serotype 9 AgI/II contributes to the bacterial anti-phagocytic properties. Alongside, participation of AgI/II in *S. suis* self-aggregation might reduce the number of bacteria activating phagocytic mechanisms [10]. Since particle size is known to affect phagocytosis, AgI/II-mediated *S. suis* aggregates could be less internalized, as was previously reported for neutrophils [25, 26]. In addition to macrophages, a certain role of neutrophils recruited to the peritoneal cavity following inoculation cannot be excluded [27]. However, neutrophil infiltration is not instant, peaking between 3 h and 6 h post-infection following inoculation of GBS [28]. If bacteria are not eliminated locally and reach the bloodstream, animals will succumb to infection. Indeed, the single animal infected with the Δ*agI/II* mutant presenting a significant blood bacterial burden died 5 days post-infection. When injected directly into the bloodstream, the Δ*agI/II* mutant causes some clinical signs, a measurable bacteremia, and 40% of mortality, suggesting a partial role of this protein in bacterial survival once in the bloodstream. AgI/II was also involved in bacterial survival using an *in vitro* whole blood killing assay. Thus, both *in vitro* and *in vivo* studies indicate that the role of AgI/II is not absolute, suggesting an implication of other bacterial factors. What these factors are, however, remains unknown given the lack of information on *S. suis* serotype 9.

Once in blood, presence of sufficiently elevated burdens will allow *S. suis* to colonize different internal organs during the systemic phase of the infection [15, 16]. DCs are important tissue-resident phagocytes central to the *S. suis* serotype 2 pathogenesis and are involved in innate immune functions such as phagocytosis and pro-inflammatory mediator production [13, 29-31]. As observed with macrophages, *S. suis* serotype 9 AgI/II confers partial protection to the bacteria against DC phagocytosis. Notably, the phagocytic capacities of DCs towards *S. suis* serotype 9 were markedly less than those of peritoneal macrophages. Though macrophages and DCs are both professional phagocytes, it was previously demonstrated that macrophages exhibit more pronounced phagocytic activities towards *Staphylococcus aureus* and *Escherichia coli* than do DCs [32]. Moreover, macrophages are better at killing ingested bacteria than DCs [32]. This concords with results obtained herein whereby DCs were less efficient at killing intracellular *S. suis* than macrophages. In fact, this difference in killing efficiency might also explain why AgI/II participated in intracellular bacterial survival in DCs but not macrophages,

since the phagosomal environment faced by *S. suis* in DCs is less harsh than that in macrophages [33]. Indeed, we previously demonstrated that though AgI/II promotes bacterial survival at different pH by participating in acid stress, its effect is less marked as the pH lowers [10],

It is interesting to compare the role of the *S. suis* serotype 9 AgI/II with that of the AspA of GAS. AspA was also reported to play a role in the resistance to phagocytic killing by murine macrophages and human neutrophils [11]. This resemblance between AgI/II and AspA, regardless of sharing only 35% amino acid sequence homology [10], suggests it might be a conserved property of AgI/II family members. However, persistence in blood was AspA-independent, indicating that while it confers anti-phagocytic properties *in vitro*, this is not necessarily the case *in vivo* [11]. By contrast, AgI/II was required for persistence of *S. suis* serotype 9 in blood and resistance to its bactericidal effect, making this the first time that an AgI/II family member is described to be involved in bacterial survival and persistence in blood.

Being an extracellular pathogen, *S. suis* has developed tools to inhibit phagocytosis and killing, including interference in complement deposition at the bacterial surface since this promotes internalization via opsonophagocytosis [13, 34]. Indeed, *S. suis* serotype 2 suilysin and cell wall modifications [13], as well as the factor H-binding protein [35], are involved in this interference. However, while AgI/II conferred anti-phagocytic properties to *S. suis* serotype 9, this rôle was complement-independent. Interestingly, unlike serotype 2 [13, 34], serotype 9 was relatively sensitive to opsonophagocytosis, with presence of complement increasing its internalization by macrophages and DCs by ten-fold. This suggests that serotype 9 lacks certain factors possessed by serotype 2 involved in the interference of complement deposition. Amongst these factors, sialic acid, present in the serotype 2 capsular polysaccharide but absent from that of serotype 9, might be involved [36, 37]. Though the role of sialic acid in the *S. suis* pathogenesis has not been clearly defined, presence of sialic acid in GBS modulates neutrophil functions and virulence [38].

Though serotype 9 is responsible for invasive disease resulting in exacerbated inflammatory responses, including sudden death [3], the inflammatory response induced by this serotype remains relatively unknown. While serotype 9 induced various pro-inflammatory mediators from murine DCs, levels were generally lower than those reported for serotype 2 [13, 29, 31]. These

results contrast with those obtained using human monocyte-derived DCs, from which serotype 9 induced greater cytokine production than serotype 2, possibly suggesting host-specific differences [39]. Nevertheless, presence of AgI/II partially modulates the induction of pro-inflammatory mediators. A similar role was observed for the SspA and SspB of *Streptococcus gordonii*, which participate in the induction of several mediators from murine DCs and from human lung epithelial cells [40]. Since very little information is available regarding the immunostimulatory properties of AgI/II family members, the cellular receptors and pathways involved remain completely unknown.

In conclusion, *S. suis* serotype 9 causes a systemic infection resulting in the development of clinical disease and host death. As with serotype 2, this infection is characterized by exacerbated inflammation induced by an uncontrolled and persistent bacterial presence in the systemic compartment. Not only does presence of AgI/II confer anti-phagocytic properties that promote resistance to phagocytic cells, but its presence also participates in innate immune cell activation, and by consequence, inflammation. As such, *S. suis* serotype 9 AgI/II is a critical virulence factor required not only for the initial steps of its pathogenesis, but also for virulence during systemic infection and development of disease. In fact, this is the first study to describe a role of an AgI/II family member in systemic bacterial disease.

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List of abbreviations

AgI/II: antigen I/II; CFU: colony forming unit; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; DC: dendritic cells; GAS: Group A *Streptococcus*; GBS: Group B *Streptococcus*; IFN: interferon; IL: interleukin; MOI: multiplicity of infection; PBS: phosphate-buffered saline; SEM: standard error of the mean; THA: THB agar; THB: Todd Hewitt broth; TNF: tumor necrosis factor.

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Figures

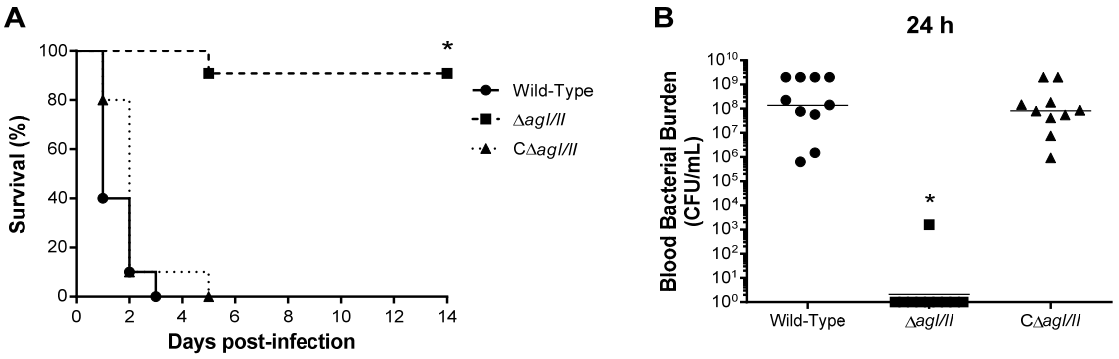

Figure 1. Presence of antigen I/II (AgI/II) is required for *Streptococcus suis* serotype 9 systemic virulence and blood persistence following intraperitoneal inoculation. Survival (**A**) and blood bacterial burden 24 h post-infection (**B**) of C57BL/6 mice following intraperitoneal inoculation of the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agl/ll*) or complemented (CΔ*agl/ll*) strain. Data represent the survival curves (**A**) or geometric mean (**B**) of 10 mice/group. * ($p < 0.01$) indicates a significant difference between survival or blood bacterial burden of mice infected with the wild-type strain and Δ*agl/ll* mutant.

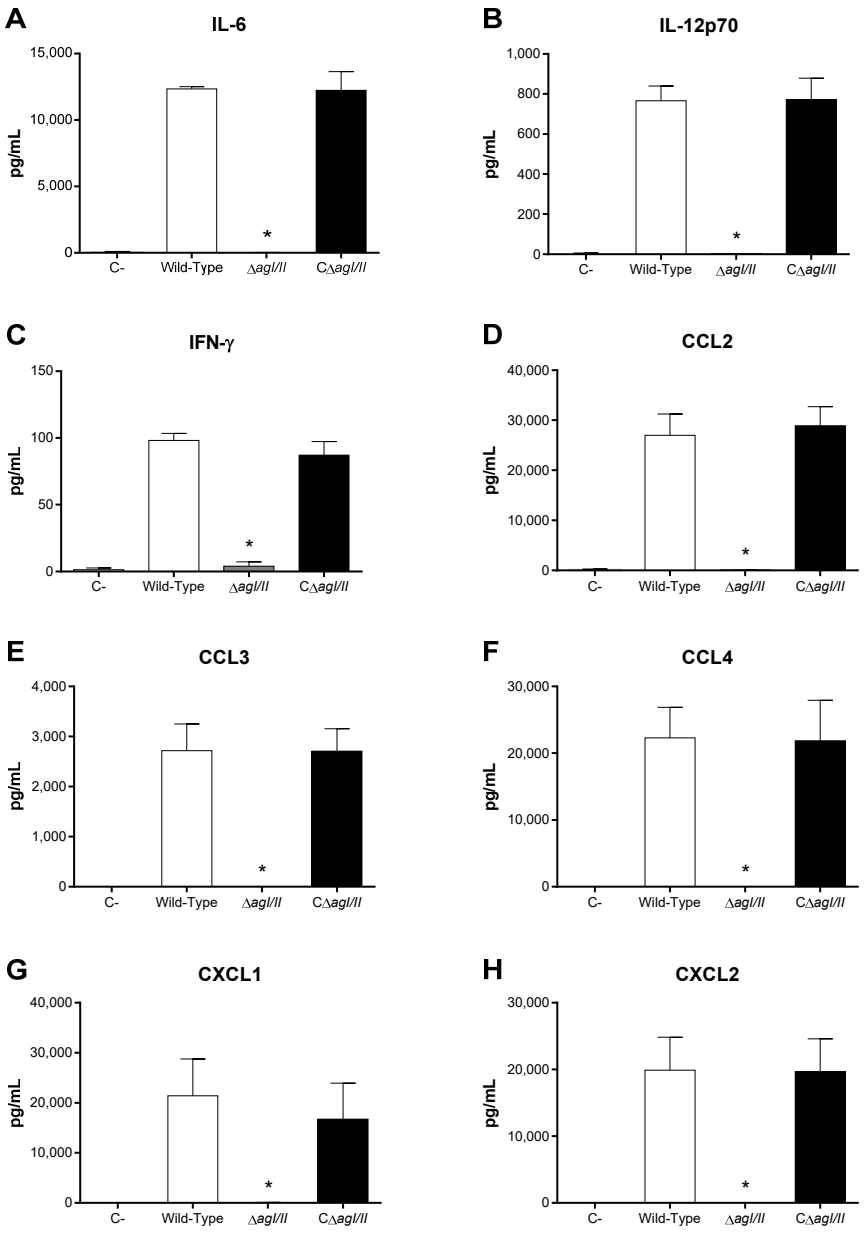

Figure 2. Reduced plasma pro-inflammatory mediator levels in the absence of antigen I/II (AgI/II) during *Streptococcus suis* serotype 9 systemic infection. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), CCL4 (**F**), CXCL1 (**G**), and CXCL2 (**H**) in mice 12 h following following mock-infection or intraperitoneal inoculation of the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agI/II*) or complemented (CΔ*agI/II*) strain. Data represent mean ± SEM (n = 8). C- denotes mock-infected mice. * ($p < 0.01$) indicates a significant difference between plasma levels of mice infected with the wild-type strain and Δ*agI/II* mutant.

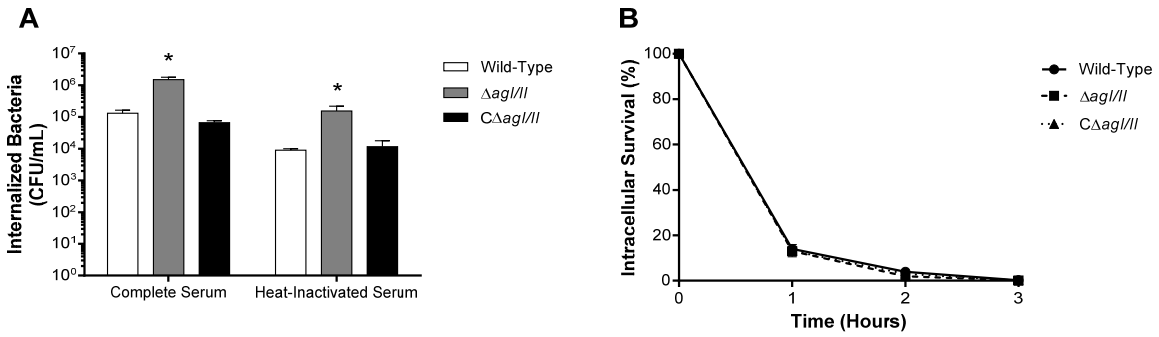

Figure 3. Presence of antigen I/II (AgI/II) promotes *Streptococcus suis* serotype 9 resistance to phagocytosis by resident peritoneal macrophages but not intracellular survival. (**A**) Phagocytosis of the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agI/II*) or complemented (CΔ*agI/II*) strains pre-opsonized with either 20% complete or heat-inactivated normal mouse serum by resident peritoneal macrophages following 1 h of infection. (**B**) Intracellular survival kinetics of the wild-type, Δ*agI/II*, and CΔ*agI/II* strains pre-opsonized with 20% complete normal mouse serum within resident peritoneal macrophages. Data represent the mean ± SEM (n = 4). * ($p < 0.01$) indicates a significant difference between the wild-type strain and Δ*agI/II* mutant.

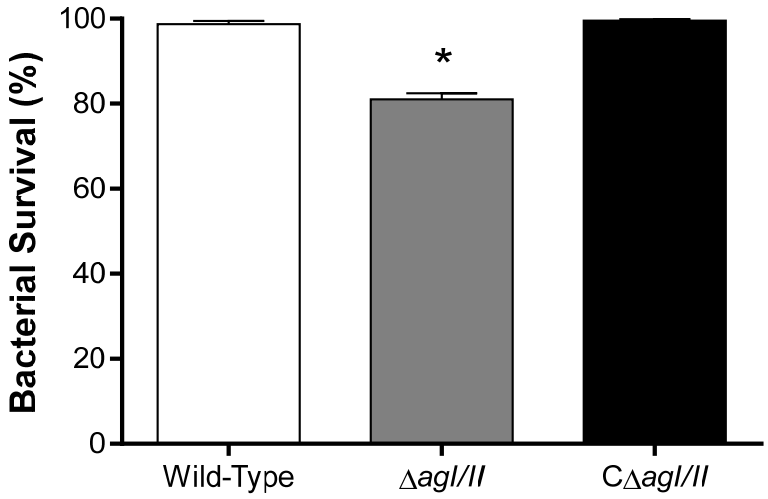

Figure 4. Presence of antigen I/II (AgI/II) promotes *Streptococcus suis* serotype 9 resistance to killing by whole blood. Resistance of wild-type, AgI/II-deficient mutant (Δ*agl/II*) or complemented (CΔ*agl/II*) strains to the bactericidal effect of murine whole blood after 4 h of incubation. Percentage of bacterial survival was calculated in comparison to bacteria in plasma alone. Data represent the mean ± SEM (n = 4). * ($p < 0.01$) indicates a significant difference between the wild-type strain and Δ*agl/II* mutant.

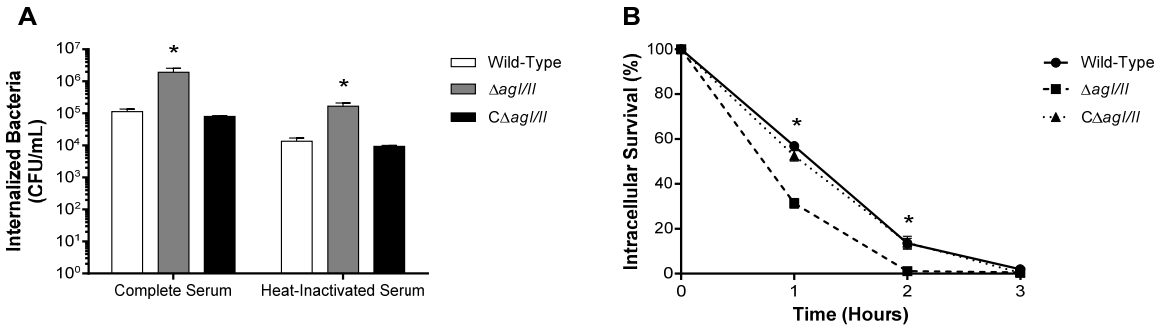

Figure 5. Presence of antigen I/II (AgI/II) promotes *Streptococcus suis* serotype 9 resistance to phagocytosis by dendritic cells (DCs) and intracellular survival. (**A**) Phagocytosis of the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agl/II*) or complemented (CΔ*agl/II*) strains pre-opsonized with either 20% complete or heat-inactivated normal mouse serum by DCs following 1 h of infection. (**B**) Intracellular survival kinetics of the wild-type, Δ*agl/II*, and CΔ*agl/II* strains pre-opsonized with 20% complete normal mouse serum within DCs. Data represent the mean ± SEM (n = 4). * ($p < 0.01$) indicates a significant difference between the wild-type strain and Δ*agl/II* mutant.

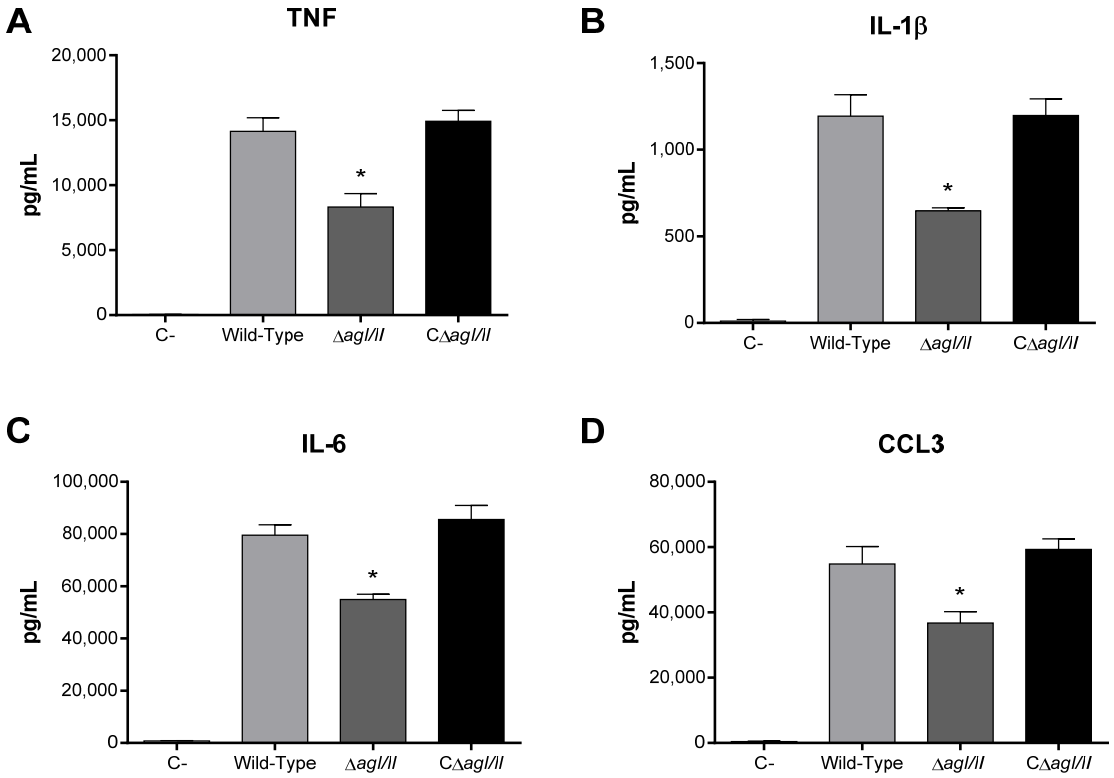

Figure 6. Presence of antigen I/II (AgI/II) modulates *Streptococcus suis* serotype 9-induced dendritic cell (DC) pro-inflammatory mediator production. Pro-inflammatory mediator production by DCs following 16 h of infection with the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agl/II*) or complemented (CΔ*agl/II*) strains pre-opsonized with 20% complete normal mouse serum, as measured by ELISA. Production of TNF (**A**), IL-1β (**B**), IL-6 (**C**), and CCL3 (**D**). Data represent the mean ± SEM (n = 4). C- denotes cells in medium alone. * ($p < 0.01$) indicates a significant difference between the wild-type strain and Δ*agl/II* mutant.

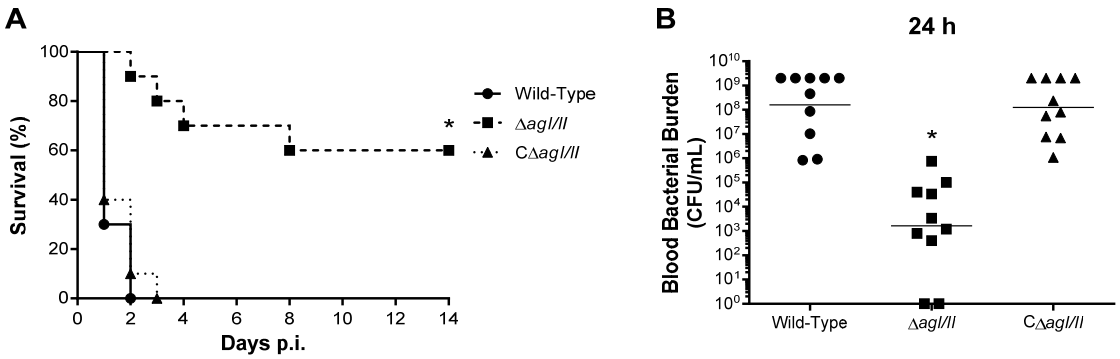

Figure 7. Presence of antigen I/II (AgI/II) participates in *Streptococcus suis* serotype 9 systemic virulence and blood persistence following intravenous inoculation. Survival (**A**) and blood bacterial burden 24 h post-infection (**B**) of C57BL/6 mice following intravenous inoculation of the *S. suis* serotype 9 wild-type, AgI/II-deficient mutant (Δ*agI/II*) or complemented (CΔ*agI/II*) strain. Data represent the survival curves (**A**) or geometric mean (**B**) of 10 mice/group. * ($p < 0.01$) indicates a significant difference between survival or blood bacterial burden of mice infected with the wild-type strain and Δ*agI/II* mutant.

ARTICLE VI

Structural analysis and immunostimulatory potency of lipoteichoic acids isolated from three *Streptococcus suis* serotype 2 strains

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-premier auteur de l'article. J'ai participé à la conception de l'étude et des approches méthodologiques (expériences avec les cellules). J'ai effecuté la préparation des bactéries, les expériences avec les cellules *in vitro* et les ELISA.J'ai aussi participé à l'analyse des résultats, à l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci. J'ai aussi effectué des corrections lors des étapes de révision pour publication.

Mise en contexte

Dans le cadre de l'objectif 1 de cette thèse, le dernier composant de surface de *S. suis* que nous avons voulu étudier est le LTA. Les LTAs sont des molécules retrouvées dans la paroi des bactéries à Gram positif, mais leur composition et leur structure varient grandement selon l'espèce. Les LTAs sont historiquement associés à l'induction du choc septique causé par les bactéries à Gram positif suite à leur reconnaissance par le TLR2 à la surface des cellules de l'hôte. Toutefois, des études récentes ont remis ce concept en question. De plus, nous n'avions aucune information sur la composition, la structure et les propriétés immunostimulatrices des LTAs de *S. suis*. Comme le bagage génétique pourrait avoir un impact sur ces paramètres, des souches appartenant aux trois des plus importants STs du sérotype 2, soit le ST1, le ST7 et le ST25 ont été utilisées. Il est important de noter que bien que le ST1 et le ST7 sont génétiquement apparentés, le ST25 est très distinct.

Abstract

Streptococcus suis serotype 2 is an important porcine and human pathogen. Lipoteichoic acid (LTA) of *S. suis* has been suggested to contribute to its virulence, and absence of D-alanylation from the *S. suis* LTA is associated with increased susceptibility to cationic antimicrobial peptides. Here, using high resolution NMR spectroscopy and MS analyses, we characterized the LTA structures from three *S. suis* serotype 2 strains differing in virulence, sequence type (ST), and geographical origin. Our analyses revealed that these strains possess–in addition to the typical type I LTA present in other streptococci–a second, mixed-type series of LTA molecules of high complexity. We observed a ST-specific difference in the incorporation of glycosyl residues into these mixed-type LTAs. We found that strains P1/7 (ST1, high virulence) and SC84 (ST7, very high virulence) can attach an 1,2-linked α-D-Glc*p* residue as branching substituent to an α-D-Glc*p* that is 1,3-linked to glycerol phosphate moieties and that is not present in strain 89-1591 (ST25, intermediate virulence). In contrast, the latter strain could glycosylate its LTA at the glycerol *O*-2 position, which was not observed in the other two strains. Using LTA preparations from wild-type strains and from mutants with an inactivated prolipoprotein diacylglyceryl transferase, resulting in deficient lipoprotein acylation, we show that *S. suis* LTAs alone do not induce Toll-like receptor 2-dependent proinflammatory mediator production from dendritic cells. In summary, our study reveals an unexpected complexity of LTAs present in three *S. suis* serotype 2 strains differing in genetic background and virulence.

Introduction

Streptococcus suis serotype 2 is a Gram-positive bacterial pathogen of pigs responsible for important economic losses to the porcine industry worldwide, causing sudden death, meningitis, and a variety of other pathologies (1). Moreover, *S. suis* is an emerging zoonotic agent responsible for septic shock and meningitis in humans, having become a public health issue, particularly in South-East Asia (2). However, *S. suis* serotype 2 strains are genotypically and phenotypically heterogeneous, both in terms of geographical distribution and virulence, with different sequence types (STs[3]), based on multilocus sequence typing (MLST), having been described (3). This molecular typing technique is based on differences in the genetic sequences of conserved house-keeping genes, in which mutations are assumed to be largely neutral (4). MLST has classified the different *S. suis* strains into a large number of STs, based on differences in these genes, which has facilitated the understanding of its evolutionary divergence and epidemiology (3,4). Data provided by the *S. suis* MLST Database revealed that 701 different STs have been identified for *S. suis* (all serotypes combined), from a total of 1528 isolates. Serotype 2 strains (which is by far the most important serotype for both pigs and humans) belong to 111 of these 701 STs. Regardless of this elevated number, these STs can be regrouped into only a few clusters called clonal complexes (CC). As depicted in Fig. S1, ST1 is the most prevalent ST in terms of number of isolates amongst serotype 2, and is the founder of the CC1. An important CC1 subgroup, founded by the ST7, has substantially grown in importance in recent years and was responsible for the 1998 and 2005 human *S. suis* outbreaks in China (Fig. S1) (5,6). As such, ST1 strains, which predominate throughout Eurasia, South America, and Australia, and ST7 strains that are present in China, are genetically similar and generally virulent/highly virulent, respectively (3,7,8). Meanwhile, the CC25 and CC28, founded by the ST25 and ST28, respectively, rank as the second and third most important clusters (Fig. S1). In fact, the ST25 and ST28 account for 95% of North American strains isolated from clinical cases of porcine and human disease (3,9). While ST25 strains are of intermediate virulence, ST28 strains are considered low virulent and have been mostly associated with secondary infections and immunocompromised individuals (8,9). Consequently, we chose ST1, ST7, and ST25 strains for our analyses because of their importance as predominant virulent *S. suis* serotype 2 strains, which are responsible for most porcine and human infections worldwide (3).

Besides peptidoglycan and lipoproteins (LPs), wall teichoic acids and lipoteichoic acids (LTA) are the major constituents of the Gram-positive cell wall. LTA has been suggested to contribute to the virulence of *S. suis,* while absence of D-alanine residues from its LTA has been associated with an increased susceptibility to the action of cationic antimicrobial peptides (10). In another study (11), LPs, which are often co-purified with LTA (12,13), were determined to be major activators of the porcine innate immune system. As of now, the detailed structure of the *S. suis* LTA remained elusive. In general, LTA contains a lipophilic anchor formed by diacyl-glycerol (DAG), which anchors these molecules to the cell membrane. The DAG is substituted with a glycosyl moiety at the *O*-3 position. These glycosyl moieties, as well as the attached complex backbone structures consisting of repetitive units (RUs), are highly variable between different species of Gram-positive bacteria. So far, five different types of LTA have been described, which are mainly characterized by the architecture of their RUs (polyglycerolphosphate (type I), complex glycosyl-glycerol-phosphate (type II + III), glycosyl-ribitol-phosphate (type IV), and glycosyl-phosphate (type V) containing polymers) (14).

We describe herein the structural analysis of LTA isolated from three *S. suis* serotype 2 strains of different background as representatives of the most clinically and epidemiologically important STs using chemical degradations, high-resolution MS analysis as well as one- and two-dimensional, homo- and heteronuclear NMR spectroscopy. Finally, the immunostimulatory properties of these well-characterized LTA preparations were evaluated in order to understand their role in the activation and modulation of the host innate immune response by *S. suis*.

Materials and Methods

Bacterial strains and growth

The well-characterized representative *S. suis* strains P1/7 (ST1; United Kingdom; pig meningitis (28)), SC84 (ST7; China; human streptococcal toxic shock-like syndrome (6)), and 89-1591 (ST25; Canada; pig septicemia/meningitis (29)) were used in this study. The different *S. suis* wild-type and mutant strains were grown as previously described in 6-liter batches (2 × 3 liters) (30) in Todd-Hewitt broth (Becton Dickinson) to late logarithmic phase (A_{600} ≈ 1) and harvested by centrifugation (10,000 × *g*, 40 min, 4 °C). Bacteria were resuspended in citric buffer (50 mM, pH 4.7) and heat-killed (10 min, 100 °C). Cells were then stored at −80 °C and subsequently lyophilized.

Construction of the* S. suis *prolipoprotein diacylglyceryl transferase (Lgt) isogenic mutants

Lgt-deficient mutants for strains P1/7, SC84, and 89-1591 were constructed as previously described (31) including DNA manipulations. Deletion of genes was confirmed by PCR and sequencing. The oligonucleotide primers used for the constructions are listed in Tab. S9. The growth of the different *lgt*-deficient mutants was determined to be similar to that of their respective wild-type strains (data not shown).

Extraction and isolation of LTA

LTA purification was performed as described elsewhere (15). Yields of LTA preparations from 6 liters of bacterial culture were: strain P1/7: 21.2 mg; strain P1/7Δ*lgt*: 20.9 mg; strain SC84: 20.2 mg; strain SC84Δ*lgt*: 30.9 mg; strain 89-1591: 18.7/15.6 mg, strain 89-1591Δ*lgt*: 20.6 mg.

Chemical treatments of LTA

Hydrazin treatment (to yield de-*O*-acyl LTA) or HF treatment (to isolate the LTA glycolipid anchor and the dephosphorylated repeats) were performed following earlier described procedures (18). Notably, de-*O*-acylated *S. suis* LTA has to be desalted by dialysis against water (MWCO: 500-1000 Da) instead of performing a size exclusion chromatography. To destroy the TLR2 activity caused by potentially co-purified LPs, LTA preparations were treated with 1% H_2O_2 for 24 h at 37 °C followed by dialysis as previously described (23).

Quantification of fatty acids

Fatty acids were extracted and quantified from LTA preparations of the Δ*lgt* mutant strains following our earlier described procedure (18), but with *n*-pentadecanoic acid (15:0; Sigma) used as an internal standard. Reported data for fatty acid ratios are the mean of two independent hydrolyses of the same LTA batch, both measured as two technical replicates. Different isoforms for unsaturated fatty acids (16:1; 18:1) are reported as one sum value.

NMR spectroscopy

Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). NMR spectroscopic measurements were performed in D_2O or deuterated 25 mM sodium phosphate buffer (pH 5.5; to suppress fast de-alanylation) at 300 K on a Bruker Avance^{III} 700 MHz (equipped with an inverse 5 mm quadruple-resonance Z-grad cryoprobe or with an inverse

1.7 mm triple-resonance Z-grad micro cryoprobe). Acetone was used as an external standard for calibration of ^{1}H (δ_H = 2.225) and ^{13}C (δ_C = 30.89) NMR spectra (32) and 85% of phosphoric acid was used as an external standard for calibration of ^{31}P NMR spectra (δ_P = 0.00). Analysis of glycolipid **1** was performed in CD_3OD and spectra were calibrated using the residual solvent peak (δ_H = 3.31, δ_C = 49.0) (32). All data were acquired and processed by using Bruker TOPSPIN V 3.0 or higher. ^{1}H NMR assignments were confirmed by 2D $^{1}H,^{1}H$-COSY and total correlation spectroscopy (TOCSY) experiments. ^{13}C NMR assignments were indicated by 2D $^{1}H,^{13}C$-HSQC, based on the ^{1}H NMR assignments. Interresidue connectivity and further evidence for ^{13}C assignment were obtained from 2D $^{1}H,^{13}C$- heteronuclear multiple bond correlation and $^{1}H,^{13}C$-HSQC-TOCSY. Connectivity of phosphate groups were assigned by 2D $^{1}H,^{31}P$-HMQC and $^{1}H,^{31}P$-HMQC-TOCSY.

Mass spectrometry

All mass spectrometric analyses were performed on a Q Exactive Plus (Thermo, Bremen, Germany) using negative ion mode. LTA fractions were diluted to a final concentration of 0.03 mg/mL in propan-2-ol/water/30 mM ammonium acetate (50:50:4, v/v/v), which was adjusted with acetic acid to pH 4.5. The HESI source was operated at −3 kV with a flow rate of 5 µL/mL using nitrogen as sheath gas at 5 au. Survey MS^1 and MS^2 spectra were acquired with a resolution of 288,000 FWHM @ m/z 200. MS^2 analyses were performed using the Triversa Nanomate (Advion, Ithaca, US) as ion source applying a spray voltage of −1.1 kV and back pressure of 1.0 psi. Precursor ions were selected with isolation window width of 1.5 Da and CID was performed with 20 NCE. Deconvoluted spectra were computed using Xtract module of Xcalibur 3.1. Software (Thermo, Bremen, Germany).

Generation of bone marrow-derived dendritic cells (DCs) and cell activation

Murine bone marrow-derived DCs were generated as previously described (21) from the femur and tibia of wild-type (C57BL/6J) or $TLR2^{-/-}$ (B6.129- *Tlr2tmKir*/J) mice. Prior to activation, cells were seeded at 1 × 10^6 cells/mL and different concentrations of the LTA preparations or cell culture medium alone (negative control) were added. Cell supernatants were collected 24 h later for quantification of secreted IL-6, TNF, CXCL1, and CCL3 by sandwich ELISA using pair-matched antibodies (R&D Systems).

Statistical analyses

Normality of data was verified using the Shapiro–Wilk test. Accordingly, parametric (unpaired *t*-test) or non-parametric tests (Mann–Whitney rank sum test), where appropriate, were performed to evaluate statistical differences between groups. $p < 0.05$ was considered statistically significant. As software, GraphPad Prism 6 was used.

Results

For the structural analyses, LTA preparations from the three *S. suis* serotype 2 strains P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25), as well as from their respective Δ*lgt* mutants, were prepared according to our previously published workflow (15). The latter strains lack the gene encoding for the lipoprotein diacylglyceryl transferase (Lgt) and are therefore deficient in lipidation of prolipoproteins (16). In a first step, we compared the ^{1}H NMR spectra of native LTA from all three strains (Fig. 1). Spectra have been recorded in deuterated 25 mM sodium phosphate buffer (pH 5.5) at 300 K to suppress fast de-alanylation. In all three ^{1}H NMR spectra, the typical NMR signals for poly-glycerolphosphate chains of type I LTA molecules (14,17) can be observed. Furthermore, all three strains are capable of modifying the glycerol *O*-2 position with alanine as indicated by the broad signal at δ_H 5.43-5.35 for proton H-2 of the alanine-substituted glycerol moieties, the signal at δ_H 4.33-4.27 for the CH-group, and the doublet at δ_H 1.63 for the CH_3-group of alanine (17). Whereas spectra of LTA from strains P1/7 and SC84 are almost identical, the spectrum of LTA from strain 89-1591 indicates a different binding position for some of the present glycosyl residues (Fig. 1). Therefore, the detailed analysis of the latter LTA will be described in the following separately. Notably, the ^{1}H NMR spectra of LTA from strains P1/7 and SC84 are virtually identical with the published ^{1}H NMR spectrum for LTA isolated from *S. suis* serotype 2 strain 31533 (10).

For a better characterization of the LTA structures, we generated defined part structures by hydrofluoric acid (HF) treatment, from which we obtained the respective lipid anchor and de-phosphorylated RUs. Afterwards, we analyzed the interconnection of the RUs using de-*O*-acylated LTA, which was obtained by hydrazine treatment, using NMR and mass spectrometry.

Preparation and structural analysis of the lipid anchor and LTA part structures from LTA of strains P1/7 and SC84

To elucidate the nature of the lipid anchor, we treated the isolated LTA of strains P1/7 and SC84 with 48% HF for two days at 4 °C according to our previously published procedure (18). This treatment cleaves all phosphodiester bonds and leads to the formation of dephosphorylated RUs as well as to the release of the glycolipid anchor from the LTA. The HF treated LTA was applied to hydrophobic-interaction chromatography (HIC) as described for the purification of intact LTA (shown for strain P1/7 in Fig. S2). Early in the gradient, the dephosphorylated RUs and other fatty acid-free part structures were eluted in fractions #6-9. To collect the lipid anchor, the later eluting UV-active fractions #26-34 were combined. The exact structure of the carbohydrate part of the glycolipid anchor of *S. suis* P1/7 and SC84 LTA was analyzed by NMR (Fig. S3; Tab. S1) and was determined as α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→ (kojibiose). Consequently, the glycolipid anchor of *S. suis* P1/7 and SC84 LTA is α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-DAG (kojibiose-diacylglycerol; **1a**; bold numbers always refer to the structures shown in Fig. 2). Early fractions were combined, lyophilized, and subsequently submitted to gel permeation chromatography (Bio-Gel P-10; shown for strain P1/7 in Fig. S4). As major molecule, we identified α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→1)-glycerol (**2**; Fig. 2) by NMR analysis (Fig. S5; Tab. S2). In minor amounts, α-D-Glc*p*-(1→1)-glycerol (**3**; Fig. 2) was present as well (NMR data in Tab. S3). Furthermore, no further substituted glycerol (**4**; Fig. 2) was observed (signals marked with # in Fig. S5).

Structural analysis of hydrazine-treated LTA of strains P1/7 and SC84

Hydrazine treatment cleaves off all ester bound residues like fatty acids and alanine and thus reduces the structural heterogeneity of LTA molecules. Therefore, the basic structural features of the LTA chain is accessible, since phosphodiester bonds remain intact (18-20). In the following, the structural investigation of hydrazine-treated LTA (LTA_{N2H4}) of *S. suis* strains P1/7 and SC84 by NMR is discussed. In Fig. 3 the $^{1}H,^{13}C$-heteronuclear single quantum correlation (HSQC) NMR of LTA_{N2H4} of strain P1/7 is depicted as an example for both strains, since almost identical spectra were obtained. The complete NMR chemical shift data are summarized in Tab. 1. LTA isolated from wild-type strains and their respective Δ*lgt* mutants are structurally identical, and the respective ^{1}H NMR spectra of native LTA preparations from Δ*lgt* mutants are depicted in Fig. S6 (for spectra of LTA preparations from wild-type strains see Fig.1).

The MS analysis of LTA_{N2H4} of *S. suis* strains P1/7 and SC84 revealed a remarkably high diversity of LTA molecules. In total, we observed >60 different LTA moieties, which could be grouped into two major structural types. One series of molecules belonged to type I LTA with the observed de-*O*-acylated lipid anchor **1b** (Fig. 2) with different numbers (X = 3 to 14) of glycerol-phosphate (GroP) repeating units. The second type of observed LTA contained, in addition, more complex RUs consisting of α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-GroP (= Y). The number of GroP repeats (X) varied from 3 to 10 and 1 to 10 RUs of structure Y were present in these LTA_{N2H4} molecules.

However, only to LTA with 4 to 9 GroP moieties multiple repeats of structure Y are added. In Fig. 4A a representative mass spectrum of the LTA_{N2H4} of strain P1/7 is depicted. In Fig. 4B the region of 800 to 2700 Da is depicted enlarged and peaks for observed LTA_{N2H4} molecules are assigned. The mass region containing molecules with higher molecular weight is shown in Fig. S7. The full list of identified LTA_{N2H4} molecules for *S. suis* strains P1/7 and SC84 is given in Tab. 2.

To further investigate the order of the different RUs, we selected different molecules for MS/MS experiments. As an example, the MS/MS spectrum for LTA_{N2H4} with X = 5 and Y = 3 (m = 2620.495 Da) is shown in Fig. 5. In this way, we could verify the consecutive order of the two different RU types X and Y. In Fig. 5A the complete overview of the MS/MS spectrum obtained in the negative ion mode is shown. Masses unequivocally presenting fragments occurring from a fragmentation starting from the de-*O*-acyl linker are labeled in blue, the ones occurring from a fragmentation starting at the terminus in red. Fragments that can exist from both cleavage directions are labeled black. In Fig. 5B, the observed fragment ions are assigned to the respective cleavage position in the molecule. A complete list of the observed fragments and their assignment is given in Table S4. Based on integration values from ^{1}H NMR spectra of LTA_{N2H4}, the chains of *S. suis* P1/7 LTA consist in average of 62% GroP (RU X), 33% RU Y, and 5% RU Y lacking Glc II. The values for LTA of *S. suis* SC84 are very similar: 57% GroP (RU X), 39% RU Y, and 4% RU Y lacking Glc II. As reference, the integral of H-1 of Glc I^{LA} (δ_H 5.17) was set to 1.0. As measures for the different repeats the following signals were used for integration: H-2 of Gro (δ_H 4.08-4.04) for RU X, H-1 of Glc I (δ_H 5.21-5.18) for RU Y, as well as H-1 of Glc I lacking Glc II (δ_H 4.97-4.94; marked with # in Fig. 3). Integration of signals was done

in ^{1}H NMR spectra obtained from LTA_{N2H4} of the WT and their respective Δ*lgt* strains and have been averaged for the evaluation of RU ratios.

Preparation and structural analysis of the lipid anchor and LTA part structures from LTA of strain 89-1591

The analysis of the lipid anchor and LTA part structures isolated from LTA of strain 89-1591 after HF treatment was done as described above. The glycolipid anchor of this strain was identified – as for the other two strains – as kojibiose-diacylglycerol (**1a** in Fig. 2). Albeit, the observed molecules representing the dephosphorylated RUs of the LTA of strain 89-1591 differed significantly from the previously observed molecules. The major present molecule was identified as α-D-Glc*p*-(1→1),α-D-Glc*p*-(1→2)-glycerol (**5**); **3** and α-D-Glc*p*-(1→2)-glycerol (**6**) were observed as well. Besides that, small amounts of **2** (resulting from the completely de-*O*-acylated glycolipid anchor), 1-*O*-Ala-glycerol (**7**) as well as unbound glycerol (**4**; structures for all molecules are depicted in Fig. 2) and alanine, are detectable in this preparation (Fig. S8). Molecule **7** is a result of the migration of the alanine moiety from *O*-2 to *O*-1 of the glycerol after phosphodiester bond cleavage (19), indicating the substitution of some Gro-*P* repeats with alanine on position *O*-2. The unbound alanine results from the decomposition of **7** during long term NMR measurement into **4** and alanine. All NMR chemical shift data of **5**, **6**, and **7** (Fig. 2) are listed in Tabs S5-S7.

Structural analysis of hydrazine-treated LTA of strain 89-1591

Hydrazine treatment of LTA isolated from *S. suis* strain 89-1591 was performed as for the other two strains. The ^{1}H,^{13}C-HSQC NMR of LTA_{N2H4} of strain 89-1591 is depicted in Fig. 6; the complete NMR chemical shift data are summarized in Tab. 3.

The MS analysis of LTA_{N2H4} of *S. suis* strain 89-1591 (Fig. 7A) revealed an even higher diversity of LTA molecules than observed for the other two investigated strains. As before, we observed one series of molecules that belonged to type I LTA with the observed de-*O*-acylated lipid anchor **1b** carrying different numbers (X = 4 to 20) of GroP repeats. In addition, more complex versions of these LTA with additionally bound hexoses have also been observed. On the one hand, these can be α-D-Glc*p* residues 1,2-linked to GroP, which would lead to the de-phosphorylated RU molecule **6** (Fig. 2). On the other hand, these residues can be α-D-Glc*p* 1,3-linked to the GroP, leading to de-phosphorylated RU molecule **3** (Fig. 2). Finally, both

glycosyl attachments can be present in the same RU, leading to de-phosphorylated RU molecule **5** (Fig. 2), which was the molecule with the highest abundance observed. Due to the multitude of combinatorial possibilities, especially with regards to the non-stoichiometric α-D-Glc*p* substituents of the glycerol *O*-2 position, which has the same additional mass as one glucose moiety within the LTA chain, the present LTA_{N2H4} molecules cannot be determined in such detail as for strains P1/7 and SC84. The mass spectrometric analysis depicted in Fig. 7A as well as two magnified sections of the spectrum (Fig. 7B,C) show this increased complexity. The full list of identified LTA_{N2H4} molecules for *S. suis* strain 89-1591 can be found in Tab. S8. In total, we identified more than 165 different LTA moieties in the LTA_{N2H4} preparation of *S. suis* strain 89-1591, all of them measured with a mass deviation of ≤ 3.5 ppm. An evaluation of RU ratios as described above for LTA_{N2H4} from ST1/ST7 strains is not possible for LTA_{N2H4} of this strain, since the signal for H-2 of Gro has too much overlap with other signals in the ^{1}H NMR spectra.

Analysis of the fatty acid composition of S. suis LTA preparations

For the analysis of the fatty acid composition, the LTA preparations isolated from the Δ*lgt* mutants have been used. These should give the most reliable values since most likely no other fatty acid containing molecules are co-purified. As the most prominent fatty acid, hexadecanoic acid (16:0) was observed in all strains. Furthermore, 12:0, 14:0, 16:1, 18:0, and 18:1 have been detected. The molar ratios were determined to be 1.0/1.9/3.2/15.1/2.9/4.9 (12:0/14:0/16:1/16:0/18:1/18:0) for P1/7Δ*lgt* LTA, 1.0/2.7/4.6/24.0/4.8/8.1 for SC84Δ*lgt* LTA, and 1.0/1.4/3.6/15.2/6.2/6.2 for 89-1591Δ*lgt* LTA.

Evaluation of the immunostimulatory properties of* S. suis *LTA preparations

The immunostimulatory properties of the different *S. suis* LTA preparations were characterized using murine bone marrow-derived DCs. DCs are innate immune cells known for their central role in the *S. suis* infection, including in the production of pro-inflammatory mediators (21). The pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF), as well as the chemokines C-X-C motif chemokine ligand (CXCL) 1 and C-C- motif chemokine ligand (CCL) 3, were selected based on the fact that they are produced in important concentrations by DCs following *S. suis* infection (21). Significant levels of all four mediators were observed following activation of DCs with concentrations of 1, 3, 10, and 30 µg/mL of native LTA preparations from the three *S. suis* wild-type strains (Fig. S9). The observed levels of these mediators were very

similar for LTA preparations from the three *S. suis* strains (P1/7, SC84, and 89-1591). Only for CCL3 was an increased level at 30 µg/ml induced by LTA of strain 89-1591 observed. Moreover, levels of IL-6, CXCL1, TNF, and CCL3 were induced by all LTA preparations in a dose-dependent manner (Fig. S9).

LPs are often co-purified alongside LTA, and bacterial LPs of other Gram-positive pathogens are important activators of the innate immune response (22). Given the elevated levels of pro-inflammatory mediators produced by DCs following activation with the *S. suis* LTA preparations, the immunostimulatory properties of LTA preparations following treatment with H_2O_2 was evaluated. H_2O_2 oxidizes the thioether bond of LPs, which abolishes immunostimulatory activity of potentially co-purified LPs (13,23). H_2O_2-treated LTA preparations only induced little IL-6, CXCL1, TNF or CCL3 production following activation with 3 or 30 µg/mL, while non-treated LTA preparations induced significantly increased activity (Fig. 8), suggesting that co-purified LPs, but not the LTA, are important inducers of pro-inflammatory mediators by DCs, and this for all three strains of *S. suis* evaluated.

In order to specifically determine the immunostimulatory potential of the LTA molecules themselves, without additive or synergistic effects of the pro-inflammatory LPs, LTA was prepared from *lgt*-deficient mutants. Lgt is required for LPs to be biological active and recognized by TLR2 (16,20). Accordingly, activation of DCs with LTA preparations from *lgt*-deficient mutants of the three *S. suis* strains led to a complete abrogation of pro-inflammatory mediator production, regardless of the concentration of LTA used ($p < 0.001$) (Fig. 8).

Taken together, these results suggested that the co-purified LPs, but not the LTA, are the main activators of DCs when using LTA preparations from the *S. suis* strains P1/7, SC84, and 89-1591. Since LPs are recognized by TLR2 following dimerization with either TLR1 or TLR6, which allows to discriminate between triacyl and diacyl motifs of LPs (24), DCs derived from wild-type and $TLR2^{-/-}$ mice were used. In accordance with the above mentioned results, TLR2-deficiency resulted in a complete abrogation of pro-inflammatory mediator production by DCs, and this regardless of the LTA concentration used (3, 10 or 30 µg/mL) ($p < 0.001$) (Fig. 9).

Discussion

The present study provides the first detailed structural characterization of LTA isolated from the important porcine and opportunistic human pathogen *S. suis*. We investigated three different serotype 2 strains: P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25). In preparation for further immunological studies, we constructed the respective Δ*lgt* mutants, which are deficient in Lgt-mediated prolipoprotein acylation (16,22), and analyzed their LTA as well. For all strains, the LTA isolated from Δ*lgt* mutants had the same chemical structure as the one isolated from the respective parental wild-type strain.

As the glycolipid anchor, we identified in LTA of all three strains kojibiose-diacylglycerol, a glycolipid anchor that has also been found in other streptococci and closely related species like *Lactococcus lactis* (19,25). When combining NMR and MS based data, we were able to show that *S. suis* strains P1/7 and SC84 produce an almost identical LTA, which is most likely the same as present in *S. suis* serotype 2 strain 31533 (which is also an ST1 strain) as judged from a published ^{1}H NMR (10). This is in line with the close relationship of ST1 and ST7 strains, since they both belong to the CC1 (7). Interestingly, these strains contained two different types of LTA. One series of LTA molecules represents a type I LTA carrying only polymeric GroP chains connected to the kojibiose-diacylglycerol lipid anchor, which are identical to those LTA molecules identified in *L. lactis* G121 (25), as well as *Streptococcus uberis* 233, *Streptococcus dysgalactiae* 2023, and *Streptococcus agalactiae* 0250 (19). The second series of observed LTA molecules comprises, in addition, more complex glycosyl-residue containing RUs consisting of α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-GroP. Similar RU constitutions are known from type II or type III LTA (14). By MS/MS experiments we could verify the consecutive order of the two different repeating unit types in these LTA. In *S. suis* strains P1/7 and SC84, a defined subset of LTA molecules with 3 to 10 GroP repeats was found to be elongated with 1 to 10 repeats of the more complex, glycosyl-residues containing units. To the best of our knowledge this is the first example of such a regulated synthesis of a mixed-type LTA.

In *S. suis* strain 89-1591, LTA molecules of two different types were also observed. The type I LTA is of the same structure as the one determined in the other two strains. The RUs of the more complex LTA type consist either of α-D-Glc*p* residues 1,2-linked to GroP, α-D-Glc*p* residues 1,3-linked to the GroP or – most prominently – both of these glycosyl attachments to GroP are present in the same repeat. Multitude combinatory possibilities, especially with

regards to the non-stoichiometric α-D-Glc*p* substituents at the glycerol *O*-2 positions in these LTA molecules, make it impossible at this stage to determine if the order of GroP repeats and more complex RUs is as regular as in strains P1/7 and SC84. The summary of the identified LTA structures considering also identified fatty acids and the presence of the possible, non-stoichiometric alanine substitution of the *O*-2 position of the glycerol moieties is depicted in Fig. 10. It is important to note that strain 89-1591 is a ST25 of the CC25, which is genetically distinct from strains of CC1. This strain also possesses lower virulence than ST1 and ST7 strains (9).

In our investigation, we showed that the pro-inflammatory potency of *S. suis* LTA molecules themselves is quite low when tested for pro-inflammatory mediator production from DCs. H_2O_2-treatment of LTA preparations as well as the use of LTA isolated from Δ*lgt*-strains lead to a complete abrogation of inflammatory activity independently of the strain, which can only be observed if LTA preparations of wild-type strains are used. This activation of DCs is totally TLR2-dependent and can therefore be ascribed to the LPs co-purified with the LTA. This is consistent with a study describing the LPs as the important activators of the swine innate immune system present in *S. suis* (11).

In summary, our study revealed an unexpected complexity of LTA molecules present in *S. suis* serotype 2 strains from different genetic and virulence backgrounds. In all investigated strains, two different kinds of LTA molecules have been identified, whereas a ST-specific difference with regard to the incorporation of glycosyl residues into the complex mixed-type LTA has been observed. Strains P1/7 and SC84 are able to attach an 1,2-linked α-D-Glc*p* residue as branching substituent to the α-D-Glc*p* 1,1-linked to the GroP. In strain 89-1591, an exclusive glycosylation at the glycerol *O*-2 position was observed. Just recently, the first enzyme required in the glycosylation process of this position in LTA of *Listeria monocytogenes* has been identified (26). The identification and analysis of respective homologous glycosyl-transferases involved in such reactions in *S. suis* as well as the analysis of the impact on bacterial physiology and virulence are currently under investigation. This will further foster the recent achievements in the understanding of the biological role of TA-glycosylation in Gram-positive bacteria (27).

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Abbreviations

Ala, alanine; CC, clonal complex; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand, DAG, diacyl-glycerol; DC, dendritic cell; Glc, glucose; Gro, glycerol; HF, hydrofluoric acid; HIC, hydrophobic-interaction chromatography; HMQC, heteronuclear multiple quantum correlation; HSQC, heteronuclear single quantum correlation; LTA, lipoteichoic acid; LTA_{N2H4}, hydrazine-treated LTA (= de-*O*-acyl LTA); ppm, parts per million; LP, lipoprotein; MLST, multilocus sequence typing; RU, repeating unit; ST, sequence type; TLR, Toll-like receptor; TOCSY, total correlation spectroscopy.

Supporting Information (Available at the Journal of Biological Chemistry Website)

Figure S1. Relationship of the different *S. suis* serotype 2 sequence types (STs) as determined by multilocus sequence typing (MLST)

Figure S2. Representative chromatogram of the hydrophobic interaction chromatography of *S. suis* P1/7 (ST1) LTA after 2 d HF treatment (UV detection at λ = 254 nm)

Figure S3. NMR analysis of the LTA glycolipid anchor preparation of *S. suis* strain P1/7 (ST1)

Figure S4. Chromatogram of a gel permeation chromatography

Figure S5. NMR analysis of dephosphorylated RUs obtained after 2 d HF treatment of LTA of *S. suis* P1/7 (ST1) and subsequent purification by HIC and gel permeation chromatography

Figure S6. ^{1}H NMR analysis of native LTA of Lgt-deficient *S. suis* serotype 2 strains

Figure S7. Mass spectrometric analysis of de-*O*-acylated LTA of *S. suis* strain P1/7 (ST1).

Figure S8. NMR analysis of dephosphorylated RUs obtained after 2 d HF treatment of LTA of *S. suis* strain 89-1591 (ST25) and subsequent purification by HIC and gel permeation chromatography

Figure S9. Pro-inflammatory mediator production by dendritic cells (DCs) following activation with different concentrations of LTA preparations from the *S. suis* strains P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25)

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Tables

Table 1. ^{1}H (700.4 MHz), ^{13}C NMR (176.1 MHz), and ^{31}P NMR (283.5 MHz) chemical shift data (δ, ppm) [*J*, Hz] for *S. suis* strain P1/7 (ST1) LTA after hydrazine treatment (de-*O*-acyl LTA). *non-resolved multiplet.

Residue (assignment)	H-1 *C-1*	H-2 *C-2*	H-3 *C-3*	H-4 *C-4*	H-5 *C-5*	H-6 *C-6*
Gro-(1→ [**Gro^{LA}**]	3.85- 3.81* 3.54- 3.50* *69.5*	3.97- 3.94* *71.3*	3.70- 3.66* 3.62- 3.58* *63.0*			
→1)-α-D-Glc*p*-(2→ [**Glc I^{LA}**]	5.17 [3.6] *96.6*	3.71- 3.67* *76.0*	3.84- 3.79* *71.8*	3.50- 3.45* *70.2*	3.73- 3.68* *72.3*	3.88- 3.84* 3.78- 3.73* *61.2*
→1)-α-D-Glc*p*-(6→*P* [**Glc II^{LA}**]	5.10- 5.07* *96.9*	3.63- 3.59* *71.8*	3.81- 3.75* *73.3*	3.58- 3.54* *69.5*	4.05- 4.01* *71.4*	4.13- 4.08* *64.7*
P→1)-Gro-(3→*P* [**Gro**]	4.00- 3.95* 3.93- 3.88* *66.8*	4.08- 4.04* *70.1*	4.00- 3.95* 3.93- 3.88* *66.8*			
P→1)-Gro [**Gro^{term}**]	3.95- 3.91* 3.89- 3.85* *67.0*	3.92- 3.89* *71.3*	3.70- 3.66* 3.63- 3.59* *62.7*			
P→1)-Gro-(3→(*Glc I*) [**Gro'**]	4.00- 3.95* 3.94- 3.88* *67.0*	4.12- 4.08* *69.9*	3.90- 3.85* 3.62- 3.57* *62.7*			
(*Gro'*)→1),(*Glc II*)→2)-α-D-Glc*p*-(6→*P* [**Glc I]**	5.21- 5.18*	3.73- 3.68*	3.90- 3.85*	3.63- 3.58*	3.84- 3.80*	4.17- 4.13*

	96.6	*75.6*	*71.8*	*69.6*	*71.2*	4.13-4.08* *64.6*
α-D-Glc*p*-(1→(*Glc I*) [**Glc II]**	5.11 [3.6] *96.5*	3.60-3.55* *71.9*	3.82-3.76* *73.3*	3.48-3.43* *70.0*	3.93-3.85* *72.4*	3.86-3.82* 3.80-3.75* *61.1*
(*Gro'*)→1),(*Glc II*)→2)-α-D-Glc*p* [**Glc I^{term}]**	5.19-5.17* *96.6*	3.70-3.66* *75.7*	3.84-3.79* *71.8*	3.49-3.44* *70.2*	3.73-3.68* *72.1*	3.88-3.84* 3.78-3.73* *61.2*
^{31}P	Gro-*P*-Glc II^{LA}/Glc I-*P*-Gro 1.54 (1.72-1.40); Gro-*P*-Gro 1.37 (1.50-1.15).					

Table 2. Mass spectrometric analysis of de-*O*-acyl LTA of *S. suis* strains P1/7 (ST1) and SC84 (ST7). Summary of calculated and observed molecular masses [Da] for LTA preparations after hydrazine treatment. For each preparation two independent MS analyses have been performed and identified molecules are listed as a combined list (A = de-*O*-acyl glycolipid anchor (**1b**); RU X = GroP; RU Y = α-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-GroP). Masses observed only in one of the two replicates are written in italic style. Accuracy of the measurement is stated as Δppm; n.d. = not detected.

		Strain P1/7		Strain SC84	
Molecule	**calculated mass [Da]**	**observed mass [Da]**	**accuracy [Δppm]**	**observed mass [Da]**	**accuracy [Δppm]**
X_3A	878.162	878.163	0.5	n.d.	-
X_4A	1032.165	1032.166	0.4	*1032.167*	1.6
X_5A	1186.169	1186.169	0.0	*1186.168*	-0.4
X_6A	1340.172	1340.171	-0.6	1340.170	-0.9
X_7A	1494.175	1494.174	-0.4	1494.173	-1.0
X_8A	1648.178	1648.177	-0.8	1648.176	-1.1
X_9A	1802.181	1802.181	0.2	1802.181	-0.2
$X_{10}A$	1956.184	1956.186	1.1	1956.186	0.8
$X_{11}A$	2110.187	2110.188	0.1	2110.187	-0.1
$X_{12}A$	2264.190	2264.192	0.8	*2264.191*	0.4
$X_{13}A$	2418.193	*2418.192*	-0.5	*2418.192*	-0.7
$X_{14}A$	2572.197	*2572.204*	2.9	n.d.	-
Y_1X_3A	1356.271	1356.270	-0.7	1356.270	-1.0
Y_1X_4A	1510.274	1510.274	-0.2	1510.273	-0.5
Y_2X_4A	1988.383	1988.385	0.9	1988.384	0.6
Y_3X_4A	2466.492	2466.494	1.1	2466.493	0.7
Y_4X_4A	2944.601	2944.602	0.3	2944.601	0.0
Y_5X_4A	3422.709	3422.707	-0.6	3422.707	-0.6
Y_6X_4A	3900.818	3900.818	-0.1	3900.808	-2.7
Y_7X_4A	4378.927	4378.929	0.4	4378.925	-0.4
Y_8X_4A	4857.036	4857.033	-0.6	4857.031	-1.0
Y_9X_4A	5335.144	n.d.	-	*5335.140*	-0.8
Y_1X_5A	1664.277	1664.276	-0.6	*1664.276*	-0.6
Y_2X_5A	2142.386	2142.389	1.5	2142.389	1.4
Y_3X_5A	2620.495	*2620.494*	-0.2	2620.494	-0.4
Y_4X_5A	3098.604	3098.601	-0.8	3098.600	-1.0
Y_5X_5A	3576.712	3576.707	-1.6	3576.708	-1.2
Y_6X_5A	4054.821	4054.810	-2.8	4054.808	-3.2
Y_7X_5A	4532.930	4532.929	-0.2	4532.928	-0.4
Y_8X_5A	5011.039	*5011.038*	-0.2	5011.035	-0.7
Y_9X_5A	5489.147	*5489.160*	2.2	*5489.142*	-1.1
Y_1X_6A	1818.280	1818.281	0.5	*1818.282*	1.1
Y_2X_6A	2296.389	2296.391	0.6	2296.390	0.3

Y_3X_6A	2774.498	2774.493	-1.7	2774.494	-1.6
Y_4X_6A	3252.607	3252.599	-2.5	3252.600	-2.2
Y_5X_6A	3730.716	3730.703	-3.5	3730.708	-2.1
Y_6X_6A	4208.824	4208.816	-1.9	4208.810	-3.4
Y_7X_6A	4686.933	4686.933	-0.1	4686.923	-2.0
Y_8X_6A	5165.042	*5165.056*	2.8	5165.038	-0.7
Y_9X_6A	5643.151	n.d.	-	*5643.149*	-0.3
$Y_{10}X_6A$	6121.259	*6121.272*	2.1	n.d.	-
Y_1X_7A	1972.284	1972.285	0.9	1972.285	0.6
Y_2X_7A	2450.392	2450.395	1.2	2450.394	0.7
Y_3X_7A	2928.501	2928.503	0.7	2928.502	0.4
Y_4X_7A	3406.610	3406.608	-0.4	3406.608	-0.4
Y_5X_7A	3884.719	3884.707	-3.0	3884.709	-2.5
Y_6X_7A	4362.827	4362.827	-0.1	4362.829	0.3
Y_7X_7A	4840.936	4840.928	-1.6	4840.929	-1.6
Y_8X_7A	5319.045	5319.046	0.1	*5319.040*	-0.9
Y_9X_7A	5797.154	n.d.	-	*5797.149*	-0.7
$Y_{10}X_7A$	6275.262	n.d.	-	*6275.257*	-0.8
Y_1X_8A	2126.287	2126.287	0.0	2126.286	-0.3
Y_2X_8A	2604.395	*2604.396*	0.0	*2604.394*	-0.5
Y_3X_8A	3082.504	3082.502	-0.8	3082.501	-1.1
Y_4X_8A	3560.613	3560.604	-2.6	3560.605	-2.4
Y_5X_8A	4038.722	4038.717	-1.1	4038.719	-0.7
Y_6X_8A	4516.831	4516.829	-0.4	4516.828	-0.7
Y_7X_8A	4994.939	*4994.947*	1.6	*4994.937*	-0.4
Y_8X_8A	5473.048	n.d.	-	*5473.045*	-0.6
Y_1X_9A	2280.290	2280.291	0.7	2280.291	0.4
Y_2X_9A	2758.399	*2758.406*	2.7	n.d.	-
Y_3X_9A	3236.507	*3236.515*	2.4	*3236.506*	-0.5
Y_4X_9A	3714.616	3714.614	-0.5	3714.614	-0.6
Y_5X_9A	4192.725	4192.717	-2.0	*4192.714*	-2.6
Y_6X_9A	4670.834	*4670.842*	1.9	*4670.828*	-1.2
Y_7X_9A	5148.942	n.d.	-	*5148.939*	-0.6
$Y_1X_{10}A$	2434.293	2434.292	-0.4	2434.291	-0.9

Table 3. ^{1}H (700.4 MHz), ^{13}C NMR (176.1 MHz), and ^{31}P NMR (283.5 MHz) chemical shift data (δ, ppm) [*J*, Hz] for *S. suis* strain 89-1591 (ST25) LTA after hydrazine treatment (de-*O*-acyl LTA). *non-resolved multiplet.

Residue (assignment)	H-1 *C-1*	H-2 *C-2*	H-3 *C-3*	H-4 *C-4*	H-5 *C-5*	H-6 *C-6*
Gro-(1→ [**Gro^{LA}**]	3.84-3.80* 3.53-3.50* *69.5*	3.97-3.94* *71.3*	3.70-3.66* 3.62-3.57* *63.1*			
→1)-α-D-Glc*p*-(2→ [**Glc I^{LA}**]	5.17-5.15* *96.6*	3.71-3.66* *76.1*	3.84-3.80* *72.0*	3.50-3.44* *70.2*	3.73-3.67* *72.4*	3.90-3.85* 3.78-3.73* *61.2*
→1)-α-D-Glc*p*-(6→*P* [**Glc II^{LA}**]	5.10 [3.6] *96.8*	3.62-3.58* *71.8*	3.79-3.74* *73.5*	3.58-3.54* *69.6*	4.05-4.01* *71.5*	4.14-4.07* *64.7*
P→1)-Gro-(3→*P* [**Gro**]	3.99-3.95* 3.93-3.87* *66.8*	4.08-4.03* *70.1*	3.99-3.95* 3.93-3.87* *66.8*			
P→1)-Gro [**Gro^{term}**]	3.95-3.91* 3.89-3.85* *67.0*	3.92-3.89* *71.3*	3.70-3.66* 3.62-3.58* *62.8*			
P→1)-Gro-(3→(*Glc I*) [**Gro'**]	4.02-3.98* 3.93-3.89* *66.8*	4.12-4.08* *70.0*	3.87-3.83* 3.58-3.54* *69.1*			
P→1),α-D-Glc*p*-(1→2)-Gro-(3→(*Glc I*) [**Gro''**]	4.08-4.03* *66.0*	4.17-4.14* *75.5*	3.99-3.96* 3.67-3.63*			

			66.9			
P→1),α-D-Glc*p*-(1→2)-Gro-(3→*P* [**Gro'''**]	4.04-4.01* *65.8*	4.14-4.11* *75.9*	4.04-4.01* *65.8*			
(*Gro'/Gro''*)→1)-α-D-Glc*p*-(6→*P* [**Glc I]**	4.97-4.93* *99.3*	3.59-3.55* *72.1*	3.86-3.79* *71.3*	3.57-3.53* *69.6*	3.78-3.73* *73.5*	4.16-4.12* 4.10-4.06* *64.6*
α-D-Glc*p*-(1→2)-Gro''/*Gro'''* [**Glc III]**	5.19-5.16*/ 5.18-5.15* *98.3/98.0*	3.57-3.53* *72.1*	3.79-3.74* *73.5*	3.44-3.39* *70.2*	3.96-3.91* *72.4*	3.90-3.85* 3.78-3.74* *61.1*
^{31}P	Gro-*P*-Glc II^{LA}/Glc I-*P*-Gro 1.55 (1.63-1.44); Gro-*P*-Gro 1.38 (1.50-1.12).					

Figures

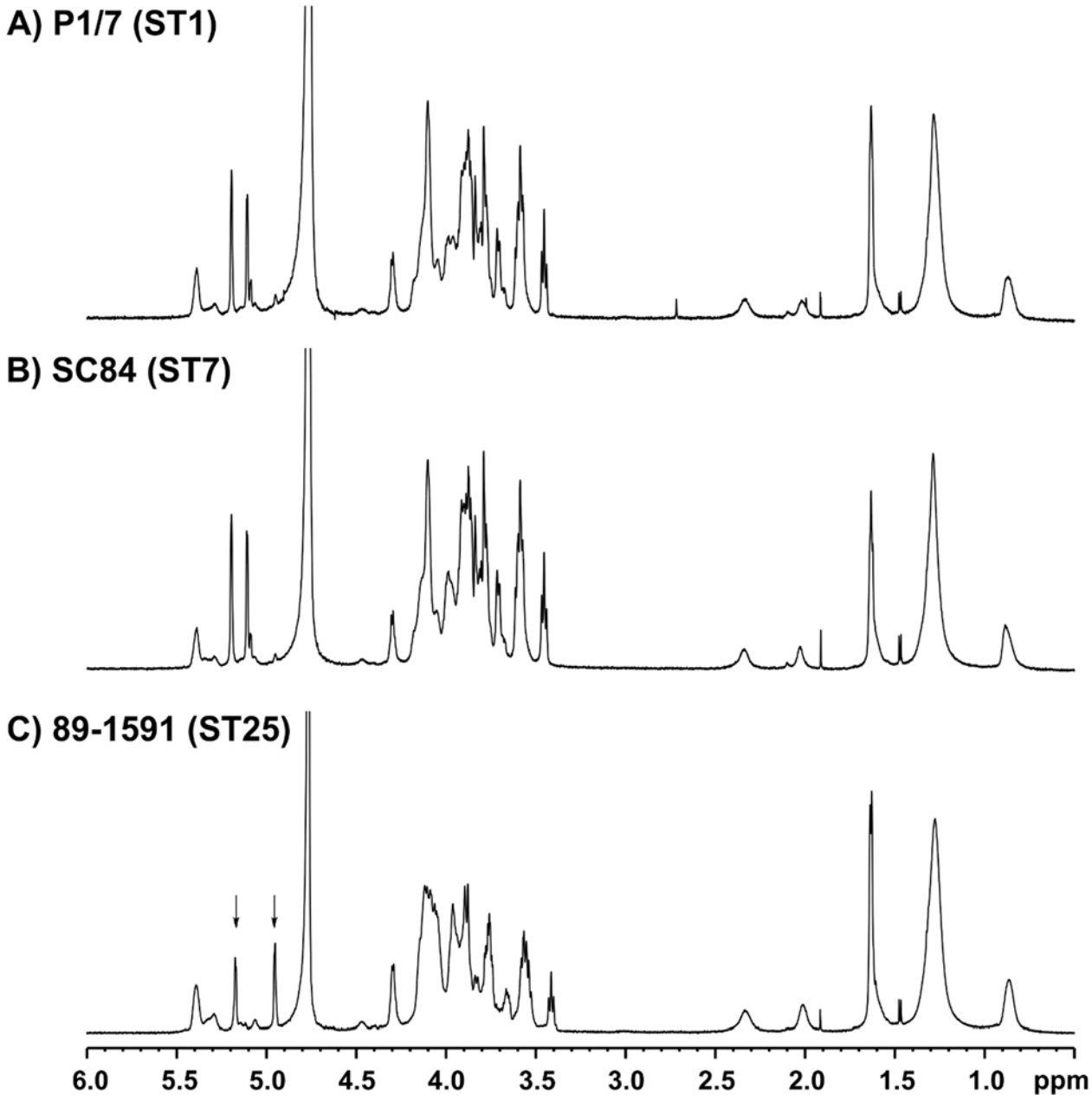

Figure 1. ^{1}H NMR analysis of native LTA of the investigated *S. suis* serotype 2 strains indicates differences in the binding position of glycosyl residues. Shown are ^{1}H NMR spectra (δ_H 6.0-0.0) isolated from *S. suis* strains **A)** P1/7 (ST1), **B)** SC84 (ST7), and **C)** 89-1591 (ST25) recorded in deuterated 25 mM sodium phosphate buffer (pH 5.5) at 300 K. Black arrows in panel C) indicate deviating major anomeric signals in LTA of strain 89-1591 compared to the other two spectra.

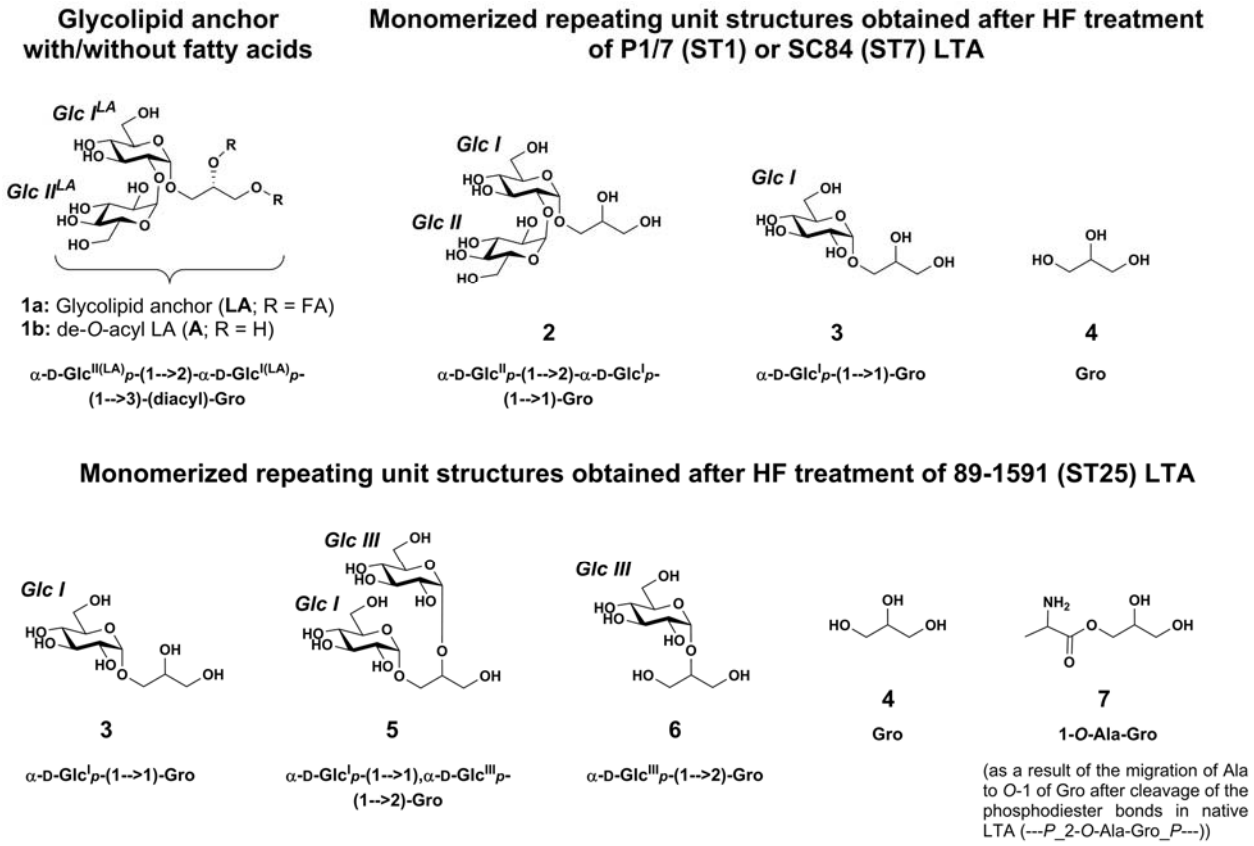

Figure 2. LTA part structures of the investigated *S. suis* strains. Compilation of structures of the observed glycolipid anchor **1a** (**LA**; R = FA (fatty acids)), its de-*O*-acylated version **1b** (**A**; R = H) and monomerized LTA repeats of different nature (**2**-**7**) isolated after HF treatment. Assignment of the different glucose moieties (*Glc I*, *Glc II*, *Glc III*, *Glc I*^{LA}, *Glc II*^{LA}) is used throughout the manuscript.

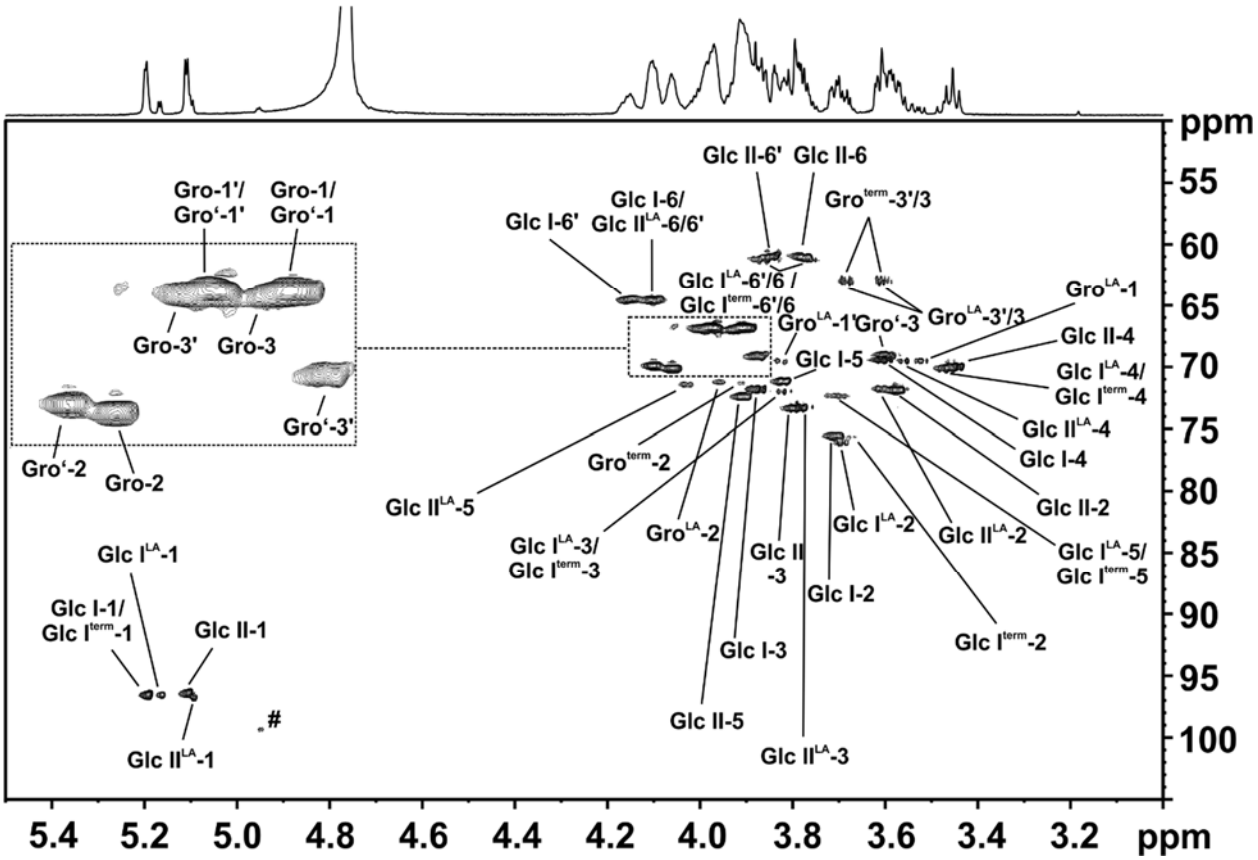

Figure 3. NMR analysis of de-*O*-acylated LTA of *S. suis* strain P1/7 (ST1). Shown is a section (δ_H 5.5-3.0; δ_C 105-50) of the $^{1}H,^{13}C$-HSQC NMR spectrum (recorded in D_2O at 300 K) obtained from hydrazine-treated LTA of *S. suis* strain P1/7 (ST1) including assignment of signals. The corresponding NMR chemical shift data are listed in Table 1. (# indicates the anomeric signal of a tiny amount of Glc I lacking Glc II)

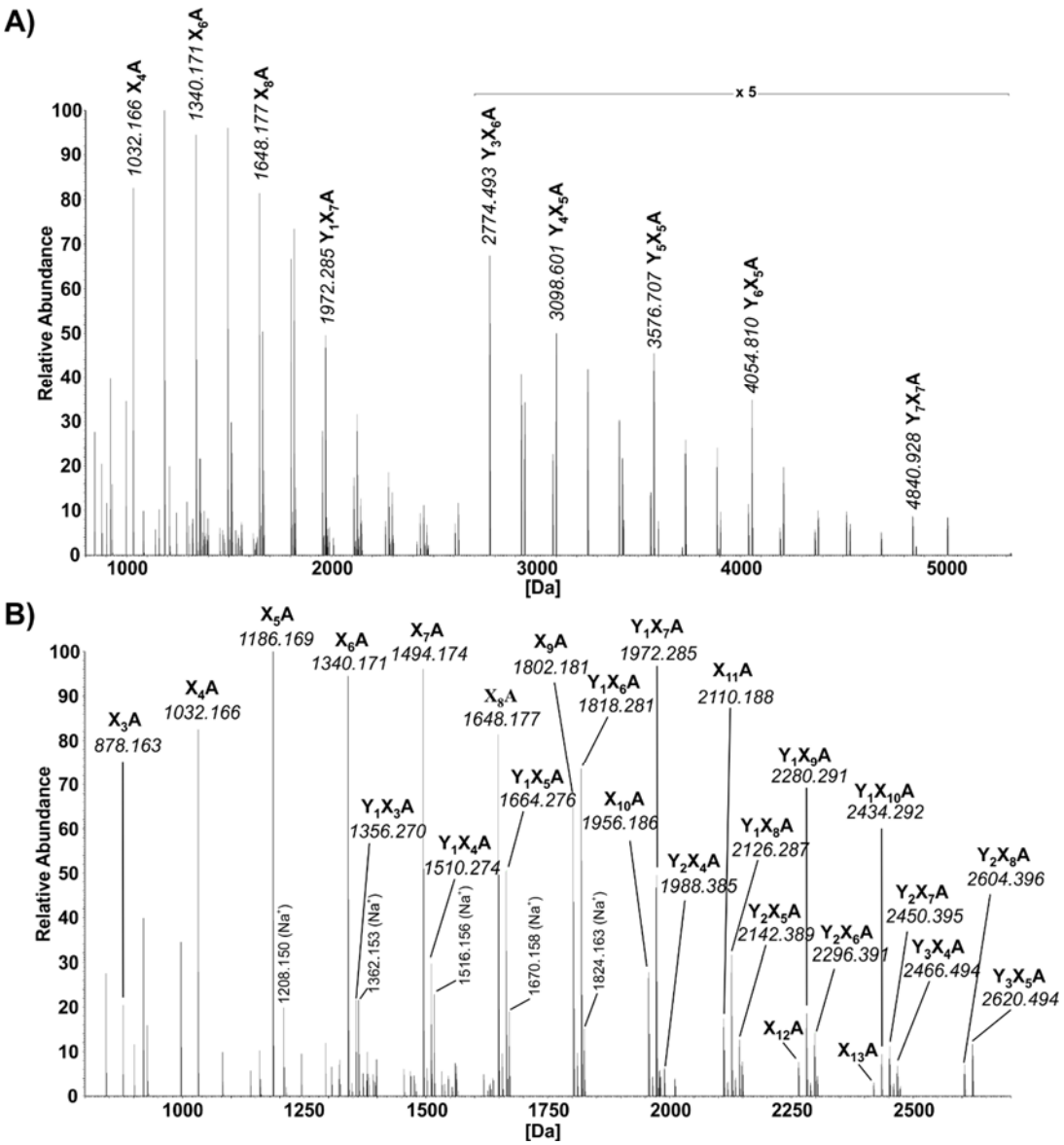

Figure 4. Molecular species distribution of de-*O*-acylated LTA of *S. suis* strain P1/7 (ST1). A) Charge-deconvoluted spectrum of a representative MS-analysis performed in the negative ion mode (the mass region of 2700 to 5300 Da has been magnified by a factor of 5). **B)** Zoom into the region between 800 and 2700 Da, including detailed assignment for molecules consisting of the de-*O*-acyl glycolipid anchor A (**1b**; Fig. 2) and different combinations of RUs X (= GroP) and Y (= α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-GroP). Details of all assigned molecular species are summarized in Tab. 2. Relative abundance for a spectral region was always normalized to the respective base peak. Additional information about molecular assignments for higher molecular species are depicted in Fig. S7.

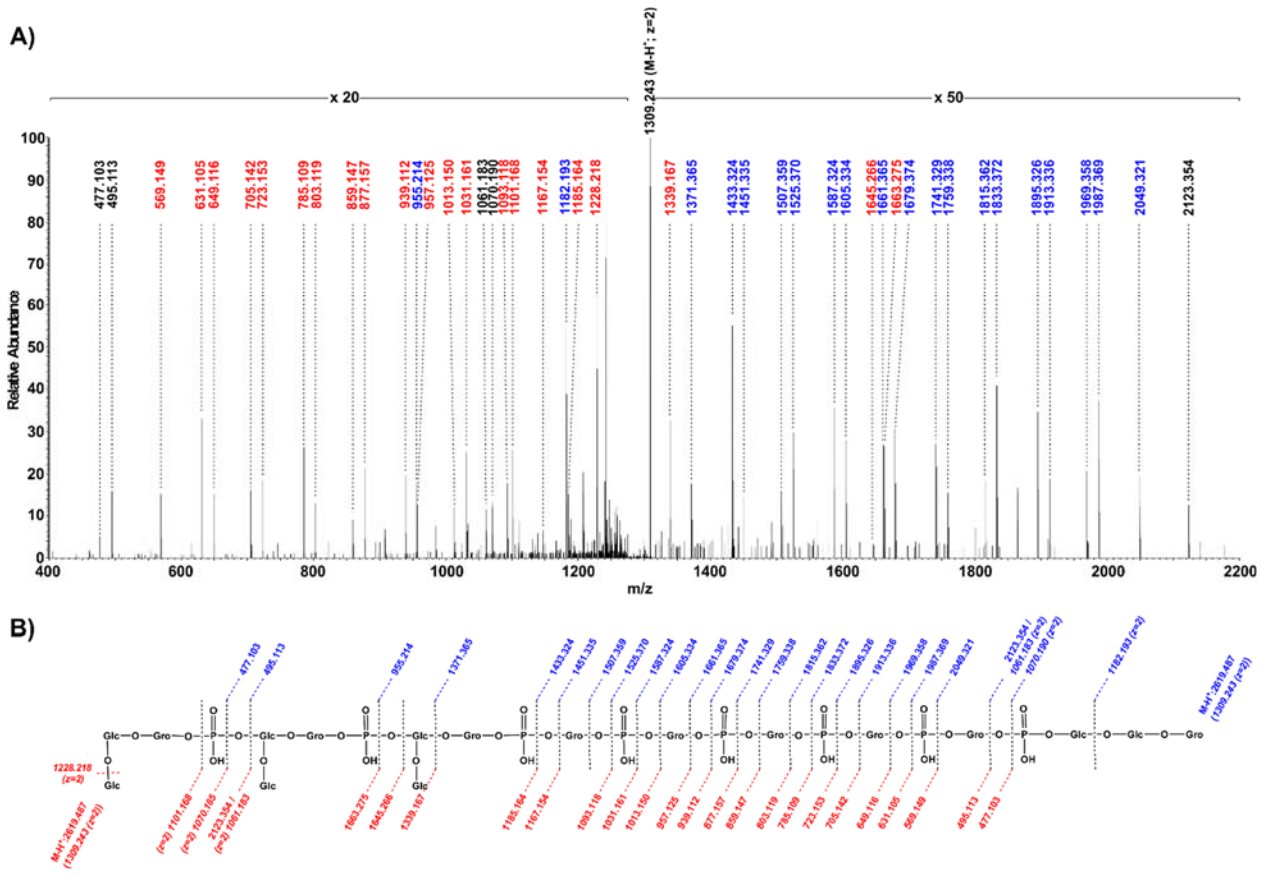

Figure 5. MS/MS analysis of de-*O*-acylated LTA molecule Y_3X_5A of *S. suis* strain P1/7 (ST1). As a representative molecule for this investigation, LTA_{N2H4} Y_3X_5A, with a calculated monoisotopic mass of 2620.495 Da has been chosen. **A)** The complete MS/MS spectrum of the doubly charged precursor *m/z* 1309.243 is shown (the mass region of 400 to 1275 Da has been magnified by a factor of 20, the mass region of 1315 to 2200 Da by a factor of 50). Fragment ions representing part structures starting from the de-*O*-acyl linker are labeled in blue, fragment ions originating from the terminus are colored in red. Fragment ions that can be produced from both cleavage directions are labeled in black. **B)** All observed fragment ions are assigned to the linearized structure model of the LTA. The complete list of observed fragments and their assignment is given in Table S4.

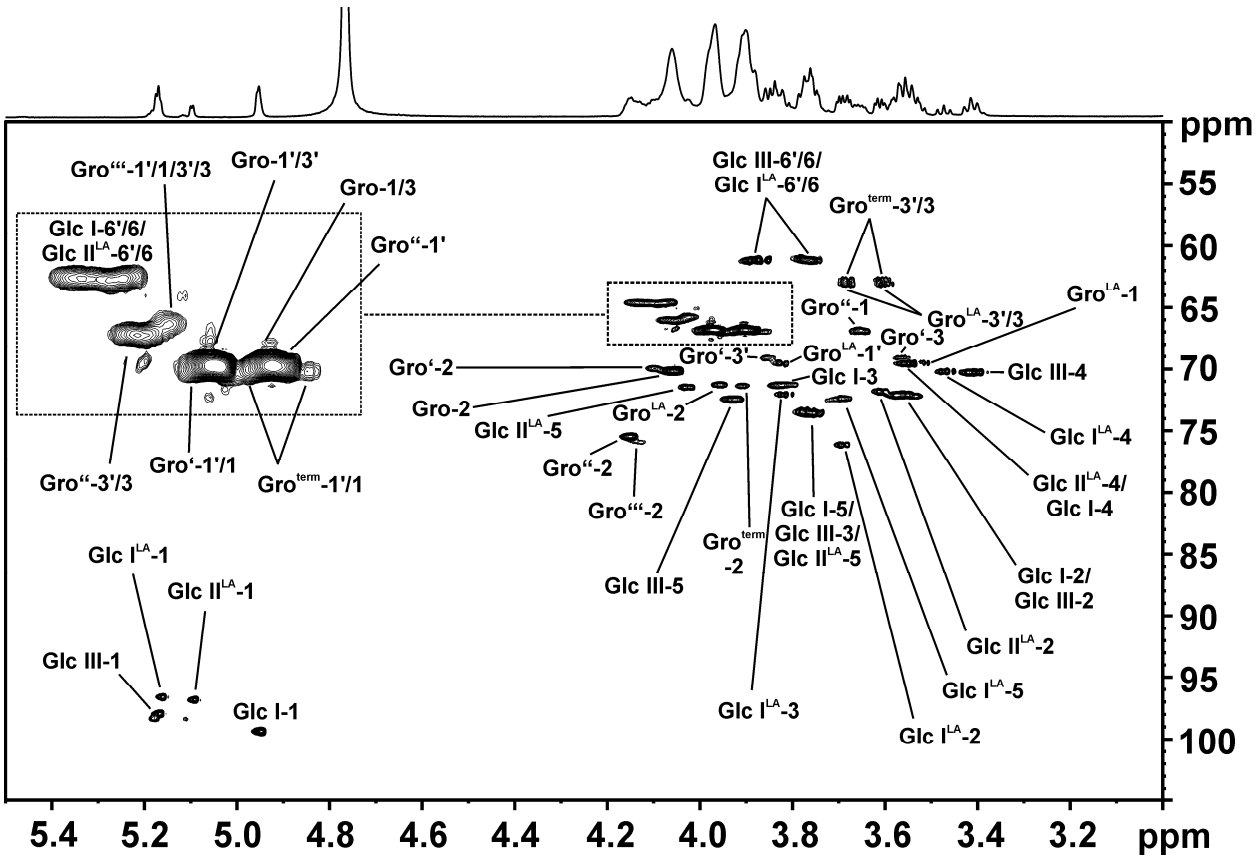

Figure 6. NMR analysis of de-*O*-acylated LTA of *S. suis* strain 89-1591 (ST25). Shown is a section (δ_H 5.5-3.0; δ_C 105-50) of the $^{1}H,^{13}C$-HSQC NMR spectrum (recorded in D_2O at 300 K) obtained from hydrazine-treated LTA of *S. suis* strain 89-1591 (ST25) including assignment of signals. The corresponding NMR chemical shift data are listed in Table 3.

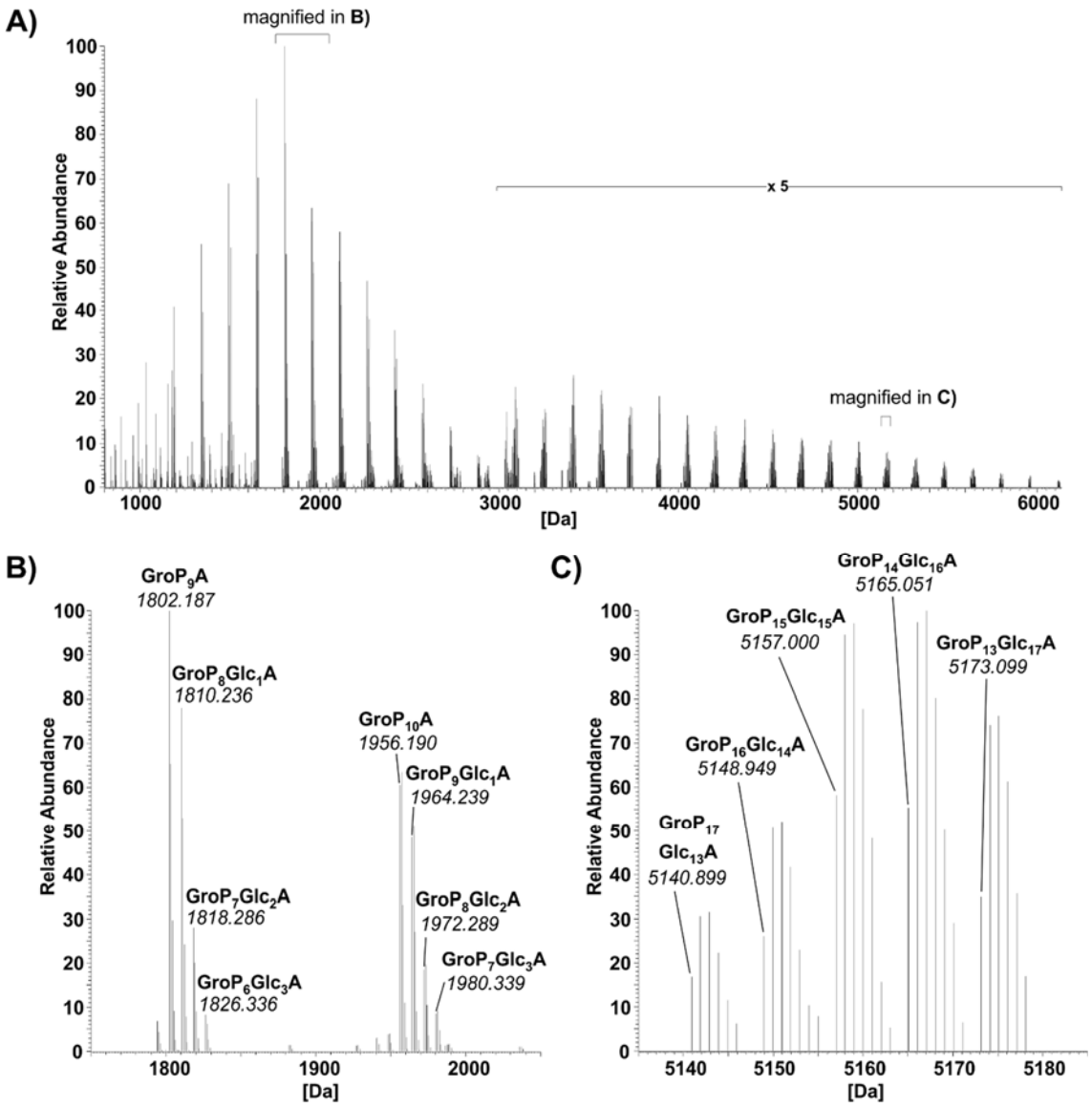

Figure 7. Molecular species distribution of de-*O*-acylated LTA of *S. suis* strain 89-1591 (ST25). A) Charge-deconvoluted spectrum of a representative MS-analysis performed in the negative ion mode (The mass region of 3000 to 6150 Da has been magnified by a factor of 5). Zoom into the regions between 1750 and 2050 Da (**B**) and 5135 and 5185 Da (**C**), including assignment of mass peaks for molecules consisting of the de-*O*-acyl glycolipid anchor A (**1b**; Fig. 2) and different numbers of GroP and Glc residues. The difference Δm = +8.05 Da corresponds to one GroP moiety less but one Glc moiety more in the overall composition. All identified molecules are listed in Tab. S8. Relative abundance for a spectral region was always normalized to the respective base peak.

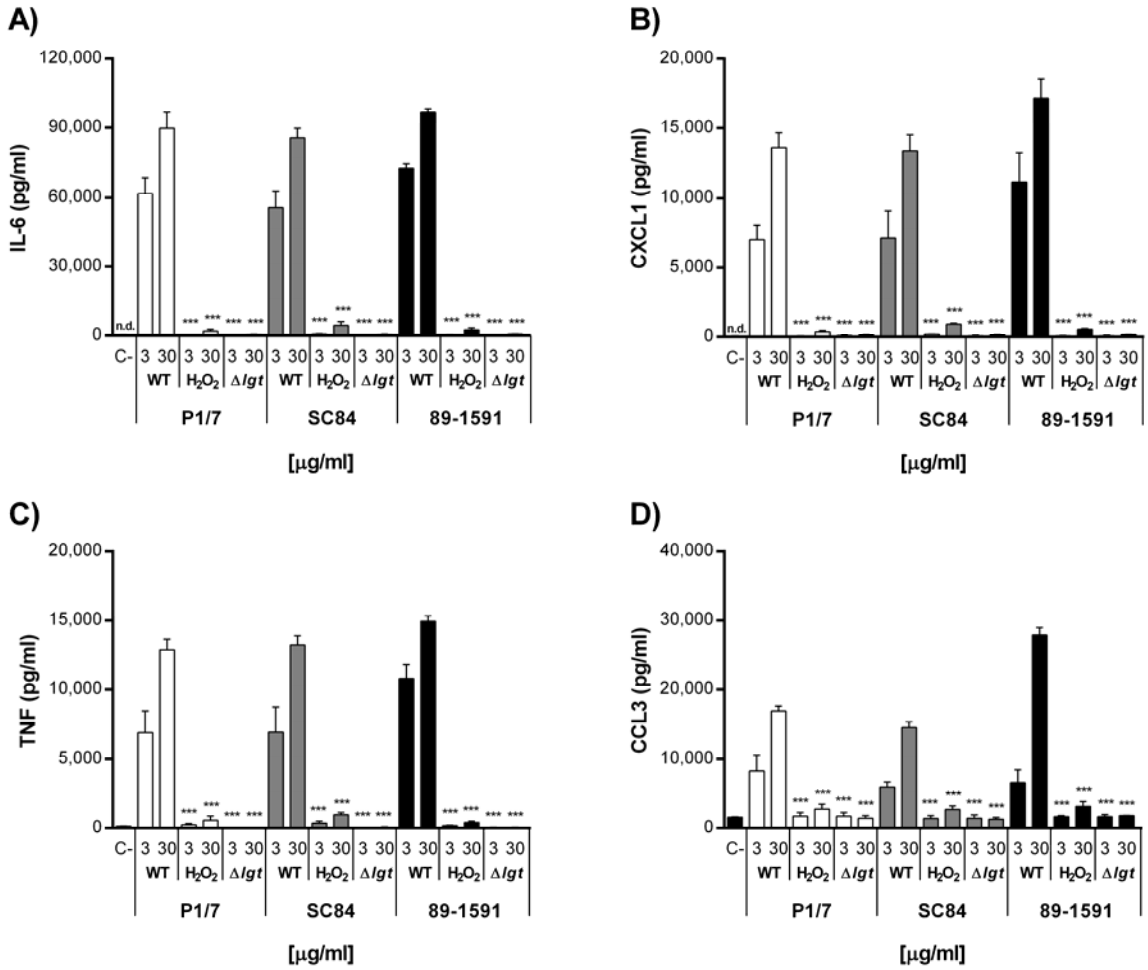

Figure 8. Pro-inflammatory mediator production by dendritic cells (DCs) following activation with the different LTA preparations from *S. suis* strains P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25). Production of **A)** IL-6, **B)** CXCL1, **C)** TNF, and **D)** CCL3 24 h following activation of DCs with 3 or 30 µg/mL of the non-treated LTA preparations from wild-type strains (WT), the LTA preparations from wild-type strains following treatment with H_2O_2 for 24 h (H_2O_2) or the LTA preparations from *lgt*-deficient mutants (Δ*lgt*). Secreted mediators were quantified by sandwich ELISA. Data represent the mean ± SEM (n = 3). C- denotes the culture medium alone; n.d. = not detected. *** ($p < 0.001$) indicates a significant difference between the WT and H_2O_2 or Δ*lgt* LTA preparations.

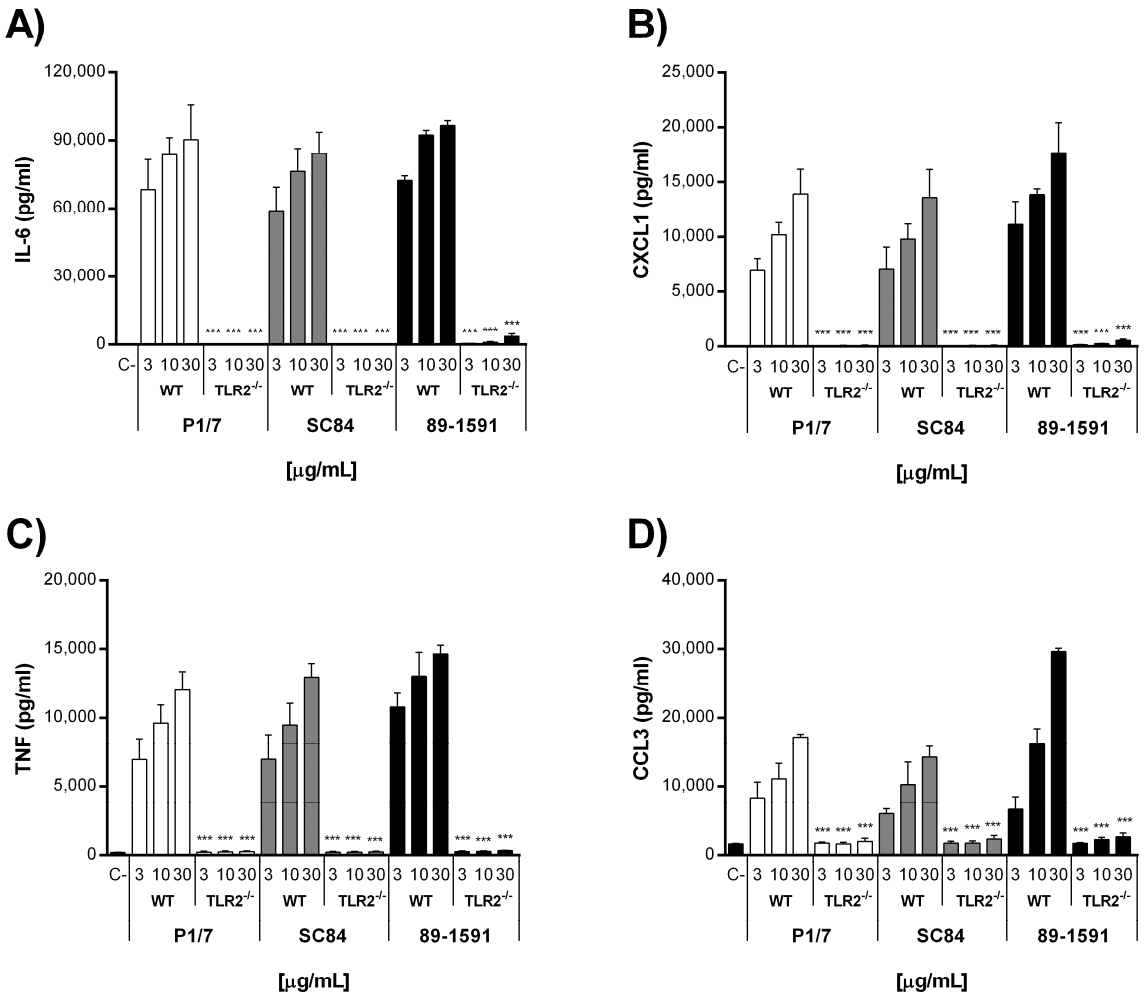

Figure 9. Pro-inflammatory mediator production by wild-type (WT) and Toll-like receptor (TLR) 2$^{-/-}$ dendritic cells (DCs) following activation with the LTA preparations from the *S. suis* strains P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25). Production of **A)** IL-6, **B)** CXCL1, **C)** TNF, and **D)** CCL3 24 h following activation of DCs derived from WT or TLR2$^{-/-}$ mice with 3, 10 or 30 μg/mL of the LTA preparations. Secreted mediators were quantified by sandwich ELISA. Data represent the mean ± SEM (n = 3). C- denotes the culture medium alone. *** (p < 0.001) indicates a significant difference between WT and TLR2$^{-/-}$ DCs.

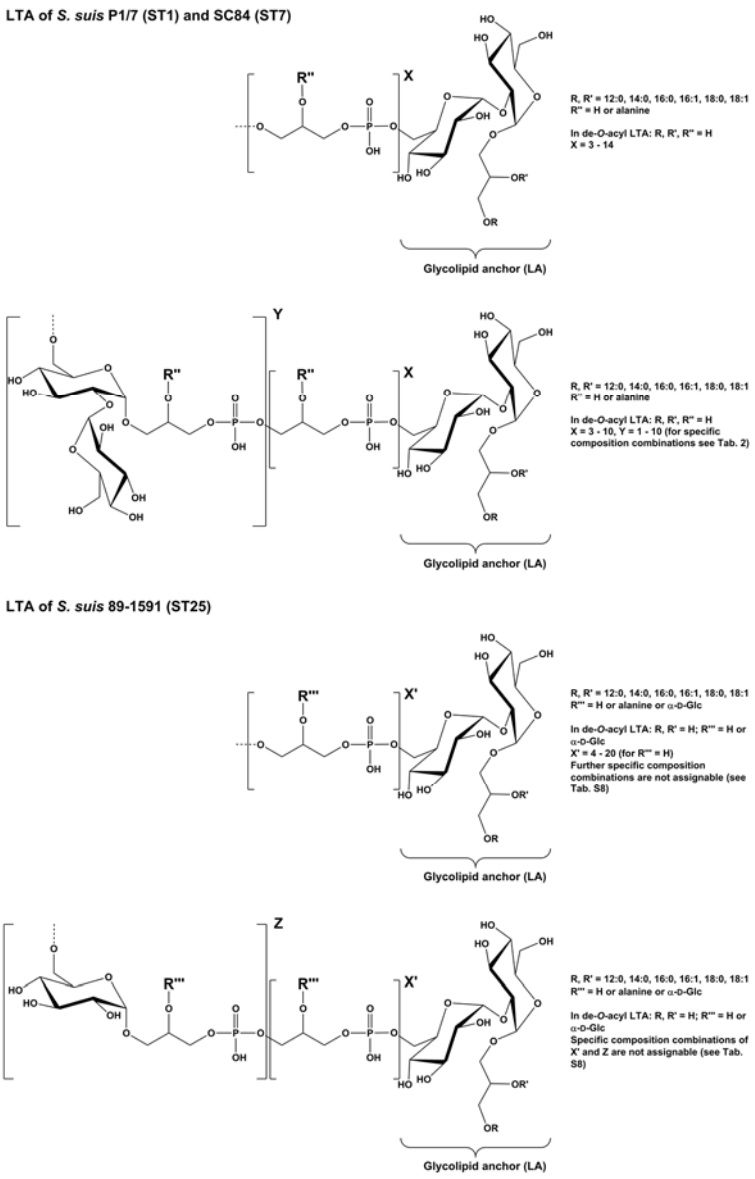

Figure 10. Chemical structures of LTA isolated from *S. suis* serotype 2 strains P1/7 (ST1), SC84 (ST7), and 89-1591 (ST25). From all three investigated strains, a type I LTA composed of kojibiose-diacylglycerol and poly-glycerolphosphate chains was isolated. All strains are capable of modifying the glycerol *O*-2 position with alanine. Exclusively in strain 89-1591, a potential glycosylation at the glycerol *O*-2 position was additionally observed. In all strains, a second kind of LTA molecule has been identified, whereas a ST-specific difference with regards to the incorporation of glycosyl residues into the complex mixed-type LTA has been observed. Strains P1/7 and SC84 are able to attach an 1,2-linked α-D-Glc*p* residue as branching substituent to the -D-Glc*p* 1,3-linked to the GroP, whereas in strain 89-1591 this branching substituent is absent.

OBJECTIF 2 – ÉTUDIER L'IMPACT DE L'ORIGINE DES SOUCHES ET DU CHOIX DES MODÈLES EXPÉRIMENTAUX SUR LES ÉTUDES DE VIRULENCE ET DE PATHOGENÈSE DE *STREPTOCOCCUS SUIS*

ARTICLE VII

The bias of experimental design, including strain background, in the determination of critical *Streptococcus suis* serotype 2 virulence factors

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé à la conception de l'étude et des approches méthodologiques et aux expériences *in vitro.* J'ai effectué les expériences *in vivo* en plus de participer à l'analyse des résultats. J'ai été responsable de l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci. J'ai aussi effectué les corrections lors des étapes de révision pour publication.

Mise en contexte

Après avoir mieux compris le rôle de différents composants retrouvés à la surface de *S. suis* dans sa pathogenèse et dans ses interactions avec les cellules de l'hôte (objectif 1), nous avons voulu investiguer quel est l'impact de l'origine des souches et des modèles expérimentaux utilisés sur les études de virulence. Tel que mentionné dans la revue de la littérature, malgré les différences importantes entre les souches de *S. suis* (nombre de sérotypes et STs élevés, entre autres), près de 40 facteurs différents ont été rapportés comme critique pour la virulence de *S. suis.* Malheureusement, la définition de ce qu'est un facteur de virulence reste ouverte à interprétation, puisqu'aucun barème n'a été établi. De plus, les approches méthodologiques et expérimentales, autant *in vitro* qu'*in vivo* diffèrent grandement entre laboratoires et peuvent affecter cette définition. C'est aussi le cas pour le choix de la souche, en raison du bagage génétique et des caractéristiques phénotypiques. Néanmoins, malgré cette problématique, très peu voire aucune étude portant sur *S. suis* n'avait été répétée entre laboratoires. Bien que cette problématique persiste depuis plusieurs années, le manque de données scientifiques a empêché de répondre de manière concrète à cette problématique.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death, septic shock, and meningitis. However, serotype 2 strains are genotypically and phenotypically heterogeneous. Though a multitude of virulence factors have been described for *S. suis* serotype 2, the lack of a clear definition regarding which ones are truly “critical” has created inconsistencies that have only recently been highlighted. Herein, the involvement of two factors previously described as being critical for *S. suis* serotype 2 virulence, whether the dipeptidyl peptidase IV and autolysin, were evaluated with regards to different ascribed functions using prototype strains belonging to important sequence types. Results demonstrate a lack of reproducibility with previously published data. In fact, the role of the dipeptidyl peptidase IV and autolysin as critical virulence factors could not be confirmed. Though certain *in vitro* functions may be ascribed to these factors, their roles are not unique for *S. suis*, probably due to compensation by other factors. As such, variations and discrepancies in experimental design, including *in vitro* assays, cell lines, and animal models, are an important source of differences between results. Moreover, the use of different sequence types in this study demonstrates that the role attributed to a virulence factor may vary according to the *S. suis* serotype 2 strain background. Consequently, it is necessary to establish standard experimental designs according to the experiment and purpose in order to facilitate comparison between laboratories. Alongside, studies should include strains of diverse origins in order to prevent erroneous and biased conclusions that could affect future studies.

Introduction

Streptococcus suis is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) [1]. Of the different described serotypes based on the presence of the capsular polysaccharide or its respective genes, serotype 2 is regarded as not only the most widespread worldwide, but also the most virulent, responsible for the majority of porcine and human cases of *S. suis* infection [2]. Using multilocus sequence typing, the distribution of the most important sequence types (STs) of *S. suis* serotype 2 has been determined worldwide [2]. Moreover, recent studies have evaluated the virulence of these important STs using well-characterized mouse models of infection, where virulence is defined based on the capacity of a strain to induce clinical disease and mortality [3, 4]. The ST7 strain responsible for the human outbreaks of 1998 and 2005 in China [5] is highly virulent whereas European ST1 strains are virulent; on the other hand, ST25 strains, typically recovered in North America, are of intermediate virulence [3].

Over the years, a multitude of virulence factors, presently totaling more than 150, have been described to be implicated in the *S. suis* serotype 2 pathogenesis in pigs and humans [6-9]. However, the lack of a clear definition regarding what constitutes a virulence factor for *S. suis*, which generally differs from one laboratory to another, and the fact that many of these have redundant roles, have greatly hindered the identification of truly “critical” virulence factors and created inconsistencies throughout the literature [9]. Indeed, of the different factors described so far, at least 76 have been reported to be implicated in virulence, while 35 of these were critical for virulence since their absence resulted in avirulence [9]. Alongside, many putative virulence factors are present in certain virulent strains but not in others, such as the suilysin, muramidase-released protein (MRP), and extracellular protein factor, which currently serve as virulence markers for Eurasian *S. suis* serotype 2 strains only, since these are often absent in North American strains [2, 9, 10]. In fact, the North American strains that do possess the MRP are associated with lower virulence [4, 10]. Moreover, the important roles played by a putative virulence factor might depend on the genetic background of the selected strain. Finally, the use of differing experimental designs, including *in vitro* assays, cell lines, and animal models, have made it extremely difficult to accurately compare results between laboratories [9].

Herein, the involvement of two *S. suis* serotype 2 virulence factors previously described as being critical were evaluated with regards to different ascribed functions using prototype strains belonging to three of the most important STs (ST1, ST7, and ST25). These proteins, which served as tools, were chosen among the more than 150 putative virulence factors described for *S. suis* on the basis of being present in strains from these three backgrounds. The dipeptidyl peptidase IV (DPPIV), originally studied using a ST7 isolate recovered from a human case during the 2005 Chinese outbreak [11], is a serine protease widely distributed in eukaryotes and bacteria that has been suggested to contribute to bacterial pathogenesis [12]. Meanwhile, the autolysin [13], originally studied using a ST378 strain recovered from a diseased pig in China [14], is a peptidoglycan hydrolase implicated in various bacterial functions such as cell wall turnover, cell division, and cell separation [13]. Consequently, the aim of this study was to determine the bias of experimental design, including strain background, in the determination of *S. suis* serotype 2 virulence factors in order to better clarify the recently highlighted controversy caused by inconsistencies plaguing this field of research.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (Permit Number: Rech-1570).

Bacterial strains and growth conditions

The three well-characterized and highly encapsulated intermediate to highly virulent prototype wild-type *S. suis* serotype 2 strains and their isogenic mutants used in this study are listed in Table 1. Strains are minimally passaged and virulence of strains is routinely tested using cell-based assays and experimental infection models. The *S. suis* strains were cultured in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada). For adhesion assays, bacterial cultures were prepared as previously described [15]. Briefly, upon reaching the mid-exponential phase, bacteria were washed twice with phosphate-buffered saline (PBS), pH 7.3, and resuspended in PBS for adhesion to fibronectin or cell culture medium (Gibco, Burlington, ON, Canada) for adhesion to porcine epithelial cells (described below). For experimental infections, early stationary phase bacteria were washed twice in PBS and resuspended in THB

[4, 16, 17]. Bacterial cultures were appropriately diluted and plated on THB agar to accurately determine bacterial concentrations. mRNA expression of the *dppIV* and *atl* genes was determined to be similar between the three wild-type strains under the growth conditions used in this study as quantified by RT-qPCR (data not shown). The *Escherichia coli* strain and different plasmids used in this study are also listed in Table 1. When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/mL and chloramphenicol at 5 µg/mL; for *E. coli*, kanamycin and spectinomycin at 50 µg/mL and chloramphenicol at 30 µg/mL.

DNA manipulations

S. suis genomic DNA was extracted using the InstaGene Matrix solution (BioRad Laboratories, Hercules, CA, USA). Mini-preparations of recombinant plasmids were carried out using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Restriction enzymes and DNA-modifying enzymes (Fisher Scientific, Ottawa, ON, Canada) were used according to the manufacturer's recommendations. Oligonucleotide primers (Table 2) were obtained from Integrated DNA Technologies (Coralville, IA, USA) and PCRs carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or with the Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using an ABI 310 Automated DNA Sequencer and the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Construction of the isogenic dipeptidyl peptidase IV and autolysin mutants

The DNA genome sequences of the wild-type *S. suis* strains were used. In-frame deletions of the *dppIV* or *atl* genes were constructed using splicing-by-overlap-extension PCRs as previously described [11, 13, 22]. Overlapping PCR products were cloned into pCR2.1 (Invitrogen, Burlington, ON, Canada), extracted with EcoRI, recloned into the thermosensitive *E. coli*–*S. suis* shuttle plasmid pSET4s, and digested with the same enzyme, giving rise to the knockout vector p4Δ*dppIV* or p4Δ*atl*. Electroporation of the three *S. suis* wild-type strains and procedures for isolation of the mutants were previously described [23]. Allelic replacement was confirmed by PCR and DNA sequencing analysis. Amplification products were purified with the QIAgen PCR Purification Kit (Qiagen) and sequenced as described above. mRNA expression of upstream and downstream genes flanking the *dppIV* and *atl* genes in the mutant strains was

confirmed by RT-PCR, validating in-frame gene deletion (data not shown). Growth of the different mutant strains was similar to that of the wild-type strains (data not shown).

S. suis *adhesion to human fibronectin (microtiter plate binding assay)*

Fibronectin adhesion assays were carried out as previously described [24]. Briefly, microtiter plates were coated with different concentrations of human plasma fibronectin (Sigma-Aldrich, St-Louis, MO, USA), ranging from 0 to 10 µg/mL, in 0.1 M carbonate buffer, pH 9.6. Formaldehyde-killed bacterial suspensions (equivalent to 1 x 10^8 colony forming units [CFU]/mL) of the different wild-type and mutant strains were added and the plates incubated for 2 h at 37°C. It was previously demonstrated that killing of *S. suis* using 0.2% formaldehyde does not affect its capacity to bind fibronectin [24]. An anti-*S. suis* serotype 2 rabbit serum followed by a horseradish peroxidase-labelled anti-rabbit IgG conjugate (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) were used. The anti-*S. suis* serum equally recognized the wild-type and mutant strains by enzyme-linked immunosorbent assay (ELISA) [24]. The enzyme substrate, 3,30,5,50-tetramethylbenzidine (Zymed, San Francisco, CA, USA) was used according to the manufacturer's instructions, the reaction stopped using 1 N H_2SO_4, and the optical density measured at 450 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA). Uncoated wells served as background controls. Casein-coated wells served as a control for non-specific adhesion of *S. suis* to protein-coated wells.

S. suis *adhesion to porcine tracheal epithelial cells*

The newborn porcine tracheal epithelial cell line (NPTr) was used and cultured until confluent as previously described [25]. Cells were infected with *S. suis* (10^6 CFU/well; multiplicity of infection [MOI]=10) by removing the cell culture medium, adding 1 mL of bacteria in cell culture medium without antibiotics, and incubating for 2 h at 37°C with 5% CO_2 as previously described [15]. Following incubation, cells were washed five times with PBS to remove non-adherent bacteria and lysed using 1 mL of sterile water. The lysates were appropriately diluted and plated on THB agar to quantify adhered bacteria. Alongside, the last wash was plated to confirm absence of non-adhered bacteria. The percentage of adhered bacteria was calculated according to the following: CFU recovered 2 h post-incubation / inoculum x 100% [11, 13].

S. suis *biofilm formation capacity*

The biofilm formation capacity of the different wild-type and mutant strains was determined as previously described [26]. Moreover, the protocol used was identical to that described by Ju *et al*., including the use of 2 mg/mL of porcine fibrinogen (Sigma-Aldrich), incubation for 24 h at 37°C, subsequent staining with crystal violet, and measurement of the optical density at 575 nm [13].

S. suis in vivo *virulence mouse infections*

A well-standardized C57BL/6 mouse model of infection was used [3, 4, 17]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering through the use of humane endpoints, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. No additional considerations or housing conditions were required. All staff members received the required animal handling training as administered by the University of Montreal Animal Welfare Committee. A total of 140 six-week-old male and female C57BL/6 mice (Jackson Research Laboratories, Bar Harbour, MA, USA) were used (10 to 15 mice/group) in this study. Mice were inoculated with $5x10^7$ CFU via the intraperitoneal route and health and behavior monitored at least thrice daily until 72 h post-infection (p.i.) and twice thereafter until the end of the experiment (14 days p.i.) for the development of clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, and lethargy. Mice were also monitored for the development of clinical signs of meningitis. Clinical scores were determined according to the grid approved by the University of Montreal Animal Welfare Committee (S1 Appendix) and required actions undertaken. Mice were immediately euthanized upon reaching endpoint criteria using CO_2 followed by cervical dislocation. No mice died before meeting endpoint criteria and all surviving mice were euthanized as described above at the end of the experiment (14 days p.i.). Blood samples were collected from the caudal vein of surviving mice 24 h p.i. and plated as previously described [4].

Statistical analyses

Significant differences were determined using the t-test, Mann-Whitney Rank sum test, one-way ANOVA, and ANOVA on ranks, where appropriate. For *in vivo* virulence experiments,

survival was analyzed using the LogRank test. A $p < 0.05$ was considered statistically significant.

Results

The S. suis *serotype 2 dipeptidyl peptidase IV and autolysin are not major fibronectin-binding adhesins, regardless of the sequence type of the strain used*

Adhesion to host extracellular matrix (ECM) components is an important and often crucial initial step of the bacterial pathogenesis [8]. Amongst the different components of the ECM is plasma fibronectin, to which both the DPPIV of a ST7 strain and the autolysin of a ST378 strain were previously reported to bind [11, 13]. Results showed similar levels of adhesion to fibronectin between the wild-type ST1, ST7, and ST25 strains, as measured by ELISA (Fig 1). The role of the DPPIV and autolysin in binding human fibronectin was then evaluated using their respective isogenic mutants. In the presence of 10 µg/mL of fibronectin (concentration shown to be optimal for *S. suis*; data not shown), no significant differences were observed between the adhesion of the Δ*dppIV* or Δ*alt* mutants and their respective wild-type strains, regardless of the ST of the strain used (Fig 1). Similar results were obtained using lower concentrations of fibronectin (data not shown). These results suggest that the DPPIV and autolysin are not major human fibronectin-binding adhesins for *S. suis* serotype 2.

The S. suis *serotype 2 dipeptidyl peptidase IV, unlike the autolysin, does not play a major role in adhesion to porcine tracheal epithelial cells*

Adhesion to host cells is a requirement for subsequent interactions, including cell activation and establishment of the disease [8]. Indeed, it was previously suggested that both the DPPIV [11] and autolysin [13] are implicated in adhesion of *S. suis* serotype 2 to the human laryngeal epithelial cell line HEp-2. Herein, adhesion of the different wild-type strains and the role of the DPPIV and autolysin in adhesion to the porcine tracheal epithelial cell line NPTr was determined after 2 h of incubation with a MOI=10 and results expressed as percentage of adhered inoculum. Adhesion of the wild-type ST1 strain was greatest, with that of the ST7 strain being intermediate, while the ST25 strain adhered the least, adhesion of which was significantly lower than that of the wild-type ST1 strain only ($p < 0.01$) (Fig 2). Moreover, results showed that the autolysin, but not the DPPIV, plays an important role in the adhesion to NPTr for the three wild-type strains ($p < 0.001$) (Fig 2). In fact, adhesion of the autolysin-deficient mutants was only 0.6%, 0.6%, and 0.4% of the inoculum for the ST1, ST7, and ST25 strains,

respectively, in comparison to 2.3%, 1.9%, and 1.4% for their respective wild-type strains. As such, adherence of the three mutant strains was reduced by more than 70%. The DPPIV mutants, however, showed no differences compared to their respective wild-type strains regarding adhesion to porcine epithelial cells.

The dipeptidyl peptidase IV is not involved in S. suis *serotype 2 biofilm formation, while the implication of the autolysin is strain-dependent*

Alongside adhesion to host ECM and cells, the capacity to form biofilm has been described as important for the *S. suis* pathogenesis, being involved in survival and propagation [8]. In order to enhance the biofilm formation capacity of the wild-type and mutant strains, culture medium was supplemented with porcine fibrinogen as previously described [26]. Results demonstrated that the wild-type ST1 strain produced significantly more biofilm than both the wild-type ST7 ($p < 0.05$) and ST25 strains ($p < 0.01$), though the ST7 strain produced more than the ST25 strain ($p < 0.01$) (Fig 3). While no data were available regarding a role of the DPPIV in biofilm formation by *S. suis*, the autolysin was previously reported to be implicated using a *S. suis* serotype 2 ST378 strain [13]. Using the same experimental design as previously used for the autolysin, the involvement of these two putative virulence factors in the capacity of the three wild-type *S suis* strains to form biofilm was evaluated. While the DPPIV was not involved in biofilm formation, regardless of the sequence type of the strain used, the autolysin of the ST7 and ST25 strains participated in biofilm formation ($p < 0.001$), but not that of the ST1 strain (Fig 3).

The S. suis *serotype 2 dipeptidyl peptidase IV and autolysin do not behave as critical virulence factors in an experimental model of S. suis serotype 2 infection*

In order to evaluate the role of the DPPIV and autolysin in virulence, a well-characterized C57BL/6 mouse model of infection was used [4, 17]. In this model, mice succumb to septic shock during the systemic infection, after which surviving mouse are susceptible of developing meningitis. While no differences in survival were observed between mice infected with the wild-type ST1 (Fig 4A) and ST7 (Fig 4B) strains, results showed that the ST7 strain induced host death more rapidly than the ST1 strain. Moreover, the wild-type ST1 and ST7 strains caused significantly more mortality than the wild-type ST25 strain (Fig 4C) ($p < 0.05$): mortality caused by the ST25 strain was delayed and, unlike with the two other wild-type strains, partially due to the development of meningitis. For the virulent European ST1 strain (Fig 4A) and the highly

virulent Chinese ST7 strain (Fig 4B), no significant role of the DPPIV and autolysin as critical virulence factors was observed. Moreover, this was also the case for the DPPIV of the intermediate virulent North American ST25 strain (Fig 4C). Meanwhile, and surprisingly, the ST25 autolysin-deficient mutant caused significantly higher mortality ($p < 0.05$) than its wild-type strain (Fig 4C). These results were confirmed in a subsequent infection (data not shown). Given these results, the *S. suis* serotype 2 DPPIV and autolysin are not significantly involved in virulence using a C57BL/6 mouse model of infection.

The S. suis *serotype 2 ST25 autolysin hinders bacterial survival in blood*

Given the higher virulence of the autolysin-deficient ST25 strain, blood bacterial burden, which when uncontrolled may be responsible for *S. suis*-induced host death [4, 17], was evaluated 24 h p.i. for the wild-type ST25 strain and its two isogenic mutants. Indeed, blood bacterial burden was significantly higher ($p < 0.01$) in mice infected with the ST25 autolysin-deficient mutant than in those infected with either the wild-type strain or the DPPIV-deficient mutant, between which burdens were similar (Fig 5). This suggests that the autolysin might somewhat hinder survival of the ST25 strain in blood.

Discussion

Though a multitude of virulence factors have been described for *S. suis* serotype 2, a clear definition of what constitutes a critical virulence factor is still lacking for this pathogen. This confusion has led to inconsistencies throughout the literature, resulting in a controversy that has only just been highlighted [9]. Consequently, the involvement of two *S. suis* serotype 2 factors, the DPPIV and autolysin, described as critical for virulence, was evaluated with regards to previously ascribed bacterial functions implicated in the *S. suis* pathogenesis using strains belonging to three important STs.

It is important to mention that the study by Ge *et al.* regarding the *S. suis* serotype 2 DPPIV was conducted using a ST7 strain (05ZYH33) isolated from the 2005 Chinese human outbreak [11]. Similarly, the ST7 strain used in the present study (SC84) was also isolated from a human case of streptococcal toxic shock-like syndrome during the same outbreak [19]. Since different isolates recovered from this outbreak have been reported to be highly similar [5], the influence of the background should be minimal between these two strains. Meanwhile, the strain originally used to study the *S. suis* serotype 2 autolysin by Ju *et al*. is a ST378 (HA9801)

according to the *S. suis* Multilocus Sequence Typing Website (http://ssuis.mlst.net) [13]. This ST was never reported beforehand and has not been reported since. To facilitate comparison of the methodologies used between the previous [11, 13] and present studies, the main experimental designs of the different assays are listed in Table 3. Since mRNA expression of *dppIV* and *atl* is similar between the three wild-type strains under the growth conditions used in this study, the differences observed herein are probably due to other variations between the strains, such as the presence/absence of putative virulence factors and/or differential expression of these factors.

In order to evaluate the reproducibility of previously published results with strains from different backgrounds, the role of the *S. suis* serotype 2 DPPIV [11] and autolysin [13] in binding human plasma fibronectin was evaluated using a virulent European ST1 strain, the highly virulent clonal ST7 strain, and an intermediate virulent North American ST25 strain. The similar capacity of the three wild-type strains to bind human plasma fibronectin suggests this characteristic might be common to *S. suis* serotype 2, indicating a possibly universal role in the pathogenesis of this bacterium. Moreover, no significant implication of the *S. suis* serotype 2 DPPIV or autolysin in binding human fibronectin was observed when using isogenic mutants, regardless of the ST and fibronectin concentration. While these results cannot exclude those previously obtained using the recombinant DPPIV and autolysin as evaluated by ELISA and Western blot, respectively, they suggest that while the recombinant proteins themselves might bind human fibronectin, their absence is not sufficient to affect binding to this ECM component by *S. suis*. This lack of role when using isogenic mutants could be the result of compensation by one or more of the 18 factors currently known to bind fibronectin other than the DPPIV and autolysin: the fibronectin/fibrinogen-binding protein [27], enolase [28], Ssa (fibronectin-binding protein) [29], MRP [30], sortase A-anchored protein [31], catabolite control protein A [32], type II histidine triad protein [33], fructose-bisphosphate aldolase, lactate dehydrogenase, oligopeptide-binding protein OppA precursor, elongation factor Tu [34], sbp2 (putative pilin subunit) [35], translation elongation factor G, phosphoglycerate mutase, phosphoglycerate kinase, pyruvate dehydrogenase E1 component alpha subunit, and chaperonin GroEL [36]. Indeed, 29 different *S. suis* serotype 2 virulence factors have been described so far as binding ECM components [8, 9], which supports bacterial redundancy [37]. This redundancy was recently demonstrated for another putative virulence factor of *S. suis* serotype 2: the deletion of a single factor H-binding protein, of which ten have been described and another six have

been proposed, is not sufficient to inhibit bacterial binding to factor H [38-41]. In fact, the simultaneous deletion of two of these genes, alongside a triple knockout for the capsular polysaccharide (also reported to bind factor H), remained insufficient to abolish binding to factor H, suggesting compensation by at least another bacterial factor, most probably one or more of these described proteins [38]. Consequently, a descriptive role for a bacterial protein alone is probably not sufficient to claim an important role in the pathogenesis of the infection, especially when other factors with redundant functions have already been described.

Adhesion to ECM components may subsequently led to interactions with host cells, which is an important step of bacterial pathogenesis [8]. It was previously demonstrated that the DPPIV of the ST7 strain and the autolysin of a ST378 strain are both implicated in adhesion to the human laryngeal epithelial cell line HEp-2 [11, 13]. Interestingly, the percentage of adhesion to epithelial cells obtained herein varied between STs, indicating a role of strain background. In fact, the ST1 and ST7 strains, which are virulent and highly virulent, respectively, adhered more than the intermediate virulent ST25 strain. Since adhesion to host cells may lead to cell invasion, differences in adhesion might influence host dissemination and virulence [6, 8]. Results obtained herein demonstrate that the DPPIV was not involved in adhesion to epithelial cells, regardless of the methodology used being similar (MOI and incubation time) and the ST being the same to that previously described [11]. However, certain differences in methodology still exist, such as the number of washes prior to cell lysis, the volume of water used to lyse the cells and, most notably, the origin of the cells used: human laryngeal epithelial cells versus porcine tracheal epithelial cells [11]. Though these two cell lines are both epithelial cells derived from the respiratory epithelium, it is impossible to ascertain that no other differences exist, such as histological differences between the trachea and larynx and the method used to immortalize the cells. Although it has been reported that adhesion to porcine and human epithelial cells by *S. suis* serotype 2 may be similar [15], the HEp-2 cells may not be an appropriate model for evaluating the role of all putative *S. suis* virulence factors since the respiratory route of infection has not been demonstrated for humans [9]. Consequently, these problems suggest that the experimental design used should be justified and the methodology standardized to ease comparison between studies and laboratories.

Meanwhile, a role of the autolysin in adhesion to host cells was confirmed when using porcine epithelial cells, and this for all three ST tested. Interestingly, it was previously reported that

absence of the autolysin resulted in adhesion of only 50% of the inoculum by the mutant strain, while 100% of the wild-type strain inoculum adhered after 3 h of incubation [13]. These results greatly differ from those obtained in this study, in which approximately only 2% of the different wild-type strain inoculums and 0.5% of the autolysin-deficient mutant inoculums adhered to the epithelial cells. In fact, the high *S. suis* adhesion levels to epithelial cells reported by Ju *et al.* have never been observed by other researchers [13]. An hypothesis explaining the results of Ju *et al.* is that the elevated initial MOI (MOI=100) and longer incubation time (3 h) may have led to bacterial replication within the wells [13]. Interestingly, despite differences in methodology and the origin of the cells, results obtained in this study arrived to the same conclusions for all three STs tested, suggesting that the role of this putative virulence factor in adhesion to epithelial cells might be universal for *S. suis* serotype 2.

Alongside adhesion to host ECM and cells, the capacity to form biofilm is an essential step of the bacterial pathogenesis involved in survival and propagation of the pathogen [8]. Interestingly, the three wild-type strains produced varying levels of biofilm, indicating a role of strain background concerning this capacity. These differences imply that choice of strain can have an important effect on the results obtained when evaluating certain characteristics or functions of *S. suis.* Moreover, the DPPIV was determined not to be involved in biofilm formation, regardless of the ST of the strain used. Though no role in biofilm formation had been attributed to this *S. suis* protein in the past, the lack of evaluation could have suggested otherwise, as exemplified by *Porphyromonas gingivalis*, for which the DPPIV is clearly involved in biofilm formation [42]. On the other hand, the autolysin was implicated in biofilm formation for the ST7 and ST25 strains, but not the ST1 strain. While the autolysin was previously reported to be implicated in biofilm formation by an ST378 strain, its absence resulted in a 25% decrease of production for the latter [13], while a decrease corresponding to nearly 70% of the biofilm formed by the wild-type ST7 and ST25 strains was observed herein. These results indicate a strain-dependent role of the *S. suis* serotype 2 autolysin with regards to this bacterial function. Consequently, these results demonstrate the impact of strain background and the bias introduced by this choice when evaluating virulence factors. This is important given that most studies regarding the evaluation of *S. suis* serotype 2 virulence factors have used ST1 or ST7 strains only.

When evaluating the implication of bacterial virulence factors, the ultimate demonstration remains the use of *in vivo* infection models. However, there exists a vast variety of *S. suis* serotype 2 animal infection models, which has complicated comparison of results. Of these the mouse is one of the most popular, with the inbred C57BL/6 breed being commonly used [3, 4, 17, 43]. Firstly, results obtained herein confirm previous studies in which the ST1 and ST7 strains were reported to both be virulent, with the ST7 strain inducing mortality more rapidly than the ST1 strain [3, 4]. Moreover, the wild-type ST25 strain caused less mortality and in a delayed time due to an important number of cases of meningitis, as previously reported [3, 4]. Unlike previously reported for a ST7 strain, results obtained herein demonstrate that the DPPIV is not implicated in virulence and host death, regardless of the ST of the strain used [11]. It is worth mentioning that unlike the C57BL/6 mice used in this study, Ge *et al*. only specify using specific pathogen free-mice [11]. It must be presumed that these mice are BALB/c since this is the breed used for the immunization experiments conducted within the same publication [11]. C57BL/6 mice, which were used in the present study, are reliable for *S. suis* studies as they exhibit a prototypical Th1 immune response and a strong pro-inflammatory response [17, 44, 45]. On the other hand, BALB/c mice are the prototypical Th2 mouse breed [44]. As such, the innate immune response differs between these two breeds: C57BL/6 mice produce higher levels of the pro-inflammatory cytokine tumor necrosis factor (TNF) and the Th1 cytokine interleukin (IL)-12p70, in comparison to BALB/c mice [44, 46]. Moreover, macrophages isolated from C57BL/6 mice produce effector molecules required for bacterial killing, including nitric oxide, whereas those from BALB/c do not, resulting in impaired bactericidal activity of the latter [44].

In addition, the route of infection may also differentially affect the conclusions. Herein, bacteria were inoculated via the intraperitoneal route (IP), while the route of inoculation used by Ge *et al*. although not clearly stated, was probably intravenous (IV) [11]. Though bacteria will reach the bloodstream following IP inoculation via lymphatic drainage, the initial cell types activated will differ: IP inoculation results in activation of peritoneal macrophages while IV injection leads to immediate stimulation of blood leukocytes [47]. Indeed, it was previously reported that the route of infection had an effect on disease development following Group B *Streptococcus* infection [48, 49]. To our knowledge, the IV route of inoculation for *S. suis* in mice has been used in only a limited number of studies [50-52], while most mouse studies have used the IP route of infection for *S. suis*, as reviewed by Segura *et al*. [9].

Meanwhile, the role of the autolysin of a ST378 strain in virulence was previously evaluated using the zebrafish model of infection [13], in which the autolysin-deficient mutant presented attenuated virulence. However, using the C57BL/6 mouse model of infection, results from the present study indicate that the autolysin does not critically contribute to virulence and does not participate in host death, independently of the ST of the strain tested. An important difference between these studies, alongside the ST of the strains used, is the experimental design and the use of animal model. Though zebrafish possess innate and adaptive immune responses [53], the genetic differences with pigs and humans are greater than those between mice and pigs or humans [54]. Although ethical regulations facilitate the use of zebrafish over mice, the former are cold-blooded, are a model in which it is more difficult to conduct central nervous system studies (meningitis being the most important pathology caused by *S. suis* serotype 2),and are limited to lethal dose 50 studies [9]. Consequently, results obtained with zebrafish are difficult to extrapolate, which may limit their use in determining *S. suis* virulence factors.

Surprisingly, autolysin-deficiency resulted in increased virulence of the ST25 strain. This was unexpected since autolysin-deficient mutants of other pathogenic Gram-positive bacteria, including for LytA of *Streptococcus pneumoniae* [55] and AtlE of *Staphylococcus epidermidis* [56], were less virulent than their respective wild-type strains. Indeed, the *S. pneumoniae* autolysin, involved in cell wall remodeling, is responsible for the release of the pneumolysin, an important virulence factor of this pathogen [57]. Consequently, in the absence of the autolysin, it is possible that cell wall remodeling and protein secretion could be altered or halted, resulting in alteration of the ST25 bacterial strain surface architecture. It is well known that certain surface proteins of *S. suis* are important activators of the host cells [6, 8], and these may, by remaining attached to the bacteria or by being differentially expressed, contribute to inflammation and host death. Moreover, absence of the autolysin resulted in increased survival of the ST25 strain in blood, suggesting that this protein could play additional functions alongside those previously described for *S. suis* [13] as well as for other pathogenic streptococci [57]. Indeed, the *Staphylococcus saprophyticus* autolysin, Aas, was shown to bind sheep erythrocytes [58], although this function has not been described for *S. suis* so far. Further investigations will be required in order to better understand these differences in virulence and the possibly unique roles of this protein in ST25 strains.

Conclusions

This study reiterates the urgent need in arriving to a consensus regarding the definition of *S. suis* serotype 2 virulence factors. Inconsistencies abound in the literature due to differences obtained between laboratories, and these have created a controversy that has only just been highlighted. The main source of these differences are variations and discrepancies in experimental design, including *in vitro* assays, cell lines, and animal models, which greatly affect the results, as demonstrated in this study for both the DPPIV and autolysin. Moreover, the use herein of different strain backgrounds has demonstrated that differences in bacterial characteristics and functions, alongside the role attributed to a virulence factor, may vary according to the *S. suis* serotype 2 strain. Consequently, it will be important to establish standard experimental designs, including methodology and appropriate cell lines and animal models, according to the experiment and purpose in order to facilitate comparison between laboratories. Alongside, studies should include strains of diverse origins in order to prevent erroneous and biased conclusions that could affect future studies. Finally, the use of alternative animal models cannot definitively exclude the role of a given *S. suis* virulence factor that may significantly contribute to disease during a natural infection in pigs. For example, the DPPIV has been reported to contribute to tissue degradation and perturbation of the host defense system [59], roles that although not critical in themselves, could significantly contribute to the final outcome of the natural infection by *S. suis*.

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Supporting Information (Available at the PLoS One Website)

S1 Appendix. Evaluation of clinical signs and scoring following intraperitoneal injection of *Streptococcus suis* serotype 2 in mice.

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Tables

Table 1. Strains and plasmids used in this study.

Strains/plasmids	General characteristics	Reference
Streptococcus suis		
P1/7	Wild-type, virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	[18]
P1/7Δ*dppIV*	Isogenic mutant derived from P1/7; in frame deletion of the *dppIV* gene	This study
P1/7Δ*atl*	Isogenic mutant derived from P1/7; in frame deletion of the *atl* gene	This study
SC84	Wild-type, highly virulent ST7 strain isolated from a case of human streptococcal toxic shock-like syndrome during the 2005 outbreak in China	[19]
SC84Δ*dppIV*	Isogenic mutant derived from SC84; in frame deletion of the *dppIV* gene	This study
SC84Δ*atl*	Isogenic mutant derived from SC84; in frame deletion of the *atl* gene	This study
89-1591	Wild-type, intermediate virulent North American ST25 strain isolated from a case of pig sepsis in Canada	[20]
89-1591Δ*dppIV*	Isogenic mutant derived from 89-1591; in frame deletion of the *dppIV* gene	This study
89-1591Δ*atl*	Isogenic mutant derived from 89-1591; in frame deletion of the *atl* gene	This study
Escherichia coli		
TOP 10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
Plasmids		
pCR2.1	Ap^r, Km^r, oriR(f1) MCS oriR (ColE1)	Invitrogen
pSET-4s	Thermosensitive vector for allelic replacement in *S. suis*. Replication functions of pG + host3, MCS oriR pUC19 lacZ Sp^R	[21]
p4Δ*dppIV*	pSET-4s carrying the construct for *dppIV* allelic replacement	This work
p4Δ*atl*	pSET-4s carrying the construct for *atl* allelic replacement	This work

Table 2. Oligonucleotide primers used for the construction of the *S. suis* dipeptidyl peptidase IV (*dppIV*) and autolysin (*atl*) mutants used in this study.

Name	Primer Sequence (5'-3')
ST1 & ST7Δ*dppIV*_1	GATCCAGCTCCAACTCCAATTC
ST1 & ST7Δ*dppIV*_2	TTGGGATCATGCACCACACC
ST1 & ST7Δ*dppIV*_3	CCCCCGGGGAAGTTCCGGCACCAATTCCAG
ST1 & ST7Δ*dppIV*_4	TCCGTCTACTTGCAAAATTCTCAATGGCAAATCCAC CTTG
ST1 & ST7Δ*dppIV*_5	TTGCCATTGAGAATTTTGCAAGTAGACGGAGGTC
ST1 & ST7Δ*dppIV*_6	CGGGATCCGTTCGGAACATACCAAAGGG
ST25Δ*dppIV*_1	CAATAAGAAGCCCAGCAAGAG
ST25Δ*dppIV*_2	GTTGCAAGTACCCTCATTTCC
ST25Δ*dppIV*_3	TCGCTTCCTTAAGCTGGTC
ST25Δ*dppIV*_4	TCCGTCTACTTGCAAAATTCTCAATGGCAAATC CACCTTG
ST25Δ*dppIV*_5	TTGCCATTGAGAATTTTGCAAGTAGACGGAGGTC
ST25Δ*dppIV*_6	GCCACTTGGTCAGACAAAG
ST1 & ST7Δ*atl*_1	CCAGTTGTAGCAGCAGAG
ST1 & ST7Δ*atl*_2	ACCAGCATGAAAAGAACAGATG
ST1 & ST7Δ*atl*_3	CATTTAACTGATGATGAAAAAG
ST1 & ST7Δ*atl*_4	ATACCAATTCATTACACCTTGCTCCTTTATGTATTTCACATGTAA
ST1 & ST7Δ*atl*_5	TTACATGTGAAATACATAAAGGAGCAAGGTGTAATGAATTGGTAT
ST1 & ST7Δ*atl*_6	GTACTTACAAAGAGCCAACAG
ST25Δ*atl*_1	GGAAGTGCTACACTACCGTC
ST25Δ*atl*_2	GACCAGCATGAAAAGAAC
ST25Δ*atl*_3	CGGAGCTGTTCCAGTT
ST25Δ*atl*_4	CAAGGCGAGTGTGGTACTCCTTTATGTATTTCACATGTAA
ST25Δ*atl*_5	TTACATGTGAAATACATAAAGGAGTACCACACTCGCCTTG
ST25Δ*atl*_6	GCAGATTTAATTACTTTCTTTAGC

Table 3. Comparison of the experimental design used in the previous and present studies to evaluate the role of the dipeptidyl peptidase IV and autolysin as virulence factors for *S. suis* serotype 2

Experimental design	Previous study – Dipeptidyl peptidase IV [11]	Previous study – Autolysin [13]	This study
Sequence type (strain)	ST7 (05ZYH33)	ST378 (HA9801)	ST1 (P1/7) ST7 (SC84) ST25 (89-1591)
Adhesion to human fibronectin	Recombinant protein ELISA assay	Recombinant protein Western blot	Whole bacteria ELISA assay
Adhesion to epithelial cells	HEp-2 (Human laryngeal cells) MOI=10 1 h or 2 h of incubation? % adhered inoculum	HEp-2 (Human laryngeal cells) MOI=100 3 h of incubation % adhered inoculum? Adhesion of wild-type strain=100%?	NPTr (Porcine tracheal cells) MOI=10 2 h of incubation % adhered inoculum
Biofilm formation	Not evaluated	Microtiter plate assay after 24 h of incubation	Microtiter plate assay after 24 h of incubation
Virulence	Mouse Unknown (SPF), possibly BALB/c? Subcutaneous or intravenous? $1x10^8$ CFU/mouse?	Zebrafish Intraperitoneal $2x10^3$ to $2x10^7$ CFU/fish	Mouse C57BL/6 Intraperitoneal $5x10^7$ CFU/mouse

Figures

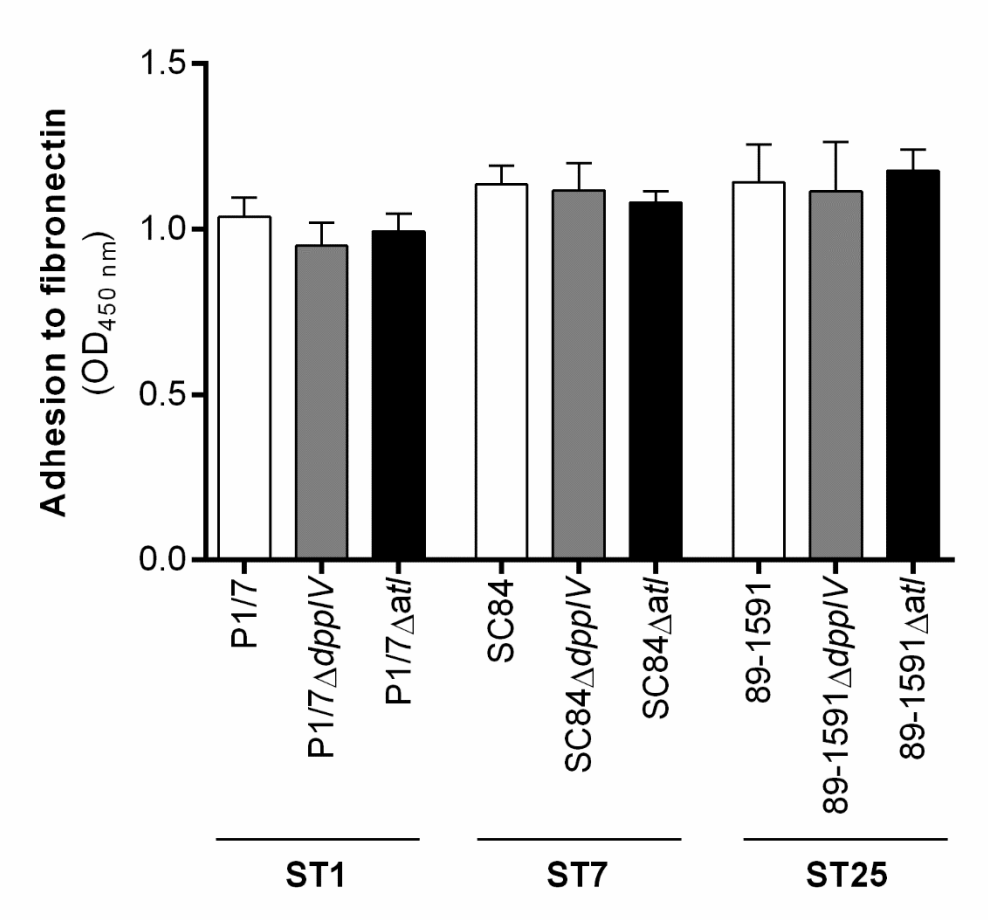

Figure 1. The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin are not involved in adhesion to fibronectin, regardless of the sequence type (ST) of the strain used. Adhesion of different wild-type strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants to human plasma fibronectin (10 µg/mL), as determined by ELISA after 2 h of incubation. The optical density (OD) was measured at 450 nm and values corrected using the appropriate controls. Results are expressed as mean ± SEM obtained from three independent experiments.

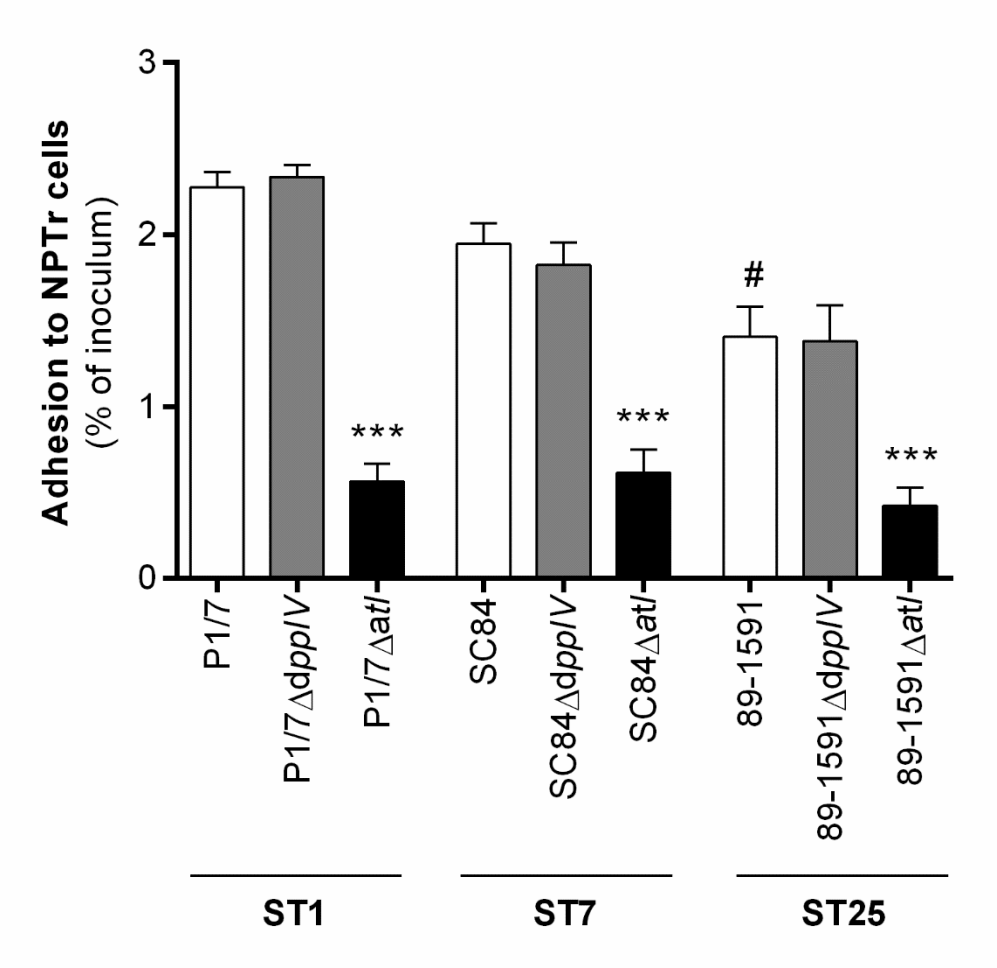

Figure 2. The *S. suis* serotype 2 dipeptidyl peptidase IV is not involved in adhesion to porcine tracheal epithelial cells, regardless of the sequence type (ST) of the strain used, unlike the autolysin. Adhesion of different wild-type strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants to porcine epithelial cells was evaluated after 2 h of incubation with bacteria (MOI=10). Results are expressed as mean ± SEM obtained from three independent experiments and represent the percentage of adhered inoculum. # indicates a significant difference ($p < 0.01$) between the wild-type ST1 strain P1/7 and ST25 strain 89-1591; *** ($p < 0.001$) between the wild-type strain and its Atl-deficient mutant.

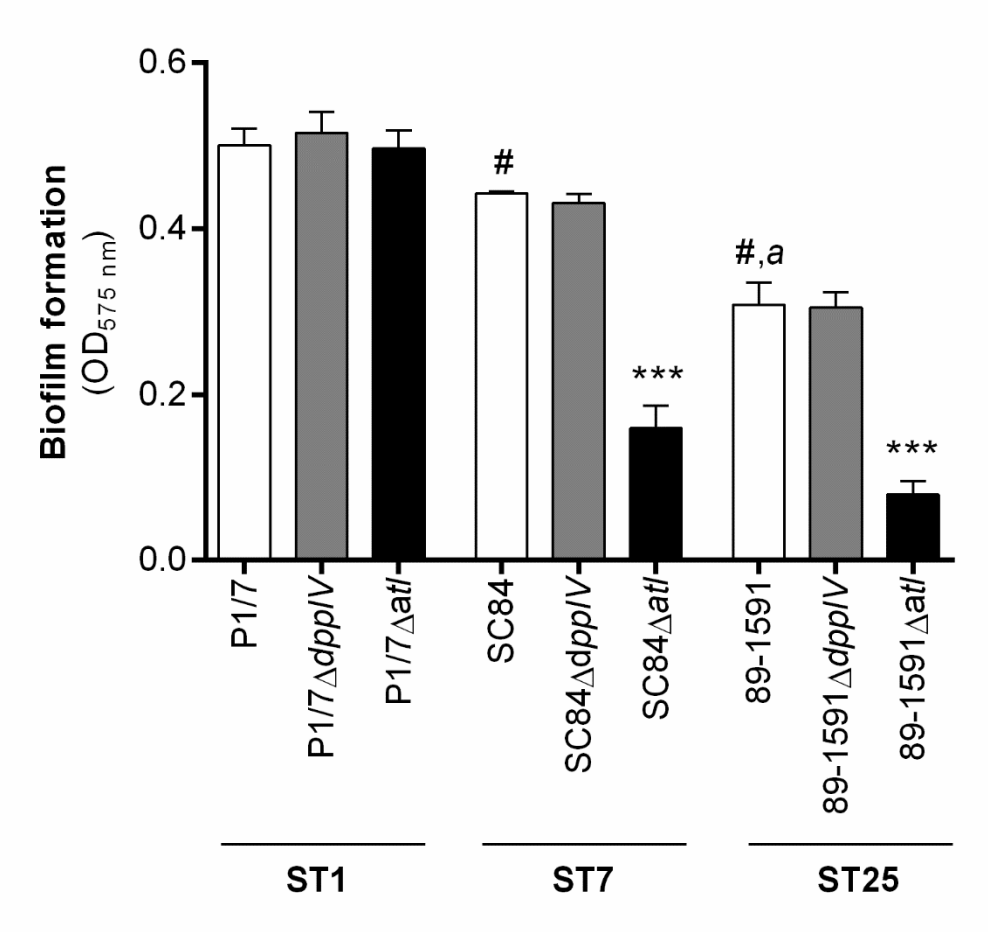

Figure 3. The dipeptidyl peptidase IV is not involved in *S. suis* serotype 2 biofilm formation, while implication of the autolysin is dependent on the sequence type (ST) of the strain used. Biofilm formation of different wild-type strains and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants in the presence of 2 mg/mL of porcine fibrinogen was evaluated after 24 h of incubation. The optical density (OD) was measured at 575 nm and values corrected using the appropriate controls. Results are expressed as mean ± SEM obtained from three independent experiments. # indicates a significant difference ($p < 0.05$) between the wild-type ST1 strain P1/7 and ST7 strain SC84 or ST25 strain 89-1591; *a* ($p < 0.01$) between the wild-type ST7 strain SC84 and ST25 strain 89-1591; *** ($p < 0.001$) between the wild-type strain and its Atl-deficient mutant.

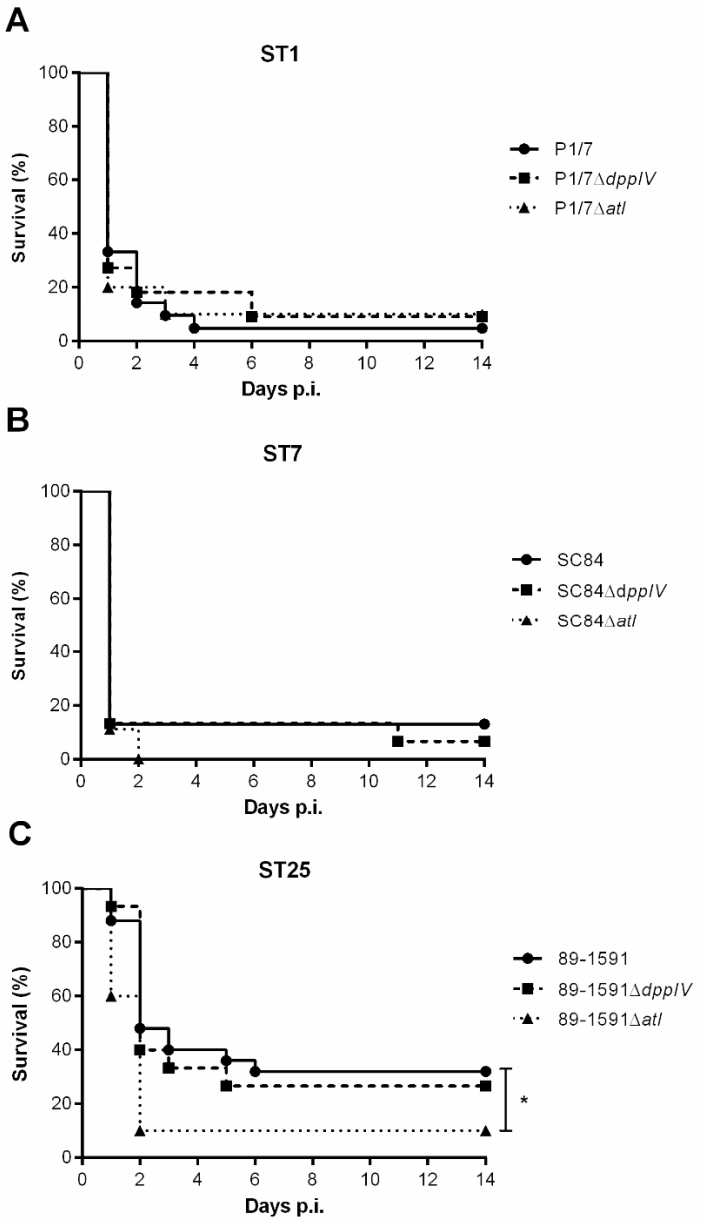

Figure 4. The *S. suis* serotype 2 dipeptidyl peptidase IV and autolysin are not implicated in host virulence in a C57BL/6 mouse model of infection, regardless of the sequence type (ST) of the strain, with the exception of a minor role for the autolysin of the ST25 strain. Survival of C57BL/6 mice infected with $5x10^7$ CFU of different wild-type and dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants by intraperitoneal inoculation. (A) P1/7 (ST1) and its mutants, (B) SC84 (ST7) and its mutants, and (C) 89-1591 (ST25) and its mutants. * indicates a significant difference ($p < 0.05$) between the wild-type ST25 strain 89-1591 and its Atl-deficient mutant.

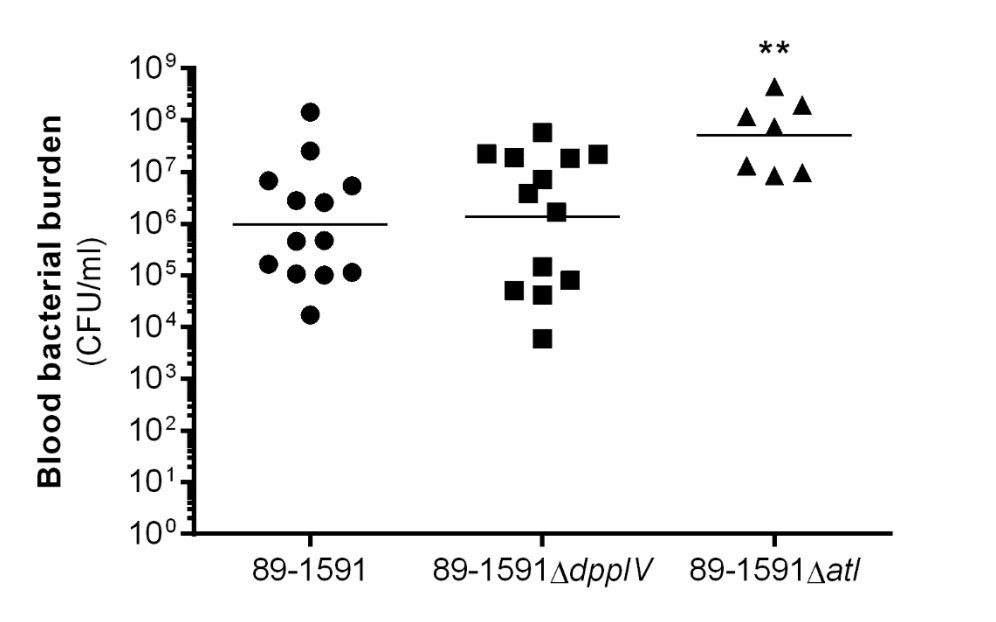

Figure 5. The autolysin of the ST25 strain, but not the dipeptidyl peptidase IV, hinders bacterial survival in the blood. Blood bacterial burden of surviving C57BL/6 mice 24 h following intraperitoneal inoculation of $5x10^7$ CFU of the ST25 strain 89-1591 and its dipeptidyl peptidase IV (DPPIV)- or autolysin (Atl)-deficient mutants. Results are expressed as geometric mean. ** indicates a significant difference ($p < 0.01$) between the wild-type ST25 strain 89-1591 and its Atl-deficient mutant.

ARTICLE VIII

Virulence studies of different sequence types and geographical origins of *Streptococcus suis* serotype 2 in a mouse model of infection

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé activement à la conception de l'étude et des approches méthodologiques. J'ai effectué toutes les expériences *in vitro* et *in vivo*, en plus de l'analyse des résultats et de l'écriture de la première version du manuscrit. J'ai aussi été impliqué dans la révision du manuscrit et j'ai aussi effectué les corrections lors des étapes de révision pour publication.

Mise en context

Dans le cadre de l'article VII, nous avons démontré que l'origine des souches utilisées et les modèles expérimentaux employés peuvent avoir un impact important sur les études de virulence. Toutefois, l'impact de l'origine des souches sur la pathogenèse de l'infection de *S. suis* restait peu connu. Étant une bactérie hautement hétérogène, autant génétiquement que phénotypiquement, un rôle de l'origine de la souche pourrait grandement affecter les résultats obtenus et les conclusions tirées, particulièrement comme ces conclusions sont régulièrement extrapolées à l'ensemble de la population de *S. suis.* Le sérotype 2 étant le plus fréquemment responsable d'infection chez le porc et chez l'humain, nous nous sommes intéressés aux STs les plus importants du sérotype 2, soit les ST1, ST7, ST25 et ST28. De plus, comme l'origine géographique (particulièrement l'Amérique du Nord vs. l'Eurasie) a longtemps été considérée comme un facteur déterminant, nous avons aussi voulu déterminer son impact sur la virulence des souches étudiées et sur la pathogenèse de l'infection causée par *S. suis.*

Abstract

Multilocus sequence typing previously identified three predominant sequence types (STs) of *Streptococcus suis* serotype 2: ST1 strains predominate in Eurasia while North American (NA) strains are generally ST25 and ST28. However, ST25/ST28 and ST1 strains have also been isolated in Asia and NA, respectively. Using a well-standardized mouse model of infection, the virulence of strains belonging to different STs and different geographical origins was evaluated. Results demonstrated that although a certain tendency may be observed, *S. suis* serotype 2 virulence is difficult to predict based on ST and geographical origin alone; strains belonging to the same ST presented important differences of virulence and did not always correlate with origin. The only exception appears to be NA ST28 strains, which were generally less virulent in both systemic and central nervous system (CNS) infection models. Persistent and high levels of bacteremia accompanied by elevated CNS inflammation are required to cause meningitis. Although widely used, in vitro tests such as phagocytosis and killing assays require further standardization in order to be used as predictive tests for evaluating virulence of strains. The use of strains other than archetypal strains has increased our knowledge and understanding of the *S. suis* serotype 2 population dynamics.

Introduction

Streptococcus suis is an important porcine bacterial pathogen and emerging zoonotic agent responsible for sepsis/septic shock and meningitis during systemic and central nervous system (CNS) infections, respectively [1]. Other infections have also been reported in both pigs and humans [2]. Of the different described serotypes based on the presence of the capsular polysaccharide or its respective genes, serotype 2 is regarded as not only the most widespread worldwide, but also the most virulent, responsible for the majority of both *S. suis* porcine and human cases of infection [2, 3]. However, the global distribution of this serotype is unequal, with cases due to this serotype being much less common in North America (NA) than in Europe and Asia (Eurasia; EA) [2]. Consequently, it has been suggested that virulence amongst *S. suis* serotype 2 strains may vary according to the geographical origin and virulence factors present, subjects that remain widely debated [1, 4].

Over the years, different approaches have been used to characterize *S. suis* serotype 2 strains, including the presence or absence of different putative virulence factors or markers [5-7]. Alongside, various DNA typing techniques, including random amplification of polymorphic DNA, amplified fragment length polymorphism, restriction fragment length polymorphism, comparative genome hybridization, and pulsed field gel electrophoresis have also been used [8-12]. Of the DNA typing techniques available, multilocus sequence typing (MLST) has emerged as one of the leading methods used for *S. suis* classification, being a reliable, generic, and reproducible technique [13-15]. More recently, a number of studies have used MLST as an approach to determining virulence profiles of *S. suis* serotype 2 strains from different sequence types (STs). The clonal ST7 strain responsible for two human outbreaks in China was demonstrated to be much more virulent than archetypal European ST1 strains using mouse models of infection [16-18]. Meanwhile, archetypal NA ST25 and ST28 strains were shown to be less virulent than the abovementioned ST1 and ST7 strains [16, 19]. Nevertheless, ST25 strains presented higher virulence than their ST28 counterparts [19]. Yet, the actual *S. suis* serotype 2 situation remains misunderstood: around 5% of NA strains (including a strain isolated from a human case) are ST1 [20], an ST typically recovered in EA [2], while strains recovered from certain human cases in Thailand and Japan have been characterized as ST25 or ST28 (commonly isolated in NA), respectively [21, 22]. Interestingly, and only adding to this complexity, whole genome sequencing recently revealed that ST25 and ST28 strains do not constitute a highly homogenous group, as was previously believed, since NA and Asian strains

clustered separately within each ST [23, 24]. Consequently, it remains unclear whether strains of the same ST have similar virulence potentials.

Experimental infections with the natural host, the pig, are possible, though difficult to achieve with a high number of strains [4]. In addition, almost all animals are carriers of *S. suis* in their tonsils, which may influence their response to the infection [4, 25]. To overcome this variation, well-established inbred mouse models of *S. suis* infection have been developed, as exemplified by C57BL/6 mice, which reproduce similar clinical signs to those observed during the systemic (septic shock) and CNS (meningitis) infections in the natural host [16, 26-28]. So far, no study has compared strains belonging to the same ST but from different origins in detail. Therefore, the aim of this study was to evaluate and compare the virulence potential of strains belonging to three important STs (ST1, ST25, and ST28) from different geographical (Americas and EA) and host (porcine and human) origins during both the systemic and CNS infections using the well-established C57BL/6 mouse model of infection.

Materials and Methods

Bacterial strains and growth conditions

The different well-encapsulated *S. suis* serotype 2 strains used in this study are listed in Table 3. Serotyping was performed with the coagglutination test using serotype 1 and 2 reference antisera, as previously described, to confirm that the strains used were serotype 2 and not serotype 1/2 [56]. Strains giving a strong positive reaction within 30 s to serotype 2 only were used, as a clear indication of presence of serotype-specific capsular polysaccharide. In addition, and for comparison purposes, an isogenic non-encapsulated mutant derived from strain P1/7, Δ*cpsF* [57], was also included during in vitro experiments. Bacteria were grown overnight on Columbia agar supplemented with 5% sheep blood (Oxoid, Nepean, ON, Canada) at 37 °C with 5% CO_2. Five milliliters of THB (Becton Dickinson, Mississauga, ON, Canada) were inoculated with isolated colonies and incubated for 8 h at 37 °C with 5% CO_2. Working cultures were prepared by inoculating 30 mL of THB with 10 µL of a 10^{-3} dilution of the 8 h cultures and incubating for 16 h at 37 °C with 5% CO_2. Bacteria were washed twice with pH 7.3 phosphate-buffered saline (PBS) and resuspended in a cell culture medium (phagocytosis and bactericidal assays) or THB (experimental mouse infections), appropriately diluted, and plated on Todd Hewitt broth agar (THA) to accurately determine bacterial concentrations using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA, USA)

S. suis *MLST*

Genomic DNA of the *S. suis* serotype 2 strain SR800 was prepared and MLST performed as previously described by sequencing the *cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS* housekeeping genes [19]. This strain was determined to be ST1 using the *S. suis* Multilocus Sequence Typing Database (http://ssuis.mlst.net). The STs of the other strains have been previously published [19, 62, 63].

S. suis s*erotype 2 phagocytosis assay*

Phagocytosis assays were performed as previously described [64] with some modifications. J774A.1 murine macrophages (ATCC TIB-67; Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 0.25% 10,000 U penicillin/streptomycin (Gibco), and cells grown at 37°C with 5% CO_2. Confluent cell cultures were scraped, washed twice with PBS, suspended in antibiotic-free medium at 1×10^5 cells/mL, and incubated for 3 h at 37 °C with 5% CO_2 to allow cell adhesion. Cells were infected by adding 1×10^7 CFU/mL of bacterial suspension in complete culture medium containing 10% of fresh mouse serum, without antibiotics (multiplicity of infection; MOI = 100). The infected cells were incubated for 1 h at 37 °C with 5% CO_2 to allow optimal phagocytosis, determined during preliminary studies (data not shown). After incubation, cell monolayers were washed twice with PBS and incubated with a medium containing 5 µg/mL penicillin G (Sigma-Aldrich, Oakville, ON, Canada) and 100 µg/mL of gentamicin (Gibco) for 1 h to kill extracellular bacteria. Supernatant controls were taken during every test to confirm the activity of the antibiotics. After antibiotic treatment, cell monolayers were washed three times with PBS, lysed with water and vigorous pipetting, and viable intracellular bacteria determined by plating appropriate dilutions as described above. Each test was repeated three times in independent experiments and the number of CFU/mL was determined. Fresh mouse serum was obtained from six- to 10-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA).

S. suis *serotype 2 bactericidal assay*

Blood was collected from six- to 10-week-old female C57BL/6 mice (Charles River Laboratories) and mixed with sodium heparin (Sigma-Aldrich). Blood was adjusted with RPMI-1640 (Gibco) to obtain 6×10^6 leukocytes/mL. Leukocytes (1×10^6 cells) were transferred to a microtube containing 1×10^6 CFU of the different *S. suis* strains (MOI = 1) and incubated for

4 h, mixing every 20 min. Assay conditions were chosen based on preliminary assays of the kinetics of *S. suis* killing by murine blood (data not shown). After incubation, cells were lysed with water and vigorous pipetting, and appropriate dilutions plated on THA to determine viable bacterial counts as described above. Resistance to bacterial killing by blood leukocytes was compared to incubation of the different bacterial strains in plasma only (similarly diluted in RPMI-1640). Each test was repeated at least three times in independent experiments and the percentage of bacteria killed determined using the following formula: 1 − (bacteria in blood/bacteria in plasma) × 100%.

S. suis *serotype 2 experimental mouse infections*

Throughout this study, six-week-old C57BL/6 mice (Charles River Laboratories) were used. A total of 650 mice were required to complete this study. To further reduce host variability, only female mice were used. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [27]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee (protocol number rech-1570) guidelines and policies, including euthanasia to minimize animal suffering, which was applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. Antibiotic treatment, previously reported as not being required in the *S. suis* mouse model of infection [26, 27], was not administered regardless of the route of inoculation and phase of infection. For systemic virulence studies, 5×10^7 CFU of the different *S. suis* serotype 2 strains, or the vehicle solution (sterile THB), were administered by intraperitoneal inoculation to groups of 15 mice. Mice were monitored at least three times daily until 72 h p.i. Mortality recorded at this time point was considered to be due to systemic infection. For the hematogenous meningitis model, animals were infected with 5×10^7 CFU or with 2×10^7 CFU for strains presenting higher virulence; all animals were monitored at least twice daily until the end of the study (14 days p.i.) for clinical signs and mortality. For the transcutaneal intracisternal model of meningitis, groups of 10 mice were anesthetized with inhaled isofluorane (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) and 20 µL of the different *S. suis* serotype 2 strains (final concentration of 2×10^5 CFU) were injected as previously described [30]. Animals were allowed to wake up, monitored every 6 h, and euthanized upon presentation of clinical signs of meningitis or at the end of the study (72 h p.i.). Controls were injected with 20 µL of the vehicle solution (sterile THB). A timeline

presenting the intraperitoneal (Figure 1A) and transcutaneal intracisternal (Figure 1B) models of inoculation used in this study is included.

Measurement of mouse blood bacterial burden

Blood bacterial titers were assessed in surviving mice 24 h, 36 h, 48 h, and five days p.i. following intraperitoneal inoculation, and 12 h and 24 h p.i. following intracisternal inoculation, by collecting 5 µL of blood from the tail vein. Proper dilutions were plated as described above.

Measurement of mouse plasma (systemic) cytokine and chemokine levels

A total of eight mice per strain were infected as described above and blood was collected from surviving mice euthanized 12 h p.i. by intracardiac puncture and stabilized with EDTA (Sigma-Aldrich) as previously described [16]. Plasma supernatants were collected following centrifugation at 10,000× *g* for 10 min, 4 °C, and stored at −80 °C. Plasmatic concentrations of TNF-α, IL-6, IL-12p70, IFN-γ, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CXCL1 (KC) were determined using a custom-made cytokine Milliplex panel (Millipore) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the MILLIPLEX Analyst 5.1 software (Upstate Group/Millipore, Etobicoke, ON, Canada).

Mouse brain histopathological studies

Upon presentation of clinical signs of meningitis or at the end of the study, mice were euthanized and brains recovered and fixed in 10% buffered formalin. After paraffin embedding, 4 µm-thick sections of the brain were stained with hematoxylin phloxine saffron (HPS) according to standard protocol and examined under light microscopy.

Measurement of mouse brain bacterial burden

Following intraperitoneal inoculation and upon presentation of clinical signs of meningitis or at the end of the study, mice were euthanized and brains aseptically recovered. This was also completed 12 h and 24 h p.i. or at the end of the study following intracisternal inoculation in groups of four to six mice. Brains were homogenized in PBS and bacterial burdens determined by plating appropriate dilutions on THA as described above.

Evaluation of systemic and brain compartmentalization and measurement of mouse brain cytokine and chemokine levels

Systemic and brain compartmentalization was evaluated following intraperitoneal inoculation of strain P1/7. Upon presentation of clinical signs of either septic shock or meningitis, mice were euthanized and plasma recovered as described above. Brains were recovered and frozen in liquid nitrogen. Extraction buffer, prepared using cOmplete Mini, EDTA-free, protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions and by adding 0.4% (w/v) CHAPS (Sigma-Aldrich), was added to the brains, which were homogenized using a POLYTRON PT 1200E system bundle (Kinematica, Lucerne, Switzerland). Brain homogenate supernatants were collected following centrifugation at 10,000× *g* for 10 min, 4 °C, and stored at −80 °C. Levels of IL-1β, IL-6, CCL2 (MCP-1), CCL3 (MIP-1α), CXCL1 (KC), and CXCL10 (IP-10) were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using pair-matched antibodies (R&D Systems, Minneapolis, MN), as previously described [57] in both plasma and brain homogenates. Once compartmentalization was determined, brain levels of these same mediators were evaluated for the other strains following intraperitoneal (upon presentation of clinical signs of meningitis or at the end of the study) and intracisternal (24 h p.i.) inoculation (n = 3 or 4 mice).

Statistical analyses

The normality of data was verified using the Shapiro–Wilk test. Accordingly, parametric (unpaired *t*-test) or non-parametric tests (Mann–Whitney rank sum test and one-way ANOVA on ranks), where appropriate, were performed to find statistical differences between groups. The log-rank (Mantel–Cox) and chi-square tests were used to compare survival and meningitis rates of studied groups, and to determine associations between blood bacterial burden and presence or absence of clinical signs of meningitis, respectively ($p < 0.05$ was considered statistically significant).

Results

Lack of correlation between In Vitro *resistance to phagocytosis and to bacterial killing by different* S. suis *ST strains*

In order to determine the capacity of different *S. suis* serotype 2 strains to resist phagocytosis by murine macrophages, bacteria were incubated with cells and 10% mouse serum for 1 h [29].

As presented in Table 1, the NA and EA ST28 strains were significantly more resistant to phagocytosis than both the ST1 ($p < 0.05$) and ST25 ($p < 0.001$) strains. Differences were not observed in phagocytosis rates between ST1 and ST25 strains nor between NA and EA ST28 strains. As expected, a significant difference was also observed between P1/7 and its non-encapsulated mutant strain, Δ*cpsF*, used as a positive control ($p < 0.001$).

To better simulate the action of the different host phagocytic cells and blood components that bacteria must confront, the bactericidal effect of blood on the different *S. suis* serotype 2 strains was evaluated. As shown in Table 1, and in contradiction to what was observed with phagocytosis, the ST1 strains were completely resistant to bacterial killing by blood leukocytes. This resistance was significantly different ($p < 0.001$) from that of all other STs. Meanwhile, with one exception in each group, the ST25 and EA ST28 strains presented an intermediate level of resistance to the bactericidal effect of blood. On the other hand, the NA ST28 strains were the least resistant to the bactericidal effect, with 30% of bacteria killed on average. In fact, these strains were the only ones to actually be killed in blood, since the number of bacteria recovered in the presence of leukocytes after incubation was lower than that present at the beginning of the incubation time (data not shown). As expected, the high percentage of bacterial killing (64%) of the non-encapsulated mutant strain, Δ*cpsF*, significantly differed from its wild-type strain, P1/7 ($p < 0.001$).

Systemic infection: An important variation of virulence among strains belonging to the same STs

A timeline summary of the infections carried out in this study was included (Figure 1). First, the survival of mice intraperitoneally inoculated with the different *S. suis* serotype 2 strains was evaluated until 72 h post-infection (p.i.), during which mortality is the result of sepsis and/or septic shock (Table 1). Interestingly, the in vitro assays used could not (phagocytosis) or only partially (killing) predicted virulence. When taken together, EA ST28, ST1, ST25, and NA ST28 strains caused 50%, 35%, 18%, and 2% of mortality on average, respectively. Indeed, mice infected with the NA ST28 strains presented the lowest mortality, which was significantly different from that of the ST1-, ST25-, and EA ST28-infected mice ($p < 0.05$). In addition, the mortality of ST25-infected mice was significantly lower than that of both ST1- and EA ST28-infected mice ($p < 0.05$), between which there was no difference. No significant difference in mortality recorded at 72 h p.i. was observed between strains of porcine or human origin. However, a

significant difference was observed when strains from different geographical origins were compared, Americas versus EA, with 15% and 33% mortality ($p < 0.001$), respectively. Regardless of statistical differences between STs, important variations amongst strains of the same ST were observed. For example, ST1 strains presented mortality rates that ranged between 0% and 80%, ST25 strains between 0% and 47%, and ST28 strains between 0% and 73%.

Bacteremia during the acute phase of systemic infection: Blood bacterial burden is associated with mortality recorded at 72 h p.i. for most strains

Mortality during the systemic infection caused by *S. suis* may be the consequence of high blood bacterial titers. Consequently, the blood bacterial burden of infected mice was measured at 24 h, 36 h, and 48 h p.i. (Figure 1). At all time points, significant differences were observed between titers of ST1-, ST25-, or EA ST28-infected mice when compared to those infected with NA ST28 strains ($p < 0.001$) (Figure 2). Indeed, it was possible to associate bacteremia with mortality 72 h p.i. for most strains. However, this was not universal, since blood bacterial titers were intermediate or high for Bassett, SR800, and LPH4 strains, while the mortality caused by these strains was low.

Inflammation during the acute phase of systemic infection: Plasma pro-inflammatory mediator levels are associated with mortality recorded at 72 h p.i. for most strains

An exacerbated systemic inflammatory response may be responsible for host death due to *S. suis*-induced septic shock. Consequently, the plasma cytokine and chemokine levels of mice infected with the different *S. suis* serotype 2 strains were determined by Luminex® at 12 h p.i. (Figure 1). This incubation time, chosen based on previous studies with ST1 strains, corresponds to the peak production of these inflammatory mediators in mice following infection with *S. suis* [26, 27]. ST1 and ST25 strains induced a significantly higher production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12p70, and interferon (IFN)-γ than NA ST28 (Figure 3) ($p < 0.01$). Significant differences were also observed between ST1- and EA ST28-infected mice for IL-6 and IFN-γ ($p < 0.05$), between ST25- and EA ST28-infected mice for IFN-γ ($p < 0.001$), and between NA and EA ST28 strains for IL-6 ($p < 0.001$) only. Interestingly, no differences were observed between ST1- and ST25-infected mice, regardless of the cytokine. The plasma concentrations of the chemokines C-C motif ligand (CCL)2, CCL3, CCL4, CCL5, and C-X-C motif ligand (CXCL)1 were also quantified (Figure 4). A significantly higher

production in ST1-, ST25-, or EA ST28-infected mice was observed for all chemokines compared to NA ST28-infected mice ($p < 0.001$). Furthermore, significant differences were also observed between ST1- or ST25- and EA ST28-infected mice for CCL3 and CCL4 ($p < 0.05$). As with cytokines, no significant differences were observed between ST1- and ST25-infected mice, regardless of the chemokine. For all of the cytokines and chemokines evaluated, significant differences were observed between ST1-, ST25-, NA, EA ST28-, and mock-infected mice ($p < 0.05$), with the exception of IFN-γ for NA and EA ST28-infected mice. Obtained data indicate that systemic inflammation is implicated in host death during the systemic infection, given that induced mediators could be associated with mortality 72 h p.i. for most strains. However, some exceptions were observed. For example, ST1 strains Bassett and SR800 and ST25 strain LPH4 induced intermediate to high levels of these inflammatory mediators yet caused little mortality. This indicates that although the ST and the geographical origin might present some kind of correlation, exceptions may occur.

Hematogenous-induced meningitis: The ST1, ST25 and Eurasian ST28 strains induce similar, yet higher rates of meningitis in infected mice than do the North American ST28 strains

Following acute systemic infection, surviving mice are susceptible of developing a CNS disease at later incubation times (Figure 1), characterized by meningitis accompanied or not by encephalitis, as evaluated by the presence of clinical signs (spatial disorientation, hyper-excitement followed by opisthotonos, circular walking with head bent to the side, sudden spinning while in recumbence, and tonicoclonic movements) and histopathology. ST1, ST25, and EA ST28 strains caused significantly more meningitis (20%, 29%, and 36%, respectively) than NA ST28 strains, which induced only 11% of meningitis ($p < 0.05$) (Table 2). As during the systemic infection, and regardless of statistical differences between STs, important variations were also observed amongst strains of the same ST (Table 2).

Typical histopathological lesions of mouse meningoencephalitis include multifocal gliosis, meningeal and/or brain suppuration, mononuclear and polymorphonuclear leukocyte infiltration, and brain hemorrhage and necrosis. All ST1, ST25, and EA ST28 strains, as well as certain of the NA ST28 strains tested, induced such lesions. Mice inoculated with Todd-Hewitt broth (THB) (Figure 5A), which served as the vehicle, did not present any clinical signs or histopathological lesions of meningoencephalitis, identical to mice infected with NA ST28

strains that did not induce meningitis (Figure 5B). Meanwhile, mice inoculated with EA ST28 strains (Figure 5C) presented moderate meningeal and brain suppuration, while ST25 strains (Figure 5D) induced severe meningeal and brain suppuration accompanied by brain hemorrhage and necrosis. Brain histopathology of mice inoculated with ST1 strains (Figure 5E) presented similar lesions to those previously reported for a European ST1 strain, 31533 [26]. Multifocal gliosis and leukocyte infiltration were also observed in the brains of mice infected with the ST1, ST25, and EA ST28 strains.

Alongside, blood bacterial burden, evaluated 5 days p.i. (Figure 1), which in this study corresponds to persistent bacteremia, was significantly higher in ST1-, ST25- or EA ST28-infected mice than in NA ST28-infected mice ($p < 0.05$) (data not shown). These persistent titers indicate that ST1, ST25, and EA ST28 strains survived in blood following the systemic infection, while NA ST28 strains generally did not. Using these data, a significant association between the presence of a blood bacterial burden at least 5 days p.i. and the presence of subsequent clinical signs of meningitis in mice infected with *S. suis* serotype 2 was observed ($p < 0.001$).

Brain bacterial burden Is only present in infected mice following the development of meningitis, regardless of the ST

The presence of bacteria in the brain is considered essential for the subsequent development of meningitis, since it is probably responsible for inducing the local inflammatory brain response. In the absence of clinical signs of meningitis, both mice infected with strains incapable of causing meningitis, i.e. lower virulence NA ST28 strains, and mice infected with strains potentially capable of causing meningitis but that never developed clinical signs of meningitis (data not shown), presented undetectable brain bacterial burdens. Contrarily, in the presence of clinical signs of meningitis (Figure 1), and regardless of the ST, high concentrations of *S. suis* were detected in the brain, ranging between 2×10^5 and 6×10^7 colony forming units (CFU)/g (Table 2).

Lower virulence North American ST28 strains remain incapable of causing meningitis following intracisternal injection into the cerebrospinal fluid

In the abovementioned experiments, it was observed that certain lower virulence NA ST28 strains (1054471 and 1088563) were incapable of causing meningitis following intraperitoneal

inoculation. It remained unclear if this was due to their relative incapacity of surviving in blood or to an intrinsic inability to induce meningitis. To answer this question, the transcutaneal intracisternal inoculation route (Figure 1) was used with a dose of 2×10^5 CFU. Negative (sterile THB or 2×10^5 CFU of heat-killed P1/7) and positive (2×10^5 CFU of P1/7) controls were also included. Previous studies showed that a virulent ST1 strain induced meningoencephalitis at this dose within 24 h p.i. in CD-1 mice [30]. Clinical signs of CNS disease were monitored and brain histopathology was carried out. Mice inoculated with either THB (Figure 6A) or heat-killed bacteria (data not shown) did not present clinical signs of meningitis or histopathological lesions 72 h p.i.; this was also the case for mice inoculated with either of the two lower virulence NA ST28 strains (Figure 6B & C). However, mice infected with the European ST1 strain P1/7 presented both clinical signs and histopathological lesions associated with meningoencephalitis within 24 h p.i. (Figure 6D), including severe meningeal and brain suppuration, multifocal gliosis, leukocyte infiltration, and brain hemorrhage and necrosis.

Since these two NA ST28 strains were incapable of inducing meningitis following intracisternal inoculation, their ability to persist in the brain was evaluated (Figure 1). It was observed that at 12 h p.i. (Figure 7A), brain bacterial burden of mice infected with these NA ST28 strains, which averaged 1×10^3 CFU/g, was significantly lower than that of P1/7-infected mice ($p < 0.01$). At 24 h p.i. (Figure 7B), this difference was even more pronounced: 6×10^7 CFU/g were detected in the brains of P1/7-infected mice compared with nearly undetectable levels (average of 1×10^2 CFU/g) in those of NA ST28-infected mice.

Though blood bacteria burden is generally absent or undetectable upon presentation of clinical signs of meningitis, its presence can result in meningitis-induced complications and worsening of disease [31]. Given the elevated brain bacterial titers detected 12 and 24 h p.i. in P1/7-infected mice, secondary bacteremia following meningitis was evaluated (Figure 1). As of 12 h p.i. (Figure 7C), biologically relevant blood bacterial titers were detected in P1/7-infected mice, which averaged 1×10^5 CFU/mL, and which were significantly higher than those of NA ST28-infected mice ($p < 0.01$). This significant difference was also observed 24 h p.i. (Figure 7D) ($p < 0.001$), at which time blood bacterial titers of P1/7-infected mice remained relatively stable, at 4×10^4 CFU/mL, while those of NA ST28-infected mice were generally undetectable.

S. suis *serotype 2-induced pro-inflammatory brain response only occurs in the presence of meningitis*

In situ protein levels of inflammatory mediators have never been quantified during *S. suis* meningitis. Firstly, it was verified that the inflammatory mediators produced in the blood during septic shock and in the brain during meningitis remained compartmentalized and separate as a result of the blood–brain barrier and blood–cerebrospinal fluid (CSF) barrier, and did not seep between systems (Figure 1). Consequently, the levels of IL-1β and IL-6 and chemokines CCL2, CCL3, CXCL1, and CXCL10 were quantified in the plasma and brain homogenates of mice infected with the European ST1 strain P1/7 (Figure 8). Remarkably, complete compartmentalization was observed during septic shock and meningitis in both the plasma and brain for all of mediators tested.

Subsequently, production of these six mediators was quantified in brain homogenates using representative strains following intraperitoneal (upon presentation of clinical signs of meningitis or at the end of the study for strains incapable of causing meningitis) and intracisternal (24 h p.i. or at the end of the study for strain incapable of causing meningitis) inoculation (Figure 9). Following intraperitoneal inoculation, none of the six pro-inflammatory mediators were detected in the brains of mice infected with the NA ST28 strain 1088563, which did not induce meningitis. Meanwhile, these mediators were highly and significantly induced in mice with meningitis and infected with the ST1 strain P1/7 and ST25 strain 89-1591, when compared with mock-infected mice ($p < 0.001$). Intermediately, the EA ST28 strain MNCM43, which caused meningitis, induced a significant production of IL-1β, CCL2, and CCL3 only ($p < 0.05$). These results were compared with those from mice infected by intracisternal inoculation. Once again, none of the six pro-inflammatory mediators tested could be detected in the brains of mice infected with the NA ST28 strain 1088563, which did not induce meningitis. Similar results were also obtained for the NA ST28 strain 1054471 (data not shown). However, these mediators were highly and significantly induced in the brains of mice infected with the strains P1/7 (ST1), 89-1591 (ST25), and MNCM43 (EA ST28) when compared with mock-infected mice ($p < 0.05$) (Figure 9). Interestingly, and differently from what was observed after intraperitoneal inoculation, similar levels of inflammatory mediators were detected in the brains of mice infected with these three strains. In fact, the Eurasian ST28 strain MNCM43 induced significantly higher levels of cytokines and chemokines following intracisternal inoculation when compared to intraperitoneal inoculation,

regardless of presenting similar clinical signs and histopathological lesions of meningoencephalitis than the ST1 and ST25 strains P1/7 and 89-1591, respectively.

Discussion

S. suis serotype 2, an important porcine pathogen and emerging zoonotic agent, is the most widespread of the described *S. suis* serotypes worldwide [2]. Recent studies have associated archetypal strains of different *S. suis* serotype 2 STs with virulence [16, 19]. However, the virulence potential of strains belonging to the same ST but from different geographical and host origins had never been investigated. In the present study, virulence of different strains was evaluated in an inbred mouse model of infection in order to reduce variability due to host heterogeneity. In addition, the predictive virulence potential of strains was also tested using in vitro assays.

Since animal models of infections are not the most straightforward approach to predict *S. suis* virulence, both logistically and ethically, different well-described in vitro assays have been developed. For example, the capacity to resist phagocytosis by immune cells, which has been largely described as being an essential step in the pathogenesis of *S. suis* serotype 2, is often associated with virulence [1]. In the present study, a clear relationship between resistance to phagocytosis by murine macrophages and virulence in the mouse model could not be established. Conditions used for phagocytosis assays in previously published studies are highly variable. Different cell types (dendritic cells, macrophages, monocytes, and neutrophils), hosts (human, mouse, and pig) and the absence or presence of normal or heat-inactivated serum have been used [32-34]. It may be hypothesized that in vitro conditions used for phagocytosis assays can influence the outcome of the test. For example, results obtained in the current study using the ST1 strain P1/7 in the presence of 10% serum showed clearly higher rates of internalization than those previously reported with the same cells in the absence of serum [33, 35]. However, whether complement or other host serum factors are responsible for this effect is not clear. In fact, the role of complement during the systemic infection caused by *S. suis* is still controversial. While an early report suggested a limited role of complement in phagocytosis and killing of well-encapsulated *S. suis* [36], another study showed that the complement system contributes to limit *S. suis* invasiveness at early stages of the infection [37]. Nevertheless, the role of complement and other conditions in *S. suis* phagocytosis tests remain to be confirmed. Finally, phagocytosis results are usually evaluated at one specific time point. The possibility

that levels of phagocytosis are similar among the strains but that certain strains are rapidly killed intracellularly cannot be ruled out.

On the other hand, a blood bactericidal assay, which more closely resembles the systemic conditions in which *S. suis* encounters different phagocytic cells and blood components [1], provided results different from those of the phagocytosis test and more closely correlated with in vivo virulence. However, correlation was only partial. For example, certain strains (Bassett, SR800, and LPH4) were highly resistant to killing by mouse blood but presented, in general, low virulence in vivo. It is possible that, as in the case with phagocytosis, the increasingly popular killing test [38-40] also needs to be optimized and compared with in vivo virulence to be validated. Confirmation of virulence of a given strain in an animal model of infection remains a requirement [16, 26-28].

Based on a study with archetypal strains, it was previously hypothesized that ST1 strains were more virulent than NA ST25, the latter being more virulent than NA ST28 strains. In addition, it was also hypothesized that EA ST28 strains may be virulent, differently from their NA counterparts. Results obtained in this study partially support this hypothesis, which might explain, to a certain extent, the greater number of serotype 2 cases recorded in Europe and in some Asian countries than in North America [2, 41]. This is especially true with ST28 strains: NA strains caused little or no mortality during the systemic infection while EA strains were virulent. Results with ST28 strains support a certain level of genetic heterogeneity of these strains as determined by whole-genome sequencing, whereby NA and EA ST28 strains clustered separately [23]. However, a critical analysis shows that, regardless of statistical differences, important variations in mortality were observed within STs. This indicates that, for strains other than NA ST28, it would be dangerous to assume that a given strain is virulent or non-virulent based only on its background. It would be important to confirm such observations using a higher number of strains. Finally, virulence of tested strains isolated from either porcine or human clinical cases of infection was similar, which supports previous genetic analyses [8].

It has been proposed that mortality during the systemic infection caused by *S. suis* may be the result of a high and uncontrolled bacteremia combined with an exacerbated pro-inflammatory response [26, 27]. For the first time, this study demonstrates that mortality is positively associated with both bacteremia and plasma pro-inflammatory mediators during the acute

systemic infection for strains other than archetypal ST1 [26] and ST25 strains [16]. Consequently, inflammatory mediators are a reflection of the host response to bacteremia. Following the systemic infection, surviving individuals are susceptible of developing a CNS infection characterized by meningitis accompanied or not by encephalitis [26]. Results also showed for the first time that meningitis can be induced by a strain belonging to an ST other than the ST1 in the mouse model of infection.

In general, virulent ST1, ST25, and EA ST28 strains persisted in circulation, whereas NA ST28 strains were mostly undetectable by the end of the systemic infection. This suggests that the latter strains are less resistant to killing, confirming results obtained using the whole blood assay in vitro. Similarly, ST1, ST25, and EA ST28 strains induced higher rates of meningitis, while most NA ST28 strains did not induce important disease. It has been demonstrated for human meningitis-inducing bacterial pathogens that a high acute blood bacterial burden is a predisposing factor for the development of meningitis [42, 43]. However, this association had not been determined with regards to persistent bacteremia, and never for *S. suis*. Differently from NA ST28 strains, persistent bacteremia was observed with ST1, ST25, and EA ST28 strains, all of which induced important, yet similar rates of meningitis. This indicates that persistent *S. suis* serotype 2 bacteremia is a prerequisite for the subsequent development of meningitis. Thus, if bacteremia can be rapidly cleared, the risk of developing subsequent meningitis, which is often more difficult to treat [41], is greatly reduced.

Alongside, excessive inflammation was also identified as a crucial step in systemic virulence [16, 26]. While ST1 and ST25 strains induced the highest levels of pro-inflammatory mediators, EA ST28 strains induced mostly chemokines, which may suggest the utilization of different mechanisms to cause inflammation and host death. In accordance, a rapid clearance of NA ST28 bacteria from the bloodstream may explain the lack of pro-inflammatory mediators induced by such strains.

Brain bacterial burden is, for most meningitis-causing bacterial pathogens, considered essential and responsible for inducing the local inflammatory response [42, 44]. Indeed, our results demonstrated that the absence or presence of meningitis is associated with the absence or presence of a minimum threshold of *S. suis* brain burden, respectively. Previous results with *Streptococcus pneumoniae* showed that elevated brain bacterial burden responsible for

meningitis can, from the CSF, enter the bloodstream: this event may lead to secondary bacteremia, resulting in systemic host inflammation and decreased host response during treatment [31, 45]. Indeed, results obtained in the current study revealed that *S. suis* meningitis can result in secondary bacteremia, which could worsen the outcome of the infection. On the other hand, the NA ST28 strains tested that did not induce meningitis via the hematogenous route of infection were also unable to induce meningitis following inoculation into the CSF. This suggests that lower virulence strains cannot persist in the CNS even if they manage to bypass clearance by blood phagocytic cells. Similarly, these strains also seem susceptible to elimination by resident CNS cells [46, 47]. Although less virulent in mouse and pig models of infection [4, 48], porcine cases of infection caused by NA *S. suis* serotype 2 strains occur in the field, suggesting the implication of environmental, management, and/or co-infection factors [25]. These stressful conditions often result in increased susceptibility to infections by secondary pathogens, such as *S. suis*, including by less virulent strains [25].

The inflammation responsible for the development of *S. suis*-induced meningitis has been barely studied. It was previously observed that mRNA levels of a few pro-inflammatory mediators are upregulated in the brains of mice during meningitis [26], but protein levels have never been quantified. In the present study, a pro-inflammatory brain response was only observed in the presence of meningitis, regardless of the ST, which suggests a probable implication of inflammatory cells in *S. suis*-induced meningitis. Accordingly, there was an absence of brain mediator production following infection with lower virulence NA ST28 strains, even when injected directly in the CNS, probably as a consequence of their reduced resistance to the local defense systems. Interestingly, production of IL-1β was clearly observed for the first time during the *S. suis* meningitis; levels of this cytokine are barely quantifiable during the systemic infection [26]. This cytokine may play an important role during the *S. suis* meningitis, as has been reported for *S. pneumoniae*, where levels of IL-1β determine the outcome and severity of the disease [49]. Albeit the source of *S. suis*-induced IL-1β remains unknown, in vitro studies have previously demonstrated that microglia and astrocytes are not responsible for this production [50, 51]. Infiltrating cells may be the source of this cytokine since monocytes have been shown to produce IL-1β when activated with *S. suis* [52]. Moreover, the production of chemokines (such as CCL2, CCL3, and CXCL1), possibly produced by resident CNS cells [50, 51], may be responsible for the infiltration of blood leukocytes into the brain. Of note, similar to what has been observed in the bloodstream, the EA ST28 strain induced higher levels of

chemokines than cytokines in the CNS. Finally, a massive production of IL-6 was also detected. When produced in the brain during *S. pneumoniae* meningitis, IL-6 is responsible for increased intracranial pressure [53]. Production of pro-inflammatory cytokines, which are known to induce neuronal death [54], are possibly responsible for the histopathological lesions observed during the *S. suis* meningitis, including brain hemorrhaging and necrosis.

Interestingly, the present results showed that systemic mediators in blood during septic shock and brain mediators in the CNS during meningitis remained completely separated and compartmentalized during both types of *S. suis* infections. Though not yet demonstrated, an important increase of brain barrier permeability possibly occurs following *S. suis* infection given the massive infiltration of leukocytes during meningitis [26]. Consequently, this observation was completely unexpected. To our knowledge, this is the first time that such a compartmentalization has ever been demonstrated. A retrospective study of meningococcal infection determined that CSF mediators remained separated from plasma during meningitis, but not septic shock [55]. Furthermore, CSF but not brain mediators were evaluated. As such, it may be hypothesized that pro-inflammatory mediators are highly concentrated in the CNS during meningitis and diluted in the bloodstream, which might explain the low systemic IL-1β levels detected during *S. suis* infection.

Conclusions

Overall, this study demonstrates that although a certain tendency is observed, *S. suis* serotype 2 virulence is difficult to predict based on ST and geographical origin alone, since some strains belonging to the same ST presented important differences in virulence, which did not always correlate with a given continent. The only exception appears to be NA ST28 strains, which induce low and transient bacteremia and low production of inflammatory mediators (in the bloodstream and in the CNS) and were generally less virulent in both systemic and CNS infection models. We have also shown that serotype 2 strains belonging to other STs than the ST1 are able to induce meningitis in the mouse model of infection. In addition, persistent and high levels of bacteremia accompanied by an elevated CNS inflammatory reaction are needed for a strain to cause meningitis. Although widely used, in vitro tests such as the phagocytosis assay must be standardized in order for these to be predictive tests that may evaluate the virulence capacities of a given strain. The blood bacterial killing assay better predicts bacteremia and virulence, though as with the phagocytosis assay, standardization is required

given that correlation with virulence was only partial. The use of strains other than archetypal strains has increased our knowledge and understanding of the *S. suis* serotype 2 population dynamics. Results obtained in the present study should, ideally, be confirmed using a greater number of strains, as well as by testing representative strains in appropriate experimental infection models in susceptible pigs.

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Tables

Table 1. Resistance to phagocytosis and bacterial killing, as well as the capacity to cause mouse mortality during the systemic infection following intraperitoneal inoculation, by different *S. suis* serotype 2 strains.

Strain	ST	Origin	Phagocytosis (CFU/mL ± SEM) [1]	Bacterial Killing (% ± SEM) [2]	Mouse Mortality 72 h p.i. (%) [3]
P1/7	1	EA	$2 \pm 0.7 \times 10^4$	0 ± 0	67
Δ*cpsF*	-	-	$2 \pm 0.5 \times 10^5$ [a]	64 ± 5 [b]	-
MGGUS2	1	NA	$2 \pm 0.9 \times 10^4$	0 ± 0	80
Bassett	1	NA	$2 \pm 1 \times 10^4$	0 ± 0	7
SR800	1	SA	$3 \pm 0.9 \times 10^4$	0 ± 0	0
MNCM06	1	EA	$2 \pm 0.9 \times 10^4$	0 ± 0	20
89-1591	25	NA	$8 \pm 5 \times 10^4$	20 ± 5	47
91-1804	25	NA	$1 \pm 0.4 \times 10^4$	34 ± 5	0
LPH4	25	EA	$6 \pm 2 \times 10^4$	2 ± 2	7
1054471	28	NA	$3 \pm 0.5 \times 10^3$	33 ± 6	0
1084708	28	NA	$8 \pm 2 \times 10^3$	27 ± 3	0
1088563	28	NA	$6 \pm 0.3 \times 10^3$	30 ± 3	0
1097205	28	NA	$8 \pm 1 \times 10^3$	27 ± 2	10
DAT254	28	EA	$6 \pm 0.9 \times 10^3$	3 ± 2	73
MNCM43	28	EA	$5 \pm 3 \times 10^3$	21 ± 3	33

[1] Phagocytosis of the different strains by murine macrophages after 1 h of incubation with 10% murine serum and a MOI = 100; [a] Significant difference between P1/7 and Δ*cpsF* ($p < 0.001$); [2] Bacterial killing of the different strains by murine whole blood after 4 h of incubation with a MOI = 1; [b] Significant difference between P1/7 and Δ*cpsF* ($p < 0.001$); [3] Mortality until 72 h post-infection (p.i.), considered as due to systemic infection; EA = Eurasia; NA = North America; SA = South America.

Table 2. Capacity to induce meningitis and brain bacterial burden after the CNS infection following intraperitoneal inoculation by different *S. suis* serotype 2 strains.

Strain	ST	Origin	Meningitis (%) [1]	Brain Burden (CFU/g) [2]
P1/7	1	EA	31	6×10^7
MGGUS2	1	NA	20	2×10^7
Bassett	1	NA	14	ND
SR800	1	SA	27	ND
MNCM06	1	EA	8	ND
89-1591	25	NA	20	2×10^5
91-1804	25	NA	27	ND
LPH4	25	EA	36	2×10^6
1054471	28	NA	0	0
1084708	28	NA	20	2×10^5
1088563	28	NA	0	0
1097205	28	NA	11	9×10^5
DAT254	28	EA	33	2×10^6
MNCM43	28	EA	30	3×10^6

[1] Mice inoculated with 5×10^7 CFU for all strains except for P1/7, MGGUS2 and 89-1591, which were inoculated with 2×10^7 CFU to ensure survival of a sufficient number of mice following the systemic infection; [2] Brain burden was determined upon presentation of clinical signs of meningitis or at the end of the study (14 days post-infection); EA = Eurasia; NA = North American; SA = South America; ND = Not determined.

Table 3. *S. suis* serotype 2 strains used in this study.

Strain	Sequence Type (ST)	Country	Clinical Feature	Reference
P1/7	1	United Kingdom	Pig, meningitis	[58]
MGGUS2	1	United States	Pig, meningitis	[19]
Bassett	1	United States	Human, meningitis	[20]
SR800	1	Argentina	Human, meningitis	[59]
MNCM06	1	Thailand	Human, meningitis	[21]
89-1591	25	Canada	Pig, sepsis/meningitis	[60]
91-1804	25	Canada	Human, endocarditis	[61]
LPH4	25	Thailand	Human, sepsis	[21]
1054471	28	Canada	Pig, meningitis	[19]
1084708	28	Canada	Pig, sepsis	[19]
1088563	28	Canada	Pig, meningitis	[19]
1097205	28	Canada	Pig, meningitis	[19]
DAT254	28	Japan	Pig, meningitis	[6]
MNCM43	28	Thailand	Human, endocarditis	[21]

Figures

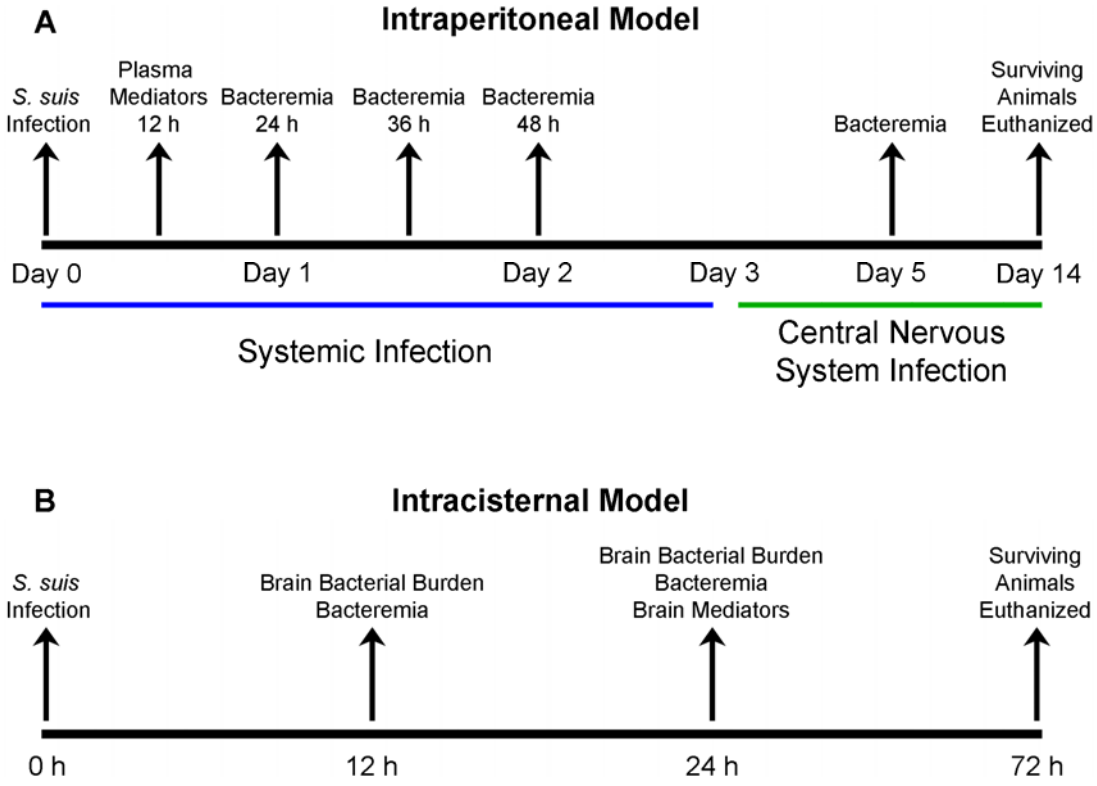

Figure 1. Timeline summary of the intraperitoneal and intracisternal mouse models of infection used throughout this study. C57BL/6 mice were infected with the different *S. suis* serotype 2 strains using the intraperitoneal route of infection (**A**) to evaluate the systemic and subsequent central nervous system infection; or the transcutaneal intracisternal route of infection (**B**) to directly evaluate the central nervous system infection.

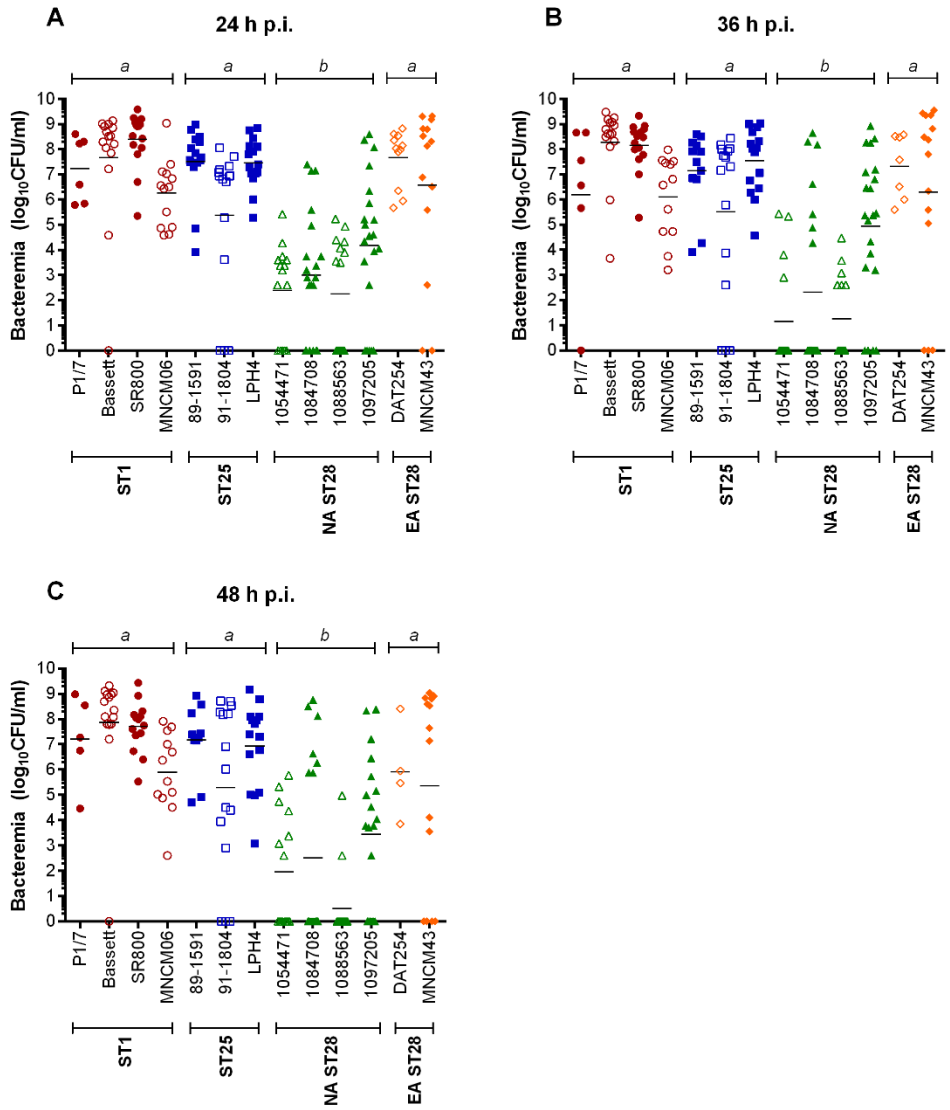

Figure 2. Blood bacterial burden is lower in North American ST28-infected mice but similar in ST1, ST25 or Eurasian ST28-infected mice during the systemic infection. C57BL/6 mice were inoculated by intraperitoneal injection with 5×10^7 CFU and blood bacterial titers evaluated 24 h (**A**); 36 h (**B**) and 48 h (**C**) post-infection (p.i.). Data of individual mice are presented as $\log_{10}$ CFU/mL with the geometric mean. Significance between groups is indicated by different letters ($p < 0.001$). Only strains for which five or more mice survived at the indicated time point are presented.

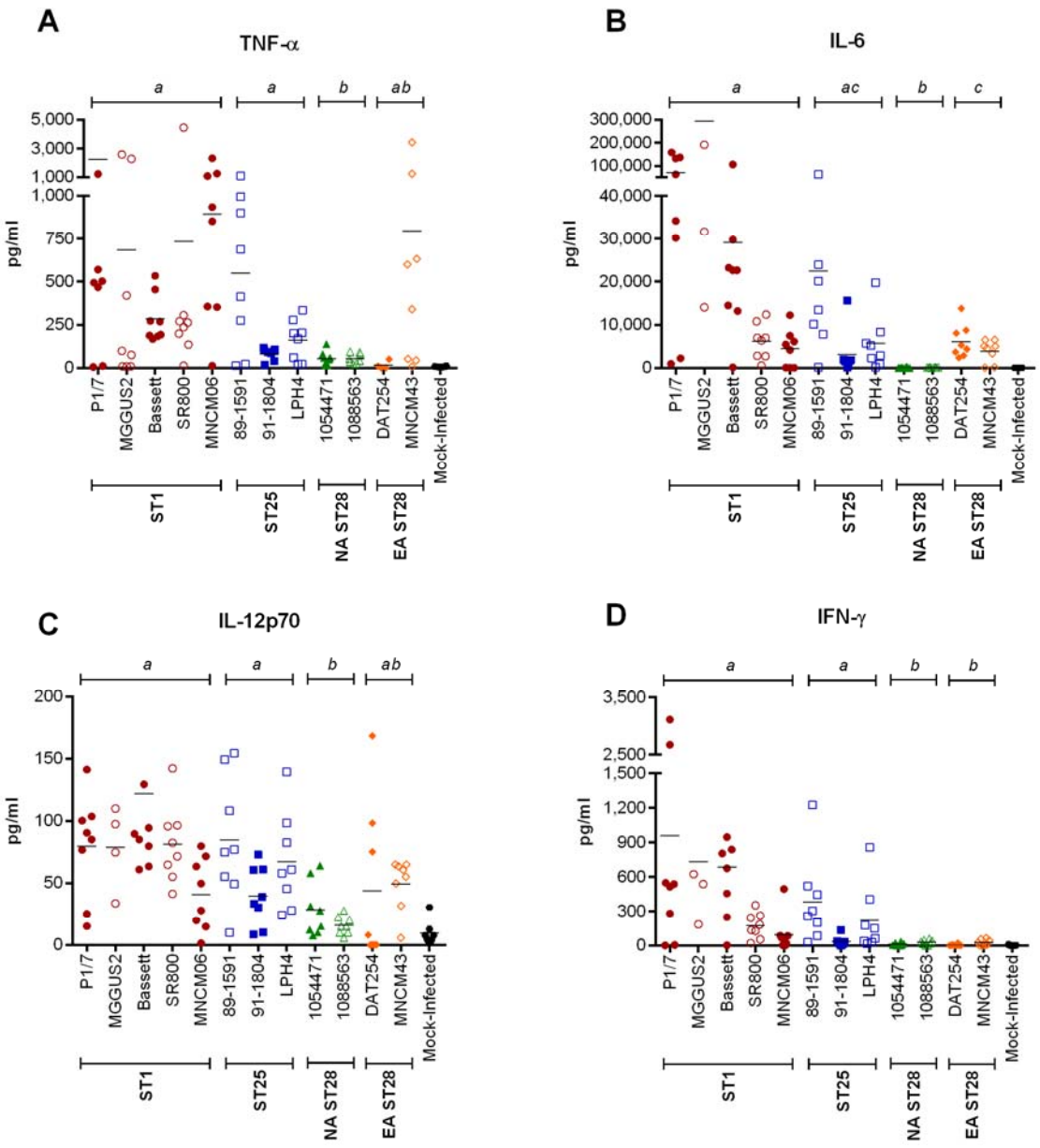

Figure 3. Plasma cytokine production is lowest in North American ST28-infected mice, intermediate in Eurasian ST28-infected mice, and highest in ST1- and ST25-infected mice during systemic infection. Plasma cytokine levels 12 h post-intraperitoneal inoculation of mock- (vehicle) or 5×10^7 CFU of *S. suis*-infected C57BL/6 mice, as determined by Luminex® for TNF-α (**A**); IL-6 (**B**); IL-12p70 (**C**); and IFN-γ (**D**). Data of individual mice are presented as pg/mL with the mean. Significance between groups is indicated by different letters ($p < 0.05$).

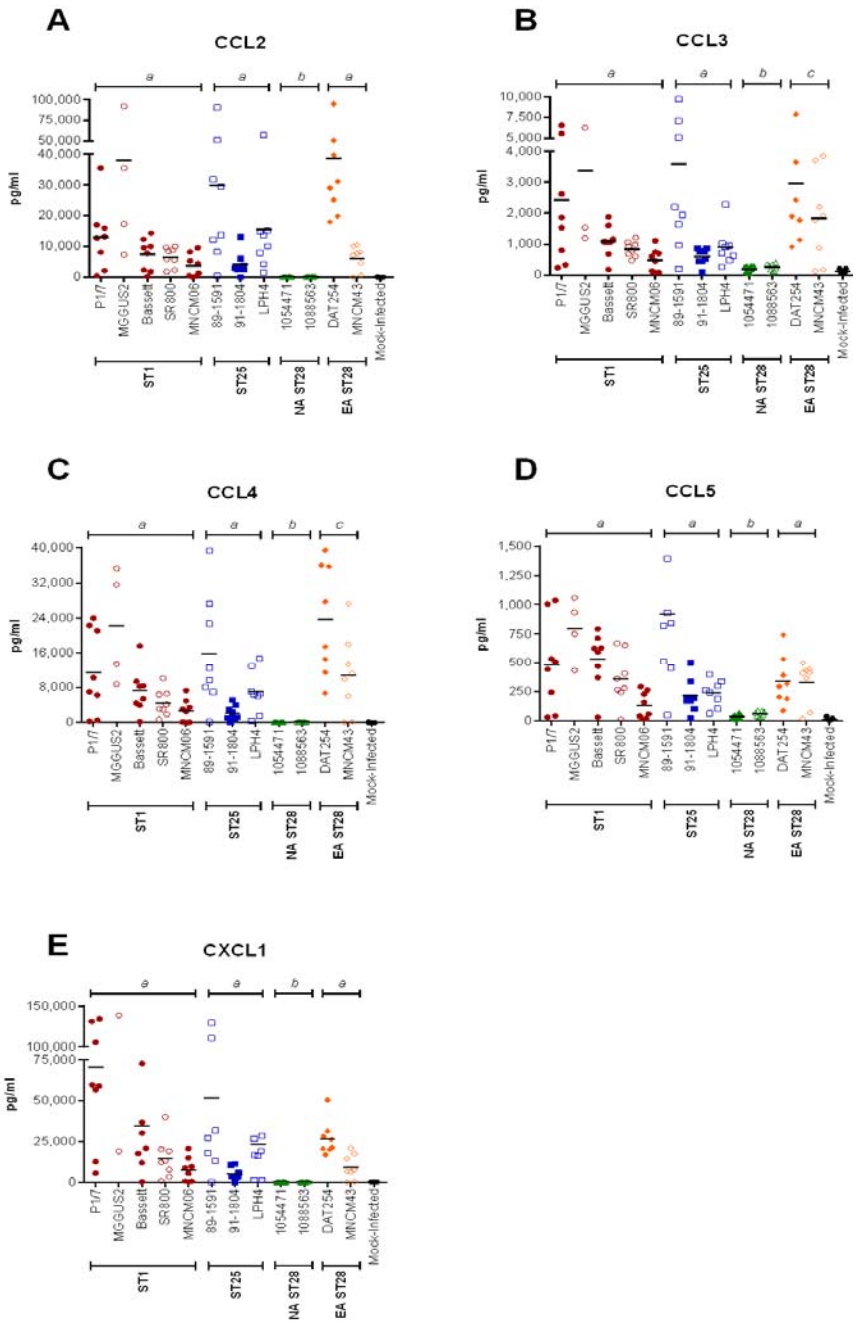

Figure 4. Plasma chemokine production is lowest in North American ST28-infected mice, intermediate in Eurasian ST28-infected mice and highest in ST1- and ST25-infected mice during the systemic infection. Plasma chemokine levels 12 h post-intraperitoneal inoculation of mock- (vehicle) or 5 × 10^7 CFU of *S. suis*-infected C57BL/6 mice, as determined by Luminex® for CCL2 (**A**); CCL3 (**B**); CCL4 (**C**); CCL5 (**D**), and CXCL1 (**E**). Data of individual mice are presented as pg/mL with the mean. Significance between groups is indicated by different letters ($p < 0.05$).

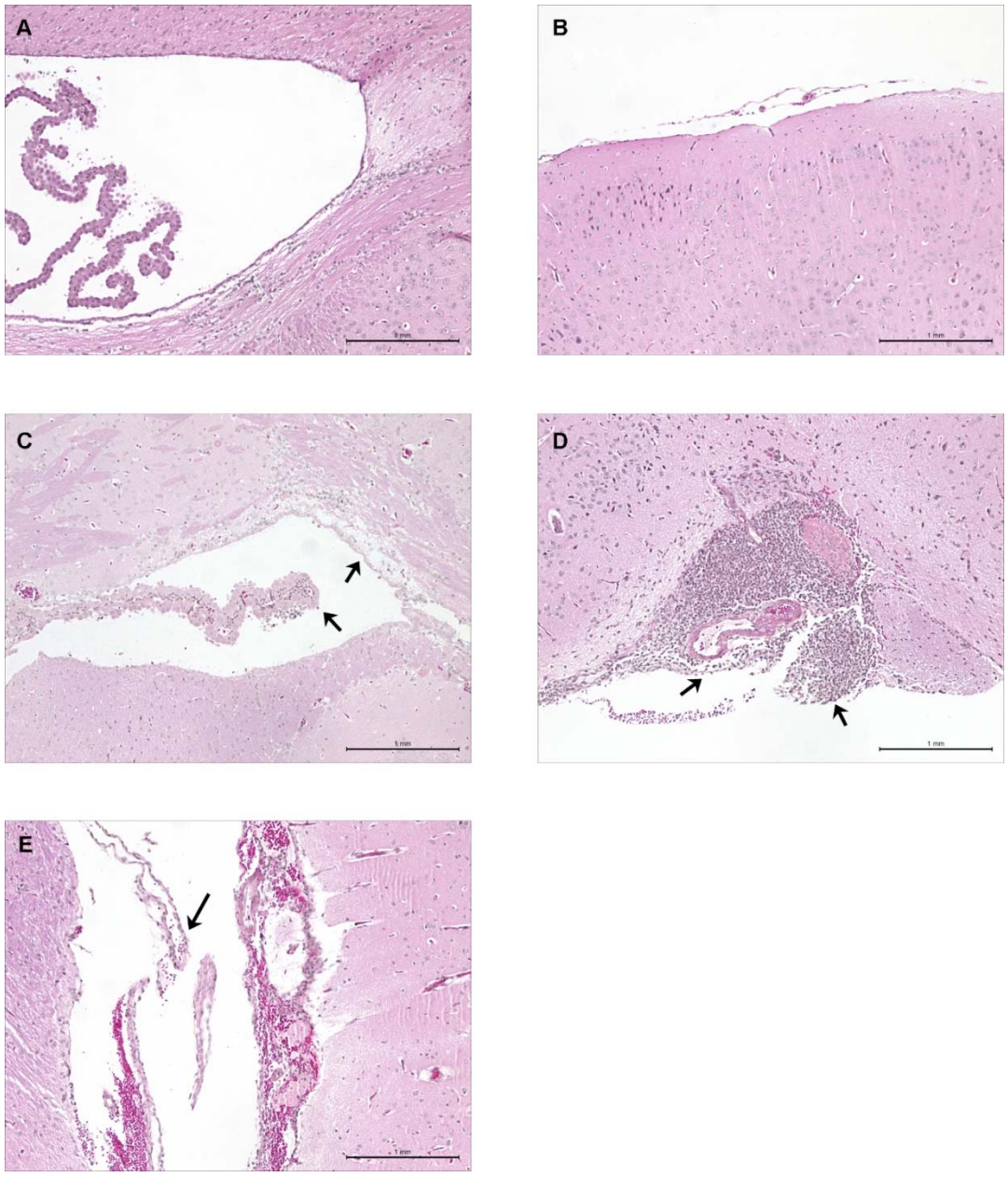

Figure 5. Histopathological studies of the brains of C57BL/6 mice infected by intraperitoneal inoculation during central nervous system infection. Presence or absence of histopathological lesions of meningitis as determined in the brains of mock-infected (vehicle) and infected mice. Micrographs of the meninges or ventricular choroid plexus of mock-infected mice (**A**); NA ST28 strain 1088563- (**B**); EA ST28 strain MNCM43- (**C**); NA ST25 strain 89-1591- (**D**); and EA ST1 strain P1/7- (**E**) infected mice. Black arrowheads indicate lesions typical of *S. suis* meningitis. HPS staining, 100x magnification. NA = North America; EA = Eurasia.

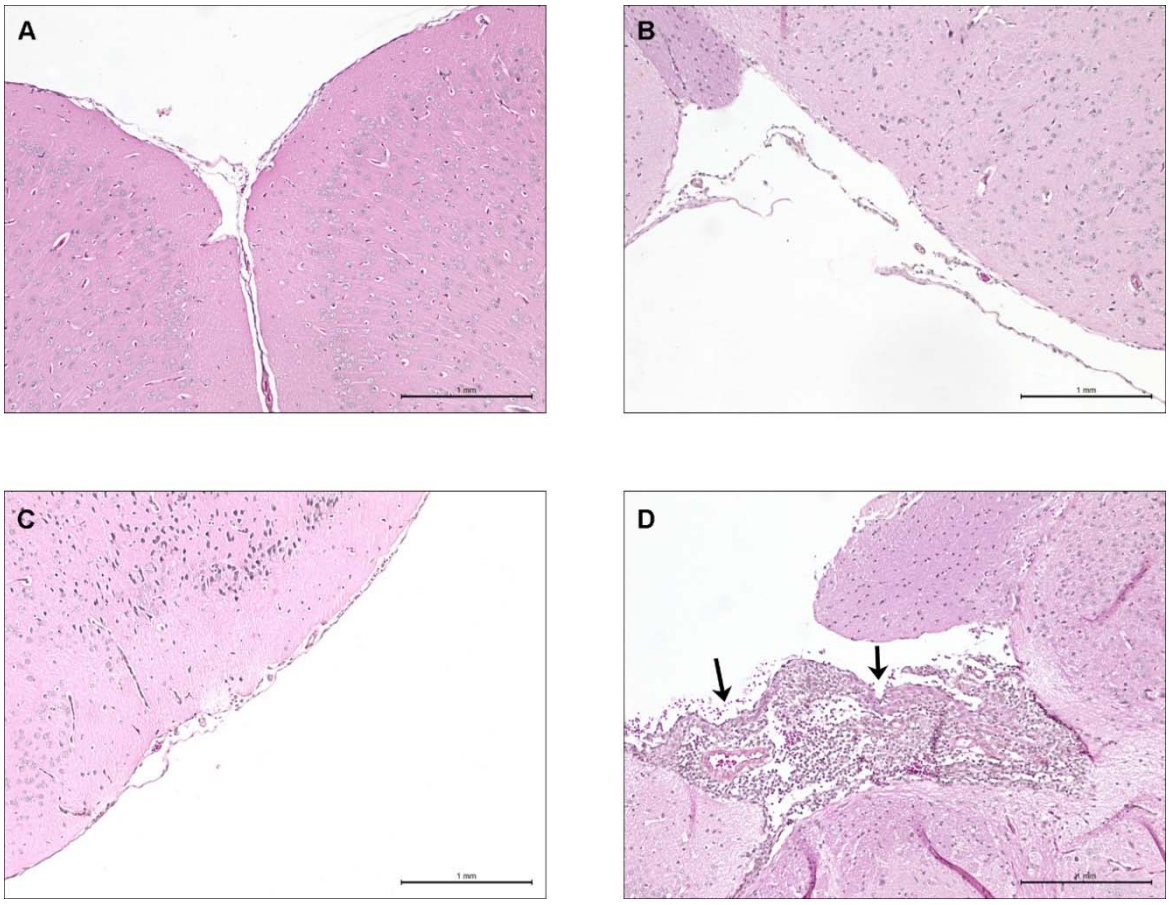

Figure 6. Lower virulence North American ST28 strains do not induce meningitis in C57BL/6 mice following intracisternal inoculation. Presence or absence of histopathological lesions of meningitis in the brains of mock-infected (vehicle) and *S. suis*-infected C57BL/6 mice following intracisternal inoculation. Micrographs of the meninges or ventricular choroid plexus of mock-infected (**A**); NA ST28 strain 1054471- (**B**); NA ST28 strain 1088563- (**C**); and EA ST1 strain P1/7- (**D**) infected mice. Black arrowheads indicate lesions typical of *S. suis* meningitis. HPS staining, 100× magnification. NA = North America; EA = Eurasia.

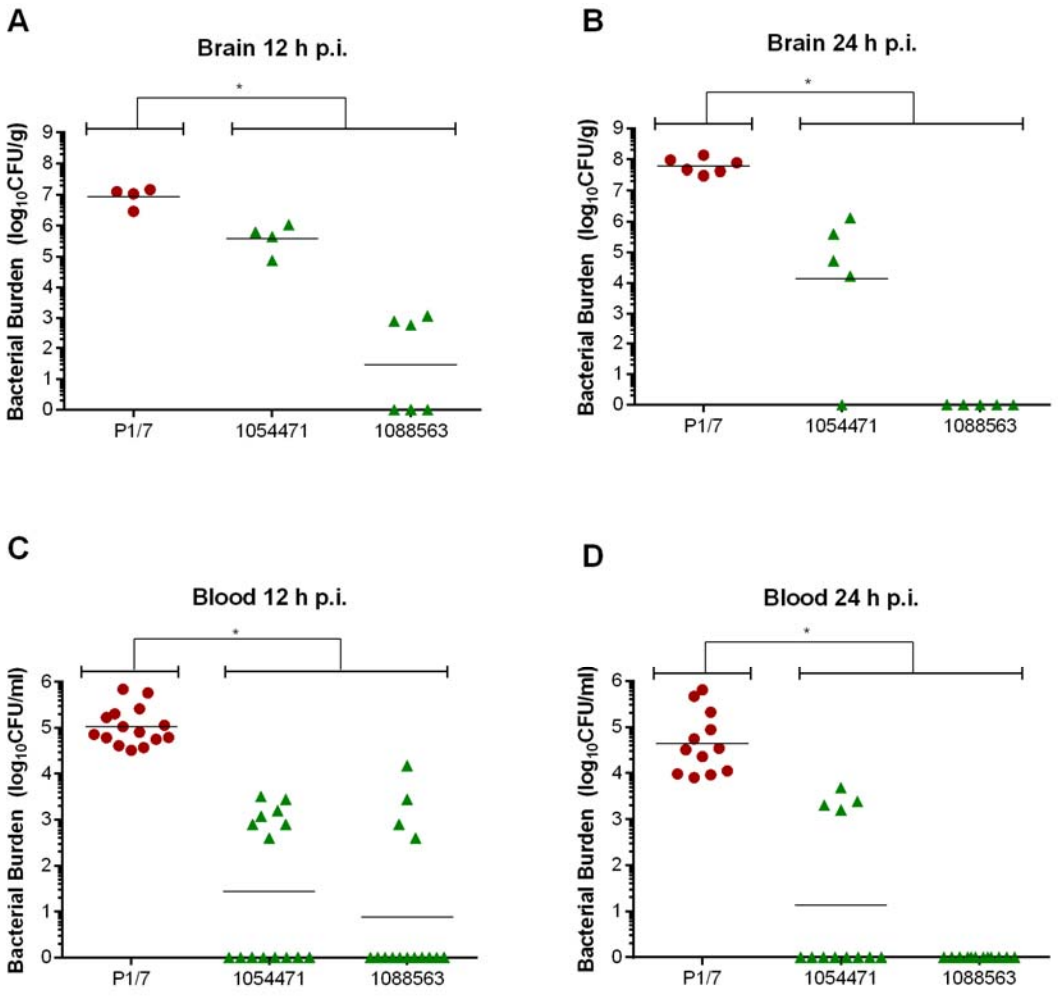

Figure 7. Brain and blood bacterial titers of lower virulence North American ST28 strains are transient, while those of the ST1 strain are persistent, following intracisternal inoculation. C57BL/6 mice were inoculated by intracisternal injection with 2×10^5 CFU and brain and blood bacterial titers evaluated 12 h (**A** & **C**) and 24 h (**B** & **D**) post-infection (p.i.). Data of individual mice are presented as $\log_{10}$ CFU/g or CFU/mL with the geometric mean. * Indicates a significant difference between the Eurasian ST1 strain P1/7 and both North American ST28 strains (1054471 and 1088563) ($p < 0.01$).

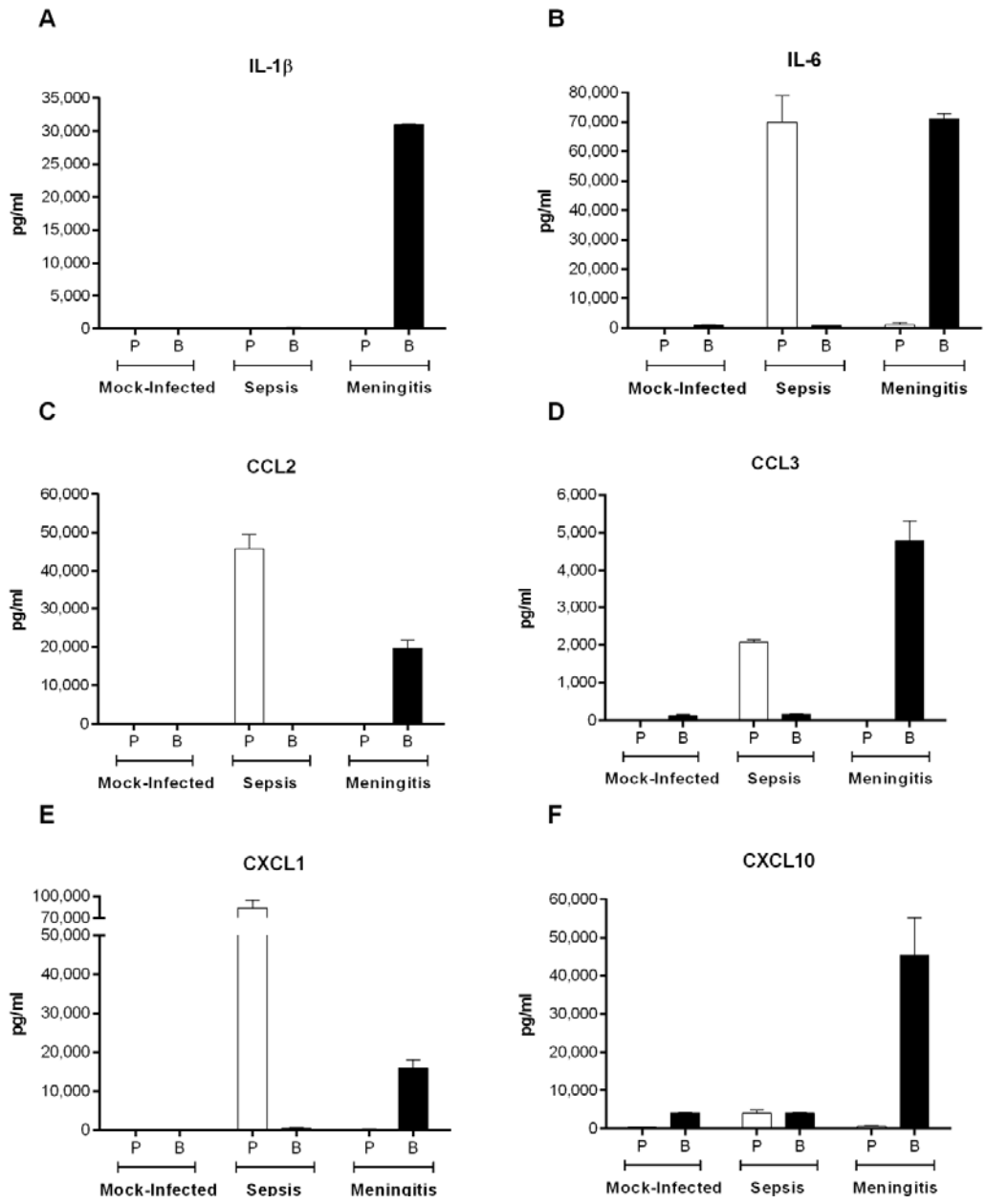

Figure 8. Plasma (P) and brain (B) homogenate cytokine and chemokines levels of mock-infected (vehicle) or C57BL/6 mice inoculated by intraperitoneal injection with *S. suis* serotype 2 European ST1 strain P/7, upon presentation of clinical signs of septic shock or meningitis, by ELISA for IL-1β (**A**); IL-6 (**B**); CCL2 (**C**); CCL3 (**D**); CXCL1 (**E**); and CXCL10 (**F**). Data are presented as mean ± SEM pg/mL.

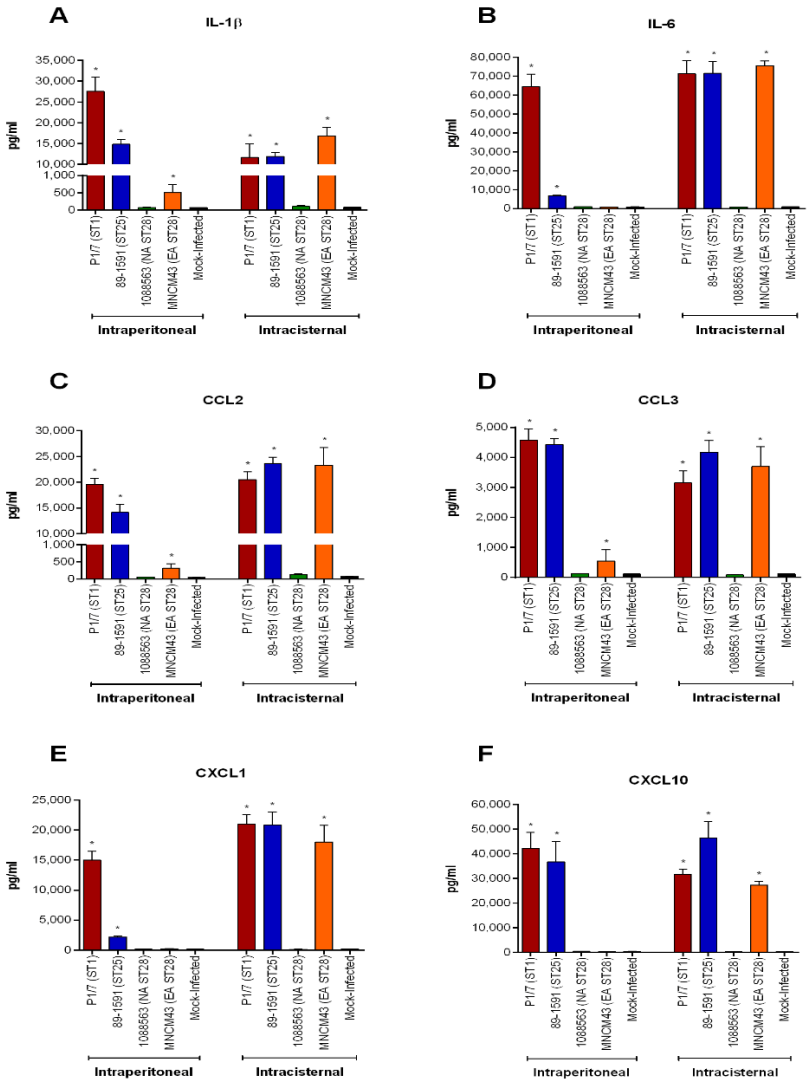

Figure 9. Production of brain cytokines and chemokines following *S. suis* serotype 2 infection only occurs in the presence of meningitis. Brain cytokine and chemokine levels of mock-infected (vehicle) or *S. suis*-infected C57BL/6 mice inoculated by intraperitoneal or intracisternal injection as determined by ELISA. Intraperitoneally injected mice were euthanized upon presentation of clinical signs of meningitis or at the end of the study (14 days post-infection) and intracisternally injected mice 24 h post-infection or at the end of the study (72 h post-infection). Brain levels of IL-1β (**A**); IL-6 (**B**); CCL2 (**C**); CCL3 (**D**); CXCL1 (**E**); and CXCL10 (**F**) following infection with EA ST1 strain P1/7, NA ST25 strain 89-1591, NA ST28 strain 1088563, or EA ST28 strain MNCM43. Data are presented as mean ± SEM pg/mL. * Indicates a significant difference with mock-infected mice ($p < 0.05$). EA = Eurasian; NA = North American.

OBJECTIF 3 – CARACTÉRISER L'INFECTION ET L'INFLAMMATION CAUSÉES PAR DIFFÉRENTS *STREPTOCOCCUS SUIS* AU NIVEAU SYSTÉMIQUE ET DU SYSTÈME NERVEUX CENTRAL

ARTICLE IX

Differential role of MyD88 signaling in *Streptococcus suis* serotype 2-induced systemic and central nervous system diseases

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur du manuscrit. J'ai été responsable de la conception de l'étude et des approches méthodologiques. J'ai effectué toutes les expériences *in vitro* et *in vivo*. J'ai aussi effectué l'analyse des résultats, l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci.

Mise en contexte

Dans le cadre de l'objectif 3, nous avons voulu mieux caractériser les infections systémiques et du SNC causées par *S. suis* et l'inflammation induite lors de celles-ci. La reconnaissance de *S. suis* par les cellules de l'hôte est une étape critique à la mise en place de la réponse immunitaire appropriée et de la réponse inflammatoire nécessaire afin de combattre l'infection, et qui peut être néfaste si exacerbée. Parmi les différents récepteurs impliqués dans cette reconnaissance, les TLRs ont été les mieux étudiés, particulièrement le TLR2. Toutefois, les études précédentes ont été effectuées *in vitro*. Ainsi, le rôle de la voie des TLRs et de la protéine adaptatrice MyD88, central à cette voie de signalisation, lors des infections systémiques et du SNC *in vivo* demeurait inconnu. De plus, l'impact de l'origine des souches de *S. suis* étudiées sur ce rôle *in vivo* était inconnu lui aussi.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and a zoonotic agent responsible for sudden death, septic shock, and meningitis, with exacerbated inflammation being the hallmark of the systemic and central nervous system (CNS) infections. However, serotype 2 strains are genetically and phenotypically heterogeneous, being composed of a multitude of sequence types (ST) whose virulence greatly varies: the virulent ST1 (Eurasia), highly virulent ST7 (responsible for the Chinese human outbreaks), intermediately virulent ST25 (North America), and low virulent/virulent ST28 (North America and Asia, respectively) are the most prevalent worldwide. However, most *S. suis* serotype 2 studies have used "classical" virulent Eurasian ST1 or ST7 strains, even though ST25 and ST28 strains account for the majority of isolates in North America. While recognition of *S. suis* by innate immune cells has been traditionally associated with the MyD88-dependent Toll-like receptor (TLR) pathway *in vitro*, particularly surface-associated TLR2, little information is available regarding the role of MyD88 or TLRs *in vivo*. Consequently, the role of MyD88 signaling and the TLR pathway during the systemic and CNS infection by strains of various backgrounds and virulence was evaluated. This study demonstrates for the first time a differential role of the MyD88 signaling in *S. suis* serotype 2-induced systemic and CNS diseases, regardless of strain background. The MyD88-dependent pathway is critical for the development of *S. suis*-induced systemic disease via its role in inflammation, which subsequently controls bacterial burden. However, and differently from what has been described *in vitro*, TLR2 and TLR4 individually do not contribute to the systemic disease, suggesting possible compensation in their absence and/or a collaborative role with other MyD88-dependent TLRs. On the other hand, CNS disease induced by *S. suis* does not necessarily require MyD88 signaling and, consequently, neither TLR2 nor TLR4, suggesting a role of other pathways. Regardless of its notable heterogeneity, recognition of *S. suis* serotype 2 appears to be similar, indicating that recognized components are conserved motifs. As such, underlying mechanisms involved in inflammatory modulation and subsequent bacterial burden control could help develop measures against this pathogen.

Introduction

Streptococcus suis is an important porcine bacterial pathogen and a zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) [1, 2]. These pathologies are the result of an exacerbated and uncontrolled inflammation, which is the hallmark of the *S. suis* systemic and central nervous system (CNS) infections [3, 4]. Of the different serotypes described based on the presence of the capsular polysaccharide or its respective genes, serotype 2 is not only the most widespread, but also the most virulent [5]. However, serotype 2 strains are genetically and phenotypically heterogeneous, classified into a multitude of sequence types (STs) using multilocus sequence typing, whose distribution greatly varies worldwide [5]. As such, *S. suis* serotype 2 strains are grouped into different STs based on shared genetic similarities that better explain the evolutionary divergence of this pathogen [6]. Virulence of the most important STs (ST1, ST7, ST25, and ST28) has been evaluated using experimental animal models of infection (mouse and pig) [4, 7-10]. The ST7 strain responsible for the human outbreaks in China is highly virulent whereas Eurasian ST1 strains are virulent [7, 11]. On the other hand, ST25 strains, typically recovered in North America, are intermediately virulent [7, 10, 12]. Meanwhile, ST28 strains, which are prevalent in both North America (NA) and certain regions of Asia (AS), are generally low virulent and virulent, respectively [4, 10, 12, 13].

While a large number of putative virulence factors have been described for *S. suis*, the majority are not universally present [14-17]. Moreover, functional redundancy has made it difficult to dissect the precise roles of such factors in the *S. suis* pathogenesis and virulence [16]. These difficulties highlight an important problem faced in *S. suis* research: since factors involved in virulence differences between strains remain poorly understood, it is difficult to generalize its pathogenesis, including induced host response. In fact, most published studies on *S. suis* serotype 2 have used “classical” virulent European ST1 strains and/or ST7 strains [16]. Though little studied, the degree of strain virulence has been suggested to correlate with its capacity to induce exacerbated inflammation, disease and, subsequently, host death [18, 19]. In fact, *S. suis* ST1, ST7, ST25, and ST28 strains have been reported to differentially regulate inflammation *in vitro* and *in vivo* [4, 7-9].

Toll-like receptor (TLRs) are immune receptors involved in inflammation [20]. This family of evolutionarily conserved membrane-associated proteins are important sensors for the

detection of pathogen-associated molecular patterns [20]. Recognition of motifs particular to individual TLRs, which are located in the extracellular or endosomal membranes, results in their activation and initiation of a signaling cascade leading to cytokine production and inflammation [21]. This includes recruitment of adaptor proteins, of which myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing-interferon (IFN)-β (TRIF) are the most important [21]. With the exception of TLR3, which recruits TRIF, all TLRs signal via recruitment of MyD88, though TLR4 can utilize MyD88 and/or TRIF [21, 22]. While each TLR recognizes a series of motifs, only certain of these have been clearly identified, including lipopolysaccharide (LPS) of Gram-negative bacteria, which activates TLR4, diacylated and triacylated bacterial lipopeptides, which are recognized by TLR2 in cooperation with TLR6 and TLR1, respectively, and single-stranded RNA and unmethylated CpG sequences of DNA, recognized by TLR7 and TLR9, respectively [21, 22]. As such, TLRs establish a combinatorial repertoire to discriminate between different microbial products, allowing the host to induce innate immune and inflammatory responses required for microbial clearance and maintenance of homeostasis.

Recognition of *S. suis* by innate immune cells involves a multitude of membrane-associated and cytoplasmic receptors, of which TLRs have been best described, particularly with phagocytes, including dendritic cells (DCs) and monocytes/macrophages [14, 15]. *In vitro*, MyD88-deficiency results in abolition of pro-inflammatory cytokine production following infection of macrophages and DCs with *S. suis* [8, 23, 24]. Being a classical extracellular pathogen, recognition of *S. suis* has been mostly associated with surface TLRs, where TLR2, in cooperation with TLR6, plays a predominant role [23-25]. In the event of internalization, however, *S. suis* nucleic acids can activate endosomal TLR7 and TLR9 following bacterial degradation in a mechanism complementary to surface-associated receptor activation, which seems to be more common with ST25 strains [8]. Meanwhile, suilysin (SLY), a cholesterol-dependent cytolysin similar to the pneumoylsin of *Streptococcus pneumoniae* and produced by ST1 and ST7 strains, has been suggested to induce tumor necrosis factor (TNF) production following recognition by TLR4 [26]. By contrast, little information is available regarding the role of TLRs *in vivo*, as data are limited to a single study, which focused on TLR2. Therein, the role of TLR2 was suggested to be strain-dependent: while beneficial for host survival by contributing to the inflammation required for clearance of a virulent classical European ST1 strain, its participation during systemic infection with the highly virulent ST7 strain was minimal [27].

Finally, the role of TLRs, including TLR2, and MyD88 signaling in the CNS remains to be elucidated.

To further our knowledge of the *S. suis* pathogenesis, the role of TLRs and MyD88 signaling during the systemic and CNS infections by strains of various backgrounds and virulence were evaluated. Herein, we demonstrated for the first time a differential role of the MyD88 signaling in *S. suis* serotype 2-induced systemic and CNS diseases, regardless of strain background. The MyD88-dependent pathway is critical for the development of *S. suis*-induced systemic disease via its role in inflammation, which subsequently controls bacterial burden. However, and differently from what has been described *in vitro*, TLR2 and TLR4 individually do not contribute to the systemic disease, suggesting possible compensation in their absence and/or a collaborative role with other MyD88-dependent. On the other hand, *S. suis*-induced CNS disease does not necessarily require MyD88 signaling and, consequently, neither TLR2 nor TLR4, suggesting a role of other pathways.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol number rech-1570).

Mice

MyD88$^{-/-}$ (B6.129P2(SJL)-*MyD88*$^{tm1.Defr}$/J), TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmKir}/J), and TLR4$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ) mice on C57BL/6 background were purchased from Jackson Research Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions alongside wild-type counterparts (C57BL/6J).

Streptococcus suis *serotype 2 strains and growth conditions*

The different encapsulated and previously well-characterized *S. suis* serotype 2 strains used in this study, belonging to the most important STs worldwide (ST1, ST7, ST25, and ST28), are listed in **Table 1**. These comprise of a classical virulent European (United Kingdom) ST1 strain (P1/7), the highly virulent ST7 strain responsible for the 2005 Chinese human outbreak (SC84),

an intermediately virulent archetypal NA (Canada) ST25 strain (89-1591), and two ST28 strains, whether a virulent AS (Japan) strain (DAT254) or a low virulent prototype NA (Canada) strain (1088563). Virulence of the different strains was previously reported in experimental animal models of infection [4, 7]. *S. suis* strains were grown overnight on Columbia agar supplemented with 5% sheep blood (Oxoid, Nepean, ON) at 37 °C with 5% CO_2. Five milliliters of Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON) were inoculated with isolated colonies and incubated for 8 h at 37 °C with 5% CO_2. Working cultures were prepared by inoculating 30 mL of THB with 10 µL of a 10^{-3} dilution of the 8 h cultures and incubating for 16 h at 37 °C with 5% CO_2. Bacteria were washed twice with pH 7.3 phosphate-buffered saline (PBS) and resuspended in cell culture medium (*in vitro* assays) or THB (experimental mouse infections), appropriately diluted, and final colony-forming units (CFU)/mL determined by plating on THB agar (THA).

Generation of bone marrow-derived dendritic cells and activation

The femur and tibia of wild-type or knockout mice were used to generate bone marrow-derived DCs as previously described [8, 28]. Cell purity was confirmed to be at least 85% $CD11c^+$ by flow cytometry [8, 29]. Prior to infection, cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different *S. suis* serotype 2 strains listed in **Table 1** (10^6 CFU/mL; initial multiplicity of infection = 1). Conditions used were based on those previously published [8, 24, 29]. Supernatants were collected 16 h post-infection (p.i.), time at which secreted cytokine levels were maximal in the absence of *S. suis*-induced cytotoxicity, for quantification of TNF and interleukin (IL)-6 by sandwich enzyme-linked immunosorbent assay (ELISA) using pair-matched antibodies (R&D Systems, Minneapolis, MN), as previously described [24]. Non-infected cells served as negative controls. The TLR1/2 and TLR4 ligands PAM_3CSK_4 (1 µg/mL; InvivoGen, Burlington, ON) and ultrapure *Escherichia coli* LPS (100 ng/mL; InvivoGen), respectively, were used as positive controls.

Streptococcus suis *serotype 2 experimental infections*

Six-week-old male and female mice were used throughout this study. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [4, 30]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously

affected since mortality was not an endpoint measurement. For systemic virulence studies, the different *S. suis* strains were administered at doses of 5 x 10^6 CFU (non-lethal dose), 1 x 10^7 CFU (standard dose) or 5 x 10^7 CFU (lethal dose) by intraperitoneal inoculation to groups of 10 mice for survival and blood bacterial burden. Mice were monitored at least thrice daily until 72 h p.i. and twice thereafter until 14 d p.i. for clinical signs of systemic disease (rough coat hair, closed/swollen eyes, prostration, depression, difficulty breathing, and lethargy). Blood bacterial burden of surviving mice was assessed at different times by collecting 5 µL of blood from the caudal vein, appropriately diluting, and plating on THA as described above. Blood bacterial burden was also measured prior to euthanasia.

For the transcutaneal intracisternal model of CNS infection, groups of 5 to 10 mice were anesthetized with inhaled isofluorane (Pharmaceutical Partners of Canada, Richmond Hill, ON) and 10 µL of 1 x 10^3 CFU/mL to 1 x 10^7 CFU/mL (final concentrations of 10 CFU to 10^5 CFU) of the different *S. suis* strains were injected as previously described [4, 31]. Animals were monitored every 8 h until 72 h p.i. and twice thereafter until 7 d p.i. and euthanized at different pre-determined time points, upon presentation of clinical signs of CNS disease (spatial disorientation, hyper-excitement followed by opisthotonos, circular walking with head tilt, sudden spinning while in recumbence, and tonicoclonic movements) or at the end of the study. Controls (mock-infected) were injected with 10 µL of the vehicle solution (sterile THB).

Measurement of plasma (systemic) pro-inflammatory mediator levels

In parallel, 8 mice per group were intraperitoneally infected with the standard dose (1 x 10^7 CFU) for each strain. Twelve hours p.i., mice were euthanized and blood was collected by intracardiac puncture and anti-coagulated with EDTA (Sigma-Aldrich, Oakville, ON) as previously described [4, 7]. Plasma was collected following centrifugation at 10 000 x *g* for 10 min at 4 ºC and stored at -80 ºC. Plasmatic concentrations of TNF, IL-6, IL-12p70, IFN-γ, C-C motif chemokine ligand (CCL) 2, CCL3, CCL4, and C-X-C motif chemokine ligand (CXCL) 1 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Analysis of plasma biochemical parameters

Wild-type and MyD88$^{-/-}$ mice (n = 3) were either mock-infected (THB) or infected with the standard dose of *S. suis* ST1 strain P1/7 intraperitoneally. Twelve hours p.i., mice were euthanized and blood was collected by intracardiac puncture and anti-coagulated with sodium heparin (Sigma-Aldrich). Plasma was collected following centrifugation at 10 000 x *g* for 10 min at 4 ºC, pooled per group, and stored at -80 ºC. Plasmatic levels of creatinine, aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) were measured using standard colorimetric assays.

Brain histopathological studies

Upon presentation of clinical signs of CNS disease or at the end of the study, animals were euthanized and brains recovered and fixed in 10% buffered formalin. After paraffin embedding, 4 µm-thick sections of the brain were stained with hematoxylin phloxine saffron following standard protocol and examined under light microscopy. Brains were examined for presence or absence of histopathological lesions of *S. suis*-induced CNS disease (massive suppuration, leukocyte infiltration, multifocal gliosis, hemorrhages, and necrosis) only.

Measurement of brain bacterial burden and pro-inflammatory mediator levels

Samples were collected upon presentation of clinical signs of CNS disease (within 24 h p.i.) for virulent strains and 24 h p.i., 72 h p.i. or upon presentation of clinical signs of CNS disease (up to 7 d p.i.) for the low virulent NA ST28 strain 1088563. Following euthanasia, brains were aseptically recovered and homogenized in PBS, from which bacterial burdens were determined by plating appropriate dilutions on THA as described above, or frozen in liquid nitrogen. For pro-inflammatory mediator evaluation, extraction buffer, prepared using cOmplete Mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, supplemented with 0.4% CHAPS (Sigma-Aldrich), was added to frozen brains, which were then homogenized using a POLYTRON PT 1200E system bundle (Kinematica, Lucerne, Switzerland). Brain homogenate supernatants were collected following centrifugation at 10 000 x *g* for 10 min at 4 °C and stored at -80 °C. Levels of IL-1β, IL-6, CCL2, CCL3, and CXCL1 were measured by ELISA as described above.

Statistical analyses

Normality of data distribution was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test and one-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test and one-way ANOVA on ranks) were performed to evaluate statistical differences between groups. Data are presented as mean ± standard error of the mean (SEM) or as geometric mean. Log-rank (Mantel-Cox) tests were used to compare survival between wild-type and knockout mice. $p < 0.01$ was considered statistically significant.

Results

Recognition of Streptococcus suis *by the MyD88-dependent pathway is required for pro-inflammatory mediator production by dendritic cells regardless of strain background*

Of the different innate immune cells, DCs are an important source of pro-inflammatory mediators following infection with *S. suis* [8, 24, 29]. Although the role of the TLR pathway in recognition of *S. suis* and induction of pro-inflammatory mediators *in vitro* has been somewhat dissected [8, 24], little information is available regarding the pathways involved in cell activation by strains from different backgrounds. A near complete abolition of TNF and IL-6 production was observed in absence of the adaptor protein MyD88, regardless of strain background ($p < 0.001$) (**Fig. 1A-B**). Meanwhile, TLR2 was partially implicated following infection with the different *S. suis* strains tested ($p < 0.01$) (**Fig. 1A-B**). By contrast, TNF and IL-6 production was TLR4-independent, regardless of strain background and capacity to secrete SLY. This result was validated using the TLR4 ligand LPS, for which a significant decrease of TNF or IL-6 production was observed in $TLR4^{-/-}$ DCs ($p < 0.001$) (**Fig. 1A-B**).

MyD88 signaling is required for host survival during Streptococcus suis *systemic infection: Modulation of systemic inflammation required for controlling blood bacterial burden*

Previous *in vitro* results suggested that the MyD88-dependent pathway might participate in modulation of the *S. suis*-induced systemic inflammation, and subsequently, host survival. Consequently, the role of MyD88 signaling in acute *S. suis* systemic infection caused by strains of different backgrounds and virulence was evaluated using a standard bacterial dose of 1 x 10^7 CFU, comparing wild-type and $MyD88^{-/-}$ mice. With the exception of the low virulent NA ST28 strain 1088563, at least 90% of $MyD88^{-/-}$ mice infected with strains P1/7 (ST1), SC84 (ST7), 89-1591 (ST25), and DAT254 (AS ST28) succumbed within 24 h of infection, with 100%

of mortality within, at the latest, 36 h (**Fig. 2A-D**). This significant difference in survival between wild-type and MyD88$^{-/-}$ mice ($p < 0.001$), both in the number of mice succumbing to infection and its speed, suggest a critical role of MyD88 signaling in host survival. By contrast, no clear role was initially observed upon infection with the low virulent NA ST28 strain 1088563, which did not induce acute clinical disease in wild-type mice and caused death in only 10% of MyD88$^{-/-}$ mice 72 h p.i. (**Fig. 2E**). Hence, survival of mice was evaluated until 14 d p.i. (**Fig. 2F**). By 4 d p.i., mortality of MyD88$^{-/-}$ mice infected with the NA ST28 strain 1088563 radically increased, with 50% of mice having succumbed to infection, before reaching 70% of mortality 10 d p.i. (**Fig. 2F**). By contrast, none of the wild-type mice succumbed to infection ($p < 0.001$) (**Fig. 2F**), suggesting that MyD88 signaling is required for host survival even following infection with lower virulence strains.

It has been suggested that host death during *S. suis* systemic infection is usually the result of an exacerbated systemic inflammatory response [3, 4]. As such, the role of MyD88 signaling on aggravated inflammation was evaluated by measuring plasma (systemic) mediators from wild-type and MyD88$^{-/-}$ mice 12 h p.i. As expected, the ST7 strain induced the highest levels of pro-inflammatory cytokines in wild-type mice, followed by the ST1 strain (**Fig. 3A-H**). The ST25 and AS ST28 strains induced intermediate levels of these mediators, whereas animals infected with the NA ST28 strain presented very low levels (**Fig. 3A-H**). Interestingly, *S. suis*-induced TNF, IL-6, IL-12p70, IFN-γ, CCL2, CCL3, CCL4, and CXCL1 production was almost completely abrogated in MyD88$^{-/-}$ mice, when compared with wild-type mice, and this for all strains ($p < 0.01$) (**Fig. 3A-H**).

Although aggravated inflammation may be a cause of death in wild-type mice, the near complete absence of inflammatory mediators in MyD88$^{-/-}$ mice may have an influence on control of bacteremia and, subsequently, host survival [4]. To study this hypothesis, bacteremia was evaluated in wild-type and MyD88$^{-/-}$ mice after infection with *S. suis*. In accordance, blood bacterial burden of MyD88$^{-/-}$ mice was significantly greater than that of wild-type mice 12 h p.i. for all strains tested ($p < 0.001$) (**Fig. 4A-E**). In fact, levels in MyD88$^{-/-}$ mice were extremely elevated, averaging 1 x 10^9 CFU/mL for the different strains, with exception of the low virulent NA ST28 strain 1088563, where levels averaged 5 x 10^7 CFU/mL, which were nonetheless greater than that of wild-type mice by 1 000-fold (**Fig. 4A-E**). Consequently, MyD88 signaling

is crucial for induction of systemic pro-inflammatory mediators that participate in clearance of *S. suis* from the bloodstream.

This critical role of MyD88 signaling in host survival via induction of inflammation required for control of blood bacterial burden was observed using a standard dose of *S. suis* causing disease. However, it was unknown whether this role was dependent on bacterial dose. As such, wild-type and MyD88$^{-/-}$ mice were infected with a non-lethal dose of *S. suis* (5 x 10^6 CFU). Under these conditions, none of the wild-type mice infected with the ST1 strain P1/7, ST7 strain SC84, ST25 strain 89-1591 or AS ST28 strain DAT254 presented clinical signs of infection or mortality (**Figure S1 in Supplementary Material**). By contrast, 100% of MyD88$^{-/-}$ mice succumbed to infection within 60 h p.i, ($p < 0.001$) (**Figure S1 in Supplementary Material**). Though mortality of MyD88$^{-/-}$ mice was generally delayed by 24 h following infection with a non-lethal dose, in comparison to the standard dose, all mice still succumbed to infection, demonstrating the importance of the role played by MyD88 signaling following systemic infection by this pathogen.

As with the standard dose, MyD88$^{-/-}$ mice infected with the non-lethal dose of the different *S. suis* strains were unable to control blood bacterial burden 12 h p.i. (**Figure S2 in Supplementary Material**). Not only were these levels significantly greater than those of wild-type mice ($p < 0.001$), but they were also similar to those obtained following infection with the standard dose (**Figure S2 in Supplementary Material**).

Mortality of* Streptococcus suis*-infected MyD88$^{-/-}$ mice is not due to inflammation-induced tissue/organ damage, unlike in wild-type mice

Unlike during infection of wild-type mice, mortality of MyD88$^{-/-}$ mice was not the consequence of exacerbated inflammation. In accordance, MyD88$^{-/-}$ mice presented no clinical signs of systemic disease following infection with the different *S. suis* strains prior to succumbing to infection (data not shown). Consequently, mortality of MyD88$^{-/-}$ mice was hypothesized to be caused by direct tissue damage due to the elevated systemic levels of *S. suis*. As such, plasmatic levels of biomarkers associated with tissue integrity or function, including creatinine (glomerular filtration), ALT (hepatocellular damage), and AST and LDH (associated with hepatocellular and muscle damage) were measured 12 h p.i. with the virulent ST1 strain P1/7. *S. suis* infection greatly increased levels of these biomarkers in wild-type mice: plasma creatinine levels were increased 2-fold, AST levels 20-fold, ALT levels 80-fold, and LDH levels

50-fold (**Table 2**). By contrast, levels remained generally unaltered in MyD88$^{-/-}$ mice following infection with *S. suis,* with exception of a 5-fold increase for LDH (**Table 2**). Together, these results suggest that MyD88$^{-/-}$ mice mice did not succumb to important inflammation-induced tissue damage characteristic of septic shock and organ failure, though the exact cause of death remains unknown.

Non-crucial role of Toll-like receptors 2 and 4 in* Streptococcus suis *systemic infection: Minor participation in modulation of systemic inflammation, blood bacterial burden, and host survival

Of the different MyD88-dependent TLRs, the surface-associated TLR2 was demonstrated to be important for recognition of *S. suis in vitro* [23, 24]. Alongside, *S. suis* SLY has been suggested to be recognized by TLR4 [26]. Consequently, implication of TLR2 and TLR4 in *S. suis* systemic infection was evaluated using strains of different backgrounds, of which the ST1 and ST7, but not the ST25 and ST28, produce SLY [32]. Using a standard dose of 1 x 10^7 CFU via intraperitoneal inoculation, no significant differences were observed between survival of wild-type and TLR2$^{-/-}$ or TLR4$^{-/-}$ mice during the acute systemic infection (until 72 h p.i.), and this regardless of the *S. suis* strain inoculated (**Fig. 5A-E**). Interestingly, similar results were also obtained when using a non-lethal (low) dose of 5 x 10^6 CFU and a lethal (high) dose of 5 x 10^7 CFU (**Figure S3 in Supplementary Material**).

To understand the role of these receptors in systemic inflammation, plasma mediators from *S. suis*-infected wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were measured. In accordance, TLR2 and TLR4 individually did not participate in *S. suis*-induced production of TNF, IL-6, IL-12p70, IFN-γ, CCL2, CCL3, CCL4, and CXCL1, regardless of the strain tested (**Fig. 6A-H**). Moreover, blood bacterial burden levels were also similar between wild-type and deficient mice 12 h p.i. with the standard (**Fig. 7A-E**), non-lethal, and lethal doses (**Figure S4 in Supplementary Material**), and this regardless of strain background. Similar results in blood bacterial burden were also observed 24 h, 48 h, and 72 h p.i. in surviving mice (data not shown). These results suggest that individually, implication of TLR2 and TLR4 in *S. suis* systemic infection is minor, and this regardless of inoculum dose. However, it is impossible to exclude a role in preventing *S. suis* from invading the epithelium and reaching the systemic circulation (bypassed in this model).

Partial implication of the MyD88 signaling in* Streptococcus suis*-induced central nervous system inflammation but not in brain burden control

Following acute *S. suis* systemic infection, surviving individuals are susceptible of developing a CNS disease at later times characterized by meningitis accompanied or not by encephalitis (meningoencephalitis) [3, 4]. In order to determine the role of MyD88 signaling, TLR2, and TLR4 in the development of *S. suis*-induced CNS disease, mice were inoculated via the transcutaneal intracisternal route directly into the cisterna magna: this well-developed model results in rapid development of clinical signs and histopathological lesions of CNS disease [4, 31]. Wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were either mock-infected (THB) or infected with a standard dose of 1 x 10^5 CFU of the different *S. suis* strains tested [4, 31]. Mice were evaluated for the development of clinical signs of CNS disease at different times following infection until the end of the experiment (7 d p.i.). Since mice were euthanized upon presentation of clinical signs, data are presented as survival curves. None of the mock-infected mice developed clinical signs of infection (data not shown) nor histopathological lesions (**Figure S5A-B in Supplementary Material**). By contrast, 100% of wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice infected with virulent strains (ST1 strain P1/7, ST7 strain SC84, ST25 strain 89-1591, and AS ST28 strain DAT254) all developed clinical signs of CNS disease within 24 h p.i. (**Fig. 8A-D**), which were confirmed by histopathology (data not shown). Indeed, wild-type and MyD88$^{-/-}$ mice infected with the virulent ST1 strain P1/7 presented similar classical histopathological lesions of CNS infection, including massive suppuration, leukocyte infiltration, multifocal gliosis, hemorrhages, and necrosis (**Figure S5C-D in Supplementary Material**).

Development of CNS disease by *S. suis* is believed to be the result of bacterial replication in the CNS [4]. Upon presentation of clinical signs of CNS disease induced by virulent *S. suis* strains (ST1 strain P1/7, ST7 strain SC84, ST25 strain 89-1591, and AS ST28 strain DAT254), similarly elevated brain bacterial burdens were observed in wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (**Fig. 9A-D**).

Results with the low virulent NA ST28 strain 1088563 were different from those obtained with more virulent strains. None of the wild-type or knockout mice presented any clinical signs of CNS disease 24 h or 72 h (3 d) p.i. (**Fig. 8E-F**). In addition, brain bacterial burden was barely or altogether undetectable in wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 24 h and 72 h p.i. (**Fig. 9E-F**). On the other hand, brain bacterial burden of MyD88$^{-/-}$ mice, though not statistically different

from that of wild-type mice 24 h p.i. (**Fig. 9E**), was significantly greater 72 h p.i. ($p < 0.001$), still in the absence of clinical signs of CNS disease (**Fig. 9F**). Five days p.i., however, certain MyD88$^{-/-}$ mice presented clinical signs of CNS disease, with 100% of MyD88$^{-/-}$ mice euthanized within 7 d p.i. (**Fig. 8F**). These animals presented similar histopathological lesions to mice infected with higher virulence strains, confirming observed clinical signs (**Figure S5E in Supplementary Material**). Furthermore, brain bacterial burden of these MyD88$^{-/-}$ mice averaged 7 x 10^7 CFU, similar to that of MyD88$^{-/-}$ mice infected with more virulent *S. suis* strains (**Fig. 9G**). Meanwhile, all wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice infected with the low virulent NA ST28 strain 1088563 remained healthy even 7 d p.i. (**Fig. 8F)**.

Presence of *S. suis* in the CNS has been suggested to be responsible for inducing a local pro-inflammatory response [4]. Consequently, given the brain bacterial burden following intracisternal inoculation of the different *S. suis* strains tested, brain pro-inflammatory mediators were measured 24 h p.i., time at which mice infected with the virulent strains presented clinical signs of CNS disease (**Fig. 10**). Levels of IL-1β, IL-6, CCL2, CCL3, and CXCL1 in the CNS of wild-type mice were similarly high between virulent strains, with the exception of CCL2 and CXCL1, of which the ST7 strain SC84 induced the highest levels (**Fig. 10A-E**). On the other hand, the low virulent NA ST28 strain 1088563 induced little pro-inflammatory mediators 24 h p.i., regardless of the mouse genotype (**Fig. 10A-E**). By contrast, levels of these mediators were significantly decreased in MyD88$^{-/-}$ mice (equivalent to at least an 80% reduction) ($p < 0.001$), regardless of the virulent strain inoculated, but not completely abrogated, suggesting a partial role of MyD88-independent signaling in induction of *S. suis* CNS inflammation (**Fig. 10A-E**).

Since the development of clinical signs of CNS disease induced by the low virulent NA ST28 strain required more time than the other strains, brain pro-inflammatory mediators induced by this strain were also evaluated in wild-type and MyD88$^{-/-}$ mice at later times. Levels increased with time for all pro-inflammatory mediators and were, with the exception of CXCL1, significantly greater in MyD88$^{-/-}$ mice 72 h p.i. ($p < 0.01$) (**Fig. 10F**). Upon presentation of clinical signs of CNS disease, levels of IL-1β, IL-6, CCL2, CCL3, and CXCL1 were significantly greater in MyD88$^{-/-}$ than in wild-type mice ($p < 0.001$) (**Fig. 10F**). In fact, these levels were similar to those observed in MyD88$^{-/-}$ mice infected with the virulent *S. suis* strains tested (P1/7, SC84, 89-1591, and DAT254) (**Fig. 10A-D**). These results confirm that though MyD88 signaling

participates in *S. suis*-induced CNS inflammation, the contribution of MyD88-independent pathways is sufficient to trigger the release of inflammatory mediators responsible for disease.

The central nervous system is extremely susceptible to* Streptococcus suis *infection

Results following intracisternal inoculation with a standard dose of 1 x 10^5 CFU of the virulent *S. suis* strains demonstrated an excessively rapid replication of *S. suis* in the CNS, with brain burdens quickly saturating at approximately 1 x 10^8 CFU. Consequently, using the virulent ST1 strain P1/7 as a representative strain, wild-type mice were infected via the intracisternal route with different doses ranging from 10 CFU to 10^5 CFU (standard dose) and brain bacterial burden was evaluated. Mice infected with doses ranging between 10^2 CFU and 10^5 CFU presented clinical signs of CNS disease within 24 h p.i. and brain bacterial burdens rapidly saturated, averaging 5 x 10^7 CFU (**Fig. 11A**). Although animals infected with only 10 CFU presented brain bacterial burdens averaging 50 CFU 24 h p.i. (**Fig. 11B**), those unable to rapidly clear bacteria eventually developed clinical signs of CNS disease, usually between 48 h and 72 h p.i. By this time, brain bacterial burdens also averaged 5 x 10^7 CFU (**Fig. 11B**). Development of CNS disease in wild-type mice inoculated with 10 CFU was confirmed by histopathology, in which lesions of CNS disease, including brain suppuration and leukocyte infiltration were observed, unlike in mock-infected mice (**Fig. 11C-D**). Consequently, these results demonstrate that the CNS is extremely susceptible to *S. suis* infection.

Based on the above results, wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were inoculated with 10 CFU of the virulent ST1 strain P1/7 via the intracisternal route and development of clinical CNS disease evaluated. Wild-type and all knockout mice developed similar clinical CNS disease, with 50% to 60% of mice presenting clinical signs between 48 h and 72 h p.i. (**Fig. 12A**). Interestingly, bacterial burden was significantly more elevated in the CNS of MyD88$^{-/-}$ than that of their wild-type counterparts 24 h p.i. ($p < 0.001$), but no significant differences were observed between wild-type and MyD88$^{-/-}$, TLR2$^{-/-}$ or TLR4$^{-/-}$ mice upon presentation of clinical disease (**Fig. 12B)**. As expected, mice that did not develop clinical CNS presented no brain bacterial burden (**Fig. 12B**).

Meanwhile, levels of IL-1β, IL-6, CCL2, CCL3, and CXCL1 in the CNS of wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice infected with 10 CFU of strain P1/7 were similar upon presentation of clinical disease, but significantly greater than those of MyD88$^{-/-}$ mice ($p < 0.01$) (**Figure S6 in**

Supplementary Material). Taken together, these results suggest that while MyD88 signaling contributes to the initiation of inflammation, if *S. suis* is not rapidly cleared, exacerbation of CNS inflammation ensues as MyD88-independent pathways are sufficient to cause *S. suis*-induced CNS disease, regardless of the inoculated bacterial dose.

Discussion

S. suis serotype 2 is an important porcine and human pathogen whose recognition has been traditionally associated with surface-associated TLR2 [23, 24]. More recently, recognition of its nucleic acids by the endosomal TLR7 and TLR9, in the event of internalization, has also been described, while a secreted cytolysin, SLY, might be recognized by TLR4 [8, 24, 26]. Though the MyD88-dependent TLR pathway was suggested to be important for recognition of *S. suis in vitro* and induction of inflammation, information regarding its role *in vivo* is very limited, and this, regardless of strain background.

Consequently, we began by evaluating the involvement of MyD88-dependent TLR pathway in *S. suis* recognition *in vitro* using DCs as an innate immune cell model. As previously described, TNF and IL-6 production by DCs was MyD88-dependent and partially TLR2-dependent following infection with a European ST1 strain [24]. In fact, this was the case for the different *S. suis* strains tested, regardless of background and virulence, indicating that while *S. suis* serotype 2 strains are genetically and phenotypically divergent, the bacterial components recognized by the MyD88-dependent pathway, including by TLR2, are relatively well-conserved. Indeed, though *S. suis* strains induce differential inflammatory host responses, as observed for levels of TNF and IL-6, the evolutionary conservation of innate sensors such as TLRs indicates that recognized motifs are much less divergent than other bacterial components, as for example, virulence factors [20]. Consequently, initial recognition by innate immune cells is probably similar between strains, while differentially expressed virulence factors subsequently modulate the induced host response.

Being a Gram-positive bacterium, recognition of *S. suis* is not usually associated with TLR4. ST1 and ST7 strains, but not ST25 and ST28 strains, secrete the toxin SLY, which is closely related to the *S. pneumoniae* pneumolysin. It was recently suggested that SLY is recognized by TLR4 in murine peritoneal macrophages [26]. Moreover, recognition of SLY by TLR4 also contributes to IFN-β production by DCs, albeit minorly [8]. Meanwhile, the minor participation

of TLR4 in CXCL1 production by DCs following infection with a virulent ST1 strain is independent of SLY [24]. Using live bacteria, we were unable to observe any role of TLR4, regardless of SLY production. These differences might be due to cell type (DCs versus peritoneal macrophages) and/or to the use of live bacteria versus SLY alone. The former contains numerous immunostimulatory components making it difficult to dissect the role of SLY alone, especially if this role is minor.

In vitro assays do not always reflect *in vivo* conditions for *S. suis* [4, 33]. Upon entering the bloodstream, *S. suis* is capable of inducing a systemic infection characterized by sepsis and septic shock [3, 4, 7]. Though inflammation is the hallmark of *S. suis* infection and is responsible for clinical signs and mortality, the pathways involved remain generally unknown. The near complete abrogation of inflammation in MyD88$^{-/-}$ mice indicates that this adaptor protein and its downstream signaling participate directly or indirectly in exacerbation of *S. suis*-induced inflammation. However, MyD88$^{-/-}$ mice presented high mortality after *S. suis* infection with a standard dose due to an uncontrolled increase in bacterial burden. Indeed, systemic bacterial replication occurred unchecked: average blood bacterial burden of surviving MyD88$^{-/-}$ mice 12 h p.i. was already high and similar to that recovered upon euthanasia of mice suffering from septic shock. Requirement of MyD88 signaling was so crucial for clearance of *S. suis* from the systemic compartment that even non-lethal doses of virulent strains caused 100% of mortality in MyD88$^{-/-}$ mice. In this regard, *S. suis* behaves differently from Group B *Streptococcus* (GBS), during infection of which the role of MyD88 signaling is dependent on the bacterial dose inoculated [34]. Confirming results obtained with virulent strains, the low virulent NA ST28 strain, incapable of inducing disease in wild-type mice, induced disease for the first time, but only in MyD88$^{-/-}$ mice, due to an inability of the host to control bacteremia. It has been suggested that a certain level of induced inflammatory mediators are required to control blood bacterial burden by participating in bacterial clearance [4]. Results from this study show that abrogated inflammation in the absence of MyD88 is as detrimental as exacerbated inflammation, which is usually responsible for host death. Consequently, as with most inflammatory diseases, balanced inflammation is required for the host to overcome *S. suis* infection, since too little inflammation results in uncontrolled bacterial replication on the one hand, while exacerbated inflammation causes tissue damage and organ failure on the other [3, 30]. However, results suggest that contribution of MyD88 signaling to *S. suis* serotype 2 pathogenesis does not depend on strain background or virulence.

Though MyD88$^{-/-}$ mice rapidly succumbed to infection, levels of systemic pro-inflammatory mediators were relatively low and mice lacked clinical signs of septic shock, suggesting that death was probably not due to inflammation-induced organ damage [3, 4]. Consequently, it was hypothesized that the elevated blood bacterial burdens of MyD88$^{-/-}$ mice, in which bacteria outnumbered leukocytes by at least 100-fold, could be directly responsible for tissue damage and organ failure. The AST/ALT ratio (ALT levels being greater than AST levels), alongside the elevated LDH levels and the fact that the ALT levels themselves were greater than 1000 U/L in wild-type mice indicate severe liver damage following *S. suis* infection, while increased creatinine indicates decreased glomerular filtration rate suggesting kidney damage and/or pre-renal problems such as severe dehydration and heart failure [35]. Though hepatocellular and muscle damage are the two main sources of plasmatic AST and LDH, severe hemolysis can also be responsible [36]. In fact, strain P1/7 possesses SLY, a well-described hemoylsin [37]. As such, plasmatic levels of these biomarkers strongly suggest that death of wild-type mice was the consequence of septic shock and organ failure due to the pro-inflammatory cytokine storm, similar to what is usually described in human cases of *S. suis* infection [35, 38, 39]. To our knowledge, this is the first report of evaluation of these biomarkers in an animal model of *S. suis* infection. By contrast, levels of these markers remained generally unaltered in MyD88$^{-/-}$ mice following infection with *S. suis*, confirming that host death in absence of MyD88 was not caused by inflammation-induced tissue and organ damage. As such, though the elevated bacterial burdens themselves are probably responsible for host death in MyD88$^{-/-}$ mice, the precise cause of death remains unknown. Similarly, even though MyD88$^{-/-}$ mice presented greater blood bacterial burden than wild-type mice following *S. pneumoniae* infection, no exact cause of death could be identified [40]. As such, further studies are required to better understand this aspect of *S. suis* infection.

The crucial role of MyD88 signaling in *S. suis* systemic infection suggested an involvement of the TLR pathway, of which surface-associated TLR2 was demonstrated to be important for recognition of *S. suis in vitro*, including in this study [23, 24]. While Lachance *et al*. previously reported that the role of TLR2 in *S. suis*-induced systemic inflammation was strain-dependent [27], we were unable to observe any role of TLR2 in host survival, systemic inflammation, and blood bacterial burden control, regardless of strain background, and this also using a standard dose. Given these differences in results, infections of wild-type and TLR2$^{-/-}$ mice with ST1 strain

P1/7 were repeated twice with similar results being obtained (data not shown). Interestingly, results obtained with *S. suis* differed from those reported for other streptococci, including GBS and *S. pneumoniae* [34, 41]. Furthermore, and unlike for GBS [34], this lack of implication of TLR2 in the *S. suis* systemic infection was not dependent on bacterial dose inoculated, since no involvement of this receptor was observed with non-lethal, standard, and lethal doses. It is worth mentioning that the dose-dependent role of TLR2 in GBS, reported using a serotype V strain, was not observed using a serotype IV strain, where TLR2-deficiency was always associated with a detrimental role and increased host death [34, 42]. Taken together, these results imply that the role of TLR2 in the *S. suis* systemic infection is far more complex than previously determined. In agreement with *in vitro* results, no role of TLR4 was observed in *S. suis* systemic infection regardless of strain background, capacity to produce SLY, and bacterial dose inoculated. Again, these results are different from those previously observed with *S. pneumoniae* [43, 44]. In the case of *S. suis*, although SLY might be recognized by TLR4, its overall contribution to the *S. suis* pathogenesis and induced host response appears to be minimal. However, an effect due to differences in the models used such as route of inoculation (intraperitoneal vs. intravenous), the mouse strain, and the age of the animals cannot be excluded.

The critical role of MyD88 signaling, but non-crucial implication of TLR2 and TLR4, during *S. suis* systemic infection is quite interesting and somewhat surprising in comparison with other streptococci. These results suggest different possibilities with regards to the role of MyD88-dependent TLRs in *S. suis* pathogenesis. Firstly, in addition to TLR2 and TLR4, at least half a dozen other TLRs are MyD88-dependent [21]. While TLR5 has not yet been described to recognize *S. suis* (flagellin being a classical ligand) [20], TLR7 and TLR9 recognize *S. suis* nucleic acids [8, 24]. Alongside, recognition of GBS ribosomal RNA by endosomal TLR13 is important for induction of pro-inflammatory mediators by macrophages [45, 46]. Consequently, it is possible that one or more of these additional MyD88-dependent TLRs are implicated in recognition of *S. suis in vivo* and induction of inflammation. Secondly, it is possible that unlike with GBS or *S. pneumoniae,* recognition of *S. suis* does not depend on a single TLR, but rather on the simultaneous collaboration of different MyD88-dependent TLRs, such that individually their role is only minimal. Finally, it is possible that absence of a single TLR is compensated by others still present. In fact, it was demonstrated that though TLR13 participates in GBS recognition, its function can be compensated by other endosomal TLRs [45].

Following *S. suis* systemic infection, surviving individuals are susceptible of developing CNS disease [3, 4]. Brain bacterial burden, local CNS inflammation, and development of clinical CNS signs were relatively similar between the different virulent *S. suis* strains tested as previously reported [4], suggesting, as during the systemic infection, a commonality in *S. suis* pathogenesis. However, unlike with other meningitis-causing bacterial pathogens, the quantity of *S. suis* required to cause CNS disease remains unknown. In fact, *S. suis* replication in the CNS of wild-type mice appeared to saturate at approximately 5 x 10^7 CFU within 24 h, and this even when initially inoculating 100 CFU. Only with the use of 10 CFU was it possible to observe lower bacterial burdens 24 h p.i.; upon presentation of clinical signs, however, bacterial loads were once again similar to those obtained using higher inoculums, indicating that the CNS is extremely susceptible to *S. suis*. In fact, it was previously reported that survival of a single intracellular *Haemophilus influenzae* may result in meningitis [47]. Consequently, this minimal bacterial quantity might be a characteristic of meningitis-causing bacterial pathogens and merits further studies in order to understand the level of risk associated with *S. suis* infection and development of CNS disease, especially since meningitis is the most common clinical feature of *S. suis* infection in both pigs and humans [1].

By contrast to the systemic compartment, MyD88 signaling was not necessarily required for development of clinical CNS signs following *S. suis* infection. To our knowledge, this is the first report of a non-critical role of MyD88 signaling in the development of clinical bacterial CNS signs and results contrast with the meningitis-causing pathogens *S. pneumoniae* and *Escherichia coli* K1 [40, 48]. Since elevated levels of bacteria in the CNS result in activation of immune cells and development of inflammation responsible for clinical signs, these aspects were further studied. Elevated brain bacterial burdens were observed upon presentation of clinical CNS signs following infection with virulent *S. suis* strains in wild-type and MyD88$^{-/-}$ mice, regardless of inoculated dose and strain background. However, prior to the development of clinical signs (24 h p.i.), brain bacterial burden of MyD88$^{-/-}$ mice was significantly greater than that of wild-type counterparts. As such, though not critical for development of clinical CNS signs, MyD88 signaling somehow participates in the initial response to clear virulent *S. suis* strains (from all backgrounds) from the CNS, even with lower inoculums. These observations are confirmed with results obtained with the low virulence NA ST28 strain: participation of MyD88 signaling is sufficient to completely clear this strain from the CNS. In fact, only in the

absence of MyD88 did clinical CNS signs develop and, unlike in wild-type mice in which bacteria are rapidly cleared, persistence of the NA ST28 strain in MyD88$^{-/-}$ mice resulted in activation of MyD88-independent pathways, inflammation and development of clinical signs. Interestingly, the low virulent NA ST28 strain was previously demonstrated to be less resistant to the bactericidal effect of whole blood [4]. This role of the MyD88 signaling in eliciting the initial response required for rapid clearance was also reported during *Staphylococcus aureus* CNS infection and brain abscess development [49].

The significant decrease in CNS inflammation in MyD88$^{-/-}$ mice following infection with virulent *S. suis* strains suggests that MyD88 signaling participates, if the pathogen is not rapidly cleared from the CNS, in exacerbation of inflammation induced by MyD88-independent pathways. In fact, and most unexpectedly, MyD88-independent inflammation alone was sufficient to cause *S. suis*-induced CNS signs. In accordance, involvement of MyD88-independent pathways has been previously reported in the host CNS response to *S. pneumoniae*, *S. aureus*, *Neisseria meningitidis*, and *Borrelia burgdorferi* [50-52]. Furthermore, the relatively low levels of CNS inflammation upon development of clinical signs, in comparison to during systemic infection, suggest that levels of inflammation required to cause CNS disease are lower, possibly due to their remaining locally. This supports previous results reporting clinical signs and histopathological lesions of CNS disease even though CNS levels of pro-inflammatory mediators were low [4]. Moreover, we previously demonstrated that *S. suis*-induced CNS inflammation remains compartmentalized due to lack of leakage into the systemic compartment, even upon presentation of clinical CNS signs [4]. Finally, differences in cell type composition between the systemic and CNS compartments might be responsible for the differential role of MyD88 signaling in the *S. suis* systemic and CNS diseases. Indeed, an organ-dependent role of MyD88 signaling was reported during polymicrobial peritonitis [53]. However, it is impossible to exclude that the clinical CNS signs observed are due to inflammation alone, and not by *S. suis*-induced tissue damage. Taken together, these results suggest that while MyD88 signaling participates in the initial response involved in clearance of *S. suis* (efficient at clearing low virulent but not virulent strains), MyD88-independent pathways are involved in inflammation causing clinical signs, which MyD88 signaling exacerbates.

Regardless of the involvement of MyD88 signaling in initiating CNS bacterial clearance, TLR2 and TLR4 were not individually implicated in this activity. However, due to the limited overall

role of MyD88 signaling in inflammation-related clinical CNS signs, results showing no implication of TLR2 and TLR4 were expected. Interestingly, an upregulation of TLR2 (but not TLR4) mRNA expression was observed in the CNS of mice following *S. suis* infection [3], but expression levels do not necessarily correlate with an actual implication. Results obtained in this study with *S. suis* contrast with those obtained with *S. pneumoniae*, where a certain implication of such receptors in brain bacterial burden control and clinical disease were reported [41, 54, 55]. Consequently, the lack of implication of TLR2 and TLR4 observed in this study could, as during the systemic infection, be the result of compensation by other MyD88-dependent receptors. Indeed, while TLR4-deficiency alone had no significant impact on development of *S. pneumoniae*-induced CNS disease, TLR2/4-deficiency resulted in attenuated immune response and worsening of disease, suggesting a synergistic role of TLR2 and TLR4 in *S. pneumoniae* recognition *in vivo* [56]. Similarly, receptor redundancy for *S. aureus* detection in the CNS was also reported [57], which might also be the case for *S. suis.*

In conclusion, and in contrast with other bacterial pathogens, this study demonstrates for the first time a differential role of MyD88 signaling in *S. suis* serotype 2-induced systemic and CNS diseases, and this regardless of strain background. Though MyD88 signaling is critical for the development of *S. suis* systemic inflammation, MyD88-independent inflammation is sufficient to cause CNS disease. By contrast, TLR2 and TLR4 alone do not contribute to *S. suis*-induced systemic or CNS inflammation, possibly suggesting compensation in their absence and/or a collaborative role of other MyD88-dependent TLRs during *S. suis* infection. These results indicate that alongside MyD88 signaling, the role of other receptors and pathways in recognition of *S. suis* and induction of inflammation should be evaluated. Though serotype 2 strains are heterogeneous and of varying virulence, their recognition by the MyD88-dependent pathway appears to be similar, indicating that the recognized components are conserved motifs. As such, underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic pathogen.

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Abbreviations

ALT, alanine transaminase; AS, Asia; AST, aspartate transaminase; CCL, C-C motif chemokine ligand; CFU, colony-forming unit; CNS, central nervous system; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; GBS, Group B *Streptococcus*; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response 88; p.i., post-infection; NA, North America; PBS, phosphate-buffered saline; SEM, standard error of the mean; SLY, suilysin; ST, sequence type; THA, Todd Hewitt broth agar; THB, Todd Hewitt broth; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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Tables

Table 1. List of *Streptococcus suis* serotype 2 strains used in this study.

Strain	General characteristics	Reference
P1/7	Classical virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	[58]
SC84	Highly virulent clonal ST7 strain isolated from a case of human streptococcal toxic shock-like syndrome during the 2005 outbreak in China	[59]
89-1591	Intermediately virulent North American ST25 strain isolated from a case of pig sepsis in Canada	[60]
DAT254	Virulent Asian (AS) ST28 strain isolated from a case of pig meningitis in Japan	[61]
1088563	Low virulent North American (NA) ST28 strain isolated from a case of pig meningitis in Canada	[32]

Table 2. Role of MyD88 signaling on plasmatic biomarkers following *Streptococcus suis* infection.

Plasmatic biomarker[1]	Wild-type C-	MyD88$^{-/-}$ C-	Wild-type *S. suis*	MyD88$^{-/-}$ *S. suis*
Creatinine (µmol/L)	5	6	12	5
Aspartate transaminase (U/L)	100	126	2 080	166
Alanine transaminase (U/L)	77	67	6 521	64
Lactate dehydrogenase (U/L)	392	361	20 510	1 664

[1]Plasma was recovered from wild-type and MyD88$^{-/-}$ mice 12 h post-inoculation with the vehicle (mock-infected; C-) or a standard dose (1 x 10^7 CFU) of the *S. suis* ST1 strain P1/7 and various biochemical parameters analyzed. Data represent a pool of 3 mice.

Figures

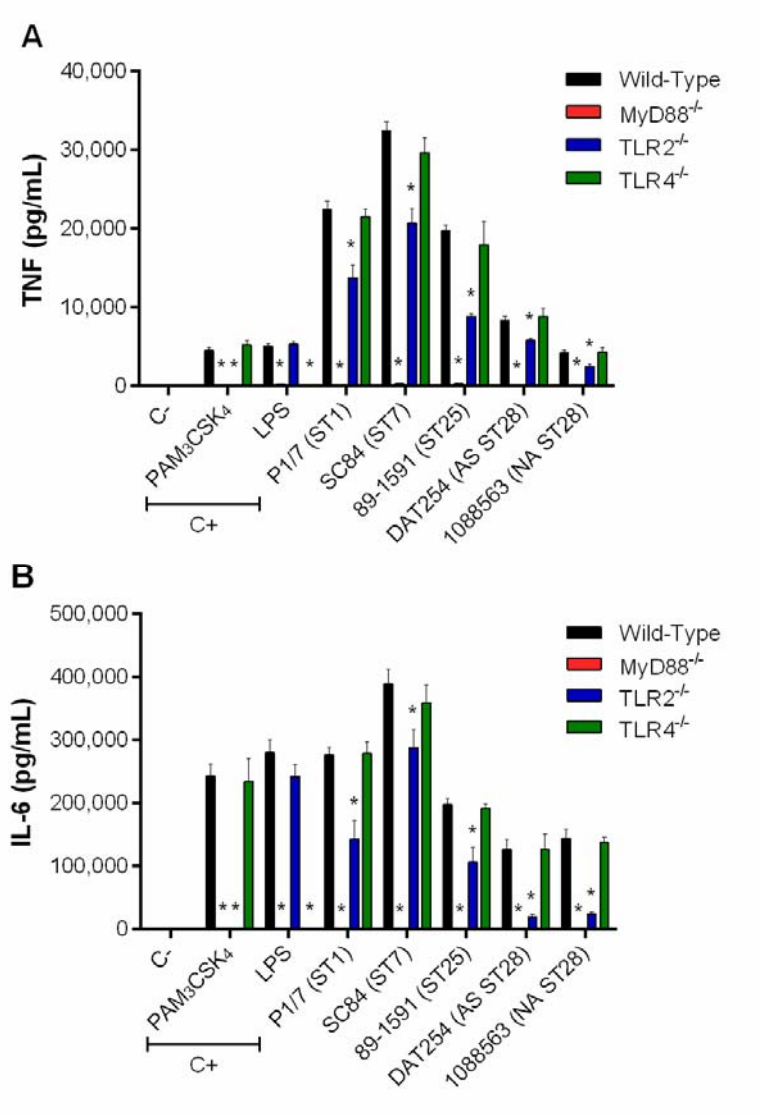


Figure 1. Recognition of *Streptococcus suis* by the MyD88-dependent pathway is required for production of tumor necrosis factor (TNF) and interleukin (IL)-6 by dendritic cells regardless of strain background. TNF (**A**) and IL-6 (**B**) production was measured by ELISA 16 h following infection of wild-type, MyD88$^{-/-}$, Toll-like receptor (TLR) 2$^{-/-}$, and TLR4$^{-/-}$ DCs with the different *S. suis* strains. The TLR1/2 ligand PAM_3CSK_4 and TLR4 ligand LPS were used as positive controls (C+). C- denotes cells in culture medium alone. Data represent mean ± SEM (n = 3). * ($p < 0.01$) indicates a significant difference between wild-type and knockout DCs.

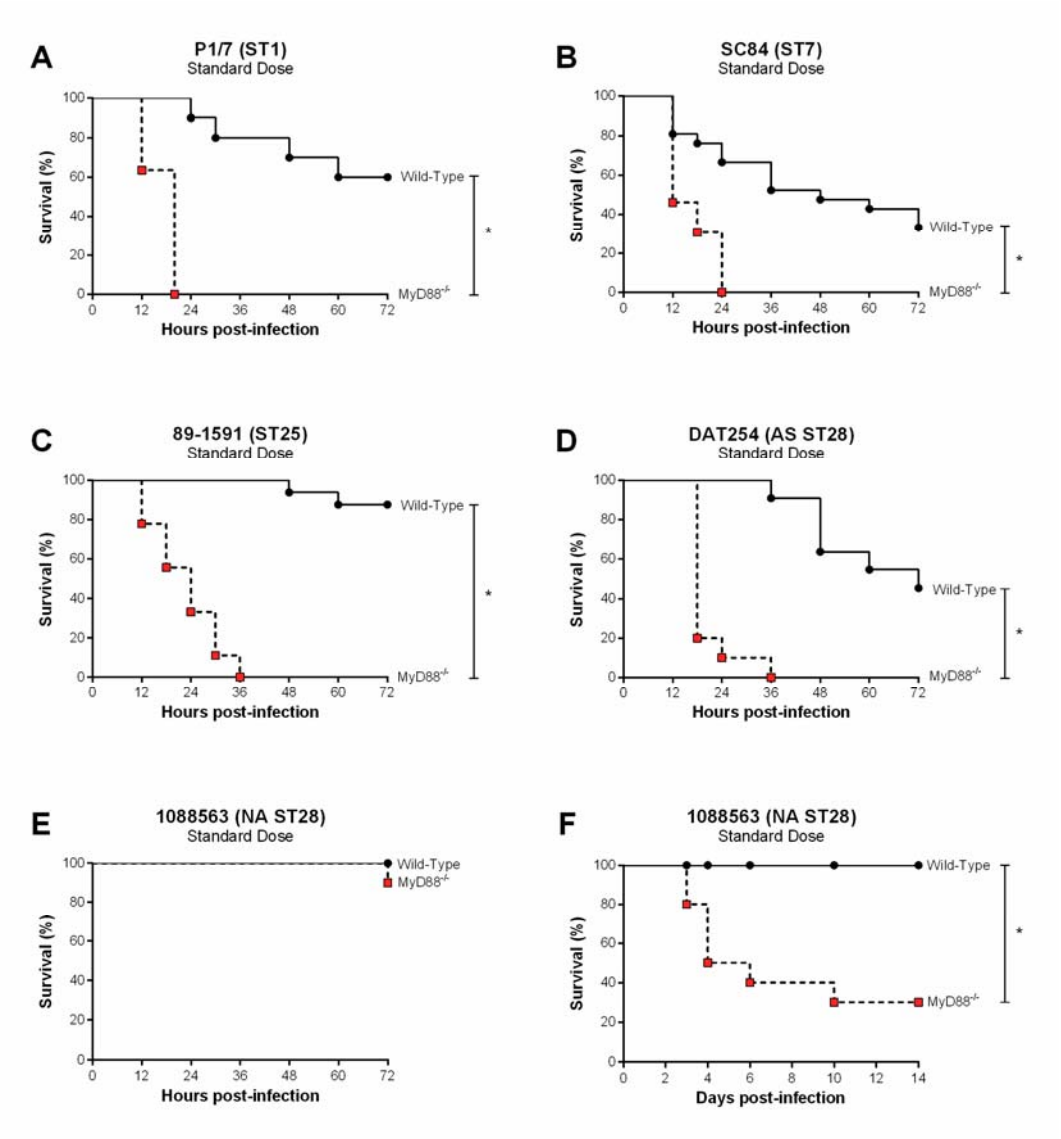


Figure 2. MyD88 signaling is required for survival following *Streptococcus suis* infection. Survival of wild-type (black) and MyD88$^{-/-}$ (red) mice infected with a standard dose of 1 x 10^7 CFU of the different *S. suis* strains by intraperitoneal inoculation: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 (**E**) during the acute systemic infection (until 72 h post-infection). Survival of wild-type and MyD88$^{-/-}$ mice infected with strain 1088563 following both the systemic and central nervous system infections (14 d post-infection) (**F**). Data represent survival curves (n = 10). * ($p < 0.01$) indicates a significant difference between survival of wild-type and MyD88$^{-/-}$ mice.

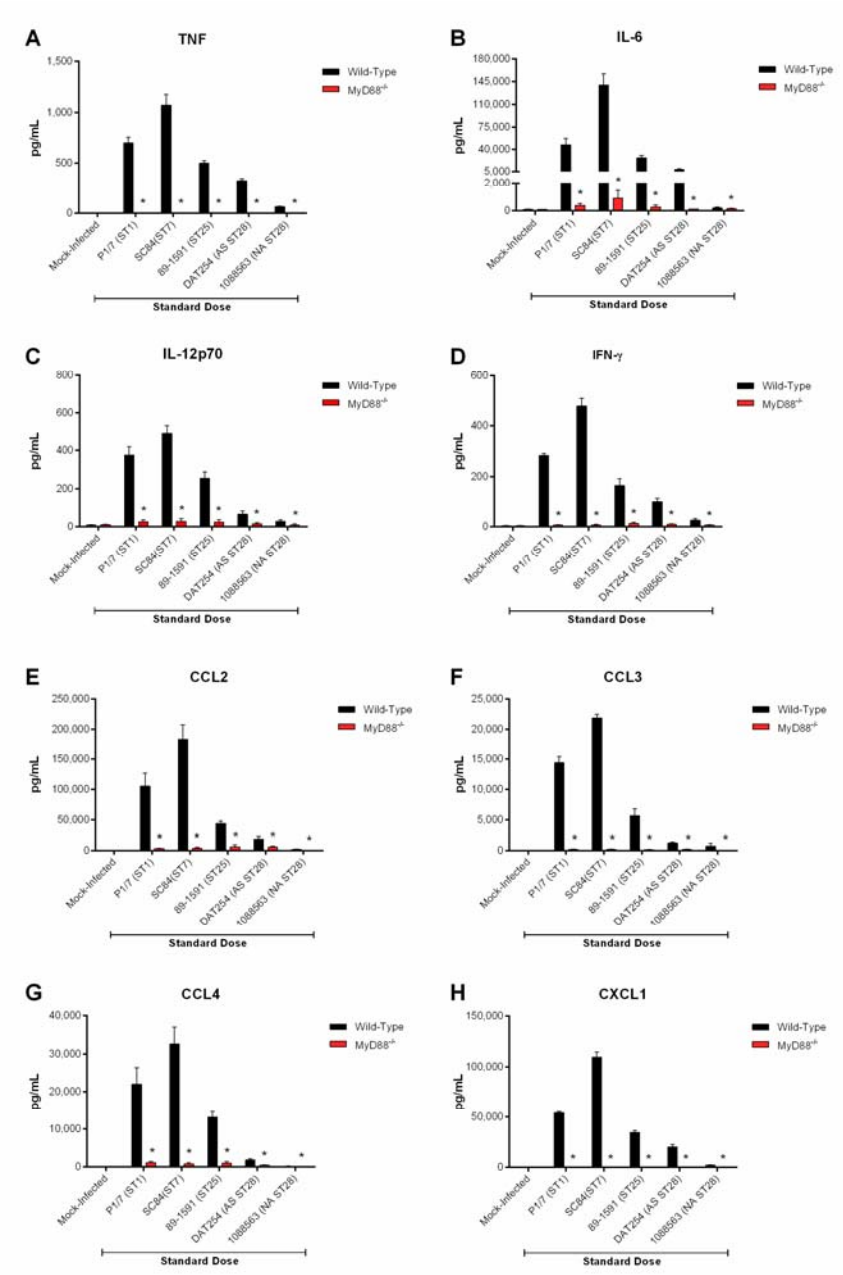


Figure 3. MyD88 signaling is required for plasma pro-inflammatory mediator production involved during *Streptococcus suis*-induced systemic inflammation. Plasma levels of TNF (**A**), IL-6 (**B**), IL-12p70 (**C**), IFN-γ (**D**), CCL2 (**E**), CCL3 (**F**), CCL4 (**G**), and CXCL1 (**H**) in wild-type and MyD88$^{-/-}$ mice 12 h following infection with a standard dose of 1 x 10^7 CFU of the different *S. suis* strains, or mock-infected, by intraperitoneal inoculation. Data represent mean ± SEM (n = 8). * ($p < 0.01$) indicates a significant difference in plasma levels between wild-type and MyD88$^{-/-}$ mice.

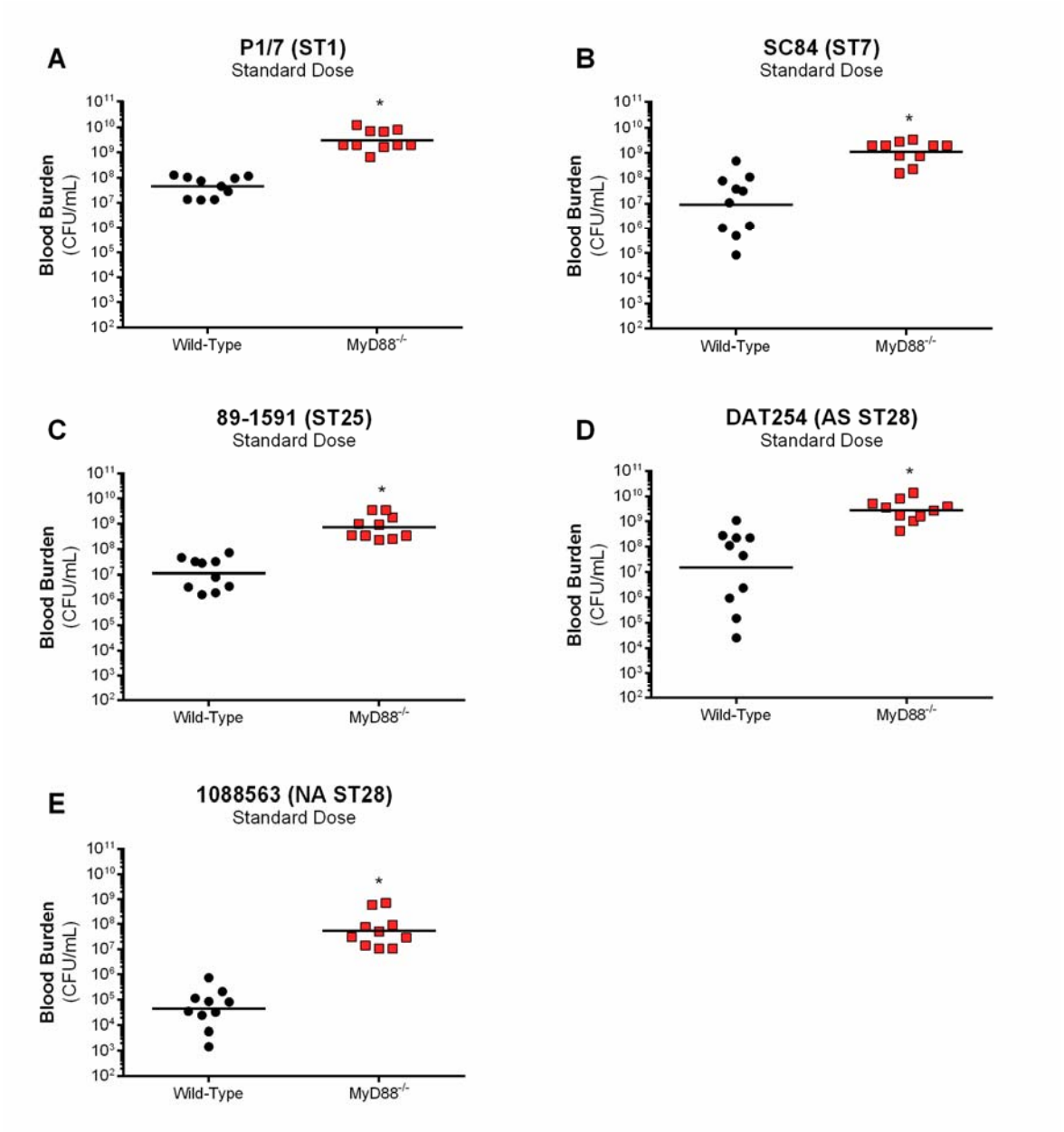


Figure 4. MyD88 signaling is required for control of blood bacterial burden following *Streptococcus suis* infection. Blood bacterial burden of wild-type and MyD88$^{-/-}$ mice 12 h following infection with a standard dose of 1 x 10^7 CFU of the different *S. suis* strains by intraperitoneal inoculation: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 (**E**). Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.01$) indicates a significant difference between blood bacterial burden of wild-type and MyD88$^{-/-}$ mice.

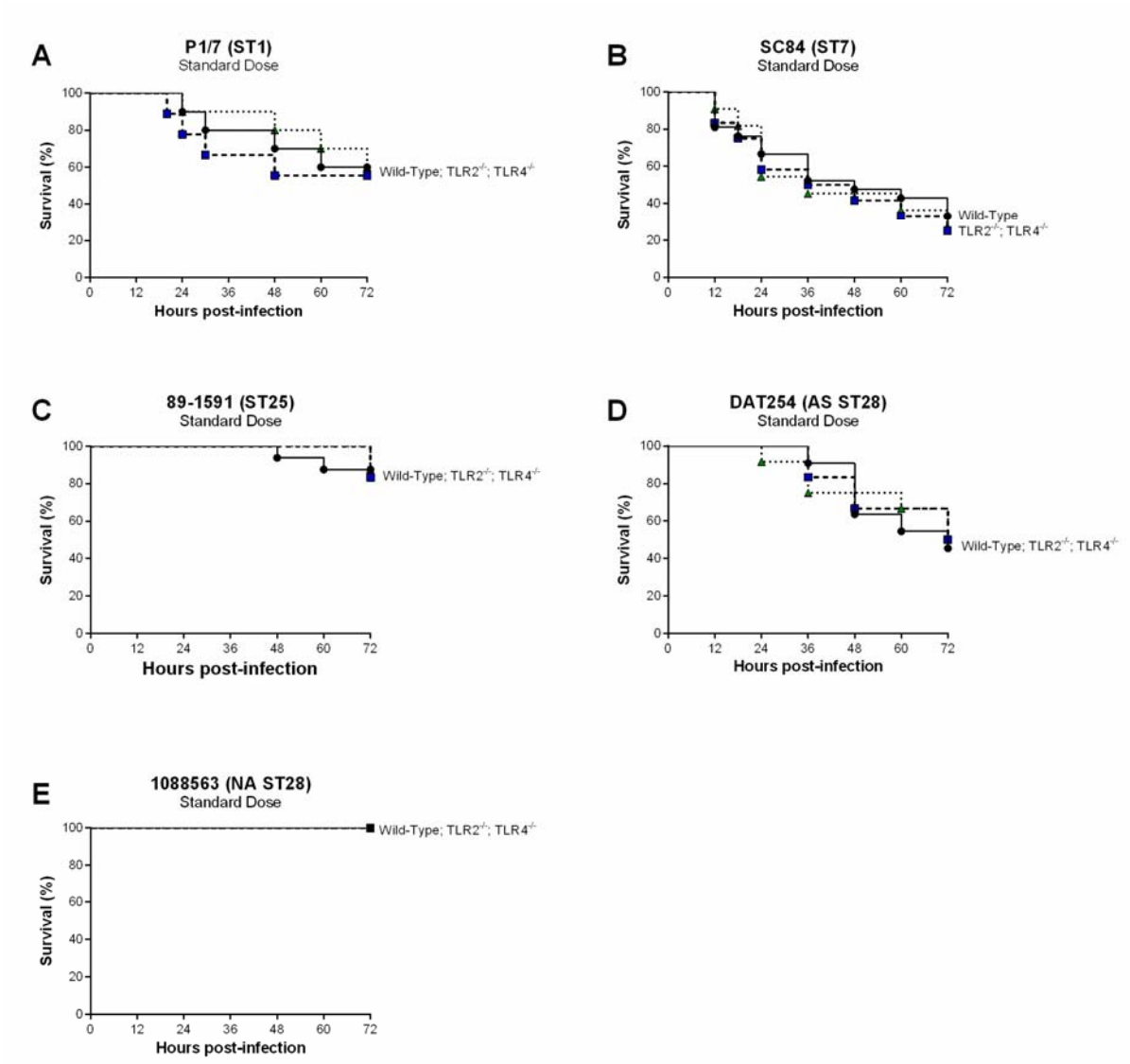


Figure 5. Toll-like receptors (TLRs) 2 and 4 are not implicted in host survival following *Streptococcus suis* infection. Survival of wild-type (black), TLR2$^{-/-}$ (blue), and TLR4$^{-/-}$ (green) mice infected with a standard dose of 1 x 10^7 CFU of the different *S. suis* strains by intraperitoneal inoculation: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 (**E**) during acute systemic infection (until 72 h post-infection). Data represent survival curves (n = 10).

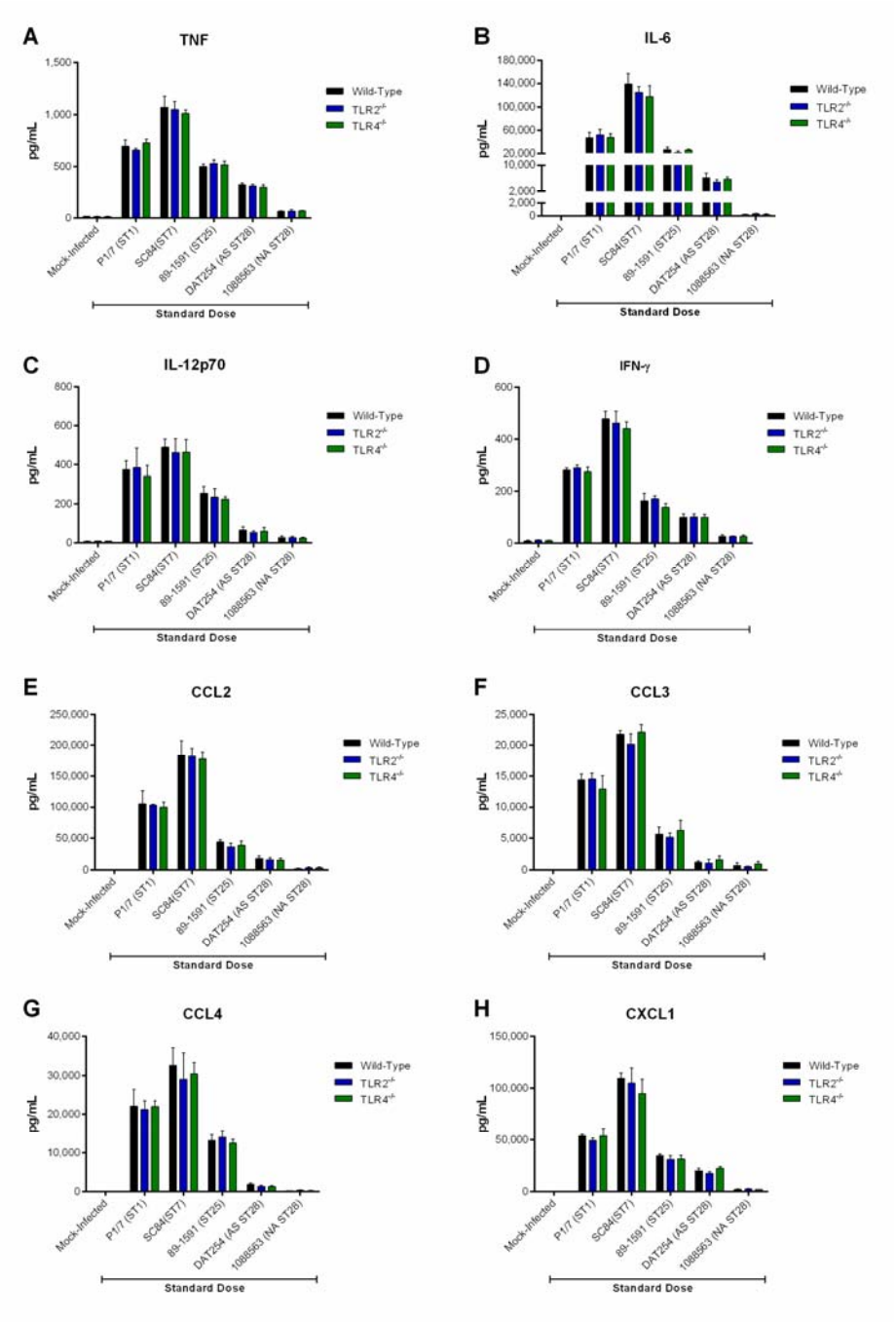


Figure 6. Toll-like receptors (TLRs) 2 and 4 are not implicated in plasma pro-inflammatory mediator production during *Streptococcus suis*-induced systemic inflammation. Plasma levels of TNF (**A**), IL-6 (**B**), IL-12p70 (**C**), IFN-γ (**D**), CCL2 (**E**), CCL3 (**F**), CCL4 (**G**), and CXCL1 (**H**) in wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 12 h following infection with a standard dose of 1 x 10^{7} CFU of the different *S. suis* strains, or mock-infected, by intraperitoneal inoculation. Data represent mean ± SEM (n = 8).

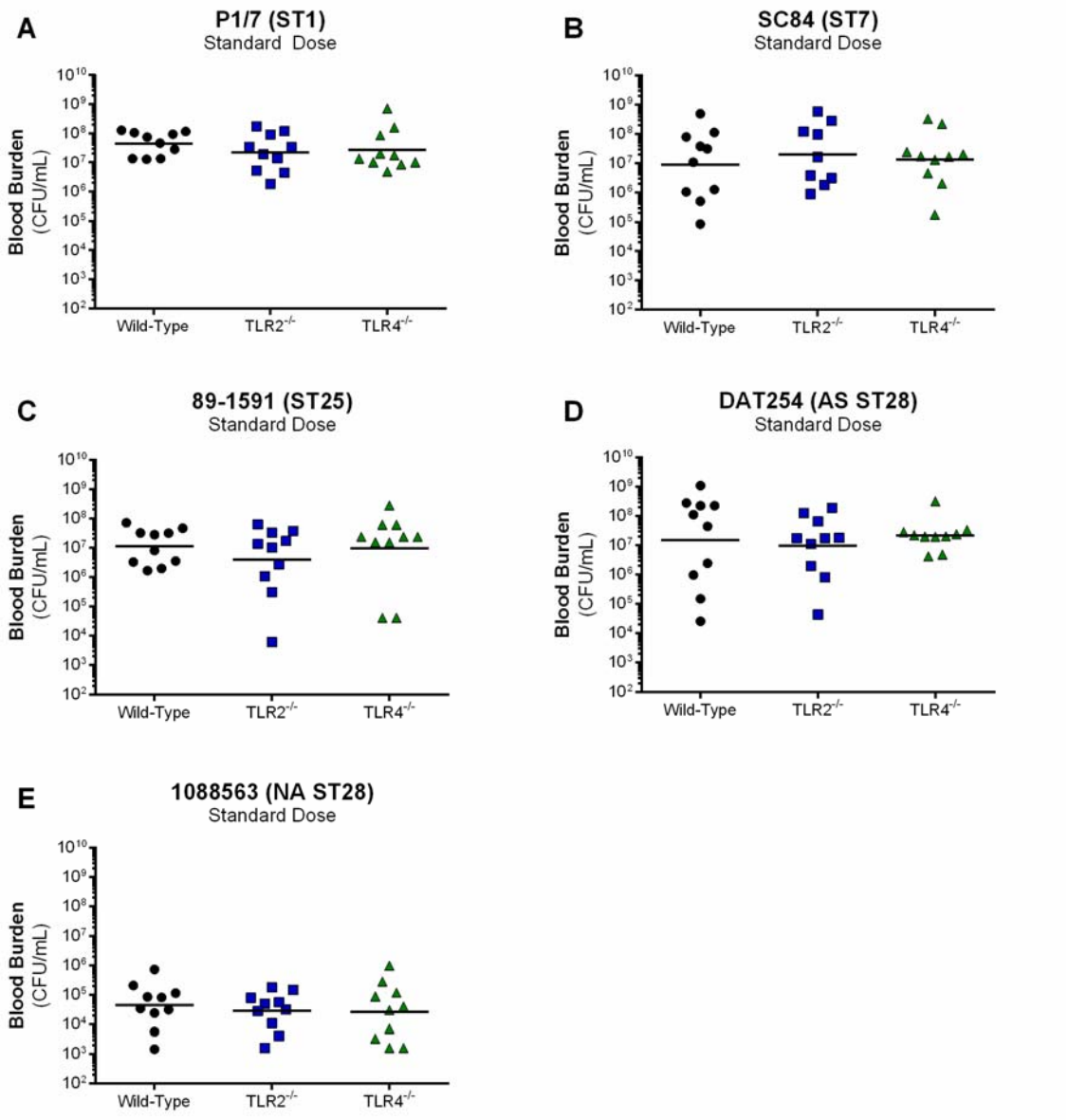


Figure 7. Toll-like receptors (TLRs) 2 and 4 are not implicated in control of blood bacterial burden following *Streptococcus suis* infection. Blood bacterial burden of wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 12 h following infection with a standard dose of 1 x 10^7 CFU of the different *S. suis* strains by intraperitoneal inoculation: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 (**E**). Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice.

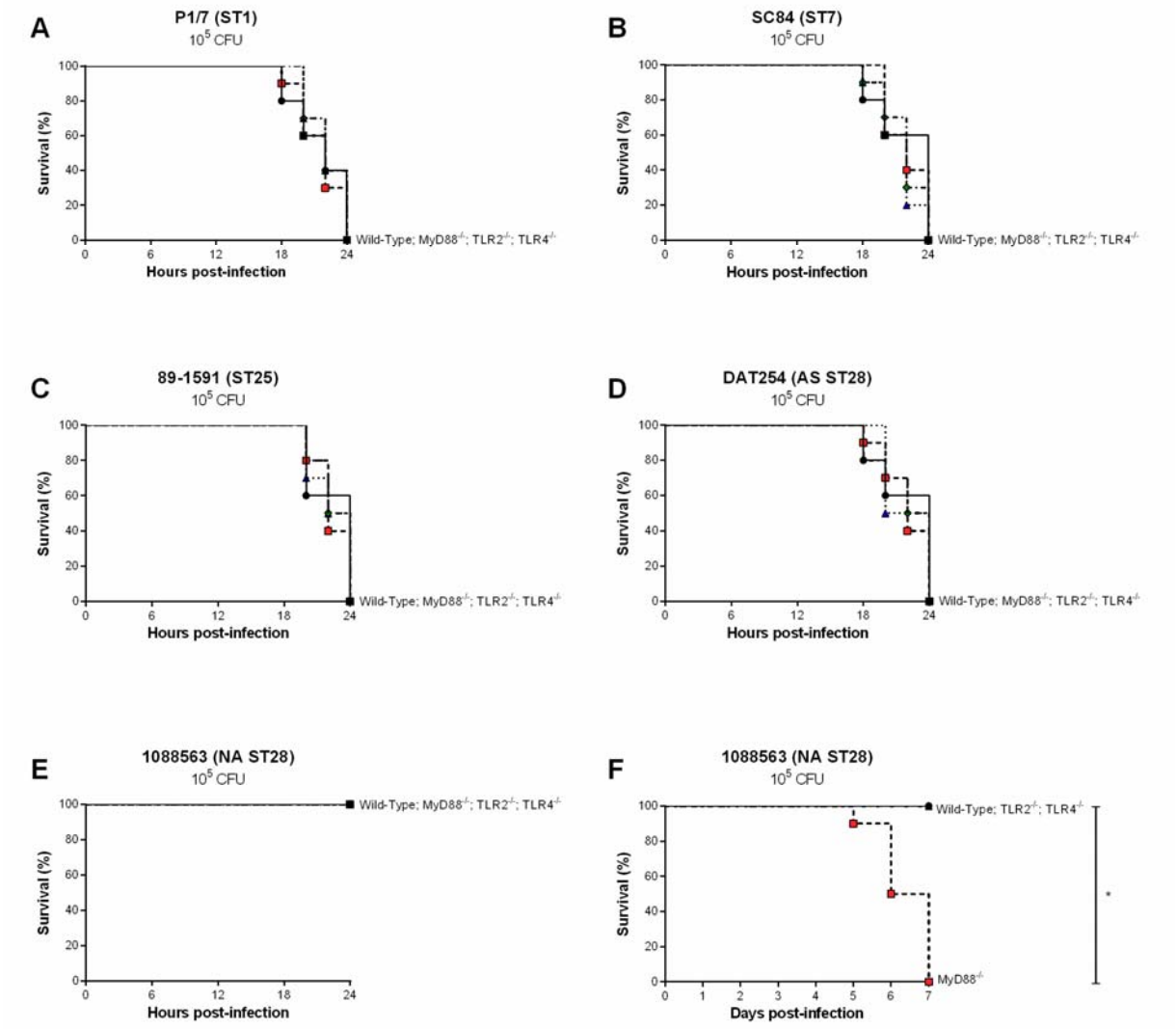


Figure 8. MyD88 signaling is not required for development of clinical central nervous system (CNS) disease following infection with virulent *Streptococcus suis*. Survival of wild-type (black), MyD88$^{-/-}$ (red), TLR2$^{-/-}$ (blue), and TLR4$^{-/-}$ (green) mice following intracisternal infection with 10^5 CFU of the different *S. suis* strains: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 24 h post-infection (**E**) and 7 d post-infection for the latter (**F**). Data represent survival curves of mice euthanized upon presentation of clinical signs of CNS disease (n = 10). * ($p < 0.01$) indicates a significant difference between survival of wild-type and MyD88$^{-/-}$ mice.

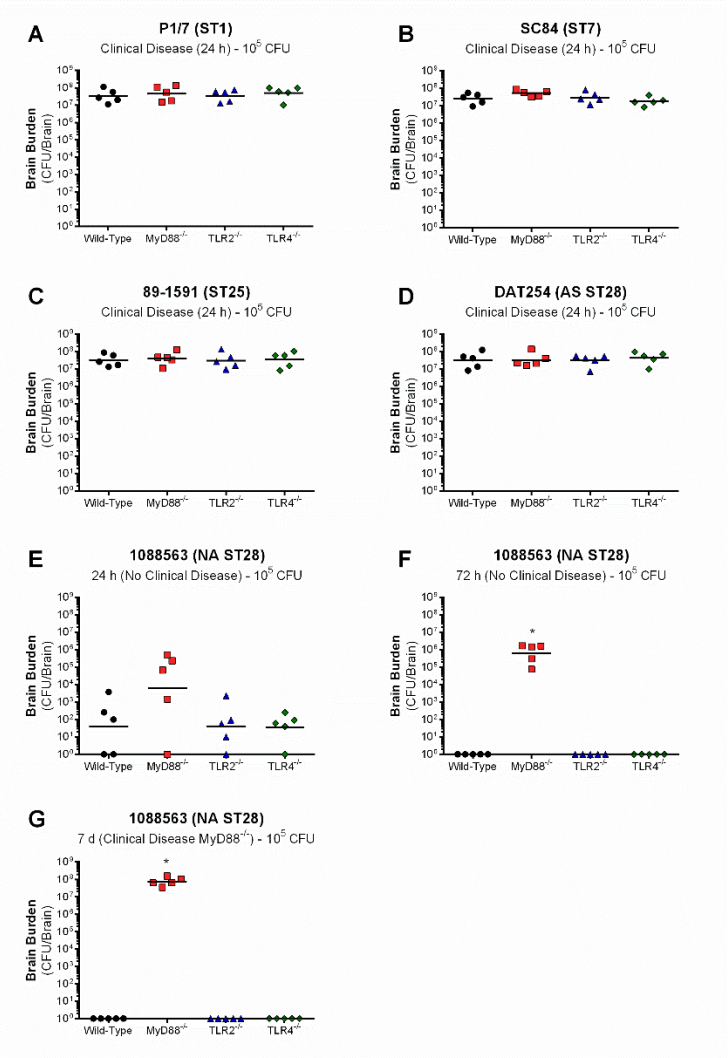


Figure 9. MyD88 signaling is not required for brain bacterial burden control following infection with a standard dose of virulent *Streptococcus suis*. Brain bacterial burden of wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 24 h following intracisternal infection with a standard dose of 10^5 CFU of the different *S. suis* strains: ST1 strain P1/7 (**A**), ST7 strain SC84 (**B**), ST25 strain 89-1591 (**C**), Asian (AS) ST28 strain DAT254 (**D**), and North American (NA) ST28 strain 1088563 (**E**), as well as 72 h post-infection for the latter (**F**). Brain bacterial burden of wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 7 d post-infection (end of the experiment; no clinical disease) and MyD88$^{-/-}$ mice upon presentation of clinical signs of central nervous system disease following intracisternal infection with strain 1088563 (**G**). Data represent geometric mean (n = 5). * ($p < 0.01$) indicates a significant difference between brain bacterial burden of wild-type and MyD88$^{-/-}$ mice.

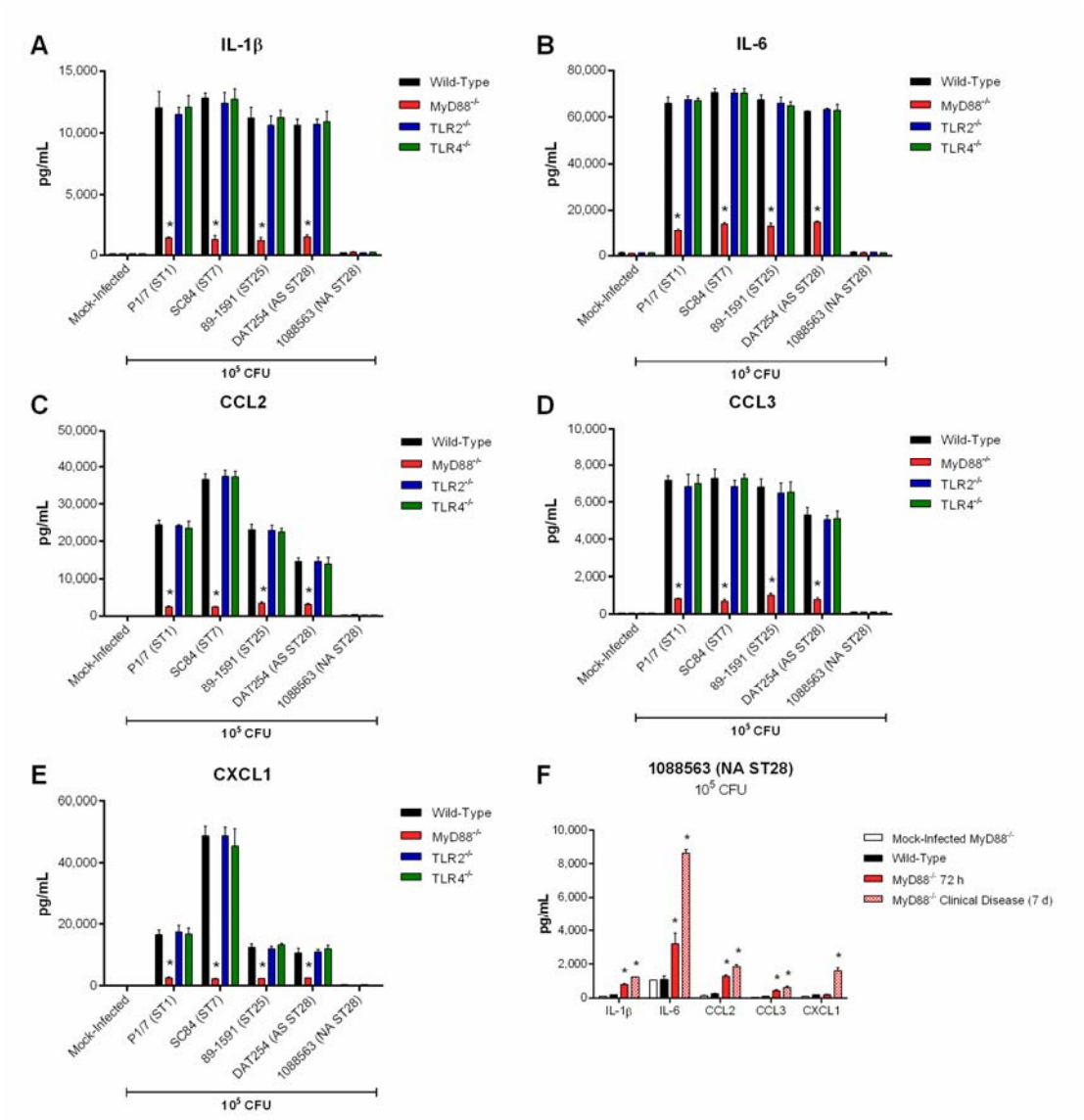


Figure 10. MyD88 signaling, but not Toll-like receptors (TLR) 2 and 4, contributes to exacerbation of brain pro-inflammatory mediator production during *Streptococcus suis*-induced central nervous system (CNS) inflammation using a standard dose. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), and CXCL1 (**E**) in wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice 24 h following intracisternal infection with a standard dose of 10^5 CFU of the different *S. suis* strains. Brain levels of these mediators in wild-type or MyD88$^{-/-}$ mice at 72 h or upon presentation of clinical signs of CNS disease following intracisternal infection with the North American (NA) ST28 strain 1088563 (**F**). Data represent mean ± SEM (n = 5). * ($p < 0.01$) indicates a significant difference in mediator levels between wild-type and MyD88$^{-/-}$ mice (**A-E**) or between 1088563-infected wild-type and MyD88$^{-/-}$ mice (**F**).

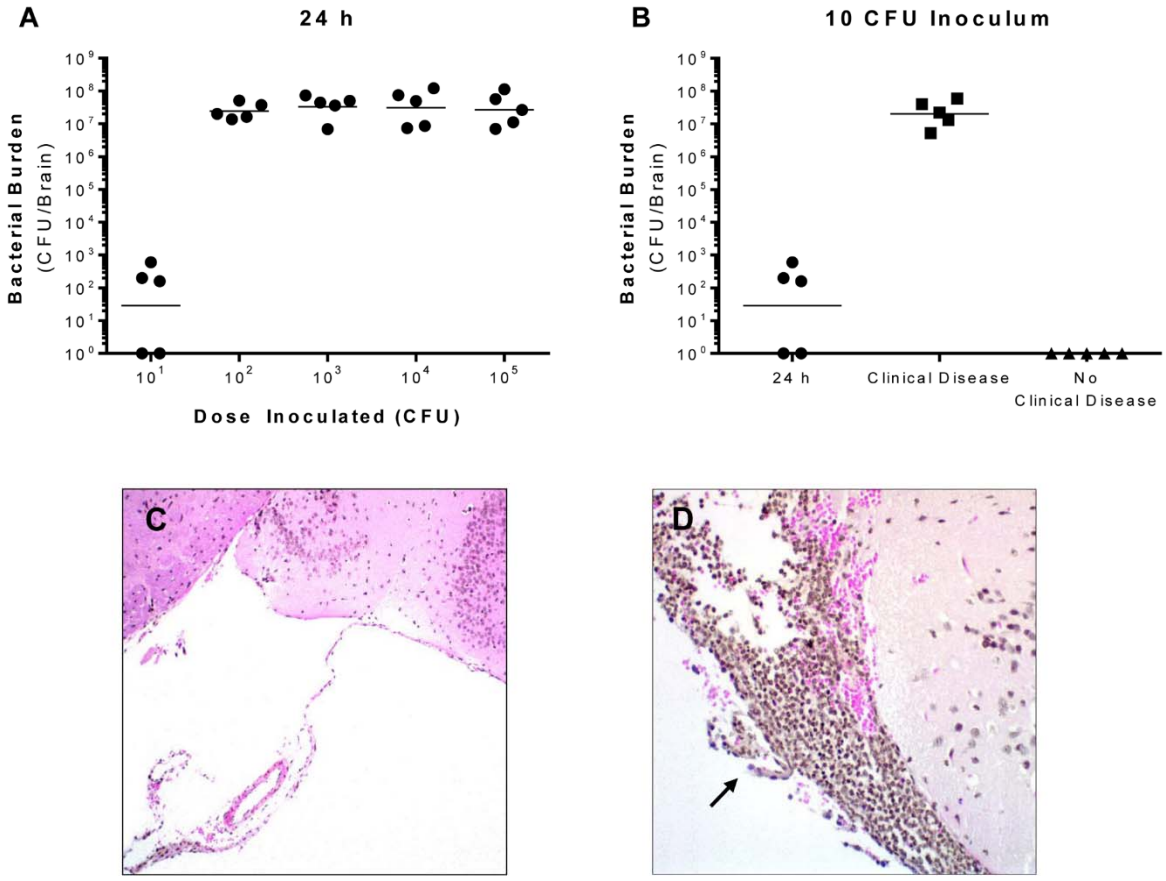


Figure 11. The central nervous system (CNS) is extremely susceptible to *Streptococcus suis* infection. Brain bacterial burden of wild-type mice 24 h following intracisternal infection with different doses of the *S. suis* ST1 strain P1/7 ranging from 10 to 10^5 CFU (**A**). Brain bacterial burden of wild-type mice at different stages following intracisternal infection with 10 CFU of the *S. suis* ST1 strain P1/7 (**B**). Data represent geometric mean (n = 5). Representative micrographs of meninges or ventricular choroid plexus following inoculation of mock-infected wild-type mice (**C**) or upon presentation of clinical signs of CNS disease induced by the *S. suis* ST1 strain P1/7-infected (10 CFU) (**D**). Black arrowheads indicate lesions typical of *S. suis*-induced CNS disease.

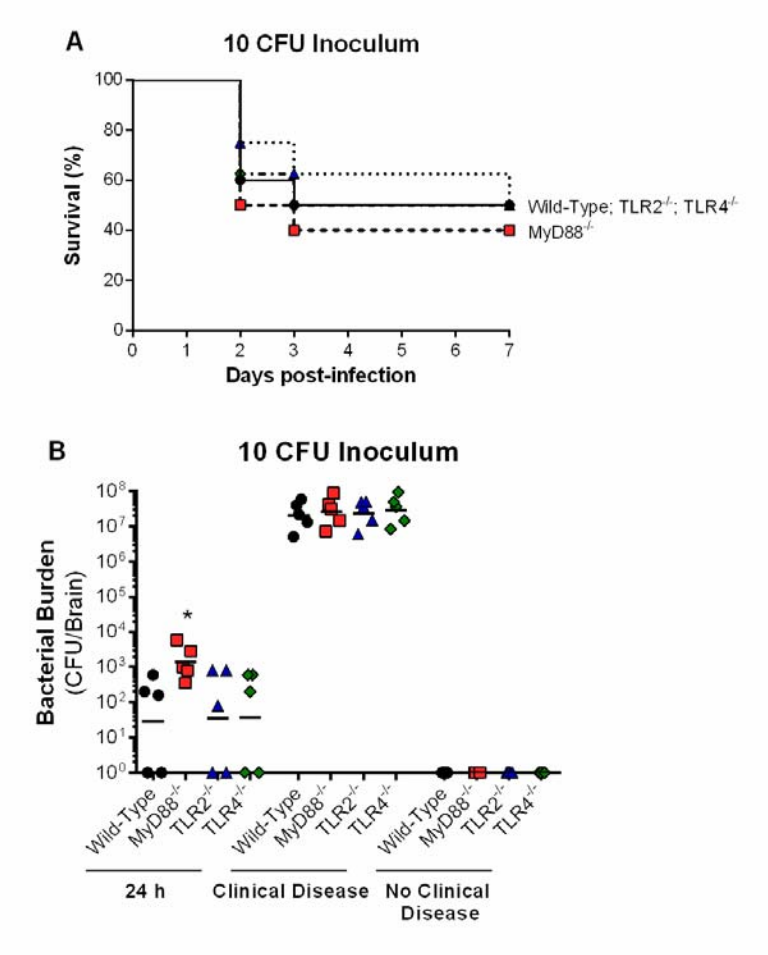
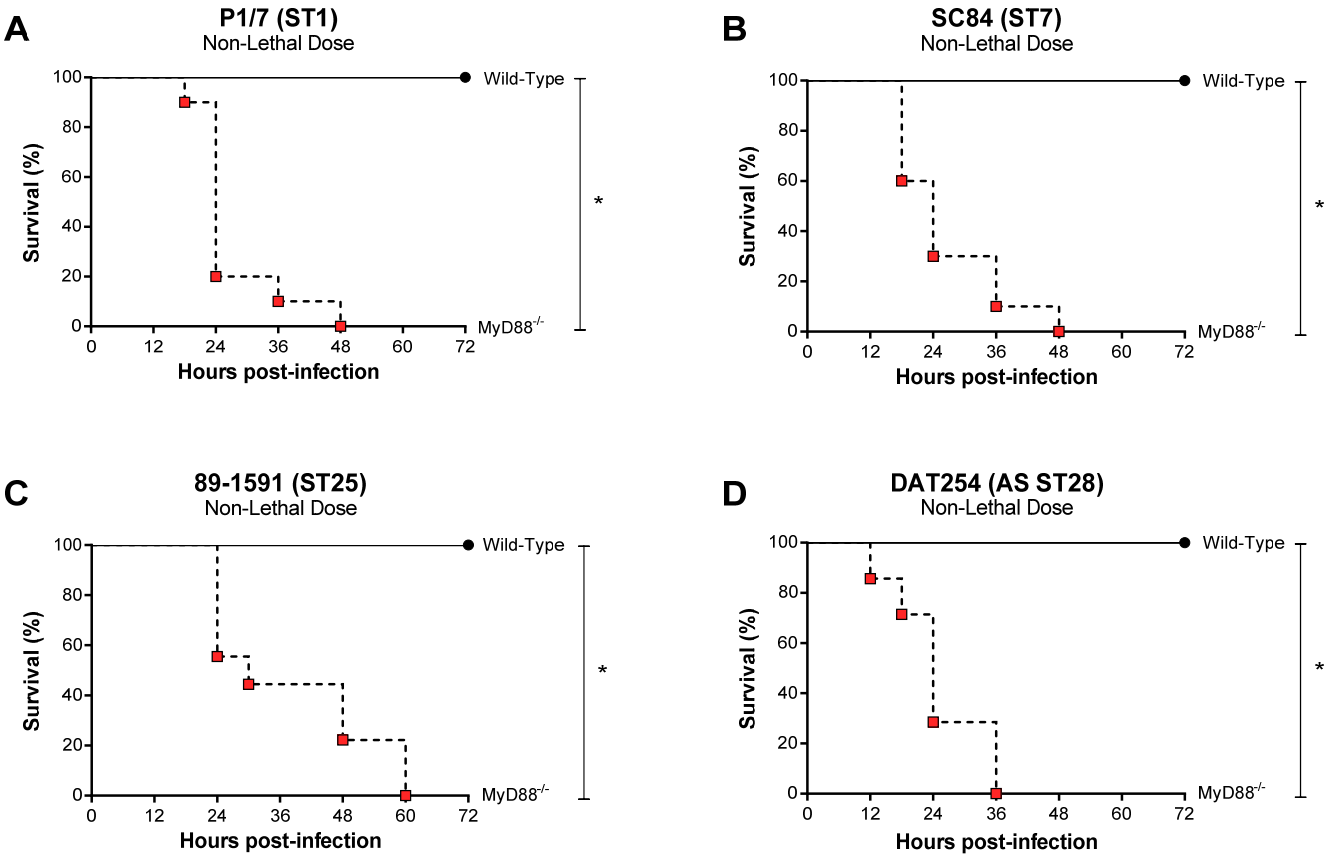
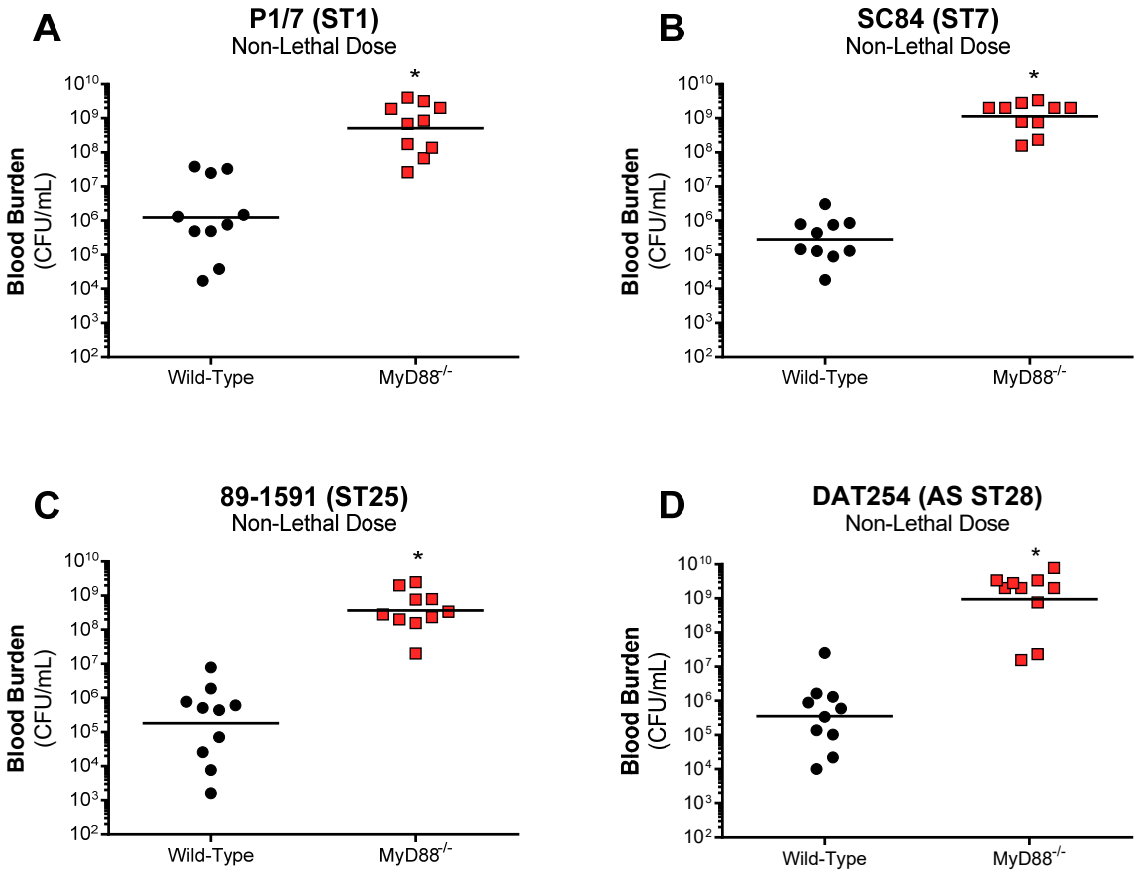


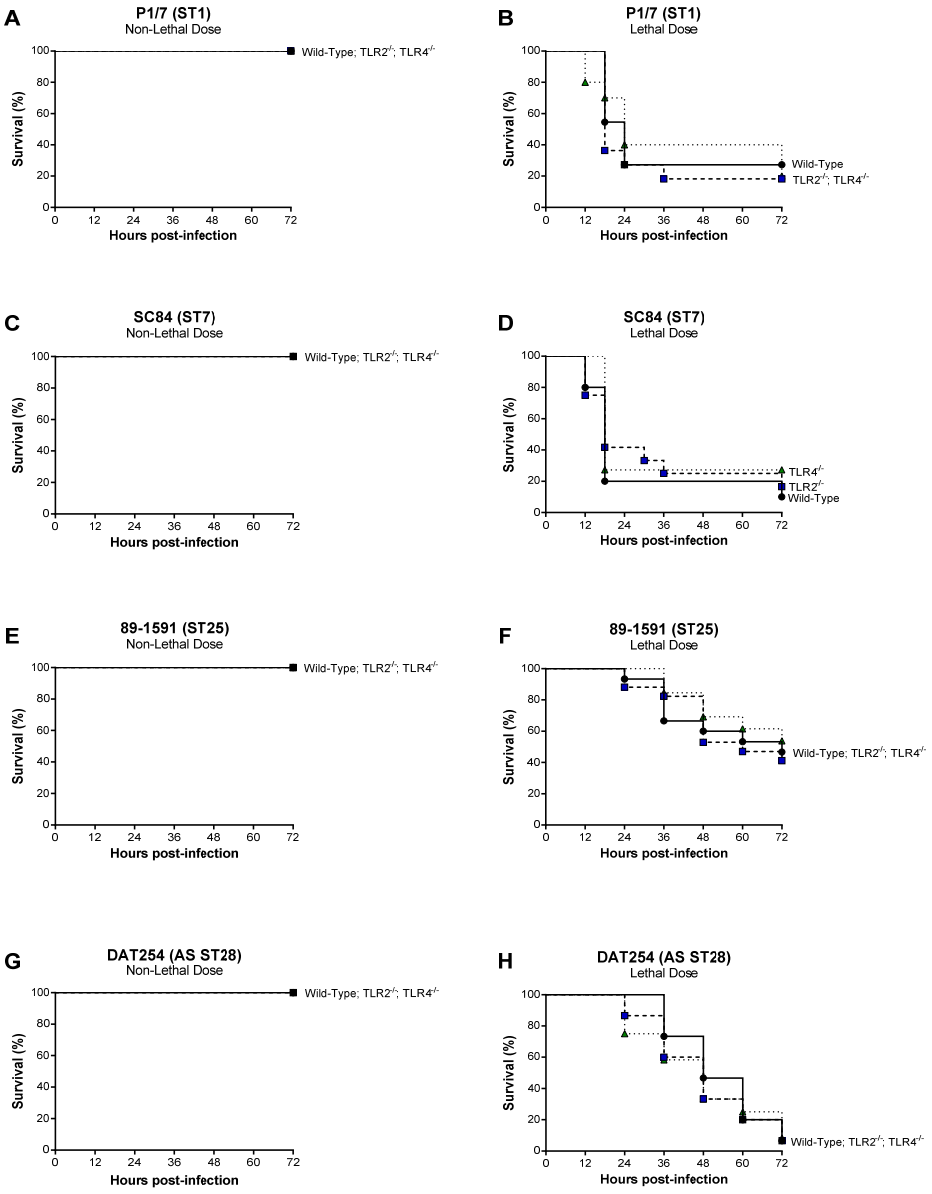
Figure 12. MyD88 signaling is not required for development of central nervous system disease following infection using a low dose of *Streptococcus suis*. Survival of wild-type (black), MyD88$^{-/-}$ (red), TLR2$^{-/-}$ (blue), and TLR4$^{-/-}$ (green) mice following intracisternal infection with a low dose of 10 CFU of the *S. suis* ST1 strain P1/7 (**A**). Data represent survival curves of mice euthanized upon presentation of clinical signs of central nervous system (CNS) disease (n = 10). Brain bacterial burden of wild-type, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice following intracisternal infection with a low dose of 10 CFU of the *S. suis* ST1 strain P1/7 (**B**). Brains were recovered 24 h post-infection, upon presentation of clinical signs of CNS disease, or at the end of the experiment (7 d post-infection; no clinical disease). Data represent mean ± SEM (n = 5). * ($p < 0.01$) indicates a significant difference between brain bacterial burden of wild-type and MyD88$^{-/-}$ mice.



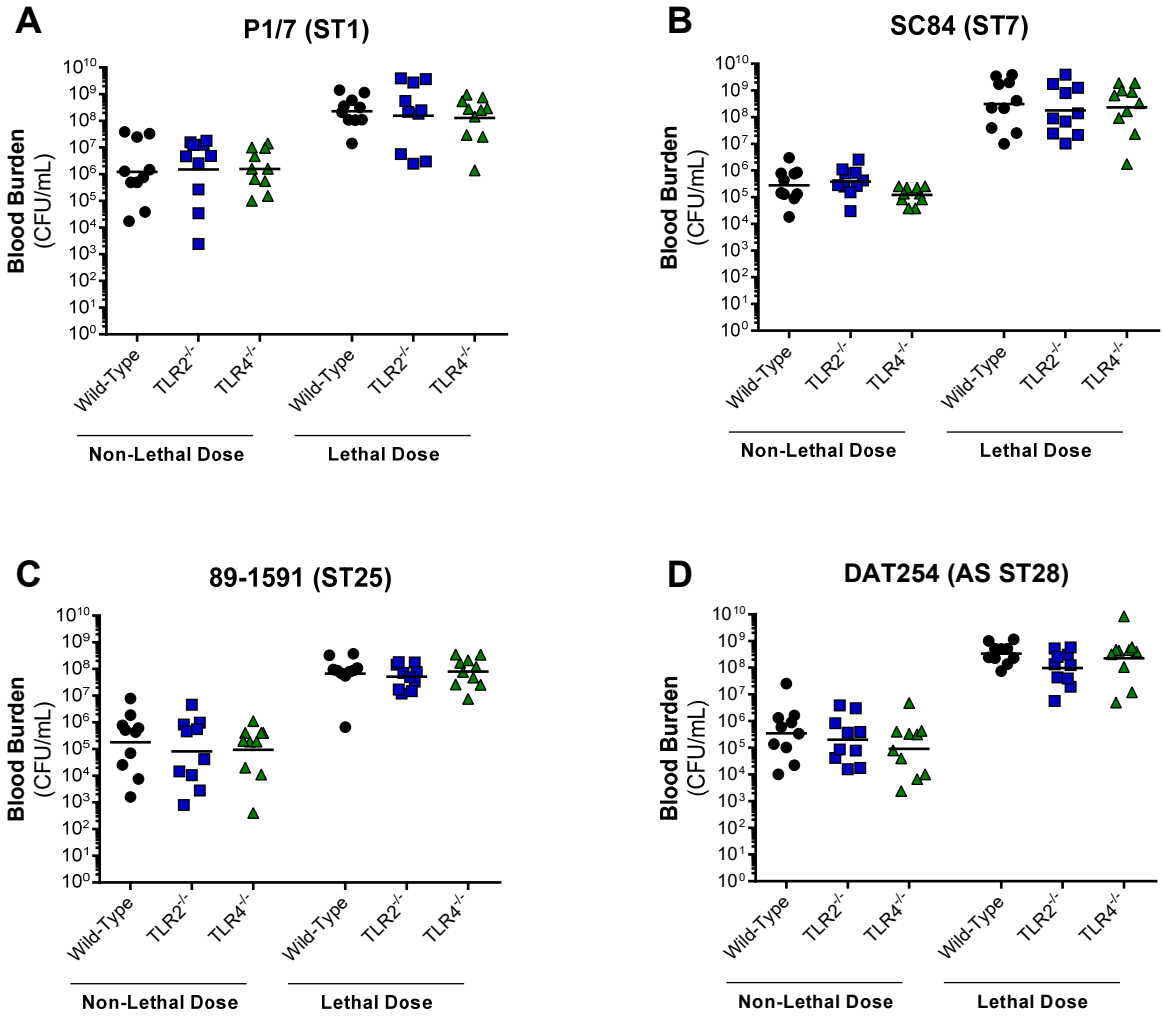
Supplementary Figure 1. MyD88 signaling is required for survival following infection with a non-lethal dose of *Streptococcus suis*. Survival of wild-type (black) and MyD88$^{-/-}$ (red) mice infected with a non-lethal dose (5 x 10^6 CFU) of the different *S. suis* strains: ST1 strain P1/7 **(A)**, ST7 strain SC84 **(B)**, ST25 strain 89-1591 **(C)**, and Asian (AS) ST28 strain DAT254 **(D)** during acute systemic infection (until 72 h post-infection). Data represent survival curves (n = 10). * ($p < 0.01$) indicates a significant difference between survival of wild-type and MyD88$^{-/-}$ mice.



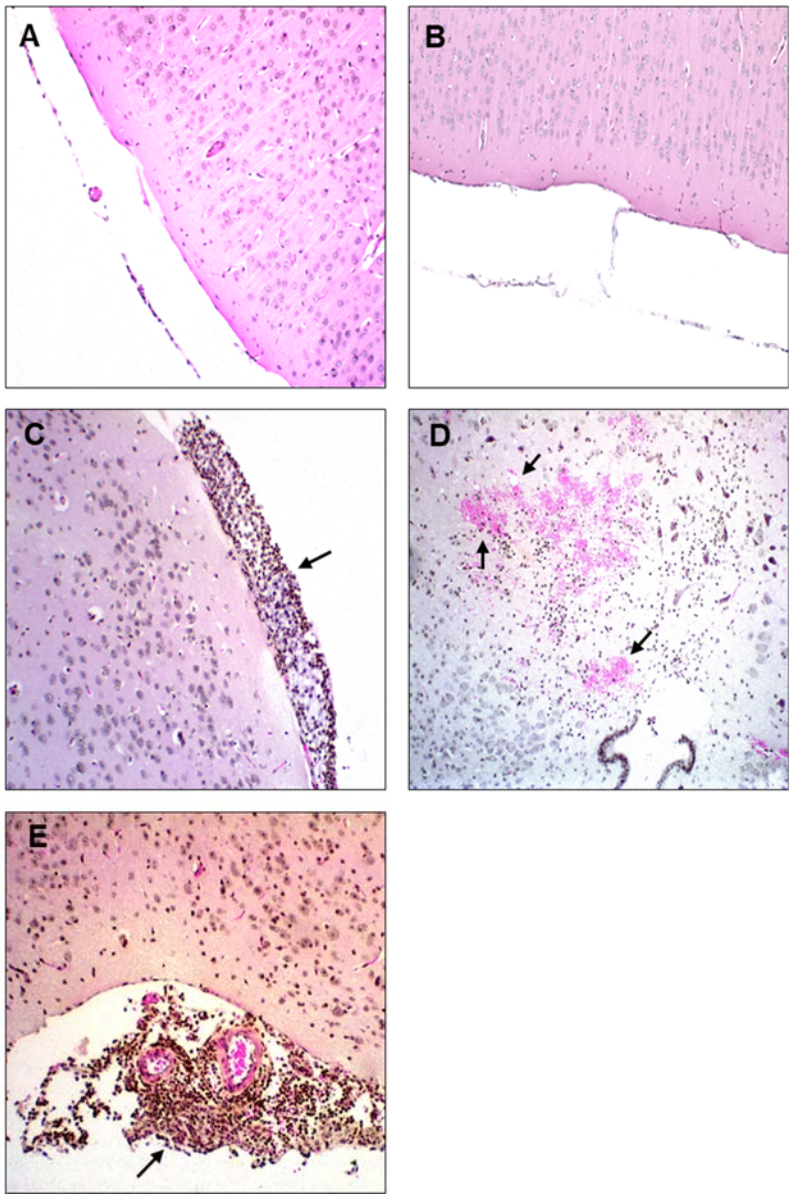
Supplementary Figure 2. MyD88 signaling is required for control of blood bacterial burden following infection with a non-lethal dose of *Streptococcus suis*. Blood bacterial burden of wild-type and MyD88$^{-/-}$ mice infected with a non-lethal dose (5 x 10^6 CFU) of the different *S. suis* strains: ST1 strain P1/7 **(A)**, ST7 strain SC84 **(B)**, ST25 strain 89-1591 **(C)**, and Asian (AS) ST28 strain DAT254 **(D)**. Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.01$) indicates a significant difference between blood bacterial burden of wild-type and MyD88$^{-/-}$ mice.



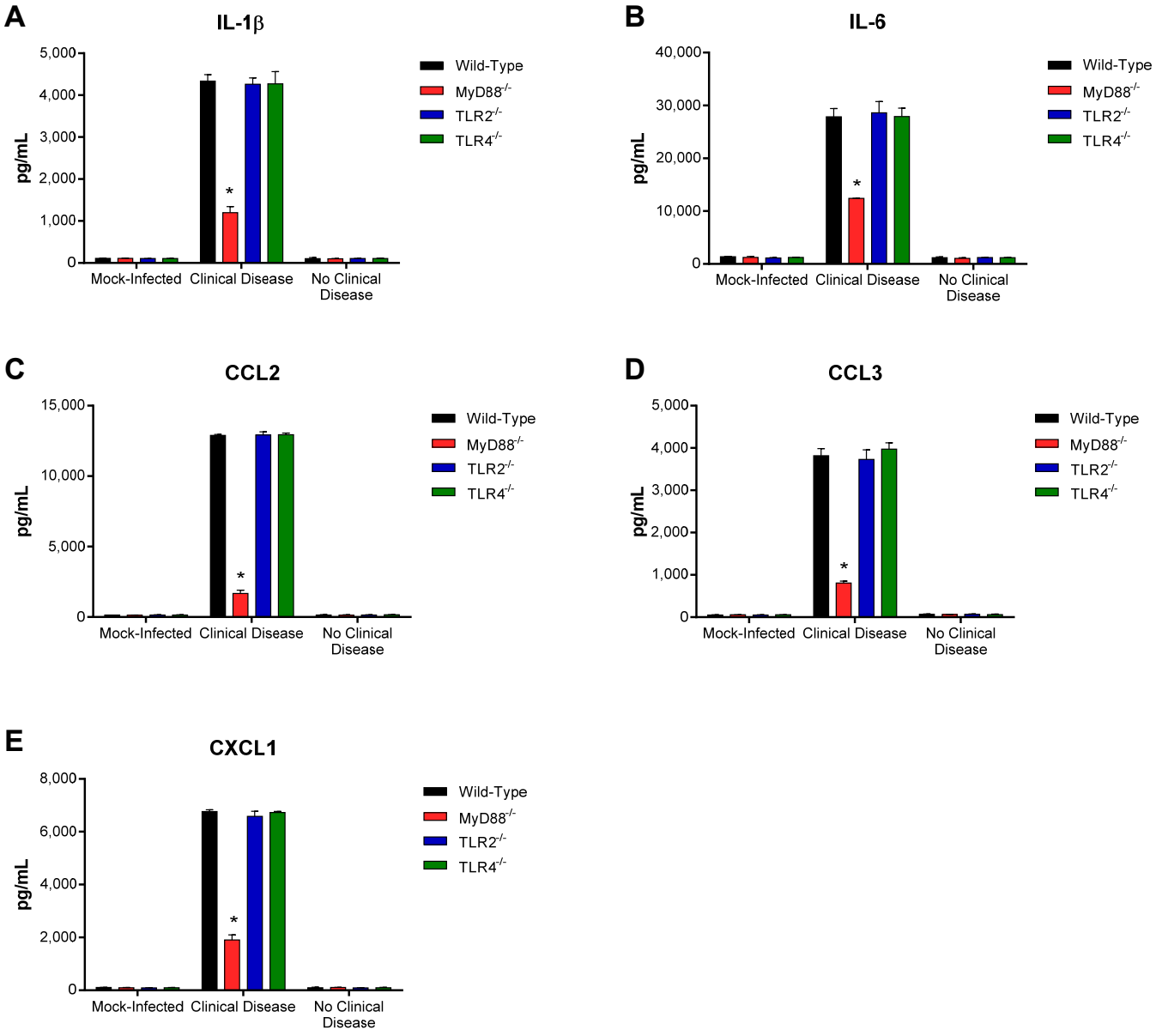
Supplementary Figure 3. Toll-like receptors (TLRs) 2 and 4 are not implicated in host survival following *Streptococcus suis* infection regardless of inoculated dose. Survival of wild-type (black), TLR2$^{-/-}$ (blue), and TLR4$^{-/-}$ (green) mice infected with either a non-lethal (5 x 10^6 CFU) or lethal (5 x 10^7 CFU) dose of the different *S. suis* strains by intraperitoneal inoculation: ST1 strain P1/7 (**A-B**), ST7 strain SC84 (**C-D**), ST25 strain 89-1591 (**E-F**), and Asian (AS) ST28 strain DAT254 (**G-H**) during the acute systemic infection (until 72 h post-infection). Data represent survival curves (n = 10).



Supplementary Figure 4. Toll-like receptors (TLRs) 2 and 4 are not implicated in control of blood bacterial burden following *Streptococcus suis* infection regardless of inoculated dose. Blood bacterial burden of wild-type, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice infected with either a non-lethal (5 x 10^6 CFU) or lethal (5 x 10^7 CFU) dose of the different *S. suis* strains: ST1 strain P1/7 **(A)**, ST7 strain SC84 **(B)**, ST25 strain 89-1591 **(C)**, and Asian (AS) ST28 strain DAT254 **(D)**. Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice.



Supplementary Figure 5. Histopathological lesions of central nervous system (CNS) disease develop following *Streptococcus suis* infection even in absence of MyD88 signaling. Histopathological studies of brains of wild-type and MyD88$^{-/-}$ mice infected with a standard dose of *S. suis* (1 x 10^5 CFU) by intracisternal inoculation. Representative micrographs of meninges or ventricular choroid plexus of mock-infected wild-type mice (**A**), mock-infected MyD88$^{-/-}$ mice (**B**), wild-type mice infected with 10^5 CFU of the ST1 strain P1/7 (**C**), MyD88$^{-/-}$ mice infected with 10^5 CFU of ST1 strain P1/7 (**D**), and MyD88$^{-/-}$ mice infected with the North American ST28 strain 1088563 (**E**). Black arrowheads indicate lesions typical of *S. suis*-induced CNS disease.



Supplementary Figure 6. MyD88 signaling contributes to exacerbation of brain pro-inflammatory mediator production during *Streptococcus suis*-induced central nervous system (CNS) inflammation using a low dose. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), and CXCL1 (**E**) upon presentation of clinical signs of CNS disease, or not, in wild-type, MyD88$^{-/-}$, Toll-like receptor (TLR) 2$^{-/-}$, and TLR4$^{-/-}$ mice following intracisternal infection with the vehicle (mock-infected) or a low dose of 10 CFU of the *S. suis* ST1 strain P1/7. Data represent mean ± SEM (n = 5). * ($p < 0.01$) indicates a significant difference in mediator levels between wild-type and MyD88$^{-/-}$ mice.

ARTICLE X

Type I interferon induced by *Streptococcus suis* serotype 2 is strain-dependent and may be beneficial for host survival

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé à la conception de l'étude et des approches méthodologiques et aux expériences *in vitro* impliquant les cellules. J'ai effectué la RT-qPCR et les expériences *in vivo*, l'analyse des résultats et l'écriture de la première version du manuscrit. J'ai aussi été impliqué dans la révision de celui-ci et j'ai effectué les corrections lors des étapes de révision pour publication.

Mise en context

Nous avons démontré l'importance de la voie de signalisation MyD88-dépendante dans la reconnaissance de *S. suis* est dans l'induction de la réponse inflammatoire exacerbée pouvant être néfaste à la survie de l'hôte dans le cadre de l'article IX. L'activation de la voie des TLRs peut mener, entre autres, à la production des IFNs de type I. Bien que les IFNs de type I soient traditionnellement associés aux infections virales, des études récentes ont démontré qu'ils peuvent aussi jouer un rôle lors des infections bactériennes en participant au contrôle de la réponse inflammatoire. De plus, il a déjà été rapporté que *S. suis* induit la production d'IFN-β dans la rate de souris infectées expérimentalement. Toutefois, le rôle que joue les IFNs de type I et les mécanismes responsables de leur production lors de l'infection à *S. suis* restent inconnus. Enfin, comme les niveaux de production d'IFN-β induits par *S. suis* varient selon la virulence de la souche, nous avons voulu évaluer l'impact de ce facteur dans le rôle des IFNs de type I et dans les mécanismes sous-jacents.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death, septic shock, and meningitis, with exacerbated inflammation being a hallmark of the infection. However, serotype 2 strains are genotypically and phenotypically heterogeneous, being composed of a multitude of sequence types (ST) whose virulence greatly varies: the virulent ST1 (Eurasia), highly virulent ST7 (responsible for the human outbreaks in China), and intermediate virulent ST25 (North America) are the most important worldwide. Even though type I interferons (IFNs) are traditionally associated with important anti-viral functions, recent studies have demonstrated that they may also play an important role during infections with extracellular bacteria. Upregulation of IFN-β levels was previously observed in mice following infection with this pathogen. Consequently, the implication of IFN-β in the *S. suis* serotype 2 pathogenesis, which has always been considered a strict extracellular bacterium, was evaluated using strains of varying virulence. This study demonstrates that intermediate virulent strains are significantly more susceptible to phagocytosis than virulent strains. Hence, subsequent localization of these strains within the phagosome results in recognition of bacterial nucleic acids by Toll-like receptors 7 and 9, leading to activation of the interferon regulatory factors 1, 3, and 7 and production of IFN-β. Type I IFN, whose implication depends on the virulence level of the *S. suis* strain, is involved in host defense by participating in the modulation of systemic inflammation, which is responsible for the clearance of blood bacterial burden. As such, when induced by intermediate, and to a lesser extent, virulent *S. suis* strains, type I IFN plays a beneficial role in host survival. The highly virulent ST7 strain, however, hastily induces a septic shock that cannot be controlled by type I IFN, leading to rapid death of the host. A better understanding of the underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

Introduction

Streptococcus suis (*S. suis*) is an important porcine bacterial pathogen and emerging zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) [1]. Of the different described serotypes, an important taxonomical classification for this pathogen based on the presence of the capsular polysaccharide (CPS) or its respective genes, serotype 2 is regarded as not only the most widespread worldwide, but also the most virulent, responsible for the majority of porcine and human cases of infection [2]. However, serotype 2 strains are genotypically and phenotypically heterogeneous, resulting in their being classified into a multitude of sequence types (STs), as determined by multilocus sequence typing, whose distribution greatly varies worldwide [2]. As such, the various *S. suis* strains belonging to serotype 2 are grouped into different STs based on shared genetic similarities that better explain the evolutionary divergence of this pathogen. Virulence of the most important STs (ST1, ST7, and ST25) has been evaluated using mouse models of infection [3, 4]. Indeed, the ST7 strain responsible for the human outbreaks of 1998 and 2005 in China is highly virulent whereas European ST1 strains are virulent; on the other hand, ST25 strains, typically recovered in North America, are of intermediate virulence [3, 5].

Of the various virulence factors described for *S. suis*, the CPS, suilysin (SLY), and cell wall modifications have been demonstrated to play important roles [6, 7]. Indeed, the CPS, which is antigenically identical for all serotype 2 strains, is a critical factor implicated in a multitude of functions, most importantly in resistance to phagocytosis by innate immune cells [8-12]; its presence also masks bacterial surface proteins responsible for host cell activation [12, 13]. Meanwhile, the SLY, a cholesterol-dependent cytolysin similar to the pneumolysin of *Streptococcus pneumoniae*, is responsible for causing cell toxicity and inducing pro-inflammatory cytokines [12, 14]. This toxin is present in serotype 2 ST1 and ST7 strains, but not in ST25 strains [14, 15]. Cell wall modifications, such as the D-alanylation of the lipoteichoic acid and N-deacetylation of the peptidoglycan, of a ST1 strain were shown to interfere with host defense and to be partially responsible for cell activation [12, 16, 17]. Finally, several cell-wall associated proteins, mainly reported for ST1 and ST7 strains, have also been described as critical virulence factors, though many of these remain controversial in the literature [7].

Recognition of *S. suis* by innate immune cells involves a multitude of membrane-associated and cytoplasmic receptors [6, 18]. Of these, the Toll-like receptor (TLR) pathway is implicated in recognition of *S. suis* by phagocytic cells, including dendritic cells (DCs) and macrophages [19], while recognition by other cells types, such as epithelial cells, remains unknown. Abrogation of MyD88, the adaptor protein central to the TLR pathway, results in near complete lack of pro-inflammatory cytokine production *in vitro* following infection with *S. suis* [13, 20]. Furthermore, being a classical extracellular pathogen, recognition of *S. suis* has been mostly associated with surface TLRs, where TLR2, in cooperation with TLR6, plays a predominant role [13, 20, 21]. Pathogen recognition by TLRs classically results in the production of pro-inflammatory cytokines via the NF-κB or interferon (IFN) pathways [19]. Pathways involved in activation of NF-κB by *S. suis* have been somewhat described in recent years [20, 22], while those regarding the IFN pathways are less known, having mainly focused on type II IFN [3, 23]. Meanwhile the capacity of *S. suis* to induce type III IFN was only evaluated once, *in vitro*, and for IFNλ1 only, with negative results obtained [24]. Nonetheless, it was recently demonstrated that expression levels of the type I IFN, IFN-β, but not those of IFN-α, are upregulated *in vivo* following infection with *S. suis* serotype 2 [3]. Moreover, this upregulation of IFN-β was significantly higher in mice infected with an intermediate virulent ST25 strain than in those infected with either a virulent ST1 strain or the highly virulent ST7 strain responsible for the human outbreaks [3]. However, no other study has addressed the production of type I IFN or its role during the *S. suis* serotype 2 infection.

Type I IFN regroups various members of the IFN family of which IFN-α, composed of sixteen different subtypes, and IFN-β, its most potent member, are the best characterized [25]. Classical production of these cytokines is the result of endosomal TLR (TLR3, TLR7, and TLR9 in mice) activation, which leads to phosphorylation and translocation of interferon regulatory factors (IRFs) to the nucleus [26]. Though IRF1 and IRF3/IRF7 are usually associated with type II and type I IFN, respectively, all three can result in transcription of type I IFNs [25, 26]. Following their production, both IFN-α and IFN-β bind a common heterodimeric receptor, the IFN-αβ receptor (IFNAR) [26]. Binding to this receptor activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, resulting in transcription of various genes associated with host defense, and modulation of the inflammatory response [26].

Even though type I IFNs are traditionally associated with important anti-viral functions, recent studies have demonstrated that they may also play an important role, particularly for IFN-β, during bacterial infections, including pathogenic streptococci [26, 27]. However, their role, whether beneficial or detrimental, may depend on the bacterial species and/or infection model [28-32]. As aforementioned, little information is available regarding type I IFN, and more specifically IFN-β, during the *S. suis* infection, which is considered a strict extracellular pathogen. Consequently, its implication in the *S. suis* serotype 2 pathogenesis was evaluated using strains of varying virulence. Herein, we demonstrated that following phagocytosis by DCs, to which intermediate virulence strains are more susceptible, *S. suis* is located within the phagosome where bacterial nucleic acids are recognized by TLR7 and TLR9, leading to activation of IRF1, IRF3, and IRF7 and production of IFN-β. When induced by intermediate, and to a lesser extent, virulent *S. suis* strains, but not by a highly virulent strain, type I IFN plays a beneficial role, being involved in the control of blood bacterial burden via modulation of systemic inflammation.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol number rech-1570).

Endotoxin-free conditions

Endotoxin (lipopolysaccharide)-free material and solutions were used for bacterial and cell culture work throughout this study.

Streptococcus suis *serotype 2 strains and growth conditions*

The different well-encapsulated *S. suis* serotype 2 strains, belonging to the most important STs (ST1, ST7, and ST25), and isogenic mutants used in this study are listed in **Table 1**. A highly virulent ST7 strain, isolated during the 2005 human outbreak in China (SC84) [33], a virulent prototype European ST1 strain (P1/7), and an intermediate virulent North American ST25 strain (89-1591) [3], were used throughout this study. Isogenic mutants derived from P1/7 (ST1) or a genotypically and phenotypically similar strain, 31533 (ST1), were also included in this study.

For comparison purposes, two additional intermediate virulent ST25 strains (91-1804 and LPH4) were used in selected experiments. Virulence of the wild-type strains was previously reported [3, 4]. *S. suis* strains were grown in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) as previously described [10], diluted in culture medium before experiments, and the number of colony-forming units (CFU)/mL in the final suspension determined by plating on THB agar.

Mice

MyD88$^{-/-}$ (B6.129P2(SJL)-*MyD88*$^{tm1.Defr}$/J), TLR2$^{-/-}$ (B6.129-*Tlr2*^{tmKir}/J), TLR3$^{-/-}$ (B6;129S1-*Tlr3*^{tm1Flv}/J), TLR4$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ), TLR7$^{-/-}$ (B6.129S1-*Tlr7*^{tm1Flv}/J), TLR9$^{-/-}$ (C57BL/6J-*Tlr9*^{M7Btlr}/Mmjax), IRF1$^{-/-}$ (B6.129S2-*Irf1*^{tm1Mak}/J), IRF3$^{-/-}$ [34], IRF7$^{-/-}$ [35], and IFNAR1$^{-/-}$ (B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax) mice on C57BL/6 background were housed under specific pathogen-free conditions alongside their wild-type counterparts (C57BL/6J). Mice were purchased from Jackson Research Laboratories (Bar Harbor, ME, USA), with the exception of IRF3$^{-/-}$ and IRF7$^{-/-}$ mice, which were provided by Dr. K. Mossman.

Generation of bone marrow-derived dendritic cells and macrophages

The femur and tibia of wild-type and knock-out mice were used to generate bone marrow-derived dendritic cells (DCs) and macrophages, as previously described [12, 36]. Briefly, hematopoietic bone marrow cells were cultured in RPMI-1640 medium supplemented with 5% (DCs) or 10% (macrophages) heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (Gibco, Burlington, ON, Canada). Complete medium was complemented with 20% granulocyte macrophage-colony stimulating factor from mouse-transfected Ag8653 cells for DCs [36] or L929 cell-derived macrophage-colony stimulating factor for macrophages [37]. Cell purity was at least 85% CD11c^{+} and F4/80^{+} cells for DCs and macrophages, respectively [12].

Streptococcus suis *infection of dendritic cells and macrophages*

Cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different *S. suis* serotype 2 strains listed in **Table 1** (10^6 CFU/mL; initial multiplicity of infection = 1). The conditions used were based on those previously published [12, 20]. Cells were harvested in TRIzol (Invitrogen, Burlington, ON, Canada) for mRNA expression 3 h, 6 h or 12 h following infection, and supernatants collected for cytokine measurement 16 h (tumor

necrosis factor (TNF), interleukin (IL)-6, IL-12p70, C-C motif chemokine ligand (CCL) 2, CCL3, and C-X-C motif chemokine ligand (CXCL) 1) or 24 h (IFN-β) post-infection (p.i.). These mediators were selected on the basis that DCs and macrophages secrete important levels of these cytokines and chemokines following infection by *S. suis* and that levels of these mediators are elevated following infection *in vivo* [12, 13, 20]. Non-infected cells served as negative controls. For neutralization of TLR9, DCs were pretreated with 5 μM ODN2088 (TLR9 inhibitor; InvivoGen, Burlington, ON, Canada) or 5 μM ODN2088-control (Ctrl) for 1 h prior to infection with *S. suis.* Different TLR ligands were used to stimulate cells as positive controls: 1 μg/mL PAM3CSK4 (TLR1/2; InvivoGen), 1 μg/mL FSL-1 (TLR2/6; InvivoGen), 10 μg/mL poly(I:C) (TLR3; Novus Biologicals, Littleton, CO, USA), 100 ng/mL ultrapure *Escherichia coli* lipopolysaccharide (TLR4; InvivoGen), 5 μg/mL imiquimod (TLR7; Novus Biologicals), and 1 μM CpG ODN1826 (TLR9; InvivoGen).

Determination of cell mRNA expression by quantitative RT-PCR (RT-qPCR)

Cell mRNA was extracted according to the manufacturer's instructions (TRIzol) and cDNA generated using the Quantitect cDNA Synthesis Kit (Qiagen, Mississauga, ON, Canada). Real-time qPCR was performed on the CFX-96 Touch Rapid Thermal Cycler System (Bio-Rad, Hercules, CA, USA) using 250 nM of primers (Integrated DNA technologies, Coralville, IA, USA) and the SsoFast Evagreen Supermix Kit (Bio-Rad). The cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 sec and 57°C for 5 sec. Melting curves were generated after each run to confirm the presence of a single PCR product. The sequences of primers used in this study are shown in **Supplementary Table 1** (**in Supplementary Material**) and were verified to have reaction efficiencies between 90 % and 110 %. The reference genes *Atp5b* and *Gapdh*, determined to be the most stably expressed using the algorithm geNorm, were used to normalize data. Fold changes in gene expression were calculated using the quantification cycle threshold (Cq) method using the CFX software manager v.3.0 (Bio-Rad). Samples from mock-infected cells served as calibrators.

Cytokine quantification in cell culture supernatants

Levels of IFN-β, TNF, IL-6, IL-12p70, CCL2 (MCP-1), CCL3 (MIP-1α), and CXCL1 (KC) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from BioLegend (Burlington, ON, Canada) for IFN-β and from R&D Systems (Minneapolis, MN, USA) for the other cytokines, according to the manufacturer's recommendations.

Phagocytosis assays

Cells were infected with the different *S. suis* strains and phagocytosis was left to proceed for different times (0.5 to 4 h) at 37°C with 5% CO_2. Multiplicity of infection and assay conditions were chosen based on previous studies regarding the kinetics of *S. suis* phagocytosis by DCs [12]. After incubation, penicillin G (5 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) and gentamicin (100 mg/mL; Gibco) were directly added to the wells for 1 h to kill extracellular bacteria. Supernatant controls were taken in every test to confirm that extracellular bacteria were efficiently killed by the antibiotics. After antibiotic treatment, cells were washed three times and sterile water added to lyse the cells. Where required, cells were pretreated with either 5 µM cytochalasin D (Santa Cruz Biotech, Dallas, TX, USA), 8 µM dynasore (Sigma-Aldrich), 1 µM bafilomycin A1 (Santa Cruz Biotech) or their vehicle, DMSO (Sigma-Aldrich), for 45 min prior to infection with bacteria, and phagocytosis allowed to proceed for 2 h. The number of CFU recovered per well was determined by plating viable intracellular bacteria on THB agar.

Streptococcus suis *DNA and RNA préparation and transfection of cells*

For bacterial RNA and DNA isolation, bacterial cultures were grown to mid-log phase. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions, including treatment with DNase I. For DNA preparation, bacteria were harvested in 10 mM Tris, 1 mM EDTA, pH 8.0, and treated with 10% SDS and 20 mg/mL proteinase K (Sigma-Aldrich) for 1 h at 37°C. DNA was isolated using phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) [28]. After isolation, bacterial DNA was treated with 10 mg/mL RNase A (Roche, Mississauga, ON, Canada) for 30 min at 37°C. DCs were transfected with 1 µg of RNA or DNA complexed or not with DOTAP liposomal transfection agent (Sigma-Aldrich) as described for transfection of bacterial extracts [28, 38].

Streptococcus suis *serotype 2 mouse model of infection*

Six-week-old wild-type C57BL/6 and $IFNAR^{-/-}$ mice were used. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [39]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. The different *S. suis* serotype 2 strains, or the vehicle solution (sterile THB), were administered at a dose of 1 x 10^7 CFU by

intraperitoneal inoculation to groups of 15 mice for survival and blood bacterial burden. Mice were monitored at least three times daily until 72 h p.i. and twice thereafter until 14 days p.i. Blood bacterial burden was assessed 12 h and 48 h p.i. by collecting 5 µL of blood from the caudal vein, appropriately diluting and plating on THB agar as described above. Blood bacterial burden was also measured prior to euthanasia.

Measurement of plasma (systemic) pro-inflammatory cytokine levels

Eight wild-type and IFNAR$^{-/-}$ mice per group were infected with each strain as described above and the blood collected 12 h p.i. by intracardiac puncture following euthanasia and stabilization with EDTA (Sigma-Aldrich) as previously described [3, 4]. Plasma supernatants were collected following centrifugation at 10 000 x *g* for 10 min at 4ºC, and stored at -80ºC. Plasmatic concentrations of TNF, IL-6, IL-12p70, CCL2, CCL3, and CXCL1 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Transmission electron microscopy

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer, pH 7.3 (Canemco & Marivac, Canton de Gore, QC, Canada). The CPS was stabilized using specific antibodies as previously described [40]. Anti-*S. suis* serotype 2 rabbit serum, produced as previously described [41], was used to gently resuspend bacteria. Next, cells were immobilized in 4% (w/v) agar in 0.1 M cacodylate buffer, pH 7.3. Pre-fixation was performed by adding 0.1 M cacodylate buffer, pH 7.3, containing 0.5% (v/v) glutaraldehyde and 0.15% (w/v) ruthenium red for 30 min. Fixation was performed for 2 h at room temperature with 0.1 M cacodylate buffer, pH 7.3, containing 5% (v/v) glutaraldehyde and 0.05% (w/v) ruthenium red. Post-fixation was carried out with 2% (v/v) osmium tetroxide in water overnight at 4˚C. Samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in an increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections were post-stained with uranyl acetate and lead citrate and examined using a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

Statistical analyses

Normality of data was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test and one-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test and one-way ANOVA on ranks), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare survival between wild-type and IFNAR$^{-/-}$ mice. Each test was repeated in at least three independent experiments. $P < 0.05$ and $p < 0.01$ values were considered as statistically significant and highly significant, respectively.

Results

Strain- and cell type-dependent induction of IFN-β by Streptococcus suis *serotype 2*

DCs and macrophages have not only been demonstrated to be important for IFN-β production during bacterial infections, but were also shown to produce high levels of other pro-inflammatory cytokines following infection with *S. suis* [12, 13, 20, 42]. Consequently, the capacity of these cells to produce IFN-β following infection with three different *S. suis* serotype 2 strains (highly virulent ST7 strain SC84, virulent ST1 strain P1/7, and intermediate virulent ST25 strain 89-1591) was evaluated. This cytokine was chosen as a representative of type I IFN since *S. suis* was previously demonstrated to upregulate levels of IFN-β expression *in vivo*, but not those of IFN-α, during acute infection [3]. Moreover, DCs and macrophages have been demonstrated to mainly produce IFN-β, but only low levels of IFN-α, following infection with pathogenic streptococci [28, 30, 38]. As shown in **Fig. 1A**, DCs expressed low levels of IFN-β 3 h after infection with *S. suis* (**Fig. 1A**). However, expression levels quickly and significantly increased, peaking at 6 h ($p < 0.05$). Importantly, levels induced by the intermediate virulent ST25 strain 89-1591 were significantly higher ($p < 0.001$) than those induced by the two more virulent strains. These levels were not specific to 89-1591, since similar high levels were also obtained using two other intermediate virulent ST25 strains (**Figure S1 in Supplementary Material**). In contrast, IFN-β expression levels of *S. suis*-infected macrophages remained relatively low and unchanged, regardless of the incubation time and strain used (**Fig. 1B**). Indeed, IFN-β expression levels by macrophages were significantly lower than those by DCs and this, for all three strains tested ($p < 0.01$). A clear induction of IFN-β expression was detected with the positive control, poly(I:C), indicating that the low response observed with *S. suis* was a consequence of the stimulus rather than the cells (**Fig. 1B**).

In order to evaluate if these differences in expression between cell types were also observed at the protein level, IFN-β was measured in the supernatant of cells 24 h after infection by ELISA (**Fig. 1C**). Indeed, IFN-β mRNA expression and protein production correlated. Results demonstrated that only DCs produce important protein levels of IFN-β following infection with *S. suis* serotype 2, which were significantly higher than control cells ($p < 0.001$) (**Fig. 1C**). However, as with mRNA expression, the intermediate virulent strain 89-1591 induced significantly higher protein levels of IFN-β by DCs than the other two *S. suis* strains ($p < 0.001$). Meanwhile, macrophages produced significant levels of IFN-β when stimulated with poly(I:C) ($p < 0.001$), but not following *S. suis* infection, confirming results observed at the transcriptional (mRNA) level (**Fig. 1C**). Based on these results, all subsequent experiments in this study were performed using DCs.

The presence of capsular polysaccharide interferes with Streptococcus suis*-induced IFN-β expression by dendritic cells, while the suilysin (when present) is partially responsible for activation*

Of the different described virulence factors for *S. suis* serotype 2, the presence of the CPS, SLY, and cell wall modifications have been reported to modulate and/or participate in cytokine production by DCs [12]. Consequently, their role in the induction of IFN-β expression by DCs was evaluated using isogenic mutants (**Fig. 2**). While the isogenic mutants used derived from two different wild-type strains (P1/7 and 31533), it was previously demonstrated that these two strains have a highly similar background (closely related virulent European ST1 strains) and induce similar levels of cytokine production by DCs [12]. Absence of the CPS in a ST1 strain resulted in a significant increase of IFN-β expression by DCs ($p < 0.001$), suggesting that its presence interferes with cell activation. On the other hand, absence of the SLY, which is a pore-forming toxin present in ST1 and ST7 strains, resulted in a significant decrease of IFN-β expression ($p < 0.01$). Meanwhile, cell wall modifications (D-alanylation of the lipoteichoic acid and the N-acetylation of the peptidoglycan) did not modulate *S. suis*-induced IFN-β by DCs. As previously described for other cytokines, levels of IFN-β induced by the ST1 strain 31533 were very similar to those observed with the prototype strain P1/7.

Recognition of Streptococcus suis *by the Toll-like receptor pathway is required for IFN-β induction in dendritic cells*

The TLR pathway has been traditionally associated with IFN-β production following pathogen recognition by the endosomal TLRs (TLR3, TLR7, and TLR9 in mice) [26]. However, being considered a classical extracellular pathogen, recognition of *S. suis* has been mostly associated with surface TLRs (TLR1, TLR2, and TLR6) [13, 20]. Consequently, the role of the TLR pathway in *S. suis*-induced IFN-β by DCs was evaluated. In the absence of the adaptor protein MyD88, used by the majority of TLRs, a significant decrease of IFN-β expression by DCs ($p < 0.001$) was observed with the three *S. suis* strains, corresponding to a near complete abrogation (**Fig. 3A**). This result suggests that *S. suis*-induced IFN-β expression by DCs is overwhelmingly MyD88-dependent since only 5 to 10% of expression remained independent of MyD88 (**Fig. 3A**).

Given the near complete MyD88-dependence of *S. suis*-induced IFN-β by DCs and the fact that the surface TLR2 [13, 20] and, possibly TLR4 via the SLY [43], may be implicated in recognition of this pathogen, their role in *S. suis*-induced IFN-β by DCs was evaluated. TLR2 was not implicated in IFN-β expression by DCs following *S. suis* infection, regardless of the strain used (**Fig. 3B**). Moreover, it was not possible to induce IFN-β expression following stimulation of wild-type DCs with PAM3CSK4 (for TLR1/2) and FSL-1 (for TLR2/6) [44], which are synthetic bacterial TLR2 ligands frequently used for most cell types (**Figure S2 in Supplementary Material**). However, TLR2 was involved in expression of the pro-inflammatory cytokines IL-6 and CXCL1 by DCs following infection with *S. suis* and stimulation by both TLR2 ligands (**Figure S3 in Supplementary Material**). These results demonstrate that though not capable of producing IFN-β following stimulation with control ligands [45], the cells remained responsive to TLR2-dependent *S. suis* stimulation. Surprisingly, TLR4 was partially implicated in IFN-β expression by DCs following infection with both the ST7 strain SC84 and the ST1 strain P1/7 ($p < 0.05$), which corresponded to a reduction of approximately 15%. TLR4 involvement was not observed with the ST25 strain 89-1591 (**Fig. 3B**). A notable difference between the ST1/ST7 and ST25 strains is the absence of SLY in the latter strain. As such, IFN-β expression by wild-type and $TLR4^{-/-}$ DCs was evaluated following infection with the SLY-deficient mutant (**Fig. 3C**). Indeed, the wild-type and SLY-deficient strains induced similar levels of IFN-β by $TLR4^{-/-}$ DCs, indicating that recognition of the SLY by TLR4 might contribute to the induction of this cytokine.

Despite the fact that *S. suis* has been described as remaining extracellularly, the largely surface TLR-independence of *S. suis*-induced IFN-β expression by DCs suggested that endosomal TLRs might participate in its induction. In accordance with *S. suis*-induced IFN-β production by DCs being mostly MyD88-dependent, the TLR3, which is MyD88-independent and recruits TRIF, was only minimally implicated, and only following infection with the SLY-negative ST25 strain 89-1591 ($p < 0.05$) (**Fig. 3D**). Meanwhile, both TLR7 and TLR9 were responsible for IFN-β expression by DCs following *S. suis* recognition, regardless of the strain (**Fig. 3D**). Indeed, their absence resulted in a 40 to 60% reduction of IFN-β expression, which was significantly lower when compared with expression by wild-type DCs ($p < 0.01$). In order to evaluate if recognition of *S. suis* by TLR7 and TLR9 was the result of a cooperative effect, DCs dually deficient for TLR7 and TLR9 were created by pretreating cells from either wild-type or TLR7$^{-/-}$ mice with the TLR9 antagonist ODN2088 or its control, ODN2088-Ctrl (**Fig. 3E**). Antagonizing wild-type DCs with ODN2088 resulted in a phenotype similar to that obtained using TLR9$^{-/-}$ DCs. Use of ODN2088-Ctrl on wild-type and TLR7$^{-/-}$ DCs confirmed the specificity of the treatment. When TLR7$^{-/-}$ DCs were antagonized with ODN2088, creating a dual TLR7$^{-/-}$/TLR9$^{-/-}$ phenotype, a greater decrease, resulting in nearly 80% abrogation of IFN-β expression by wild-type DCs ($p < 0.001$), was observed regardless of the strain. Results of IFN-β by DCs following stimulation with the different purified or synthetic TLR ligands are presented in **Figure S2 in Supplementary Material**.

The interferon regulatory factors 1, 3, and 7 play important though partially redundant roles in IFN-β expression by dendritic cells following* Streptococcus suis *infection

Activation of the TLR pathway via the endosomal TLRs usually leads to phosphorylation of IRF3/IRF7 and subsequent production of IFN-β [26]. Moreover, though IRF1 is typically associated with induction of type II IFN, crosstalk within the cell and/or simultaneous activation of various cellular pathways may result in IRF1 phosphorylation leading to IFN-β induction [46]. Consequently, given that TLR7 and TLR9 are the main TLRs responsible for *S. suis*-induced IFN-β expression by DCs, the expression levels of IRF1, IRF3, and IRF7 in DCs was determined (**Fig. 4A**). All three strains of *S. suis* induced upregulation of both IRF1 and IRF7, but not IRF3, with no significant differences amongst strains. However, IRF7 expression was significantly more upregulated than that of IRF1 ($p < 0.05$). Subsequently, in order to evaluate the role of IRF1, IRF3, and IRF7 in *S. suis*-induced IFN-β expression by DCs, cells isolated

from wild-type and knock-out mice infected with the three strains of *S. suis* were evaluated. All three IRFs were significantly implicated in *S. suis*-induced IFN-β expression by DCs ($p < 0.01$), with reductions ranging between 40 and 70% (**Fig. 4B**).

Streptococcus suis-*induced IFN-β by dendritic cells requires internalization and phagosome maturation*

Previous studies with group A *Streptococcus* (GAS) and group B *Streptococcus* (GBS) have demonstrated that internalization of the pathogen and maturation of the phagosome are required for IFN-β production by DCs [28, 38]. However, and differently from *S. suis*, these two pathogens are well-known to be internalized by phagocytes [47]. Given that the endosomal TLR7 and TLR9 are implicated in *S. suis*-induced IFN-β expression by DCs, it was hypothesized that internalization could be a critical step, even for this classical extracellular pathogen whose CPS protects against phagocytosis [10-12, 48]. However, no study has evaluated the capacity of DCs to internalize strains of *S. suis* other than ST1. Consequently, the kinetics of internalization of the three *S. suis* strains by DCs was evaluated. In accordance with previous studies [12], the well-encapsulated ST1 strain P1/7 was poorly internalized, even after 4 h of infection (**Fig. 5A**). Similar results were obtained with the ST7 strain SC84, for which information was previously unavailable. On the other hand, the intermediate virulent ST25 strain 89-1591, which induces the highest levels of IFN-β, was surprisingly and significantly more internalized by DCs than the ST1 and ST7 strains ($p < 0.01$). Since the CPS is the most important anti-phagocytic factor possessed by *S. suis* serotype 2, these results could have suggested that the strain 89-1591 was less encapsulated. However, it was possible to observe, using transmission electron microscopy, that the ST1 strain P1/7 (**Figure S4A in Supplementary Material**) and the ST25 strain 89-1591 (**Figure S4B in Supplementary Material**) are similarly well-encapsulated, suggesting that the greater internalization of strain 89-1591 by DCs is not the result of a thinner CPS.

In order to determine if *S. suis* internalization is indeed required for IFN-β expression by DCs, cells were pretreated with cytochalasin D, an inhibitor of actin polymerization, or its vehicle, DMSO. In the absence of actin polymerization, IFN-β expression was completely abrogated ($p < 0.001$) following infection with all three strains of *S. suis* (**Fig. 5B**). In contrast to IFN-β expression, actin polymerization was only partially implicated in DC expression of IL-6 and CXCL1 following infection with the *S. suis* strain P1/7 (**Figure S5 in Supplementary Material**).

Once internalized by phagocytes, the pathogen will find itself within the phagosome, which must undergo maturation [49]. Amongst the different proteins involved in internalization is the GTPase dynamin, which is required in the case of coated endosomal vesicles [50]. As such, dynamin frequently contributes to endosomal signaling of IFN-β [28, 51]. Indeed, when inhibiting dynamin activity using the inhibitor dynasore, a near complete abrogation of IFN-β expression ($p < 0.001$) was observed (**Fig. 5C**). However, though dynamin was essential for *S. suis*-induced IFN-β expression by DCs, its rôle was not internalization-dependent since internalization levels of all three *S. suis* strains did not differ when inhibiting dynamin (**Fig. 5D**).

Following phagosome formation, destruction of the pathogen requires acidification of the compartment, which occurs following fusion with the lysosome [52]. This fusion, resulting in the creation of the phagolysosome, leads to acidification of the vesicle in which vacuolar-type H^+ ATPases are implicated [53]. Inhibition of these vacuolar-type H^+ ATPases using bafilomycin A1 resulted in a significant ($p < 0.001$) and near complete abrogation of *S. suis*-induced IFN-β expression by DCs (**Fig. 5C**). It should be noted that bafilomycin A1 treatment did not affect internalization of *S. suis* (**Fig. 5D**).

Streptococcus suis ***nucleic acids are responsible for inducing IFN-β expression by dendritic cells following endosomal delivery***

The requirement of internalization and phagosome maturation in *S. suis*-induced IFN-β expression by DCs suggests that destruction of the pathogen within the phagolysosome is necessary. TLR7 and TLR9 recognize single-stranded RNA and unmethylated CpG motifs of DNA, respectively [54, 55]. To evaluate this hypothesis, bacterial RNA and DNA, isolated from all three *S. suis* strains, were complexed or not with DOTAP liposomal transfection agent which allows for delivery within the phagosome. As shown in **Fig. 6**, both *S. suis* RNA (**Fig. 6A**) and DNA (**Fig. 6B**) induced significant levels of IFN-β in DCs ($p < 0.001$), but only when complexed with DOTAP. Similarly, the capacity of poly(I:C) (**Fig. 6A**) and CpG (**Fig. 6B**) to induce IFN-β by DCs was significantly increased when complexed with DOTAP ($p < 0.001$). Moreover, levels of IFN-β expression were similar between the different *S. suis* strains and between RNA and DNA.

Streptococcus suis*-induced type I interferon by dendritic cells modulates autocrine cytokine production*

Once produced, type I IFN, including IFN-β, will bind to its receptor, IFNAR, located on the surface of most cell types, including DCs [26]. Consequently, type I IFN can modulate autocrine production of other inflammatory mediators. As such, the production of certain inflammatory cytokines known to be induced by *S. suis* [20], by DCs derived from wild-type and IFNAR$^{-/-}$ mice, was compared (**Fig. 7**). A significant role of type I IFN ($p < 0.01$) was observed in TNF, IL-6, IL-12p70, and CCL2 (**Fig. 7A-D**) production induced by the ST1 strain P1/7 and the ST25 strain 89-1591, but not by the ST7 strain SC84. On the other hand, no type I IFN-downstream modulation of CCL3 and CXCL1 production was observed for any of the strains tested.

Type I interferon is beneficial for host survival during the* Streptococcus suis *serotype 2 systemic infection: Implication in the modulation of systemic inflammation which controls blood bacterial burden

The *in vitro* production of IFN-β by DCs observed in this study, coupled with the upregulation of this cytokine in mice infected with *S. suis* [3], suggests that IFN-β may be implicated in the balance and/or exacerbation of the systemic inflammation induced by this pathogen, and subsequently, host survival. Consequently, the role of type I IFN during the *S. suis* systemic infection caused by the three strains was evaluated using a well-standardized intraperitoneal C57BL/6 mouse model of infection comparing wild-type and IFNAR$^{-/-}$ mice [39]. No role of type I IFN was observed in mouse survival during the systemic infection with the highly virulent ST7 strain SC84, with wild-type or IFNAR$^{-/-}$ mice, with both equally succumbing to the infection (**Fig. 8A**). Meanwhile, type I IFN played a significant role in the survival of mice infected with the virulent ST1 strain P1/7 ($p < 0.05$), with IFNAR$^{-/-}$ mice succumbing at a greater rate than their wild-type counterparts (45% of wild-type mice survived the systemic infection vs. only 10% of the IFNAR$^{-/-}$ mice) (**Fig. 8B**). Interestingly, no role of type I IFN was observed during early systemic infection (first 72 h p.i.) with the intermediate virulent ST25 strain 89-1591 (**Fig. 8C**), which induced high levels of IFN-β *in vitro*. However, given the lower virulence of this strain, which caused only 10 to 20% of mortality at 72 h p.i., this result was not entirely surprising. Less virulent *S. suis* strains are known to cause a delayed mortality, usually by meningitis, as a result of persistent bacteremia [4]. As such, survival of mice was evaluated until 14 days p.i. (**Fig. 8D**). Four days p.i., mortality in the wild-type group increased but then remained stable until the end of the experiment. On the other hand, IFNAR$^{-/-}$ mice were significantly more

susceptible to the infection than their wild-type counterparts ($p < 0.01$) (**Fig. 8D**). Taken together, these results suggest that type I IFN plays a beneficial role during the *S. suis* infection, at least for the ST25 and, to a certain extent, the ST1 strains, but not with the ST7 strain.

Host death during the *S. suis* systemic infection is usually the result of uncontrolled blood bacterial burden resulting from excessive bacterial growth, concomitant with an exacerbated systemic inflammatory response [4, 56]. As such, the role of type I IFN on aggravated inflammation was evaluated by measuring plasma (systemic) cytokines of both wild-type and IFNAR$^{-/-}$ mice 12 h p.i., as previously described (**Fig. 9**) [4, 39]. For TNF, IL-6 , IL-12p70, CCL2, CCL3, and CXCL1 (**Fig. 9A-F**), a significant decrease in the levels produced by IFNAR$^{-/-}$ mice, in comparison to their wild-type counterparts, was only observed for the strains P1/7 and 89-1591 ($p < 0.05$), with the difference being more pronounced in mice infected with the strain 89-1591.

The second factor responsible for host death during the *S. suis* systemic infection is uncontrolled blood bacterial burden [4]. No differences were observed between the acute blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice early after infection (12 h), regardless of the strain (**Fig. 10A**). However, since differences in mortality only became important at later time points, the effect of type I IFN during the *S. suis* infection was possibly not immediate, but rather delayed. Indeed, blood bacterial burden of mice infected with the ST1 strain P1/7 and the ST25 strain 89-1591, but not of those infected with the ST7 strain SC84, was significantly higher in IFNAR$^{-/-}$ mice 48 h p.i. than in their wild-type counterparts ($p < 0.05$) (**Fig. 10B**). Consequently, type I IFN is implicated in the modulation of systemic inflammatory mediators required for control of blood bacterial burden.

Discussion

S. suis serotype 2, an important porcine and emerging human pathogen, has always been considered a prototypical extracellular bacterium whose CPS confers important anti-phagocytic properties [6, 18]. Consequently, recognition of *S. suis* by the innate immune response was long thought to occur following interaction of bacterial lipoproteins (or other cell wall components) with surface-associated receptors, in particular TLR2 [13, 20, 21, 57]. More recently, however, partial implication of the endosomal TLR9 and cytoplasmic nucleotide-binding oligomerization domain-containing protein (NOD) 2 in recognition of *S. suis* by DCs

was reported [20], suggesting that *S. suis* or its products might also activate cellular intracellular pathways (in-out signals). Nonetheless, the implications of such intracellular receptors in the pathogenesis of the infection caused by this bacterial pathogen were, so far, unknown.

Since *S. suis* was previously reported to upregulate levels of IFN-β *in vivo* [3], but not IFN-α, the ability of DCs and macrophages, which are usually important sources of pro-inflammatory mediators, to produce IFN-β following *S. suis* infection was evaluated. Meanwhile, information regarding type III IFN is limited to a single *in vitro* study [24]. In response to *S. suis*, DCs produced higher levels of IFN-β than macrophages, suggesting different activation levels and/or intrinsic differences in cytokine production by both cell types, resulting in part from the activation of varying signaling pathways and cascades [38]. Since results obtained with positive controls revealed that DCs and macrophages are both able to induce high IFN-β levels, the differential activation of these two cell types by *S. suis* reflects an intrinsic feature of this pathogen, a characteristic than can be extended to other pathogenic streptococci [28, 38].

Lachance *et al*. suggested that *in vivo* levels of IFN-β were inversely associated with virulence of the strain used [3], a fact that was confirmed in this study with DCs, where the intermediate virulent ST25 strain 89-1591 induced higher levels of IFN-β than the virulent ST1 and ST7 strains. This inverse association is not a trait unique to the strain used, since two other intermediate virulent ST25 strains, as well as an additional ST1 strain (31533), presented results similar to their respective prototypical ST strains. To our knowledge, the inversed association of virulence with IFN-β induction has not been previously described for other bacterial pathogens. However, this association does not apply to strains of highly virulent to virulent phenotypes, as ST7 and ST1 strains induce similar levels of IFN-β.

The TLR pathway has been traditionally associated with IFN-β induction following recognition by the endosomal TLRs [26], yet, as aforementioned, recognition of *S. suis* has been mostly demonstrated to occur via surface TLRs [13, 20]. *S. suis*-induced IFN-β by DCs was MyD88-dependent, indicating that the TLR pathway is almost exclusively implicated in its production. However, even though production of many *S. suis*-induced pro-inflammatory cytokines by DCs has been reported to be mainly TLR2-dependent [20], we were unable to induce TLR2-dependent IFN-β expression by DCs. Though TLR2 activation can result in IFN-β production by macrophages, it was previously suggested that this is not the case for DCs [58,

59]. On the other hand, a partial role of TLR4 was observed in IFN-β expression induced by ST1/ST7 strains, but not by ST25 strains: this activation may be related to SLY production. Indeed, this toxin was previously reported to be recognized by TLR4 expressed on peritoneal macrophages [43].

The limited contribution of surface TLRs to *S. suis*-induced IFN-β expression by DCs suggested that endosomal TLRs, of which TLR7 and TLR9 are MyD88-dependent [55], might participate in its induction. The involvement of endosomal TLRs in recognition of *S. suis* has been little evaluated since it has been considered an extracellular pathogen. Consequently, it was unexpected that the TLR7 and TLR9 were equally and primarily responsible for *S. suis*-induced IFN-β. Though TLR7, and to a lesser extent TLR9, were responsible for IFN-β production following recognition of GBS [38], no individual TLR could be identified for GAS [28]. Even though *S. suis* and GBS share a similarity in this regard, the pathogenesis of these two encapsulated bacteria greatly differs: the most important difference between these two pathogens is that while *S. suis* is a classical extracellular bacterium protected from phagocytosis by its CPS, well-encapsulated GBS is highly internalized [47]. In the case of GBS, IFN-β production by DCs was shown to be completely dependent on IRF1 and only partially IRF7-dependent [38]. Interestingly, IRF1, IRF3, and IRF7 were all implicated in *S. suis*-induced IFN-β by DCs, suggesting a partial redundancy in signaling pathways, not observed for GBS. Indeed, localization of pathogens within the phagosome usually triggers IRF1 and IRF7 [38]. Furthermore, IFN-β induced by TLR9 agonists results in IRF1 activation via a phagosome-dependent pathway [60]. Participation of IRF3 may be the result of TLR3 or TLR4 activation by SLY-negative and SLY-positive strains, respectively, via a MyD88-independent, TRIF-dependent pathway [46]. While IRF3 was partially implicated in *S. suis*-induced IFN-β by DCs, its expression was not modulated following infection with *S. suis*, unlike that of IRF1 and IRF7. This suggests that other processing steps could be important for regulation of IRF3. Indeed, it was previously reported that unlike IRF1 and IRF7, expression of IRF3 is constitutive, with phosphorylation, rather than transcription modulating its activation [35]. Moreover, feedback loops resulting from crosstalk between pathways could also be responsible for implication of IRF3 [35]. In agreement with activation of these intracellular pathways, *S. suis*-induced IFN-□ levels inversely correlate with strain-dependent capacity to resist phagocytosis. This hypothesis is supported by the significantly higher levels of IFN-β observed in this study when using the non-encapsulated ST1 mutant strain, which was previously demonstrated to be highly

internalized by DCs [20], and by the complete abrogation of IFN-β induction following blockage of actin-dependent internalization. Interestingly, non-encapsulated *S. suis* strains have traditionally been shown to increase cytokine induction by hindering recognition of surface cell wall components, responsible for cell activation by surface-associated receptors [12, 13, 20], mechanism that would not be involved in IFN-β modulation.

Previous studies with GAS and GBS have demonstrated that internalization of the pathogen and maturation of the phagosome are required for IFN-β production by DCs [28, 38]. Results obtained in this study demonstrate that though dynamin is required for IFN-β production, this protein is implicated in early pre-acidification steps of phagosome maturation rather than in phagosome formation as evidenced by lack of effect on *S. suis* internalization. Subsequent acidification of the phagosome is required for IFN-β expression by DCs following infection with *S. suis*, suggesting that bacterial processing via hydrolytic degradation is essential for the liberation of TLR7 and TLR9 ligands [28, 38]. These results indicate that bacterial nucleic acids were the ligands of TLR7 and TLR9. Indeed, both bacterial RNA, and to a lesser extent, DNA, from GAS and GBS are also responsible for IFN-β production by DCs following stimulation of TLR7 and TLR9 [28, 38]. In contrast to GAS and GBS, however, the *S. suis* RNA and DNA induced similar levels of IFN-β, suggesting that both nucleic acids have comparable stimulatory effects. This is supported by the dual implication of TLR7 and TLR9 in *S. suis*-induced IFN-β production by DCs. Nucleic acids isolated from the three *S. suis* strains induced similar levels of IFN-β by DCs, indicating that regardless of virulence, the different *S. suis* strains possess similar stimulatory properties and differences observed would be mainly attributed to intracellular bacterial levels. The importance of nucleic acid localization within the endosome following internalization and pathogen degradation as critical steps of IFN-β induction by *S. suis* is further evidenced by the complete lack of IFN-β expression when the nucleic acids were not complexed with DOTAP. In agreement, while the TLR agonists poly(I:C) and CpG induced IFN-β in the absence of DOTAP, being powerful activators, their potency was significantly increased when complexed with DOTAP.

Once produced, type I IFN will bind to its receptor, IFNAR, located on the surface of most cell types, including DCs [26]. This autocrine effect modulates *S. suis*-induced pro-inflammatory cytokines by DCs, as well as distal production of cytokines and chemokines by other cells as observed *in vivo* during *S. suis* infection. The IFNAR downstream effect is complex as

evidenced by a lack of effect on DC chemokine production at the single-cell level [61]. Yet, when analyzing the global systemic response, the release of the chemokines CCL3 and CXCL1 is indeed modulated by the type I IFN pathway. This pathway was previously demonstrated to amplify TNF signaling following infection with GBS, *S. pneumoniae*, and *E. coli* [29]. Pro-inflammatory cytokine signaling is the result of a cascade triggered by TNF, among other mediators, leading to production of IL-6 and IL-12p70 [62]. An amplification of these downstream cytokines by type I IFN is thus expected. Similarly, type I IFN was shown to modulate the recruitment of myeloid cells by influencing CCL2 signaling during infection with the intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* [63, 64]. Given that IFNAR downstream signaling is complex, it could be interesting to evaluate the role of the SOCS protein family since they are important regulators of the JAK-STAT pathway associated with IFNAR [65]. Indeed, previous studies have demonstrated that *S. suis* induces upregulation of SOCS3 in mice and pigs following infection, with SOCS3 having been reported to block the catalytic site of JAK, thus inhibiting phosphorylation of STAT and suppressing IFNAR-induced effects [3, 65, 66]. Taken together, these results indicate a mechanism complementary to surface-associated receptor activation whereby higher internalization of *S. suis* leads to increased IFN-β induction and subsequent regulation of the pro-inflammatory loop. Our data also suggest that this IFN-□-modulated inflammatory response contributes to control bacterial burden during *S. suis* infection and improves the clinical outcome of infected animals.

It has been previously reported with other pathogens that the induction of IFN-β may be either beneficial or detrimental for the host, as shown using experimental infections. For example, a similar beneficial role was described for GAS [28], GBS [29, 38], and *S. pneumoniae* [29-31]. On the other hand, the induction of a strong type I IFN response may also be considered a key factor in early progression of invasive *S. pneumoniae* beyond the lung during development of invasive pneumococcal disease [32]. Moreover, type I IFN is associated with suppression of the innate response to certain bacterial infections, such as *L. monocytogenes* and *Francisella tularensis*, resulting in hindered bacterial clearance and deleterious host effects [46].

Taken together, type I IFN is produced by the host following *S. suis* infection and contributes to a regulated inflammatory response. In the case of the intermediate virulent ST25 strain, the

elevated IFN-β production modulates systemic pro-inflammatory mediators and appears responsible for the decreased blood bacterial burdens, which ultimately results in a reduction of meningitis and increased host survival. Indeed, it was previously reported that persistent blood bacterial burden is a prerequisite for the development of *S. suis* meningitis [4]. Albeit lower levels of IFN- production, a beneficial effect is also noticed after infection with the virulent ST1 strain. In contrast, type I IFN is unable to counteract the exacerbated inflammatory response and/or bacterial burden induced by the highly virulent ST7 strain. This observation might be related to its genetic particularities, including the presence of a pathogenicity island, and its capacity to induce exaggerated inflammation unparalleled by other *S. suis* strains, resulting in streptococcal toxic shock-like syndrome characterized by a cytokine storm [67-69].

In conclusion, this study demonstrates that, depending on the virulence level of the strain, type I IFN is involved in host defense during the *S. suis* infection by participating in clearance of blood bacterial burden and/or modulation of systemic inflammation. Results also showed that the lower virulence of the North American serotype 2 ST25 strains might be related to a lower resistance to phagocytosis that would lead to increased intracellular receptor activation with consequent IFN-β induction. Underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important zoonotic infection.

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Abbreviations

CCL, C-C motif chemokine ligand; CFU, colony-forming unit; CPS, capsular polysaccharide; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; GAS, group A *Streptococcus*; GBS, group B *Streptococcus*; IFN, interferon; IFNAR, interferon-α/β receptor; IL, interleukin; IRF, interferon regulatory factor; JAK, Janus kinase; p.i., post-infection; SLY, suilysin; SOCS,

suppressor of cytokine signaling; ST, sequence type; STAT, signal transducer and activator of transcription; THB, Todd Hewitt broth; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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Supporting Information (Available at the Frontiers in Immunology Website)

Table S1. Primer sequences used for real-time qPCR

Figure S1. Intermediate virulent *Streptococcus suis* ST25 strains induce high levels of IFN-β expression by dendritic cells

Figure S2. Ligands of the different Toll-like receptors evaluated in this study, with the exception of TLR2, induce IFN-β by dendritic cells

Figure S3. IL-6 and CXCL1 induced by *Streptococcus suis* and bacterial TLR2 ligands partially requires TLR2 expression by dendritic cells

Figure S4. The intermediate virulent *Streptococcus suis* strain 89-1591 is highly encapsulated

Figure S5. *Streptococcus suis*-induced IL-6 and CXCL1 expression by dendritic cells is partially internalization-dependent

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Tables

Table 1. *Streptococcus suis* serotype 2 strains used in this study.

Strain	General characteristics	Reference
P1/7	Virulent European ST1 strain isolated from a case of pig meningitis in the United Kingdom	[70]
P1/7Δ*cpsF*	Isogenic non-encapsulated mutant derived from P1/7; in frame deletion of *cpsF* gene	[12]
31533	Virulent European ST1 strain isolated from a case of pig meningitis in France	[71]
31533Δ*sly*	Isogenic suilysin-deficient mutant derived from 31533; in frame deletion of *sly* gene	[72]
31533Δ*dltA*	Isogenic D-alanylation of lipoteichoic acid-deficient mutant derived from 31533; in frame deletion of *dltA* gene	[17]
31533Δ*pgdA*	Isogenic N-deacetylation of peptidoglycan-deficient mutant derived from 31533; in frame deletion of *pgdA* gene	[16]
SC84	Highly virulent ST7 strain isolated from a case of human streptococcal toxic shock-like syndrome during the 2005 outbreak in China	[33]
89-1591	Intermediate virulent North American ST25 strain isolated from a case of pig sepsis in Canada	[73]
91-1804	Intermediate virulent North American ST25 strain isolated from a case of human endocarditis in Canada	[74]
LPH4	Intermediate virulent Asian ST25 strain isolated from a case of human sepsis in Thailand	[75]

Figures

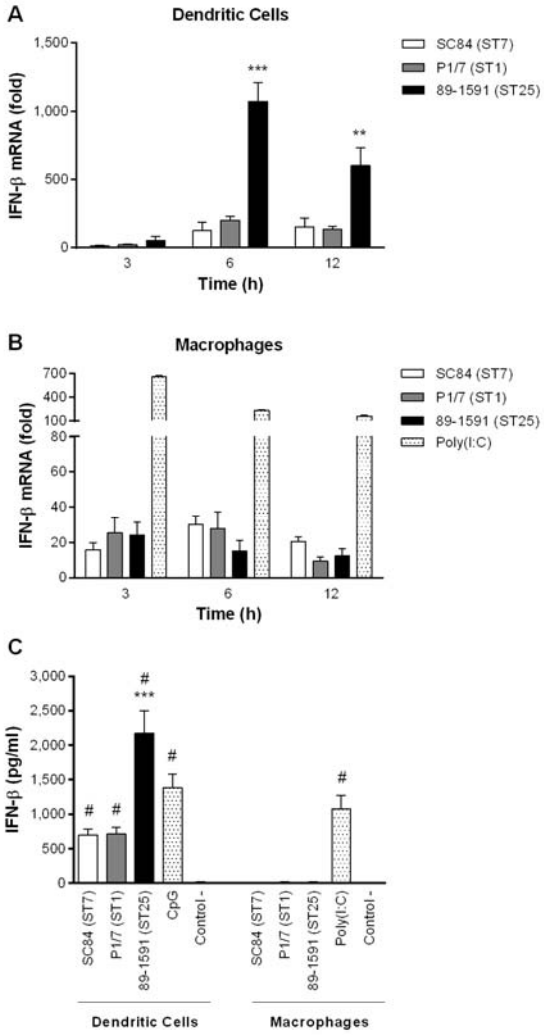

Figure 1. Dendritic cells (DCs) produce higher levels of IFN-β than macrophages following infection with *Streptococcus suis* serotype 2. IFN-β mRNA expression kinetics, measured by RT-qPCR, following infection of DCs **(A)** and macrophages **(B)**, with the highly virulent ST7 strain SC84, virulent ST1 strain P1/7, and intermediate virulent ST25 strain 89-1591. IFN-β protein production by DCs and macrophages was measured by ELISA 24 h following infection with the different *S. suis* strains **(C)**. Data represent the mean ± SEM (n = 4). ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between 89-1591 and P1/7 or SC84; # ($p < 0.001$) between *S. suis* or CpG and the negative control (control -) for DCs or between poly(I:C) and negative control (control -) for macrophages.

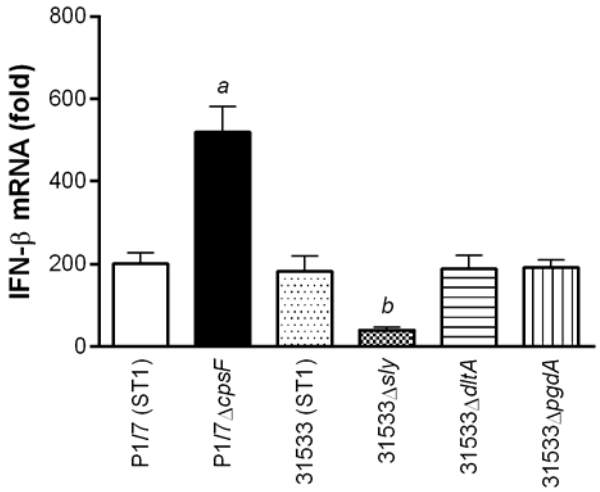

Figure 2. The presence of the capsular polysaccharide interferes with *Streptococcus suis*-induced IFN-β expression by dendritic cells, while the suilysin is partially responsible for its activation. Role of the capsular polysaccharide (CPS), suilysin (SLY), and cell wall modifications (D-alanylation of the lipoteichoic acid, Δ*dlta* or N-deacetylation of the peptidoglycan, Δ*pgdA*) in IFN-β mRNA expression by dendritic cells 6 h following infection with the wild-type or mutant *S. suis* strains. Data represent the mean ± SEM (n = 4). *a* ($p < 0.001$) indicates a significant difference between P1/7 and P1/7Δ*cpsF*; *b* ($p < 0.01$) between 31533 and 31533Δ*sly.*

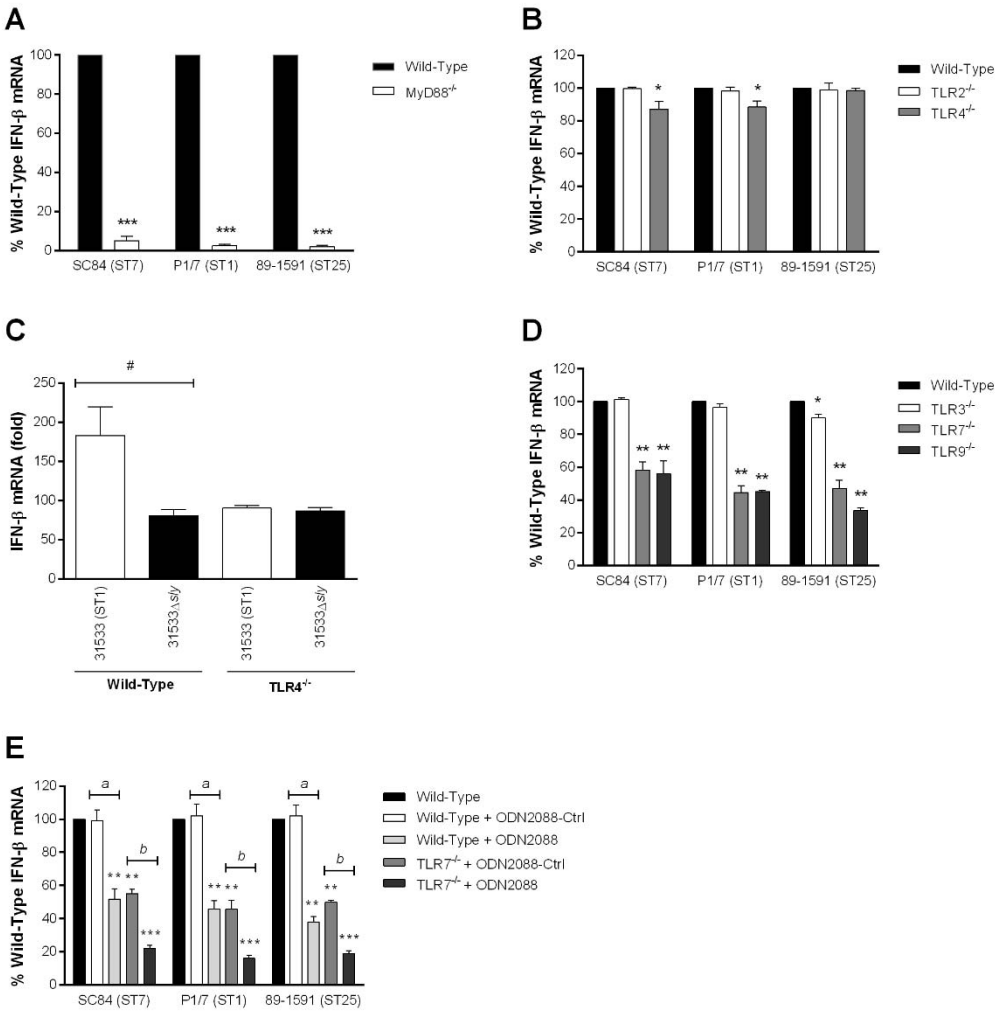

Figure 3. Recognition of *Streptococcus suis* by the Toll-like receptor (TLR) pathway is required for induction of IFN-β expression by dendritic cells (DCs). IFN-β mRNA expression induced by the different *S. suis* strains 6 h following infection of DCs deficient for MyD88 **(A)**, TLR2 or TLR4 **(B)**, or for the endosomal TLR3, TLR7, or TLR9 **(D)**. The suilysin (SLY) is responsible for TLR4-dependent IFN-β expression by DCs **(C)**. The cooperative role of TLR7 and TLR9 was evaluated using wild-type or TLR7$^{-/-}$ cells pretreated with the TLR9 antagonist ODN2088 or its control, ODN2088-Ctrl, resulting in TLR7$^{-/-}$ cells non-responsive for TLR9 (dual deficiency) **(E)**. Data represent the mean ± SEM (n = 4). * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) indicates a significant difference between expression by wild-type and deficient DCs; # ($p < 0.01$) between 31533 and 31533Δ*sly*; *a* ($p < 0.01$) between expression by wild-type DCs pretreated with ODN2088-Ctrl or ODN2088; *b* ($p < 0.01$) between expression by TLR7$^{-/-}$ DCs pretreated with ODN2088-Ctrl or ODN2088.

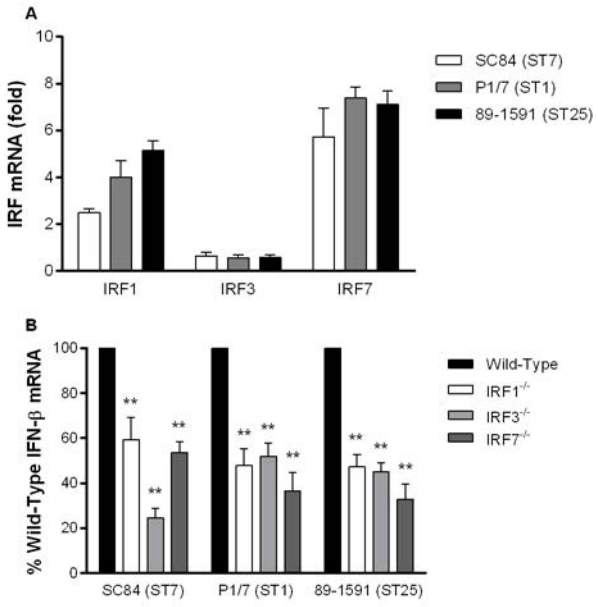

Figure 4. The interferon regulatory factors (IRFs) 1, 3, and 7 are involved in IFN-β expression by dendritic cells (DCs) following infection with *Streptococcus suis*. *S. suis*-induced IRF1, IRF3, and IRF7 mRNA expression by DCs 6 h following infection with the different strains **(A)**. IFN-β mRNA expression induced by the different *S. suis* strains following infection of IRF1$^{-/-}$, IRF3$^{-/-}$, or IRF7$^{-/-}$ DCs in comparison with cells from wild-type mice **(B)**. Data represent the mean ± SEM (n = 4). ** ($p < 0.01$) indicates a significant difference between expression by wild-type and deficient DCs.

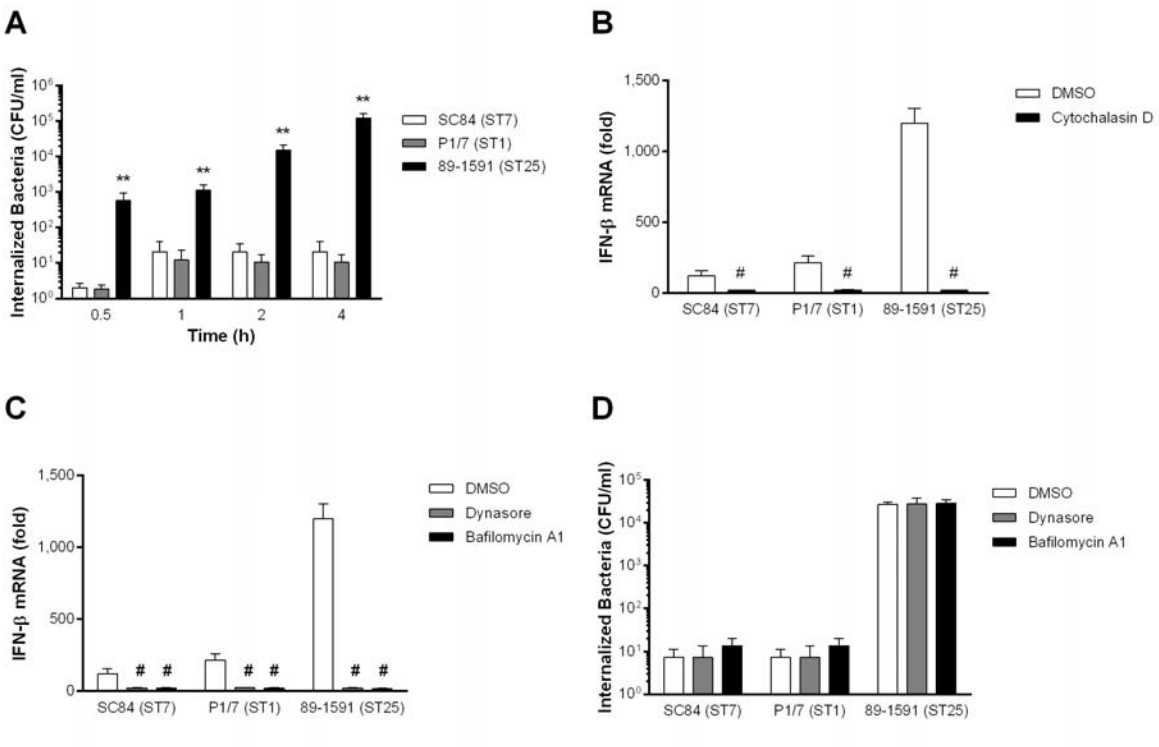

Figure 5. *Streptococcus suis*-induced IFN-β expression by dendritic cells requires internalization and phagosome maturation. Internalization kinetics of different *S. suis* strains by dendritic cells (DCs) **(A)**. Implication of actin polymerization (5 µM cytochalasin D) **(B)**, dynamin (8 µM dynasore), and vacuolar-type H^+ ATPase-dependent phagosome acidification (1 µM bafilmoycin A1) **(C)** on IFN-β mRNA expression by DCs 6 h following infection with *S. suis*. Effect of dynamin remodeling and phagosome acidification on internalization of *S. suis* by DCs 2 h following infection **(D)**. Data represent the mean ± SEM (n = 3). ** ($p < 0.05$) indicates a significant difference between 89-1591 and P1/7 or SC84; # ($p < 0.001$) between DCs treated with DMSO (vehicle) and DCs treated with the inhibitors (cytochalasin D, dynasore or bafilomycin A1).

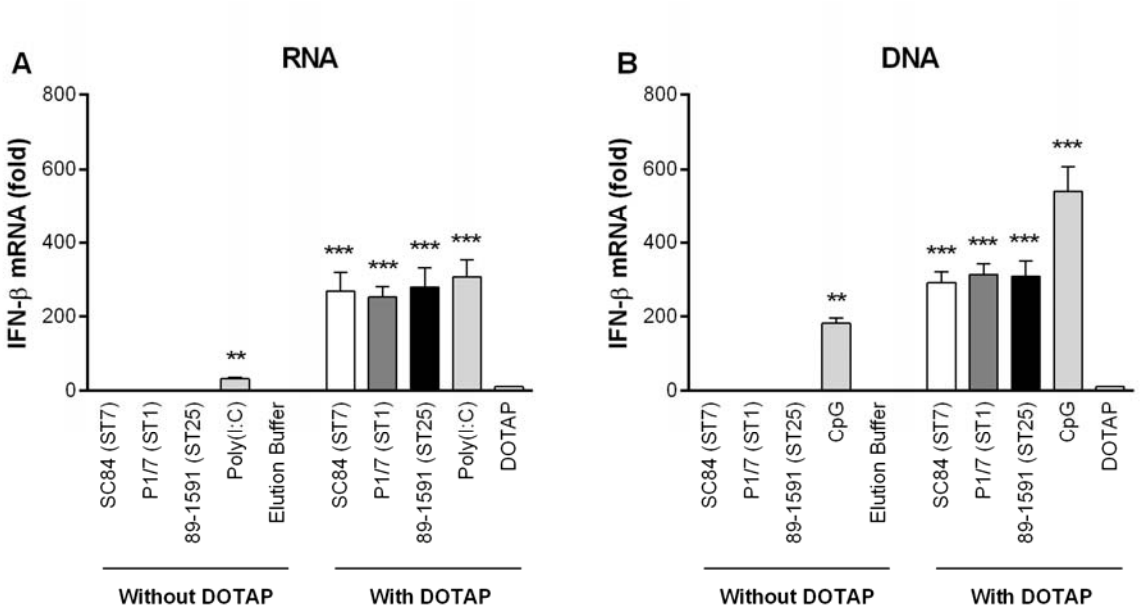

Figure 6. The *Streptococcus suis* nucleic acids are responsible for inducing IFN-β expression by dendritic cells following phagosomal delivery. IFN-β mRNA expression by dendritic cells 6 h following transfection with RNA **(A)** or DNA **(B)** isolated from the different *S. suis* strains, poly(I:C) or CpG. Nucleic acids were complexed or not with DOTAP liposomal transfection agent prior to transfection of dendritic cells. Data represent the mean ± SEM (n = 3). ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference with the elution buffer (negative control) or DOTAP alone (vehicle).

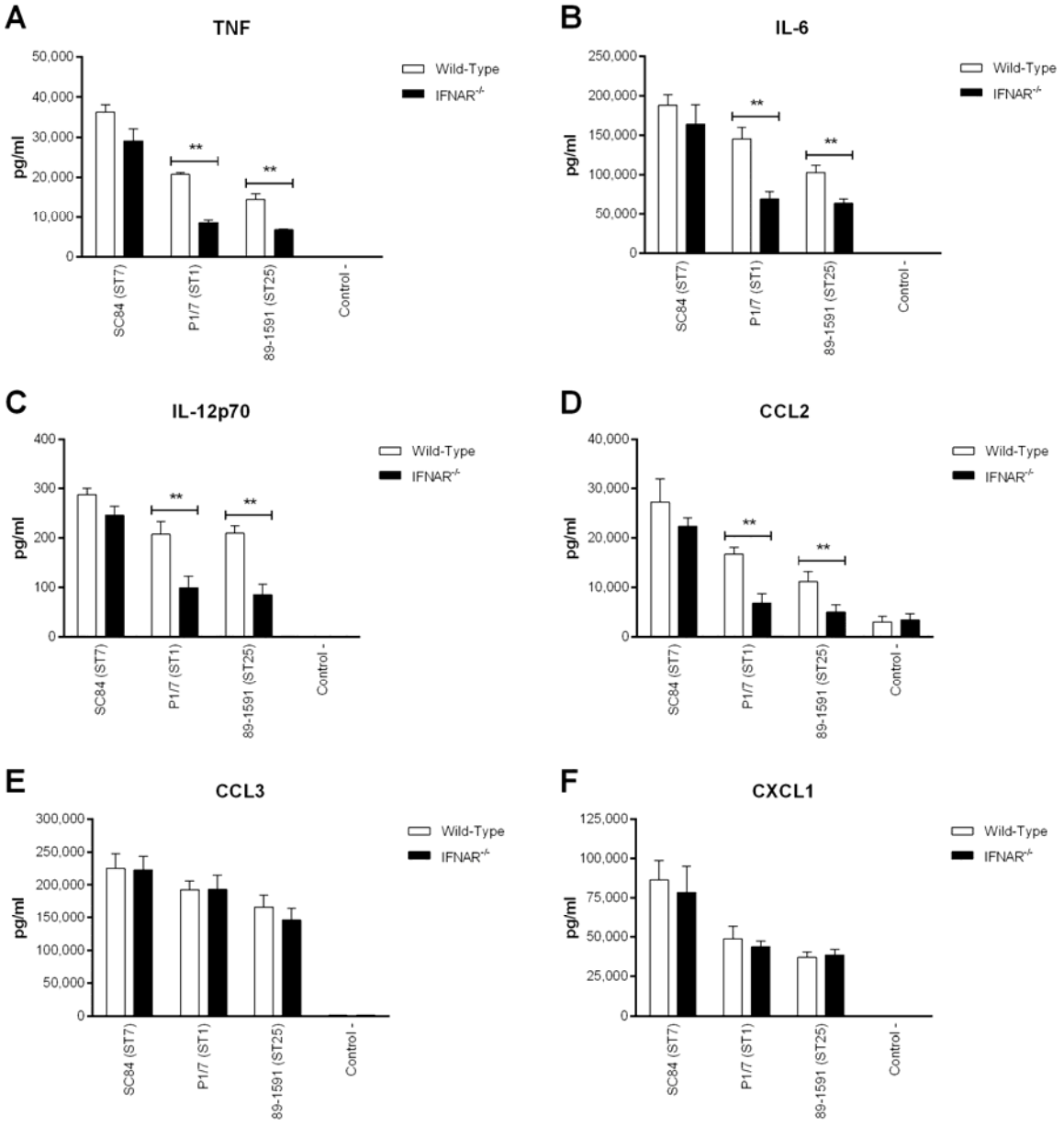

Figure 7. *Streptococcus suis*-induced type I interferon produced by dendritic cells modulates autocrine cytokine production. Production of TNF **(A)**, IL-6 **(B)**, IL-12p70 **(C)**, CCL2 **(D)**, CCL3 **(E)**, and CXCL1 **(F)** by dendritic cells 16 h following infection with the different *S. suis* strains. Data represent the mean ± SEM (n = 4). ** ($p < 0.01$) indicates a significant difference in cytokine production between wild-type and IFNAR$^{-/-}$ DCs.

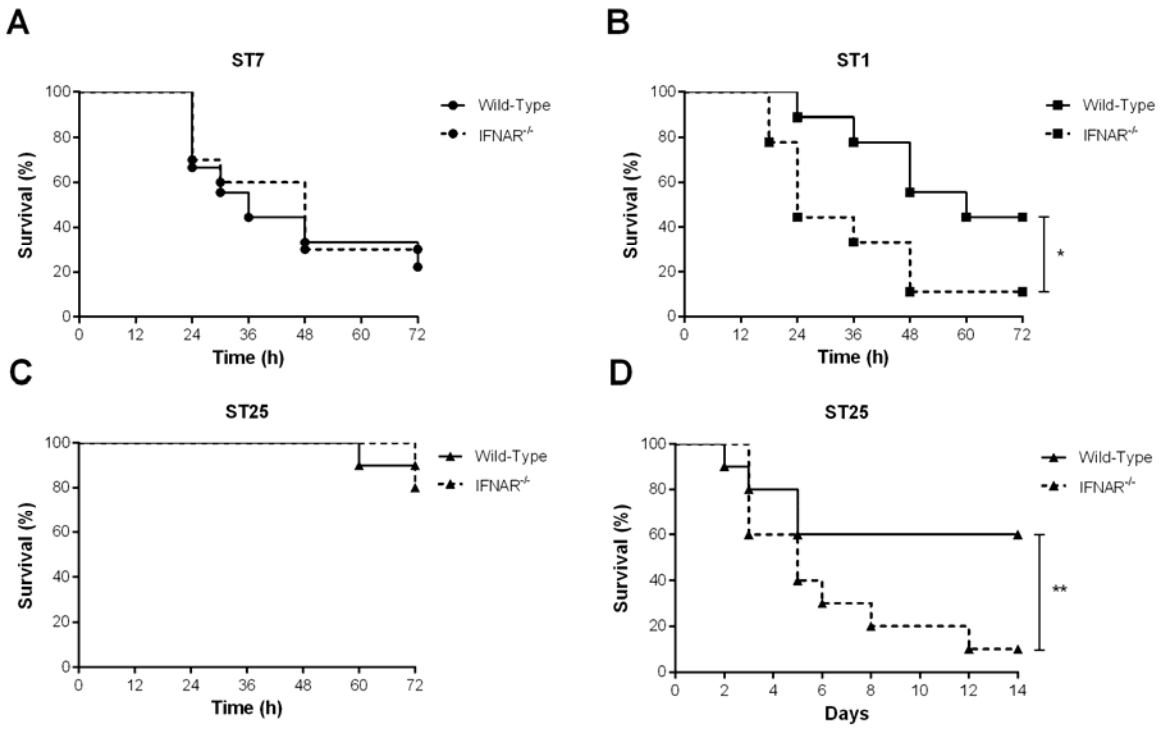

Figure 8. Type I interferon is beneficial for host survival following infection with intermediate virulent and virulent *Streptococcus suis* serotype 2 strains. Survival of wild-type and IFNAR$^{-/-}$ mice infected with the different *S. suis* strains: the highly virulent ST7 strain SC84 **(A)**, the virulent ST1 strain P1/7 **(B)**, and the intermediate virulent ST25 strain 89-1591 **(C)** during the systemic infection (until 72 h post-infection). Survival of wild-type and IFNAR$^{-/-}$ mice infected with strain 89-1591 following both the systemic and central nervous system infections (14 days post-infection) **(D)**. Data represent survival curves (n = 15). * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between survival of wild-type and IFNAR$^{-/-}$ mice.

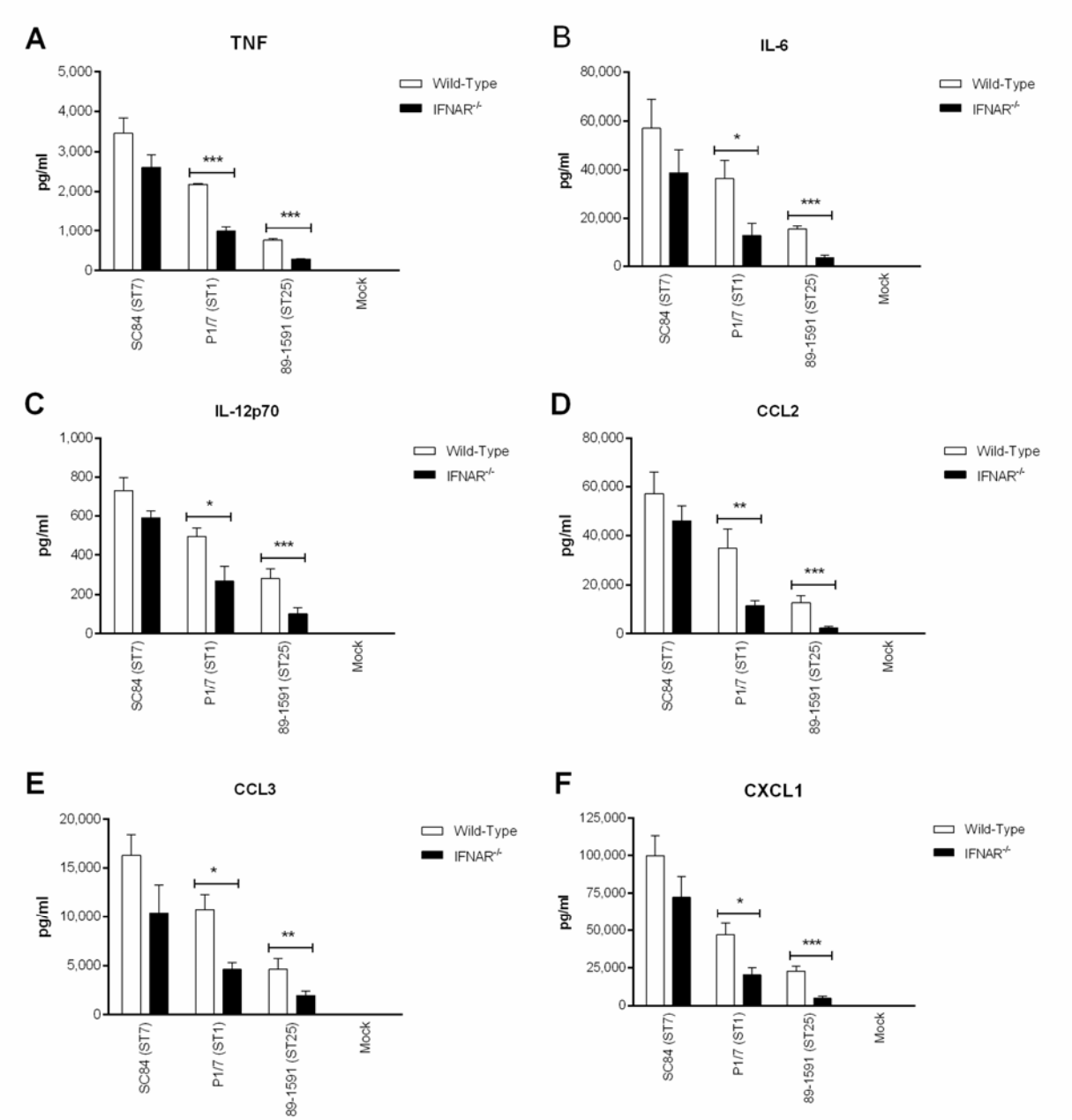

Figure 9. Type I interferon modulates plasma pro-inflammatory cytokines involved in *Streptococcus suis*-induced systemic inflammation. Plasma levels of TNF **(A)**, IL-6 **(B)**, IL-12p70 **(C)**, CCL2 **(D)**, CCL3 **(E)**, and CXCL1 **(F)** in wild-type and IFNAR$^{-/-}$ mice 12 h following infection with the different *S. suis* strains. Data represent the mean ± SEM (n = 8). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate a significant difference in plasma levels between wild-type and IFNAR$^{-/-}$ mice.

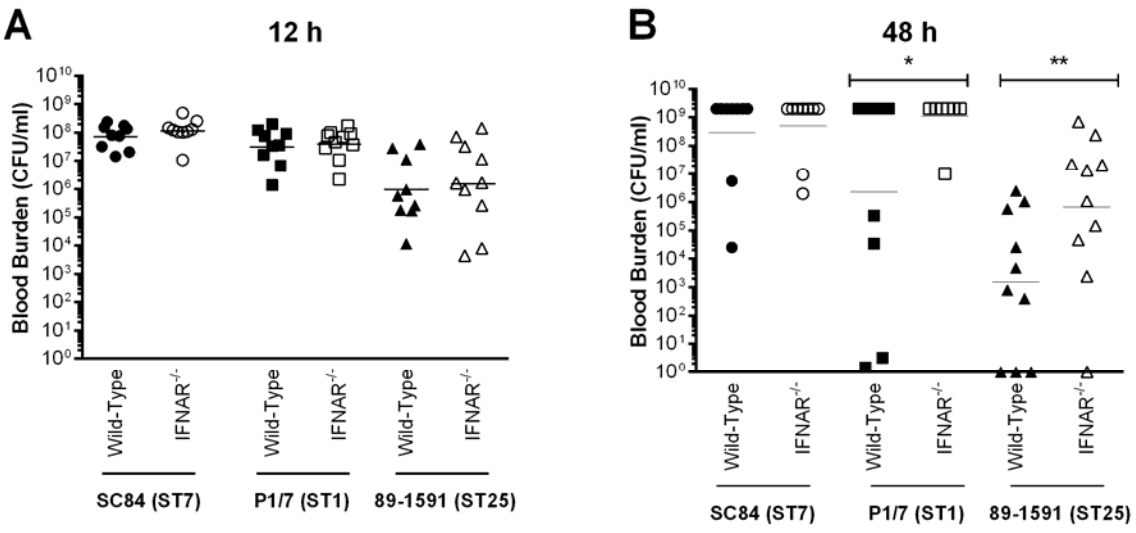

Figure 10. Type I interferon is required for control of blood bacterial burden following infection with intermediate virulent and virulent *Streptococcus suis* serotype 2 strains. Blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice infected with the different *S. suis* strains 12 h post-infection **(A)** or 48 h post-infection **(B)**. Data represent the geometric mean (n = 15). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between blood bacterial burden of wild-type and IFNAR$^{-/-}$ mice.

ARTICLE XI

# Monocytes and neutrophils equally contribute to *Streptococcus suis* serotype 2-induced systemic inflammation and disease but play differential roles during central nervous system disease

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Manuscrit en préparation pour soumission à *Frontiers in Immunology*

Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur du manuscrit. J'ai été responsable de la conception de l'étude et des approches méthodologiques. J'ai effectué toutes les expériences, l'analyse des résultats, l'écriture de la première version du manuscrit et j'ai été impliqué dans la révision de celui-ci.

Mise en contexte

Les travaux présentés dans le cadre des articles IX et X ont démontré que la réponse inflammatoire induite par *S. suis* peut causer la mort de l'hôte si elle n'est pas finement contrôlée. En effet, bien que l'inflammation soit bénéfique et nécessaire pour combattre l'infection causée par *S. suis* son exacerbation est la caractéristique principale des infections systémiques et du SNC et des pathologies causées. Plusieurs types cellulaires sont impliqués dans la réponse inflammatoire systémique et du SNC. Bien que le rôle des macrophages et des DCs ait été quelque peu étudié, celui des neutrophiles et des monocytes restait inconnu lors des infections à *S. suis*, et ce malgré leur importance dans la circulation sanguine où ils sont les phagocytes prédominants et le fait qu'ils infiltrent massivement le SNC lors de la méningite/méningoencéphalite. Ainsi, nous avons voulu mieux caractériser leurs rôles et leurs contributions à la pathogenèse de l'infection systémique et du SNC causées par *S. suis.*

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and a zoonotic agent responsible for sudden death, septic shock, and meningitis. These pathologies are the consequence of elevated bacterial replication leading to exacerbated and uncontrolled inflammation, which is a hallmark of the *S. suis* systemic and central nervous system (CNS) infections. Neutrophils and monocytes are important innate immune blood cells involved in a variety of functions, including pro-inflammatory mediator production. Moreover, monocytes are composed of two main subsets, whether the shorter-lived inflammatory monocytes and the longer-lived patrolling monocytes. However, regardless of their dominance in blood and that *S. suis*-induced meningitis is characterized by a massive infiltration of monocytes and neutrophils into the CNS, their role during the *S. suis* systemic and CNS diseases remains unknown. As such, the role of inflammatory and patrolling monocytes was determined using $CCR2^{-/-}$ and $Nr4a1^{-/-}$ mice, respectively, while neutrophils were depleted using anti-Ly6G neutralizing antibodies. Results demonstrated that neutrophils, and to a lesser extent inflammatory monocytes, participate in *S. suis*-induced systemic disease via their role in inflammation required for bacterial burden control. Meanwhile, inflammatory monocytes contributed to the exacerbation of *S. suis*-induced CNS inflammation while neutrophils participated in CNS bacterial burden control. However, development of clinical CNS disease was independent of both cell types, and this regardless of inoculated dose, suggesting that resident immune cells are mostly responsible for *S. suis*-induced CNS inflammation and clinical disease. By contrast, implication of patrolling monocytes was minimal throughout the *S. suis* infection. Consequently, this study demonstrates that while monocytes and neutrophils equally contribute to *S. suis*-induced systemic inflammation and disease, they play differential roles during the CNS disease.

Introduction

Streptococcus suis is one of the most important porcine bacterial pathogens and is a zoonotic agent mainly responsible for sudden death (pigs), septic shock (humans), and meningitis (both species) [1, 2]. These pathologies are characterized by an exacerbated and uncontrolled inflammation, which is a hallmark of the *S. suis* systemic and central nervous system (CNS) infections [3, 4]. Of the different serotypes described, serotype 2 is the most virulent and widespread worldwide [5].

Following colonization of the upper respiratory tract of pigs, *S. suis* may reach the bloodstream by breaching the mucosa or other poorly understood mechanisms [6]. Infection of humans occurs via skin wounds or at the intestinal interface following contact with diseased animals and/or raw or undercooked infected pork products [6]. In the bloodstream, *S. suis* resists killing by phagocytes, which results in bacteremia, organ dissemination, and development of systemic infection [7]. Moreover, activation of innate immune cells leads to an exacerbated inflammation responsible for sepsis leading to sudden death in pigs and septic shock in humans [1]. If untreated, *S. suis*-induced systemic inflammation may result in host death [1]. Moreover, if bacteria are not rapidly cleared from the bloodstream, they can reach the blood-brain barrier or blood-cerebrospinal fluid barrier, which they then cross [7]. In the CNS, recognition by innate immune cells leads to the recruitment of infiltrating cells and an excessive production of pro-inflammatory mediators, resulting in meningitis [7].

The interactions between *S. suis* and innate immune cells, particularly phagocytes, have been somewhat dissected, with studies mostly focusing on macrophages and conventional dendritic cells (DCs), which are mainly tissue-resident cells [8-15]. However, not only are monocytes and neutrophils the main phagocytes in blood [16], but they are sources of pro-inflammatory mediators and play important roles during bacterial infection [17, 18]. Moreover, meningitis induced by *S. suis* is characterized by a massive infiltration of both cell types into the CNS [2, 3, 19, 20]. Yet, their role during *S. suis*-induced systemic and CNS diseases has been little studied. In fact, information is limited to only a handful of studies *in vitro* [21-24].

Though historically viewed as simply being precursor cells responsible for replenishing the tissue-resident macrophage and DC populations, monocytes are mature effector cells involved in a variety of functions, of which phagocytosis and pro-inflammatory mediator production are

the most important [25]. Though composed of morphologically and phenotypically heterogeneous subsets with different roles, these subsets are similar between humans, pigs, and mice [25]. The two main subsets are the shorter-lived “classical” inflammatory monocytes ($Ly6C^{high}CCR2^{high}CX_3CR1^{low}$ in mice) that infiltrate inflamed tissues to trigger local immune responses and the longer-lived “non-classical” patrolling/resident monocytes ($Ly6C^{low}CCR2^{low}CX_3CR1^{high}$ in mice) that home to non-inflamed tissues and repopulate tissue-resident cells during homeostatic conditions [26]. While egress of inflammatory monocytes from the bone marrow and their mobilization requires C-C chemokine receptor (CCR) type 2 (CCR2), differentiation and survival of patrolling monocytes depends on the transcription factor nuclear receptor subfamily 4 group A member 1 (Nr4a1) [27, 28]. More recently, however, these roles have become much less clearly defined. Indeed, though fully differentiated upon exiting from the bone marrow, current research suggests that monocytes can shift between subsets in peripheral blood [25, 29]. Though the role of inflammatory monocytes depends on the pathogen, playing a beneficial role during *Listeria monocytogenes* and *Escherichia coli* K1 infections, but no role during *Streptococcus pneumoniae* meningitis, that of patrolling monocytes during bacterial infection remains virtually unknown [27, 30, 31].

Meanwhile, neutrophils representing the most abundant innate immune cells in blood [16]. As such, they play important roles in bacterial clearance and immune responses, including phagocytosis and killing, degranulation, neutrophil extracellular trap formation, and pro-inflammatory mediator production [18]. Moreover, neutrophils migrate to infected tissues where their presence is often decisive to the outcome. Indeed, while neutrophils play a beneficial role during Group B *Streptococcus* (GBS) and *S. pneumoniae* infection via their participation in inflammation required for bacterial control and clearance, they play a dual role (beneficial and detrimental) during bacterial sepsis depending on the stage of disease [30, 32-34].

To further our knowledge of the *S. suis* pathogenesis, the role of inflammatory and patrolling monocytes, as well as neutrophils, during the systemic and CNS infections was evaluated. We demonstrated that neutrophils, and to a lesser extent inflammatory but not patrolling monocytes, participate in *S. suis*-induced systemic disease via their role in inflammation, which subsequently controls bacterial burden. Meanwhile, inflammatory monocytes contribute to the exacerbation of *S. suis*-induced CNS inflammation while neutrophils participate in CNS bacterial burden control, although development of clinical CNS disease is independent of both

cell types. Consequently, monocytes and neutrophils have differential roles during the *S. suis*-induced systemic and CNS diseases.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol number Rech-1570).

Mice

CCR2$^{-/-}$ (B6.129S4-*Ccr2*^{tm1Ifc}/J) and Nr4a1$^{-/-}$ (B6.129S2-*Nr4a1*^{tm1Jmi}/J) mice on C57BL/6 background were purchased from Jackson Research Laboratories (Bar Harbor, ME, USA) and housed under specific pathogen-free conditions alongside C57BL/6J wild-type counterparts.

Streptococcus suis *serotype 2 strain and growth conditions*

The well-characterized and highly encapsulated classical virulent *S. suis* serotype 2 P1/7 strain, isolated from a case of pig meningitis in the United Kingdom, was used throughout this study [35]. *S. suis* was grown overnight on Columbia agar supplemented with 5% sheep blood (Oxoid, Nepean, ON, Canada) at 37 °C with 5% CO_2. Five milliliters of Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) were inoculated with an isolated colony and incubated for 8 h at 37 °C with 5% CO_2. Working cultures were prepared by inoculating 30 mL of THB with 10 µL of a 10^{-3} dilution of the 8 h culture and incubating for 16 h at 37 °C with 5% CO_2. Bacteria were washed twice with phosphate-buffered saline, pH 7.3, resuspended in THB, and final colony-forming units (CFU)/mL determined by plating on THB agar (THA).

Neutrophil depletion

Mice were intraperitoneally injected with 0.5 mg of rat monoclonal anti-mouse Ly6G antibody (clone 1A8) or rat IgG2a isotype control (clone RTK2758) (BioLegend, Burlington, ON, Canada) 24 h prior to infection with *S. suis* [36, 37]. To confirm depletion, 50 µL of peripheral blood was collected at different times following injection, anti-coagulated with EDTA and treated with a FcR-blocking reagent (Fc*γ*III/II Rc Ab, BD Pharmingen, Mississauga, ON, Canada) for 15 min on ice. Cells were stained with FITC-conjugated anti-Ly6G (clone 1A8) or isotype antibody as

control for 45 min on ice and erythrocytes lysed using a 155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA solution. Samples were then analyzed on a BD FACS Fusion using the FACSDiva software (BD Biosciences, Mississauga, ON, Canada). $Ly6G^+$ cells were depleted beyond 85% (**Table S1 and Figure S1 in Supplementary Material**).

Streptococcus suis *serotype 2 experimental infections*

Six-week-old male and female mice were used throughout this study. Mice were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [4, 38]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an endpoint measurement. For systemic virulence studies, 1 x 10^7 CFU of *S. suis* were administered by intraperitoneal inoculation to mice for survival and blood bacterial burden. Mice were monitored at least thrice daily until 72 h post-infection for clinical signs of systemic disease (rough coat hair, closed/swollen eyes, prostration, depression, difficulty breathing, and lethargy). Blood bacterial burden of surviving mice was assessed at different times post-infection by collecting 5 µL of blood from the caudal vein, appropriately diluting, and plating on THA as described above. Blood bacterial burden was also measured prior to euthanasia.

For the transcutaneal intracisternal model of CNS infection, mice were anesthetized with inhaled isofluorane (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) and 10 µL of 1 x 10^3 CFU/mL or 1 x 10^7 CFU/mL (final concentrations of 10 CFU and 10^5 CFU, respectively) of *S. suis* were injected as previously described [4, 39]. Animals were monitored every 8 h until 72 h post-infection and euthanized at different pre-determined time points, upon presentation of clinical signs of CNS disease (spatial disorientation, hyper-excitement followed by opisthotonos, circular walking with head tilt, sudden spinning while in recumbence, and tonicoclonic movements) or at the end of the study. Controls (mock-infected) were injected with 10 µL of the vehicle solution (sterile THB).

Measurement of plasma (systemic) pro-inflammatory mediator levels

In parallel, additional groups of mice were intraperitoneally infected with 1 x 10^7 CFU of *S. suis* as described above. Mice were euthanized 12 h post-infection [4, 40] and blood was collected

by intracardiac puncture and anti-coagulated with EDTA (Sigma-Aldrich, Oakville, ON, Canada). Plasma was collected following centrifugation at 10 000 x *g* for 10 min at 4 ºC and stored at -80 ºC. Plasmatic concentrations of interleukin (IL)-6, IL-12p70, interferon (IFN)-γ, C-C motif chemokine ligand (CCL) 3, CCL4, CCL5, C-X-C motif chemokine ligand (CXCL) 1, and CXCL2 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Measurement of brain bacterial burden and pro-inflammatory mediator levels

Samples were collected at different pre-determined time points, upon presentation of clinical signs of CNS disease or at the end of the experiment (72 h post-infection). Following euthanasia, brains were aseptically recovered and homogenized in phosphate-buffered saline, pH 7.3, from which bacterial burdens were determined by plating appropriate dilutions on THA as described above, or directly frozen in liquid nitrogen. For pro-inflammatory mediator evaluation, extraction buffer, prepared using cOmplete Mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and supplemented with 0.4% CHAPS (Sigma-Aldrich), was added to frozen brains, which were then homogenized using a POLYTRON PT 1200E system bundle (Kinematica, Lucerne, Switzerland). Brain homogenate supernatants were collected following centrifugation at 10 000 x *g* for 10 min at 4 °C and stored at -80 °C. Levels of IL-6, CCL2, CCL3, and CXCL2 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad) as described above, while levels of IL-1β and CXCL1 were measured by sandwich enzyme-linked immunosorbent assay using pair-matched antibodies (R&D Systems, Minneapolis, MN, USA) as previously described [10].

Statistical analyses

Normality of data distribution was verified using the Shapiro-Wilk test. Accordingly, parametric (unpaired t-test and one-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test and one-way ANOVA on ranks) were performed to evaluate statistical differences between groups. Data are presented as mean ± standard error of the mean (SEM) or as geometric mean. Log-rank (Mantel-Cox) tests were used to compare survival between groups of mice. $P < 0.05$ was considered statistically significant.

Results

Inflammatory but not patrolling monocytes are implicated in host survival during Streptococcus suis *systemic infection: Partial role in modulation of the systemic inflammation required for blood bacterial burden control*

Previous studies suggested that monocytes interact with *S. suis* [21, 23, 41]. However, these studies were conducted *in vitro* and have not evaluated the differential contribution of monocyte subsets. Consequently, the role of inflammatory and patrolling monocytes was evaluated during the acute *S. suis* systemic infection following intraperitoneal inoculation of a standard dose of 1 x 10^7 CFU using CCR2$^{-/-}$ and Nr4a1$^{-/-}$ mice, which are required for inflammatory and patrolling monocyte mobilization and survival, respectively [27, 28]. Following the acute systemic disease (72 h post-infection), survival of CCR2$^{-/-}$ mice was significantly less than that of wild-type and Nr4a1$^{-/-}$ mice ($p < 0.05$), whose survival was similar (**Fig. 1**).

Though host death during *S. suis* systemic infection is usually due to an exacerbated inflammatory response, a certain level of inflammation is required for bacterial elimination [8]. As such the role of monocytes in systemic inflammation was evaluated by measuring plasma mediators from wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice 12 h post-infection, corresponding to the time when production is maximal [4, 38]. As previously described, *S. suis* induced high plasmatic levels of pro-inflammatory mediators in wild-type mice (**Fig. 2**) [4, 38]. While levels in wild-type and Nr4a1$^{-/-}$ mice were similar, they were significantly lower in CCR2$^{-/-}$ mice ($p < 0.05$), with a 20% to 50% reduction depending on the mediator (**Fig. 2**).

Given these differences in systemic inflammation and that inflammation participates in bacterial clearance, bacteremia of wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice was evaluated at different times following infection. No differences were observed in blood bacterial burden early after infection (6 h and 12 h post-infection) (**Fig. 3A-B**). However, since inflammation must first be induced to have an effect, this was not entirely surprising. At later times (24 h and 48 h post-infection), however, blood bacterial burden was significantly greater in CCR2$^{-/-}$ mice than in wild-type and Nr4a1$^{-/-}$ mice ($p < 0.01$) (**Fig. 3C-D**). This suggests that inflammatory monocytes are indirectly implicated in blood bacterial burden control via modulation of inflammation.

Neutrophils are required for host survival during the* Streptococcus suis *systemic infection: Critical role in modulation of systemic inflammation necessary for blood bacterial burden control

The partial contribution of monocytes in host survival during *S. suis* systemic infection suggested that other innate immune cells are also involved. Since neutrophils are the most abundant innate immune cells in blood, their role was evaluated. Neutrophils were depleted from wild-type mice by injection of anti-Ly6G neutralizing antibody or its isotype control 24 h prior to infection with a standard dose of 1 x 10^7 CFU of *S. suis*. Treatment was sufficient to deplete neutrophils beyond 85%, with low counts persisting for at least 72 h (**Table S1 in Supplementary Material**). Depletion of neutrophils resulted in significantly less survival during the acute systemic infection (until 72 h post-infection), with only 20% of mice remaining alive by 48 h post-infection ($p < 0.01$) (**Fig 4**).

To better explain these differences in survival, the role of neutrophils in inflammation was evaluated by measuring plasma mediators 12 h post-infection. Neutrophil depletion resulted in a significant reduction of all systemic pro-inflammatory mediators evaluated, equivalent to a 50% to 75% decrease ($p < 0.01$) (**Fig. 5**). Given this important modulation of systemic inflammation by neutrophils, their role on blood bacterial burden was determined. In accordance, depletion of neutrophils resulted in significantly greater bacteremia as of 6 h post-infection ($p < 0.01$) (**Fig. 6**), which is prior to maximum inflammatory production [38]. This rapid increase in burden following depletion suggests that neutrophils are probably implicated in blood bacterial burden control both via modulation of inflammation and direct killing mechanisms.

Streptococcus suis *induces a massive release of pro-inflammatory chemokines from the central nervous system*

Following the acute *S. suis* systemic infection, surviving individuals are susceptible of developing a CNS disease of which meningitis is the hallmark [3, 4]. This disease is the result of an exacerbated local inflammatory response in the CNS and is characterized by a massive infiltration of peripheral immune cells, namely monocytes and neutrophils [19, 38]. This inflammatory response is composed of not only pro-inflammatory cytokines, but also of chemokines, which are responsible for the recruitment of these peripheral immune cells [4]. However, production of these chemokines has always been evaluated upon presentation of

clinical CNS disease, making it difficult to understand timeline. Consequently, the production of CCL2, CCL3, CXCL1, and CXCL2, important for the chemoattraction of monocytes and neutrophils, was evaluated at different times following mock-infection (sterile THB) or inoculation of *S. suis* via the transcutaneal intracisternal route as this well-developed model results in rapid development of clinical signs and histopathological lesions of CNS disease [4, 39]. Since chemokines levels were similar in mock-infected regardless of time (data not shown), average values are presented (**Fig. 7**). On the other hand, levels of CCL2, CCL3, CXCL1, and CXCL2 were already significantly higher 6 h post-infection ($p < 0.01$) (**Fig. 7**). Levels increased with time before reaching maximum values upon presentation of clinical disease (between 18 h and 24 h post-infection) (**Fig. 7**). These results suggest that an early and important release of chemokines from the CNS following *S. suis* infection may be responsible for the massive infiltration of monocytes and neutrophils present during clinical disease.

Differential role of monocytes and neutrophils during* Streptococcus suis*-induced central nervous system disease

Given the elevated levels of chemokines observed in the CNS following *S. suis* infection and the massive monocytes and neutrophil infiltrates during clinical disease [3, 4], the role of monocytes and neutrophils in the development of *S. suis*-induced CNS disease was evaluated. Wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice (monocytes) or wild-type mice treated with isotype control or anti-Ly6G (neutrophils) were mock-infected or infected with a standard dose (1 x 10^5 CFU) of *S. suis* using the transcutaneal intracisternal route and the development of clinical signs of CNS disease evaluated. Since mice were euthanized upon presentation of clinical signs, data are presented as survival curves. None of the mock-infected mice developed clinical signs of infection (data not shown). By contrast, 100% of wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice infected with *S. suis* developed clinical signs of CNS disease within 24 h post-infection (**Fig. 8A**). Similar results were obtained following neutrophil depletion, with 100% of isotype control- or anti-Ly6G-treated mice developing clinical signs of CNS disease within 24 h of *S. suis* infection (**Fig. 8B**).

Development of CNS disease by *S. suis* is usually due to bacterial presence and replication in the CNS, which leads to inflammation [4]. As such, brain bacterial burden was evaluated early following infection (6 h) and upon presentation of clinical signs of *S. suis*-induced CNS disease (between 18 h and 24 h post-infection). Burden was similar between wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice 6 h post-infection, averaging 5 x 10^3 CFU (**Fig. 9A**). Furthermore, no differences

were observed upon presentation of clinical CNS disease (**Fig. 9A**). While, depletion of neutrophils had no effect on brain bacterial burden 6 h post-infection, bacterial concentration was significantly greater in neutrophil-depleted mice upon presentation of clinical signs of CNS disease ($p < 0.05$), regardless of similar susceptibility to the infection (**Fig. 9B**). Notably, brain bacterial burden increased with time regardless of mouse genotype or treatment (equivalent to a ten thousand-fold increase between 6 h and presentation of clinical disease), indicating rapid and efficient replication of *S. suis* within the host CNS.

Since presence of *S. suis* in the CNS is responsible for inducing a local pro-inflammatory response [4], brain pro-inflammatory mediators were measured 6 h post-infection and upon presentation of clinical CNS disease (between 18 h and 24 h post-infection). Levels of IL-1β, IL-6, CCL2, CCL3, CXCL1, and CXCL2 were significantly greater 6 h following infection than in mock-infected mice ($p < 0.05$), but were similar between wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice (**Fig. 10**). Upon presentation of clinical CNS disease, however, they were significantly lower in the CNS of CCR2$^{-/-}$ mice than in that of wild-type and Nr4a1$^{-/-}$ counterparts ($p < 0.05$), which presented similar levels (**Fig. 10**). It is worth noting that the reduction in CCR2$^{-/-}$ mice was equivalent to a 25% decrease at most (**Fig. 10**). By contrast, only CXCL1 and CXCL2 production was significantly increased 6 h post-infection in the CNS of neutrophil-depleted mice ($p < 0.05$) (**Fig. 11**). Upon presentation of clinical CNS disease, levels of IL-1β, IL-6, CCL2, CCL3, CXCL1, and CXCL2 were significantly higher following anti-Ly6G treatment with a 300% increase in CXCL1 and CXCL2 production ($p < 0.05$) (**Fig. 11**).

This differential role of inflammatory monocytes and neutrophils in *S. suis*-induced CNS disease was observed using a standard dose of *S. suis*. Since inoculation of high bacterial loads might influence the role or outcome observed (due to elevated initial burden), the minimal bacterial dose capable of inducing CNS disease (10 CFU) was inoculated via the intracisternal route (Auger *et al.* Submitted). Wild-type, CCR2$^{-/-}$, and Nr4a1$^{-/-}$ mice or isotype control- and anti-Ly6G-treated mice all developed similar clinical CNS disease, with 50% to 60% of mice presenting clinical signs between 36 h and 72 h post-infection (**Figure S2 in Supplementary Material**). Moreover, similar patterns in brain bacterial burden, both early (24 h post-infection) and upon presentation of clinical CNS disease (between 36 h and 72 h post-infection), were observed as with the standard dose (**Figure S3 in Supplementary Material**). Notably, only certain mice presented brain bacterial burden 24 h post-infection, corresponding to the mice

that eventually developed clinical disease, while no burden was detected in the CNS of mice that never presented clinical disease by the end of the experiment (72 h post-infection) (**Figure S3 in Supplementary Material**). Finally, patterns of IL-1β, IL-6, CCL2, CCL3, CXCL1, and CXCL2 production in the CNS of mice were also similar to the standard dose (**Figure S4 and S5 in Supplementary Material**). Taken together, these results demonstrate that while inflammatory monocytes and neutrophils have differing roles during *S. suis*-induced CNS disease, development of clinical CNS disease is independent of their presence.

Discussion

Diseases caused by *S. suis* serotype 2 are the consequence of elevated bacterial replication usually leading to exacerbated systemic and CNS inflammatory responses. Though monocytes and neutrophils are the predominant phagocytes in blood and massively infiltrate the CNS during meningitis, their role during the *S. suis* systemic and CNS infections *in vivo* remains unknown.

Once in the bloodstream, *S. suis* can replicate and disseminate, resulting in innate host cell activation and induction of an inflammatory response [3, 4, 40]. Results obtained herein demonstrate that inflammatory monocytes and neutrophils actively participate in *S. suis*-induced systemic inflammation, since all pro-inflammatory mediators evaluated were reduced in their absence. This concords with *in vitro* studies using THP-1 monocytes, in which secretion of tumor necrosis factor (TNF) and CCL2 was observed following *S. suis* infection [23]. Furthermore, this is the first study to report a role monocytes and neutrophils in pro-inflammatory mediator production during *S. suis* infection *in vivo*. In fact, to our knowledge, no other study has ever evaluated pro-inflammatory mediator production by neutrophils whatsoever.

Alongside, absence of neutrophils and monocytes resulted in increased blood bacterial burdens. Since a certain level of inflammation is required for bacterial clearance [8], this indicates that their contribution to systemic inflammation is necessary for host outcome during *S. suis* infection. In addition to this indirect role in bacterial clearance, neutrophils also appear to be directly involved in early elimination of *S. suis* from the bloodstream. Indeed, blood bacterial burden was already greater in neutrophil-depleted mice 6 h post-infection, at which time phagocytic and killing mechanisms induced by inflammation have probably not been

optimally activated since *S. suis*-induced inflammation peaks around 12 h post-infection [38]. This supports *in vitro* studies having shown that neutrophils are more efficient at killing *S. suis* than monocytes without prior priming [21, 22].

Even though inflammatory monocytes and neutrophils contribute to *S. suis*-induced inflammation, mortality of CCR2$^{-/-}$ and neutrophil-depleted mice was greater, indicating that these two cell types play a beneficial role during *S. suis* systemic infection. Likewise, inflammatory monocytes play a beneficial role during Group A *Streptococcus* infection by participating in systemic bacterial elimination via yet unknown mechanisms [42]. Moreover, neutrophils play a crucial role during GBS, *S. pneumoniae*, and *Staphylococcus aureus* infection via direct (killing) and/or indirect (inflammation) bacterial control [32, 43, 44]. Though induced inflammation is required for bacterial clearance, its exacerbation is detrimental to the host and causes death [3, 4, 40]. While associated with a “beneficial role”, participation of inflammatory monocytes and neutrophils to the exaggerated inflammation that usually lead to death in wild-type infected animals cannot be ruled out. Similar results were observed in absence of MyD88 signaling, critical for *S. suis*-induced inflammation [Auger *et al.* Submitted]. This indicates that the systemic inflammatory response is precariously balanced: too little inflammation results in uncontrolled bacterial replication on the one hand [8], while exacerbated inflammation causes tissue damage and organ failure on the other [3, 38]. In the present study, CCR2$^{-/-}$ or neutrophil-depleted mice clearly presented higher mortality not necessarily due to exaggerated inflammation. As such, mice probably died from tissue and organ damage directly caused by the uncontrolled levels of systemic bacteria. Indeed, *S. suis* possesses numerous cytotoxic factors, including the toxin suilysin, that may cause organ failure [45].

Unlike inflammatory monocytes, no significant contribution of patrolling monocytes to the *S. suis*-induced systemic infection was observed. Being traditionally associated with patrolling, tissue repair, and homeostatic functions [17, 46], this was not entirely surprising. However, knowledge of their role in bacterial infections is very limited. In fact, this is one of only a handful of studies having investigated their role. It was reported, however, that patrolling monocytes support *Porphyromonas gingivalis* survival and infection-driven bone resorption by hindering neutrophil infiltrating and bacterial clearance [47].

Following systemic infection, surviving individuals are susceptible of developing a life-threatening CNS disease [3, 4]. Inflammatory monocytes (but not patrolling monocytes) were partially implicated in *S. suis*-induced CNS inflammation, but not in brain bacterial burden control, with no influence on development of clinical CNS disease. While absence of inflammatory monocytes resulted in higher mortality during *E. coli* K1 meningitis due to reduced modulation of the local inflammatory response, they were not involved in *S. pneumoniae* meningitis [30, 31].

Meanwhile, neutrophils were involved in control and elimination of *S. suis* from the CNS, but surprisingly not in CNS inflammation. In fact, their depletion increased pro-inflammatory mediator production. Likewise, though neutrophils participated in elimination of *E. coli* K1 from the CNS, brain pro-inflammatory mediator production was also increased in their absence [31]. By contrast, neutrophils contribute to the local CNS inflammatory response during *S. pneumoniae* meningitis required to eliminate bacterial burden [30]. As such, it was previously suggested that increased brain bacterial burden in absence of neutrophils further activates resident immune cells, resulting in amplification of their production [31].

Indeed, the development of clinical CNS disease in the absence of monocytes or neutrophils suggests that resident CNS cells, most probably microglia and astrocytes, are mainly responsible for local inflammation. Not only do they produce IL-6, CCL2, and CXCL1 following *S. suis* infection *in vitro* [48, 49], but they are also associated with CCL2 expression *in vivo* [3]. Furthermore, these results confirm that the CNS is extremely sensitive to *S. suis* infection, with only 10 CFU being sufficient to cause CNS disease (Auger *et al.* Submitted), and that it responds not only quickly, but also aggressively to eliminate *S. suis*, as evidence by the elevated levels of pro-inflammatory mediators.

Results confirmed that infiltration of inflammatory monocytes and neutrophils into the CNS is the consequence and not the cause of extremely high levels of chemokines produced within. Furthermore, infiltrating monocytes may contribute not only to their own recruitment, given the decrease in CCL2 production in their absence (a key chemokine involved in inflammatory monocyte recruitment) [17], but also that of neutrophils, via CCL3, CXCL1, and CXCL2 production. Indeed, CCL3 has been previously reported to participate in recruitment of neutrophils to the CNS during *Haemophilus influenzae* type b and *S. pneumoniae* infections

[50, 51], whereas CXCL1 and CXCL2 are potent neutrophil chemoattractants [52]. In accordance, lack of CXCL2 reduces recruitment of neutrophils to the CNS during *H. influenzae* type b meningitis [50].

In conclusion, neutrophils, and to a lesser extent inflammatory monocytes, participate in the inflammatory response required for clearance of *S. suis* during the systemic infection. By contrast, they partially contribute to *S. suis*-induced CNS inflammation and bacterial elimination, respectively. However, their overall role in clinical CNS disease is redundant. This indicates that even though they massively infiltrate into the CNS because of the elevated chemokine production, resident immune cells are mostly responsible for *S. suis*-induced CNS inflammation and clinical disease. Consequently, future studies will be necessary to better understand their role and the underlying mechanism involved.

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Abbreviations

CCL, C-C motif chemokine ligand; CCR, C-C chemokine receptor; CFU, colony-forming unit; CNS, central nervous system; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; GBS, group B *Streptococcus*; IFN, interferon; IL, interleukin; Nr4a1, nuclear receptor subfamily 4 group A member 1; SEM, standard error of the mean; THA, Todd Hewitt broth agar; THB, Todd Hewitt broth; TNF, tumor necrosis factor.

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Tables

Table S1. Percentage of neutrophils in peripheral blood following treatment with anti-Ly6G neutralizing antibody or its isotype control.

Treatment	Time post-injection (h)		
	24	48	72
Isotype Control	7.8 ± 0.2	7.2 ± 0.6	7.6 ± 0.4
Anti-Ly6G	0.8 ± 0.3*	0.9 ± 0.4*	1.2 ± 0.3*

Data represent mean ± SEM (n = 3). * ($p < 0.05$) indicates a significant difference between groups.

Figures

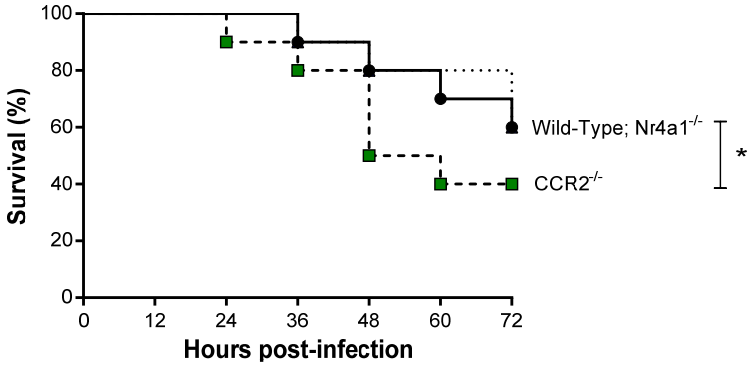

Figure 1. Inflammatory but not patrolling monocytes are implicated in host survival during *Streptococcus suis* systemic infection. Survival of wild-type (black), CCR2$^{-/-}$ (green) or Nr4a1$^{-/-}$ (blue) mice infected with *S. suis* by intraperitoneal inoculation during the acute systemic infection (until 72 h post-infection). Data represent survival curves (n = 10). * ($p < 0.05$) indicates a significant difference between survival of wild-type and CCR2$^{-/-}$ mice.

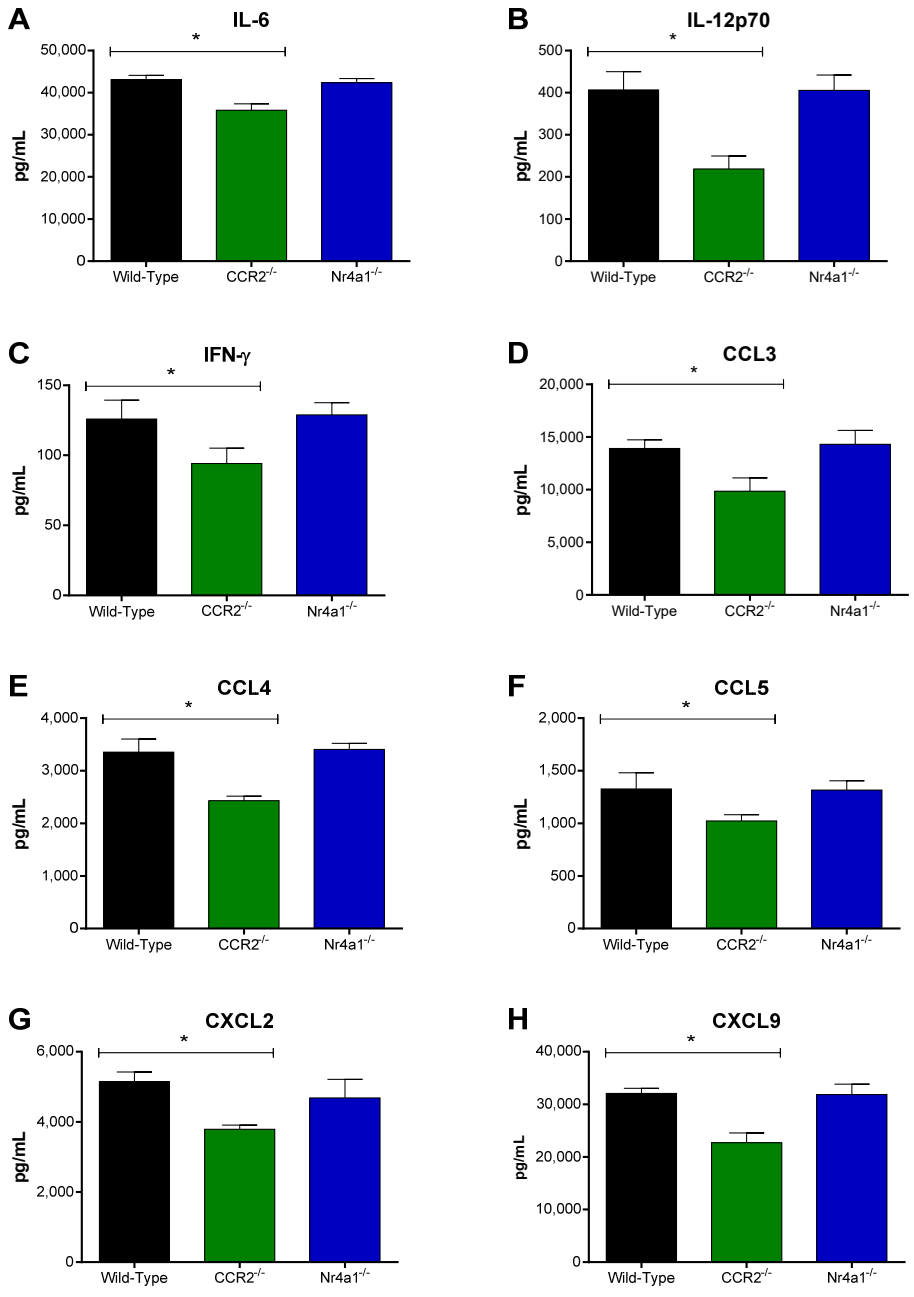

Figure 2. Inflammatory but not patrolling monocytes contribute to plasma pro-inflammatory mediator production involved in *Streptococcus suis*-induced systemic inflammation. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL3 (**D**), CCL4 (**E**), CCL5 (**F**), CXCL2 (**G**), and CXCL9 (**H**) in wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice 12 h following infection with *S. suis* by intraperitoneal inoculation. Data represent mean ± SEM (n = 8). * ($p < 0.05$) indicates a significant difference in plasma levels between wild-type and CCR2$^{-/-}$ mice.

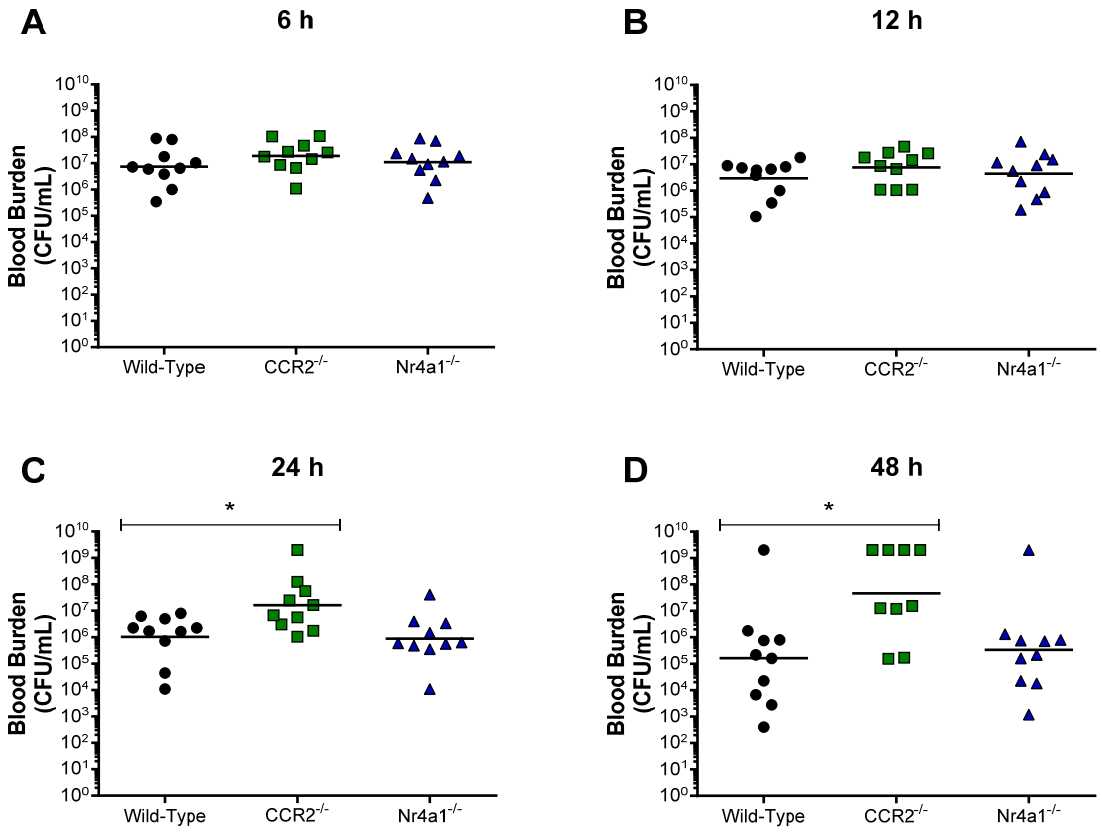

Figure 3. Inflammatory but not patrolling monocytes participate in blood bacterial burden control following *Streptococcus suis* infection. Blood bacterial burden of wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice 6 h (**A**), 12 h (**B**), 24 h (**C**), and 48 h (**D**) following infection with *S. suis* by intraperitoneal inoculation. Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.05$) indicates a significant difference between blood bacterial burden of wild-type and CCR2$^{-/-}$ mice.

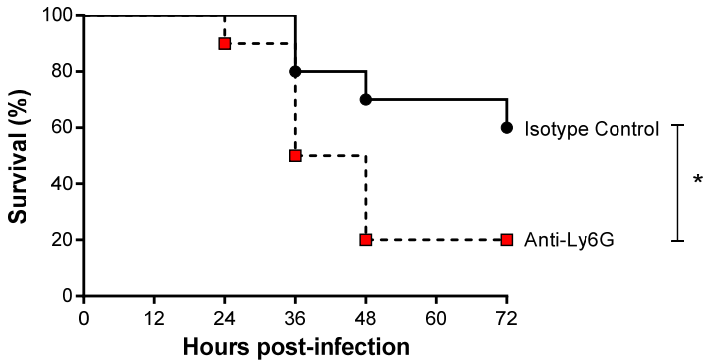

Figure 4. Neutrophils are required for host survival during *Streptococcus suis* systemic infection. Survival of wild-type mice pre-treated with either isotype control (black) or anti-Ly6G neutralizing antibody (red) and infected with *S. suis* by intraperitoneal inoculation during the acute systemic infection (until 72 h post-infection). Data represent survival curves (n = 10). * ($p < 0.05$) indicates a significant difference between survival of isotype control- and anti-Ly6G-treated mice.

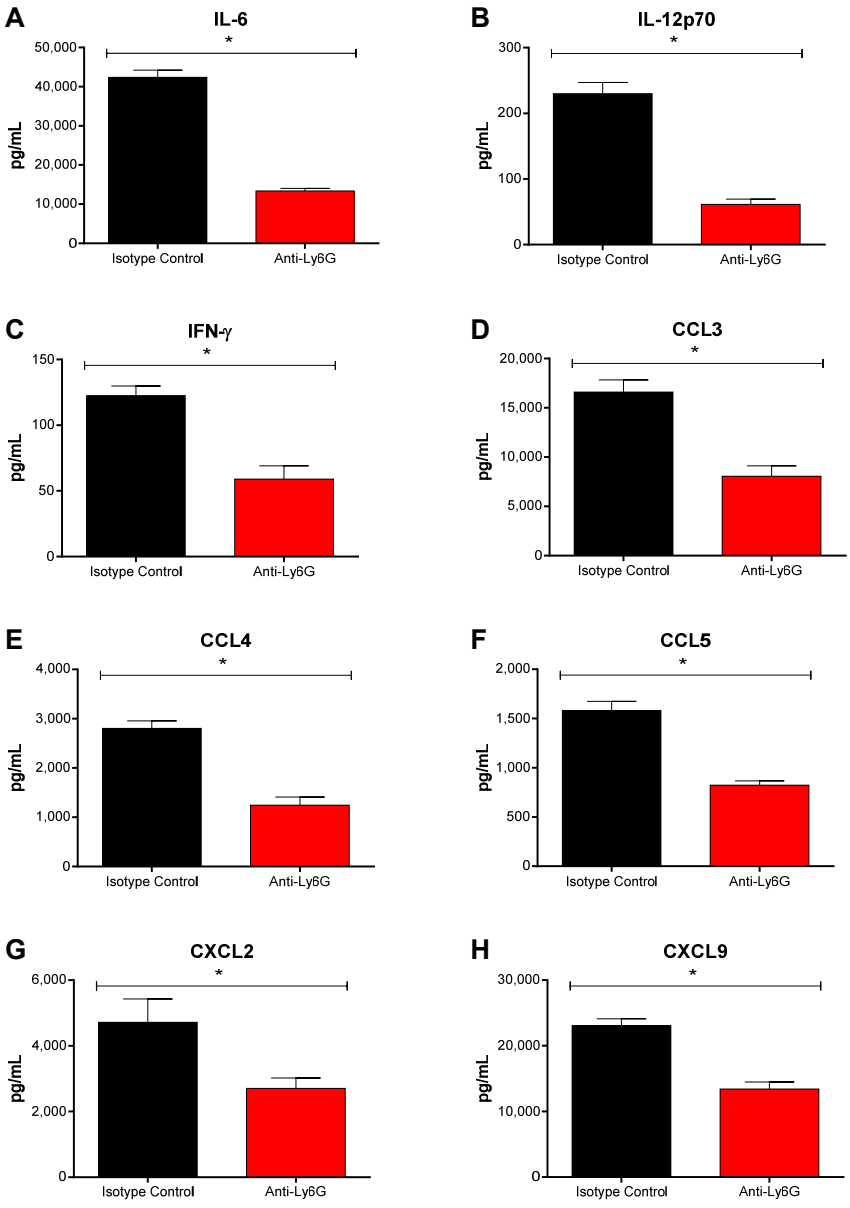

Figure 5. Neutrophils contribute to plasma pro-inflammatory mediator production involved in *Streptococcus suis*-induced systemic inflammation. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL3 (**D**), CCL4 (**E**), CCL5 (**F**), CXCL2 (**G**), and CXCL9 (**H**) in wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody and infected with *S. suis* by intraperitoneal inoculation. Data represent mean ± SEM (n = 8). * ($p < 0.05$) indicates a significant difference in plasma levels between isotype control- and anti-Ly6G-treated mice.

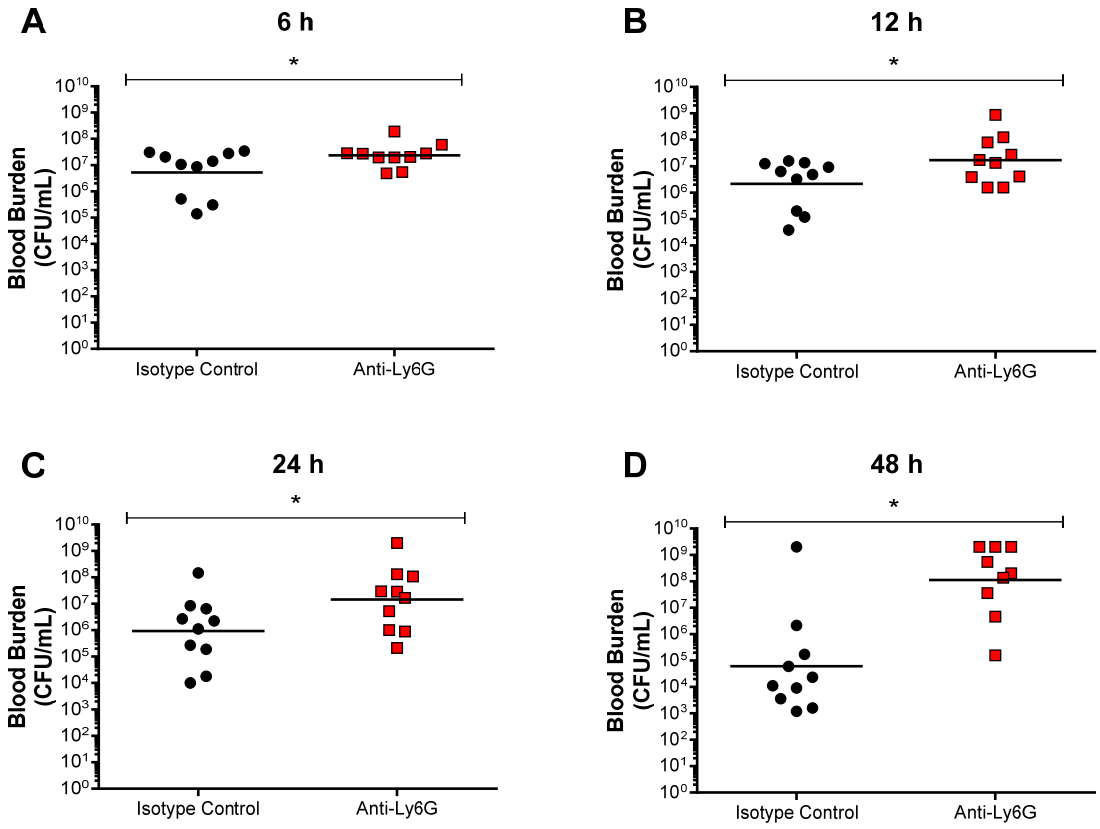

Figure 6. Neutrophils participate in blood bacterial burden control following *Streptococcus suis* infection. Blood bacterial burden of wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody 6 h (**A**), 12 h (**B**), 24 h (**C**), and 48 h (**D**) following infection with *S. suis* by intraperitoneal inoculation. Data represent geometric mean (n = 10). A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to average burden upon euthanasia, was attributed to euthanized mice. * ($p < 0.05$) indicates a significant difference between blood bacterial burden of isotype control- and anti-Ly6G-treated mice.

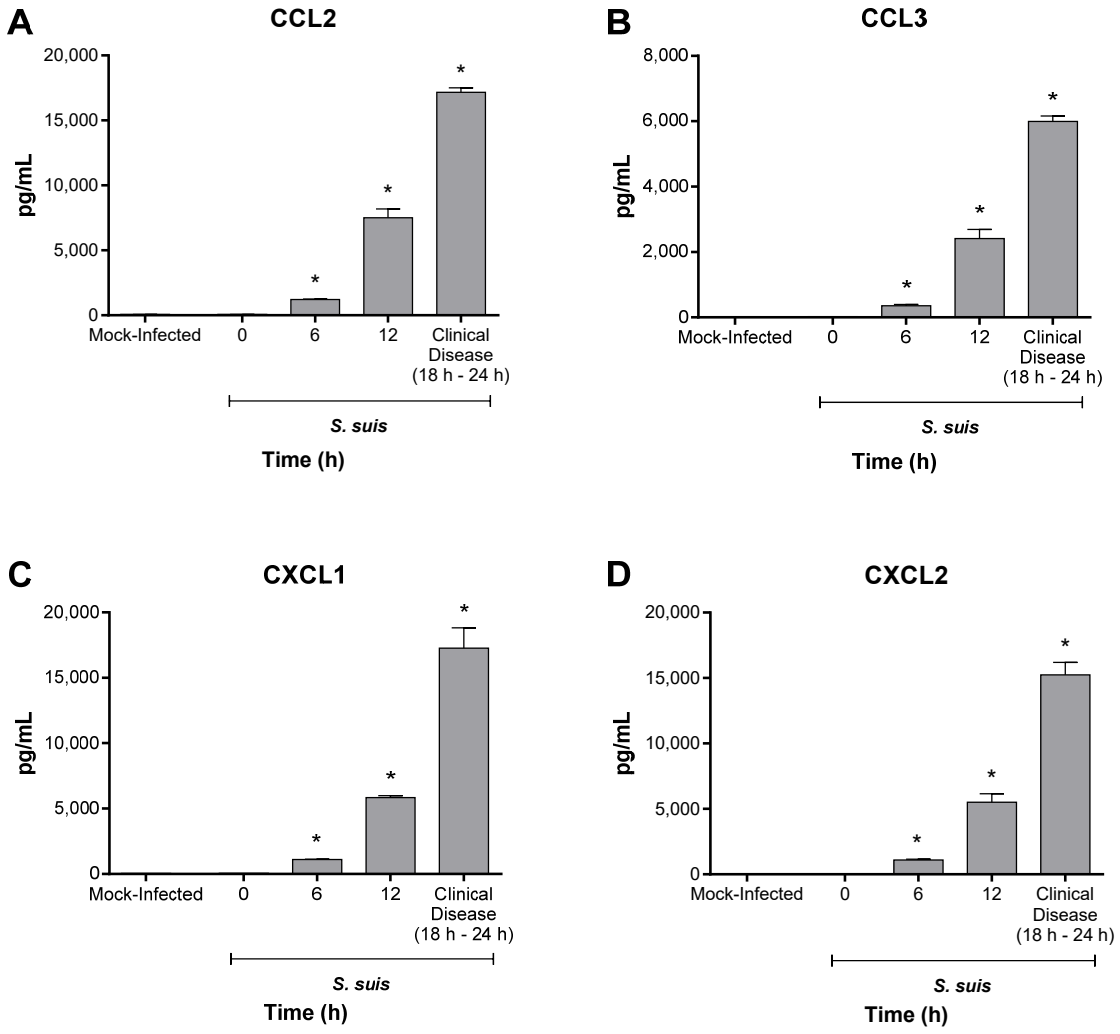

Figure 7. *Streptococcus suis* induces a massive release of pro-inflammatory chemokines from the central nervous system. Brain levels of CCL2 (**A**), CCL3 (**B**), CXCL1 (**C**), and CXCL2 (**D**) in wild-type mice at different times following mock-infection (THB) or intracisternal infection with 10^5 CFU of *S. suis*. Data represent mean ± SEM (n = 5). * ($p < 0.05$) indicates a significant difference in production with 0 h.

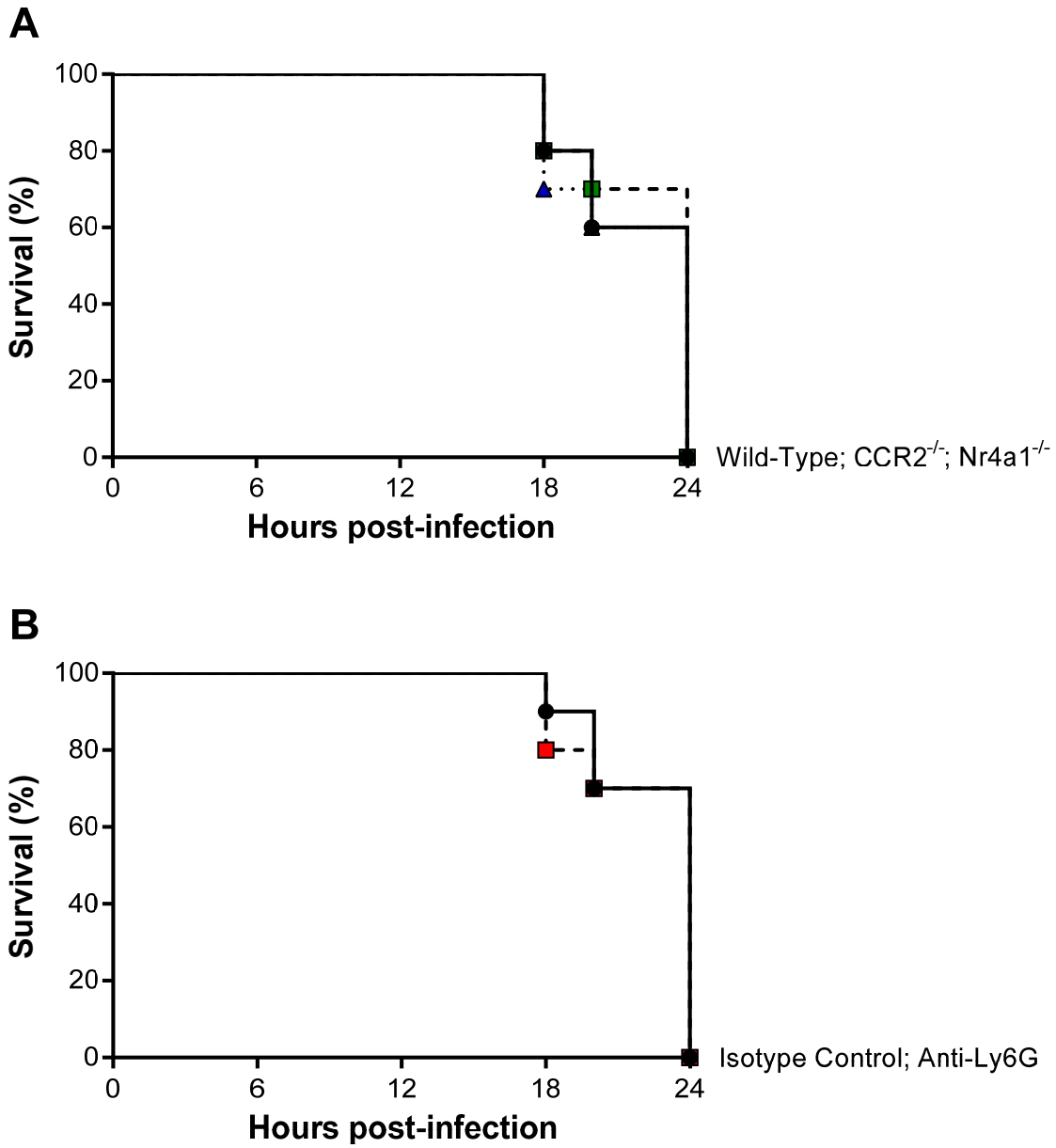

Figure 8. Monocytes and neutrophils are not required for the development of clinical central nervous system (CNS) disease following *Streptococcus suis* infection. Survival of wild-type (black), CCR2$^{-/-}$ (green) or Nr4a1$^{-/-}$ (blue) mice (**A**) or wild-type mice pre-treated with either isotype control (black) or anti-Ly6G neutralizing antibody (red) (**B**) following intracisternal infection with 10^5 CFU of *S. suis*. Data represent survival curves of mice euthanized upon presentation of clinical signs of CNS disease (n = 10).

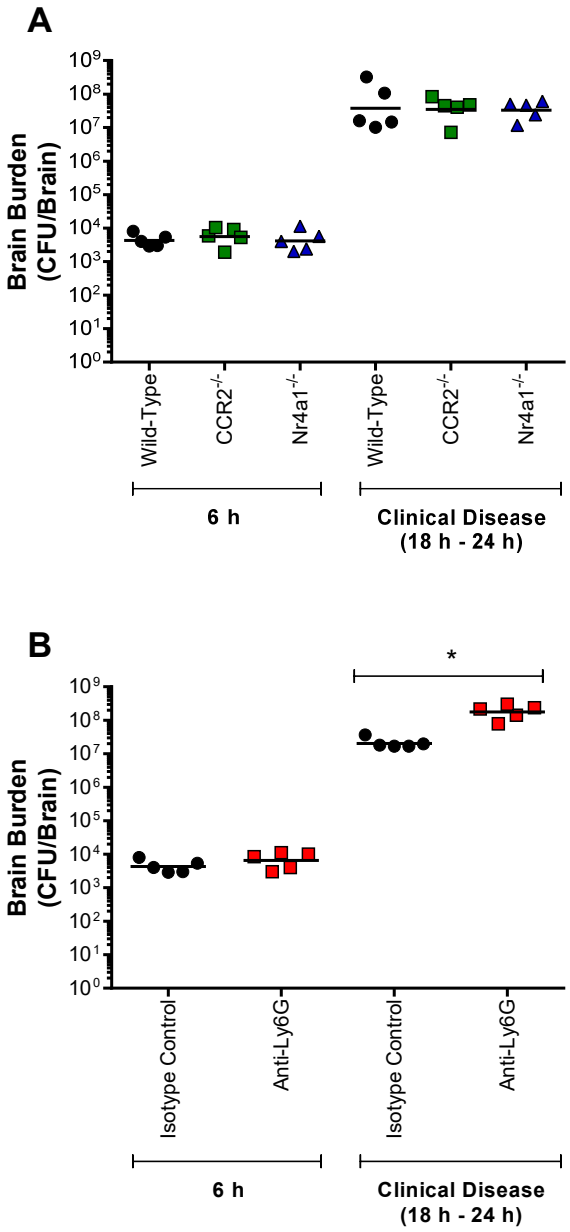

Figure 9. Neutrophils but not monocytes participate in brain bacterial burden control following *Streptococcus suis* infection. Brain bacterial burden of wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice (**A**) or wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody (**B**) 6 h following intracisternal infection with 10^5 CFU of *S. suis* or upon presentation of clinical central nervous system disease. Data represent geometric mean (n = 5). * ($p < 0.05$) indicates a significant difference between blood bacterial burden of isotype control- and anti-Ly6G-treated mice.

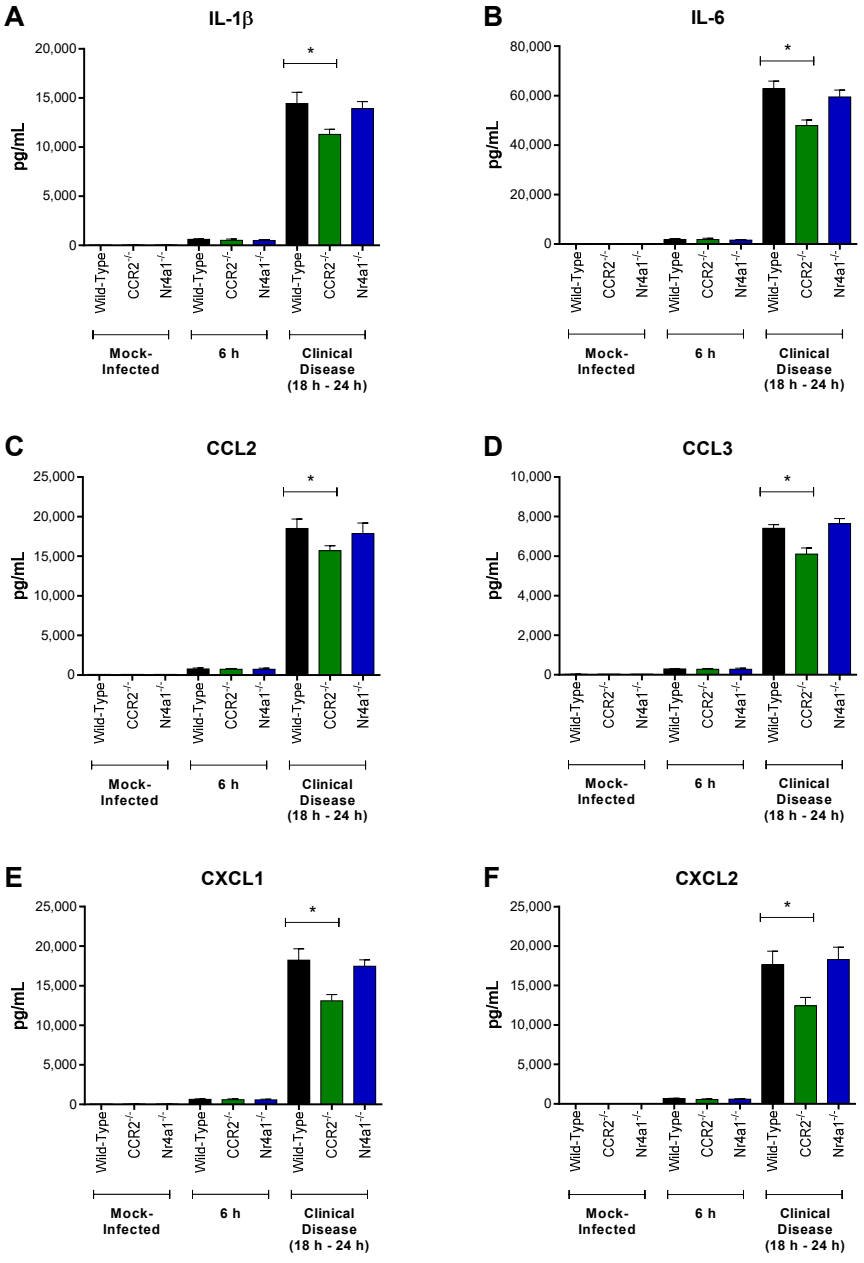

Figure 10. Inflammatory but not patrolling monocytes contribute to *Streptococcus suis*-induced central nervous system (CNS) inflammation. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), CXCL1 (**E**), and CXCL2 (**F**) in wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice following intracisternal mock-infection (THB) or 6 h following infection with 10^5 CFU of *S. suis* or upon presentation of clinical CNS disease. Data represent mean ± SEM (n = 5). * ($p < 0.05$) indicates a significant difference in mediator levels between wild-type and CCR2$^{-/-}$ mice.

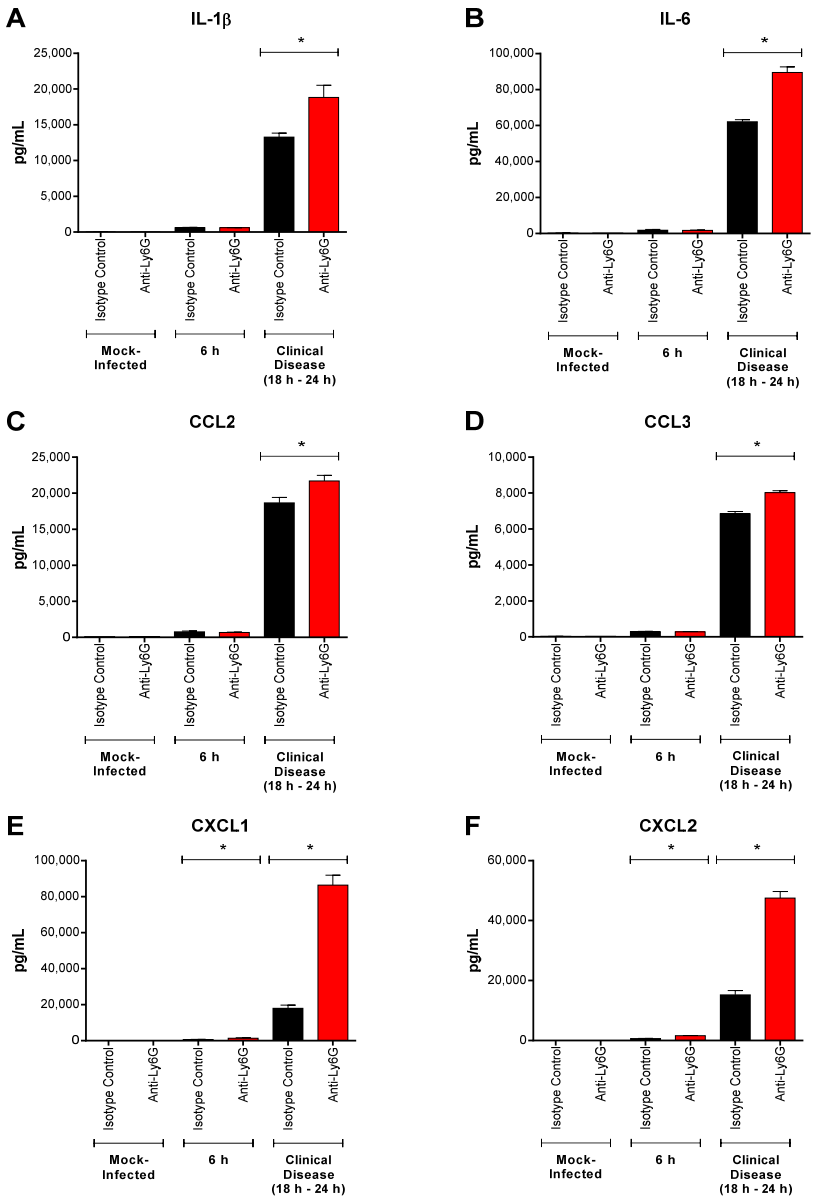

Figure 11. Presence of neutrophils modulates *Streptococcus suis*-induced central nervous system (CNS) inflammation. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), CXCL1 (**E**), and CXCL2 (**F**) in wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody mice following intracisternal mock-infection or 6 h following infection with 10^5 CFU of *S. suis* or upon presentation of clinical CNS disease. Data represent mean ± SEM (n = 5). * ($p < 0.05$) indicates a significant difference in mediator levels between isotype control- and anti-Ly6G-treated mice.

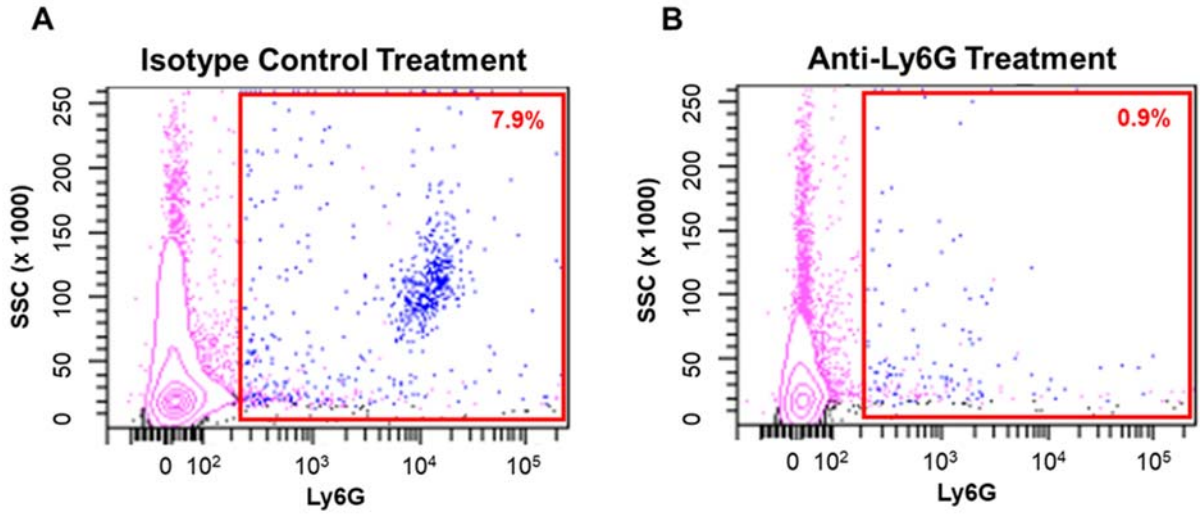

Figure S1. Efficiency of *in vivo* neutrophil depletion. Wild-type mice were pre-treated with either isotype control (mock-treated) (**A**) or anti-Ly6G monoclonal neutralizing antibody (**B**) 24 h prior to infection. Peripheral blood was collected at different times following injection and cells were stained with FITC-conjugated anti-Ly6G. Representative dot plots showing neutrophil percentages in mock-treated or anti-Ly6G-treated mice as analyzed by FACS.

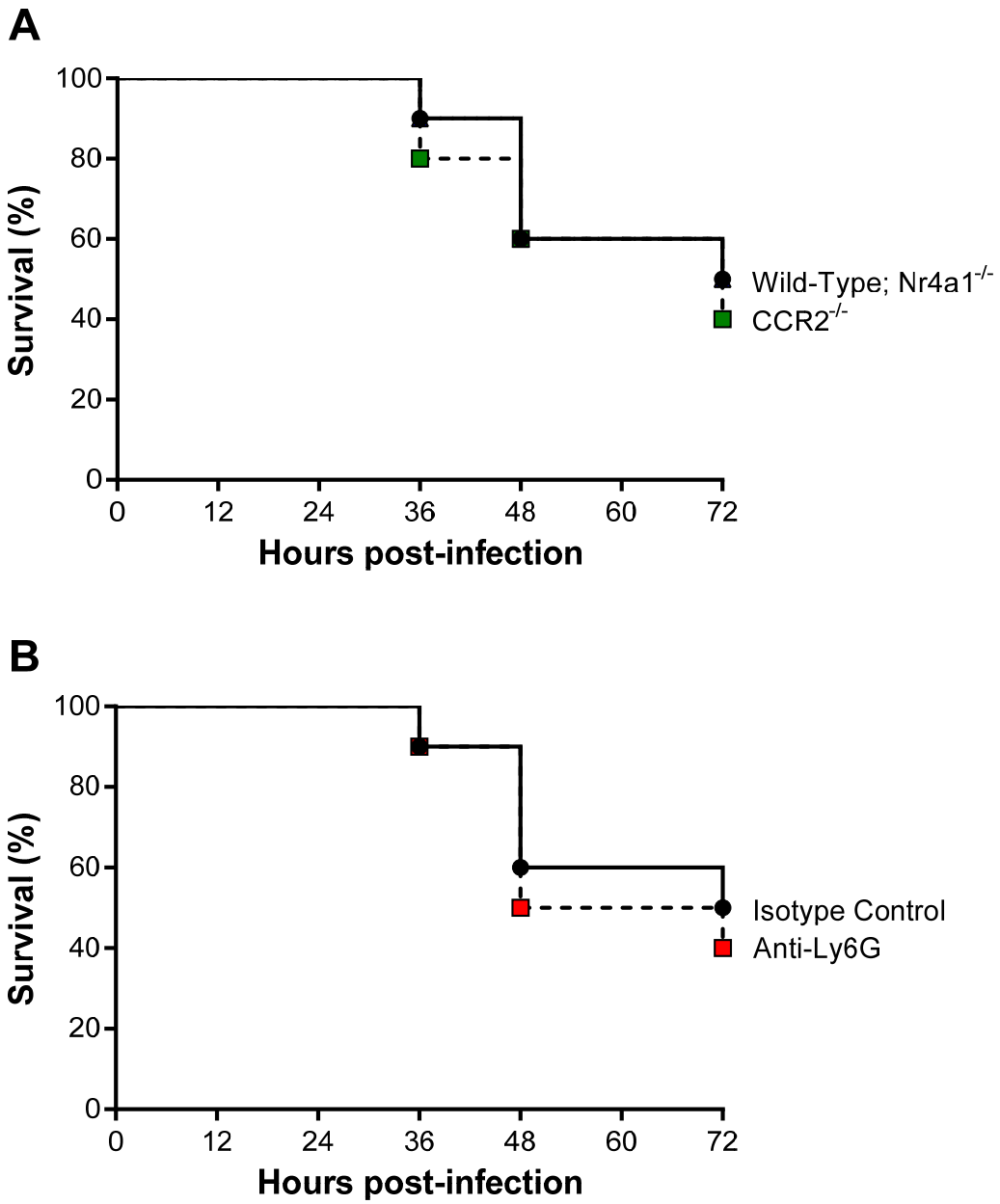

Figure S2. Monocytes and neutrophils are not required for the development of clinical central nervous system (CNS) disease following infection with a low dose of *Streptococcus suis*. Survival of wild-type (black), CCR2$^{-/-}$ (green) or Nr4a1$^{-/-}$ (blue) mice (**A**) or wild-type mice pre-treated with either isotype control (black) or anti-Ly6G neutralizing antibody (red) (**B**) following intracisternal infection with 10 CFU of *S. suis*. Data represent survival curves of mice euthanized upon presentation of clinical signs of CNS disease (n = 10).

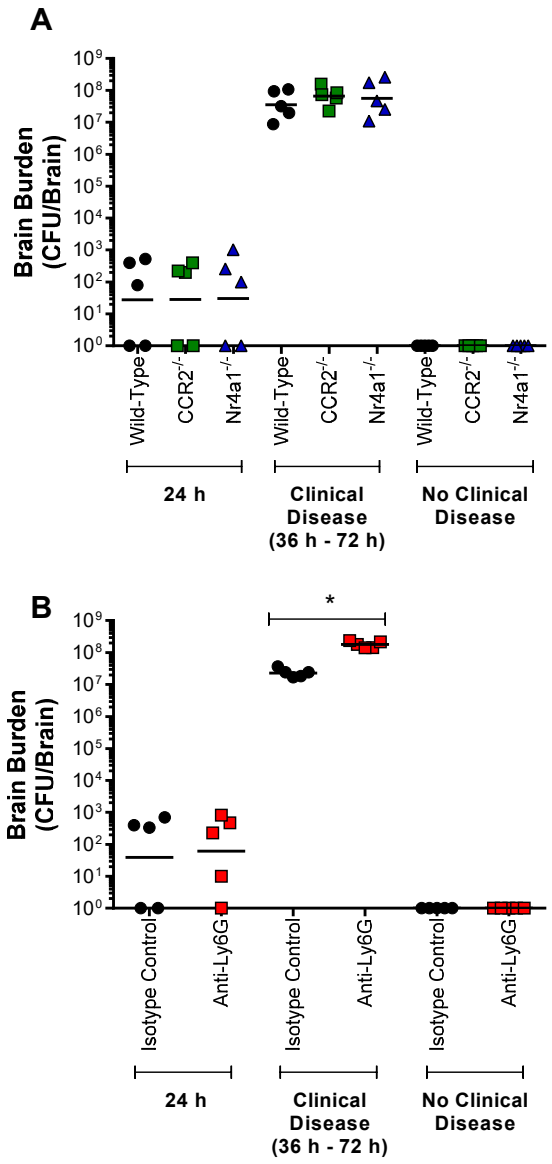

Figure S3. Neutrophils but not monocytes participate in brain bacterial burden control following infection with a low dose of *Streptococcus suis*. Brain bacterial burden of wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice (**A**) or wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody (**B**) 24 h following intracisternal infection with 10 CFU of *S. suis*, upon presentation of clinical central nervous system disease or at the end of the infection (no clinical disease). Data represent geometric mean (n = 5). * ($p < 0.05$) indicates a significant difference between blood bacterial burden of isotype control- and anti-Ly6G-treated mice.

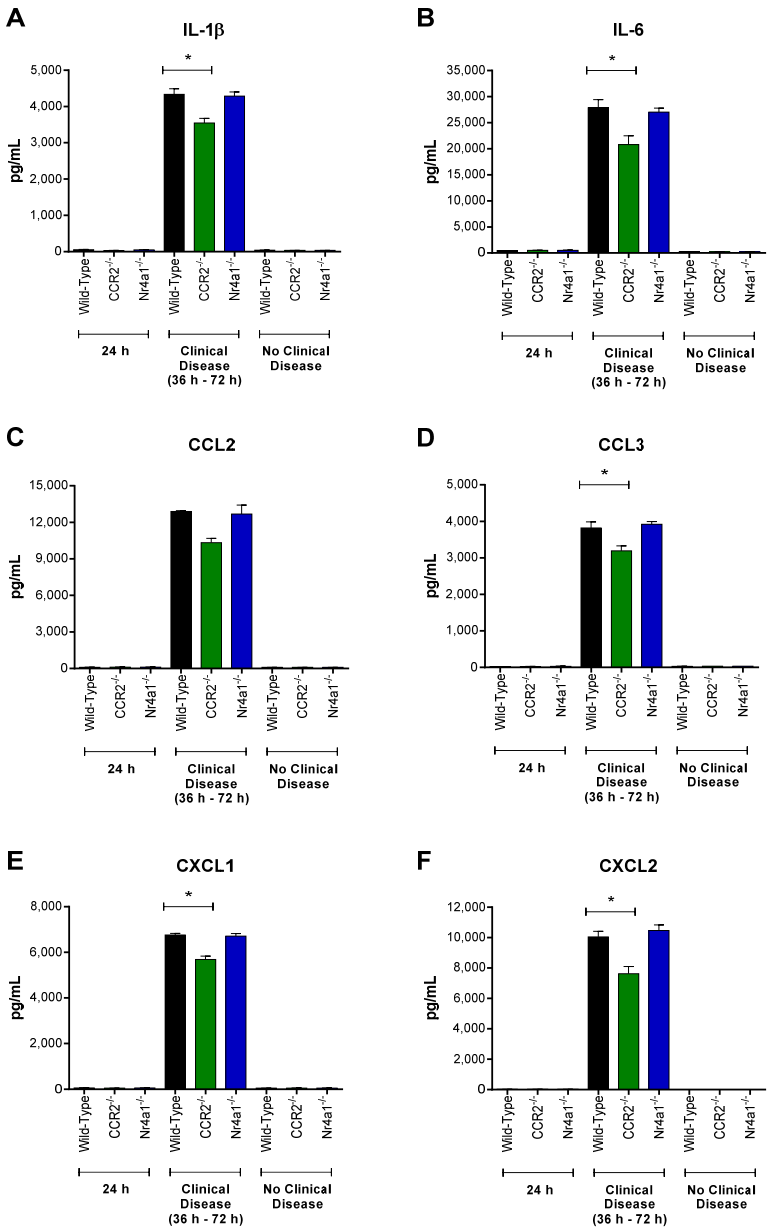

Figure S4. Inflammatory but not patrolling monocytes contribute to *Streptococcus suis*-induced central nervous system (CNS) inflammation following infection with a low bacterial dose. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), CXCL1 (**E**), and CXCL2 (**F**) in wild-type, CCR2$^{-/-}$ or Nr4a1$^{-/-}$ mice 24 h following intracisternal infection with 10 CFU of *S. suis*, upon presentation of clinical CNS disease or at the end of the infection (no clinical disease). Data represent mean ± SEM (n = 5). * ($p < 0.05$) indicates a significant difference in mediator levels between wild-type and CCR2$^{-/-}$ mice.

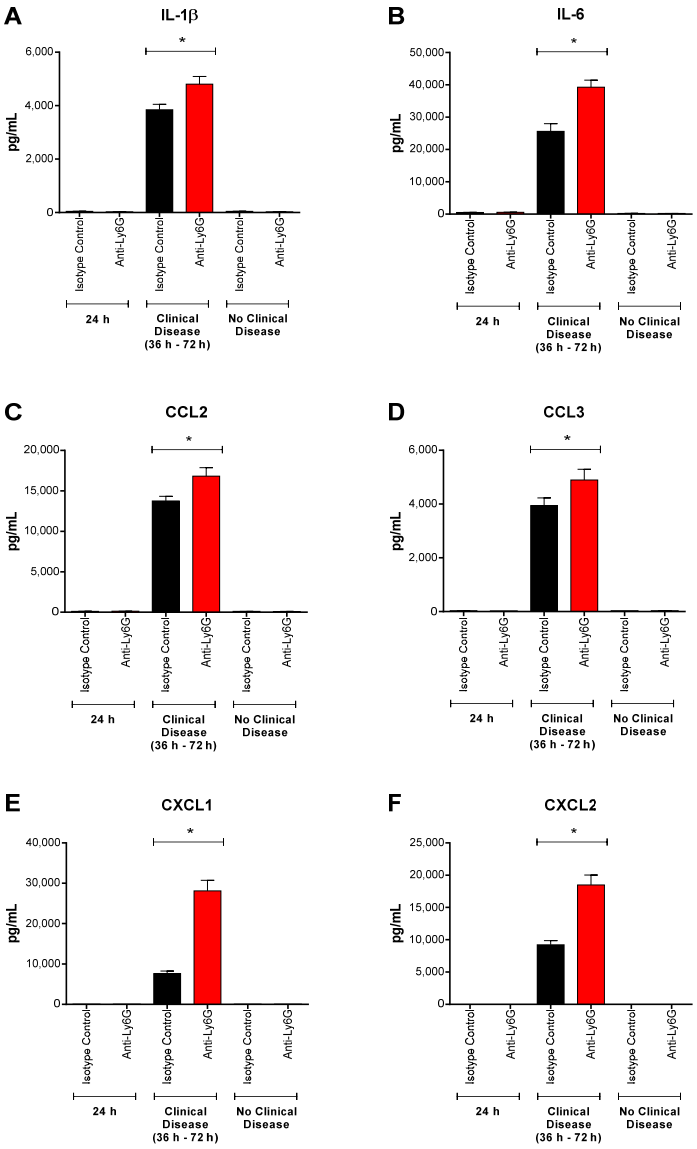

Figure S5. Presence of neutrophils modulates *Streptococcus suis*-induced central nervous system (CNS) inflammation following infection with a low bacterial dose. Brain levels of IL-1β (**A**), IL-6 (**B**), CCL2 (**C**), CCL3 (**D**), CXCL1 (**E**), and CXCL2 (**F**) in wild-type mice pre-treated with either isotype control or anti-Ly6G neutralizing antibody 24 h following intracisternal infection with 10 CFU of *S. suis*, upon presentation of clinical CNS disease or at the end of the infection (no clinical disease). Data represent mean ± SEM (n = 5). * ($p < 0.05$) indicates a significant difference in mediator levels between isotype control- and anti-Ly6G-treated mice.

ARTICLE XII

Interactions of *Streptococcus suis* serotype 2 with human meningeal cells and astrocytes

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis le premier auteur de l'article. J'ai participé à la conception de l'étude et des approches méthodologiques et j'ai effectué toutes les expériences, l'analyse des résultats et l'écriture de la première version du manuscrit. J'ai aussi été impliqué dans la révision de celui-ci et j'ai aussi effectué les corrections lors des étapes de révision pour publication.

Mise en context

Après avoir mieux caractérisé le rôle et la contribution des neutrophiles et des monocytes dans la pathogenèse des infections systémiques et du SNC causées par *S. suis* (article XI), nous nous sommes concentrés sur le rôle des cellules résidentes du SNC, car très peu d'information était disponible. Bien que la méningite/méningoencéphalite se définit par une inflammation des méninges, les interactions de *S. suis* avec celles-ci n'avaient jamais été évaluées. De plus, les astrocytes forment la membrane gliale limitante superficielle sous-jacente aux méninges. Ils peuvent donc interagir avec *S. suis* une fois que la bactérie à traverser les méninges. Enfin, comme l'origine des souches de *S. suis* étudiée peut avoir un effet sur les interactions observées, nous avons inclus des souches de sérotype 2 de STs et d'origines géographiques différents.

Abstract

Background: *Streptococcus suis* serotype 2 is an important porcine pathogen and emerging zoonotic agent responsible for meningitis, of which different sequence types predominate worldwide. Though bacterial meningitis is defined as an exacerbated inflammation of the meninges, the underlying astrocytes of the *glia limitans superficialis* may also be implicated. However, the interactions between this pathogen and human meningeal cells or astrocytes remain unknown. Furthermore, the roles of well-described virulence factors (capsular polysaccharide, suilysin and cell wall modifications) in these interactions have yet to be studied. Consequently, the interactions between *S. suis* serotype 2 and human meningeal cells or astrocytes were evaluated for the first time in order to better understand their involvement during meningitis in humans.

Results: *S. suis* serotype 2 adhered to human meningeal cells and astrocytes; invasion of meningeal cells was rare however, whereas invasion of astrocytes was generally more frequent. Regardless of the interaction or cell type, differences were not observed between sequence types. Though the capsular polysaccharide modulated the adhesion to and invasion of meningeal cells and astrocytes, the suilysin and cell wall modifications only influenced astrocyte invasion. Surprising, *S. suis* serotype 2 induced little or no inflammatory response from both cell types, but this absence of inflammatory response was probably not due to *S.suis*-induced cell death.

Conclusions: Though *S. suis* serotype 2 interacted with human meningeal cells and astrocytes, there was no correlation between sequence type and interaction. Consequently, the adhesion to and invasion of human meningeal cells and astrocytes are strain-specific characteristics. As such, the meningeal cells of the leptomeninges and the astrocytes of the *glia limitans superficialis* may not be directly implicated in the inflammatory response observed during meningitis in humans.

Background

Streptococcus suis is an important porcine bacterial pathogen and emerging zoonotic agent responsible for sepsis and meningitis [1]. Of the thirty-five described serotypes, based on the presence of the capsular polysaccharide (CPS) or its respective genes, serotype 2 is regarded as the most widespread and virulent, responsible for the majority of porcine and human cases of infection worldwide [2]. In fact, 97% of the approximately 1 300 serotyped *S. suis* cases of infection in humans were caused by the serotype 2, of which nearly 70% presented clinical signs of meningitis [2]. Furthermore, this pathogen is the first cause of adult bacterial meningitis in Vietnam, the second most common in Thailand, and the third most frequent cause of community-acquired bacterial meningitis in Hong Kong [2]. The use of multilocus sequence typing has identified four predominate serotype 2 sequence types (ST): the ST1 in Eurasia, the ST7, responsible for the human outbreaks of 1998 and 2005, in China, and the ST25 and ST28 in North America [2]. In addition, porcine and human cases of infection have been attributed to ST25 and ST28 strains in Thailand and Japan, though less frequently [2]. Moreover, differential interactions have been observed between STs using various cell types from different species [3, 4].

Of the multitude of *S. suis* serotype 2 virulence factors described [1], the CPS, suilysin (SLY) and cell wall modifications have been well-characterized. The CPS is a critical factor implicated in a multitude of functions, most importantly in the resistance to phagocytosis [5, 6]. Meanwhile, the SLY is a hemolysin responsible for causing cell cytotoxicity [7]. Finally, cell wall modifications, such as the D-alanylation of the lipoteichoic acid (LTA) and N-deacetylation of the peptidoglycan (PGN), are known to interfere with the immune response; the D-alanylation of the LTA was also shown to modulate adhesion to and invasion of endothelial cells [6, 8, 9]. The interactions between *S. suis* serotype 2 and certain cells of the central nervous system (CNS) have been studied, including porcine brain microvascular endothelial cells (pBMEC) and porcine choroid plexus epithelial cells (CPEC), as well as murine microglia and astrocytes [4, 10-12]. However, few studies have used human cells: only the interactions with human BMEC (hBMEC) and choroid plexus papilloma cells have been reported [13, 14], while those with meningeal cells and other cells of the human CNS remain unknown. The meninges are composed of the dura mater and leptomeninges: the latter are formed of the pia mater and arachnoid mater together with the trabeculae that traverse the cerebrospinal fluid (CSF)-filled subarachnoid space (SAS) [15]. The pia mater overlies the *glia limitans superficialis*, a layer of

compact astrocytes that surrounds the brain and forms a barrier [16]. While astrocytes play a crucial role in cerebral homeostasis, they also participate in inflammation, though this role has only begun to be studied [17, 18]. Consequently, astrocytes possess a multitude of pattern recognition receptors involved in the innate immune response, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [18]. As such, these cells are a source of certain inflammatory mediators [18].

Penetration of the CPEC that form the blood-CSF barrier by *S. suis*, from the underlying blood vessels, is a proposed portal of entry into the CNS [14, 19]. Consequently, the leptomeninges and underlying astrocytes of the *glia limitans* are possibly implicated in both porcine and human *S. suis* infections, which are characterized by meningitis (inflammation of the leptomeninges) often accompanied by inflammation of the surrounding CNS tissues, such as the *glia limitans* [20]. The interactions of different important meningitis-causing human bacterial pathogens with these cells have already been studied [21-24].

In this study, the interactions between different *S. suis* serotype 2 strains and human meningeal cells or astrocytes, including certain well-characterized virulence factors, were evaluated for the first time in order to better understand the implication of the meninges and underlying CNS tissue during meningitis in humans.

Materials and Methods

Bacterial strains and growth conditions

The well-encapsulated *S. suis* serotype 2 strains and isogenic mutants for different well-characterized virulence factors used in this study are listed in **Table 1**. Bacteria were grown overnight on Columbia Blood Agar containing 5% sheep blood (v/v) at 37ºC with 5% CO_2 (Oxoid, Basingstoke, UK). Five ml of Todd-Hewitt Broth (THB; Becton Dickinson, Swindon, UK) were inoculated and incubated for 8 h at 37ºC with 5% CO_2. Working cultures were prepared by inoculating 30 ml of THB with 10 µl of a 10^{-3} dilution of the 8 h cultures, incubated for 16 h at 37ºC with 5% CO_2. Bacteria were washed twice with pH 7.3 phosphate-buffered saline (PBS), resuspended in cell culture medium, appropriately diluted, and plated on Todd-Hewitt Broth Agar (THA) to accurately determine concentrations. The *N. meningitidis* serogroup B strain MC58 [44], used as a positive control, was grown on supplemented GC agar and bacterial suspensions prepared in cell culture medium, as previously described [24].

Human meningeal cell and astrocyte cultures

Human meningioma cells, obtained from surgically removed tumors as previously described, express the characteristic markers of desmosomal desmoplakin, epithelial membrane antigen, vimentin, and cytokeratin [24]. The cells were grown in Dulbecco's Modified Eagles Medium containing Glutamax-1 and sodium pyruvate (Lonza, Slough, UK) supplemented with 10% fetal calf serum (v/v) (FCS; Lonza) and seeded in flasks pre-coated with 5 mg/cm^2 of type I collagen from rat tail (Becton-Dickinson); culture passages 2 to 10 were used [24]. SVGmm human fetal astrocyte cells were grown in Eagle's Minimal Essential Medium (Lonza) supplemented with 10% FCS (v/v) [45].

Infection of human meningeal cells and astrocytes

Human meningeal cells and astrocytes were grown to confluence in 24-well cell culture plates, averaging 4x10^4 cells/well and 1x10^5 cells/well, respectively. Cell monolayers were maintained overnight in medium containing 1% FCS (v/v) and washed twice with warm PBS prior to bacterial challenge, in triplicate, with the different *S. suis* serotype 2 wild-type or mutant strains, or *N. meningitidis*. Based on preliminary assays, 1 ml of 4x10^4 CFU/well for meningeal cells and 1x10^5 CFU/well for astrocytes (multiplicity of infection [MOI]=1) was added for bacterial adhesion. For bacterial invasion, host cell cytotoxicity and inflammatory activation, 4x10^6 CFU/well for meningeal cells and 1x10^7 CFU/well for astrocytes (MOI=100) was added. Monolayers were incubated for 9 h (adhesion and invasion) or 24 h (cell cytotoxicity and inflammatory activation) at 37°C with 5% CO_2 as previously described [21-23].

Bacterial adhesion and invasion measurement

After incubation, cells were washed four times with warm PBS, lysed using 250 µl/well of 1% saponin (w/v) (Sigma-Aldrich, Dorset, UK) in PBS and incubated for 15 min at 37°C with 5% CO_2. Bacterial adhesion was measured by plating the lysates onto THA and incubating overnight at 37°C with 5% CO_2. Bacterial invasion was carried out using the antibiotic protection assay [5]: cell medium was removed after 9 h, monolayers were washed twice with warm PBS and 1 ml of medium containing 5 µg/ml penicillin G (Sigma-Aldrich) and 100 µg/ml of gentamicin (Sigma-Aldrich) was added for 90 min at 37°C with 5% CO_2 to kill extracellular bacteria. The last wash was plated to confirm antibiotic activity.

Host cell cytotoxicity assay

Lactate dehydrogenase (LDH) release was measured 24 h post-infection using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, UK) according to instructions. Non-infected monolayers were included for measurement of spontaneous LDH release and maximum LDH release was induced using the lysis reagent included in the kit. The absorbance was read at 492 nm using the iMark Absorbance Reader (Bio-Rad, Hercules, CA, USA).

Host cell inflammatory activation

The measurement of the pro-inflammatory cytokine IL-6 and chemokines CCL2, CCL5 and CXCL8 was carried out by enzyme-linked immunosorbent assay as previously described [22].

Statistical analyses

Unpaired t-tests, Mann-Whitney rank sum tests and one-way ANOVA, where appropriate, were performed to find statistical differences between groups. $p<0.05$ was considered statistically significant.

Results and Discussion

S. suis *serotype 2 adheres to but rarely invades human meningeal cells and these interactions are modulated by the CPS*

Though a high blood bacterial load is often regarded as crucial for the development of meningitis by extracellular bacterial pathogens (ex. *Escherichia coli*) [25], this remains unknown for *S. suis*. Nonetheless, bacteria must breach one of the physiological barriers that separate the brain: the blood-CSF barrier is the preferred site of entry into the CNS for most meningitis-causing human bacterial pathogens, which subsequently reach the CSF and the meningeal cells with which they may interact [19]. Bacterial adhesion to the cell surface is considered a critical host-pathogen interaction. Consequently, the adhesion of the *S. suis* strains, belonging to different STs, to human meningeal cells was measured. Since primary human leptomeningeal cells cannot be reliably cultured, previously established human leptomeningioma cell cultures were used [26]. These cells present the same cytological and morphological structures as their primary counterparts [24] and have been used with various meningitis-causing human bacterial pathogens [21, 23]. The ST1, ST7, ST25, and ST28 strains all adhered to meningeal cells, with associated bacteria ranging between $5x10^4$ colony forming units (CFU) and $1x10^7$ CFU, equivalent to 1 and 2 500 bacteria/cell, respectively (**Fig. 1A**).

Though significant differences were not observed between STs, there were differences between strains: P1/7 and 89-1591 adhered significantly less and more than the other strains ($p<0.01$), respectively. These differences suggest the partial implication of strain-specific factors [1], which remain largely unknown. However, nearly all virulence studies have been conducted using ST1, and more recently, ST7 strains [1]. Consequently, very little is known regarding ST25 and ST28-specific virulence factors, including those implicated in adhesion. The adhesion levels of *S. suis* are similar to those reported for group B *Streptococcus* (GBS) serotypes III and V, yet *S. suis* adhered more strongly than other important encapsulated human bacterial pathogens, such as *Streptococcus pneumoniae* serotype 2, *Haemophilus influenzae* type b and *E. coli* K1, but less so than *Neisseria meningitidis* serogroup B, when similar initial concentrations were used [21, 23]. This adhesion capacity of *S. suis* serotype 2 had also been observed when porcine tracheal epithelial cells, human lung epithelial cells, hBMEC or pBMEC were infected with ST1 strains [11, 13, 27, 28]. Importantly, this is the first time that the interactions between *S. suis* serotype 2 ST25 or ST28 strains and CNS cells, other than the BMECs, have been studied, regardless of the species. Unlike with human and porcine intestinal epithelial cells [29], no differences were observed between adhesion of human or porcine *S. suis* serotype 2 strains to human meningeal cells.

Since *S. suis* adhered to meningeal cells, its capacity to subsequently invade the cells was evaluated. Bacterial invasion ranged between $2x10^3$ CFU and $7x10^3$ CFU for the different strains, equivalent to 0.05 and 0.2 intracellular bacterium/cell, respectively, with the exception of the ST7 strain, for which $8x10^4$ CFU were recovered, equivalent to 2 intracellular bacteria/cell (**Fig. 1B**). Unfortunately, the definition of bacterial invasion remains controversial and subjective, with no criterion allowing to determine with certainty if a bacterial strain is invasive [30]. Though the number of internalized bacteria required for *S. suis* serotype 2 to induce meningitis remains unknown, it was previously suggested that the survival of a single intracellular *H. influenzae* may result in meningitis [31]. Consequently, biologically relevant invasion was defined as 1 intracellular bacterium/cell in this study. As such, only the ST7 was capable, in theory, of meningeal cell invasion: this capacity could be the result of unique factors, since important differences have been observed in the genome of this strain when compared to ST1 and ST25 strains [32]. The general inability of *S. suis* to invade meningeal cells is a characteristic shared with GBS, *S. pneumoniae* and *H. influenzae* [21, 23]. By contrast, *N. meningitidis* and *E. coli* invaded meningeal cells [23]. It was previously demonstrated that

though *S. suis* serotype 2 invaded porcine tracheal epithelial cells, it did not invade human lung epithelial cells or hBMEC; invasion of pBMEC remains controversial however [11, 13, 27, 28, 30]. Breaching of the meningeal cell barrier via the paracellular route cannot be discarded however, since this route is difficult to evaluate in the absence of transwell inserts and measuring of the transepithelial electrical resistance. Although described for intestinal epithelial cells [29], this route of entry has not yet been described for *S. suis* in the CNS, where the bacteria were reported to use the transcellular route (cellular invasion) [11, 14, 33]. Surprisingly, a correlation between meningeal cell adhesion and invasion by the strains studied was not observed, suggesting that different bacterial and/or host cell factors are probably involved in these interactions.

Of the different well-characterized *S. suis* serotype 2 virulence factors, the CPS has, amongst its various properties, been described to modulate the adhesion to and invasion of epithelial and endothelial cells [27, 29, 30, 34]. Meanwhile, the SLY has more recently been suggested to be involved in the adhesion to and invasion of human epithelial cells at sub-cytolytic concentrations [35]. Finally, the roles of cell wall modifications, including the D-alanylation of the LTA and N-acetylation of the PGN, in adhesion and invasion have been studied using pBMEC and porcine tracheal epithelial cells [8, 28]. Given these properties, the role of these factors on the adhesion to and invasion of meningeal cells by *S. suis* was determined. The adhesion of *S. suis* to meningeal cells was significantly modulated by the CPS ($p<0.001$) (**Fig. 1C**): the absence of the CPS increased adhesion by tenfold. Meanwhile, the SLY and cell wall modifications had no effect. The absence of the CPS also significantly increased invasion of meningeal cells (**Fig. 1D**), by one hundredfold ($p<0.001$), such that the intracellular bacteria/cell ratio was of 10. These modulations by the CPS, though expected since it is known to mask various surface proteins implicated in adhesion and invasion, are similar to those obtained using pBMEC and porcine tracheal epithelial cells [11, 28, 34]. In fact, P1/7 was non-invasive when encapsulated, but highly invasive in the absence of its CPS. Regulation of CPS expression may play an important role in bacterial-host interactions *in vivo* [1]. On the other hand, the SLY did not modulate the adhesion to and invasion of pBMEC or porcine tracheal epithelial cells by *S. suis*, as was observed in the present study [11, 28]. Meanwhile, the D-alanylation of the LTA modulated the adhesion to and invasion of pBMEC, but not of porcine tracheal epithelial cells [8, 11, 28]. Consequently, different receptors may be implicated in adhesion and invasion according to the cell type and/or species.

S. suis *serotype 2 adheres to and invades human astrocytes and these interactions are modulated by the CPS and, to a certain extent, by the SLY and cell wall modifications*

In the case where *S. suis* serotype 2 would modulate its CPS, the pathogen may penetrate the meningeal cells of the pia mater, as observed in this study, in order to reach the astrocytes of the *glia limitans superficialis* [21]. As with meningeal cells, no significant differences were observed between adhesion of the different STs to astrocytes. Indeed, adhesion ranged between $1x10^5$ CFU and $5x10^6$ CFU, equivalent to 1 and 50 bacteria/cell, respectively (**Fig. 2A**). Moreover, the strains P1/7, LPH4 and 1088563 adhered similarly, but significantly less than the other strains ($p<0.05$). The adhesion levels of *S. suis* serotype 2 to human astrocytes are similar to those previously reported for GBS serotypes III and V [21].

Since all of the *S. suis* strains adhered to astrocytes, their subsequent invasion capacity was evaluated. Unlike with meningeal cells, multiple strains were, in theory, capable of biologically relevant invasion (**Fig. 2B**). Indeed, at least $1x10^5$ CFU, equivalent to 1 intracellular bacterium/cell, were recovered following infection with the different strains, with the exception of P1/7 and 89-1591. Differences were nonetheless observed between the strains capable of invasion: 31533 and 1088563 invaded the cells significantly more than did SC84, LPH4 or MNCM43 ($p<0.01$). These differences suggest that invasion of astrocytes by *S. suis* is a strain-specific characteristic: as mentioned previously however, the characteristics of ST25 and ST28 strains remain largely unknown. These results are different from those obtained using murine astrocytes, which did not internalize the *S. suis* serotype 2 strains 31533 (ST1) and SC84 (ST7), also used in this study [4]. On the contrary, invasion of human astrocytes by GBS was reported as infrequent since the intracellular bacterium/cell ratio recovered following infection was much lower than 1 [21]. As with meningeal cells however, *S. suis* adhesion to and invasion of astrocytes did not correlate.

Since *S. suis* interacted with astrocytes, the role of the CPS, SLY and cell wall modifications were evaluated. Adhesion to human astrocytes was significantly modulated by the CPS ($p<0.001$) (**Fig. 2C**), its absence increasing adhesion by tenfold. Meanwhile, the absence of the SLY and cell wall modifications did not modulate the adhesion of *S. suis* to astrocytes. The absence of the CPS also significantly increased the invasion capacity of astrocytes ($p<0.001$) (**Fig. 2D**), for which the intracellular bacteria/cell ratio recovered was of 5, equivalent to a tenfold increase. This greatly differs from murine astrocytes, which *S. suis* did not invade even

in the absence of CPS [4]. Unlike with meningeal cells however, the absence of the SLY and D-alanylation of the LTA significantly decreased invasion of astrocytes by *S. suis* ($p<0.01$): the strain was no longer capable of biologically relevant cell invasion. Meanwhile, as with meningeal cells, the N-acetylation of the PGN had no effect. These results are in accordance with previous studies: sub-cytolytic levels of SLY increased the invasion capacity of *S. suis* serotype 2 in epithelial cells [35], while, as mentioned above, the D-alanylation of the LTA modulated the invasion of pBMEC [8].

S. suis *serotype 2 induces little inflammatory response from infected human meningeal cells*

S. suis-induced inflammation is a characteristic of the infection that can lead to an exacerbated inflammatory response and host death [36]. Furthermore, meningitis, the hallmark of the *S. suis* CNS infection, is defined as an inflammation of the meninges in which the surrounding CNS tissue may be implicated [36]. Consequently, an understanding of the role of these cells in the production of inflammatory mediators following *S. suis* serotype 2 infection is crucial.

Human meningeal cells have been previously reported to produce the pro-inflammatory cytokine interleukin (IL)-6 and chemokines C-C motif ligand (CCL)2, CCL5 and C-X-C motif ligand (CXCL)8 in response to meningeal pathogens, but not IL-1α, IL-1β, IL-10, IL-12, tumor necrosis factor (TNF)-α, CCL3 or CCL4 [21-23]. Consequently, only the production of IL-6, CCL2, CCL5, and CXCL8 was quantified 24 h following infection with the different *S. suis* strains. Meningeal cells infected with *N. meningitidis*, used as a positive control, significantly produced all four mediators ($p<0.01$). However, no production of IL-6, CCL2 or CXCL8 was observed when cells were infected with *S. suis*, regardless of the strain (data not shown). Interestingly, a significant production of CCL5 was induced, but by both ST1 strains only ($p<0.01$) (**Fig. 3**). The levels were significantly greater when cells were infected with 31533 than with P1/7 ($p<0.01$). This production of CCL5 corroborates with previous results in which elevated levels of the mediator were observed in the CSF of human patients with bacterial meningitis [37]. Furthermore, CCL5 is implicated in the chemoattraction of monocytes, which massively infiltrate the SAS and meninges during *S. suis* meningitis [36]. An absence of these four inflammatory mediators was also observed when human meningeal cells were infected with GBS or *S. pneumoniae* [21, 23]. By contrast, these cells produced important levels of the four mediators following infection with *N. meningitidis* and *H. influenzae*, but only produced

CCL2 and CXCL8 following infection with *E. coli* [23]. However, *S. suis* serotype 2 induced IL-6, CCL2 and CXCL8 from hBMEC and IL-6 and CXCL8 from pBMEC, suggesting a possible cell and/or species specificity [38, 39].

Given the relative absence of the inflammatory mediators evaluated, the *S. suis*-induced cytotoxicity was evaluated (**Table 2**). Cytotoxicity levels were below 10% following infection with the different strains, suggesting that unlike with the hBMEC and pBMEC, the SLY, produced by the ST1 and ST7 strains but not by the ST25 or ST28 strains, was not toxic for human meningeal cells. Consequently, the absence of the meningeal cell inflammatory mediators evaluated was probably not due to cell death. It is possible that human meningeal cells do not express certain of the various receptors implicated in the *S. suis* serotype 2 recognition, including TLRs (TLR2, TLR4, TLR9, and possibly, TLR3) and NLRs (NOD2) [40]. Of these however, the TLR2, often considered the most important to the *S. suis* serotype 2 pathogenesis, and TLR4, are present on the cell surface [1, 41].

S. suis *serotype 2 does not induce an inflammatory response from infected human astrocytes*

Human astrocytes produce various inflammatory mediators following bacterial infection, including IL-6, CCL2, CCL5, and CXCL8 [21]. Consequently, these mediators were quantified following infection with the different *S. suis* strains. As with meningeal cells, *N. meningitidis* induced a significant production of all four mediators ($p<0.01$). Meanwhile, no production of IL-6, CCL2, CCL5 or CXCL8 was observed when cells were infected with *S. suis*, regardless of the strain (data not shown). Interestingly, these results vary from those obtained using murine astrocytes, which were an important source of TNF-α, IL-6, CCL2, and CXCL1 following *S. suis* serotype 2 infection [4]. Furthermore, recognition of *S. suis* serotype 2 by murine astrocytes was mainly TLR2-dependent, since the expression of TLR1, TLR4 and TLR6 was barely modulated following infection [4]. Meanwhile *S. suis* serotype 2-induced TNF-α and CCL2 were partially dependent on TLR2 in murine astrocytes [4]. By contrast, in the present study, no CCL2 production was observed when human astrocytes were infected with *S. suis* serotype 2, while production of TNF-α was not determined. Consequently, the species from which the cells originate appears to play an important role on the results obtained, as the receptors present may vary, which could influence the subsequent inflammatory response [42, 43].

Given the absence of the inflammatory mediators measured, *S. suis*-induced astrocyte cytotoxicity was also evaluated (**Table 2**). In contrast to the lack of meningeal cell death following infection with *S. suis*, astrocytes were generally more sensitive to infection. The ST1, ST7 and ST25 strains induced important cytotoxicity, which ranged between approximately 30 and 70%, while the ST28 strains caused little cytotoxicity (less than 15%). The SLY may be partially responsible for the *S. suis*-induced astrocyte cell death since the ST1 and ST7 strains induced higher cytotoxicity levels than did the SLY-negative strain LPH4. Furthermore, the differences observed between the ST1 and ST25 strains suggest that strain-specific factors are implicated in these interactions. Nevertheless, the *S. suis*-induced astrocyte cell death was probably not responsible for the absence of the inflammatory mediators evaluated, since *N. meningitis* caused near total cell death, with 90% cytotoxicity, yet induced important levels of these inflammatory mediators.

Conclusions

S. suis serotype 2 interacted with human meningeal cells and astrocytes via the adhesion to both cell types; invasion of meningeal cells was rare however, whereas that of astrocytes was generally more frequent. Furthermore, these interactions were largely modulated by the CPS, and to a certain extent, by the SLY and cell wall modifications. Regardless of the fact that *S. suis* interacted with meningeal cells and astrocytes, little or no production of the inflammatory mediators evaluated was observed. This suggests that the meningeal cells of the leptomeninges and the astrocytes of the underlying *glia limitans superficialis* may not be directly implicated in the inflammatory response observed during *S. suis* meningitis in humans. Nevertheless, the use of a microarray or proteomic assay investigating a larger number and greater variety of inflammatory mediators induced by *S. suis* serotype 2 in these cell types may yield further details. Alongside, a given ST could not be correlated with a specific interaction since important variations were observed between strains within a single ST. Consequently, the interactions with meningeal cells and astrocytes, though important for the pathogenesis, are not ST-dependent but rather a characteristic of *S. suis* serotype 2 that varies according to the strain.

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Abbreviations

CCL: C-C motif ligand; CFU: Colony forming unit; CNS: Central nervous system; CPEC: Choroid plexus epithelial cells; CPS: Capsular polysaccharide; CSF: Cerebrospinal fluid; CXCL: C-X-C motif ligand; FCS: Fetal calf serum; GBS: Group B *Streptococcus*; hBMEC: Human brain microvascular endothelial cells; IL: Interleukin; LDH: Lactate dehydrogenase; LTA: Lipoteichoic acid; MOI: Multiplicity of infection; NOD: Nucleotide-binding oligomerization domain; NLR: NOD-like receptors; pBMEC: Porcine brain microvascular endothelial cells; PBS: Phosphate-buffered saline; PGN: Peptidoglycan; SAS: Subarachnoid space; SLY: Suilysin; ST: Sequence type; THA: Todd-Hewitt Broth Agar; THB: Todd-Hewitt Broth; TLR: Toll-like receptor; TNF: Tumor necrosis factor; v/v: Volume/volume; w/v: Weight/volume.

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Tables

Table 1. *S. suis* serotype 2 strains used in this study

Strain	Sequence type	Country	Host	Phenotype	Reference
P1/7	1	United Kingdom	Pig	Wild-Type	[46]
P1/7Δ*cpsF*	1	-	-	Non-encapsulated mutant	[6]
31533	1	France	Pig	Wild-Type	[47]
31533Δ*sly*	1	-	-	Suilysin-deficient mutant	[7]
31533Δ*dltA*	1	-	-	D-alanylation of lipoteichoic acid-deficient mutant	[8]
31533Δ*pgdA*	1	-	-	N-deacetylation of peptidoglycan-deficient mutant	[9]
SC84	7	China	Human	Wild-Type	[48]
89-1591	25	Canada	Pig	Wild-Type	[49]
LPH4	25	Thailand	Human	Wild-Type	[50]
1088563	28	Canada	Pig	Wild-Type	[51]
MNCM43	28	Thailand	Human	Wild-Type	[50]

Table 2. *S. suis* serotype 2-induced human meningeal cell and astrocyte cytotoxicity, 24 h following infection

Strain	Sequence type	Presence of SLY	% cytotoxicity ± SEM (n=3)	
			Meningeal cells	Astrocytes
P1/7	1	Yes	0.1 ± 0.1	38.2 ± 3.4
31533	1	Yes	3.4 ± 1.2	63.3 ± 0.6
SC84	7	Yes	9.1 ± 2.6	35.3 ± 6.2
89-1591	25	No	0.0 ± 0.0	71.5 ± 1.5
LPH4	25	No	6.1 ± 2.2	28.9 ± 6.0
1088563	28	No	0.0 ± 0.0	12.0 ± 3.9
MNCM43	28	No	0.0 ± 0.0	1.9 ± 1.0
N. meningitidis	Not applicable	Not applicable	Not determined	88.6 ± 0.9

Figures

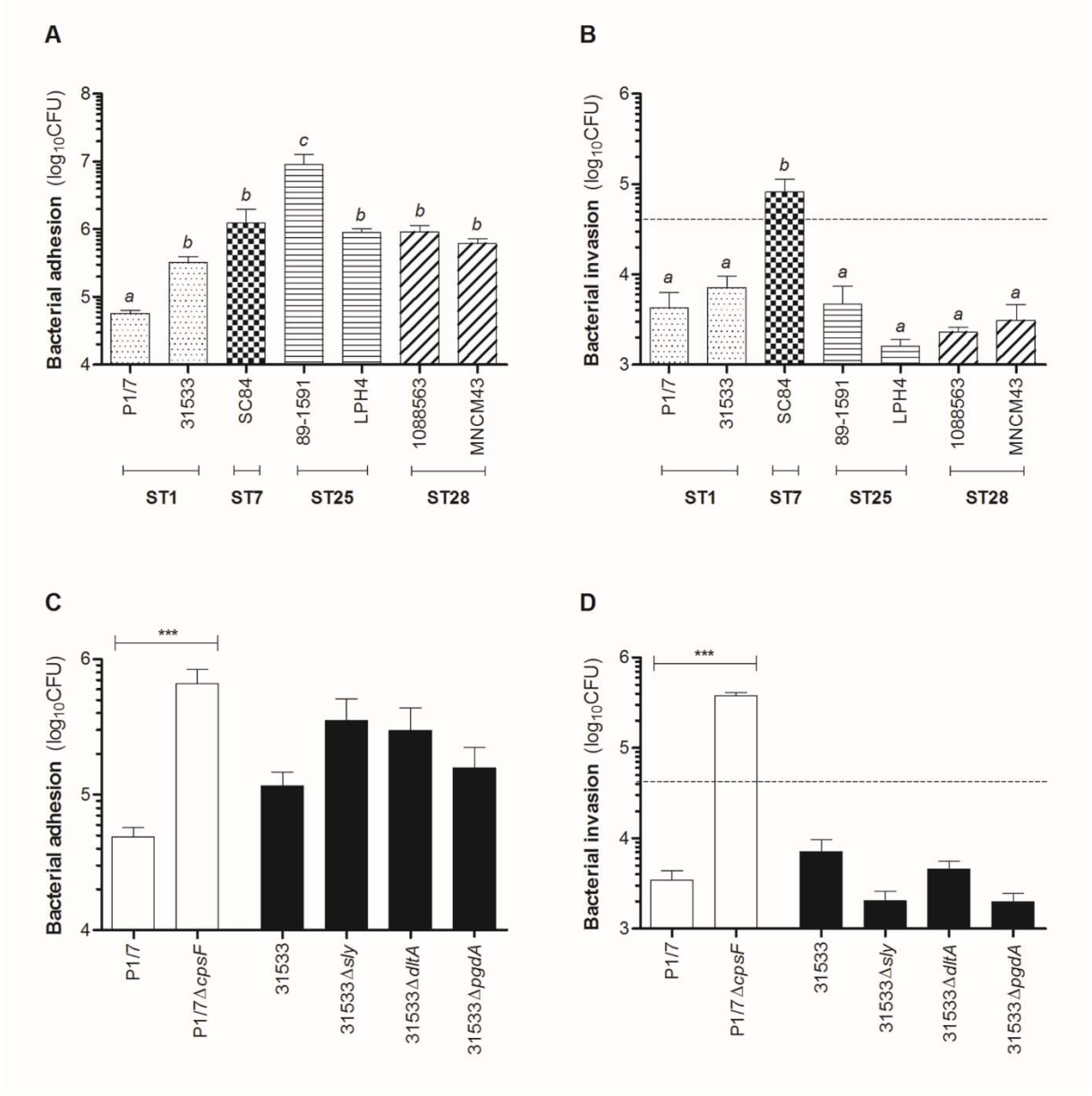

Figure 1. Interactions of *S. suis* serotype 2 with human meningeal cells. Bacterial adhesion (A) and invasion (B) of human meningeal cells by the *S. suis* strains belonging to different sequence types (STs), and adhesion (C) and invasion (D) of human meningeal cells by the mutant strains. Results are expressed as $\log_{10}$ mean ± SEM CFU obtained from three independent experiments. Dotted lines indicate a ratio of 1 intracellular bacterium/cell. The use of different letters (a, b or c) indicates a significant difference between groups ($p<0.01$); *** ($p<0.001$).

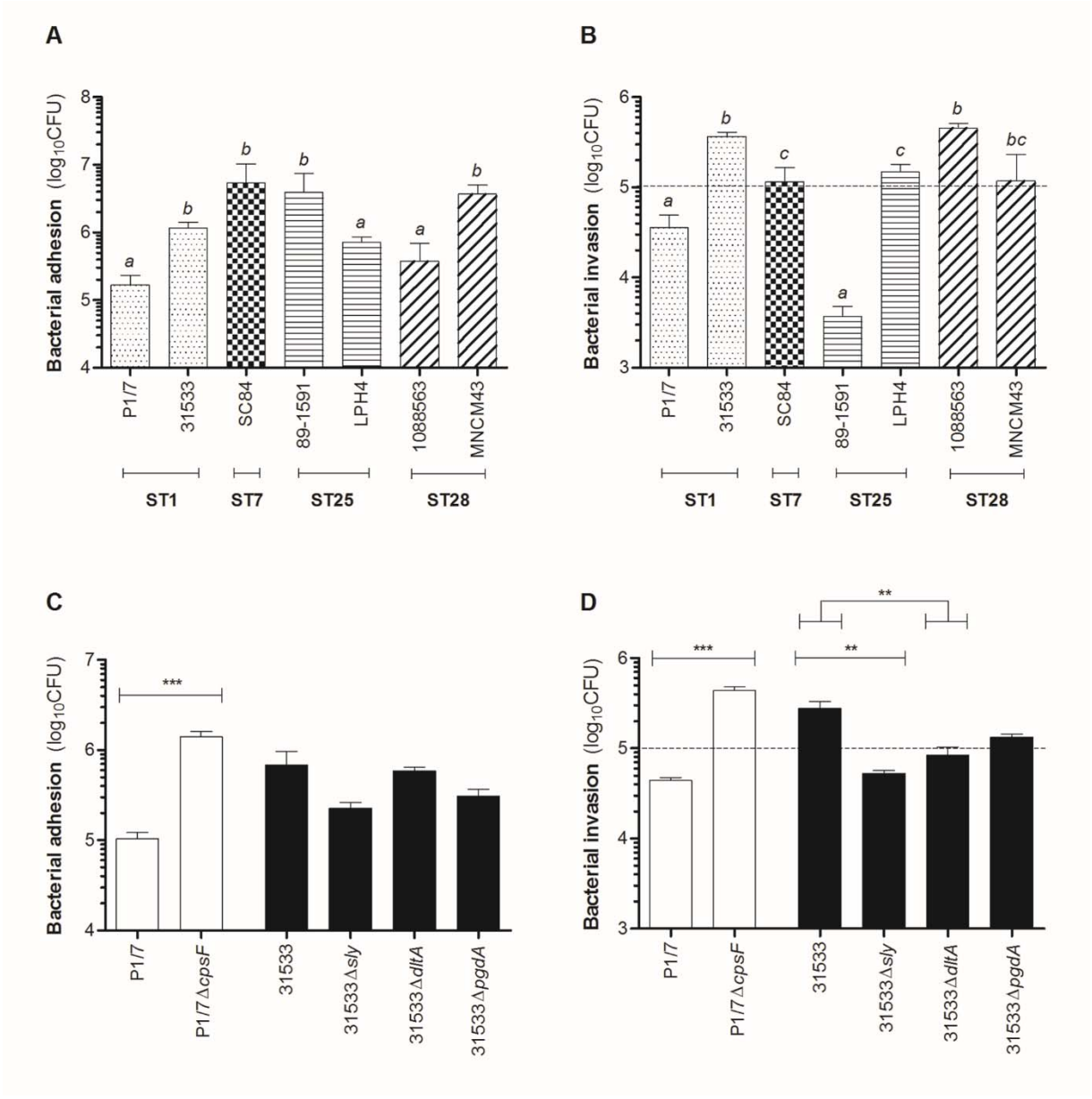

Figure 2. Interactions of *S. suis* serotype 2 with human astrocytes. Bacterial adhesion (A) and invasion (B) of human astrocytes by the *S. suis* strains belonging to different sequence types (STs), and adhesion (C) and invasion (D) of human astrocytes by the mutant strains. Results are expressed as $\log_{10}$ mean ± SEM CFU obtained from three independent experiments. Dotted lines indicate a ratio of 1 intracellular bacterium/cell. The use of different letters (a, b or c) indicates a significant difference between groups ($p<0.01$); ** ($p<0.01$); *** ($p<0.001$).

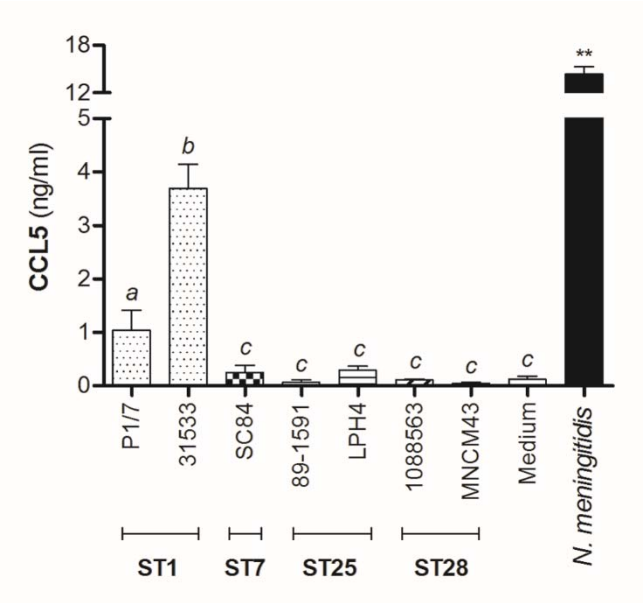

Figure 3. Only *S. suis* serotype 2 ST1 strains induce CCL5 from infected human meningeal cells. CCL5 production from human meningeal cells infected with the *S. suis* strains belonging to different sequence types (STs). Results are expressed as mean ± SEM ng/ml obtained from three independent experiments. The use of different letters (a, b or c) indicates a significant difference between groups ($p<0.01$). ** indicates a significant difference between *N. meningitidis* and the medium ($p<0.01$).

IV. DISCUSSION

Les infections causées par *S. suis* sont un sérieux problème en médecine vétérinaire et en santé humaine. En effet, non seulement *S. suis* est l'un des pathogènes bactériens les plus importants du porc, responsables de pertes économiques substantielles à l'industrie porcine mondialement, mais le nombre de cas humains augmente de manière exponentielle [1, 2]. Malgré les avancées scientifiques en médecine vétérinaire et en médecine humaine, l'incidence de cette bactérie n'a pas diminué; au contraire, le nombre de cas porcins a augmenté globalement [2]. De plus, *S. suis* possède une multitude de facteurs de virulence au cours de son évolution, dont plusieurs composants de surface, afin de pouvoir causer la maladie [10]. Il est donc nécessaire de mieux comprendre tant les mécanismes impliqués dans la pathogenèse de *S. suis* que la réponse de l'hôte à la suite de cette infection, afin d'établir davantage de stratégies de contrôle efficace. Bien que les recherches aient amélioré nos connaissances des interactions entre *S. suis* et son hôte, il y a toujours un manque crucial d'information sur sa pathogenèse et sa virulence.

En effet, la CPS, le LTA et une panoplie de protéines de surface, dont l'AgI/II et les lipoprotéines, sont des composants exprimés à la surface de la bactérie, avec des rôles potentiels ou décrits dans la colonisation et/ou dans l'infection systémique ou au niveau du SNC [10]. Toutefois, la définition de ce qu'est un facteur de virulence est ouverte à l'interprétation et dépend grandement des modèles expérimentaux utilisés, tant *in vitro* qu'*in vivo* [8]. De plus, le choix de la souche, en raison de son bagage génétique et de ses caractéristiques phénotypiques, pourrait également influencer le résultat [8], ce qui a créé une controverse n'ayant pas été testée lorsque cette thèse a débuté.

D'autre part, bien que le rôle des macrophages et des cellules dendritiques ait été étudié, du moins en partie [99, 128, 139, 148, 367], le rôle d'autres cellules phagocytaires, telles que les monocytes et les neutrophiles lors de l'infection à *S. suis*, est peu connu. De plus, même si l'inflammation exacerbée est une caractéristique des infections à *S. suis* et est responsable, du moins en partie, de la mort de l'hôte [1], les voies de signalisation impliquées lors de l'infection *in vivo*, dont celles des TLRs, et la fonction de différents médiateurs pro-inflammatoires n'avaient pas été déterminées. Enfin, bien que la méningite soit la pathologie la plus fréquemment causée par *S. suis* [2], nos connaissances de l'infection du SNC, dont les acteurs cellulaires et les médiateurs pro-inflammatoires, étaient très limitées au début de cette thèse.

Finalement, la plupart des études portant sur *S. suis* ont été effectuées à l'aide d'un nombre très limité de souches dites "classiques" de sérotype 2, c'est-à-dire des souches d'origines européennes appartenant au ST1 ou des souches ST7 responsables des éclosions humaines de 1998 et 2005 en Chine [8, 10]. Alors que ces souches prédominent en Europe et en Chine, 95% des souches nord-américaines diffèrent grandement de celles-ci [7]. De plus, les sérotypes 9 et 14 sont responsables d'un nombre croissant de cas porcins et humains mondialement [3]. Cependant, très peu d'études ont évalué leur virulence et ont investigué leurs facteurs de virulence et leur immunopathogenèse auparavant.

Dans un premier temps, cette thèse a permis de mieux caractériser le rôle et les fonctions de composants de surface de *S. suis* dans la pathogenèse de son infection. De plus, elle a permis de mieux comprendre l'impact de l'origine de la souche étudiée, dont son bagage génétique et ses caractéristiques phénotypiques, sur les conclusions tirées, incluant la virulence, et comment celles-ci peuvent affecter nos connaissances dans ce domaine de recherche. Dans un second temps, cette thèse a également disséqué la réponse de l'hôte face à *S. suis* et certains mécanismes cellulaires et moléculaires impliqués dans les infections systémiques et du SNC causées par ce pathogène.

Ainsi, la discussion des résultats présentés dans le cadre de cette thèse se divise en quatre grands axes :

1. Le rôle de composants de surface de *S. suis* dans sa pathogenèse de l'infection;
2. L'impact de l'origine des souches de *S. suis* et du choix des modèles expérimentaux sur les études de virulence et de pathogenèse;
3. La caractérisation de l'infection et de la réponse inflammatoire causées par différents *S. suis* au niveau systémique;
4. La caractérisation de l'infection et de la réponse inflammatoire causées par différents *S. suis* au niveau du SNC.

1. Le rôle de composants de surface de *Streptococcus suis* dans sa pathogenèse de l'infection

Comme décrit dans la revue de littérature, *S. suis* possède une panoplie de composants de surface, dont plusieurs sont des facteurs de virulence impliqués dans sa pathogenèse. Cette pathogenèse se divise en trois grandes étapes, soit la colonisation, l'infection systémique et l'infection du SNC. Malgré le nombre de publications portant sur ce sujet, il y avait tout de même un manque important d'information lorsque cette thèse a débuté.

1.1. La capsule polysaccharidique

La classification la plus fréquemment utilisée pour *S. suis* est celle des sérotypes, qui est basée sur l'antigénicité de la CPS présente à la surface bactérienne [2]. De plus, selon les études effectuées avec des souches de sérotype 2, la CPS est un facteur de virulence critique, car les mutants non-encapsulés ne sont pas en mesure de survivre dans le sang et de se disséminer. Bien que ses fonctions aient été extensivement étudiées au cours des dernières années, ces études ont été effectuées avec des souches de sérotypes 2 dites "classiques" appartenant au ST1 (Europe) ou au ST7 (responsable des éclosions humaines en Chine) [8]. Ainsi, bien que la structure et composition de la CPS, variant entre sérotypes, puissent influencer ses propriétés fonctionnelles, très peu d'information était disponible pour les autres sérotypes.

1.1.1. Effet de la délétion de gènes du locus capsulaire

Au cours des années, la délétion des gènes *cpsB*, *cpsE*, *cpsF*, *cpsG*, *cpsJ*, *cpsL*, *neuB* et *neuC* du sérotype 2 a été effectuée [92, 93, 139-141]. Peu importe quelle protéine est encodée par ces gènes, que ce soit une glycosyltransférase, une sialyltransférase ou une protéine impliquée dans la synthèse de l'acide sialique, leur délétion résulte en un phénotype non-encapsulé. Nous avons confirmé que ce phénomène n'est pas spécifique au sérotype 2, à l'aide de mutants déficients pour le gène *cpsG* chez le sérotype 9 (**Article I**) et les gènes *cpsB* et *neuC* chez le sérotype 14 (**Annexes – Article VI**). De plus, un phénotype similaire a été obtenu pour une souche de sérotype 2 nord-américaine appartenant au ST25 (génétiquement très distincte des souches ST1 et ST7), à la suite de la délétion du gène *cpsF* (**Article II**). En effet, la délétion de ces gènes a mené à la perte de la CPS, et ce malgré les différences de structure et de composition. Considérant que les fonctions des gènes *cpsA* à *cpsD* sont conservées dans tous les sérotypes [54], il n'est pas surprenant d'obtenir un phénotype non-encapsulé chez le mutant

du sérotype 14. Toutefois, la non-encapsulation, à la suite de la délétion des gènes codants pour des glycosyltransférases, démontre que l'absence d'une protéine interrompt le processus de synthèse de la CPS. Ainsi, la synthèse de la CPS chez *S. suis* est hautement régulée et nécessite la présence et la fonctionnalité de toutes les protéines codées par le locus capsulaire, peu importe le sérotype ou le bagage génétique de la souche. Cela indique que même si les sérotypes ont évolué de manière distincte [46], certaines caractéristiques sont conservées. Ceci est supporté davantage par le fait que la modification du lien α-2,6 de la chaîne latérale d'acide sialique en α-2,3 (retrouvé chez GBS de type III et V) cause une perte de la CPS chez *S. suis*, au contraire de GBS [404]. De plus, la non-encapsulation en absence de l'acide sialique démontre que celui-ci est nécessaire à la synthèse de la CPS chez *S. suis*. Ceci pourrait être dû à l'incapacité de la flippase ou de la polymérase à reconnaître les sous-unités, ce qui nuit à l'exportation et à la polymérisation de la CPS, respectivement.

1.1.2. Facteur de virulence critique indépendamment du sérotype

Tel que mentionné, la CPS des souches de sérotype 2 a longtemps été considérée comme un facteur de virulence critique, car les mutants non-encapsulés sont avirulents [10]. Toutefois, les différences dans la structure et la composition de la CPS ont été proposées comme pouvant influencer ce rôle, telle que la présence d'acide sialique (chez les sérotypes 1, 1/2, 2 et 14, entre autres) [10, 405]. Bien que les CPSs des sérotypes 2 et 14 se ressemblent, il y a tout de même quelques différences subtiles [129, 130]. Toutefois, elles diffèrent grandement de celle du sérotype 9, qui contient du glucitol, du phosphate et un sucre 4-céto labile, mais qui ne contient pas d'acide sialique [131]. Néanmoins, malgré ces différences, nous avons déterminé à l'aide de mutants non-encapsulés que les CPSs des sérotype 9 (**Article I**) et 14 (**Annexes – Article VI**) sont des facteurs de virulence critiques requis pour leur survie et leur persistance dans le sang. Ces données démontrent que la CPS de *S. suis* demeure un facteur de virulence critique, peu importe sa structure ou sa composition et pourrait suggérer que la présence physique de la CPS perturbe directement ou indirectement les radeaux lipidiques [145]. En effet, la CPS du sérotype 2 a été décrite comme empêchant l'accumulation de lactosylcéramide à la surface de la cellule de l'hôte, interférant ainsi dans la reconnaissance de *S suis* par des macrophages [145]. De plus, il a été suggéré que la présence de la CPS en soi confère une charge négative à la surface bactérienne, qui repousse la charge négative de la cellule de l'hôte, modulant ainsi les interactions [405, 406]. Toutefois, cela pourrait interférer dans la capacité d'infecter les cellules stromales, supportant davantage une modulation de la présence

et/ou l'épaisseur de sa CPS. Ainsi, lors de l'évolution de *S. suis*, sa CPS a été conservée, donnant un avantage à la bactérie lors des interactions hôte-pathogène.

1.1.3. Rôle de l'acide sialique

Le rôle critique, mais similaire que joue la CPS des sérotypes 2 (**Article II**), 9 (**Article I**) et 14 (**Annexes – Article VI**), suggère que l'acide sialique n'est probablement pas nécessaire pour la virulence de ce pathogène. Des résultats antérieurs n'ont rapporté aucune différence de virulence dans un modèle murin à la suite d'un traitement de *S. suis* sérotype 2 avec de la sialidase (qui clive l'acide sialique) ou avec la lectine SNA-I (qui lie l'acide sialique et la rend inaccesible pour effectuer ses fonctions) [136]. Toutefois, nos résultats devront être confirmés à l'aide d'un mutant dépourvu d'acide sialique, mais exprimant toujours la CPS à sa surface, en raison des autres différences de composition et de structure entre les CPSs des sérotypes 2, 9 et 14. Il est à noter qu'il n'est présentement pas possible d'évaluer le rôle de la sialyltransférase, car l'absence de ce gène est létale pour la bactérie en raison d'une accumulation intracellulaire d'acide sialique [407].

1.2. L'antigène I/II : Nouveau facteur de virulence du sérotype 9

Bien que les AgI/II sont des protéines multimodales impliquées dans la colonisation de la plupart des streptocoques humains [12] et qu'ils soient présents chez les sérotypes 2 et 9 de *S. suis*, leur rôle dans la pathogenèse de l'infection de cette bactérie n'avait jamais été étudié auparavant. Dans un premier temps, nous avons démontré, à l'aide d'un mutant déficient pour l'AgI/II, que ce dernier joue un rôle important dans les premières étapes de la pathogenèse du sérotype 9, soit dans la colonisation des voies respiratoires supérieures et des amygdales du porc (**Article IV**). Ces résultats démontrent que l'AgI/II est un nouveau facteur de virulence pour *S. suis*. Toutefois, son rôle est sérotype-dépendent. En effet, bien qu'il participe à l'infection par des souches de sérotype 9, cela n'est pas le cas pour des représentants du sérotype 2. Cette différence entre sérotypes suggère que le sérotype 2 possède d'autres facteurs ou composants ayant des fonctions ou des rôles similaires à ceux de l'AgI/II. Entre autres, au moins 28 différents facteurs ont été décrits au sein du sérotype 2 comme liant la MEC et au moins 34 sont activement impliqués dans l'adhésion aux cellules épithéliales [8]. Ainsi, l'un ou plusieurs de ces facteurs compensent pour l'absence de l'AgI/II, démontrant que *S. suis* sérotype 2 a développé des mécanismes de redondance. Au contraire, le rôle critique que joue l'AgI/II chez le sérotype 9 suggère que ce sérotype n'a pas développé autant de

facteurs compensatoires. Toutefois, le manque d'information sur les facteurs de virulence du sérotype 9 et sur sa pathogenèse limite nos connaissances sur la redondance chez ce dernier.

Dans un deuxième temps, nous avons démontré qu'en plus de son rôle lors de la colonisation, l'AgI/II du sérotype 9 est un facteur de virulence critique lors de l'infection systémique, étant requis pour sa survie dans le sang, pour ses interactions avec les phagocytes et le développement de la maladie clinique (**Article V**). En effet, en absence de l'AgI/II, le sérotype 9 active moins les cellules immunitaires et est plus rapidement éliminé de l'hôte.

1.3. La structure et la composition des acides lipotéichoïques

Les LTAs sont des composants intégraux de la paroi cellulaire des bactéries à Gram positif [11]. Contrairement au peptidoglycane, la structure et la composition du LTA de *S. suis* n'avaient jamais été investiguées auparavant [162]. À l'aide de résonance magnétique nucléaire à haute résolution et d'analyses par spectrométrie de masse, nous avons déterminé la structure et la composition du LTA de trois souches de virulence, d'origine géographique et de ST différents de *S. suis* sérotype 2 (**Article VI**). Le LTA des souches ST1 et ST7, qui sont plus virulentes, est très similaire et contient deux types de LTA, soit du type I et du type II ou III. Ceci n'est pas particulièrement surprenant, car ces deux STs sont génétiquement hautement apparentés, étant tous deux membres du CC1 [6]. Quant au LTA de la souche ST25 (virulence intermédiaire), celui-ci contient du type I identique à celui des souches ST1 et ST7, tandis que l'autre type est une molécule complexe caractérisée par une substitution à la position O-2 du glycérol. Ainsi, il existe peut-être un lien entre la complexité du LTA et la virulence de la souche. De plus, la structure et la composition des LTAs de *S. suis* est beaucoup plus complexe que celles d'autres espèces bactériennes qui, pour la plupart, ne synthétisent qu'un seul type [11]. En effet les LTAs retrouvés chez GAS, GBS, *S. aureus* et *L. monocytogenes* sont tous de type I (le type de LTA le plus simple) [11]. Comme la plupart des études investiguant les LTAs n'ont utilisé qu'une seule souche, souvent une souche de référence, il existe très peu d'information sur la diversité du LTA à l'intérieur d'une espèce, et encore moins sur son implication dans la pathogenèse. Toutefois, l'absence de LTA de *S. pneumoniae*, dont le LTA est composé d'un polymère complexe de type IV, n'affecte pas sa croissance, mais plutôt sa virulence et sa capacité d'adhérer aux cellules épithéliales [408]. Ce serait donc le premier exemple d'un LTA de type mixte aussi complexe à être rapporté. Toutefois, d'autres études seront nécessaires afin de mieux comprendre le rôle du LTA dans

la virulence de *S. suis* et l'impact qu'exerce ces différences de composition et de structures sur la virulence de la souche et sur sa pathogenèse.

1.4. Rôles de ces composants de surface dans les premières étapes de la pathogenèse

1.4.1. Colonisation des voies respiratoires supérieures

Plusieurs étapes sont requises pour que *S. suis* puisse coloniser les voies respiratoires supérieures du porc, tel que décrit dans l'article de synthèse de Segura *et al* [9]. Parmi celles-ci, il y a les interactions avec les protéines de la MEC et de la salive, sans oublier la capacité de la bactérie à former un biofilm afin d'assurer sa persistance [9]. En effet, l'AgI/II du sérotype 9 participe à une panoplie de fonctions nécessaires pour sa colonisation, dont l'autoagrégation, l'agrégation à la glycoprotéine 340 et l'adhésion aux protéines de la MEC. De plus, l'AgI/II favorise la formation de biofilm, permettant à la bactérie d'échapper aux défenses de l'hôte (**Article IV**).

1.4.2. Adhésion aux et invasion des cellules de l'hôte

La présence de la CPS du sérotype 2 interfère dans l'adhésion aux cellules de l'hôte et à leur invasion [10]. Nous avons démontré un rôle similaire pour les CPSs des sérotype 9 et 14 envers les cellules épithéliales de la trachée du porc (**Article II**). Cet effet n'est probablement pas dû à leurs composition ou structure en raison des différences importantes, mais plutôt à la charge négative que confère la présence de la CPS à la surface bactérienne, ce qui repousse la charge négative de la cellule de l'hôte (empêchant ainsi l'adhésion et l'invasion de celles-ci par *S. suis*) [405, 406]. Toutefois, malgré une encapsulation similaire, le sérotype 9 adhère plus aux cellules que les sérotypes 2 et 14, comme cela avait également été démontré auparavant avec des cellules épithéliales intestinales porcines et humaines [409]. Cela suggère que même en présence de CPS, les adhésines du sérotype 9 sont partiellement exposées ou que le sérotype 9 possède des adhésines différentes aux autres sérotypes. En effet, l'adhésine SadP est présente tant chez le sérotype 2 que chez le sérotype 9, mais leurs séquences en acides aminés sont très différentes, ce qui pourrait induire des différences dans leurs fonctions [251, 409].

Au contraire, la présence de l'AgI/II favorise l'adhésion du sérotype 9 aux cellules épithéliales de la trachée du porc (**Article IV**). Ceci pourrait être due à ses interactions avec les protéines de la MEC, dont le collagène et le fibrinogène, ou via un rôle direct en tant qu'adhésine. En

effet, l'adhésion de *S. gordonii* aux cellules épithéliales est médiée par la reconnaissance de l'intégrine β1 par son AgI/II, tandis que l'AgI/II de GAS (AspA) est impliquée dans l'adhésion de cette bactérie par des mécanismes peu connus [410]. Il est intéressant de noter que le sérotype 9 adhère plus aux cellules épithéliales de la trachée et que l'AgI/II participe dans l'adhésion à celles-ci. De plus, en absence de l'AgI/II, l'adhésion du sérotype 9 aux cellules épithéliales de la trachée est très similaire à celle du sérotype 2 (**Article IV**). Ainsi, l'AgI/II pourrait être l'une des adhésines faiblement masquées par la présence de la CPS du sérotype 9. Cela expliquerait, en plus du phénomène de redondance, pourquoi l'AgI/II joue très peu de rôles chez le sérotype 2. Des expériences avec un double mutant du sérotype 2 n'exprimant pas de CPS ni d'AgI/II permettrait de mieux étudier ce phénomène.

Une fois adhéré à la surface de la cellule, *S. suis* peut l'envahir. Tout comme pour l'adhésion, la CPS interfère dans l'invasion des cellules épithéliales de la trachée de porc par les sérotypes 2, 9 et 14 (**Article I**). Ceci indique que les facteurs impliqués dans l'adhésion et l'invasion diffèrent probablement. L'AgI/II, quant à lui n'est pas impliqué dans l'invasion de ces cellules, bien que les AgI/II d'autres streptocoques, dont l'AspA de GAS, le sont [410]. Ainsi, le fait que la CPS du sérotype 9 masque moins bien les adhésines, mais pas les facteurs impliqués dans l'invasion, indique davantage que l'AgI/II ne contribue pas à l'invasion par le sérotype 9. De plus, l'invasion des cellules épithéliales intestinales est plus élevée par un mutant non-encapsulé du sérotype 2 que du sérotype 9, ce qui suggère que différents facteurs sont impliqués dans les interactions hôte-bactérie dans les tractus respiratoires et intestinaux [409].

Puisque la présence de la CPS interfère globalement avec la capacité de *S. suis* à adhérer aux cellules épithéliales et à les envahir, l'hypothèse avait été émise que *S. suis* peut moduler la présence et/ou l'épaisseur de sa CPS afin de pouvoir interagir avec celles-ci [9, 10]. Bien que notre étude se soit concentrée sur la survie dans le sang, nous avons démontré que l'environnement et les pressions auxquelles font face *S. suis* peuvent mener à la modulation de l'expression de sa CPS (**Article III**). Ces résultats aident à expliquer comment *S. suis* envahit les cellules épithéliales malgré la présence de sa CPS épaisse.

1.4.3. La voie orale comme porte d'entrée

Une propriété fort intéressante de l'AgI/II du sérotype 9 est qu'il confère une importante résistance au stress acide (pH acides) à la bactérie (**Article IV**). Au cours des dernières

années, il est devenu évident que l'infection humaine par *S. suis* ne s'effectue pas seulement via des lésions cutanées, mais que l'ingestion d'aliments crus ou peu cuits contaminés par ce pathogène peut en être la cause [3, 31]. Ainsi, la résistance au stress acide pourrait permettre à la bactérie de survivre au pH de l'estomac. En effet, nous avons démontré que *S. suis* peut survivre à pH 3 pour plus de 20 min. Cela lui permettrait d'atteindre les intestins, auxquels le sérotype 9 adhère de manière importante [409]. En combinaison avec d'autres facteurs prédisposants, tels que l'alcoolisme, qui influence la fonction de barrière des jonctions serrées des cellules épithéliales [411], la présence de l'AgI/II pourrait favoriser les infections via la voie orale.

1.5. Rôle lors de l'infection systémique

1.5.1. Résistance à la phagocytose et à l'effet bactéricide

Nous avons démontré que la CPS de souches appartenant au ST1 et au ST25 du sérotype 2, de même que pour celle du sérotype 14, est nécessaire pour la résistance à l'effet bactéricide du sang entier, car elle confère des propriétés anti-phagocytaires à la bactérie (**Article II** & **Annexes – Article VI**). En effet, en absence de sa CPS, *S. suis* est rapidement éliminé da la circulation sanguine *in vivo*. De plus, elle est un facteur anti-phagocytaire des sérotype 1 et 1/2 (**Annexes – Article V**) face aux macrophages. Toutefois, comme décrit dans la revue de la littérature, les CPS des sérotypes 14 et 1 et celle des sérotypes 2 et 1/2, contiennent toutes de l'acide sialique et ne diffèrent que par la modification d'un galactose en galactosamine, due à un seul polymorphisme dans le gène *cpsK*. Cependant, nous avons observé des fonctions similaires pour le sérotype 9, bien que sa CPS soit très différente, sans présence d'acide sialique (**Article II**). Cela indique que d'autres motifs que l'acide sialique sont probablement responsables de ces propriétés.

Malgré ces propriétés partagées avec les autres souches de sérotypes 2, une souche ST25 nord-américaine, de virulence intermédiaire et bien encapsulée, est hautement phagocytée par les DCs murines (**Article I**). En fait, son internalisation est identique que sa CPS soit présente ou non. De manière similaire, la présence de CPS à la surface du sérotype 9 n'empêche pas entièrement sa phagocytose par les macrophages (**Article II**). Ainsi, il est fort probable que même si présente, la CPS masque de manière différente les composants sous-capsulaires responsables de l'activation de la cellule, dont des mécanismes phagocytaires. Toutefois, ces différentes souches et sérotypes sont résistants à l'effet bactéricide des leucocytes sanguins,

et ce de manière égale. Cependant, les tests de phagocytose ont été effectués à l'aide de macrophages, tandis que les neutrophiles et les monocytes sont les principaux phagocytes du sang [19]. En effet, la capacité des macrophages à internaliser et tuer les pathogènes bactériens est beaucoup plus grande que celle des neutrophiles et monocytes [412]. De plus, lors des tests bactéricides avec du sang entier *in vitro* ou dans la circulation sanguine *in vivo*, il y a présence du complément, qui n'est pas utilisé lors de la plupart des tests de phagocytose. Des études précédentes ont démontré que même en présence de la CPS, *S. suis* est plus sensible à la phagocytose par les DCs murines et les neutrophiles porcins à la suite du dépôt du complément à sa surface [91, 139]. En effet, plusieurs mécanismes de *S. suis* interfèrent dans le dépôt du complément, tels que la production de suilysine et de la protéine liant le facteur H et par des modifications de la paroi bactérienne, entre autres [139, 143, 229]. Nous avons confirmé la sensibilité de *S. suis* au complément et avons démontré que ce n'est pas une caractéristique spécifique au sérotype 2 : en présence de complément, l'internalisation du sérotype 9 par les macrophages péritonéaux et par les DCs a augmenté d'un facteur d'au moins dix (**Article V**).

Ce rôle critique que joue la présence de la CPS dans le sang et dans la virulence a été conforté à l'aide d'une souche de *S. suis* naturellement non-encapsulée en raison d'une mutation ponctuelle dans le gène *cpsF* (**Article III**). Le phénotype sauvage a été retrouvé à la suite de passages successifs de la souche dans le sang de souris. Ainsi, *S. suis* peut restaurer l'expression de sa CPS face à certaines pressions environnementales. En effet, plusieurs régulateurs ou protéines peuvent influencer l'expression des gènes de synthèse de la CPS *in vitro*, dont la protéine régulatrice du métabolisme du carbone, le régulateur CovR, le régulateur global CodY et la di-adénosine monophosphate phosphodiestérase [10]. De plus, l'épaisseur de la CPS et la concentration d'acide sialique sont modulées lorsque la bactérie se retrouve dans le sang [136]. Bien que l'expression, la production et le maintien de la CPS exigent un coût énergétique de la part de la bactérie, celle-ci peut être bénéfique dans plusieurs situations.

En plus de sa CPS, l'AgI/II du sérotype 9 contribue à sa survie dans le sang et dans les organes internes en conférant des propriétés anti-phagocytaires, similaires à la CPS (**Article V**). Toutefois, la contribution partielle de l'AgI/II dans la résistance à l'effet bactéricide du sang suggère qu'il y a d'autres facteurs importants d'impliqués. Il est intéressant de noter qu'en absence de l'AgI/II, la résistance du sérotype 9 à l'effet bactéricide du sang entier est similaire

à celle du mutant non-encapsulé (**Article II** & **Article V**). Ceci indique qu'au moins un autre facteur, présentement inconnu, y joue un rôle. De manière similaire à la CPS, l'absence d'AgI/II rend le sérotype 9 avirulent suite à l'inoculation par voie intrapéritonéale chez un modèle murin, de la même manière qu'un mutant non-encapsulé. Toutefois, à la suite de l'inoculation par voie intraveineuse, une diminution de la virulence du mutant déficient pour l'AgI/II a été observée, mais pas une perte totale. Il serait donc pertinent de comparer ces résultats au rôle de la CPS à la suite de l'inoculation d'un mutant non-encapsulé par voie intraveineuse. Cela permettrait de mieux comprendre l'importance relative de ces deux facteurs chez ce sérotype encore peu étudié.

De manière intéressante, la capacité d'internalisation du sérotype 9 par les DCs est moindre qu'avec des macrophages. Toutefois, l'AgI/II protège la bactérie contre la phagocytose par ces deux types cellulaires également. Bien qu'ayant des fonctions anti-phagocytaires, l'AgI/II n'interfère pas dans le dépôt du complément à la surface de *S. suis*. Ceci indique que son rôle anti-phagocytaire est dû à un effet physique (il est possible que son rôle dans l'autoagrégation bactérienne y participe [**Article IV**]) ou qu'il bloque les mécanismes phagocytaires de la cellule, tel que la déstabilisation des radeaux lipidiques [145]. De plus, un rôle de l'AgI/II dans la survie intracellulaire de *S. suis* n'a été rapporté qu'avec les DCs. Ceci pourrait être expliqué par le fait que les macrophages sont plus efficaces à tuer les bactéries ingérées [412]. Ainsi, l'environnement intracellulaire auquel fait face *S. suis* est moins extrême chez les DCs que chez les macrophages, en raison de leurs rôles principaux [313]. Bien que les DCs et macrophages soient tous deux des phagocytes professionnels et des APCs, les macrophages sont les phagocytes par excellence tandis que les DCs sont les meilleures APCs qui existe [313, 412]. Ce rôle de l'AgI/II contraste avec celui de la CPS, qui même s'il est un facteur anti-phagocytaire, n'a pas d'impact majeur sur la survie intracellulaire de *S. suis* [92, 405]. De plus, le rôle que joue l'AgI/II comme facteur anti-phagocytaire et dans la survie intracellulaire pourrait aussi avoir un effet sur le développement de la réponse immunitaire adaptative. En effet, la présence de la CPS interfère dans la présentation antigénique (**Annexes – Article I**) [139, 146].

1.5.2. Induction de la réponse inflammatoire et immunomodulation

En plus de ces autres fonctions, la présence de la CPS du sérotype 2 est associée avec une diminution de la réponse immunitaire innée via l'activation des cellules et la reconnaissance

bactérienne [10]. Nous avons démontré que c'est aussi le cas pour les sérotypes 9 et 14 (**Article I**). Cela suggère que cette caractéristique, partagée entre les sérotypes, est un mécanisme évolutif qu'a développé *S. suis,* lui permettant de freiner la mise en place de la réponse immunitaire requise pour son élimination. Ces résultats confirment aussi que ce sont plutôt les composants de la surface bactérienne qui en sont responsables, dont les lipoprotéines [37, 139, 148]. En effet, bien que les LTAs aient été décrits comme activant les cellules de l'immunité innée de l'hôte à la suite de leur reconnaissance par le TLR2, nous avons démontré que les LTAs de *S. suis* sérotype 2 n'ont pas de propriétés immunostimulatrices, peu importe le STs, du moins face aux DCs (**Article VI**). Ce sont en fait les lipoprotéines co-purifiées avec les LTAs qui seraient responsables de cette activité à la suite de leur reconnaissance par le TLR2.

De plus, nous avons démontré pour la première fois que la production d'IFN-β est modulée par la présence de la CPS (**Article X**). Toutefois, au contraire de la plupart des autres médiateurs, sa production n'est pas induite par des composants de la surface bactérienne, mais plutôt par ses acides nucléiques, dépendant donc de l'internalisation de la bactérie. Ainsi, sa production par une souche ST25 nord-américaine de sérotype 2, qui est hautement phagocytée, est similaire à son mutant non-encapsulé (**Article II**).

L'immunomodulation des chimiokines CCL2 et CCL3 par la CPS est un cas particulier. Contrairement aux cytokines pro-inflammatoires (TNF, IL-1β, IL-6, IL-12p70) et même aux autres chimiokines (CXCL1, CXCL9, CXCL10), CCL2 et CCL3 sont induits par les CPSs des sérotypes 2 et 14 elles-mêmes, à la suite de l'activation des DCs et des macrophages [37, 148]. En effet, des études antérieures ont démontré qu'en absence de CPS (mutant non-encapsulé), la production de CCL2 induite par le sérotype 2 chez des macrophages murins est diminuée [37, 148]. Cependant, chez les DCs, aucune différence de production de CCL2 et CCL3 n'avait été observée en absence de la CPS [139]. Nous avons confirmé ces résultats chez les DCs, et ce pour les sérotypes 2, 9 et 14. Cela suggère que les inducteurs ne sont pas masqués par la CPS, peu importe le sérotype, ou que ce sont des facteurs sécrétés.

D'autre part, les résultats que nous avons obtenus suggèrent que l'AgI/II est une protéine immunostimularice responsable de l'activation de cellules et de la production de médiateurs pro-inflammatoires. En effet, sa présence contribue à la production de TNF, d'IL-1β, d'IL-6 et

de CCL3. Toutefois, ces résultats devront être confirmés à l'aide de l'AgI/II purifié. D'autres membres de la famille des AgI/II ont été rapportés comme participant à l'induction de la réponse inflammatoire, dont SspA et SspB de *S. gordonii* [282]. Cependant, les récepteurs impliqués dans la reconnaissance de l'AgI/II et les voies de signalisation activées restent à être élucidées.

2. L'impact de l'origine des souches de *Streptococcus suis* et du choix des modèles expérimentaux sur les études de virulence et de pathogenèse

Comme mentionné dans la revue de la littérature, le nombre de publications portant sur *S. suis* et s'intéressant à l'étude de ses facteurs de virulence et de sa pathogenèse a augmenté de manière exponentielle au cours des dernières années [8]. Toutefois, la définition de ce qu'est un facteur de virulence reste ouverte à interprétation, puisqu'aucun barème n'a été établi, ce qui a créé un énorme problème dans ce domaine. De plus, nos connaissances actuelles de la pathogenèse de *S. suis* sont des généralisations basées sur des souches dites "classiques" de sérotype 2 (souches ST1 ou ST7), malgré l'importante hétérogénéité de ce pathogène [8, 10]. Ainsi, nous nous sommes intéressés à l'impact qu'a l'origine de la souche, ainsi qu'au choix des modèles expérimentaux sur les études de virulence et de pathogenèse de *S. suis*.

2.1. L'impact de l'origine de la souche

2.1.1. Effet du sérotype

Bien qu'il existe présentement 29 "vrais" sérotypes de *S. suis* (les sérotypes 20, 22 et 26 appartenant à *Streptococcus parasuis*, le sérotype 33 à *Streptococcus ruminantium* et les sérotypes 32 et 34 à *Streptococcus orisratti* [46] suite à des analyses génétiques), la plupart d'entre eux ont été très peu étudiés [10, 46]. Nous avons démontré dans le cadre de cette thèse que les résultats obtenus ne peuvent pas toujours être généralisés. En effet, bien que la CPS soit un facteur de virulence critique pour les sérotypes 2, 9 et 14 (**Article I**, **Article II** & **Annexes – Article VI**), son rôle dans les interactions avec les cellules de l'hôte varie. Elle est un facteur anti-phagocytaire important pour les sérotypes 1, 1/2, 2 et 14 face aux macrophages, mais elle protège moins bien que le sérotype 9 (**Article I** & **Annexes – Article V**). De manière similaire, le rôle de l'AgI/II, dans les premières étapes de la pathogenèse, est complètement différent pour les sérotypes 2 et 9 (**Article IV**). Si nous n'avions étudié que le sérotype 2, nous en aurions conclu que l'AgI/II joue très peu de fonctions chez *S. suis*.

Il est important de noter que les sérotypes 2, 9 et 14 sont considérés comme étant virulents en raison du grand nombre de cas cliniques qu'ils causent mondialement [3]. À l'opposé, plusieurs sérotypes sont responsables de très peu de cas cliniques [4]. Il est fort probable que les conclusions obtenues avec des sérotypes virulents ne s'appliquent pas aux sérotypes moins virulents. Ainsi, les résultats obtenus dans le cadre d'une étude avec un sérotype particulier ne

s'appliquent pas nécessairement aux autres sérotypes. Cela est donc très imprudent de généraliser les conclusions pour cette bactérie si hétérogène.

2.1.2. Effet du type allélique

Comme mentionné, des souches de différents sérotypes peuvent appartenir au même ST [6]. Toutefois, nous nous sommes intéressés à l'effet de différents types alléliques au sein du sérotype 2 seulement. Les ST1 et ST7, qui regroupent les souches classiques européennes et les souches responsables des éclosions humaines, respectivement, ont été les plus étudiées [8, 10]. En effet, le nombre d'études ayant utilisé des souches ST25 et ST28 (fréquemment associés avec l'Amérique du Nord, la Thaïlande et la Japon) est très limité.

Nous avons démontré que des souches du même ST peuvent présenter des résultats très différents. Bien que ces différences ne soient pas marquées pour des souches rapprochées (par exemple les souches ST1 et ST7), ceci n'est pas le cas pour des souches plus distinctes. En effet, la structure du LTA des souches ST1 et ST7 étudié est très similaire, mais elle diffère grandement de celle d'une souche ST25 (**Article VI**). De plus, cette souche ST25 forme moins de biofilms et adhère moins aux cellules épithéliales que les souches ST1 et ST7 (**Article VII**). Finalement, même si la DPPIV et l'autolysine sont présentes chez ces différents STs, la DPPIV possède des rôles semblables, contrairement à l'autolysine (**Article VII**).

En plus de ces différences, nous avons démontré des souches du même ST peuvent interagir différemment avec les cellules de l'hôte. En effet, bien que la CPS soit un facteur de virulence critique pour toutes les souches de sérotype 2 étudiée, son rôle dans les interactions avec les DCs varie grandement. Les résultats obtenus avec une souche ST25 et son mutant non-encapsulé sont identiques lors des interactions avec les cellules (**Article II**). De plus, les souches ST28 sont plus résistantes à la phagocytose par les macrophages que le sont les souches ST1 et ST25 (**Article VIII**). Toutefois, les souches ST25 et ST28 sont plus sensibles à l'effet bactéricide du sang entier que les souches ST1 (**Article VIII**). Néanmoins, aucune différence n'a été observée dans leurs interactions avec les cellules de méninges et les astrocytes (**Article XII**). Cela indique que les différences entre souches de différents STs ne sont pas nécessairement constantes et peuvent varier d'un type cellulaire à l'autre, compliquant davantage les généralisations.

Finalement, la virulence des souches varie également. En effet, bien que les souches ST1 sont dites plus virulentes que les souches ST25, et les souches ST25 plus virulentes que les souches ST28 [7], nous avons démontré que certaines souches ST25 causent plus de mortalité chez un modèle murin que les souches ST1, de même que certaines souches ST28 (**Article VIII**) [7]. De plus, même si une souche virulente induit plus de médiateurs pro-inflammatoires, les niveaux de production par un type cellulaire en particulier peuvent varier selon les interactions. C'est le cas de l'IFN-β, qui est induit en plus grande quantité par une souche ST25 que par une souche ST1, au contraire des autres médiateurs produits par l'hôte. Toutefois, nous avons démontré que la production d'IFN-β est particulière et dépend de l'internalisation de la souche (**Article X**). De manière similaire, bien que les souches ST1 induisent plus d'IL-6 par les cellules épithéliales et de CCL5 par les cellules de méninges que les souches ST25, la production de CXCL8 est similaire entre ces STs (**Article XII** & **Annexes – Article IX**). Enfin, les souches ST1 et ST7 induisent des niveaux systémiques similaires d'IL-1, ce qui n'est pas le cas *in vitro* lorsque les DCs et macrophages sont infectés (**Annexes – Article X**). Ces résultats démontrent pourquoi il est nécessaire d'utiliser des souches de STs différents, afin de confirmer si les résultats sont spécifiques ou communs à un ou plusieurs STs.

2.1.3. Effet de la provenance géographique

Une des plus importantes différences rapportées depuis plusieurs années au sein des souches de sérotype 2 est l'effet de la provenance géographique [31]. En effet, le nombre plus faible d'infections en Amérique du Nord, à l'opposé de l'Europe et de l'Asie, a été suggéré comme étant dû à une virulence moindre des souches nord-américaines [3]. Bien que nos résultats supportent cette hypothèse, il faut garder en tête que les souches européennes et asiatiques, de même que celles de l'Amérique du Sud (**Annexes – Article IV**), bien que toutes de sérotype 2, appartiennent généralement aux ST1 et/ou au ST7, tandis que 95% des souches nord-américaines sont des ST25 et ST28 [3, 7].

À l'intérieur d'un même ST, l'origine géographique semble tout de même jouer un rôle. En effet, nous avons démontré que les souches nord-américaines et asiatiques appartenant aux ST25 et aux ST28 diffèrent grandement génétiquement (**Annexes – Article VIII**) [413]. Malgré leurs similitudes au niveau des gènes de ménage, ces souches ont évolué séparément et sont regroupées de manière distincte. Cela se traduit par des différences de virulence importantes : bien que les souches ST28 nord-américaines soient peu virulentes dans des modèles

expérimentaux, les souches provenant de l'Asie le sont (**Article VIII**). Quant aux ST25, très peu d'études se sont attardées sur la virulence de souches de l'Asie, en comparaison à celles de l'Amérique du Nord. *In vitro*, nous avons démontré que les propriétés associées à la présence de la CPS du sérotype 2 de ST1 et/ou ST7, déterminées à l'aide de souches de l'Europe ou de l'Asie, ne s'appliquent pas nécessairement aux souches nord-américaines (**Article II**). Ainsi, l'ensemble de ces résultats démontrent, encore une fois, qu'il est dangereux de généraliser en se basant uniquement sur la classification de la souche.

Outre pour le sérotype 2, très peu d'études ont évalué l'effet de l'origine géographique. Toutefois, il a été rapporté très récemment que les souches nord-américaines de sérotype 9 pourraient être moins virulentes que celles de l'Europe [60]. Ainsi, il est possible que le rôle de l'AgI/II du sérotype 9 en tant que facteur de virulence critique, déterminée à l'aide d'une souche nord-américaine, ne soit pas applicable à toutes les souches de sérotype 9. En effet, il est fort probable que les souches européennes possèdent des facteurs de virulence en plus, ou du moins différents de ceux retrouvés chez les souches nord-américaines.

2.2. Impact du choix des modèles expérimentaux *in vitro* et *in vivo*

2.2.1. *In vitro*

(A) Utilisation de la bactérie entière versus des composants purifiés ou recombinants

Plusieurs rôles ou fonctions des facteurs de virulence de *S. suis* ont été décrits à l'aide de composants purifiés ou recombinants [8, 10]. Bien que cela permette de confirmer leur rôle précis dans les interactions ou fonctions, l'interprétation des résultats doit être faite avec prudence, car ce même rôle ne sera pas nécessairement observé lorsque la bactérie entière est utilisée. Cela n'enlève rien au rôle du facteur, mais démontre qu'il n'est pas nécessairement critique. En effet, l'AgI/II des sérotype 2 et 9 sont très similaires, partageant 95% d'homologie de leur séquence protéique. Ainsi, les deux protéines se comportent de manières similaires lorsqu'utilisées seules. Toutefois, aucun rôle majeur n'a été observé pour l'AgI/II des souches de sérotype 2, contrairement au sérotype 9 (**Article IV**). Comme mentionné précédemment, ceci est dû à la compensation chez le sérotype 2 par d'autres facteurs partageant des fonctions similaires. En effet, bien que la DPPIV et l'autolysine ont été décrites comme liant la fibronectine humaine [177, 179], nous n'avons pas observé de différence entre un mutant déficient et la souche sauvage, et ce même avec des souches de trois types alléliques différents (**Article VII**).

Un autre exemple est la suilysine. Cette toxine a été décrite comme induisant la production de TNF à la suite de sa reconnaissance par le TLR4 des macrophages [331]. Toutefois, aucun rôle du TLR4 dans la reconnaissance du sérotype 2 n'a été démontré à l'aide de la bactérie entière, peu importe le ST étudié (**Article IX** & **Annexes – Article X**). Cela ne veut pas dire que la suilysine n'est pas reconnue, mais plutôt qu'en présence d'une panoplie d'autres composants immunostimulateurs retrouvés à la surface de *S. suis*, la contribution de cette reconnaissance à la production inflammatoire est mineure. Ainsi, un facteur de virulence peut être impliqué dans la virulence sans être critique pour la survie de la bactérie. Il est donc nécessaire d'avoir une vue d'ensemble lors des expérimentations.

(B) Les cellules de l'hôte utilisées

Les lignées cellulaires sont utilisées de routine pour plusieurs raisons, dont leur faible coût, leur immortalisation et leur disponibilité commerciale. Toutefois, leur désavantage majeur est qu'elles ont été modifiées lors du processus d'immortalisation, ce qui peut avoir des effets importants sur leurs fonctions et leurs propriétés [8]. Au contraire, les cellules primaires sont plus représentatives des cellules retrouvées chez l'hôte, mais varient entre animaux et même entre expériences et nécessitent l'utilisation constante d'animaux [8]. Ainsi, ces deux types cellulaires ont des points forts et faibles. Cependant, cela complique la comparaison des résultats, puisque les types cellulaires et leur provenance diffèrent d'une étude à l'autre et l'utilisation de l'un ou de l'autre peut créer un biais lors des expériences.

De plus, l'hôte duquel proviennent les cellules semble aussi avoir un effet sur les résultats obtenus. Bien que le porc soit l'hôte naturel de *S. suis*, il existe peu d'outils pour effectuer des études chez le porc [8]. Ainsi, plusieurs études, dont la plupart de celles réalisées dans cette thèse, utilisent des cellules d'origine murine. D'autre part, plusieurs études ont utilisé des cellules humaines. En effet, l'utilisation de cellules épithéliales des voies respiratoires humaines, telles les HEp-2, est très commune, mais n'est pas nécessairement pertinente, étant donné que l'infection par les voies respiratoires n'a jamais été démontrée chez l'humain [8]. Par conséquent, lors d'études des voies respiratoires, des cellules porcines devraient être utilisées. En effet, aucun rôle de la DPPIV dans l'adhésion aux cellules épithéliales n'a pas été observé avec des cellules porcines, au contraire de cellules humaines (**Article VII**) [179]. Enfin, les cellules d'origines murines ont été démontrées à plusieurs reprises comme capable d'interagir avec *S. suis,* de manière similaire aux cellules du porc ou de l'humain [139, 146].

Toutefois, il peut y avoir des différences et celles-ci doivent être prises en compte lors des analyses. En effet, nous avons démontré que le sérotype 9 induit moins de médiateurs pro-inflammatoires par les DCs murines, que par les DCs humaines (**Article I**) [147]. Cela pourrait suggérer des différences inter-espèces, bien que l'origine tissulaire des cellules diffère également (cellules hématopoïétiques versus monocytes sanguins).

Finalement, l'utilisation d'un seul type cellulaire ou de plusieurs types à la fois peut avoir un impact majeur sur les résultats obtenus. L'utilisation unique permet de décortiquer les mécanismes cellulaires et de mieux comprendre les interactions entre la cellule et le pathogène. Toutefois, elle ne tient pas compte de la communication intercellulaire et de l'effet qu'a cette communication sur leurs fonctions. En effet, nous avons démontré que le sérotype 9 est plus sensible à la phagocytose par les macrophages, que les sérotype 2 et 14, mais qu'il résiste également à l'effet bactéricide du sang entier (**Article I**). De manière similaire, les souches ST28 de sérotype 2 sont plus résistantes à la phagocytose par les macrophages que les souches les souches ST1, mais elles résistent peu à l'effet bactéricide du sang (**Article VIII**). De plus, l'ajout de composants retrouvés chez l'hôte, tels que le complément, peut affecter grandement les résultats obtenus. Nous avons démontré que la pré-opsonisation des bactéries avec du complément de souris naïves augmente la phagocytose par les DCs et les macrophages d'un facteur de dix, ce qui peut affecter les conclusions tirées (**Article V**).

Ainsi, le choix des cellules de l'hôte utilisées est un facteur déterminant lors des expériences et doit être fait de manière à répondre adéquatement à la question de recherche. Il serait donc pertinent de créer une liste de types cellulaires ou de lignées cellulaires appropriées pour l'étude de *S. suis,* selon l'expérience à réaliser, afin de réduire les variations entre laboratoires.

2.2.2. *In vivo*

Bien que plusieurs modèles expérimentaux d'infection à *S. suis* ont été développés au cours des années, le porc et la souris sont les deux plus fréquemment utilisés [8]. Toutefois, plusieurs autres espèces, dont le lapin, le cochon d'Inde et le poisson zèbre ont aussi été utilisées. Le choix de l'animal utilisé peut avoir un effet marqué sur les résultats obtenus. En effet, malgré une étude antérieure effectuée chez le poisson zèbre [177], nous avons démontré que l'autolysine ne contribue pas à la virulence globale de *S. suis* chez la souris (**Article VII**). De plus, la race de porc ou la souche de souris peut aussi jouer un rôle important [8]. En effet, la

DPPIV joue un rôle critique dans la virulence d'une souche ST7 de sérotype 2 chez les souris BALB/c [179], mais aucunement chez les souris C57BL/6 (**Article VII**). Bien que les souris BALB/c soit couramment utilisées, elles sont moins représentatives de la réponse naturelle chez le porc, car elles ont une réponse Th2, au contraire des C57BL/6 qui ont une réponse immunitaire prototype Th1 et une forte réponse pro-inflammatoire requise pour surmonter l'infection à *S. suis* chez le porc [41, 414].

En plus du modèle animal, la voie d'inoculation peut avoir un effet important. Nous avons démontré qu'en absence de l'AgI/II, le sérotype 9 est avirulent à la suite d'une inoculation par voie intraperitonéale, et que sa virulence est atténuée par voie intraveineuse (**Article V**). Ceci s'explique par les types cellulaires rencontrés initialement, soit les macrophages de la cavité péritonéale et les leucocytes sanguins, respectivement. Comme nous l'avons démontré dans plusieurs études de cette thèse, *S. suis* résiste de manière différente aux macrophages, aux DCs et aux leucocytes sanguins (**Article I**, **Article II**, **Article V** & **Article IX**).

Ainsi, le choix des souches utilisées peut grandement influencer les résultats obtenus. En effet, nous avons démontré que l'origine de la souche est un biais important. De plus, comme le choix des modèles expérimentaux employés peut avoir un impact sur l'étude, ceux-ci devraient être choisis afin de répondre à la question de recherche. Dans le but d'éviter des conclusions erronées ou généralisées il serait important d'établir des critères d'évaluation afin de définir comment déterminer ce qu'est un facteur de virulence putatif ou d'étudier la pathogenèse de l'infection en réduisant au minimum le biais expérimental.

2.3. La redondance des facteurs de virulence de *Streptococcus suis*

S. suis est un pathogène, chez qui nous avons démontré le concept de redondance. En effet, plusieurs résultats obtenus dans le cadre de cette thèse supportent ce concept, dont l'AgI/II chez le sérotype 2 (**Article IV**), la DPPIV et l'autolysine (**Article VII**) et la Zmp (**Annexes – Article VII**). Cette redondance est nécessaire pour la survie bactérienne, car elle permet d'augmenter la résistance aux différents stress auxquels font face la bactérie [415]. Cela inclut, entre autres, l'évasion du système immunitaire et la réponse inflammatoire et démontre que *S. suis* est un pathogène très complexe nécessitant encore beaucoup de recherche.

3. La caractérisation de l'infection et de la réponse inflammatoire causées par différents *Streptococcus suis* au niveau systémique

L'infection systémique causée par *S. suis* débute lorsque le pathogène atteint la circulation sanguine, dans laquelle il peut se répliquer, persister, puis se disséminer. Cela cause l'activation des cellules de l'immunité innée, qui vont répondre par la production d'une réponse inflammatoire. Cette réponse s'exacerbera si la bactérie n'est pas rapidement éliminée du compartiment systémique, menant au développement de maladies graves et potentiellement mortelles, tel que le sepsis et le choc septique [10, 286]. Toutefois, cette infection n'avait été que très peu caractérisée auparavant. Ainsi, nous avons évalué (1) le rôle de la signalisation MyD88-dépendante dans la reconnaissance de *S. suis*, (2) la contribution des monocytes et des neutrophiles sanguins et (3) le rôle de médiateurs sur la modulation immunitaire lors de l'infection systémique, à l'aide d'un modèle de sepsis/choc septique chez des souris C57BL/6. Il est important de garder en tête que bien que l'inflammation exacerbée induite par *S. suis* soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes.

3.1. Rôle de la signalisation MyD88-dépendante dans la reconnaissance de *Streptococcus suis* et dans la réponse inflammatoire induite

Parmi les différents PRRs, les TLRs sont non seulement les mieux étudiés, mais les plus importants pour la reconnaissance de bactéries extracellulaires, dont *S. suis* [13]. La protéine adaptatrice MyD88 est essentielle à la transduction du signal de tous les TLRs, à l'exception du TLR3 [155]. Bien que quelques études se sont intéressées au rôle des TLRs dans la reconnaissance de *S. suis* auparavant, elles se sont limitées à des études *in vitro* [148, 367].

L'importance de la signalisation MyD88-dépendante dans la reconnaissance de *S. suis* par les macrophages et les DCs a été démontrée auparavant à l'aide de souches appartenant au ST1 du sérotype 2 seulement [148, 367]. Nous avons confirmé que la reconnaissance des souches de sérotype 2, peu importe le ST (ST1, ST7, ST25 et ST28) et la proximité génétique et phénotypique, nécessite la signalisation MyD88-dépendante, puisque la production de médiateurs pro-inflammatoires est pratiquement abolie en son absence (**Article IX** & **Article X**). C'est aussi le cas pour les sérotypes 9 et 14 par les DCs (**Article I**). Cette dépendance, presque exclusive de la reconnaissance de *S. suis* par MyD88 suggère que les composants

reconnus par les cellules et responsables de leur activation initiale sont conservés. En effet, bien que les différentes souches et sérotypes étudiés induisent des niveaux de médiateurs pro-inflammatoires variables, ceux-ci sont la conséquence des facteurs de virulence exprimés de manière différentielle et impliqués dans la modulation subséquente de la réponse inflammatoire de l'hôte. Ainsi, bien que les sérotypes et même les souches de *S. suis* aient évolué de manières distinctes, certains motifs ont été conservés au cours de cette évolution, ce qui semble être le cas pour d'autres streptocoques pathogènes tels le GBS et *S. pneumoniae* [416, 417].

Les résultats que nous avons obtenus *in vitro* suggèrent un rôle important de la signalisation MyD88-dépendante *in vivo*. En effet, son absence *in vivo* cause la mort subite de l'hôte, en raison d'une abrogation quasi totale de la réponse inflammatoire systémique requise pour le contrôle et l'élimination de *S. suis* (**Article IX**). Ce rôle est tellement important que même des doses sous-létales causent la mort de 100% des animaux en son absence. Bien que l'inflammation exacerbée induite par *S. suis* soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes. Ainsi, la balance inflammatoire est nécessaire au maintien de l'homéostasie. En fait, nous n'avions jamais été capables d'induire la maladie clinique dans un modèle expérimental avec une souche ST28 nord-américaine de sérotype 2 (considérée comme étant peu virulente) auparavant (**Article VIII**) [7]. Toutefois, comme ces souches sont rapidement éliminées de la circulation sanguine, le manque d'activation du système immunitaire innée, en l'absence de MyD88, leur permet de s'établir et de persister. Ces résultats démontrent que les souches ST28 nord-américaines ne sont pas non-virulentes, mais que leur faible virulence nécessite une immunosuppression de l'hôte. Ceci corrobore les résultats qui démontrent une plus grande susceptibilité des porcs à *S. suis* lors d'une infection au préalable par d'autres pathogènes viraux ou bactériens induisant une immunosuppression [29, 30].

Étant une bactérie extracellulaire, la reconnaissance de *S. suis* s'effectue principalement à la surface des cellules par le TLR2. Nous avons démontré que la reconnaissance de *S. suis* par le TLR2 à la surface des DCs compte pour 50% de la production de médiateurs pro-inflammatoires, et ce, que ce soit pour les sérotypes 2 (peu importe le ST), 9 et 14 (**Article I** & **Article IX**). À ce jour, la production de tous les médiateurs inflammatoires induits, à l'exception

de l'IFN-β, dépend en partie, de la reconnaissance de *S. suis* par le TLR2, et ce par les DCs, les macrophages et les astrocytes [126, 148, 367]. Cela démontre que les composants reconnus sont partagés, du moins en partie, entre les souches de *S. suis*. Au contraire, le TLR2 n'est pas impliqué dans la production d'IFN-β à la suite de la reconnaissance de *S. suis* par les DCs (**Article X**). Toutefois, il avait été suggéré que le TLR2 ne peut pas mener à la production d'IFN-β par les DCs, contrairement aux macrophages [418, 419].

Comme pour la plupart des bactéries à Gram positif, ce sont les lipoprotéines de *S. suis* qui ont été suggérées comme étant reconnues par le TLR2. En effet, nous avons démontré que les lipoprotéines de surface, et non le LTA, induisent la production de TNF, d'IL-1β, d'IL-6, de CCL3 et de CXCL1 des DCs (**Article VI** & **Annexes – Article X**). Ces résultats suggèrent que les lipoprotéines ont évolué de manière conservée entre les souches de *S. suis*. Ceci pourrait s'expliquer par leurs fonctions multiples, dont plusieurs sont critiques à la survie de la bactérie et au maintien de son homéostasie [164]. Il existe tout de même une certaine diversité, tel que démontré par les lipoprotéines du sérotype 9 qui ont des propriétés immunostimulatrices plus fortes que celles du sérotype 2 [105]. Par contre, l'activation plus importante des cellules d'origines humaines par le sérotype 9 indique que la reconnaissance des lipoprotéines par le TLR2 est dépendante de l'espèce [105, 147]. En effet, bien que les domaines intracellulaires du TLR2 humain et murin partagent 84% d'homologie, seulement 65% de la séquence primaire des domaines extracellulaires, responsables de la reconnaissance du motif, est similaire [420]. En contrepartie, l'hôte a développé des mécanismes afin de reconnaître les motifs conservés des lipoprotéines, plus particulièrement le motif diacylé, qui est indispensable à leur maturation [13].

Bien que la reconnaissance de *S. suis in vitro* dépende en partie du TLR2, nous n'étions pas en mesure de démontrer de rôle dans l'inflammation systémique *in vivo*, peu importe la souche utilisée (**Article IX**). De plus, la dose de bactéries inoculée n'a eu aucun effet sur ce résultat, au contraire de GBS type V [416]. Bien que le TLR2 soit important pour la reconnaissance des bactéries à Gram positif, aucun rôle n'a été observé lors de l'infection causée par GBS de type IV [421], démontrant que son implication n'est pas universelle.

Contrairement au TLR2, nos résultats *in vitro* indiquent que le TLR4 est peu impliqué dans la reconnaissance de *S. suis.* Bien qu'il ait été suggéré que la production de médiateurs

inflammatoires induite par la suilysine chez les macrophages dépend de sa reconnaissance par le TLR4 [331], nous avons été incapables de reproduire ces résultats à l'aide de DCs, et ce avec des souches de sérotypes 2 et du sérotype 14 (**Article I** & **Article IX**). En effet, nous avons obtenu des résultats similaires entre des souches exprimant ou non la suilysine, lorsque les cellules sont infectées avec la bactérie vivante. De plus, la suilysine recombinante n'induit pas la production d'IL-1β (**Annexes – Article X**). Bien que plusieurs publications aient rapporté que la pneumolysine, qui ressemble à la suilysine, induit la production de médiateurs pro-inflammatoires suite à sa reconnaissance par le TLR4, une étude récente a démontré le contraire lorsque la bactérie entière est présente [422, 423].

En raison des résultats *in vitro*, nous n'étions pas surpris d'observer que le TLR4 ne joue aucun rôle *in vivo*, et ce peu importe le bagage génétique de la souche de sérotype 2 utilisée (**Article IX**). Néanmoins, ces expériences étaient nécessaires pour confirmer les résultats *in vitro*, car, comme nous avons démontré avec certains facteurs de virulence, les résultats obtenus à l'aide d'un seul type cellulaire ne sont pas toujours représentatifs.

Le rôle critique de la signalisation MyD88-dépendante dans la reconnaissance de *S. suis*, mais le rôle partiel du TLR2 et nul du TLR4 indiquent l'implication d'autres TLRs dans la reconnaissance de ce pathogène [155]. Bien que le TLR9 ait été rapporté comme pouvant participer dans sa reconnaissance par les DCs [367], aucune autre information n'était disponible. Nous avons démontré que le TLR 7 et le TLR9 sont nécessaires pour la production d'IFN-β par les DCs (**Article X**). En effet, bien qu'étant un pathogène extracellulaire, un faible pourcentage des bactéries est tout de même internalisé par les cellules. Toutefois, certaines souches, dont les souches ST25 du sérotype 2, sont moins résistantes à la phagocytose et seront par conséquent plus internalisées [424, 425]. Ces bactéries sont dégradées, à la suite de l'acidification de l'endosome, ce qui mène au relâchement des acides nucléiques. Leur reconnaissance par les TLR7 et TLR9, qui se retrouvent dans la membrane des endosomes, mène à la production d'IFN-β. De plus, nous avons démontré que ce mécanisme n'est pas spécifique à l'IFN-β, comme l'ADN et l'ARN de *S. suis* peuvent induire la production d'IL-1β (**Annexes – Article X**). Bien que la production de certains médiateurs pro-inflammatoires induits par *S. suis*, dont l'IL-6 et le CXCL1, sont majoritairement indépendant de la phagocytose (**Article X**), nous démontrons que des mécanismes de reconnaissance intracellulaire phagocytose-dépendant complémentaires à l'activation via les récepteurs associés à la surface

cellulaire permettent à l'hôte de mieux répondre à ce pathogène. Un phénomène similaire a été rapporté pour GAS, GBS et *S. pneumoniae* [17, 338, 350], suggérant une évolution du système immunitaire de l'hôte face à des motifs conservés et partagés par ces pathogènes afin d'optimiser sa réponse lors de l'infection.

L'ensemble de ces résultats *in vitro* et *in vivo* démontre que la reconnaissance de *S. suis* est multifactorielle et implique une panoplie de mécanismes de surface et intracellulaires qui se complémentent et qui permettent à la cellule de répondre le plus adéquatement possible lorsqu'elle fait face à ce pathogène, afin de favoriser son élimination. En effet, le rôle critique que joue MyD88 dans l'inflammation systémique induite par *S. suis*, mais pas via les TLR2 et TLR4, est non seulement surprenant, mais très intéressant, car il contraste avec ceux des autres streptocoques. En effet, le TLR2 joue un rôle important dans l'induction de la réponse inflammatoire induite par GBS et par *S. pneumoniae* et détermine la résultante de l'infection [416, 417]. Cela suggère donc deux possibilités. Premièrement, en plus des TLR2 et TLR4, la réponse implique d'autres TLRs MyD88-dépendants, dont au moins une demi-douzaine d'autres TLRs [155]. Bien que le TLR5 n'ait jamais été décrit comme reconnaissant *S. suis*, les TLR7 et TLR9, tel que décrit plus haut, reconnaissent ses acides nucléiques. De plus la reconnaissance de l'ARN ribosomal de GBS par le TLR13 est importante pour l'induction de médiateurs pro-inflammatoires des macrophages [426]. Ainsi, il est possible qu'un ou plusieurs de ces TLRs MyD88-dépendant soit aussi nécessaires pour la reconnaissance de *S. suis in vivo*. Deuxièmement il est fort possible que la reconnaissance de *S. suis*, contrairement à GBS et à *S. pneumoniae*, ne dépend pas d'un seul TLR, mais d'une collaboration simultanée de plusieurs TLRs. En effet, bien que le TLR13 participe à la reconnaissance de GBS, son rôle est compensé par d'autres TLRs endosomaux [426]. Ainsi, en l'absence de l'un d'entre eux, il est possible que les autres compensent. Il est important de garder en tête qu'*in vivo* tous les types cellulaires sont présents en même temps [13]. L'expression des TLRs varie grandement d'un type cellulaire à l'autre. Par exemple, les macrophages expriment fortement le TLR3, mais faiblement le TLR9, à l'opposé des DCs [427]. Comme seul le rôle des TLRs dans l'activation des DCs et des macrophages a été étudié, nous ne savons pas leur importance pour les autres cellules présentes, dont les monocytes, les neutrophiles, les cellules NK, et les lymphocytes B et T. Ainsi, d'autres études seront nécessaires pour identifier ces récepteurs et les cellules qui les portent avant de mieux comprendre leurs rôles dans la pathogenèse de l'infection systémique causée par *S. suis.*

3.2. Contribution des monocytes et des neutrophiles en tant qu'acteurs cellulaires

Les neutrophiles et les monocytes sont les deux phagocytes les plus importants dans le sang et jouent des rôles dans la réponse inflammatoire systémique, face aux pathogènes bactériens [19]. Les monocytes sont composés de deux sous-types principaux, soit les monocytes inflammatoires et les monocytes patrouilleurs [293]. Malgré leur prédominance en circulation, leurs rôles, n'avaient jamais été investigués auparavant.

Tel que mentionné dans la revue de la littérature, la survie et la différenciation des monocytes patrouilleurs nécessitent le facteur de transcription Nr4a1 [294]. À l'aide de souris déficiente pour celui-ci, nous avons démontré que les monocytes patrouilleurs ne contribuent peu ou pas à l'infection systémique par *S. suis* (**Article XI**). Étant traditionnellement associés à des fonctions de patrouille, de réparation tissulaire et de fonctions homéostatiques [18, 300], ces résultats n'étaient pas particulièrement surprenants. Toutefois, il existe présentement très peu d'information sur leur rôle lors d'infections bactériennes. En effet, ils ont été surtout étudiés dans le contexte du cancer et des maladies inflammatoires non-infectieuses [300]. Cependant comme ils supportent la survie de *Porphyromonas gingivalis* et la résorption osseuse [428], il était tout de même important d'étudier leur rôle afin d'évaluer leur contribution potentielle.

À l'opposé, les monocytes inflammatoires et les neutrophiles sont requis pour la survie de l'hôte lors de l'infection systémique induite par *S. suis* (**Article XI**). Ceci est plus marqué pour les neutrophiles que pour les monocytes inflammatoires. En effet, ces deux types cellulaires participent à la réponse inflammatoire qui est nécessaire à l'élimination de *S. suis* de la circulation sanguine. Ainsi, les médiateurs pro-inflammatoires qu'ils produisent vont promouvoir l'activation des mécanismes de phagocytose et de « killing », que peuvent effectuer ces mêmes cellules ou d'autres phagocytes. En effet, la diminution d'IFN-γ en leur absence va réduire l'activation des macrophages, qui est critique pour augmenter leur capacité à effectuer la phagocytose [293]. Cela indique aussi que les neutrophiles et les monocytes inflammatoires participent à l'activation directe ou indirecte des cellules NK, qui sont la source majoritaire d'IFN-γ lors des premières heures de l'infection à *S. suis* [352, 429]. De plus, les monocytes inflammatoires et les neutrophiles participent à leur propre chimiotaxie, en raison de la réduction des niveaux plasmatiques des nombreuses chimiokines induites.

La capacité des monocytes inflammatoires à produire des médiateurs pro-inflammatoires à la suite de l'infection à *S. suis* concorde avec une étude précédente ayant rapporté la production de TNF et de CCL2 par les monocytes humains THP-1 [100]. Toutefois, nos résultats sont les premiers à démontrer un rôle des monocytes inflammatoires et des neutrophiles *in vivo*, et le tout premier en ce qui concerne les neutrophiles. De plus, ces résultats se rapportent à ceux obtenus avec d'autres streptocoques. En effet, les monocytes inflammatoires jouent un rôle bénéfique dans le cadre de l'infection à GAS en participant à l'élimination des bactéries par des mécanismes inconnus [430], tandis que les neutrophiles sont nécessaires pour le contrôle de la charge bactérienne lors des infections à GBS et *S. pneumoniae* [431, 432]. Toutefois, dans le cadre de ces études, les auteurs n'ont étudié que l'effet de leur absence sur la charge bactérienne et la survie de l'hôte. Leur rôle sur la réponse inflammatoire induite n'a pas été étudié. Ainsi, notre étude apporte une information manquante dans la littérature sur les infections causées par les streptocoques.

Comme très peu d'information est disponible sur le rôle des différentes cellules de l'immunité innée lors de l'infection à *S. suis*, ces résultats nous permettent de mieux comprendre sa pathogenèse. En effet, outre cette étude, seul le rôle des cellules NK a été investigué à ce jour. Tout comme les monocytes inflammatoires et les neutrophiles, les cellules NK participent à la réponse inflammatoire systémique, mais pas à l'élimination du pathogène. Ceci indique que leur rôle est probablement plus limité à l'activation d'autres types cellulaires impliqués dans la phagocytose et le « killing ». Ainsi, il sera nécessaire d'effectuer d'autres études portant sur les différents types cellulaires afin de mieux comprendre l'infection causée par *S. suis.*

En plus de leur rôle dans la modulation de la réponse inflammatoire, nous avons aussi démontré que les neutrophiles participent directement à l'élimination de *S. suis* (**Article XI**). En effet, dès 6 h post-infection, une bactériémie plus élevée a été mesurée chez les souris déficientes en neutrophiles. Comme la réponse inflammatoire systémique induite par *S. suis* atteint son maximum à environ 12 h post-infection [102, 286], la réponse inflammatoire n'a pas eu le temps d'activer de manière optimale les mécanismes responsables de la phagocytose à 6 h post-infection. Cela ne veut pas dire qu'il n'y a aucune activation cellulaire, mais suggère que les neutrophiles participent directement au « killing » de *S. suis.* En effet, bien que la CPS confère d'importantes propriétés anti-phagocytaires, des études *in vitro* ont démontré que les neutrophiles sont les phagocytes sanguins les plus efficaces pour tuer *S. suis* [90, 91].

3.3. Modulation de l'immunité innée et de la réponse inflammatoire par les médiateurs qu'induisent *Streptococcus suis*

La réponse inflammatoire systémique qu'induit *S. suis* est composée d'une panoplie de cytokines et chimiokines pro-inflammatoires (**Article VIII**) [102, 286]. Leur production mène à l'activation des différentes cellules de l'hôte, ce qui cause une cascade d'amplification pouvant avoir pour conséquence l'exacerbation de la réponse inflammatoire et la mort de l'hôte. Toutefois, une quantité équilibrée de ces médiateurs est nécessaire au maintien de l'homéostasie et à combattre l'infection, car leur absence permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes. Nous savons que le TNF est la première cytokine à être produite, suivie rapidement par l'IL-1β. Ensemble, elles vont mener à l'activation de la cascade pro-inflammatoire, et donc à la production d'IL-6, de CXCL8 et d'IL-12p70, en plus de diverses chimiokines [340]. Malgré ces connaissances, le rôle de ces médiateurs, sur le développement de l'infection à *S. suis* et sur la modulation de la réponse inflammatoire, n'a pas été étudié auparavant *in vivo*.

Dans le cadre de cette thèse, nous nous sommes principalement intéressés à l'IL-1β et à l'IFN-β (**Article X** & **Annexes – Article X**). Bien que les mécanismes cellulaires impliqués dans leur production par les DCs infectées avec *S. suis* varient, ces deux médiateurs ont des effets pléiotropiques sur la réponse inflammatoire. Nous avons démontré qu'ils jouent des rôles bénéfiques pour l'hôte en participant à l'amplification de la réponse inflammatoire nécessaire au contrôle et à l'élimination de *S. suis* du compartiment systémique, car en leur absence les animaux succombent à une bactériémie non-contrôlée. Fait intéressant, l'IL-1β induite par *S. suis* n'est pas nécessaire pour sa propre régulation (absence d'une boucle de rétrorégulation), suggérant que les niveaux atteints lors des premières étapes sont suffisants pour activer la suite de la cascade.

Comme ces résultats sont similaires à ceux obtenus avec GAS, GBS et *S. pneumoniae* [15-17, 433-435], cela suggère que l'activation et l'amplification de la cascade inflammatoire est bénéfique pour combattre les infections par ces bactéries. De plus, l'absence de TNF, lors des infections à GAS et *S. pneumoniae,* cause des effets similaires et une survie réduite de l'hôte [430, 436]. Ainsi, il semblerait que l'absence d'un médiateur de la cascade inflammatoire ou de sa signalisation a pour conséquence une abrogation de celle-ci et une susceptibilité accrue de

l'hôte face à l'infection. Cela suggère que la cascade inflammatoire est bénéfique pour l'hôte, bien qu'elle soit aussi responsable de la mort de l'hôte lors de son exacerbation.

3.4. L'équilibre précaire de la balance inflammatoire lors de l'infection systémique

Les résultats obtenus dans le cadre de cette thèse réitèrent que l'inflammation exacerbée, induite par une présence bactérienne non-contrôlée, est responsable de la mort de l'hôte, lors de l'infection systémique causée par *S. suis* (**Article V** & **Article VII**) [102, 286]. Cependant, les résultats obtenus en absence de la signalisation MyD88-dépendante, de monocytes inflammatoires, de neutrophiles, d'IL-1β, ou d'IFN-β démontrent qu'ils ont tous un rôle bénéfique. En effet, ils contribuent à la survie de l'hôte via leurs rôles dans la réponse inflammatoire nécessaire à l'élimination de *S. suis* (**Article X**, **Article XI**, **Article XII** & **Annexes – Article X**). Ainsi, ces résultats paraissent contradictoires à première vue. Toutefois, il faut aussi prendre en compte qu'ils participent simultanément à l'exacerbation de l'inflammation, qui peut être bénéfique pour combattre l'infection ou contribuer à la mort de l'hôte. En effet, lorsque présents (c'est-à-dire chez des souris de type sauvage par exemple), les animaux meurent tout de même, probablement d'inflammation exacerbée, mais ceci reste à être évalué à l'aide d'un traitement anti-TNF, par exemple.

Ainsi, la réponse inflammatoire systémique est un équilibre précaire. D'un côté, trop peu d'inflammation mène à une réplication bactérienne non-contrôlée responsable de dommages directs à l'hôte via la sécrétion de composants toxiques, entre autres, dont la suilysine. De l'autre côté, l'inflammation exacerbée cause des dommages tissulaires et une dysfonction multiorganes menant au choc septique (**Figure 11**). Par ce fait, il existe un seuil correspondant à l'inflammation contrôlée requise pour que l'hôte puisse combattre l'infection, sans se causer de dommages importants. Ceci démontre que la balance inflammatoire est délicate, mais qu'elle est la clé pour les traitements lors de l'infection par ce pathogène.

De plus, cette balance inflammatoire explique parfaitement pourquoi une souche virulente cause plus de mortalité qu'une souche moins virulente. Lors de l'infection causée par les souches ST7 de sérotype 2 (responsables des éclosions humaines en Chine), il est suggéré que l'inflammation induite dépasse toujours ce seuil d'équilibre, en raison de ses propriétés intrinsèques la rendant plus virulente [61, 437]. En effet, ces souches possèdent un îlot de pathogénicité de 89 K codant pour une panoplie de facteurs de virulence qui sont responsables

de la suractivation des cellules de l'immunité innée [438, 439]. C'est pour cela que l'absence d'un seul médiateur, que ce soit l'IL-1β ou l'IFN-β par exemple, n'est pas suffisante pour freiner l'exacerbation de la réponse inflammatoire qu'elle induit (**Article** X & **Annexes – Article X)**. De plus, la charge bactérienne, relativement similaire entre les animaux de type sauvage infectés avec des souches virulentes ou très virulentes indique que l'exacerbation de l'inflammation n'est pas due à une présence excessive de bactéries dans le compartiment systémique, mais plutôt à une virulence plus élevée de la souche. Comme mentionné précédemment, ceci indique que les composants conservés et partagés entre les différentes souches sont responsables de l'activation initiale de la réponse immunitaire innée, mais que les facteurs de virulence spécifiques modulent cette réponse. Il est toutefois impossible d'exclure que l'inflammation seule et non les dommages tissulaires directement causés par la bactérie sont responsables de la mort de l'hôte. Cet aspect devra donc être évalué dans des études futures.

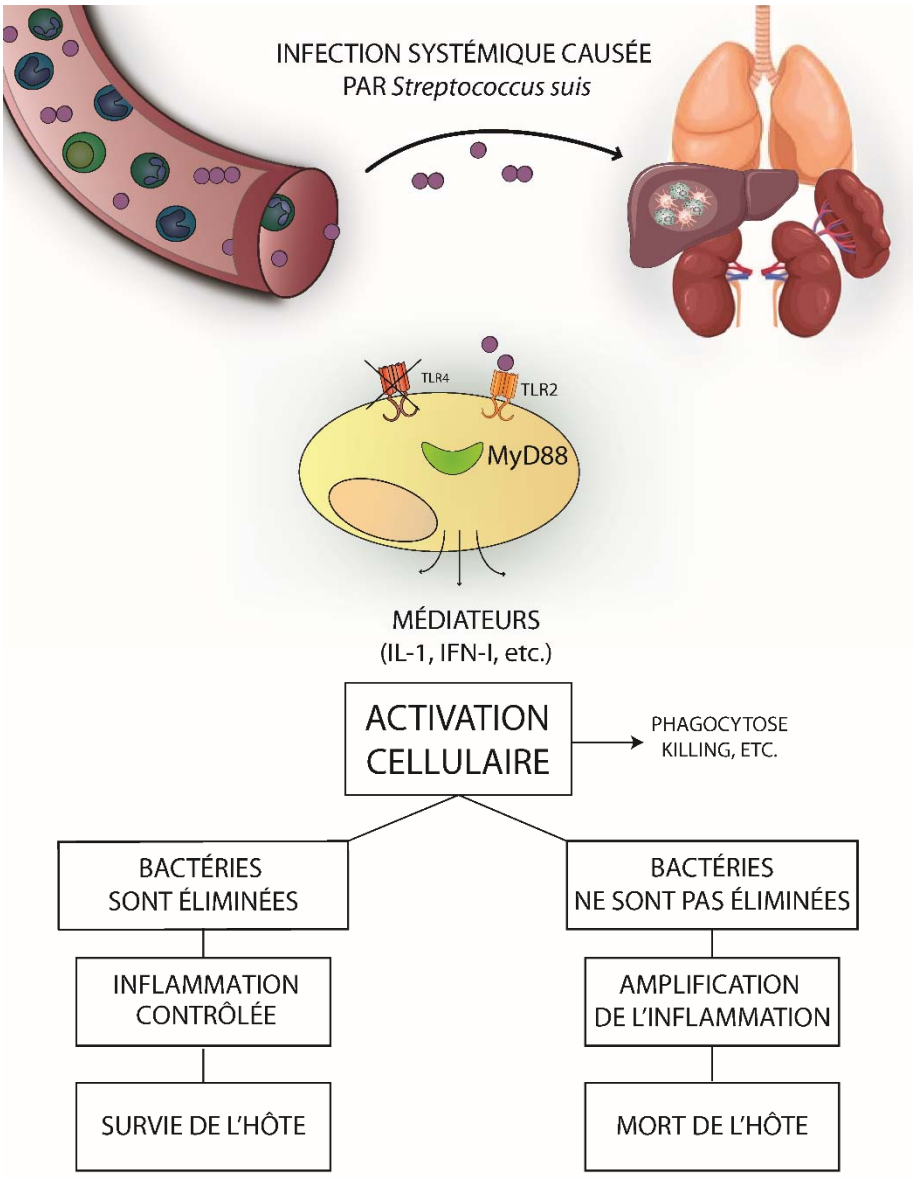

Figure 11. Schématisation de l'infection systémique causée par *Streptococcus suis* et de la réponse inflammatoire induite lors de celle-ci. Lorsque dans la circulation sanguine, *S. suis* va activer les cellules immunitaires, dont les monocytes et les neutrophiles. Leur activation, en partie par le TLR2, mais pas par le TLR4, mène au recrutement de MyD88 et à la production d'une réponse inflammatoire composée de divers médiateurs-pro-inflammatoires, dont l'IL-1 et les IFN de type I. Cette inflammation va activer des mécanismes de phagocytose et de « killing » nécessaire à l'élimination de la bactérie. L'élimination rapide de *S. suis* va favoriser la survie de l'hôte. Au contraire, la persistance de la bactérie entraînera une amplification exacerbée de la réponse inflammatoire, ce qui causera la mort de l'hôte.

4. La caractérisation de l'infection et de la réponse inflammatoire causées par différents *Streptococcus suis* au niveau du système nerveux central

Si l'individu survit à l'infection systémique, il est susceptible de développer une infection du SNC caractérisée par une méningite et/ou une méningoencéphalite. Bien que la méningite soit la pathologie la plus importante lors de l'infection à *S. suis,* chez le porc et chez l'humain, très peu d'information était disponible concernant celle-ci au début de cette thèse. Comme lors de l'infection systémique, il faut garder en tête que bien que l'inflammation exacerbée induite par *S. suis* soit responsable de la mort de l'hôte, au contraire, l'absence d'inflammation permet à la bactérie de se répliquer de manière non-contrôlée, causant ainsi des dommages directs aux organes. Ainsi, nous avons caractérisé la réponse inflammatoire lors de cette infection, en plus d'avoir déterminé le rôle de la signalisation MyD88-dépendante dans la reconnaissance de *S. suis* et la contribution des monocytes et des neutrophiles infiltrants à l'aide de deux modèles de méningoencéphalite chez des souris C57BL/6 (voie hématogène et voie intracisternale).

4.1. Caractérisation de la réponse inflammatoire locale

4.1.1. Importance de la bactériémie persistante

Plusieurs facteurs ont été rapportés comme nécessaires pour le développement de la méningite bactérienne causée par des pathogènes humains, dont une bactériémie persistante [108, 111]. Toutefois, ce facteur n'avait jamais été étudié pour *S. suis*. À l'aide de souches de sérotype 2 de virulences et de types alléliques différents, nous avons démontré que la bactériémie persistante est, en effet, un prérequis pour le développement de méningite à *S. suis* (**Article VIII**). Ainsi, si l'hôte est capable d'éliminer rapidement les bactéries de la circulation sanguine, le risque de développer une méningite est grandement réduit. Ceci est important, car la méningite est très difficile à traiter. De plus, chez les individus survivant à la méningite, le développement de séquelles, telles que la perte auditive, est fréquent [1, 287].

4.1.2. La réponse pro-inflammatoire

Bien que la méningite induite par *S. suis* soit due, du moins en partie, à une inflammation localisée, celle-ci n'avait jamais été caractérisée auparavant. En mesurant les protéines présentes dans les broyats de cerveau de souris infectées, nous avons démontré que la réponse inflammatoire locale lors de l'infection du SNC par *S. suis* est composée de différents médiateurs pro-inflammatoires. En effet, une forte production d'IL-1β et d'IL-6 a pu être

mesurée, de même que de CCL2, de CCL3, de CXCL1, de CXCL2 et de CXCL10, avec des niveaux semblables entre les souches virulentes de sérotype 2 étudiées, peu importe leur origine (**Article VIII** & **Article IX**). La production de ces médiateurs est très rapide et résulte en des niveaux excessivement élevés. En effet, dès 6 h post-infection (voie intracisternale), les niveaux des différents médiateurs mesurés sont équivalents à ceux retrouvés dans le SNC de souris infectées avec *S. pneumoniae,* présentant des signes cliniques de méningite [299]. De plus les niveaux induits par *S. suis* en présence de signes cliniques sont environ cinq cents fois plus élevés que ceux retrouvés lors de la méningite à *E. coli* K1 pour une dose similaire [392].

Malgré la production élevée d'IL-1β, nous n'avons pas détecté de production d'IL-18 (donnée non-publiée), normalement associée avec cette dernière en raison de mécanismes de maturation similaires. Ceci pourrait suggérer que la production d'IL-1β, induit par *S. suis* dans le SNC, est indépendante des voies impliquant les inflammasomes, car le clivage par la caspase-1, activée suite à l'assemblage des inflammasomes, mène aussi à la maturation d'IL-18 [440]. Bien que nous ayons démontré que la production d'IL-1β par les DCs, à la suite de l'infection par *S. suis,* nécessite la présence de différents inflammasomes pour sa maturation (**Annexes – Article X**), les types cellulaires présents dans le SNC varient grandement du compartiment systémique [25]. Toutefois, les voies indépendantes de l'inflammasome ont été très peu caractérisées à ce jour, la mieux connue étant le clivage par la protéinase-3 retrouvée à la surface des neutrophiles et des monocytes/macrophages, qui sont tous deux présents en grands nombres lors de la méningite causeé par *S. suis* [440]. Il a été rapporté que la production d'IL-1β par les cellules de la microglie, lors de la méningite à *S. pneumoniae*, nécessite l'inflammasome NLRP3 [394]. De manière intéressante, nous avons aussi constaté l'absence d'IFN-γ (donnée non-publiée), qui est induit par la production d'IL-18 [441]. En effet, la production d'IFN-γ lors de l'infection à *S. pneumoniae* est dépendante des inflammasomes [442, 443]. Finalement, nous n'avons pas détecté de TNF ou d'IL-12p70 (données non-publiées), toutes deux associées avec une réponse Th1 [441]. L'absence de TNF est surprenante étant donné que ce médiateur joue un rôle important lors de la méningite à *S. pneumoniae* et cause des dommages tissulaires, dont la mort neuronale [444]. Toutefois, il est impossible d'exclure que ces résultats sont dus à un biais méthodologique.

Les niveaux élevés des différentes chimiokines dans le SNC, lors de l'infection à *S. suis,* pourraient expliquer l'infiltration massive de monocytes et de neutrophiles de la circulation sanguine, qui peuvent à leur tour contribuer à leur production (boucle d'amplifcation). En effet, la chimiokine CCL2 est nécessaire au recrutement des monocytes inflammatoires. Au contraire, CCL3 a été rapportée comme participant au recrutement des neutrophiles au SNC lors des infections à *H. influenzae* type b et *S. pneumoniae* [445, 446], tandis que l'absence de CXCL1 et de CXCL2 a grandement réduit leur recrutement dans le SNC, lors de l'infection par *H. influenzae* type b [445].

4.1.3. La compartimentation de la réponse inflammatoire

Une des caractéristiques de la méningite bactérienne est une augmentation de la perméabilité des barrières du SNC [108]. Ceci est également le cas lors de l'infection à *S. suis* [447]. Cependant, même en sa présence, nous avons démontré que les médiateurs pro-inflammatoires du sang et du SNC restent compartimentés (**Article VIII**). C'est la première fois que ce phénomène est rapporté dans le cadre d'une méningite infectieuse, mais ce résultat inattendu pourrait expliquer des particularités de la méningite causée par *S. suis*, dont pourquoi la production de médiateurs pro-inflammatoires est plus importante lors de l'infection à *S. suis,* en comparaison à celle induite par *S. pneumoniae* ou *E. coli* K1 [299, 392]. En effet, bien que les niveaux de médiateurs induient sont similaire globalement, ils ne restent localisés que lors de l'infection à *S. suis*.

De plus, bien que la plupart des souches virulentes du sérotype 2 induisent des niveaux élevés et semblables des différents médiateurs, la souche MNCM43 a induit des niveaux relativement faibles. Toutefois, elle a causé des signes cliniques et des lésions histopathologiques comparables à ceux causés par des souches ayant induit des niveaux beaucoup plus élevés de médiateurs (**Article VIII**). Ces résultats suggèrent donc que le seuil nécessaire pour causer la maladie est moins élevé si les médiateurs inflammatoires restent localisés que s'ils se dispersent.

4.1.4. La susceptibilité du système nerveux central à *Streptococcus suis*

Bien que la quantité de bactéries nécessaires pour induire la méningite à *S. suis* n'a jamais été déterminée, il avait été suggéré que la présence d'une seule bactérie de *H. influenzae* type b serait suffisante [448]. Lors de la méningite causée par *S. suis*, nous avons pu constater que

la quantité de bactéries présentes dans le SNC est relativement la même peu importe la dose inoculée, soit autour de 5 x 10^7 UFC (**Article VIII**, **Article IX** & **Article XI**). Cela suggère une réplication bactérienne rapide (près de mille fois l'inoculum en moins de 24 h) qui atteint une saturation. À l'aide d'inocula différents, nous avons démontré qu'une dose de 10 UFC doit être administrée afin d'éviter une saturation rapide du SNC (**Article IX**). Toutefois, une charge bactérienne d'environ 5 x 10^7 UFC (atteinte entre 36 h et 72 h à la suite de l'inoculation), est tout de même nécessaire pour la présentation de signes cliniques de méningite. Cela indique que le SNC est hautement susceptible à l'infection par *S. suis*. Comme la bactériémie est un prérequis pour le développement de la méningite à *S. suis*, la présence de quelques bactéries sera suffisante pour causer la maladie.

4.2. Rôle de la signalisation MyD88-dépendante et -indépendante dans la reconnaissance de *Streptococcus suis*

En comparaison avec l'infection systémique, encore moins d'information est disponible par rapport au rôle des voies de signalisation lors de l'infection du SNC par *S. suis*. En effet, il avait été rapporté que la production de TNF et d'IL-6 ou de CCL2 par la lignée de cellules de la microglie BV-2 et par des astrocytes murins dépend en partie du TLR2 [395]. De plus, l'expression du TLR2, mais pas du TLR4, augmente dans différentes régions du cerveau lors de l'infection à *S. suis* et est associée à la microglie [286]. Toutefois, le rôle *in vivo* de cette signalisation était inconnu au début de cette thèse.

Nous avons démontré que l'absence de MyD88 n'affecte pas le développement de la maladie du SNC par des souches virulentes de *S. suis*, et ce peu importe le ST de sérotype 2 étudié (**Article IX**). Toutefois, il y a une diminution importante (supérieure à 80%) de la production locale de médiateurs pro-inflammatoires, pas de la charge bactérienne dans le cerveau lors de la maladie clinique. De plus, l'utilisation d'une dose très faible (10 UFC) n'a eu aucun effet sur les résultats obtenus. Ceci suggère que l'inflammation MyD88-dépendante est responsable de l'exacerbation de la réponse inflammatoire locale, mais que les voies indépendantes de MyD88 sont suffisantes, à elles seules, pour causer la méningite, même si elles n'induisent que 10% à 20% de l'inflammation totale. Il est toutefois impossible d'exclure que l'inflammation induite est un effet direct de *S. suis* et non un effet indirect suite à la destruction tissulaire par la bactérie elle-même. Des études futures seront donc nécessairement afin de décortiquer la contribution différentielle de ces deux phénomènes.

Les résultats de reconnaissance de *S. suis* dans le SNC suggèrent, comme lors de l'infection systémique, que les motifs bactériens reconnus par les TLRs sont relativement bien conservés entre les souches de *S. suis*. Ces composants immunostimulateurs pourraient fort probablement être les mêmes que dans le compartiment systémique. Bien qu'il n'y ait pas de différence dans la charge bactérienne cérébrale lors de la méningite clinique en absence de MyD88, à des temps précédant le développement de la maladie, nous avons pu constater que MyD88 participe à l'élimination de la bactérie du SNC. Ainsi, l'activation des cellules phagocytaires résidentes à la suite de l'inflammation induite par la voie MyD88-dépendante, joue un rôle déterminant dans le développement de la maladie.

En effet, nous n'avons jamais été capables d'induire la méningite dans un modèle murin à la suite de l'inoculation d'une souche ST28 nord-américaine de faible virulence auparavant, et ce même par voie intracisternale (**Article VIII**). Par contre, en absence de MyD88, les cellules résidentes du SNC sont incapables d'éliminer la souche, qui se réplique rapidement, induisant ainsi une réponse inflammatoire locale qui mène au développement de la méningite. Ainsi, ces résultats démontrent que, bien que la signalisation MyD88-dépendante participe à l'initiation de la réponse inflammatoire nécessaire à l'élimination de *S. suis*, cette réponse n'est efficace que pour des souches de faible virulence.

Cependant, bien que MyD88 soit important pour l'élimination initiale de *S. suis* et qu'il soit impliqué dans l'exacerbation de la réponse inflammatoire, les TLR2 et TLR4 n'y contribuent que minimalement individuellement. Toutefois, puisque l'absence de MyD88 n'affecte pas le développement de la maladie clinique, ces résultats ne sont pas surprenants. À notre connaissance, ceci est la première fois que la voie MyD88-dépendante n'est pas critique pour le développement de la maladie dans le SNC lors d'une infection bactérienne.

Bien que les voies MyD88-indépendantes ont été peu évaluées dans le cadre de la reconnaissance de *S. suis*, des études *in vitro* ont démontré que NOD2 y participe pour les DCs [367]. De plus, la voie NOD2-RIP2 est impliquée dans la réponse inflammatoire du SNC lors des infections à *S. pneumoniae*, à *N. meningitidis* et à *B. burgdorferi* [385, 449]. En effet, NOD2 est exprimé par les cellules de la microglie et par les astrocytes [450]. De plus, bien que les rôles du TLR3 et de la protéine adaptatrice TRIF dans l'inflammation du SNC induite par *S. suis* demeurent inconnus, il a été rapporté que *S. suis* induit l'expression du TLR3 dans le

cerveau de souris infectées [21]. Le TLR3 est exprimé par diverses cellules, dont les BMECs de la BBB, la microglie, les astrocytes et les neurones [450].

Même si nos résultats suggèrent le contraire, différents inflammasomes, dont NLRP3 et AIM2 pourraient aussi y contribuer [451-454]. En effet, l'inflammasome AIM2 joue un rôle critique lors de l'infection du SNC par *S. aureus* [455]. De plus, les cellules des méninges et du plexus choroïdien expriment une panoplie de récepteurs « éboueurs », dont l'activation induit la production de médiateurs inflammatoires [456]. Finalement, le « C-type lectin receptor » SIGN-R1 participe à la phagocytose de *S. pneumoniae* par la microglie, induisant, lui aussi, une réponse inflammatoire [444]. Ainsi, d'autres études seront nécessaires afin d'évaluer le rôle de ces voies lors de l'infection du SNC par *S. suis*.

4.3. Contribution des cellules résidentes du système nerveux central et des cellules infiltrantes

Le SNC est composé de divers types cellulaires, dont la plupart peuvent participer à la réponse inflammatoire locale [25]. De plus, la méningite causée par *S. suis* se caractérise entre autres par une infiltration massive de monocytes et de neutrophiles [22, 286]. Malgré cela, le rôle de ces différentes cellules a été très peu étudié à ce jour. En effet, les études *in vitro* se sont concentrées sur les BMECs qui forment la BBB [112, 117, 149, 191, 457], sur la microglie [125, 395] et sur les astrocytes [126], tandis qu'aucune étude n'a été effectuée *in vivo*.

4.3.1. Les cellules résidentes

Bien que la BBB représente une plus grande surface d'entrée au SNC [111], l'expression rapide de molécules inflammatoires dans le plexus choroïdien, à la suite de l'infection par *S. suis* et l'inflammation souvent observée dans celui-ci chez les porcs, indique que la BCSFB pourrait être une porte d'entrée aussi importante [20, 286].

À l'aide des cellules des leptoméninges humaines (les cellules de la membrane arachnoïdienne et de la pie-mère), nous avons démontré que *S. suis* sérotype 2 interagit avec celles-ci, mais que ces interactions sont généralement indépendantes du ST et varient selon la souche (**Article XII**). Bien que la CPS masque un peu les adhésines impliquées, *S. suis* adhère considérablement à la surface des cellules des méninges. Comme les souches encapsulées adhèrent aux cellules, ces résultats suggèrent que les adhésines impliquées dans les

interactions avec les cellules de méninges diffèrent probablement de celles jouant un rôle au niveau des cellules épithéliales respiratoires ou de l'intestin. Il a été démontré que l'expression des gènes impliqués dans le métabolisme du carbone de *S. suis* diffèrent grandement lorsque présent dans le LCR, en comparaison au sang, suggérant une adaptation du pathogène à cet environnement [458]. Cette capacité d'adhésion de *S. suis* est similaire à celle de GBS types III et V, plus grande que *S. pneumoniae* sérotype 2, *H. influenzae* type b et *E. coli* K1, mais moindre que celle de *N. meningitidis* [378, 459]. Ces différences démontrent que les adhésines responsables varient grandement entre les différentes bactéries causant la méningite.

Toutefois, l'invasion des cellules des méninges est fortement inhibée par la présence de la CPS. Ainsi, comme pour l'épithélium, *S. suis* module probablement la présence et/ou l'épaisseur de sa CPS, afin de pouvoir franchir les méninges. Au contraire de l'épithélium respiratoire, le LCR est un milieu très riche en nutriments [25]. Ainsi, il est non seulement favorable à la réplication de *S. suis*, ce qui pourrait augmenter davantage le nombre de bactéries adhérées et favoriser son invasion, mais aussi à la modulation de sa CPS, puisque sa présence n'est pas critique.

Cependant, ces interactions de *S. suis* avec les cellules des méninges résultent en une faible activation de celles-ci. En effet, à l'exception de CCL5, aucun autre médiateur évalué n'a pu être détecté, et ce uniquement par les souches appartenant au ST1. De manière similaire, GBS et *S. pneumoniae* n'induisent pas la production de médiateurs [378, 459]. Comme *N. meningitidis* et *H. influenzae* induisent la production de médiateurs par ces cellules [376, 378], cela démontre que ce n'est pas une incapacité des cellules à en produire. Il se peut donc que *S. suis* ait développé des mécanismes évitant l'activation de ces cellules et permettre sa persistance dans le LCR pour ensuite favorisier sa dissémination dans le cerveau. Comme les méninges sont le site d'une infiltration massive de cellules périphériques et qu'une forte expression d'IL-1β y a été observée (**Article VIII**) [286], il se peut que ce soit plutôt les macrophages périvasculaires et des méninges qui soient activés par la présence de la bactérie et qui sont responsables de l'inflammation du SAS. De plus, la production de CCL5 des leptoméninges, à la suite de l'infection par des souches ST1 pourrait elle aussi, contribuer à la chimiotaxie des monocytes infiltrants [286, 460].

Si *S. suis* envahit les leptoméninges, la bactérie va se retrouver dans la membrane gliale limitante superficielle. Cette couche de tissu compacte est peuplée d'astrocytes [24]. Bien que certaines interactions de *S. suis* avec des astrocytes aient déjà été rapportées, celles-ci ont été étudiées avec des cellules d'origines murines [126, 127]. À l'aide de cellules humaines, nous avons démontré que *S. suis* adhère à leur surface, mais moindrement qu'aux cellules épithéliales, et qu'il les envahit (**Article XII**). Bien que la présence de CPS inhibe partiellement ces interactions, la plupart des souches peuvent envahir les astrocytes, même lorsqu'encapsulées. Toutefois, la suilysine et la D-alanylation du LTA, mais pas la *N*-désacétylation du peptidoglycane, participent à l'invasion des astrocytes. En effet, des concentrations sous-létales de suilysine augmentent la capacité de *S. suis* sérotype 2 à envahir les cellules épithéliales [461], tandis que le D-alanylation du LTA module l'invasion des BMECs porcines [158]. Un mécanisme particulier que nous avons pu constater est que *S. suis* cause la mort des astrocytes, et ce indépendamment de la présence ou de l'absence de la suilsyine. Cela indique que certains composants de *S. suis*, soit de surface ou sécrétés, sont relativement partagés entre les souches virulentes. Cependant, la capacité des astrocytes humains à produire une réponse inflammatoire à la suite de l'infection à *S. suis* n'a pas été évaluée.

Ces différences entre les astrocytes humains et murins en termes d'invasion et d'internalisation (les astrocytes murins internalisant peu *S. suis*) peuvent être dues à l'hôte à partir duquel les cellules ont été isolées ou à l'utilisation de cellules primaires versus une lignée cellulaire [126, 127]. Comme discuté à la section 2 de la discussion, ces différences peuvent affecter les résultats obtenus. Toutefois, comme il est difficile d'obtenir des astrocytes humains primaires, l'utilisation d'une lignée cellulaire demeure la seule option pour l'instant. L'implication de ces résultats dans le cadre de l'infection du SNC par *S. suis* est illustrée à la **Figure 12**.

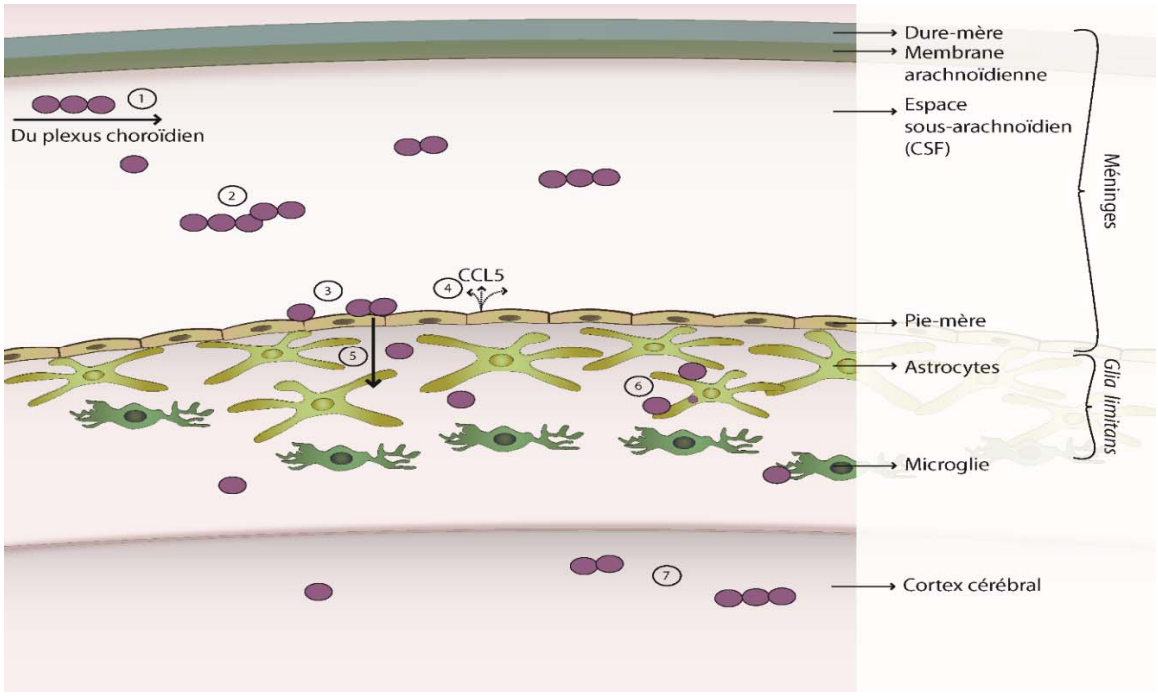

Figure 12. Modèle proposé des interactions de *Streptococcus suis* 2 avec les cellules des méninges et les astrocytes humains. (1 & **2)** Si *S. suis* traverse les cellules épithéliales du plexus choroïdien, la bactérie va se retrouver dans le liquide céphalorachidien (LCR) dans lequel elle pourra se répliquer. **(3)** La bactérie peut aussi adhérer à la surface des cellules des méninges. **(4)** Ces interactions mèneront à une forte induction de CCL5 par les souches ST1. **(5)** À la suite de son adhésion, *S. suis* peut envahir les cellules des méninges en modulant la présence de sa capsule polysaccharidique, atteignant ainsi la membrane gliale limitante superficielle. **(6)** Dans la membrane gliale limitante superficielle, *S. suis* rencontrera des astrocytes, auxquels il peut adhérer puis les envahir et/ou causer la mort cellulaire. **(7)** Après ces évènements, *S. suis* pourra se disséminer dans le cortex cérébral et induire une réponse inflammatoire exacerbée.

4.3.2. Les cellules infiltrantes

Tel que mentionné précédemment, les monocytes et les neutrophiles sont les deux types cellulaires qui infiltrent massivement le SNC lors de la méningite causée par *S. suis* [22, 286]. Bien que l'absence de monocytes inflammatoires ou patrouilleurs ou de neutrophiles n'ait aucun rôle sur le développement de la maladie, les monocytes inflammatoires et les neutrophiles participent tout de même à l'infection (**Article XI**). Au contraire, et comme pour l'infection systémique, les monocytes patrouilleurs semblent ne contribuer que peu (ou pas) à l'infection du SNC. De plus, nous avons démontré que le rôle des monocytes inflammatoires et des neutrophiles varie grandement. En effet, les monocytes inflammatoires participent en partie à la réponse inflammatoire locale, mais pas à l'élimination de la charge bactérienne, que

ce soit à des temps courts ou en présence de signes cliniques. Toutefois, la production de médiateurs pro-inflammatoires en leur absence est réduite d'au maximum 25%, suggérant un rôle minoritaire. Bien que l'absence de monocytes inflammatoires augmente la mortalité à la suite de la méningite par *E. coli* K1 en raison d'une modulation réduite de la réponse inflammatoire [392], ces cellules ne sont pas impliquées dans la méningite causée par *S. pneumoniae* [299].

À l'opposé, les neutrophiles participent à l'élimination de la charge bactérienne du SNC, mais plus tardivement. Nous avons été très surpris de voir que l'absence des neutrophiles ne cause pas une diminution de la réponse inflammatoire locale, mais plutôt une augmentation importante de celle-ci, en particulier des chimiokines CXCL1 et CXCL2 (d'environ 300%), qui sont toutes deux critiques pour leur chimiotaxie. Des résultats similaires ont été obtenus avec une faible dose bactérienne (10 UFC), indiquant que la dose n'a pas d'effet marqué sur leurs rôles. Ceci pourrait suggérer que les neutrophiles ne contribuent pas majoritairement à l'inflammation, ce qui serait plutôt surprenant. Toutefois, nos résultats indiquent que comme les neutrophiles participent à l'élimination de *S. suis* du SNC, l'hôte promeut leur infiltration, bénéfique. Ainsi, les cellules résidentes tentent de les recruter de manière si importante, qu'en leur absence, elles continuent d'amplifier la réponse inflammatoire (boucle de rétrocontrôle négatif) [392]. Il est donc impossible d'exclure un rôle des neutrophiles dans la réponse pro-inflammatoire locale dans ce modèle. En effet, les neutrophiles sont une source importante d'IL-1β [299]. Bien que les neutrophiles contribuent à la réponse inflammatoire locale responsable de l'élimination de la bactérie du SNC lors de la méningite à *S. pneumoniae* [299], un rôle similaire à celui que nous avons obtenu a été rapporté pour *E. coli* : les neutrophiles participent à son élimination du SNC, mais en leur absence, il y a une augmentation de la réponse inflammatoire [392].

En raison de leur rôle important dans son élimination, il a été rapporté que *S. pneumoniae* a développé des mécanismes pour retarder l'infiltration des neutrophiles au SNC. En effet, la production de CXCL2 par la microglie est inversement corrélée à l'expression du CD14 à leur surface [462]. Ainsi, la bactérie induit une augmentation de l'expression du CD14, et réduit la chimiotaxie des neutrophiles. Comme les neutrophiles participent à l'élimination de *S. suis* et que l'expression du CD14 augmente dans les différentes régions du cerveau lors de l'infection à *S. suis* [286], un mécanisme similaire pourrait être en jeu.

L'ensemble de ces résultats suggère que ce sont les cellules résidentes et non les monocytes et les neutrophiles infiltrants, qui sont majoritairement responsables de cette réponse inflammatoire du SNC. Toutefois, comme mentionné ci-dessus, nous ne pouvons pas exclure le rôle des neutrophiles dans notre modèle. En effet, les niveaux élevés de chimiokines seraient responsables pour leur infiltration. Toutefois, les cellules résidentes qui sont impliquées restent inconnues. Nous avons démontré, du moins à l'aide de cellules humaines, que ce ne sont pas les leptoméninges (**Article XII**). Comme mentionné précédemment, la microglie et les astrocytes produisent de l'IL-6, du CCL2 et du CXCL1 à la suite de l'infection par *S. suis in vitro* [125, 126, 395]. Par contre, la seule information que nous avons *in vivo* est que l'expression de CCL2 est associée à la microglie et moindrement aux astrocytes [286]. Une étude récente a démontré que suite à leur infection avec *S. suis*, les astrocytes sécrètent des composés solubles qui activent la microglie et favorisent la production d'oxyde nitrique par cette dernière [127]. D'autres cellules résidentes pouvant jouer un rôle sont les macrophages périvasculaires, ceux des méninges et ceux du plexus choroïdien, de même que les mastocytes. Bien qu'ils soient dispensables pour les défenses de l'hôte lors de la méningite à *S. pneumoniae*, les mastocytes sont une source de médiateurs pro-inflammatoires lors de l'infection à ce pathogène [463].

4.4. La réponse inflammatoire du système nerveux central : Bénéfique, mais à quel point?

Bien que l'hôte induise une réponse inflammatoire locale dans le SNC lors de l'infection à *S. suis*, sont induction est généralement plus néfaste que bénéfique pour sa survie. En effet, et au contraire de l'infection systémique, l'inflammation du SNC n'a pas a être exacerbée pour être dommageable. Bien que la réponse induite se doive d'être équilibrée, il est beaucoup plus difficile de contrôler cette inflammation, car son amplification est très rapide et reste localisée. De plus, la quantité d'inflammation requise pour causer des dommages tissulaires est moindre que dans le compartiment systémique. Ces résultats démontrent que l'infection causée par *S. suis* se divise en deux phases distinctes avec des mécanismes très différents, ce qui rend ce pathogène particulier.

En conclusion, si *S. suis* n'est pas éliminé rapidement à la suite de son arrivée dans le SNC, sa présence va activer les cellules résidentes via des mécanismes MyD88-dépendants. Sa persistance va enclencher des voies MyD88-indépendantes, dont l'inflammation sera

exacerbée par la signalisation MyD88-dépendante. Bien que les monocytes inflammatoires et les neutrophiles infiltrants contribuent à l'infection du SNC, ils sont une conséquence de l'inflammation induite par les cellules résidentes, probablement la microglie, les macrophages et/ou les astrocytes (**Figure 13**). Ainsi, des études subséquentes seront nécessaires afin de mieux décortiquer les mécanismes impliqués.

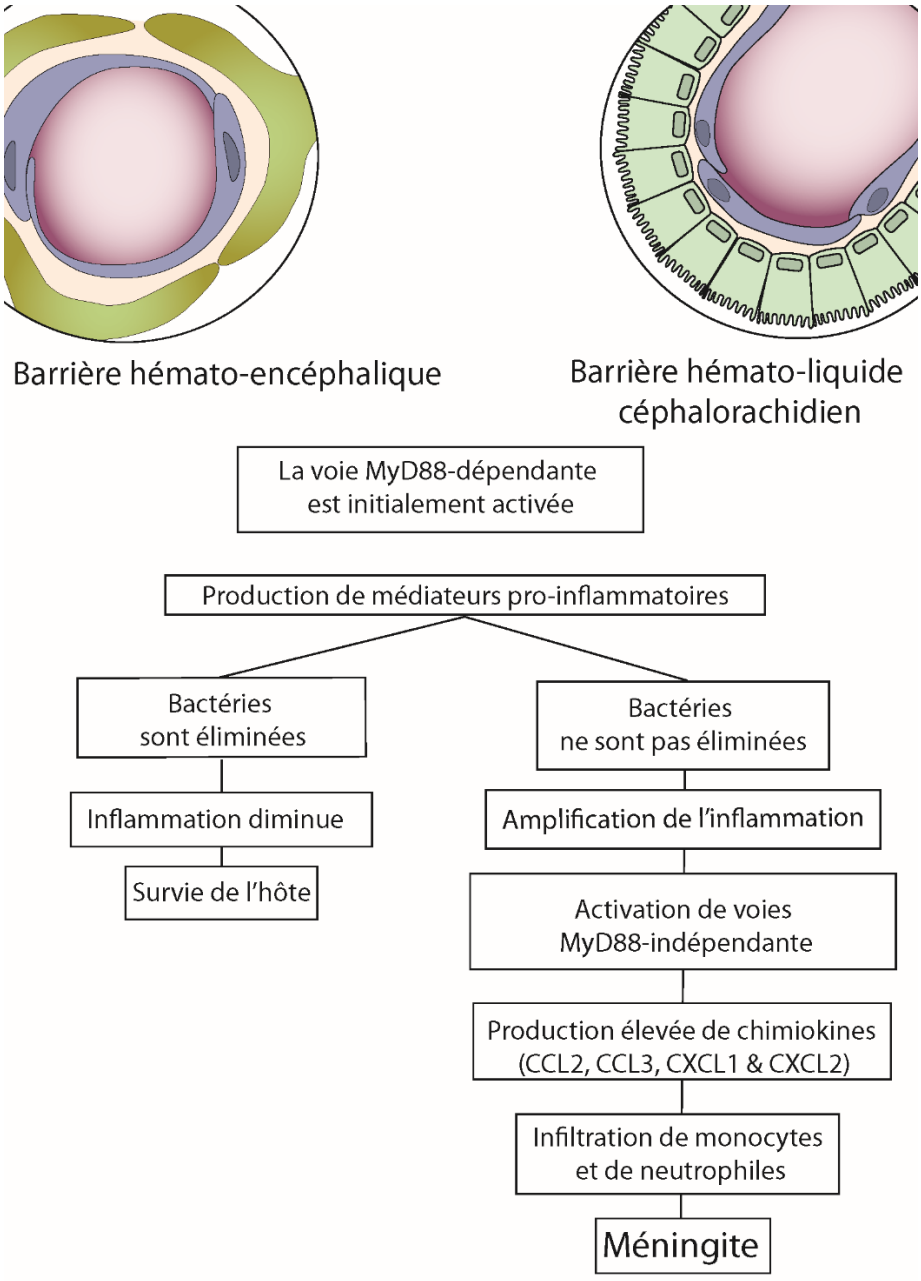

Figure 13. Schématisation de l'infection du système nerveux central causée par *Streptococcus suis* et de la réponse inflammatoire induite lors de celle-ci. *S. suis* envahit le SNC en franchisant soit la BBB ou la BCSFB. Dans le SNC, la bactérie activera initialement le signalisation MyD88-dépendante, ce qui mènera à la production de médiateurs pro-inflammatoires. L'élimination rapide de la bactérie favorisera la survie de l'hôte. Cependant, si la bactérie persiste, il y aura amplification de la réponse inflammatoire par des voies MyD88-dépendante et -indépendante. Cela aura pour effet la production élevée de médiateurs, dont les chimiokines CCL2, CCL3, CXCL1 et CXCL2. Leur production recrutera les monocytes et les neutrophiles, qui infiltreront. L'inflammation exacerbée causera ainsi le développement de la méningite et sera néfaste pour l'hôte.

V. CONCLUSIONS ET PERSPECTIVES

Conclusions générales

Les travaux réalisés dans le cadre de cette thèse ont permis d'approfondir nos connaissances au sujet des composants de *S. suis*, de sa virulence et de sa pathogenèse. Nous avons démontré que les composants de surface, de même que leurs rôles et propriétés, peuvent différer selon l'origine (le sérotype, le ST et le lieu géographique) en raison du bagage génétique et des caractéristiques phénotypiques. En effet, bien que la présence de la CPS soit requise pour le développement de la maladie, elle ne masque pas les composants immunostimulateurs et les adhésines de manière équivalente entre les souches, ce qui a un effet sur les interactions avec les cellules de l'hôte. De plus, l'AgI/II est un facteur de virulence important du sérotype 9, mais pas du sérotype 2, et est impliqué dans sa colonisation et lors de l'infection systémique. Enfin, bien que la composition et la structure du LTA varient, il possède très peu de propriétés immunostimulatrices, au contraire des lipoprotéines.

Nous avons démontré à plusieurs reprises que le choix de la souche et du modèle expérimental peut créer un biais important dans les études de virulence et de pathogenèse et affecter les conclusions. Il est donc primordial de les choisir judicieusement, afin de répondre à la question de recherche. Bien que certains aspects puissent être extrapolés à des souches de *S. suis* d'autres origines, la généralisation des conclusions est imprudente et peut causer de la confusion et/ou de la controverse.

La reconnaissance de *S. suis* par les cellules de l'hôte s'effectue de manière similaire, en raison des composants conservés, et mène à l'induction d'une réponse inflammatoire, à laquelle participe les monocytes et les neutrophiles. Toutefois, l'amplitude de cette réponse sera modulée par le bagage génétique et les caractéristiques phénotypiques de la souche. Les souches non ou peu virulentes seront donc rapidement élimées par l'immunité innée, tandis que les souches plus virulentes exacerberont la réponse induite. Dans le compartiment systémique, la voie des TLRs est requise pour l'induction de cette inflammation. Bien que bénéfique pour l'hôte, l'inflammation doit être équilibrée : trop peu d'inflammation permet à la bactérie de se répliquer excessivement, tandis qu'une inflammation exacerbée cause des dommages tissulaires.

Enfin, nous avons démontré que le SNC est très sensible à l'infection par *S. suis*. De plus, il répond rapidement et agressivement à sa présence par l'entremise d'une réponse inflammatoire exacerbée, qui restera localisée et qui sera, plus souvent qu'autrement, néfaste pour l'hôte. Au contraire du compartiment systémique, la reconnaissance de *S. suis* dans le SNC ne nécessite pas la signalisation MyD88-dépendante. De plus, les monocytes et les neutrophiles infiltrants ne sont pas responsable du déclenchement de la maladie. En effet, leur infiltration est une conséquence des niveaux élevés de chimiokines. Cela indique que ce sont les cellules résidentes qui sont responsables de cette inflammation exacerbée. Toutefois, les leptoméninges ne semblent pas y participer.

Perspectives

Les prochaines étapes de recherche à la suite de cette thèse incluent :

- La création de mutants bien encapsulés, mais n'exprimant pas l'acide sialique dans leur CPS, afin de confirmer l'absence de rôle critique dans les interactions avec les cellules de l'hôte et dans la virulence.
- Confirmer le rôle et les fonctions de l'AgI/II du sérotype 9 observés, à l'aide de souches européennes de virulence plus élevée.
- Étudier le rôle de l'AgI/II lors de l'infection du SNC causée par des souches de sérotype 9.
- Étudier le rôle, les fonctions et les propriétés des lipoprotéines de surface de souches de *S. suis* d'origines différentes.
- Identifier les différences génétiques et phénotypiques responsables des différences de virulences entre les souches nord-américaines et asiatiques appartenant au ST25 et au ST28.
- Étudier le rôle des autres TLRs dans la reconnaissance de *S. suis in vitro* et identifier lequel/lesquels est/sont impliqué(s) dans l'induction de la réponse inflammatoire systémique *in vivo.*
- Étudier le rôle des voies de signalisation MyD88-indépendantes, dont celle de NOD2 et de TRIF, dans la réponse inflammatoire au niveau du SNC, lors de l'infection causée par *S. suis.*
- Déterminer le rôle de la microglie et des macrophages périvasculaires, des méninges et du plexus choroïdien dans la réponse inflammatoire du SNC lors de l'infection causée par *S. suis.*
- Confirmer que l'infiltration massive des monocytes et des neutrophiles dans le SNC, lors de l'infection causée par *S. suis,* est la conséquence des niveaux élevés de chimiokines et non la cause.

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VII. ANNEXES

ANNEXES - ARTICLE I

Streptococcus suis serotype 2 infection impairs IL-12 production and the MHC-II-restricted antigen presentation capacity of dendritic cells

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-premier auteur de l'article. J'ai participé activement à la réalisation des expériences *ex vivo* et de FACS et à l'analyse des résultats. J'ai aussi effectué les corrections lors des étapes de révision pour publication.

Abstract

Streptococcus suis is an important swine pathogen and emerging zoonotic agent. Encapsulated strains of *S. suis* modulate dendritic cell (DC) functions, leading to poorly activated $CD4^+$ T cells. However, the antigen presentation ability of *S. suis*-stimulated DCs has not been investigated yet. In this work, we aimed to characterize the antigen presentation profiles of *S. suis*-stimulated DCs, both *in vitro* and *in vivo.* Upon direct activation *in vitro, S. suis*-stimulated murine bone marrow-derived DCs preserved their antigen capture/processing capacities. However, they showed delayed kinetics of MHC-II expression compared to LPS-stimulated bmDCs. Meanwhile, splenic DCs from infected mice exhibited a compromised MHC-II expression, despite an appropriate expression of maturation markers. To identify potential interfering mechanisms, CIITA and MARCH1/8 transcription was studied. *S. suis*-stimulated DCs maintained low levels of *Ciita* at early time points, both *in vitro* and *in vivo*, which could limit their ability to increase MHC-II synthesis. *S. suis*-stimulated DCs also displayed sustained/upregulated levels of *March1/8*, thus possibly leading to MHC-II lysosomal degradation. The bacterial capsular polysaccharide played a partial role in this modulation. Finally, IL-12p70 production was inhibited in splenic DCs from infected mice, a profile compatible with DC indirect activation by pro-inflammatory compounds. Consequently, these cells induced lower levels of IL-2 and TNF in an antigen-specific $CD4^+$ T cell presentation assay and blunted T cell CD25 expression. It remains unclear at this stage whether these phenotypical and transcriptional modulations observed in response to *S. suis* in *in vivo* infections are part of a bacterial immune evasion strategy or rather a feature common to systemic inflammatory response-inducing agents. However, it appears that the MHC-II-restricted antigen presentation and Th1-polarizing cytokine production capacities of DCs are impaired during *S. suis* infection. This study highlights the potential consequences of inflammation on the type and magnitude of the immune response elicited by a pathogen.

Introduction

Streptococcus suis is one of the most important bacterial pathogens in pigs causing meningitis, septicemia, and sudden death [1]. It is responsible for major economic losses to the swine industry worldwide, and yet there is currently no real effective vaccine available to control infections caused by this bacterium [2]. *S. suis* is also an emerging zoonotic agent that can cause meningitis and septicemia. High mortality rates have been observed in humans, particularly in cases of streptococcal toxic shock-like syndrome (STSLS) in Asia [1]. Similarly, mice infected with *S. suis* have been shown to develop a strong systemic inflammatory response within 6 hours post-infection, and septicemia leading to death within 48 hours [3-5]. *S. suis* is an encapsulated bacterium, and a total of 35 serotypes have been defined based on the antigenicity of their capsular polysaccharides (CPS) [2]. Serotype 2 is the most virulent for both pigs and humans, and most studies have been performed with this serotype [1]. *S. suis* possesses several virulence factors [6], among which the CPS is clearly critical for the pathogenesis of *S. suis* infections [7].

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs); they connect innate and adaptive immunity [8, 9]. During an infection, DC maturation can be initiated indirectly by inflammatory mediators released by innate immune cells (indirectly activated mature DCs; indir-mDCs) or through direct contact with the pathogen (directly activated mature DCs; dir-mDCs) [10]. In both instances, DC maturation is characterized by the expression of cell surface molecules, particularly the MHC class II (MHC-II) molecules and costimulatory molecules, such as CD86 [10, 11]. DCs that have captured a pathogen then process it and load its derived antigenic peptides on their MHC-II molecules [12], forming peptide-MHC-II complexes (pMHC-II) that will be exported from the endosomal peptide-loading compartments to the cell surface [12, 13]. The whole process is usually complete within 1 to 3 h [14]. These pMHC-II will then be recognized by an antigen-specific T cell receptor (TCR) [15, 16]. Specific pMHC-II recognition is the first signal for $CD4^+$ T cell activation and is essential for the induction of the adaptive response [17]. The second signal determines the ability of the antigen-specific $CD4^+$ T cell to expand, and involves binding of the costimulatory molecules on the naive T cell [17, 18]. Finally, the third signal for $CD4^+$ T cell activation is conveyed by DC-derived cytokines that will induce T cell polarization toward different $CD4^+$ T helper lineages with distinct effector functions [18,

19]. Host protection against infections caused by *S. suis* is mediated primarily by opsonophagocytosis, a process favoured by type 1 IgG subclasses. These antibody subclasses with high protective potential are mainly associated with Th1-type immune responses [2]. Interleukin (IL)-12 is known as the primary cytokine for differentiation of the Th1 subset [20]. However, indir-mDCs do not secrete IL-12 in situations where dir-mDCs do and are thus unable to induce functional T cell responses [20, 21].

Different antigenic peptides can be loaded either on newly synthesized or on recycling MHC-II molecules [14]. MHC-II transcription is tightly regulated by the Class II Major Histocompatibility Complex Transactivator (CIITA); this master regulator induces *de novo* transcription of MHC-II genes [13, 21]. Upon exposure to a Toll-like receptor (TLR) ligand, a transient increase in MHC-II synthesis has been observed as early as 1 hour after challenge [14]. However, CIITA transcription (and thus the ensuing MHC-II synthesis) is severely reduced within hours [22, 23], as well as the uptake of new antigens for processing [8, 22]. Independently from CIITA control, MHC-II expression also undergoes regulation at the protein level [13]. The trafficking of MHC-II molecules and their cell surface expression are regulated, among other mechanisms, via ubiquitination by ubiquitin ligases of the membrane-associated RING-CH (MARCH) family, particularly MARCH1 and MARCH8 [11, 13, 15]. In fact, ubiquitination by MARCH1 of the transmembrane glycoproteins MHC-II and CD86 is known to lead to lysosomal degradation of these molecules in immature DCs [11]. However, MARCH1/8 expression is downregulated in dir-mDCs [11, 21, 24]. It has been suggested that, while MARCH1 activity allows the turnover of various pMHC-II in immature DCs, termination of MARCH1 expression in dir-mDCs would considerably prolong the half-life of pMHC-II and CD86, and enhance the stability of pMHC-II derived from the activating pathogen [11]. Such regulation processes would allow the DC to present large and stable amounts of relevant pMHC-II, thereby increasing its ability to activate an antigen-specific $CD4^+$ T cell in an efficient manner [22, 23, 25]. In contrast, indir-mDCs retain their capacity to present new antigens and have a high pMHC-II turnover rate (thus reducing the stability of relevant pMHC-II derived from the pathogen) as they do not downregulate MARCH1 synthesis [26, 27].

S. suis recognition by DCs has been reported to occur essentially through TLR2 [28]. Encapsulated strains of *S. suis* have been shown to modulate DC functions in a variety of ways. First of all, these strains exhibit anti-phagocytic properties in murine, porcine and

human DCs [7, 9, 29, 30]. They also modulate expression of the maturation markers CD80/CD86 and MHC-II in murine and porcine DCs [9, 29] and CD83/CD86 in human DCs [30]. Moreover, encapsulated strains are known to impair cytokine production/release by DCs from all three species; the CPS most probably hinders the recognition of immunogenic cell wall components [9, 29, 30]. However, the antigen presentation ability of *S. suis*-stimulated DCs has never been investigated thoroughly. Interference of *S. suis* with the signals required for antigen presentation in DCs could have profound consequences on the development of the ensuing adaptive response [31]. This could account, at least in part, for the weak $CD4^+$ T cell activation [32], the low primary and memory humoral responses observed in both mice and pigs [2, 33], as well as for some of the difficulties experienced in developing an effective vaccine to control *S. suis* disease in swine [2]. Finally, the low antibody titers obtained against a bystander antigen (ovalbumin, OVA) in *S. suis*-preinfected mice [32], suggest that the antigen presentation machinery is altered. It is hypothesized here that *S. suis* interferes with the ability of DCs to present antigens to $CD4^+$ T cells via the MHC-II pathway, and thus compromises the development of an efficient adaptive immune response. The purpose of the present study was to investigate in an *in vitro*, *in vivo* and *ex vivo* mouse model, the signals involved in antigen presentation, from antigen capture and processing to T cell-polarizing cytokine production, in *S. suis*-stimulated DCs. A nonencapsulated *S. suis* serotype 2 mutant was also included in the study to dissect the role of the CPS regarding the MHC-II pathway.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol number rech-1399).

Bacterial strains

Streptococcus suis serotype 2 virulent strain P1/7 and its isogenic nonencapsulated mutant strain Δ*cpsF* [29] were used. *S. suis* strains were grown on sheep blood agar plates at 37°C for 18 h, and isolated colonies were used as inocula for Todd–Hewitt Broth (THB;

Becton Dickinson, Mississauga, ON, Canada). For *in vitro* experiments, 5 ml of inoculated THB were incubated 16 h at 37 °C with agitation; working cultures were then obtained by inoculating 300 µl of these cultures in 10 ml of THB and incubating for 5 h at 37 °C with agitation. For the *in vivo* experiment, 5 ml of inoculated THB were incubated for 8 h at 37˚C with agitation; working cultures were obtained by inoculating 10 µl of a 10^{-3} dilution of these cultures in 30 ml of THB and incubating for 16 h at 37˚C with agitation. Both bacterial growth protocols were standardized to obtain late-logarithmic bacteria timely synchronized for *in vitro* or *in vivo* infections. Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.3, and appropriately diluted in complete cell culture medium for *in vitro* assays or THB for the *in vivo* experiment. The number of CFU/ml in the final suspension was determined by plating samples onto THB agar using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA).

Mice and CD4⁺ T cell hybridoma

C57BL/6 female mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were used at 5 to 6 weeks of age. The BO97.10 $CD4^+$ T cell hybridoma specific for the $OVA_{323-339}$ peptide on $I\text{-}A^b$ (a kind gift from M. Desjardins) was maintained in Kappler Marrack complete medium, as previously described [34].

Reagents

Anti-mouse antibodies (BD Pharmingen, Mississauga, ON, Canada; unless otherwise noted) used for FACS analysis were as follows: FITC-conjugated anti-CD25 (7D4), Alexa488-conjugated anti-CD11c (N418; BioLegend, San Diego, CA), PE-conjugated anti-$I\text{-}A^b$ (AF6-120.1) and anti-CD86 (GL1), PE/Cy5-conjugated anti-CD3 (145-2C11) and anti-CD11c (N418; BioLegend), and APC-conjugated IL-12p40 (C15.6) and anti-CD11c (HL3).

Generation of mouse bone marrow-derived dendritic cells (bmDCs)

Cells were generated from naive mice as previously described [28]. Briefly, bone marrow was removed from femurs and tibiae. After red blood cell lysis (eBioScience, San Diego, CA), total bone marrow cells (2.5×10^5 cells/ml) were cultured in RPMI 1640 supplemented with 5% heat-inactivated FBS, 10 mM HEPES, 20 µg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine and 50 µM 2-ME (Gibco, Invitrogen, Burlington, ON, Canada). Complete medium was complemented with 20% GM-CSF from

a mouse GM-CSF-transfected cell line (Ag8653) as a source of GM-CSF [35]. Cells were cultured for 7 days at 37°C in a 5% CO_2 incubator, and fresh medium was added on days 3 and 5. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were harvested on day 8, washed, and used as immature DCs for the studies in complete medium containing 5% GM-CSF. Cell purity routinely comprised 86-90% $CD11c^{high}$, as determined by FACS analysis and as previously reported [28].

In vitro *analysis of OVA uptake and processing by bmDCs*

BODIPY dye (DQ-OVA, Molecular Probes, Invitrogen), a self-quenching molecule that is degraded into peptides that exhibit a bright, photostable fluorescence after uptake and intracellular processing, was used as described before to determine cellular internalization and processing of OVA by DCs [36]. Briefly, bmDCs were suspended at 5 x 10^6 cells/ml in RPMI complete medium (without antibiotics) and incubated with *S. suis* strain P1/7 (5 x 10^7 CFU/ml; initial MOI:10) for 45 minutes. Then, bmDCs were washed and incubated with 50 µg/ml of OVA labeled with BODIPY dye or with non-labeled OVA for 15 min at 37 °C in complete medium. After three washing steps with cold PBS to remove non-internalized OVA, cells were suspended in PBS with 5% FBS and incubated at 37 °C (time 0). Processing of OVA into peptides after internalization was assayed at different times ranging from 0 to 60 min by FACS. The culture conditions and concentrations of DQ-OVA were determined based on pretrials (data not shown). Non-infected cells served as negative control.

In vitro *bmDCs stimulation assay*

A total of 1 x 10^6 cells in RPMI complete medium (without antibiotics) were seeded into 24-well flat bottom plates (500 µl/well). Cells were allowed to rest for 1 h at 37˚C with 5% CO_2. Afterward, cells were stimulated in technical duplicates for each condition with *S. suis* strains P1/7 and Δ*cpsF* (1 x 10^6 CFU; initial MOI:1; final volume 1 ml). Conditions used were based on those already published [28]. Cells were harvested after the desired incubation time for FACS and RT-qPCR analysis. Unstimulated cells served as negative control at each time point, while cells treated with the TLR4-ligand lipopolysaccharide (LPS) at 1 µg/ml (from *Escherichia coli* 0127:B8, [Sigma-Aldrich, Oakville, ON, Canada]) were used as positive control at each time point.

***Isolation of splenic DCs from naïve mice and stimulation* in vitro**

Spleen cells were obtained from a pool of 10 naive mice. Spleens were harvested, perfused with RPMI complete medium (Gibco), and pressed gently through a sterile fine wire mesh. After red blood cells lysis (eBioscience), total splenocytes were suspended in 2 mM EDTA-PBS solution and separated using Lympholyte-M density gradient (Cedarlane Labs, Burlington, ON, Canada). Low-density cells at the interphase were isolated, and then further FACS-purified for CD11c+ (APC-conjugated) with the FACSAria Fusion flow sorter (BD Biosciences, San Jose, CA) using "low-recovery high-purity" sorting settings. A purity of >98% was obtained. Cells were allowed to rest for 1 h at 37˚C with 5% CO_2. Afterward, cells were stimulated with *S. suis* strains P1/7 and Δ*cpsF* (initial MOI:1) or LPS at 1 µg/ml for 2 h, 4 h and 18 h.

In vivo *infection model and isolation of splenic DCs*

On the day of the experiment, mice were injected i.p. with 1 ml of the bacterial suspension (5 x 10^7 CFU of *S. suis* strain P1/7) or sterile vehicle solution (THB), as previously standardized in our laboratory [3]. The use of 5 x 10^7 CFU was the optimal dose to induce a positive bacteremia, elicit clinical signs of disease and a strong inflammatory response within 6 hours. Spleens from naive and infected mice were harvested 3 or 6 h postinfection and processed individually (see number of animals in figure legends). Infected mice showing clinical signs of septic disease (e.g. ruffled coat, prostration, and depression) were selected for the experiments. At the time of euthanasia, blood was collected by terminal cardiac puncture and bacteremia (the number of CFU/ml) was determined by plating samples onto THB agar using an Automated Spiral Plater. Infected mice had a positive bacteremia (~ 1-3 x 10^8 CFU/mL of blood) as previously reported [32, 37]. For purification of splenic DCs, spleens (from either naive or infected mice) were harvested and total splenocytes were separated using Lympholyte-M density gradient as described above. Low-density cells at the interphase were purified by MACS positive selection using CD11c MicroBeads and MS columns (Miltenyi Biotec, Auburn, CA) as per the manufacturer's recommendations. The enriched $CD11c^+$ cells had >86-90% purity and similar yield for both naive and infected mice by FACS analysis using CD11c antibody (**Figure 2A**).

Ex vivo *splenic DC stimulation assay*

Splenic DCs from naive or infected mice were isolated at 6 h post-infection as described above. A total of 5 x 10^5 cells in RPMI complete medium with antibiotics (100 µg/ml gentamycin) were seeded into 48-well flat bottom plates (250 µl/well). Cells were allowed to rest for 1 h at 37˚C with 5% CO_2. Afterward, cells were stimulated either with CpG oligodeoxynucleotides at 1 µM (ODN 1826; Invivogen, San Diego, CA) or LPS at 1 µg/ml (final volume 500 µl). Plates were incubated for 24 h, before supernatant harvesting for cytokine quantification. Unstimulated cells served as negative controls or basal expression.

Ex vivo *antigen presentation assay*

Splenic DCs from naive or infected mice were isolated at 6 h post-infection as described above. A suspension of 1 x 10^5 cells/ml in Kappler Marrack complete medium with antibiotics (100 µg/ml gentamycin) were seeded into 48-well flat bottom plates (250 µl/well). Cells were allowed to rest for 1 h at 37˚C with 5% CO_2. Afterward, $CD4^+$ T cells (BO97.10) were added to the wells (DC: T cell ratio of 1: 3, final volume 500 µl) in Kappler Marrack complete medium containing OVA (500 µg/ml; grade VII, Sigma). Cocultures incubated with medium alone served as negative controls, while cocultures treated with LPS (1 µg/ml) and OVA served as positive controls. Coculture plates were incubated for 24 h. The supernatant was harvested for ELISA testing, while cells were collected for FACS analysis. Single cell cultures (either DCs alone or T cells alone) were also included as controls. Culture conditions were based on *in vitro* pretrials (**Figure S1 in Supplementary Material** and data not shown).

FACS analysis

To determine cellular internalization and processing of OVA by DCs, FACS was performed after each incubation time using a Cell Lab Quanta™ SC MPL MultiPlate Loader instrument (Beckman Coulter, Mississauga, ON, Canada). Twenty thousand gated events were acquired per sample. Data are expressed as the increase in the mean fluorescence intensity (MFI) over time and were analyzed using Cell Lab Quanta Collection/Analysis software. For evaluation of MHC-II and CD86 cell surface expression *in vitro*, bmDCs were harvested at each time point, washed and fixed (eBioScience). Cells were then treated with a FcR-blocking reagent (FcγIII/II Rc Ab, BD Pharmingen) for 15 min on ice. Cells were stained with PE/Cy5-conjugated anti-CD11c (45 min on ice), then washed and

stained with either PE-conjugated anti-CD86 or anti-MHC-II I-A^b (45 min on ice). After washing steps, cells were suspended in sorting buffer for FACS analysis. Splenic DCs from the *in vivo* experiment were similarly treated for evaluation of MHC-II and CD86 expression. *In vitro*-stimulated splenic DCs were stained only with PE-conjugated anti-CD86 or anti-MHC-II I-A^b as they had already been stained with APC-conjugated anti-CD11c for FACS sorting. For IL-12p40 intracellular staining, splenic DCs were surface stained with Alexa488-conjugated anti-CD11c (45 min on ice), fixed and permeabilized using the IC Fixation/Permeabilization kit (eBioScience) as per the manufacturer's recommendation. Following fixation and permeabilization, cells were stained (20 min at room temperature) with APC-conjugated anti-IL-12p40. For evaluation of CD25 expression on DC-T cell cocultures, cells were harvested, washed, blocked and surface stained with FITC-conjugated anti-CD25 (45 min on ice), before washing steps and staining with PE/Cy5-conjugated anti-CD3 or APC-conjugated anti-CD11c. For the *in vitro* experiment with splenic DCs, flow cytometry analysis was performed with the FACSAria™ Fusion flow sorter (BD Biosciences) and data was analyzed using the FACSDiva software. For all other experiments, flow cytometry was performed using a BD Accuri™ C6 cytometer (BD Biosciences, Mississauga, ON, Canada). At least thirty thousand gated events were acquired per sample, and data analysis was performed using BD Accuri™ C6 software. Quadrants were drawn based on single stain and isotype controls and were plotted on logarithmic scales.

Quantitative real-time PCR

BmDCs and splenic DCs from the *in vitro* and *in vivo* experiments were washed, transferred in QIAzol (1 ml/10^6 cells; Qiagen, Mississauga, ON, Canada) and kept at -80˚C after each time point. Total cellular RNA was later extracted and quantified by spectrophotometry (Nanodrop ND-1000). Total RNA (500 ng) was reverse-transcribed with the QuantiTect® Reverse Transcription kit (Qiagen) as per the manufacturer's recommendations. The cDNA was amplified using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, Hercules, CA). A real-time thermal cycler CFX96 (Bio-Rad) was used for amplification of target cDNA, and quantitation of differences between the different groups was calculated using the $2^{-\Delta\Delta Ct}$ method. Peptidylprolyl isomerase A (PPIA) was used as the normalizing gene to compensate for potential differences in cDNA amounts. The unstimulated DCs were used as the calibrator reference in the analysis. Each sample was run in triplicates and a no-template control without cDNA was run for every primer set. The

primers used for amplification of the different target cDNA (Integrated DNA Technologies, Coralville, IA) are as follows: CIITA, forward 5'-AACCTGCGCTGACCTCCCGTGTAA-3' and reverse 5'-GCTCCCTTTCCTGGCTCTTGTTGC-3'; MARCH1, forward 5'-CAGATGACCACGAGCGAAAG-3' and reverse 5'-CCAATAGCCACCACAACCAG-3'; MARCH8, 5'-TGGCTTCATGTTGTTCCCTTTATTTC-3' and reverse 5'-CAGCCGTGCCTTGCCAGTC-3'; PPIA, 5'-TGCTGGACCAAACACAAACGGTTC-3' and reverse 5'-CAAAGACCACATGCTTGCCATCCA-3'.

Cytokine quantification by ELISA

IL-12p70 and IL-10 levels in *ex vivo* supernatants, as well as IL-2, TNF, IFN-γ and IL-10 levels in DC-T cell coculture supernatants, were measured by sandwich ELISA using pair-matched antibodies (R&D Systems, Minneapolis, MN) as per the manufacturer's recommendations. Twofold dilutions of recombinant murine cytokines were used to generate the standard curves. Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of the cytokine. Absorbance was measured at 450 nm.

Cytokine and chemokine quantification by Luminex

IL-6, G-CSF, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1ß), CCL5 (RANTES), CXCL2 (MIP-2), and CXCL9 (MIG) levels in *ex vivo* supernatants were determined using a liquid multiarray system (Bio-Plex Pro™) according to the manufacturer's instructions. Commercial multiplex-coated beads (custom-made cytokine panel), biotinylated Abs, and Beadlyte microtiter 96-well filter plates were obtained from Bio-Rad. Data were collected with Bio-Plex Manager™ software and analyzed with Bio-Plex® MAGPIX system. Standard curves for each cytokine and chemokine were obtained using the reference concentrations supplied by the manufacturer.

Statistical analysis

Data for the *in vitro* kinetic experiments were analyzed for significance using a two-way analysis of variance (ANOVA). For the *in vivo* transcription profile of splenic DCs and the coculture experiments, a one-way ANOVA was used. All pairwise comparisons were run, and *p*-values were Bonferroni-adjusted. The FACS data for the *in vivo* experiments and data for the *ex vivo* stimulation experiments were analyzed using Student's unpaired *t*-

test. A $P < 0.05$ was considered as statistically significant. Data are expressed as mean ± SEM. All statistical analyses were performed using the IBM SPSS Statistics software.

Results

S. suis *does not interfere with soluble antigen uptake and processing in bmDCs*

The ability of *S. suis*-stimulated bmDCs to capture and process a reporter antigen linked to a self-quenching molecule, DQ-OVA, was first investigated. Although bmDCs preincubated with *S. suis* tended to express lower mean fluorescence intensity (MFI) of DQ-OVA than unstimulated cells at all time points, there was no statistically significant difference (**Figure S2 in Supplementary Material**). Similar data were obtained when using different DQ-OVA doses, bacterial MOI and incubation times (data not shown). *S. suis*-stimulated bmDCs thus appear to have intact soluble antigen capture and processing capacities.

In vitro *stimulation of bmDCs with the encapsulated strain of* S. suis *induces an enhanced expression of MHC-II, but with a delayed kinetics*

MHC-II cell surface expression (T cell activation signal 1) was investigated in naive and *S. suis*-stimulated bmDCs at 1, 2, 4, 12, and 18 h after stimulation. Moreover, as *S. suis* is an encapsulated bacterium, the impact of CPS on MHC-II expression was evaluated by comparing the wild-type (WT) strain P1/7 with its nonencapsulated mutant, Δ*cpsF*. LPS-treated bmDCs served as positive controls for the expected kinetics of MHC-II expression in bmDCs responding to a TLR ligand (dir-mDCs). While LPS-treated bmDCs significantly increased their MHC-II expression within 1 h, *S. suis*-stimulated cells only reached such high percentages of MHC-II^{+} cells at late time points (**Figure 1**). Overall, the total MFI levels were similar among treatments, but a similar delay in the increase of MFI levels was observed in *S. suis*-stimulated cells when considering the MHC^{low} subpopulation (P1, see **Figure S3 in Supplementary Material**). In fact, bmDCs stimulated with the WT strain showed no significant increase of MHC-II expression at early time points, in contrast to Δ*cpsF*-stimulated cells. A statistically significant difference was observed between the two strains at 4 h, thus suggesting that the presence of the CPS on *S. suis* plays a partial role in the delayed maturation process underwent by *S. suis*-stimulated bmDCs.

S. suis *induces splenic DC maturation in vivo but with a low intensity of MHC-II expression on the cell surface*

MHC-II expression was investigated in purified splenic DCs in an *in vivo* mouse model. Unfortunately, as nonencapsulated *S. suis* mutants are rapidly cleared from circulation [6], *in vivo* investigation of the role of this virulence factor was impossible and the experiment had to be conducted with the WT strain only. Splenic DCs have never been studied in the context of *S. suis* infection; we thus evaluated the maturation profile of these cells during an acute systemic infection, along with their MHC-II expression. Splenic DCs derived from *S. suis*-infected mice showed an increased expression (at both % and MFI levels) of the DC maturation marker CD86 (T cell activation signal 2) compared to naive mice (**Figure 2B**). This suggests that initiation of the DC maturation process occurs quickly in splenic DCs during a systemic infection with *S. suis*. Moreover, the percentage of splenic DCs expressing MHC-II molecules on their cell surface remained elevated after infection and was therefore in agreement with this DC maturation profile (**Figure 2C**). However, DCs from *S. suis*-infected mice showed a lower MFI. This observation could imply that the total number of MHC-II molecules on the surface of each MHC-II^{+} cell was reduced; in spite of the initiation of the DC maturation process, *S. suis* could downregulate the expression of MHC-II molecules on splenic DCs *in vivo*.

BmDCs and splenic DCs stimulated in vitro with **S. suis** *have different maturation profiles compared to splenic DCs from infected mice*

As bmDCs and splenic DCs from infected mice were found to differ in their patterns of MHC-II expression levels we investigated whether these differences were inherent to the cell type or were rather due to complex interactions occurring *in vivo* (i.e. direct vs indirect activation). We stimulated naive splenic DCs *in vitro* and evaluated their MHC-II expression kinetics in response to *S. suis.* All treatments yielded high percentages of MHC-II^{+} and CD86^{+} cells, but analysis of the MHC-II^{high} subpopulation revealed a consistently lower MHC-II MFI for WT *S. suis*-stimulated cells, as compared to cells stimulated with LPS or the nonencapsulated mutant (**Figure 3** and **Figure S4 in Supplementary Material**). This maturation profile is similar to those observed at short incubation times in bmDCs (**Figure 1B**). However, while bmDCs upregulate at late time points their MHC-II and CD86 expression (**Figure 1** and [29]), WT *S. suis*-stimulated splenic DCs still displayed low MHC-II MFI levels as late as 18 h **(Figure 3B**). Splenic DCs stimulated *in vitro* with the WT strain also maintained low CD86 MFI levels (**Figure 3B**),

which contrasts with the maturation profile obtained in splenic DCs from infected mice (**Figure 2B**). These results suggest that, while *S. suis*-DC interactions might partly depend on the cell origin, other complex modulations are also likely to be involved *in vivo*.

S. suis-*stimulated DCs maintain low transcriptional levels of CIITA, both* in vitro *and* in vivo

Given the key role of CIITA in *de novo* transcription of MHC-II genes and its potential implication in the modulation of MHC-II expression by *S. suis*, the transcriptional expression of CIITA was evaluated in *in vitro*-stimulated bmDCs as well as in splenic DCs derived from infected mice. *Ciita* expression was investigated in bmDCs stimulated with the WT strain and its nonencapsulated mutant for 1, 2, or 4 h. Interestingly, *S. suis*-stimulated bmDCs showed globally low levels of *Ciita* for both strains (**Figure 4A**). No significant difference was observed between the WT strain and its nonencapsulated mutant. In contrast, under our assay conditions, LPS-treated bmDCs showed *Ciita* upregulation at 2 and 4 h, as reported previously [23]. The expression of *Ciita* mRNA was also investigated *in vivo* at 3 and 6 h after infection, with splenic DCs derived from *S. suis*-infected mice showing low levels of *Ciita* compared to naive mice (**Figure 5A**). These results might reflect a bacterial strategy to limit the ability of the DC to increase MHC-II synthesis and thus possibly, impair presentation of relevant pathogen-derived peptides.

S. suis-*stimulated DCs upregulate their transcriptional expression of MARCH1 and MARCH8, both in vitro and in vivo, with the CPS playing a partial role in this regulation*

To identify additional mechanisms potentially responsible for MHC-II modulation, transcriptional expression of the ubiquitin ligases MARCH1 and MARCH8 was evaluated. As these molecules are involved in the fate (either lysosomal degradation or recycling) of pMHC-II at the cell surface, these molecules were promising regulatory candidates to investigate. BmDCs were stimulated *in vitro* with *S. suis* strains P1/7 and Δ*cpsF* for 1, 2, or 4 h. LPS-treated bmDCs showed *March1* and *March8* downregulation, as is expected in bmDCs responding to a TLR ligand. Meanwhile, transcription of these genes was poorly downregulated or even upregulated in *S. suis*-stimulated bmDCs (**Figure 4B** and **C**). In fact, incubation with the WT strain led to *March1* levels significantly higher than those observed in LPS-treated bmDCs at all time points. As MARCH1 is known to ubiquitinate the costimulatory molecule CD86 as well as the MHC-II molecules, CD86 cell surface

expression was analyzed by FACS to corroborate these results. *S. suis*-stimulated bmDCs displayed percentages of $CD86^+$ cells that remained similar to those found in unstimulated cells, until at least 4 h, which is thus in agreement with the sustained/upregulated levels of *March1.* Meanwhile, LPS-stimulated cells showed an increased expression of CD86 (**Figure S5 in Supplementary Material**). Similarly, the WT strain induced significantly higher *March8* levels than those obtained with LPS at early time points in bmDCs. As for the role of the CPS, cells stimulated with the nonencapsulated mutant quickly downregulated *March1* and *March8*, and their expression levels became significantly different from those obtained with the WT strain at 2 h (**Figure 4B** and **C**). These results suggest that the presence of the CPS allows *S. suis* to hamper antigen presentation by promoting ubiquitination of MHC-II, and thus lysosomal degradation of these molecules early after infection. *March1* and *March8* expression levels were also investigated *in vivo* at 3 and 6 h after infection. Splenic DCs derived from *S. suis*-infected mice showed sustained levels until at least 3 h for *March1*, and 6 h for *March8* (**Figure 5B** and **C**). These results might support the previous *in vitro* findings or reflect just as well DC indirect activation.

S. suis *infection interferes with IL-12 production in splenic DCs but does not impair other cytokine production*

As Th1 immune responses are known to be protective against *S. suis* infection, the production of IL-12, the key Th1-polarizing signal 3, by splenic DCs was evaluated *in vivo* at 6 h after infection. The p40 subunit of IL-12 (also described as a subunit of IL-23) was detected by IC-FACS. Splenic DCs derived from *S. suis*-infected mice showed significantly higher percentages of $IL\text{-}12p40^+$ cells than naive controls, but their MFI levels were overall reduced (**Figure 6A**). To detect IL-12p70 (the bioactive heterodimeric form), splenic DCs from either naive or infected mice were cultured *ex vivo* with CpG or LPS for 24 h and the supernatants were tested by ELISA. DCs from infected mice failed to produce IL-12p70 in both instances, while their naive counterparts produced significant amounts of this cytokine (**Figure 6B**). Meanwhile, unstimulated DCs, derived from both naive and infected mice, showed no significant production of IL-12p70. Interestingly, IL-10 production remained unchanged in DCs derived from *S. suis*-infected mice in response to CpG or LPS (**Figure 6B**). This thus led to an altered IL-10/IL-12 ratio, as reported previously in *S. suis*-stimulated bmDCs [29]. To confirm that this inhibition was IL-12p70 specific and had no effect on cytokine production in a more general way, we also measured other cytokine

levels by Luminex in these *ex vivo* supernatants. Following stimulation with CpG or LPS, DCs from infected mice were found to release similar or higher amounts of IL-6, G-CSF, CCL2, CCL3, CCL4, CCL5, CXCL2, and CXCL9, as compared to naive DCs (**Figure 7**). Thus, mere cytotoxicity does not seem to be the underlying reason for DC impaired IL-12p70 production. In fact, these data rather suggest that DC exposure to inflammatory compounds during the acute phase of *S. suis* infection modulates the T cell polarizing signal 3.

S. suis *infection impairs the MHC-II-restricted antigen presentation capacity of splenic DCs*

Finally, we analyzed whether *S. suis* infection could interfere with MHC-II-restricted antigen presentation. To this aim, we cocultured for 24 h splenic DCs derived from either naive or infected mice with the T cell hybridoma BO97.10, specific for $OVA_{323–339}$ epitope on $I\text{-}A^b$. The production of IL-2, TNF, IFN-γ, and IL-10 was measured in the supernatants by ELISA, as a way to evaluate $CD4^+$ T cell activation in response to processing and presentation of the $OVA_{323-339}$ peptide by DCs. $CD4^+$ T cells incubated with DCs from infected mice showed significantly lower IL-2 production than $CD4^+$ T cells exposed to naive DCs (**Figure 8**). This diminished $CD4^+$ T cell response was further supported by the reduced expression (at both % and MFI levels) of the CD25 receptor (IL-2 receptor chain) that was observed by FACS on $CD3^+$ cells after 24 h of coculture with DCs from infected mice. Meanwhile, CD25 expression remained unchanged on $CD11c^+$ cells (**Figure 9**). DCs derived from *S. suis*-infected mice also induced lower TNF production by $CD4^+$ T cells as compared to their naive counterparts (**Figure 8**). While *S. suis* infection did not alter the OVA-induced production of IFN-γ, it is interesting to note that these cells also failed to upregulate their IFN-γ production to the levels obtained with the positive control. No increased production of the regulatory cytokine IL-10 was detected in the cocultures, as was observed with the splenic DCs cultured *ex vivo* (**Figure 6B**).

Single cell cultures (either DCs or T cells alone) were included as controls and showed no significant cytokine production. Moreover, the WT strain ability to induce polyclonal $CD4^+$ T cell activation was investigated in *S. suis* infected-cocultures without OVA and was found to be negligible (data not shown). Interestingly, bmDCs infected *in vitro* with *S. suis* (thus yielding dir-mDCs) and cocultured with BO97.10 cells induced no clear difference in OVA-induced IL-2 and TNF levels, compared to non-infected cocultures (**Figure S1 in**

Supplementary Material). Hence, these results suggest that indirect activation of DCs during the acute, pro-inflammatory phase of *S. suis* infection leads to suboptimal $CD4^+$ T cell activation, while direct activation of DCs by *S. suis* itself might not interfere with this process to the same extent.

Discussion

While *S. suis* is known as a potent inducer of proinflammatory mediators during acute/peracute systemic infections [3, 6, 28], evidence of its ability to impair various immune cell functions and prevent the development of an effective adaptive immune response is not less substantial [6, 29, 32]. In this regard, *S. suis* modulation of DC functions have been documented in mice, swine and humans [9, 28-30] and $CD4^+$ T cells were shown to be poorly activated during *S. suis* infection [32]. However, a comprehensive evaluation of the DC antigen presentation ability has yet to be performed. This prompted us to evaluate MHC-II-restricted antigen presentation by DCs, as well as the other signals required for $CD4^+$ T cell activation.

TCR recognition of a specific antigenic peptide presented on a MHC-II molecule constitutes the first signal for $CD4^+$ T cell activation. In order for an extracellular pathogen such as *S. suis* to have its antigenic peptides enter the MHC-II pathway in a DC, it must first be captured and processed by the APC. Therefore, we investigated the ability of *S. suis*-stimulated bmDCs to capture and degrade a soluble protein into peptides, using DQ-OVA. The CPS of *S. suis* is well known for conferring resistance to phagocytosis [7, 29, 38] and it has been shown to inhibit entry of latex beads in macrophages [7]; we thus expected *S. suis*-stimulated DCs to display impaired capture and, consequently processing of OVA. However, our observations led us to conclude that, in DCs, *S. suis* does not significantly affect this phase of antigen presentation. *S. suis* CPS has been reported to block phagocytosis of bacterial size particles (such as latex beads), yet other endocytic pathways might remain unaltered. In fact, macropinocytosis has been described as a far more important endocytic process in DC-mediated antigen presentation to T cells [8]. It should also be noticed that once *S. suis* is internalized (albeit at low levels [9, 39]) it is rapidly degraded intracellularly, thereby supporting the results of normal processing of DQ-OVA.

The MHC-II pathway involves a series of complex intracellular events that eventually lead to the trafficking of pMHC-II to the cell surface. The expression of MHC-II molecules on *S. suis*-stimulated DCs had only been studied thus far at long incubation times (16 h), like a mere cellular maturation marker [9, 29]. However, the events that take place in the first few hours following stimulation appear to be decisive for the antigen repertoire selection, at least for dir-mDCs as these cells process microbial antigens at the time of the TLR stimulus only [27]. Hence, we sought to investigate MHC-II expression in DCs responding to *S. suis* at short incubation times. Unfortunately, due to the lack of immunological tools available for *S. suis,* loading of MHC-II molecules with *S. suis*-derived peptides could not be evaluated directly in the present study, and expression of MHC-II molecules on the cell surface (e.g. independently of the antigenic fragments they bore) was investigated instead. Through comparison with LPS-treated cells, *in vitro* kinetic studies evidenced a delayed increase of MHC-II$^+$ cells in *S. suis*-stimulated bmDCs. Interestingly, this delay appeared to be more pronounced with the encapsulated strain, suggesting a role of the CPS in this modulation by either limiting bacterial internalization and/or hindering the recognition of immunogenic cell wall components by TLRs at the cell surface level [9, 29]. We cannot undermine here the limitations of comparing *S. suis* with a pure TLR4 ligand, as a live pathogen is bound to interact with DCs in a much more complex way than the latter. However, for the sake of comparison with literature, LPS offered interesting perspectives: this ligand has been used extensively in molecular studies of the MHC-II pathway (in contrast to TLR2 ligands), and has also proven to be useful in the context of *S. suis* for normalizing the expression of DC maturation markers [30]. The reduction of the MHC-II expression levels on naïve splenic DCs stimulated *in vitro* with encapsulated *S. suis* or splenic DCs derived from *S. suis*-infected mice supports the relevance of the *in vitro* findings and is consistent with a modulation of MHC-II expression by encapsulated *S. suis*. Moreover, it has been reported that the timing of DC-antigen encounter and the subsequent changes induced by DC maturation can dramatically affect the outcome of vaccination [10].

To get an insight into the mechanisms potentially involved in the modulation of MHC-II expression in *S. suis*-stimulated DCs, transcriptional expression of CIITA, MARCH1, and MARCH8 were evaluated. Low *Ciita* mRNA levels were observed in *S. suis*-stimulated bmDCs and splenic DCs from *S. suis*-infected mice. These results suggest that *S. suis* impairs the ability of DCs to increase synthesis of new MHC-II molecules shortly after they

encounter the pathogen. The fact that newly synthesized MHC-II molecules constitute the primary source for antigen presentation [10] underscores the likelihood that *S. suis* disturbs the optimal timing between processing of pathogen-derived antigens and MHC-II loading/trafficking, through *Ciita* modulation. Similarly, monocytes/macrophages infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis* BCG [40, 41] or *Brucella abortus* [42] were shown to downregulate the expression of IFN-γ-stimulated *Ciita* and MHC-II genes at very early time points in a way to prevent recognition by T cells and establish a chronic infection. Yet, this is the first time that *Ciita* regulation is observed in DCs with an extracellular pathogen. As for the transcription of the ubiquitin ligases MARCH1/8, our results suggest that the encapsulated strain of *S. suis* hijacks MARCH1/8-mediated MHC-II ubiquitination in DCs, thereby promoting lysosomal degradation of these molecules with obvious consequences on antigen presentation to $CD4^+$ T cells. Modulation of *March1* expression and subsequent MHC-II downregulation has already been described in *Francisella tularensis*-infected-macrophages and attributed to a PGE_2-inducible host factor capable of inducing IL-10 [43]. However, it appears unlikely that *S. suis* would use a similar mechanism to modulate *March1* expression in DCs since the effect of IL-10 on antigen presentation is MARCH1-independent in this cell type [11]. Our *in vivo* results support, at least in part, a modulation of *March1/8* expression by *S. suis,* as sustained transcription levels of these molecules were observed in splenic DCs derived from infected mice, without involving IL-10 upregulation. In a murine model of multiple organ dysfunction syndrome (MODS), which is characterized by the loss of control over systemic inflammatory responses, the protein expression levels of MHC-II and functional immune activities of DCs were also inversely related to *March1* expression. The authors suggested that MARCH1-mediated ubiquitination and aberrant degradation of MHC-II on DC surfaces affected the pathological progression of MODS [44].

Modulation of MHC-II expression in *S. suis*-stimulated DCs cannot be analyzed without discussing the maturation profile of the cells. Although a clear and unanimous definition of mature and immunogenic DCs is yet to be determined, phenotypically mature DCs are usually defined, regardless of their activation mode, by their high surface expression of appropriate “maturation markers” such as MHC-II, CD80, CD86, CD40, and CCR7 [20, 21]. BmDCs were most probably activated directly in our *in vitro* experiments, as autocrine production of inflammatory mediators is likely negligible in a closed system at short incubation times. However, the delayed MHC-II kinetics and low CD86 expression we

observed at early time points suggests that *S. suis* failed to induce optimal cellular maturation at those times. Upregulation of *March1/8* in *S. suis*-stimulated bmDCs could be responsible for their impaired MHC-II and CD86 expression, but other regulatory mechanisms must be involved, as bmDCs stimulated with the nonencapsulated mutant still displayed low CD86 expression despite an adequate downregulation of *March1/8.* Meanwhile, splenic DCs from infected mice showed high percentages of $CD86^+$ cells but, low *Ciita* expression and sustained mRNA levels of *March1/8*, with concomitant reduction in expression levels of MHC-II. These last results support our *in vitro* findings, even though splenic DCs form a heterogeneous population of DC subsets with distinct maturation phenotypes, levels of infection, and T cell priming capacities [45]. *S. suis* could also interact differently with DCs of various origins, as *in vitro*-stimulated bmDCs and splenic DCs were found to differ in their MHC-II expression profile at late time points. Finally, our *in vivo* approach possibly yielded both dir-mDCs and indir-mDCs as splenocytes had the opportunity to encounter either the pathogen itself or the inflammatory mediators released by innate immune cells during the acute phase of the infection.

The last signal required for full $CD4^+$ T cell activation is the production of polarizing cytokines by the APC. Host protection against *S. suis* is highly dependent on antibodies associated with Th1 immune responses [2]. However, encapsulated *S. suis* serotype 2 has been shown to induce, in murine and human DCs *in vitro,* a high IL-10/IL-12 ratio, which indicates the potential to polarize T cell responses toward Th2/Treg, or at least to impair optimal T cell activation [29, 30]. Actually, *S. suis* systemic infection induces a weak Th1 response with low levels of TNF, IFN-γ and IL-2 as well as production of IL-10. *S. suis* CPS is known to interfere with $CD4^+$ T cell activation [32]. Here, we found that splenic DCs derived from *S. suis*-infected mice specifically failed to secrete IL-12p70 in response to *ex vivo* stimulation with CpG or LPS, thus revealing that the infection primarily leads, in fact, to indir-mDCs. Consequently, these cells elicited a significant inhibition of IL-2 production by $CD4^+$ T cells in an antigen presentation assay, along with low levels of TNF and IFN-γ. These results were further supported by the downregulated expression of CD25 we observed on $CD3^+$ cells, which reflects inhibition of T cell activation [46] in response to *S. suis* infection. Meanwhile, the fact that $CD11c^+$ cells from infected mice expressed similar levels of CD25 as their naive counterparts might suggest that the infection does not induce regulatory DCs [47, 48] or Th17-polarizing DCs [49]. Nevertheless, the slight increase of $IL\text{-}12p40^+$ cells that we detected by IC-FACS in splenic DCs from infected mice might still

reflect initiation of a Th17 response – rather than representing a bioactive IL-12p70 production – as IL-12p70 shares its subunit p40 with IL-23, a key cytokine for Th17 and Th1 responses against extracellular bacteria [19, 50]. Interestingly, direct activation of porcine or mouse bmDCs by *S. suis* results in increased levels of IL-23 production *in vitro* [29, 51]; once again suggesting possible generation of both dir-mDCs and indir-mDCs during *in vivo S. suis* infection.

Similarly, *M. tuberculosis* have been reported to impair IL-12p70 secretion and antigen presentation to $CD4^+$ T cells, thus diverting T cell differentiation toward Tregs or Th2 cells that are not productive toward an intracellular pathogen like *M. tuberculosis* [52, 53]. Impaired antigen presentation ability of DCs has also been demonstrated for *Salmonella enterica* [54, 55], and *Brucella suis* [56], all intracellular pathogens. Albeit these mechanisms seem to be generalized to several intracellular pathogens, this is the first time that *in vitro* and *in vivo* transcriptional regulation of MHC-II, *ex vivo* DC antigen-presentation and downstream effects on T cell activation are evaluated in the context of an infection with an extracellular pathogen. To the best of our knowledge, only one study has previously reported that bmDCs exposed *in vitro* to live *Streptococcus mutans* fail to drive antigen-specific T-cell proliferation [57], which is different from what was observed with *S. suis* dir-mDCs in this study. Finally, *in vivo* experiments conducted in parallel with another gram-positive encapsulated bacterium, Group B *Streptococcus* (GBS), have shown that both pathogens have similar surface expression profiles of MHC-II and CD86 and transcription levels of antigen presentation genes (unpublished observations); yet GBS-infected mice developed optimal primary and memory T cell responses [58]. It thus remains to further investigate whether the observed modulation of IL-12p70 is a feature common to systemic inflammatory response-inducing agents, a particular bacterial immune evasion strategy, or even a combination of both mechanisms.

This work offers a comprehensive study of the signals involved in antigen presentation in DCs responding to *S. suis,* and their consequences on $CD4^+$ T cell activation. We have shown that *S. suis* modulates MHC-II expression, both *in vitro* and *in vivo*. *S. suis* CPS was found to be only partly accountable for these transcriptional profiles. In fact, a variety of factors defining the strain pathogenicity might be involved in the modulation of the APC functions by systemic inflammatory response-inducing pathogens. Nevertheless, this is the first report of an *in vivo* transcriptional kinetics study of CIITA and MARCH1/8 in DCs

responding to a live bacterial pathogen. More importantly, we described, in splenic DCs from infected mice, a cytokine secretion profile that is compatible with indirect activation by inflammatory mediators during the acute phase of *S. suis* infection. These cells have an impaired MHC-II-restricted antigen presentation capacity and an altered cytokine profile, which could lead to the formation of heterogeneous Th cells [21]. This study thus highlights the potential consequences of inflammation on the type and magnitude of the immune response elicited by a pathogen. Better characterization of dir-mDCs and indir-mDCs profiles will help understand in the future how pathogens modulate T cell activation signals 1 and 2.

Abbreviations

bmDC, bone marrow-derived dendritic cell; CIITA, Class II Major Histocompatibility Complex Transactivator; CPS, capsular polysaccharide; dir-mDC, directly activated mature DC; indir-mDC, indirectly activated mature DC; MARCH, membrane-associated RING-CH; OVA, ovalbumin; pMHC-II, peptide-MHC-II complexes; WT, wild-type.

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Supporting Information (Available on the Frontiers in Immunology Website)

Figure S1. *S. suis*-stimulated bmDCs preserve their capacity to induce CD4+ T cell activation.

Figure S2. *S. suis*-stimulated bmDCs preserve their antigen capture and processing capacities.

Figure S3. BmDCs enhance their MHC-II cell surface expression with a delayed kinetics upon stimulation with *S. suis.*

Figure S4. Naïve splenic DCs show low intensity of MHC-II expression upon *in vitro* stimulation at short incubation times with the encapsulated strain of *S. suis*.

Figure S5. BmDCs do not enhance their CD86 cell surface expression at short incubation time points following stimulation with *S. suis*.

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Figures

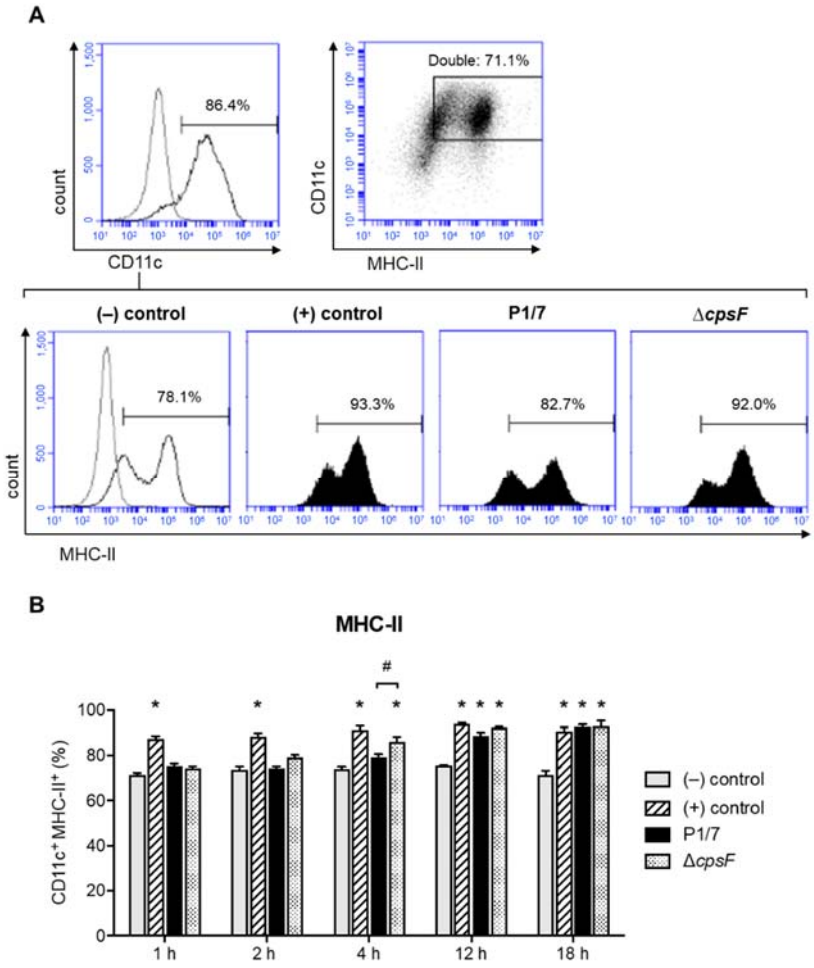

Figure 1. BmDCs enhance their MHC-II cell surface expression with a delayed kinetics upon stimulation with *S. suis* WT strain. Cells were stimulated for 1, 2, 4, 12, or 18 h with *S. suis* WT strain P1/7 or the non-encapsulated mutant Δ*cpsF* (initial MOI:1) in technical duplicates. Unstimulated cells served as negative (–) control for basal expression at each time point. Cells stimulated with LPS (1 µg/ml) were used as positive (+) control. Cells were harvested and fixed after each incubation time. Once the last incubation time was over, cells were surface stained for CD11c and MHC-II, and analyzed by FACS. Events are gated on $CD11c^+$ cells. (**A**) Representative histograms have been selected for this figure (at time = 4 h). The gray lines are isotype controls. At least thirty thousand gated events were acquired per sample and data analysis was performed using BD Accuri™ C6 software. Quadrants were drawn based on PE/Cy5- and PE-control stains and plotted on logarithmic scales, a representative dot plot is displayed. (**B**) Data are expressed as mean ± SEM (% of positive cells) and are from 3 independent experiments. **P* < 0.05 indicates a statistically significant difference compared to (–) control cells. *#P* < 0.05 indicates a statistically significant difference between P1/7-stimulated bmDCs and Δ*cpsF*-stimulated bmDCs.

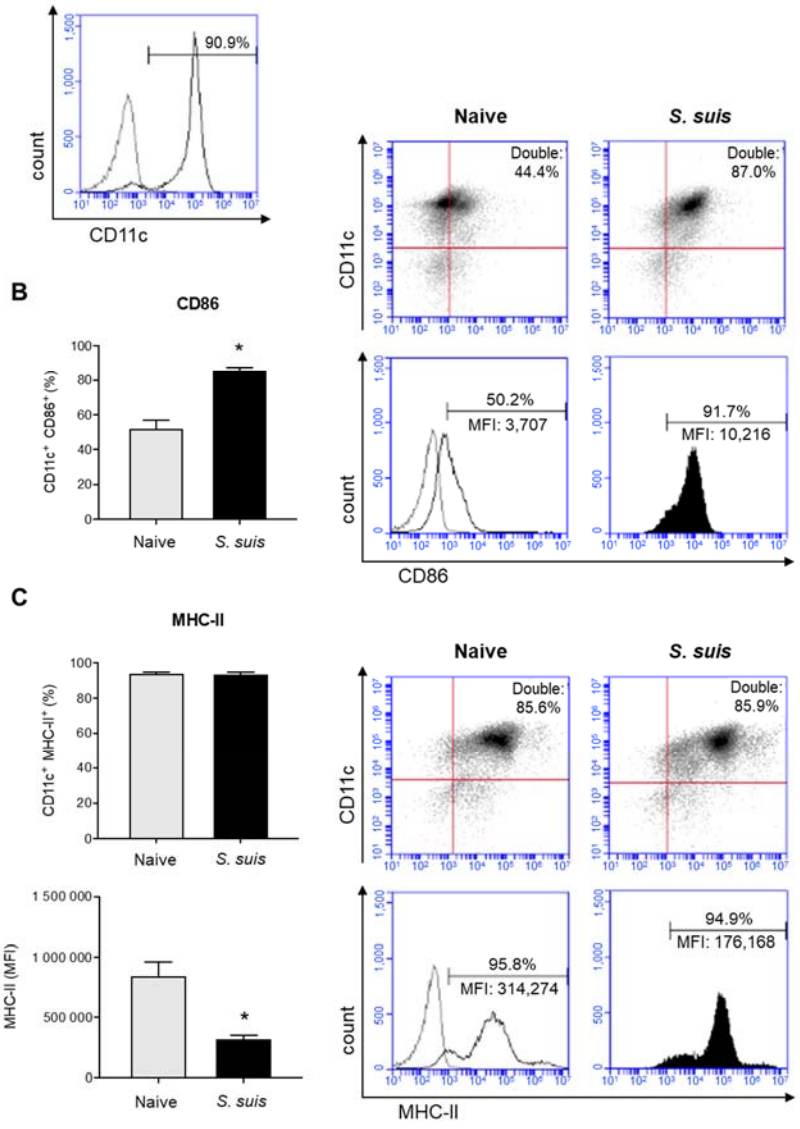

Figure 2. Splenic DCs become phenotypically mature but show a reduction in their MHC-II expression levels during *S. suis in vivo* infection. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 6 h after infection (n = 2 to 3 per group X 2 independent experiments). Splenic DCs were purified by MACS positive selection and cells were surface stained for FACS analysis. CD11c purity is shown in (**A**). The expression of (**B**) CD86 and (**C**) MHC-II was evaluated. Data are expressed as mean ± SEM (of % double positive cells or of mean fluorescence intensity [MFI]. In the latter case, events are gated on CD11c+ cells). Representative dot plots and histograms have been selected for this figure. The gray lines on the histograms are isotype controls. Thirty thousand events were acquired per sample and data analysis was performed using BD Accuri™ C6 software. Quadrants were drawn based on PE/Cy5- and PE-control stains and plotted on logarithmic scales. *P < 0.05 indicates a statistically significant difference compared to naive cells.

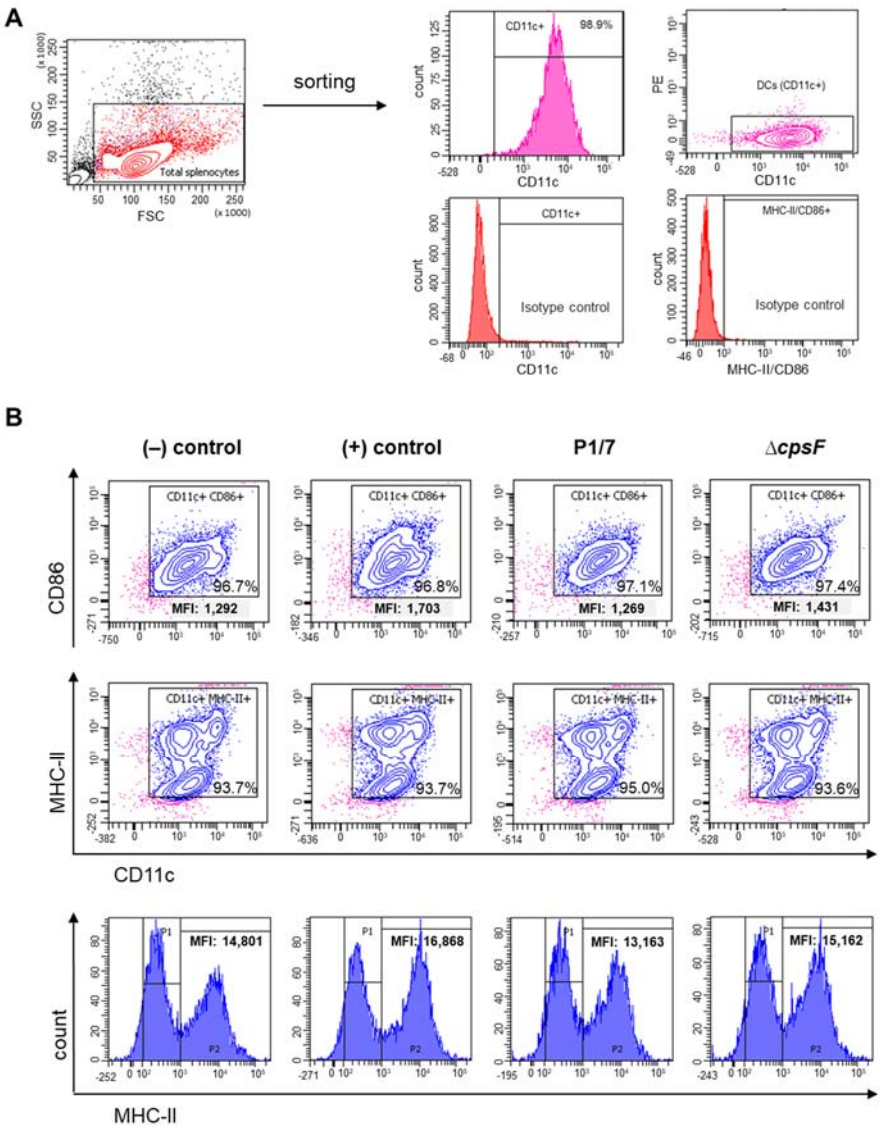

Figure 3. Naïve splenic DCs show low intensity of MHC-II expression upon *in vitro* stimulation with the encapsulated strain of *S. suis*. Spleen cells from a pool of 10 naive C57BL/6 mice were FACS-purified and $CD11c^+$ cells (APC-conjugated anti-CD11c) were sorted with the BD FACSAria™ Fusion flow sorter as illustrated in (**A**). (**B**) Purified cells were stimulated for 2, 4 or 18 h with *S. suis* WT strain P1/7 or the non-encapsulated mutant Δ*cpsF* (initial MOI:1). Unstimulated cells served as negative (–) control for basal expression at each time point. Cells stimulated with LPS (1 µg/ml) were used as positive (+) control. Cells were harvested and surfaced stained for CD86 or MHC-II. Representative density plots have been selected for this figure (at time = 18 h). Histograms of MHC-II expression (gated on $CD11c^+$ cells) have also been included to illustrate variations in the MFI of the MHC-II^{high} population (P2, at time = 18 h). Data analysis was performed using FACSDiva software. Density plots were drawn based on APC- and PE-control stains and plotted on logarithmic scales.

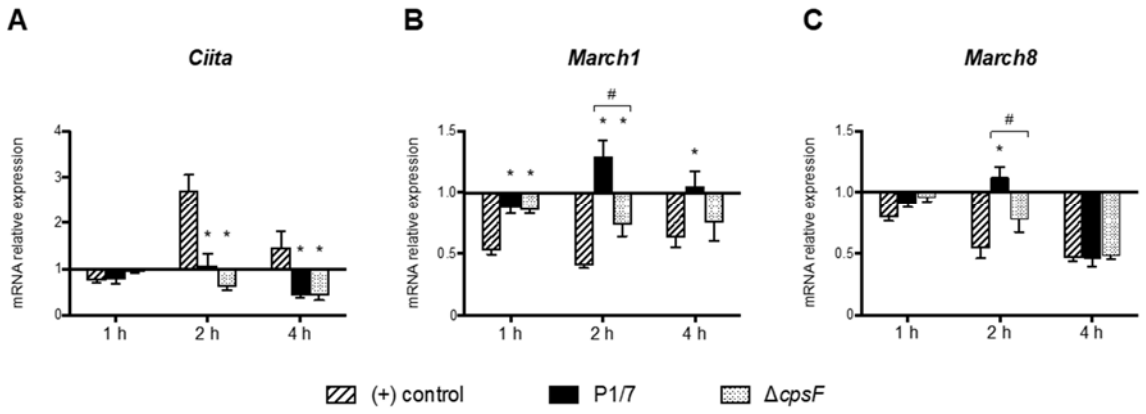

Figure 4. *S. suis*-stimulated bmDCs and directly-activated bmDCs have distinct CIITA, MARCH1 and MARCH8 transcriptional profiles. BmDCs were stimulated for 1, 2, or 4 h with *S. suis* WT strain P1/7 or the non-encapsulated mutant Δ*cpsF* (initial MOI:1) in technical duplicates. Total cellular RNA was extracted and analyzed by RT-qPCR for (**A**) *Ciita*, (**B**) *March1* and (**C**) *March8* mRNA expression. Expression is illustrated as fold level over unstimulated cells [(–) control)]. Cells stimulated with LPS (1 µg/ml) served as positive (+) control. Data are expressed as mean ± SEM from 4 independent experiments. **P* < 0.05 indicates a statistically significant difference compared to (+) control cells. *#P* < 0.05 indicates a statistically significant difference between P1/7-stimulated bmDCs and Δ*cpsF*-stimulated bmDCs.

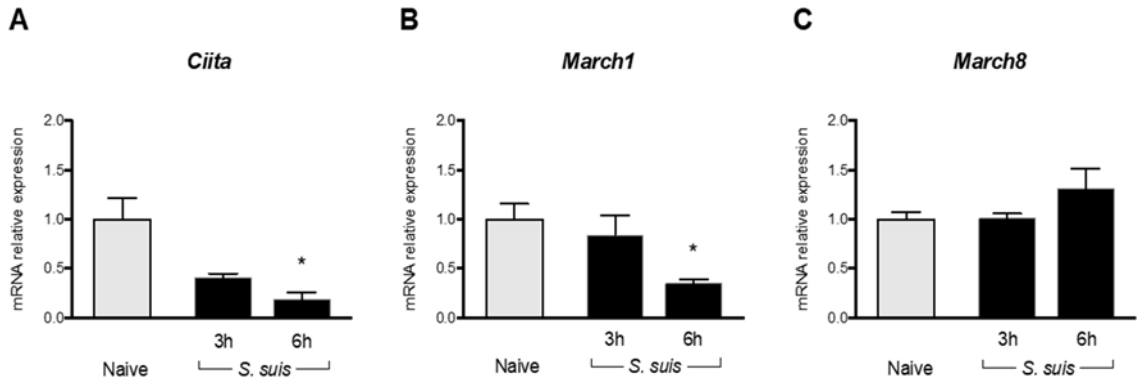

Figure 5. Splenic DCs show reduced transcription of CIITA and sustained/upregulated transcription of MARCH1 and MARCH8 during *S. suis* infection. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 3 or 6 h after infection (n = 2 to 3 per group X 2 independent experiments). Splenic DCs were purified by MACS positive selection, then total cellular RNA was extracted and analyzed by RT-qPCR for (**A**) *Ciita*, (**B**) *March1* and (**C**) *March8* mRNA expression. Expression is illustrated as fold level as compared to expression in naive mice. Data are expressed as mean ± SEM. *P < 0.05 indicates a statistically significant difference compared to naive cells.

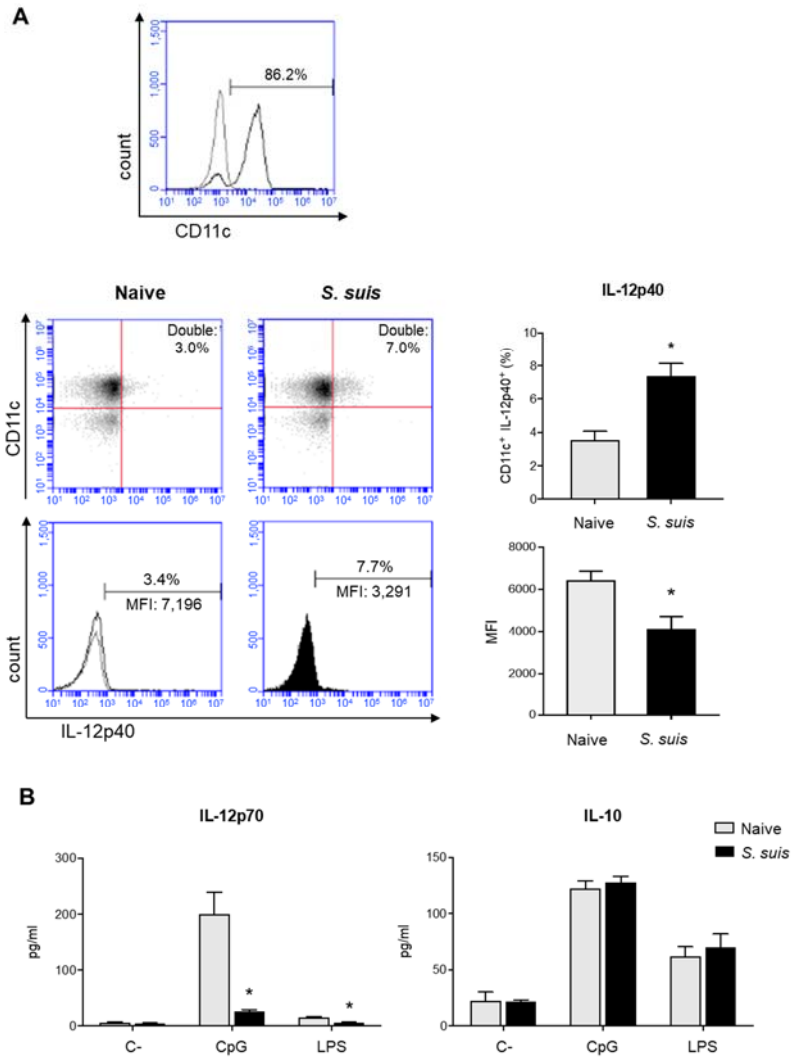

Figure 6. Splenic DCs have an impaired IL-12p70 secretion capacity following *S. suis* infection. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 6 h after infection (n = 8 per group). Splenic DCs were purified by MACS positive selection and CD11c purity is shown in (**A**). Cells were stained for IC-FACS analysis of IL-12p40 expression. Events are gated on total cells and data are expressed as mean ± SEM (% of double positive cells or of mean fluorescence intensity [MFI]). Representative dot plots and histograms have been selected for this figure. The gray line on the histogram is the isotype control. Thirty thousand gated events were acquired per sample and data analysis was performed using BD Accuri™ C6 software. Quadrants were drawn based on Alexa488- and APC-control stains and plotted on logarithmic scales. (**B**) Cells were cultured for 24 h *ex vivo* with CpG oligodeoxynucleotides (1 µM) or LPS (1 µg/ml). Supernatants were harvested and IL-12p70 and IL-10 levels were quantified by ELISA. Unstimulated cells served as negative controls (C-) for basal expression. Data are expressed as mean ± SEM (in pg/ml). $^*P < 0.05$, indicates a statistically significant difference compared to naive cells.

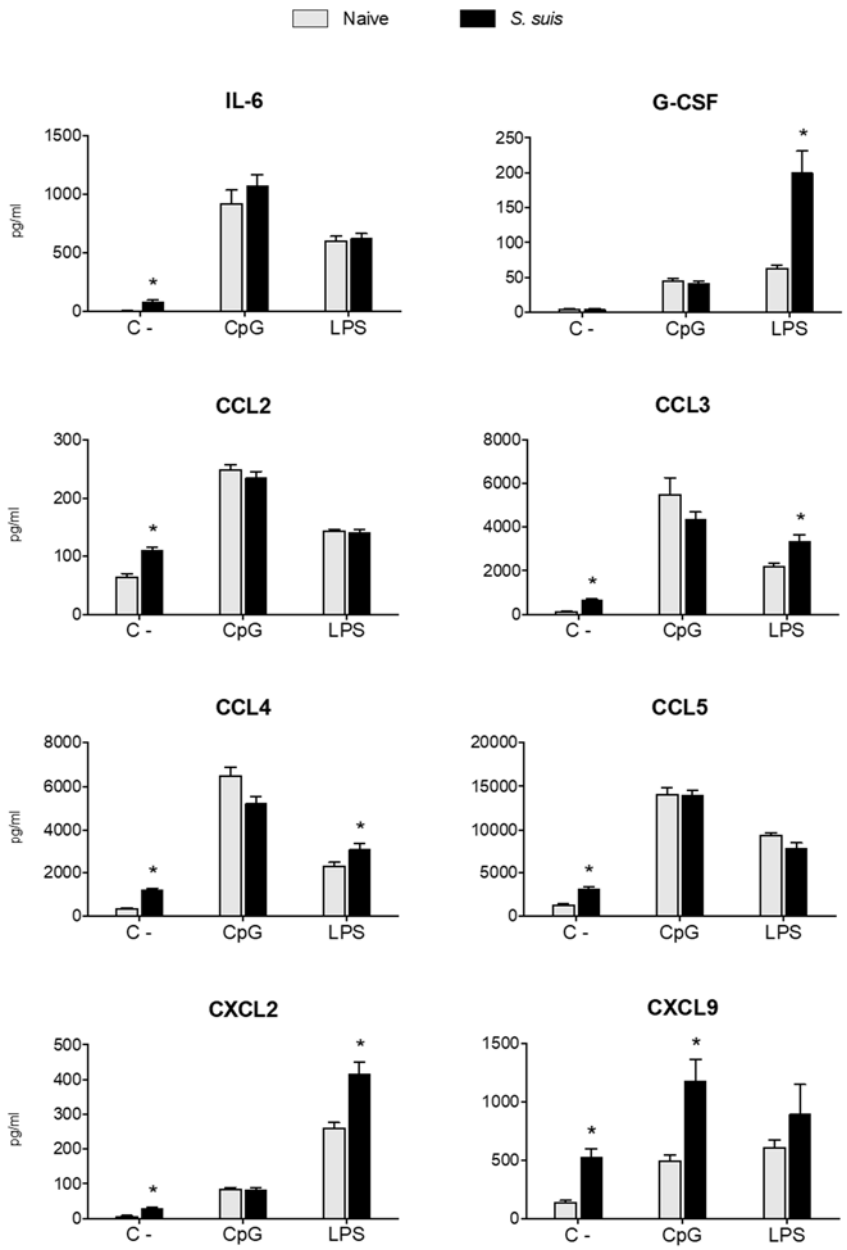

Figure 7. Splenic DCs maintain their secretion capacity of various cytokines and chemokines following *S. suis* infection. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 6 h after infection (n = 8 per group). Splenic DCs were purified by MACS positive selection. Cells were cultured for 24 h *ex vivo* with CpG oligodeoxynucleotides (1 µM) or LPS (1 µg/ml). Supernatants were harvested, and cytokine and chemokine levels were quantified by Luminex. Unstimulated cells served as negative controls (C-) for basal expression. Data were collected with Bio-Plex Manager™ software and analyzed with Bio-Plex® MAGPIX system. Data are expressed as mean ± SEM (in pg/ml). $^*P < 0.05$, indicates a statistically significant difference compared to naive cells.

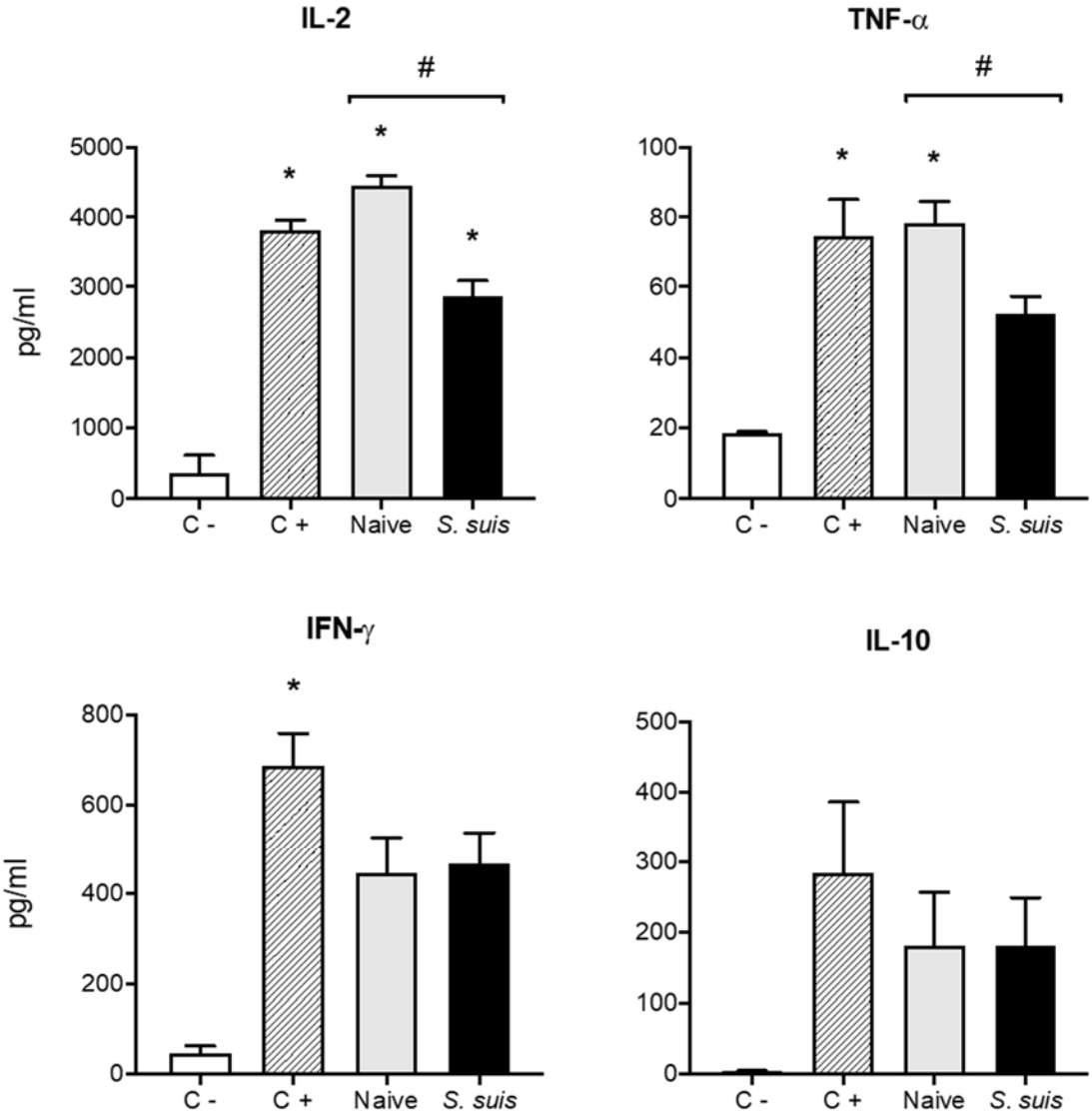

Figure 8. Splenic DCs from infected mice induce low Th1 cytokine production by CD4⁺ T cells. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 6 h after infection (n = 8 per group). Splenic DCs were purified by MACS positive selection and cocultured 24 h *ex vivo* with BO97.10 cells (DC: T cell ratio of 1: 3) in Kappler Marrack complete medium containing OVA (500 µg/ml) and antibiotics (100 µg/ml gentamycin). Supernatants were harvested and IL-2, TNF, IFN-γ, and IL-10 levels were quantified by ELISA. Cocultures incubated with medium alone served as negative controls (C-), while cocultures treated with LPS (1 µg/ml) and OVA served as positive controls (C+). Data are expressed as mean ± SEM (in pg/ml). **P* < 0.05 indicates a statistically significant difference compared to unstimulated control cells. # *P* < 0.05, indicates a statistically significant difference between cells derived from naive and infected mice.

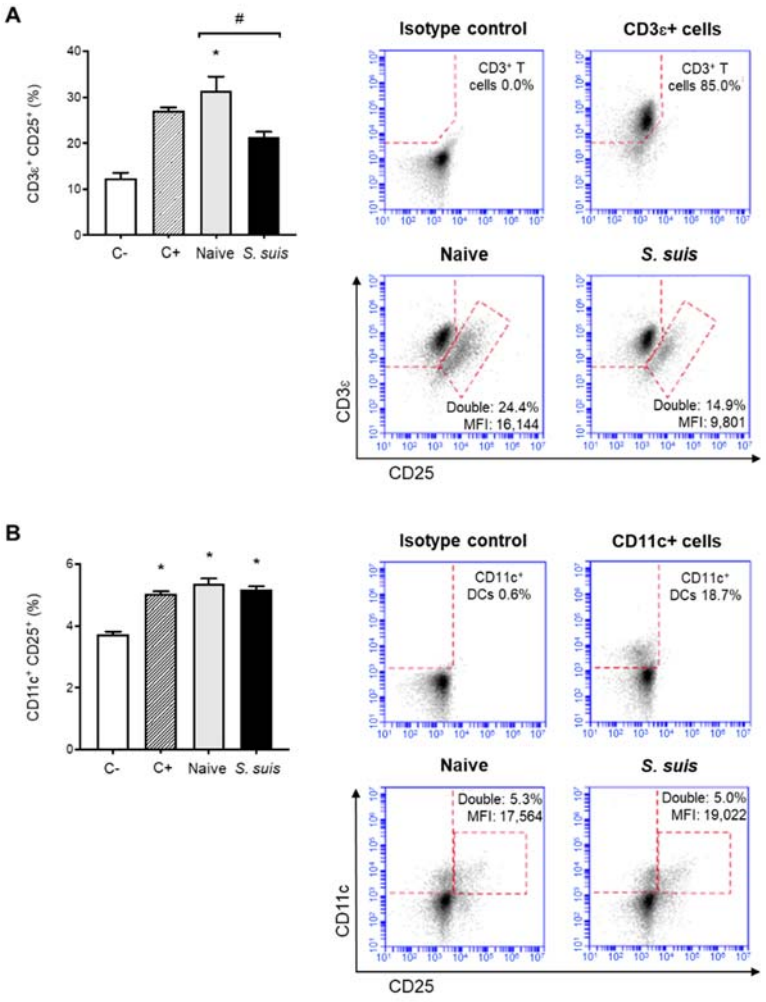

Figure 9. Splenic DCs from infected mice induce low CD25 expression on CD3⁺ cells but not on CD11c⁺ cells. C57BL/6 mice were injected intraperitoneally with 5 x 10^7 CFU of *S. suis* WT strain P1/7. Spleens from mock-infected (naive) and infected mice were harvested 6 h after infection (n = 8 per group). Splenic DCs were purified by MACS positive selection and cocultured 24 h *ex vivo* with BO97.10 cells (DC: T cell ratio of 1: 3) in Kappler Marrack complete medium containing OVA (500 µg/ml) and antibiotics (100 µg/ml gentamycin). Cocultures incubated with medium alone served as negative controls (C-), while cocultures treated with LPS (1 µg/ml) and OVA served as positive controls (C+). Cells were harvested and surface stained for FACS analysis of CD25 expression on (**A**) CD3□⁺ or (**B**) CD11c⁺ cells. Events are gated on total cells and data are expressed as mean ± SEM (% of double positive cells). Representative dot plots have been selected for this figure, including mean fluorescence intensity [MFI] values. Thirty thousand gated events were acquired per sample and data analysis was performed using BD Accuri™ C6 software. Quadrants were drawn based on FITC-, PE/Cy5-, and APC-control stains and plotted on logarithmic scales. **P* < 0.05 indicates a statistically significant difference compared to unstimulated control cells. # *P* < 0.05, indicates a statistically significant difference between cells derived from naive and infected mice.

ANNEXES - ARTICLE II

Streptococcus suis, an important pig pathogen and emerging zoonotic agent – An update on the worldwide distribution based on serotyping and sequence typing

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-premier auteur de cet article de synthèse. J'ai participé activement à la recension de la littérature, à l'écriture de la première version du manuscrit et à la révision de celui-ci. J'ai aussi participé aux corrections lors des étapes de révision pour publication.

Abstract

Streptococcus suis is an important pathogen causing economic problems in the pig industry. Moreover, it is a zoonotic agent causing severe infections to people in close contact with infected pigs or pork-derived products. Although considered sporadic in the past, human *S. suis* infections have been reported during the last 45 years, with two large outbreaks recorded in China. In fact, the number of reported human cases has significantly increased in recent years. In this review, we present the worldwide distribution of serotypes and sequence types (STs), as determined by multilocus sequence typing, for pigs (between 2002 and 2013) and humans (between 1968 and 2013). The methods employed for *S. suis* identification and typing, the current epidemiological knowledge regarding serotypes and STs and the zoonotic potential of *S. suis* are discussed. Increased awareness of *S. suis* in both human and veterinary diagnostic laboratories and further establishment of typing methods will contribute to our knowledge of this pathogen, especially in regions where complete and/or recent data is lacking. More research is required to understand differences in virulence that occur among *S. suis* strains and if these differences can be associated with specific serotypes or STs.

Introduction

Streptococcus suis is one of the most important pathogens in the porcine industry causing septicemia, meningitis and many other infections.[1] In addition, it is an emerging zoonotic agent responsible for septicemia with or without septic shock, meningitis and other less common infections in humans.[1] During the last decade, the number of reported human cases due to *S. suis* has dramatically increased, and while most sporadic human cases of infection appear to be due to close occupational contact with pigs/pork products, particularly in Western countries (farmers, veterinarians, butchers, food processing workers, etc.), two epidemics were recorded in China in 1998 and 2005.[1] As of 2006, the number of human cases reported in Asia has increased.[2, 3] In fact, in some Asian countries, the general population is at risk.[2] However, an update on the distribution of the different serotypes and sequence types (STs), as determined by multilocus sequence typing (MLST), of strains responsible for infections in both pigs and humans from around the world have not been recently compiled. Yet, knowledge of this distribution is necessary in order to not only understand the current situation regarding *S. suis*, but also to evaluate areas where knowledge is lacking. This review covers the global distribution of the *S. suis* serotypes and STs responsible for infections reported in pigs from January 1st, 2002 to December 31st, 2013. A complete review on serotypes from humans since the first description in 1968 was also carried out. However, data of the STs from human cases are available only since 2002. Complete reviews have already covered the different virulence factors implicated in the pathogenesis of the infection caused by this important pathogen[4, 5], and this will not be further addressed in this review.

Brief description of the general aspects of infection in pigs and human

The natural habitat of *S. suis* is the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts.[6] With almost 100% of pig farms worldwide having carrier animals, *S. suis* is one of the most important bacterial pig pathogens. Transmission of *S. suis* among animals is considered to be mainly through the respiratory route.[6] Of the various manifestations of the disease, septicemia and meningitis are by far the most striking features, but endocarditis, pneumonia and arthritis can also be observed.[7] Nevertheless, in peracute cases of infection, pigs are often found dead with no premonitory signs of disease.[7] Presumptive diagnosis of infection in pigs

is usually based on clinical signs and macroscopic lesions.[8] Confirmation of the infection is mandatory and must be achieved by isolation and characterization of the pathogen.[8]

Since first described in Denmark in 1968[9], over 1 600 human cases of *S. suis* infection have been reported with many more probably never diagnosed or misdiagnosed. *S. suis* is the most common cause of adult meningitis in Vietnam, the second most common in Thailand, and the third most frequent cause of community-acquired bacterial meningitis in Hong Kong.[10-12] Different from pigs, the main route of entry of *S. suis* in humans is thought to be through contact of cutaneous lesions, most usually on the hands and arms, with contaminated animals, carcasses or meat.[3] However, this situation seems to be different in some Asian countries where the oral route is taken into consideration since many cases of infection have been reported after ingestion of contaminated raw pork products.[13] In Western countries, infections in humans most usually occur sporadically.[1] After an incubation period that ranges from a few hours to days[13], *S. suis* usually produces meningitis in humans, but is also responsible for cases of endocarditis, pneumonia, peritonitis, arthritis, and other less common diseases usually related to generalized septicemia.[14, 15] In addition, peracute infections with shock and a high mortality rate have been described, particularly in the case of the streptococcal toxic shock-like syndrome (STSLS) closely associated with the 2005 Chinese epidemic, but also observed independently of this outbreak.[16]

Identification of *S. suis*

S. suis is an encapsulated Gram-positive bacterial coccus that occurs singly, frequently in pairs, or occasionally in short chains. The organism grows well in aerobiosis, but growth is enhanced by microaerophilic conditions. The majority of strains are alpha-hemolytic on bovine and sheep blood agar plates after 24 hours of incubation at 37ºC.

Pig and human strains isolated from clinical cases of infection

For clinical cases in pigs, isolation and identification of strains is relatively easy since successful identification can be achieved by a minimum of biochemical tests and confirmed by serotyping (see below).[17] However, the use of rapid multi-test biochemical kits may be misleading as some strains of *S. suis* can be misidentified.[18] Although *S. suis* field isolates readily grow on media used for culturing meningitis-causing bacteria and

veterinary diagnostic laboratories easily identify this pathogen, many human diagnostic laboratories are less aware of this pathogen and may misidentify it as enterococci, *S. pneumoniae*, *Streptococcus bovis*, viridans group streptococci (e.g. *Streptococcus anginosus* and *Streptococcus vestibularis*) or even *Listeria monocytogenes*.[14, 18-22] In many cases, the initial Gram stain presumptive diagnosis of the cerebrospinal fluid (CSF) specimen is pneumococcal meningitis. This confusion may have led to the misdiagnosis of *S. suis* infections in the past. Many cases were diagnosed retrospectively after the isolates were initially misidentified.[23] More recently, PCR tests have been used to directly detect *S. suis* DNA from CSF samples with a sensitivity considerably higher than direct culture, especially if antibiotics have been used.[2] However, most PCR tests used for humans detect serotype 2 strains only and will not detect infections caused by other *S. suis* serotypes (see below). Instead of the serotype 2 PCR test, it is advisable to use other validated PCR tests that allow the detection of the *S. suis* species (see below).

Strains isolated from clinically healthy pigs

S. suis is a normal inhabitant of the oral cavity of pigs. The biochemical identification of isolates recovered from clinically healthy pigs (mainly from tonsil samples) is difficult to achieve due to the presence of other streptococci that are part of the normal oral microflora and that are phenotypically similar to *S. suis*. For this reason, molecular biology techniques have been developed during the last decade to allow the detection and identification of *S. suis* strains, such as a PCR assay targeting the specific house-keeping gene encoding for the glutamate dehydrogenase (*gdh*).[24] Although widely used, the *gdh* PCR test can sometimes fail to correctly identify certain *S. suis* isolates.[25] Using this test, it has also been reported that there was a risk of misidentifying *Streptococcus gallolyticus* as *S. suis*.[26]

Serotyping

Definition of serotypes

Serotyping, which should be a part of the routine identification of *S. suis* strains recovered from diseased pigs and humans, will provide further confirmation regarding the pathogen's identity. A total of 35 serotypes of *S. suis* have been described and are defined based on the antigenicity of their capsular polysaccharide (CPS).[6] Evidence accumulated throughout the years has demonstrated a high level of genetic diversity in the *S. suis*

species, as shown by sequence analysis of the 16S rRNA and *cpn60* genes: these two methods suggested that serotypes 32 and 34 were divergent, being clustered together at a significant distance from the other serotypes. This led to the suggestion that these two serotypes might be reclassified as a different species (*Streptococcus orisratti*).[27, 28] However, field strains belonging to these serotypes have been isolated from diseased pigs in recent years in Canada and China.[25, 29, 30] More recently, further studies have suggested that the reference strains of additional serotypes (20, 22, 26, and 33) do not belong to the *S. suis* species.[31] As such, there is an urgent need for a consensus in defining whether a serotype belongs to *S. suis* or not.[32]

In order to understand the important roles of the CPS in serotyping and in the interactions of *S. suis* with the host, knowledge regarding the specific structure that confers capsular properties to each individual serotype is necessary. Van Calsteren *et al.* have described the composition and structures of the *S. suis* serotypes 2 and 14 CPS.[33, 34] Still, the chemical composition and structure of the CPS of the serotypes other than 2 and 14 are presently unknown.

Serological methods

Proper serological typing, which is one of the most important features of the *S. suis* infection diagnosis, must be performed using either a coagglutination test, capillary precipitation test or with Neufeld's capsular reaction using reference antisera.[35, 36] Details on how to perform the coagglutination test have already been published.[37] This test is preferred by many laboratories, especially in North America.[17] Some serotypes cross-react, indicating the presence of common antigenic determinants. This cross-reaction is probably due to similar or closely related structural features of the CPS. To date, the following cross-reactions have been described: serotype 1/2 with serotypes 1 and 2, serotypes 6 and 16, serotypes 2 and 22, and serotypes 1 and 14.[35, 38] In some cases, absorption is recommended in order to obtain monospecific antisera.[17] However, levels of cross-reactions for some serotypes vary with field strains (M. Gottschalk, 2014, unpubl. observations).

Serotyping by multiplex PCR

The idea of molecular serotyping by PCR amplification of serotype specific *cps* genes using either a simplex or multiplex PCR is attractive due to the fact that animals are not

used for serum production, ease of development and effectiveness. Early studies reported the use of assays targeting some serotypes.[39, 40] Liu *et al*.[41] reported a new protocol using four multiplex PCR assays allowing the detection of the 33 serotypes of *S. suis* (serotypes 1 to 31, 33 and 1/2), but not those related to *S. orisratti* (serotypes 32 and 34). Strains reacting in serology with more than one serotype could be confirmed using this technique. More recently, Okura *et al*. developed a PCR for all 35 described serotypes of *S. suis*.[42] Regardless of the PCR test used, a major disadvantage is the fact that the serotypes 2 and 14 cannot be distinguished from serotypes 1/2 and 1, respectively, since both of these serotype pairs do not possess unique *cps* genes.[43] This represents a major problem for pig isolates since serotypes 1, 1/2, 2, and 14 are commonly isolated in swine.[3, 10] The use of specific antisera is mandatory for such strains. For this reason, only serologically confirmed isolates of serotypes 2 and 14 recovered from pigs were considered in the present review. Although it is also necessary to confirm isolates from humans, it may represent a less important drawback since serotype 1/2 has never been isolated from humans and serotype 1 has been reported in only three non-serologically confirmed cases (at least with both serotypes 1 and 14 antisera), which is important considering these serotypes cross-react (see above).[44, 45] For this reason, isolates from humans reported in the literature as being serotype 2 or 14 based only on PCR reactions (although not serologically confirmed) will be considered as such in the present review.

"Serotyping" by biochemical identification

In the past, some reported cases of *S. suis* infection in humans have been attributed to serotype 2 based on the biochemical analyses obtained using the rapid multi-test commercial kits mentioned above. While many of these kits claim to differentiate between serotype 1 and 2 strains based on sugar fermentation, there is still no evidence of a correlation between a specific serotype and its biochemical properties.[17, 20, 46] As a result, some human cases have been reported as serotype 2 while others were reported as serotype 1, but since the serotypes of these strains have not been confirmed using antisera (and not even by PCR), their serotypes are herein reported as "unconfirmed by reference antisera or PCR tests".

Non-typable strains

Some *S. suis* isolates do not agglutinate with any of the typing antisera directed against the 35 serotypes and are identified as non-typable isolates.[29] Non-typable *S. suis* strains

may correspond to either truly encapsulated strains that belong to novel, not yet described, encapsulated serotypes, or to non-encapsulated strains, which are impossible to serotype using the serological methods based on CPS antigens. The proportion of non-typable isolates varies between studies depending on the number of serotypes detected using antisera.

Using antisera against all 35 serotypes, Gottschalk *et al*. demonstrated that 89% of non-typable *S. suis* strains presented high surface hydrophobicity, suggesting that they were poorly or non-encapsulated.[25, 47] This was confirmed using transmission electron microscopy, demonstrating that highly hydrophobic strains (74-93%) were non-encapsulated.[25] More than 40% of these non-encapsulated strains have been recently shown to belong to known serotypes using a multiplex PCR for all 35 serotypes.[42] It is also difficult to be certain if these strains were already non-encapsulated when causing disease, or if they lost their CPS during isolation and culture. It has been previously reported that 34% of isolates belonging to serotype 1/2 or 2 recovered from cases of endocarditis in Japan were non-encapsulated due to deletions and insertions in the genes of the CPS locus.[48] It was concluded that although the CPS is considered an important virulence factor for *S. suis*, loss of capsular production might be beneficial to *S. suis* in the course of infective endocarditis. In fact, non-encapsulated strains were shown to possess not only high adhesion properties to mammalian cells, but also a capacity to form biofilms.[47] Since the sites of isolation of these non-typable strains were similar to those of the most important serotypes (meninges/brain, joints, heart, and lungs), their potential virulence capacity should not be disregarded.

Multilocus sequence typing

Being a pathogen capable of causing sporadic cases of infection and epidemics in both pigs and humans, the global surveillance of *S. suis* is very important in order to better understand the epidemiology of this bacterial species.[49] Though different methods based on DNA have been used for the surveillance of *S. suis*, these methods are effective for short-term epidemiology only as they are based on non-characterized genomic differences between isolates.[49] In contrast, MLST distinguishes a large number of genotypes while using genetic variations that accumulate very slowly, in housekeeping genes, and has

allowed global and long-term epidemiology for many important meningitis-causing bacteria by determining the STs present within a population.[49, 50]

In 2002, King *et al*. established a model of MLST for *S. suis* using seven different house-keeping genes (*cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS*).[51] Since being established, this MLST model has been used by multiple laboratories throughout the world to determine the STs of *S. suis* strains isolated from pig and human cases of infection. When used alongside serotyping, MLST allows gathering further information about the genetic diversity of the *S. suis* strains within the different serotypes. The popularity of this typing method for *S. suis* continues to increase due to the ease in carrying out the technique (PCR and sequencing) and due to the fact that the data are transferrable and comparable from one laboratory to another. Thanks to the *S. suis* MLST Database, the global distribution of the different *S. suis* STs can be shared and compared. When the MLST data take into consideration the serotypes, it is important that a trustable and complete serotyping system has been applied to the field strains, otherwise the final data are difficult to interpret. More recently, studies have begun combining data obtained from MLST with the presence or absence of different *S. suis* virulence-associated markers at the gene and protein levels including the suilysin (SLY, encoded by the *sly* gene), muramidase-released protein (MRP, encoded by the *mrp* gene), extracellular factor (EF, encoded by the *epf* gene), and different pili in order to compare STs data with phenotypic characteristics.[52, 53]

Methodology used in the present REVIEW

In the present review, literature searches were completed using the following databases: Medline (Pubmed; http://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://thomsonreuters.com/web-of-science/) and Science Direct (http://www.sciencedirect.com/). Relevant articles in English were used while references in other languages were considered when available. MLST results were obtained from the *S. suis* MLST Database (http://ssuis.mlst.net/). Regarding the distribution of serotypes, the results reported for clinical cases in pigs correspond only to those where serotyping was carried out using antisera and not PCR, while for human cases, reports where serotyping was performed by either antisera or PCR (for serotypes 2 and 14) are both reviewed. This consideration is primarily based on the fact that serotype 1 and serotype 1/2 isolates have not yet been recovered (or confirmed) from human cases of infection. It is important to note

that when the correlation between STs and serotypes are reported in this review, it is impossible to confirm the methodology used for the serotyping for many cases.[51] We decided to take the data into consideration, especially for those included in the *S. suis* MLST Database, since the number of cases used for this review would otherwise have been highly limited. For the distribution of STs, the ST complexes represent hyperinvasive lineages to which two or more STs belong as a result of genetic proximity and are based on the MLST phylogeny diagram presented by Lachance *et al.*[54] STs considered to be unrelated to another ST according to this phylogeny diagram are identified as being 'unrelated' to any known ST complex. With the exception of the serotype distribution of *S. suis* human cases, which comprises all cases reported since first described in 1968, distribution of serotypes for clinical cases in pigs and distribution of all STs are based on data published between January 1st, 2002 to December 31st, 2013.

Worldwide distribution of serotypes

Diseased pigs

In order to fully appreciate the prevalence of human infections and the zoonotic potential of *S. suis*, one must understand the situation at the farm level since transmission from diseased pigs or pork-derived products is a prerequisite for infection of humans. The data compiled from studies since January 1st, 2002 are shown in Table 1.

While *S. suis* is part of the normal microflora of pigs and many studies have focused on carriage in healthy pigs, these studies were not included in this review since the serotype distribution in healthy carriers greatly differs from that of clinical strains, with serotype 2 being much less frequently isolated.[55-61]. In addition, since *S. suis* is a normal inhabitant of the upper respiratory tract,[6] most of these studies identified only a small part of the real population of each carrier. Finally, in the case where species-specific PCR tests were not used, many of the "untypable" strains detected probably did not belong to the *S. suis* species., The contribution of these commensal strains to the risk posed to humans in close-contact with pigs or with pork-derived products remains to be further studied[14, 62, 63] and seems to occur particularly in immunocompromised patients. The situation may be different when animals are not really "healthy carriers" but rather convalescent animals, carrying in their tonsils virulent strains. However, this situation is almost impossible to define in field studies.

During the last 12 years, more than 4 500 serologically-confirmed strains recovered from diseased pigs have been reported (Table 1). Globally, the predominant *S. suis* serotypes isolated from clinical cases in pigs are, in decreasing order, serotypes 2, 9, 3, 1/2, and 7, along with 15.5% of the strains being non-typable by serotyping. However, there is a clear geographical effect on the distribution of serotypes (see below) and these figures are influenced by the number of published studies.

Almost 70% of studies on worldwide isolates recovered from diseased pigs are from North America (Table 1). Nevertheless, this is not an indication of a higher number of cases but simply of a higher number of published reports and studies. In fact, 97% of North American data are from Canada and the rest from the United States of America (USA), with no data from Mexico. In North America, serotype 2 is the most prevalent in Canada, while in the USA, it is serotype 3. Meanwhile, the second most prevalent serotype in Canada is serotype 3 and is serotype 2 in the USA. However, only slight differences in percentages have been observed in both countries, demonstrating a similar distribution when the data are combined: both serotypes 2 and 3 are the two most prevalent serotypes isolated from clinical pig cases in North America with 24.3% and 21.0% of prevalence, respectively, followed by serotypes 1/2, 8 and 7. Similar distributions of serotypes in Canada and the USA may be explained by a fluid movement of animals between the two countries.

In South America, only two studies have been published, both from Brazil, which report serotype 2 as being the most prevalent with a mean of 57.6% of cases, followed by, in decreasing order of prevalence, serotypes 1/2, 14, 7, and 9 (Table 1). Interestingly, although no clinical cases in pigs have been published, human cases have been reported in other South American countries (see below).[64-67] On the other hand, no human cases have yet been reported in Brazil.

In Asia, where the vast majority of human cases have been reported (see below), studies on clinical cases in pigs account for only 14.0% of the data available and are provided from China and South Korea only. The most prevalent serotypes in infected pigs are, in decreasing order of prevalence, serotypes 2, 3, 4, 7, and 8 (Table 1). The South Korean study surprisingly reported serotypes 3 and 4 as being predominant, followed by serotype 2 at a low prevalence of only 8.3%. This anomaly may be due to the fact that the paper reported a relatively low number of cases (only 20 isolates) from animals with acute

polyserositis only.[68] Even more disturbing is the fact that the information available regarding the status of *S. suis* infections in pigs in Asia is the most scarce of the three continents, while this is the continent where cases of zoonosis occur the most frequently in the general population. Although many hospitals and microbiologists in both Thailand and Vietnam are aware of *S. suis* infections when it comes to diagnosis[2], the four veterinary studies published from these countries investigated healthy or slaughterhouse pigs only.[59, 60, 69, 70] Similarly, there have been 10 human *S. suis* cases reported in Japan between 1994 and 2009, but no complete study on the distribution of isolates from clinically ill pigs including all serotypes has been recently published. In fact, data from the last epidemiological studies in Japan date back to between 1987 and 1991, more than 20 years ago, which predate the human cases reported in this country and illustrate the necessity to gather data in order to increase our knowledge of the current epidemiological situation.[71] In Hong Kong, where *S. suis* has been reported as the third most common culture-confirmed cause of community-acquired bacterial meningitis, there have been 47 human cases from 1983-2001 and 21 from 2003-2005[12], yet there is a complete absence of epidemiological data regarding *S. suis* in pigs, with the exception of two studies investigating the prevalence of *S. suis* in pork meat sold in wet markets.[72, 73] In Singapore, the Philippines, Laos, and Cambodia, where human cases have occurred during the last decade (Table 2), there are no data available on the epidemiology of *S. suis* infections in pigs.

Important pig producing European countries, such as Denmark, Belgium, France, Germany, Italy, and the United Kingdom have not recently reported the distribution of serotypes recovered from clinical cases in pigs: the last reports from these countries regarded strains isolated between 1990 and 2000.[55, 74] Before the year 2000, serotype 2 was the most common serotype recovered in Italy, France and Spain, whereas serotype 9 was more frequently found in the Netherlands, Germany and Belgium.[76] This lack of information is of high importance. The only two countries with more recent data are Spain and the Netherlands. In fact, in Spain, serotype 2 is no longer the most prevalent serotype, but the second behind serotype 9 and followed by serotypes 7, 8, and 3.[56, 75, 76] In the Netherlands, between 2002 and 2007, serotype 9 was still the most prevalent, followed by serotypes 2, 7, 1, and 4.[77] Although serotype 9 is the most prevalent serotype in Spain and in the Netherlands and was also prevalent in many European countries before the year 2000, no human cases associated with this serotype have yet been reported.

Taken together, these two European countries provide a relatively similar serotype distribution with serotypes 9, 2, 7, 8, and 3 in order of importance (Table 1). In studies previous to 2002, serotype 1 also appeared to be prevalent in countries such as Belgium and the United Kingdom[76]; however, it is not clear if this is the current situation. The situation is similar in Denmark where only serotype 7 isolates were characterized during the last twelve years and where the last serological study regarded isolates recovered in 1995-1996.[78, 79] For the time period covered in this review, two reports from France and Italy have been published, although the data from none of these was considered since one of them used serotyping by PCR, detecting serotypes 1 (and 14), 2 (and 1/2), 7, and 9 only and, thus, did not meet the inclusion criterion (see above), while the other studied the distribution of serotypes in healthy animals.[57, 80] Interestingly, human cases have emerged in other European countries in many of which few or no studies on *S. suis* isolation from diseased pigs have been conducted. In Serbia, a recent prevalence study was published using isolates from swab samples taken from dead animals, clinically healthy pigs, slaughterhouse pig carcasses, and butchers' knives, but it was impossible to associate corresponding serotypes to the diseased animals only.[81] In Croatia, only one study pertaining to the antimicrobial susceptibility of *S. suis* type 2 isolates was published,[82] but no epidemiological studies on the distribution of serotypes is available. Moreover, in Greece and Poland, where human cases have been reported, there are still no pig data available. As such, there is an urgent need to evaluate fresh data on the prevalence of *S. suis* in clinical isolates from pigs in Europe where many countries are amongst the more important pig producers in the world.

Finally, there is also a lack of relatively recent serotyping data from Australia and New Zealand. In Australia, where three human cases have been recently reported, the studies concerning serotype distribution in pigs predate these human cases, thus showing that there were once active surveillance studies in this country.[83, 84] One human case of *S. suis* was also reported in New Zealand[85], but no recent studies regarding isolates recovered from diseased pigs have been published in this country.

In summary, there are countries where the serotype distribution of field strains recovered from diseased pigs has been systematically undertaken during the last 12 years, generating data that may influence any analysis of the worldwide distribution of serotypes. There is an urgent need for new data from European countries on the serotype

distributions of *S. suis* strains isolated from diseased pigs, especially considering the fact that currently available vaccines are bacterins, which are supposed to confer serotype-specific protection. Some countries with an important pig production, such as Brazil, have few studies on pig disease and no human cases declared. Other countries commonly report human cases, but data from diseased animals are almost absent.

Human cases

One of the goals of this review was to compile the serotype distribution of *S. suis* infections in humans, which has not been completed since 1989,[86] and to update the total number of cases from recent reviews on human cases, all of which are excellent comprehensive reviews regarding the clinical features of *S. suis* infections in humans.[3, 18, 87] In the present study, and differently from clinical pig isolates, serotype 2 and 14 isolates were considered to be as such even if identified by PCR, which is unable to differentiate them from serotypes 1/2 and 1, respectively. This consideration is primarily based on the fact that serotype 1 and serotype 1/2 isolates have not yet been recovered (or confirmed) from human cases. A total of 1 642 cases have been reported worldwide as of December 31st, 2013 (Table 2).

Of these cases, serotype 2 was the most frequently reported with 74.7%, followed by serotype 14 with 2.0%. It is important to note that 262 (21.5%) serotype 2 strains and 2 (6.0%) serotype 14 strains were identified by PCR only. The most striking aspect regarding human cases of infection is that 377 cases (23.0%) of all reported cases either do not specify the serotype in the case report or employed a method for serotype identification that was judged inadequate ("biochemical serotyping"). Even though many of these reports claim to be caused by serotype 2, which is probably true, serotyping, or at the very least PCR, should be performed if the strains are still available in order to increase our knowledge of *S. suis* infections in humans. Meanwhile, the remaining five human cases of infection were caused by the following serotypes: 4[14], 5[88], 16[89], 21[67], and 24[88]. Since 2011, human *S. suis* cases of infection have been reported for the first time in Cambodia, Chile, French Guiana, Poland, and South Korea.

The vast majority of human cases have occurred in Asia, which account for more than 90% of all reported cases, particularly in Vietnam, Thailand and China. These three countries alone account for 83.6% of all cases worldwide. However, in China, almost all

cases were described during the 1998 and 2005 outbreaks.[16], [90] In East and Southeast Asia, *S. suis* zoonosis should be considered endemic due in part to the high density of pigs, relatively high number of backyard-type production farms, slaughtering practices with the use of few preventive measures, presence of wet markets, and consumption of ill pigs and/or consumption of uncooked or undercooked pork products.[3] In Thailand, most of the reported cases were caused by serotype 2, while serotype 14 is the second highest, the latter accounting for 21 cases of meningitis and sepsis out of a total of 530 cases. However, infections by other serotypes, such as a serotype 5 peritonitis and a serotype 24 sepsis have also been reported. In Vietnam, where *S. suis* is now the most frequent cause of adult bacterial meningitis[10], most cases are also due to serotype 2, six cases of meningitis were caused by serotype 14, and one case of peritonitis by serotype 16. The diversity of infections caused by serotypes other than serotype 2 could be explained by the awareness of diagnosticians to *S. suis* infections. Consequently, this could also explain why Vietnam and Thailand have the largest number of reported cases and a higher probability of encountering atypical cases on a more regular basis. It is also interesting that the patients who suffered infections by serotypes 5, 16 and 24 were also suffering from cirrhosis: a likely explanation is that these infections were the result of immunocompromisation. The compilation of human cases in China includes sporadic cases and the two *S. suis* serotype 2 epidemics, the first in Jiangsu province in 1998, where 14 deaths out of 25 cases occurred[16], and the second in Sichuan province in 2005, where 38 deaths out of 215 cases were reported. This second epidemic still remains the largest outbreak of *S. suis* in humans.[90] These epidemics were unprecedented for *S. suis* infections in humans considering the high incidence of systemic disease, the low number of cases of meningitis, and the high rate of mortality observed.[1] The patients presented cases consisting of either sepsis, meningitis, or STSLS, based on the presence of the following symptoms: sudden onset of high fever, diarrhea, hypotension, blood spots and petechial, clear erythematous blanching rash, and dysfunction of multiple organs, such as acute respiratory distress syndrome, liver and heart failure, disseminated intravascular coagulation, and acute renal failure.[1] Even though China has the largest pig production in the world and is the site of the 1998 and 2005 outbreaks that attracted much attention from the scientific community, the number of reported cases is considerably lower than in Thailand and Vietnam. One possible explanation is that dishes containing raw pork and blood, very popular in Thailand and Vietnam, are uncommon in

China. In fact, since the last outbreak in 2005, only 4 other sporadic human cases were reported in mainland China.[91, 92]

Human cases were also reported in Cambodia, Hong Kong, Japan, Laos, Singapore, South Korea, and Taiwan. It is of interest to note that in Hong Kong, *S. suis* serotype 2 was reported as the third most frequent cause of adult bacterial meningitis.[12, 93] There is also a human case of infection from the Philippines, although it was not officially reported in this country. In fact, even though the infection was diagnosed as *S. suis* serotype 2 meningitis and treated in the USA, the patient had just returned from a seven month vacation in the Philippines where he had consumed raw pork, making this an Asian case and considered as such in this study.[94]

The second continent where most human infections have been described is Europe, accounting for 8.5% of all reported human cases (Table 2), with approximately 71.4% of all European human cases occurring in countries with a highly developed pig industry: the Netherlands, United Kingdom, France, and Spain. Cases were also reported in Austria, Belgium, Croatia, Denmark, Germany, Greece, Ireland, Italy, Poland, Portugal, Serbia, and Sweden. Surprisingly, no cases have yet been reported in Russia, a country with an increased developing swine production. Human cases of *S. suis* infection were reported for the first time in Europe, with the first ever case in Denmark, in 1968. It is interesting to note that all cases reported before 1983 were from Western Europe.[9, 93] Only two countries consider *S. suis* infections in humans as an industrial disease: the United Kingdom and France since 1983 and 1995, respectively.[18] This recognition led to legislation and regulations which may have contributed to reducing the number of cases in both of these countries, with the last human case in the United Kingdom being reported in 2001. In France, although few cases have been reported since 1995, most were related to wild boar hunters. It is known that wild boars also carry *S. suis*.[95] Consequently, hunters should be aware of the necessary precautions when preparing the carcasses, which present the greatest risk.[96, 97] Most human cases of infection contracted from wild boars were serotype 2, with two cases of meningitis caused by serotypes 4 and 14 in the Netherlands, one case of meningitis by serotype 14 in Denmark, and one case of septicemia by serotype 14 in the United Kingdom.[14, 98, 99]

Although being the continent with the highest number of *S. suis* infection reports from diseased pigs (Table 1), only a few sporadic cases of *S. suis* infection in humans have been reported in North America. As previously mentioned, the isolation rate of *S. suis* serotype 2 from diseased pigs is considerable lower than that of Europe or Asia. The lower number of pig cases of disease due to this serotype may be a reason for the lower number of human cases of infection. It has been suggested that *S. suis* strains of serotype 2 from Canada and the USA present lower virulence properties than those from Europe and Asia.[54] For these two countries, confirmed human cases were all serotype 2, with the exception of one serotype 14 case of meningitis in Canada.[100] In the USA, two of the cases were from the continent while the third was from Hawaii, an island in the Pacific Ocean importing pigs from Asia. While the case in Hawaii is considered American (unlike the case from the Philippines), it would perhaps be more appropriate to compare the features of this infection with those from Asia. In fact, the strain recovered from Hawaii presents phenotypic characteristics typical of Asian strains (M. Gottschalk, 2014, unpubl. data).

Sporadic cases were also reported elsewhere throughout the world, such as those in South America (Argentina, Chile and French Guiana), and Oceania (Australia and New Zealand), but when combined, they only account for 0.8% of all reported cases. As was shown by Wertheim *et al*.,[3] sporadic cases of *S. suis* infections in humans are encountered in most regions where pig rearing is important, with notable exceptions being Mexico and Brazil. In the case of Mexico, even though one study evaluated upper respiratory tract colonization of slaughterhouse workers[101] (see below), no official reports on clinical pig cases or human infections are available, which is alarming, knowing full well that in previous studies, clinical strains of *S. suis* isolated from pig and human cases were included.[74, 102] Despite the lack of available reports, this infection is very common in Mexican farms (M. Gottschalk, 2014, unpubl. information). As such, action is required in order to remedy the troubling situation in this country which has well-developed veterinary diagnostic laboratories. It remains to be seen if the absence of human cases is due to a lack of awareness of *S. suis* as a zoonotic pathogen in these regions when diagnosing bacterial infections, or if these strains are of lower virulence, as with some strains from Canada and the USA.

Table 3 shows the frequency of the clinical manifestations of infection by confirmed serotype, illustrating that the serotypes 2 and 14 are involved in similar proportions in meningitis (50-70%) and septicemia (20-25%). Septic shock was defined as a category comprising sepsis, septicemia, bacteremia, and/or STSLS, while meningitis comprises all meningeal-related symptoms. Serotype 2 infections also represented 2.9% of the cases where other afflictions where diagnosed such as endocarditis, septic arthritis, pneumonia, peritonitis, pulmonary edema, myocarditis and other infections. Serotypes 4 and 21 were isolated from cases of meningitis, serotypes 5 and 16 from cases of peritonitis, and serotype 24 from a case of sepsis.

Why reported strains isolated from human cases are almost exclusively serotypes 2 and 14 is puzzling. While the serotypes 3, 9 and 1/2 are amongst the most highly prevalent in diseased pigs after serotype 2, at least in North America, no human infections due to these serotypes have yet been reported in any country. These differences in virulence between pig and human isolates may be partially due to serotype-specific CPS structural features, for which the CPS structures are still unknown, except for serotypes 2 and 14, and/or due to other serotype-specific bacterial factors involved in pathogenesis. We could speculate that the serotypes 2 and 14 are more virulent than the other serotypes based on the observation that four out of the five cases caused by other serotypes (5, 16 and 24) occurred in patients suffering from cirrhosis, which in turn may have immunocompromised them and made them susceptible to serotypes uncommon in human infections.[88, 89] These differences could also be explained by varying serotype-specific colonization abilities. However, many cases caused by serotypes 2 and 14 have also been described in individuals with predisposing conditions. More research is required in order to understand how differences in virulence of *S. suis* strains occurs and if these differences can be associated with specific serotypes.

Sub-clinically infected humans: Zoonotic potential to workers?

Sub-clinically infected pigs are carriers of *S. suis* mainly in the tonsils.[6] However, this information is not clearly available for humans. Only a handful of studies have investigated *S. suis* subclinical infection in humans as displayed in Table 4, either by serology (antibodies) or by bacterial detection.

Three of these were serological studies, with two using an indirect ELISA with *S. suis* serotype 2 whole bacteria (formalin-inactivated or not) as antigen (Table 4). In New Zealand, the authors examined sera from 70 pig farmers, 96 dairy farmers, 107 meat inspectors and 16 veterinary students.[103] Twenty-one percent of pig farmers, 10% of meat inspectors and 9% of dairy farmers (most of them also raising pigs on their farms) were positive for "anti-*S. suis* serotype 2 antibodies". It is impossible to be certain if these titers were specific for serotype 2 since cross-reacting antibodies with other *S. suis* serotypes and/or with other bacterial species were probably detected. Antibody titers were associated with longer occupational exposure for both pig farming and meat inspection. In the USA, using a similar antigen, sera from 73 pig-exposed and 67 non-pig-exposed adults from Iowa were titrated. Ten percent of pig-exposed workers were found to be positive compared to only one positive individual (1.5%) from the non-pig-exposed group.[104] Once again, the authors concluded that positive titers were associated with either working with pigs or living on a pig farm for more than ten years, and also found that study participants who worked with both finishing and nursery pigs had 8.8 times the odds of having a positive titer when compared to non-exposed participants. The third serological study, from the Netherlands, is interesting since the authors chose to perform Western Blot analysis, with higher specificity, targeting two virulence-related markers from *S. suis* serotype 2 that are widely present among virulent strains in that country, the MRP and EF proteins, in sera from 102 veterinarians and 191 pig farmers.[105] Results showed that 6% of veterinarians were positive for anti-MRP and 2% were positive for anti-EF, while 1% of pig farmers were positive for anti-MRP and 0.5% were positive for anti-EF. It is important to note that these antigens have not been reported to be present in bacterial species other than *S. suis*. These three serological studies, while the titers obtained cannot be directly compared, illustrate the zoonotic potential of *S. suis* based on the presence of antibodies possibly generated through long-term exposure and/or exposure-related subclinical infections. It should be noted that the presence of antibodies does not guarantee a carriage status for positive individuals.

In addition to these serological studies, three studies have directly focused on carriage in the upper respiratory tract of humans by trying to isolate *S. suis* from pharyngeal or tonsil swabs of pig-exposed workers. (Table 4) In Italy, 10 volunteers from slaughterhouse were swabbed and two were found to be positive for *S. suis* serotype 2.[106] In Mexico, the tonsils from 69 slaughterhouse workers were swabbed and 4 were found to be

positive.[101] Of the four strains isolated, one was serotype 2, another was serotype 27, and two were non-typable by serotyping. The virulence of these four strains was also evaluated in mice and it was determined that three of them were mildly virulent (30 to 80% mortality) and one of the non-typable strains was highly virulent (70-90% mortality). Finally, in Germany, the tonsils from 132 meat workers (from either a slaughterhouse or meat processing factory) and 140 controls were swabbed and seven workers were found to be positive carriers of *S. suis* serotype 2 while none of the controls showed positive results. For the seven workers found to be positive carriers, four were retested three weeks later and were confirmed to still be positive carriers, indicating that the bacteria probably remained in the tonsils of healthy individuals for a relatively long period of time, which was also shown to be the case in pigs.[6] However, since these individuals were continuously exposed to pork products, repeated recolonization could not be ruled out.[2] All of the positive workers were employed for an average of 9.9 years ranging from three to 21 years. These three studies regarding the nasopharyngeal carriage all report positive samples in pig-related meat workers and none in the population without occupational exposure to pig. It is also very important to note that of the 13 strains isolated from healthy workers, 84.6% were serotype 2.

Taken together, these studies illustrate the zoonotic potential through long term exposure, either by demonstrating the presence of antibodies towards *S. suis* or by isolating *S. suis* from tonsils or pharynx in pig-exposed workers. It must be noted that the positive rates in the studies of carriers at the upper respiratory tract are probably underestimated since detection of the pathogen was achieved using isolation methods, which are considered as having low sensitivity. Studies using molecular techniques (species-specific PCR) would probably present a significantly higher sensitivity. Exposure to *S. suis* may lead to subclinical infections, induction of antibodies, serious disease, as was the case in the human cases discussed previously, or just an insignificant and transitory atypical colonization of the mucosal membranes within the respiratory route.[107, 108] The annual risk of developing meningitis due to *S. suis* in the Netherlands was estimated to be 3.5/100 000 for slaughterhouse workers, 2.7/100 000 for pig breeders, and 1.2/100 000 for butchers.[14] Compared to the non-exposed general population (0.002/100 000), the risk for slaughterhouse workers is 1 500 times higher. In the United Kingdom, the risk for butchers is even higher.[109] The annual incidence for the occupational group (direct contact with pigs) in Hong Kong was 32/100 000, 350 times higher than that of the general

population (0.09/100 000) and 30 times higher than the homologous group in the Netherlands.[73] In addition, a less dramatic difference between the occupational group and the general population was observed in Hong Kong (350 times higher in Hong Kong versus 1 500 times in The Netherlands). All these differences may be due, at least in part, to a closer contact between people and pork carcasses in Asia.[2] Also, a case-control study conducted in Vietnam from 2006 to 2009 showed that eating high risk dishes common in Vietnam, such as fresh (Tiết Canh) or undercooked blood, tonsils, tongue, stomach, intestines, and uterus from pigs within two weeks prior to admission was the most significant risk factor for *S. suis* infection, followed by exposure to pigs, pork products and preparation of pork in the presence of skin lesions within two weeks prior to admission.[62] The authors also investigated the presence of *S. suis* in pork by-products collected from slaughterhouses and wet/retail markets in Vietnam, and found an 11% contamination rate with *S. suis* serotype 2 in internal organ samples. Unfortunately, they were unable to demonstrate *S. suis* carriage rates in 1 022 healthy individuals or patients without *S. suis* infection using a PCR targeting *S. suis* serotype 2 and 1/2. A PCR for the detection of *S. suis* may have been more sensitive and could possibly have identified potential carriers of other serotypes.[101] The authors suggested that their results, which include 6 positive rectal swabs from patients, strengthen the hypothesis that the gastrointestinal tract may be a route of entry for *S. suis*, which is in agreement with cases following the ingestion of raw pork meat. Similarly, data from Thailand suggest a high incidence rate (6.2/100 000) of *S. suis* infection in the general population in 2010, mostly related to consumption of raw pork products.[110]

Worldwide distribution of sequence types

Global distribution of sequence types

As presented in Fig. 1, different serotype 2 STs predominate in different regions of the world. The ST1 is mostly associated with disease in both pigs and humans in Europe (though the ST20 is important in the Netherlands), Asia (Cambodia, mainland China, Hong Kong, Japan, Thailand, and Vietnam) and Argentina (Tables 5 and 6). Meanwhile, the ST7, responsible for the 1998 and 2005 epidemics, is mostly endemic to mainland China (Tables 5 and 6). North American cases greatly vary from those in Eurasia, with most strains being either ST25 or ST28, two STs also recovered in Thailand and Japan, respectively (Tables 5 and 6). Finally, the ST101 to 104 are endemic to Thailand and

appear to be more and more commonly isolated from human cases, especially the ST104 (Table 6). As such, it can be easily observed that the current distribution of the *S. suis* serotype 2 STs greatly varies throughout the world, though data have only been available for a little over a decade, from only a few countries and mostly only for the serotype 2.

Nevertheless, there is an important issue regarding the serotypes attributed to the different strains typed by MLST. Many of the studies, including most of the strains from the *S. suis* MLST Database, do not specify the method used for serotyping. In other cases, such as the study of King *et al*.[51], the authors did not necessarily confirm the serotypes of the strains used. As explained above, the use of PCR for serotyping of serotypes 2 and 14 strains is an inappropriate method, especially for clinical pig cases, even though cases serotyped using this method were still considered for this part of the review. As such, in the cases where more than one serotype was identified for a single ST, it is possible that the serotypes were misidentified and remain to be confirmed using reference antisera. Hence, it is important to keep in mind these discrepancies when analyzing the distribution of STs, particularly for the clinical pig cases. On the other hand, if confirmed, it would be extremely interesting to study strains of different serotypes sharing the same ST, since capsular switching has not been clearly demonstrated for this pathogen.

Diseased pigs

In contrast to the serotype distribution in diseased pigs (section 6.1), results where the serotype was identified by either serological methods, PCR or unidentified methods and where the ST was determined using the MLST method described by King *et al.*, were taken into consideration for the worldwide distribution of the STs of clinical cases in pigs.[51]

In North America, the majority of MLST studies conducted on *S. suis* strains isolated from diseased pigs have been serotype 2 (Table 5). It was determined that 44% of North American strains are ST25, 51% ST28 and only 5% are ST1.[52] In Canada, the proportion of ST25 and ST28 is similar with 54% and 46%, respectively, but in the USA, 75% of strains were shown to be ST28 and only 10% ST25, while the remaining 15% are ST1.[52] As with North American *S. suis* strains, the majority of European studies have been conducted using serotype 2 strains (Table 5). Most of these studies have demonstrated

that ST1 strains are predominately isolated from diseased pigs in the Netherlands, Spain and the United Kingdom.[51, 77, 111] King *et al*. had already associated the serotype 2 ST1 strains with invasive infections.[51] Nevertheless, many strains of serotype 9 have also been recovered from diseased pigs and typed.[77, 111] In both the Netherlands and Spain, serotype 9 isolates were identified as belonging to the ST16, where they represent 43% of strains in the Netherlands.[77] Unlike some countries in Europe, where the serotype 9 is as important as the serotype 2, most *S. suis* strains isolated from diseased pigs in Asia are serotype 2, representing 90% of cases in mainland China.[112] However, as mentioned above, there are relatively few reports of isolation from diseased pigs in Asia. Of these serotype 2 cases, the predominant STs are ST1, ST7 and ST28 (Table 5). In mainland China, Chen *et al.* demonstrated that 22% of serotype 2 strains are ST1 and 77% are ST7.[112] Meanwhile, the few ST28 strains recovered in that country were mostly associated with cases of pneumonia.[113] Regarding Japan, ST1 and ST28 strains isolated from cases of endocarditis in diseased pigs account for 8% and 76% of serotype 2 strains, respectively.[114]

Human cases

With 97% of all serotype confirmed human cases of *S. suis* infection due to serotype 2, the determination of the STs responsible for these cases becomes highly important (Table 6). Globally, ST1 strains have been described as mostly responsible for *S. suis* serotype 2 human cases, particularly in South America, Europe and Asia, but also one case in North America.[10, 77, 80, 115-117] Nevertheless, multiple other STs have been described worldwide, though these appear to be endemic to certain geographical regions. For example, the ST20 is important in the Netherlands and France but not in the rest of Europe.[77] ST7 strains, to which belong the strains responsible for the 1998 and 2005 Chinese epidemics, were isolated from human patients only in mainland China and Hong Kong.[116, 117] Meanwhile, ST25 and ST28 strains have been particularly associated with human cases in North America and Japan, respectively.[53, 116, 118] The situation is particular in Thailand, where ST1 and ST104 strains are predominant, causing mainly meningitis and non-meningitis cases, respectively. [53, 115, 119] A few cases of ST25, ST28, ST101, ST102 and ST103 have also been described[115]. Interestingly, ST101 to 104 are so far endemic to Thailand only.

Though *S. suis* serotype 14 infections are less frequent in humans than serotype 2 cases, representing 2% of all the serotype-confirmed cases, the number of human infections caused by this serotype appears to be increasing. The ST105 is prevalent in Southeast Asia, particularly in Vietnam and Thailand. In the latter country, 92% of human serotype 14 cases are caused by this ST (Table 6).[10, 120]

Only three human *S. suis* cases, other than those caused by serotypes 2 and 14, have been typed by MLST (Table 6), and all three were described as newly identified STs. Serotype 5, 16 and 24 human cases of infection were identified as ST181, ST106 and ST221, respectively.[88, 89] Interestingly, no data has yet been published on the possible presence of these three newly identified STs for human isolates in diseased pigs.

Association between pig and human sequence types

Albeit no study has yet associated STs of strains isolated from human cases with those from diseased pigs in the same geographical region, it would seem reasonable to suggest that this association exists, particularly for the serotype 2, as most human strains appear to originate from contact with either pigs or pork by-products. In North America, where serotype 2 ST25 and ST28 strains are predominately isolated from diseased pigs, it is of no surprise that human ST25 cases were identified.[52, 116, 121] Interestingly, no cases due to ST28 have been diagnosed in humans. It has been suggested that ST25 strains from pigs are more virulent than their ST28 counterparts,[52] which may explain this situation. Moreover, it is interesting to note that even though ST1 strains account for only 5% of isolates from diseased pigs, a human case caused by an ST1 strain was reported in the USA. [122] It is possible that ST1 strains were imported from pigs from Europe and Asia. Being more virulent than their ST25 and ST28 counterparts, it may be hypothesized that a higher number of cases in pigs due to ST1 strains will appear in the near future in North America. Furthermore, similar STs have been described in some Asian countries for both diseased pigs and humans. For example, in Japan, ST1 and ST28 strains have been isolated from both species, while ST1 and ST7 strains have been identified for human and pig isolates in mainland China.[53, 112, 114, 116, 118, 119, 123] The situation is similar in Europe where serotype 2 ST1 clonal complex strains are predominately isolated from diseased pigs in Spain, Italy, the Netherlands, and the United Kingdom and

where most human cases have also been typed as belonging to this complex.[51, 77, 80, 111, 124, 125]

This association between pig and human strains within a geographical region, though not definitive and currently only reflecting the situation for the serotype 2, confirms the results obtained by Chatellier *et al*. whereby using randomly amplified polymorphic DNA, they concluded that strains isolated from pigs and humans could not be genotypically distinguished and were similar.[126]

Association between sequence types and virulence markers

Presently, the most popular virulence markers used in association with STs are the SLY (*sly*), MRP (*mrp*) and EF (*epf*). It is important to note that although the genotypes of strains belonging to other serotypes have been reported, these factors are mainly associated with serotype 2 strains.

Serotype 2 ST1 strains recovered from both clinical pig and human cases have for the most part been genotyped/phenotyped as *sly*+*mrp*+*epf*+/SLY+MRP+EF+, regardless of the geographic origin (whether it be mainland China, Japan, North America or Spain for both diseased pigs and human cases), which is identical to the serotype 2 ST7 strains isolated from diseased pigs and humans in mainland China.[52, 111, 113, 114, 116, 127] Nevertheless, other important genetic differences vary between ST1 and ST7 strains including the presence of a 89K pathogenicity island in ST7 strains.[128] These ST1 complex strains interestingly differ from not only the human serotype 2 ST104 strains of Thailand which are *sly*+*mrp*-*epf*- but also from the human serotype 2 ST20 strains recovered in the Netherlands that were *epf*-. [77, 115, 116] Also of interest is a human case from Spain where the serotype 2 strain isolated was typed as being a ST3, and presented a large variant of the *mrp*, identified as *mrp** (which has a higher molecular weight), though being *sly*+*epf*+.[125, 129] In Europe, it was determined that strains isolated from diseased pigs belonging to the ST16 complex differ from the ST1 complex strains in being *mrp** rather than *mrp*, while in Spain, the endemic ST123 and ST125 are both *mrp*- and *epf*-.[111] As for ST25 strains isolated from diseased pigs in North America, these were identified as being SLY-MRP-EF-, while the ST28 isolated from North America, mainland China and Japan were *sly*-*mrp*+*epf*- or SLY-MRP+EF-.[52, 113, 114] Though currently not as widely used as the above mentioned virulence

markers, different pili (*srtB*, *srtC*, *srtD*, *srtF*, and *srtG*) have also been associated with different STs. It was identified that ST1 strains isolated from both diseased pigs and human cases of serotype 2 infections in Japan and Thailand were *srtBCD*+ and *srtF*+ but *srtG*-.[53] Meanwhile North American ST25 strains isolated from diseased pigs and human cases were *srtF*- and *srtG*+ and ST28 strains isolated from diseased pigs and human cases from North America and Japan were *srtF*+ and *srtG*+. [52, 114]

It still remains difficult to be certain of the association between STs and virulence markers as being universal, but it appears to be representative of populations within a region and may be a useful diagnostic tool with methods identifying the genotypic or phenotypic presence or absence of the different virulence markers.

Concluding Remarks

S. suis serotype 2 still remains the most isolated serotype and the one most associated with disease in both pigs and humans in most parts of the world. Due to its endemic status in Southeast Asia and the two epidemics it caused in Jiangsu (1998) and Sichuan (2005), China, characterized by STSLS and high mortality rates, this bacterium should no longer be considered merely an important pig pathogen, but also an important and emerging zoonotic agent. Much progress still remains regarding the definition of a “true” *S. suis* serotype and the adoption and use of a complete serotyping system. In this regard, the recent availability of new complete PCR serotyping systems will allow any laboratory in the world to serotype *S. suis* isolates without the need of specific antibodies. The identification of this pathogen by medical and veterinary laboratories significantly varies among countries, and in only a few regions both are well developed. In some important pig producing countries in the Americas, such as Canada, the USA, Mexico, and Brazil, diagnostic laboratories in human medicine should be more aware of the zoonotic potential of *S. suis* due to occupational exposure to pigs and/or pork-derived products. The scientific community should request that the specific serotype of each reported case in humans be identified using acceptable techniques to keep useful epidemiological data. On the other hand, in some countries in Asia where human cases are routinely reported, the number of studies provided by diagnostic laboratories working in veterinary medicine should be significantly increased, since there are almost no data regarding strains recovered from diseased animals. The same applies to European countries which are

important pig producers and from which no data regarding the distribution of serotypes from diseased animals were reported in the last twelve years, hampering the use of species-specific protective bacterins.

Though the MLST data currently available for *S. suis* strains isolated from both diseased pigs and human cases of infection come from different countries throughout the world, there still remains a long way to go before a complete picture of the current situation of *S. suis* can be obtained. Yet, this picture is necessary as it could hint at both dominating and emerging STs and could possibly help identify epidemic strains quickly and to categorize the zoonotic potential of specific STs. With the dissemination of information about STs across the internet, particularly thanks to the *S. suis* MLST Database, it would be important to set up a complete and reliable serotype identification of the different strains added. Albeit this database is a great tool that facilitates the sharing of knowledge, the lack of many details pertaining to each strain such as the serotype, method used for serotyping, health status of the host, and host itself, limit the full potential of the STs as was demonstrated in this review where many strains could not be included. Furthermore, in the absence of identification of the serotyping method, it is impossible to confirm if the serotype indicated is correct. Nevertheless, the global distribution of *S. suis*, particularly serotype 2 strains greatly varies, an aspect that could be useful in developing diagnostic and preventive tools specific for a particular geographical distribution. Despite the fact that studies have recently associated virulence markers and profiles with given STs, the biological significance of these associations still needs to be studied, as only a handful of studies have compared STs from different geographical origins. In fact, some STs found in North America and described as low virulent are frequently isolated from patients in Asia. There is an urgent need to compare virulence properties of strains of similar STs from different geographical origins. Finally, though still tentative, the possibility of associating strains from diseased pigs and human cases in a given region, as determined based on serotypes and STs, would provide further epidemiological support for the zoonotic potential of this pig pathogen, demonstrating the need for proper hygiene practises in order to reduce the risk of zoonotic infections. With more and more laboratories using a complete serotyping system and MLST as a tool for *S. suis* classification, we will one day have the complete picture regarding the *S. suis* distribution.

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Tables

Table 1. Worldwide distribution of serotypes for reported clinical *S. suis* cases of infection in pigs by country from January 1st, 2002 to December 31st, 2013

Country	Clinical Cases	Predominant Serotypes[1] (Frequency in %)			References
WORLDWIDE	4711	2 (27.9%)	9 (19.4%)	3 (15.9%)	
NORTH AMERICA	3 162 (67.1%)	2 (24.3%)	3 (21.0%)	1/2 (13.0%)	
Canada	3 065	2	3	1/2	[25, 29]
United States	97	3	2	7	[130]
SOUTH AMERICA	125 (2.7%)	2 (57.6%)	1/2 (9.6%)	14 (8.8%)	
Brazil	125	2	1/2	14	[131, 132]
ASIA	659 (14.0%)	2 (44.2%)	3 (12.4%)	4 (5.6%)	
Mainland China	639	2	3	4	[30, 112, 133, 134]
South Korea	20	3	4	2, 8, 22	[68]
EUROPE	765 (16.2%)	9 (61.0%)	2 (18.4%)	7 (6.7%)	
Netherlands	99	9	2	7	[77]
Spain	666	9	2	7	[56, 75, 76]

[1]Only the three most predominant serotypes, which were identified by coagglutination (or an equivalent method using reference antisera), are shown in this table.

Table 2. Worldwide distribution of reported clinical *S. suis* cases of infection in humans by country until December 31st, 2013

Country	Reported Cases	Confirmed Serotypes[1]			Unconfirmed[3] or Unknown Serotypes[4]	References
		2	14	Others[2]		
WORLDWIDE	1 642	1 227 (74.7%)	33 (2.0%)	5 (0.3%)	377 (23.0%)	
NORTH AMERICA	8 (0.5%)	7 (87.5%)	1 (12.5%)	0	0	
Canada	5	4	1	-	-	[7, 20, 100, 135]
United States	3	3	-	-	-	[121, 122, 136]
SOUTH AMERICA	9 (0.5%)	2 (22.2%)	0	1 (11.1%)	6 (66.7%)	
Argentina	4	1	-	1	2	[64, 67, 137, 138]
Chile	4	-	-	-	4	[65, 66]
French Guiana	1	1	-	-	-	[139]
ASIA	1 481 (90.2%)	1 133 (76.5%)	29 (2.0%)	3 (0.2%)	316 (21.3%)	
Cambodia	13	13	-	-	-	[140]
Mainland China	245	245	-	-	-	[16, 90, 91, 141-143]
Hong Kong	69	53	-	-	16	[12, 73, 92, 93, 107, 144-146]
Japan	11	10	-	-	1	[118, 147-150]
Laos	1	-	-	-	1	(Unpubl.[5])
Philippines (USA)	1	-	-	-	1	[94]
Singapore	3	-	-	-	3	[151-153]
South Korea	4	-	-	-	4	[154-157]
Taiwan	7	2	2	-	3	[21, 22, 158]
Thailand	553	292	21	2	238	[11, 13, 23, 26, 45, 63, 88, 110, 115, 119, 120, 159-169]

Vietnam	574	518	6	1	49	[10, 62, 89, 170-174]
EUROPE	140 (8.5%)	84 (60.0%)	3 (2.1%)	1 (0.7%)	52 (37.1%)	
Austria	1	-	-	-	1	[175]
Belgium	4	3	-	-	1	[176-179]
Croatia	2	-	-	-	2	[44]
Denmark	8	6	1	-	1	[9, 98, 180-183]
France	19	8	-	-	11	[95-97, 184-198]
Germany	9	8	-	-	1	[19, 86, 108, 199-204]
Greece	2	-	-	-	2	[205, 206]
Ireland	1	-	-	-	1	[207]
Italy	3	2	-	-	1	[208-210]
Netherlands	51	39	1	1	10	[14, 211-219]
Poland	1	-	-	-	1	[220]
Portugal	1	-	-	-	1	[221]
Serbia	5	-	-	-	5	[222]
Spain	13	4	-	-	9	[125, 223-233]
Sweden	1	1	-	-	-	[234]
United Kingdom	19	13	1	-	5	[99, 235-248]
OCEANIA	4 (0.2%)	1 (25.0%)	0	0	3 (75.0%)	
Australia	3	-	-	-	3	[249, 250]
New Zealand	1	1	-	-	-	[85]

[1]Serotypes were identified by coagglutination (or an equivalent method using reference antisera) or by PCR specific reaction for serotype 2 (and 1/2) or for serotype 14 (and 1).

[2]Serotypes other than serotypes 2 and 14. See main text and Table 3 for details.

[3]Serotypes were determined based on biochemical identification so are reported as "unconfirmed"

[4]Strain serotype was not mentioned in the publication and is thus considered as "unknown".

[5](H. Wertheim, 2014, personal communication)

Table 3. Clinical manifestations of reported clinical *S. suis* cases of infection in humans by serotypes confirmed by coagglutination

Confirmed Serotypes	Reported Cases	Clinical Manifestations			
		Meningitis	Septic Shock[1]	Other[2]	Unknown[3]
2	867	590 (68.1%)	233 (26.9%)	25 (2.9%)	19 (2.2%)
14	28	14 (50.0%)	6 (21.4%)	-	8 (28.6%)
4	1	1	-	-	-
5	1	-	-	1	-
16	1	-	-	1	-
21	1	1	-	-	-
24	1	-	1	-	-

[1]Septic shock includes bacteremia, sepsis, septicemia, and streptococcal toxic shock-like syndrome cases.
[2]Other clinical manifestations such as endocarditis, septic arthritis, pneumonia, peritonitis, pulmonary edema, myocarditis, etc.
[3]Clinical manifestations were either not specified or could not be associated with a particular serotype.

Table 4. Studies exploring human carriage and exposure to *S. suis* in risk groups

Study (Reference)	Technique Employed/ Target	Risk Groups	Positive Carriage or Exposure [Positive/Tested]	Serotypes Found
Serological				
New Zealand, 1989 ([103])	Indirect ELISA / Serotype 2 whole bacteria	Pig farmers	15/70 (21.4%)	-
		Meat inspectors	11/107 (10.3%)	
		Dairy farmers	9/96 (9.4%)	
Netherlands, 1999 ([105])	Western Blot / Serotype 2 MRP/EF	Veterinarians	MRP: 6/100 (6.0%) EF: 2/100 (2.0%)	-
		Pig farmers	MRP: 2/190 (1.1%) EF: 1/190 (0.5%)	
Upper Respiratory Tract Colonization (Isolation)				
Italy, 1989 ([106])	Tonsil swabs	Slaughterhouse workers	2/10 (20%)	2
Mexico, 2001 ([101])	Tonsil swabs	Slaughterhouse workers	4/69 (5.8%)	2, 27, NT[1]
Germany, 2002 ([108])	Pharyngeal swabs	Slaughtering and meat processing workers	7/132 (5.3%)	2

[1]Non-typable strain (NT).

Table 5. Determined sequence types of reported clinical *S. suis* cases of infection in pigs from January 1st, 2002 to December 31st, 2013

Country	Serotypes[1]	ST	ST Complex	Number of Cases	References
NORTH AMERICA					
Canada	2	1	1	2	[124]
		25	25	28	[52, 124]
		28	28	18	[52]
		145	25	1	MLST Database
	23	80	Unrelated	1	[51]
	24	68	Unrelated	1	[51]
	25	69	Unrelated	1	[51]
	27	72	Unrelated	1	[51]
	28	75	Unrelated	1	[51]
	29	92	Unrelated	1	[51]
	30	77	Unrelated	1	[51]
	31	70	Unrelated	1	[51]
	Not Specified	13	Unrelated	Not Specified	[51]
United States	2	1	1	3	[52]
		25	25	28	[52]
		28	28	33	[52]
EUROPE					
Denmark	4	54	53/54	1	[51]
	5	53	53/54	1	[51]
	6	55	Unrelated	1	[51]
	8	87	87	1	[51]
	9	82	Unrelated	1	[51, 124]
	10	78	Unrelated	1	[51]
	11	91	Unrelated	1	[51]
	12	74	Unrelated	1	[51]
	13	71	Unrelated	1	[51, 123]
	16	73	Unrelated	1	[51]
Finland	2	37	Unrelated	1	[51]
	Not Specified	27	27	Not Specified	[51]
		38	Unrelated	1	[51]

France	2	1	1	4	[116]
		140	Unrelated	1	MLST Database
		141	1	1	MLST Database
		142	28	1	MLST Database
		143	1	1	MLST Database
		144	1	1	MLST Database
		229	25	1	MLST Database
		230	25	1	MLST Database
	14	231	Unrelated	1	MLST Database
	15	81	Unrelated	1	MLST Database
Germany	1/2	100	Unrelated	1	[251]
	2	1	1	5	[251]
		25	25	1	[251]
		28	28	2	[251]
		97	Unrelated	1	[251]
	3	95	Unrelated	1	[251]
	7	29	29	7	[124, 251]
		89	16	1	[251]
	9	93	Unrelated	1	[251]
		96	Unrelated	1	[251]
		98	16	2	[251]
		99	16	1	[251]
Italy	2	1	1	1	[80]
	9	138	Unrelated	1	MLST Database
Netherlands	1	1	1	4	[77, 124]
		13	13/149	2	[51, 77]
		149	13/149	1	[77]
		156	1	1	[77]
	1/2	1	1	1	[77, 124]
	2	1	1	21	[77, 124]
		20	147	19	[77]
		29	29	1	[77]
		134	1	1	MLST Database
		146	1	1	MLST Database

		3	15	16	1	[77]
			35	27	1	[51]
		4	17	147	3	[77]
		7	29	29	5	[77]
			135	29	1	MLST Database
			136	16	1	[77]
			150	Unrelated	1	[77]
			153	Unrelated	1	MLST Database
			218	Unrelated	1	[77]
		8	87	87	1	[77]
			198	16	1	[77]
		9	1	1	2	[77, 124]
			15	16	1	[77]
			16	16	38	[77]
			136	16	2	[77]
			148	1	1	[77]
			151	16	1	[77]
			152	16	1	MLST Database
			154	16	1	MLST Database
			155	16	1	[77]
			182	Unrelated	1	[77]
			184	Unrelated	1	MLST Database
			189	Unrelated	1	[77]
			220	Unrelated	1	[77]
		15	81	Unrelated	1	[51]
		Not Specified	183	Unrelated	1	MLST Database
Spain		1	1	1	2	[111]
		1/2	1	1	2	MLST Database [111] MLST Database
			28	28	1	MLST Database
			64	94	1	[51]
		2	1	1	31	[111]
			5	1	1	[51]

		27	27	1	MLST Database
		28	28	1	MLST Database
		86	1	1	MLST Database
		124	1	1	[111]
	3	14	147	1	[51]
		15	16	1	[51]
		27	27	1	MLST Database
		89	16	1	[51]
	4	16	16	1	[51, 124]
	5	17	147	1	[51]
	9	16	16	2	[51, 124]
		59	123	1	[51]
		123	123	8	[111]
		125	123	9	[111]
		361	Unrelated	1	MLST Database
		367	Unrelated	1	MLST Database
	14	1	1	1	[111] MLST Database
	15	65	Unrelated	2	[51]
	27	65	Unrelated	2	[51]
United Kingdom	1	1	1	5	[116, 124] MLST Database
		12	11	1	[51]
		13	13/149	1	[51]
	1 or 14	10	1	1	[51]
	2	1	1	37	[116, 124] MLST Database
		2	1	5	[51]
		9	1	1	[51]
		25	25	7	MLST Database
		28	28	5	MLST Database
		29	29	1	MLST Database
		30	28	1	MLST Database
	3	27	27	2	[51]
		29	29	1	MLST Database

		31	28	2	[51]
		33	27	1	[51]
		42	Unrelated	1	[51]
	4	23	87	3	[51]
	5	39	Unrelated	1	MLST Website
		44	Unrelated	1	[51]
	7	29	29	6	MLST Database
		34	225	1	[51]
		83	29	1	[51]
	8	1	1	1	MLST Database
	9	46	Unrelated	1	[51]
		48	Unrelated	2	[51]
		50	Unrelated	1	[51]
	11	88	Unrelated	1	[51]
	14	1	1	14	MLST Database
	15	43	43/52	1	[51]
		45	Unrelated	1	[51]
		49	Unrelated	1	[51]
		52	43/52	1	[51]
	16	41	Unrelated	2	[51] MLST Website
		47	Unrelated	1	[51]
	28	21	87	1	[51]
	Not Specified	40	Unrelated	1	MLST Website
		51	Unrelated	1	MLST Website
		90	Unrelated	1	MLST Website
ASIA					
Mainland China	2	1	1	49	[112, 113, 127]
		7	7	216	[112, 113, 116, 123, 127, 252]
		25	25	1	[113]
		28	28	31	[112, 113, 127]
		86	1	1	[127]
		117	27	27	MLST Database

	162	28	1	[127]
	223	1	1	MLST Database
	228	7	1	MLST Database
	242	1	1	MLST Database
	244	7	1	MLST Database
	245	28	1	MLST Database
	289	1	1	[112]
	290	Unrelated	1	MLST Database
	352	Unrelated	1	MLST Database
	353	Unrelated	1	MLST Database
	354	Unrelated	1	MLST Database
	355	Unrelated	1	MLST Database
	418	Unrelated	1	MLST Database
	419	Unrelated	1	MLST Database
3	224	Unrelated	1	MLST Database
7	129	29	1	MLST Database
	225	225	1	MLST Database
	335	Unrelated	1	MLST Database
	420	Unrelated	1	MLST Database
9	222	Unrelated	1	MLST Database
	226	226/227	1	MLST Database
	227	226/227	1	MLST Database
	239	239/241	1	MLST Database
	241	239/241	1	MLST Database
	417	Unrelated	1	MLST Database
11	260	Unrelated	1	MLST Database
	263	Unrelated	1	MLST Database
13	262	Unrelated	1	MLST Database
27	258	Unrelated	1	MLST Database
31	261	27	1	MLST Database
	265	27	1	MLST Database
Not Specified	29	29	1	[112]
	118	Unrelated	1	[112]
	156	1	2	[112]

		264	Unrelated	1	MLST Database
		266	Unrelated	1	MLST Database
		267	Unrelated	1	MLST Database
		303	Unrelated	1	MLST Database
		383	Unrelated	2	MLST Database
		421	Unrelated	1	MLST Database
		422	Unrelated	1	MLST Database
Japan	1	1	1	1	[53, 114]
	2	1	1	5	[53, 114]
		28	28	48	[53, 114]
		324	28	1	[114]
	3	108	94	1	[53]
		117	27	1	[53]
	7	29	29	1	[53]
		118	Unrelated	1	[53]
	11	108	94	1	[53]
Vietnam	9	390	Unrelated	1	MLST Database

[1]Serotypes were identified by coagglutination (or an equivalent method using reference antisera), by PCR or, sometimes, by undefined methods.

Table 6. Determined sequence types of reported clinical *S. suis* cases of infection in humans from January 1st, 2002 to December 31st, 2013

Country	Serotype[1]	ST	ST Complex	Number of Cases	References
NORTH AMERICA					
Canada	2	25	25	3	[53], [116]
	14	6	1	1	[51]
United States	2	1	1	1	[53]
		25	25	1	[121]
SOUTH AMERICA					
Argentina	2	1	1	1	(Unpubl.2)
French Guiana	2	1	1	1	[139]
EUROPE					
France	2	20	147	2	[53], [116]
Italy	2	1	1	1	[80]
		134	1	2	[80], [208]
Netherlands	2	1	1	14	[77], [124]
		20	147	11	[77], [124]
		134	1	1	[77]
		146	1	1	[77]
	14	6	1	1	[51]
Spain	2	3	1	1	[125]
United Kingdom	2	1	1	1	[53]
	14	2	1	1	MLST Database
ASIA					
Cambodia	2	1	1	13	[140]
Mainland China	2	1	1	11	[116], [252]
		7	7	210	[116], [124], [252]
	14	1	1	1	[123]
Hong Kong	2	1	1	14	[51], [117]
		9	1	12	[51], [117]
		25	25	1	[117]
Japan	2	1	1	7	[53], [118]
		28	28	1	[118]

Thailand	2	1	1	123	[53]
		25	25	17	[53], [115]
		28	28	4	[53], [115]
		101	225	1	[119]
		102	25	2	[53]
		103	25	6	[53], [115]
		104	225	45	[53]
		126	1	3	[115]
	5	181	Unrelated	1	[88]
	14	11	11	1	[53]
		105	1	19	[110, 120]
		127	1	1	[120]
	24	221	221/234	1	[88]
Vietnam	2	1	1	56	[10, 124]
		107	1	1	[10]
	14	105	1	1	[10]
	16	106	Unrelated	1	[89]

[1]Serotypes were identified by coagglutination (or an equivalent method using reference antisera) or by PCR. However, it is impossible to distinguish between serotypes 1 and 14 and serotypes 1/2 and 2 by PCR, so serotypes remain to be confirmed.
[2](M. Gottschalk, 2014, unpubl. data)

Figures

Figure 1. Worldwide distribution of the most important *S. suis* serotype 2 sequence types (STs) isolated from both clinical pig and human cases of infection.

ANNEXES - ARTICLE III

Atypical *Streptococcus suis* in man, Argentina

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Je suis co-auteur de l'article. J'ai participé à la recension de la littérature.

Abstract

Streptococcus suis is an important swine and zoonotic agent. We report for the first time the isolation of a *S. suis* serotype 21 from a patient, who presented peritonitis. Although this serotype has already been recovered from diseased pigs, it is rather usually isolated from clinically healthy pigs. The patient denied any recent occupational or even occasional contact with swine or other animals and had no history of eating raw or undercooked pork. Atypical serotypes of *S. suis* rarely present in humans may be difficult to identify biochemically and may sometimes be under-diagnosed.

Introduction

Streptococcus suis is a major swine pathogen responsible for important economic losses to the swine industry worldwide [1]. It is also an emerging zoonotic agent of meningitis, endocarditis and streptococcal toxic shock-like syndrome, among other infections [2]. Since the recent recognition of the high prevalence of *S. suis* human disease in South East and East Asia, the interest of the scientific community on this pathogen has significantly increased [3]. Among the thirty-five serotypes that have been described based on capsular antigens, the serotype 2 is the most frequently isolated from diseased pigs and humans in most countries [1]. Human cases of *S. suis* infections have been documented in several European and Asian countries as well as in North and South America, Australia and New Zealand [2]. In Western countries, *S. suis* infections in humans have usually been restricted to workers in close contact with pigs or pork by-products. However, in South East and East Asia, this pathogen affects not only that population at higher risk but also the general population, and it represents a significant public health concern [3]. In some Asian countries, the backyard type of production of swine as well as open meat markets are very popular. In humans, it is believed that people can become infected through skin lesions handling infected pork meat [2]. However, it has been recently suggested that the oral route of infection may predominate in countries where dishes are prepared with raw pork meat and/or blood, such as Thailand and Vietnam [4]. On the other hand, in some Latin-American countries, although the backyard type of production of pigs is also common, very few reports on human infection are available [5, 6]. In the present case we report a peritonitis case caused by *S. suis* serotype 21 in a patient in Argentina, South America, without known contact with swine or pork-derived products. It is the first time that this atypical serotype is reported in humans.

Case report

A 62 year old man from Paraná, province of Santa Fe, Argentina, who had a history of tobacco and alcohol abuse was admitted to the hospital as an emergency case with the symptoms of acute abdomen. Ten days previously to his admission to the hospital, the patient developed abdominal distention accompanied by a significant upper abdominal pain. The patient's family reported he had been suffering from gastrointestinal bleeding four days before admittance and he was suspected to be diabetic.

On admission, physical examination, chest and abdominal radiography, abdominal ultrasonography, routine serum laboratory tests (including complete white blood cell count, standard liver and renal function tests), ascitic fluid cell count, ascitic and blood cultures and fresh urine sediment were performed. Physical examination revealed jaundice, hepatosplenomegaly and ascites. On neurologic examination the patient was vigil and disoriented and his vital signs were stable. Temperature was 38,9 ºC, pulse rate 130 beats per minute, and blood pressure 110/70 mm Hg. Laboratory findings were 2,900 leukocytes/μL (70% neutrophils); 94,000 platelets/μL; hemoglobin concentration serum 13,20 g/dL; glucose 195 mg/dL; blood urea nitrogen 42 mg/dL; creatinine 0,96 mg/dL; serum bilirrubin 3,01 mg/dL, ALT 35 U/L; AST 70 U/L; serum albumin 2,66 g/dL, and extension of prothrombin time to 22 sec.

Spontaneous bacterial peritonitis was suspected. Abdominal paracentesis was performed resulting in evacuation of turbid milky fluid. The analysis of ascitic fluid demonstrated a protein level of 16 g/L; 1,340 cells/uL (90% neutrophils); a lactate dehydrogenase level of 221 U/L and amylase level of 34 U/L. Samples of blood and ascetic fluid (10 mL each) were inoculated in aerobic and anaerobic blood culture bottles at the patient's bedside. Gram staining was performed and no organisms were observed.

Empirical treatment with intravenous ceftriaxone (2g/day) was started with the diagnosis of spontaneous bacterial peritonitis associated with liver cirrhosis. After 48 h of incubation, blood and ascitic cultures bottles were positive and an aliquot was plated into sheep blood agar and chocolate agar and incubated at 35 ºC in a 5% CO_2 enriched atmosphere. After 24 h incubation, cultures demonstrated growth of α-hemolytic streptococci. An API Strep (bioMérieux Marcy l'Etoile, France) was inoculated according to the manufacturer´s recommendations and after 48 h incubation gave a profile number of 0240453 and an identification of *Streptococcus pneumoniae* with 58,7% probability, *Streptococcus suis* with 20,7% probability and an "unacceptable identification" confidence level.

Species identification was performed by sequence analysis of 16S rRNA gene as described [7]. Serotyping was performed by co-agglutination test with all reference antisera as previoulsy described [8]. The isolate was confirmed by polyvalent and monovalent reagents to be a serotype 21.

Antimicrobial drug susceptibility testing, performed according to guidelines of the Clinical and Laboratory Standards Institute, indicated susceptibility to penicillin, ceftriaxone, chloramphenicol, tetracycline, erythromycin and vancomycin. Diagnostic paracentesis was repeated every 2 days. Infection was considered resolved when all signs of infection had disappeared, polymorphonuclear cell count in ascitic fluid had decreased to a level less than 250 cells/uL, and ascitic fluid cultures were negative for bacterial growth. Antibiotic therapy was maintained for 48 hours after resolution of infection. The patient denied any recent occupational or even occasional contact with swine or other animals and had no history of eating raw or undercooked pork.

Discussion

S. suis is considered an emerging zoonotic agent. Although meningitis, endocarditis and, more recently, toxic shock-like syndrome are commonly described in humans, other types of infections have also been reported [2]. More precisely, spontaneous peritonitis as that described in the present report have previously been reported, mainly in Vietnam and Thailand [9-12]. One case was caused by a serotype 5, one by serotype 16 and serotype 2 was involved in the two other cases. In the present case report, and for the first time, a serotype 21 was isolated from a case of human peritonitis. The reference strain of this serotype had originally been isolated from the tonsils of a clinically healthy pig [13]. In fact, it has been shown that this serotype is predominant in tonsils of clinically healthy pigs [13]. However, sixteen strains have also been recovered from ill pigs between 2008 and 2011 in Canada [14]. This indicates that this serotype is potentially virulent.

The only two previous reports of *S. suis* isolated from humans in Latin America are, as the present case, from Argentina [5, 6]. Additional strains of *S. suis* serotype 2 have been isolated from patients in this country (unpublished data). Since the swine production in Argentina is relatively small compared to other countries (such as Brazil and Mexico, for example), the significant isolation rate of this pathogen in this Latin-American country is probably the consequence of a good surveillance system and good knowledge of the pathogen by local diagnostic laboratories. To note that half of serotypes other than serotype 2 (and especially serotypes 9 to 22) are usually not identified as *S. suis* by rapid multitest identification system [15], such as the API 20 Strep, as it was the case of the present report. Direct PCR detection

of *S. suis* serotype 2 (the most common serotype recovered from humans) [16] would have also misidentified this case.

Interesting, the association between human *S. suis* infection and occupational exposure has been largely reported in Europe and in the few cases of North America [3]. In Vietnam, the proportion of patients reported to have occupational exposures was lower than reported in European patients but it remained an important independent risk factor [4]. In the present case report, the patient could not remember any contact with swine or pork-derived products. A similar case was also described in Italy [17]. A patient with *S. suis* infection may be unaware or have no memory of previous exposure to animals. Latent infection with a reactivation many years later has also been described [18]. *S. suis* may become an opportunistic pathogen in persons who are under stress or who have immunodeficiency, and it has been increasingly isolated from mammalian species other than pigs, from birds, and from the environment. The patient in this case has a history of alcohol consumption, which has been described as a predisposal factor [4].

As a conclusion, we described the first case of *S. suis* serotype 21 infection in humans. Diagnostic laboratories should be aware that different serotypes of *S. suis* may be involved in human disease and that their identification requires different and complementally approaches, and absence of awareness recent known exposure to pigs or pork-derived products is possible.

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ANNEXES - ARTICLE IV

Streptococcus suis serotype 2 strains isolated in Argentina (South America) are different from those recovered in North America and present a higher risk for humans

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Je suis co-auteur de l'article. J'ai participé à la recension de la littérature.

Abstract

Introduction: *Streptococcus suis* serotype 2 is an important swine pathogen and emerging zoonotic agent causing meningitis and septicemia/septic shock. Strains are usually virulent (Eurasia) or of intermediate/low virulent (North America). Very few data regarding human and swine isolates from South America are available.

Case Presentation: Seventeen new human *S. suis* cases in Argentina (16 serotype 2 strains and a serotype 5 strain) are reported. Alongside, 14 isolates from pigs are analyzed: 12 from systemic disease, one from lungs and one from tonsils of a healthy animal. All human serotype 2 strains and most swine isolates are sequence type (ST) 1, as determined by multi-locus sequence typing and present a *mrp*$^{+}$/*epf*$^{+}$/*sly*$^{+}$ genotype typical of virulent Eurasian ST1 strains. The remaining two strains (recovered from swine lungs and tonsils) are ST28 and possess a *mrp*$^{+}$/*epf*$^{-}$/*sly*$^{-}$ genotype typical of low virulence North American strains. Representative human ST1 strains as well as one swine ST28 strain were analyzed by whole genome sequencing and compared with genomes from GenBank. ST1 strains clustered together with three strains from Vietnam and this cluster is close to another one composed of 11 strains from the United Kingdom (UK).

Conclusion: Close contact with pigs/pork products, a good surveillance system, and the presence of potentially virulent Eurasian-like serotype 2 strains in Argentina may be an important factor contributing to the higher number of human cases observed. In fact, Argentina is now fifth among Western countries regarding the number of reported human cases after the Netherlands, France, the UK, and Poland.

Introduction

Streptococcus suis is an important swine pathogen mainly causing septicemia, meningitis, and arthritis [1]. In addition, it is an emerging zoonotic agent responsible for septicemia with or without septic shock and meningitis [1]. During the last decade, the number of human cases due to *S. suis* has increased, and while most sporadic human cases of infection occur following close occupational contact with pigs/pork products, particularly in Western countries, important outbreaks have been recorded in Asia. In the latter, the general population is at risk due to consumption of raw pork products as part of traditional dishes. Indeed, *S. suis* infections are considered among the most frequent causes of adult meningitis in Asia [2].

Although different serotypes have been described, serotype 2 is the most commonly isolated from diseased pigs; it also represents more than 95% of human cases worldwide [3]. However, important differences in virulence of isolates within this serotype have been described [4]. It has been shown that Eurasian strains generally present a higher virulence potential than those from Canada and the United States of America (USA), two countries that together are the second most important swine producers worldwide after China [1].

Multilocus sequence typing (MLST) has been used worldwide to determine the sequence types (STs) of *S. suis* strains, thus allowing to gather further information regarding their genetic diversity and evolution [3]. More recently, studies have begun combining data obtained from MLST with the presence or absence of different *S. suis* serotype 2 virulence-associated markers such as the muramidase released-protein (*mrp*), the extracellular protein factor (*epf*), and the suilysin (*sly*), to compare ST data with pathotypes. ST1 strains, which are usually more virulent and possess a genotypic $mrp^{+}/epf^{+}/sly^{+}$ pathotype profile, are mostly present in Eurasia, whereas ST25 (intermediate virulence) and ST28 (low virulence) strains with a $mrp^{-}/epf^{+/-}/sly^{-}$ profile predominate in North America [3, 5]. Interestingly, human cases reported in North America represent less than 0.5% of described cases worldwide, compared to more than 8% in European countries, confirming differences in virulence of *S. suis* serotype 2 isolates [3, 4]. Very few data are available concerning serotype 2 strains recovered from diseased pigs in South America (Doto et al., 2016; Martinez et al., 2003), where the swine industry is either well-developed (such as in Brazil) or in clear expansion (such as in Argentina).

Herein, we describe new human cases of *S. suis* recovered from diseased patients in Argentina. We have also studied strains isolated from diseased pigs in this country. MLST and pathotype characteristics of strains were determined and representative strains recovered from humans were further analyzed by whole genome sequencing (WGS).

Case Report

We present the characteristics of 33 *S. suis* strains isolated in Argentina (Table 1). We report and analyze 17 new human cases of *S. suis* in Argentina isolated between 1995 and 2016. An additional strain from a previously reported human patient [5] was also included. All but one of the human strains were isolated from cases of meningitis; the remaining strain was recovered from a patient presenting septic arthritis. Of the 17 cases of meningitis, 10 strains were isolated exclusively from the cerebrospinal fluid (CSF), while the remaining strains were isolated from both CSF and blood. In the latter cases, only one isolate per patient was further characterized. Three isolates were recovered from female patients and thirteen patients, from rural areas, recalled having had contact with swine, pork or pork-derived products. Four patients did not recall this information, whereas one patient assured having no such contact.

We also studied 14 *S. suis* serotype 2 strains recovered from pigs as pure cultures in non-related farms: 12 were from systemic disease (meningitis, arthritis or endocarditis). In addition, a strain isolated from the lungs of an animal with pneumonia and another strain isolated from the tonsils of a clinically healthy animal were included.

Investigations

Identification of *S. suis* by PCR and multiplex PCR was performed as previously described [6]. Strains positive for serotype 2 were further differentiated from serotype 1/2 by coagglutination test [7]. All but one strain from human cases of infection were serotype 2; the remaining strain (only fatal case) was characterized as serotype 5. No further characterization of this strain was completed.

To evaluate pathotype based on the presence or absence of the traditional virulence factor genes *mrp*, *epf*, and *sly*, specific PCRs were performed on serotype 2 strains as previously described [8]. Interestingly, all serotype 2 strains isolated from humans present the *mrp*$^+$/*epf*$^+$/*sly*$^+$ profile usually associated with virulent Eurasian serotype 2 strains [3]. The *epf*$^+$

genotype obtained referes to the variant encoding the 110 kDa protein [9]. All but two strains recovered from pigs also presented this pathotype. The two exceptions were the strain isolated from lungs and that recovered from tonsils of a clinically healthy animal, which both presented the *mrp*$^{+}$*/epf*$^{-}$*/sly*$^{-}$ profile usually found in North America.

Results from MLST studies performed as previously described [10] confirm such results. All strains presenting the *mrp*$^{+}$*/epf*$^{+}$*/sly*$^{+}$ pathotype were ST1, similar to most virulent Eurasian strains [3]. The two swine strains presenting the *mrp*$^{+}$*/epf*$^{-}$*/sly*$^{-}$ profile (one recovered from lungs and the other from tonsils of a clinically healthy animal) were ST28, which is usually associated with low virulent strains in Canada and the USA [3].

Since almost all Argentinean serotype 2 strains from humans and diseased pigs included in this study were ST1, we further compared nine available human ST1 strains and one porcine ST28 strain by WGS. Phylogenetic analyses were performed and compared with the available assembled GenBank genome sequence read data of 26 ST1 strains from the United Kingdom (UK; 13 strains), the Netherlands (1 strain, reference strain), China (3 strains), and Vietnam (9 strains) (Table 2). In addition, a Chinese serotype 8 strain was also included as a control outsider. The sequences of strain P1/7 (UK), GZ1 (China), BM407 (Vietnam), and R735 (the Netherlands) are completely finished. The methodology used was that previously described [11]. For each strain, a 500 bp library was constructed and then sequenced using the Illumina Hiseq 4000 system (Illumina, San Diego, CA, USA) to produce 150 bp paired-end reads. The high throughput read data were mapped to the reference genome of *S. suis* ST1 strain P1/7 (Accession number: NC_012925) using SOAP2 and SNPs detected using SOAPsnp v1.03 [12]. For the assembled genome sequences, SNPs were called using MUMmer v3.23 [13]. These SNP patterns were distilled and concatenated using an automatic pipeline as previously described [11]. Based on this, a phylogenetic tree was constructed using the maximum likelihood method and GTRGAMMA substitution model via RAxML software (version 7.2.8) with 1000 bootstrap replications [14]. (Fig. 1). The sequencing data were deposited in the GenBank database (Accession number SRP079937).

Approximately 1.36 to 1.63 Gb high-quality read data was obtained for each strain and covered 680 to 858 (769 ± 45) fold of the complete genome of P1/7. A phylogenetic study showed that all of the ST1 strains clustered tightly together when compared to the ST28 strain P706 and

ST308 (RC1, China) strain (Fig. 1(a)). Within the ST1 cluster, the 9 Argentinean ST1 strains tested clustered together with 3 other strains from Vietnam, with this cluster being close to another one composed of 11 strains from the UK (Fig. 1(b)). These two clusters are relatively far away from other European (UK and the Netherlands) and Asian (China and Vietnam) strains.

Discussion

It has been clearly shown that serotype 2 strains isolated in North America (Canada and USA) are phenotypically and genotypically highly different from those recovered in Europe and Asia [15]. Preliminary results with archetypal strains [15], as well as more recent results with field strains [4, 16], showed that North American *S. suis* serotype 2 strains are, in general, of low virulence. It has been strongly suggested that the relatively low number of human cases in both Canada and the USA (which together represent the second largest swine producing region worldwide after China) are probably due to the lower virulence properties of *S. suis* ST25 and ST28 strains that predominate in these countries.

Very few data are available regarding strains isolated in South America. A few reports on serotype 2 strains recovered from diseased pigs in Brazil have been published [17-19], though no human case has yet been reported. Human cases have been reported in Argentina [5, 20, 21] and, only once, in Chile [22], but strains were not further characterized. Although not a traditional pork producing country, Argentina has significantly increased its swine production in the last few years, having presently more than 4,000,000 pigs (http://www.porcinos.org.ar/). In this study, we analyzed 18 *S. suis* strains isolated from humans, 17 of which were serotype 2. With the addition of another case for which the strain was not available [21] and a serotype 21 strain previously described by us [20], this number is considerably higher than in the important swine producing countries that are Canada and USA. Three different reasons may explain these differences: a) the presence of backyard types of swine production; b) a good surveillance system in rural hospitals, and c) the presence of potentially virulent strains, when compared to those found in North America.

Relatively close contact with pigs/pork products and animals slaughtered at home cannot be ruled out as a possible factor contributing to this atypically high prevalence. More than 75% of farms in Argentina have less than 10 sows (http://www.porcinos.org.ar/), indicating a relatively

high number of backyard family types of production with which human *S. suis* infections have been traditionally associated [23]. Most patients described in this study had contact with swine/pork products and/or live in rural areas. However, it is important to note that typical dishes using raw blood or meat (such as are commonly consumed in some Asian countries) do not exist in the Argentinean culture [24]. In addition, and since the first human case of *S. suis* has been described more than 10 years ago [5], a good surveillance system for the characterization of alpha-hemolytic bacteria recovered from cases of meningitis in rural hospitals has been established. Each suspicious isolate is immediately sent to the National Institute of Microbiology for further identification. Consequently, close contact with pigs/pork and a good surveillance system may have contributed to the proper identification of these cases.

However, in the present study, we also demonstrated that serotype 2 strains recovered from either ill patients or systemically diseased pigs in Argentina are mostly ST1 strains with a typical *mrp*$^{+}$/*epf*$^{+}$/*sly*$^{+}$ genotype that characterizes virulent Eurasian strains. This highly differs from lower virulence ST25/ST28 North American strains, which have a *mrp*$^{+/-}$/*epf*$^{-}$/*sly*$^{-}$ genotype [15]. By using WGS, results showed that two strains from the UK (S90C and S11N) are clustered together in lineage 1 and significantly diverged from other strains of lineage 2 (Fig. 1(b)). These two lineages may have evolved from the ancestor of ST1 before separating, with the lineage 2 having become dominant. Within this lineage, most strains could be clustered into three main groups with the exception of the Chinese strain ZGST1. Argentinean strains and three strains from Vietnam were clustered into group 1. Strains from the UK, two Chinese strains, other Vietnamese strains, and the old reference strain from the Netherlands were clustered into group 2 and 3, respectively. A reasonable hypothesis is that the ST1 originated in Europe (UK) where a dominant lineage evolved before spreading to other countries maybe via the introduction of animals from genetic companies. In many countries, including China, Vietnam, and Argentina, the advanced breeds of pigs have been introduced from European countries like the UK and Denmark. Interestingly, the strains in these three groups of lineage 2 were all isolated in more than one country from different continents. It would appear that these groups diverged before the spreading and transmitted, in parallel, to different countries.

Interestingly, all strains from diseased pigs in this study were also ST1. Since some genetic breeders from North America have also been incorporated in Argentina, it is possible that North-American-like serotype 2 strains are also present. In fact, one strain isolated from a diseased

pig (pneumonia, lungs) and another recovered from tonsils of a clinically healthy pig were typical ST28 strains with a *mrp*$^{+}$*/epf/sly*$^{-}$ genotype profile identical to that found in Canada and the USA [15]. In this study, the strain isolated from lungs grouped far from all ST1 strains (Fig. 1(a)). These strains are most probably of low virulence; it has been previously reported that *S. suis* is not a primary cause of pneumonia and isolates recovered from lungs are often low virulent [1]. It is important to note that these strains are also able to induce serious disease in Canada and the USA. The most important difference between these countries and Argentina, from the disease status point of view, is the absence of the most important swine virus from this South American country: the porcine reproductive and respiratory syndrome virus, which is considered one of the most important predisposing factors for *S. suis* infection [1]. In the absence of this virus, only virulent *S. suis* strains are usually able to cause important disease in swine. Finally, transmission of ST1 strains from South America to North America is probably low since pig flow from genetic companies and breeders is usually from Europe and North America to South America.

Finally, we report the first human case of serotype 5 in Argentina (the only fatal case in the present study). Although this is the first report of this serotype in South America, one case of septic arthritis and one case of peritonitis have been previously described in Sweden and Thailand, respectively [25, 26]. In addition, one human case of arthroplasty infection with streptococcal toxic shock-like syndrome was caused by a non-encapsulated strain belonging to this serotype in the USA [27].

In conclusion, and in addition to the probably close contact with pigs/pork products and a good surveillance system, the presence of potentially virulent Eurasian-like serotype 2 strains in Argentina may play an important risk factor contributing to the higher number of human cases observed. In fact, and with this report, Argentina is now among the Western countries with the highest number of reported human cases after the Netherlands, France, and the UK, with a similar number of cases as Poland [28]. However, the three former European countries began the identification of *S. suis* in humans more than 15 years before Argentina, so the total number of human cases in this South American country may have been underestimated. Further studies in other South American countries, such Brazil, where the swine production is very important, should be performed.

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Tables

Table 1: *S. suis* strains isolated from humans or pigs in Argentina included in this study.

Strain	Source‡	Clinical manifestation	Serotype	Host	Date	Gender	Swine/pork contact	Pathotype	ST
285*	CSF, blood	Meningitis	2	H	1995	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
284	CSF, blood	Meningitis	2	H	1995	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
263*	CSF, blood	Meningitis	2	H	2003	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
178*	CSF, blood	Meningitis	2	H	2003	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
247*	CSF, blood	Meningitis	2	H	2003	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
2376#	CSF	Meningitis	2	H	2004	F	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
12*	CSF, blood	Meningitis	2	H	2009	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
83	CSF, blood	Meningitis	2	H	2009	M	Unknown	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
88	CSF	Meningitis	2	H	2009	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
245*	CSF	Meningitis	2	H	2012	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
486*	CSF, blood	Meningitis	2	H	2012	M	Unknown	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
371*	CSF, blood	Meningitis	2	H	2013	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
473*	CSF	Meningitis	2	H	2013	F	Unknown	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
42	CSF, blood	Meningitis	2	H	2014	F	Unknown	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
130/15	CSF	Meningitis	2	H	2015	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
695-15	CSF	Meningitis	2	H	2015	M	Yes	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
136-16	Joint	Arthritis	2	H	2016	M	No	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
15†	CSF	Meningitis	5	H	2014	M	Yes	ND	ND
P156	Spleen	Septicemia	2	S	2000	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P232	Joint	Arthritis	2	S	2001	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
050798	Brain	Meningitis	2	S	2001	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P421	Joint	Arthritis	2	S	2002	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
130387	Brain	Meningitis	2	S	2002	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P517	Brain	Meningitis	2	S	2003	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
477	Heart	Endocarditis	2	S	2003	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P613	Brain	Meningitis	2	S	2005	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P655	Heart	Endocarditis	2	S	2005	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1
P574	Joint	Arthritis	2	S	2005	N/A	N/A	*mrp*$^+$/*epf*$^+$/*sly*$^+$	1

P611	Brain	Meningitis	2	S	2005	N/A	N/A	*mrp*$^{+}$*/epf*$^{+}$*/sly*$^{+}$	1
P706*	Lungs	Pneumonia	2	S	2006	N/A	N/A	*mrp*$^{+}$*/epf/sly*$^{-}$	28
50703-8	Brain	Meningitis	2	S	2010	N/A	N/A	*mrp*$^{+}$*/epf/sly*$^{-}$	1
BE 21§	Tonsils	None	2	S	2014	N/A	N/A	*mrp*$^{+}$*/epf/sly*$^{-}$	28

‡When two source of isolations are mentioned for the same patient, only one strain was characterized; *Strains studied by whole genome sequencing (see Table 2); [#][5]; †Fatal case; §Clinically healthy pig; ST: sequence type (as evaluated by multilocus sequence typing); CSF: cerebrospinal fluid; ND: not determined; N/A: not applicable.

Table 2: List of strains used for comparison of the whole genome sequencing of *S. suis* from Argentina (see Table 1).

Strain number	Country	ST	Year	Host	Clinical signs
S90C	UK	1	2010	Pig	Diseased
S11N	UK	1	2010	Pig	Diseased
S98Y	UK	1	2010	Pig	Diseased
S13B	UK	1	2010	Pig	Diseased
S16E	UK	1	2010	Pig	Diseased
S15U	UK	1	2010	Pig	Diseased
S13F	UK	1	2010	Pig	Diseased
P1/7	UK	1	1980	Pig	Diseased
S92D	UK	1	2010	Pig	Diseased
S17G	UK	1	2010	Pig	Diseased
S16D	UK	1	2010	Pig	Diseased
S16B	UK	1	2010	Pig	Diseased
S12U	UK	1	2010	Pig	Diseased
R735	Netherlands	1	1963	Pig	Meningitis
YS1	China	1	2011	Pig	Healthy
GZ1	China	1	2005	Human	Meningitis
ZGST1	China	1	2015	Human	Meningitis
RC1[#]	China	308	2005	Pig	Healthy
BM237a	Vietnam	1	2001	Human	Meningitis
BM407	Vietnam	1	2004	Human	Meningitis
BM190a	Vietnam	1	2000	Human	Meningitis
BM264a	Vietnam	1	2002	Human	Meningitis
BM346B	Vietnam	1	2003	Human	Meningitis
BM478	Vietnam	1	2014	Human	Meningitis
BM224C	Vietnam	1	2001	Human	Meningitis
BM461	Vietnam	1	2014	Human	Meningitis
BM424a	Vietnam	1	2004	Human	Meningitis

[#]Serotype 8 (outgroup); UK: United Kingdom

Figures

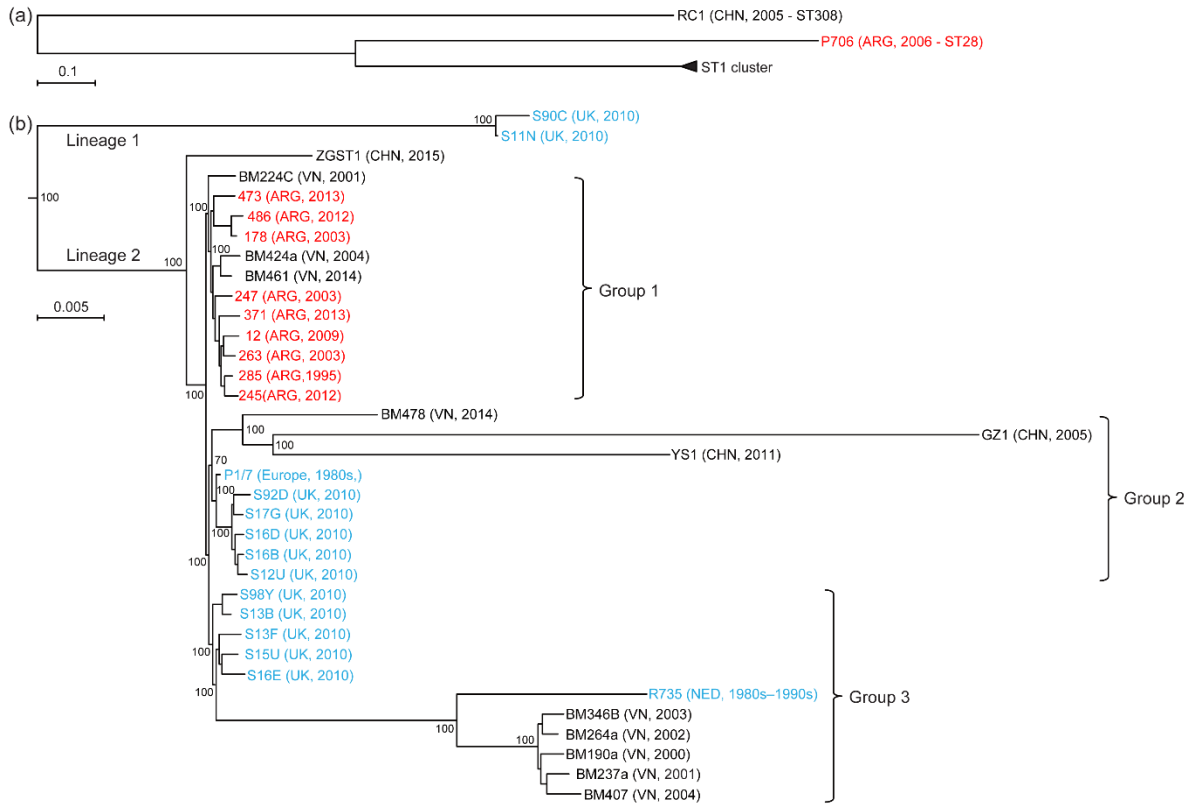

Figure. 1. Phylogenetic relationship of ST1 strains. (a) Phylogenetic tree of all strains (including sequence type) used in this study. The ST1 cluster was compressed and is marked by a triangle. (b) Phylogenetic tree of ST1 strains included in the present study compared to available data from GenBank. The numbers on the branches correspond to the bootstrap values. Strains from different areas are represented with different colors: Asia (black), Europe (blue), and Argentina (red). Geographical origins and year of isolation are included, in parentheses, after the strain name.

ANNEXES - ARTICLE V

A single amino acid polymorphism in the glycosyltransferase CpsK defines four *Streptococcus suis* serotypes

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Détails sur le rôle du candidat dans la conception de l’article :

Je suis co-auteur de l’article. J’ai effectué les expériences *in vivo* et j’ai participé à l’analyse des résultats.

Abstract

The capsular polysaccharide (CPS) is the major virulence factor of the emerging zoonotic pathogen *Streptococcus suis*. CPS differences are also the basis for serological differentiation of the species into 29 serotypes. Serotypes 2 and 1/2, which possess identical gene content in their *cps* loci, express CPSs that differ only by substitution of galactose (Gal) by *N*-acetylgalactosamine (GalNAc) in the CPS side chain. The same sugar substitution differentiates the CPS of serotypes 14 and 1, whose *cps* loci are also identical in gene content. Here, using mutagenesis, CPS structural analysis, and protein structure modeling, we report that a single amino acid polymorphism in the glycosyltransferase CpsK defines the enzyme substrate predilection for Gal or GalNAc and therefore determines CPS composition, structure, and strain serotype. We also show that the different CPS structures have similar antiphagocytic properties and that serotype switching has limited impact on the virulence of *S. suis*.

Introduction

Streptococcus suis is a major swine pathogen and an increasingly recognized agent of zoonotic disease [1]. At least 29 *S. suis* serotypes are defined based on a serological reaction directed against the capsular polysaccharide (CPS), a crucial virulence factor with antiphagocytic properties [2, 3, 4, 5]. Strains of serotype 2 are highly prevalent worldwide and frequently isolated from diseased swine [1]. Some serotype 2 genetic lineages such as sequence type (ST) 1, common in European and Asian countries, are highly virulent [1]. Clonal serotype 2 strains belonging to ST7, another highly virulent genotype, were responsible for two major outbreaks of *S. suis* human disease that affected hundreds of patients in China [6]. Other serotype 2 genetic lineages such as ST25 and ST28 are considered less virulent [7], although strains belonging to both ST25 and ST28 have caused human disease [1]. Strains of serotype 14 are also often associated with zoonotic disease [1]. One recurring problem for diagnostics laboratories is that strains of zoonotic serotypes 2 and 14 cross-react in the coagglutination test (the most commonly used *S. suis* serotyping scheme) with strains of non-zoonotic serotypes 1/2 and 1, respectively [8, 9, 10].

CPS biosynthesis in *S. suis* appears to proceed through the flippase/polymerase (Wzx/Wzy)-dependent pathway originally described for lipooligosaccharides biosynthesis [12, 13], in which an initial monosaccharide is linked as a sugar phosphate to a membrane-associated lipid carrier by an initial sugar transferase, followed by sequential addition of sugar residues by specific glycosyltransferases. The repeating units are then translocated across the cytoplasmic membrane by Wzx, polymerized to form the lipid-linked CPS by Wzy, and finally attached to the peptidoglycan by the membrane protein complex [11]. Pioneering work by Smith *et al.* identified that all genes needed for *S. suis* serotype 2 CPS biosynthesis cluster in a single *cps* locus [12, 14]. Further studies identified *cps* loci in all other *S. suis* serotypes [15, 16]. In addition to genes encoding various different glycosyltransferases, polymerases, transferases and translocases, the *cps* loci of some serotypes also contain genes encoding additional enzymes involved in modifications of sugar residues, or in the biosynthesis and linkage of sialic acid to the CPS side chain [12, 14, 15, 16].

We have recently determined the CPS structures of serotypes 2, 1/2, 14 and 1. The serotype 2 CPS contains galactose (Gal), glucose, *N*-acetylglucosamine, rhamnose, and sialic acid [17], while the serotype 14 CPS possesses Gal, glucose, *N*-acetylglucosamine, and sialic acid [18].

The serotype 1/2 CPS differs from the serotype 2 CPS and the serotype 1 CPS from the serotype 14 CPS by a single substitution of the Gal residue bearing the sialic acid in the serotypes 2 and 14 CPS side chains by an *N*-acetylgalactosamine (GalNAc) residue [16, 19] (Fig. 1a-d).

Interestingly, despite the aforementioned differences in CPS sugar composition and structure and the fact that all other serotypes possess a "serotype-specific" gene, serotype pairs 2 and 1/2, and 1 and 14 have identical *cps* gene content (Fig. 1e) [16]. Thus, there is no specific glycosyltransferase permitting to explain the differential addition of Gal or GalNAc to the CPS side chains of these serotypes [16]. To investigate the issue in more detail, we recently sequenced the genomes of seven strains each of serotypes 2 and 1/2, and seven strains each of serotypes 14 and 1. We found that the only consistent difference in the *cps* loci of strains of these serotype pairs was a nonsynonymous single-nucleotide polymorphism (SNP) in codon 161 of gene *cpsK*, predicted to result in a single amino acid difference in the glycosyltransferase CpsK (W161 in serotypes 2 and 14, and C161 in serotypes 1/2 and 1) [20].

Here, we tested the hypothesis that this single amino acid polymorphism is the key factor influencing the sugar residue (Gal or GalNAc) added to the CPS repeating unit by either CpsK variants. We show that polymorphic CpsK variants define expression by *S. suis* strains of either serotype 2 or 1/2 CPSs, or either serotype 14 or 1 CPSs, and that it is possible to achieve serotype switching of field strains of serotypes 2 and 1/2, and 14 and 1 solely by replacing amino acid 161 of CpsK. We also report that serotype switching does not modify the virulence of the strains in an experimental infection model.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 2. Well-characterized clinical isolates of serotype 2 (strain P1/7) [37], serotype 14 (strain DAN13730) [18], serotype 1/2 (strain 2651) [19] and serotype 1 (strain 1659834) were used. *S. suis* field strains and mutants were grown in Todd-Hewitt broth (THB) or agar (THA) (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37°C. *E. coli* strains were grown in Luria-Bertani broth or agar at 37°C. When needed, antibiotics (Sigma-Aldrich, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *S. suis*, spectinomycin at 100 µg/ml; for *E. coli*, kanamycin and spectinomycin at 50 µg/ml and ampicillin at 100 µg/ml.

DNA manipulations

S. suis genomic DNA was purified using InstaGene Matrix (BioRad, Mississauga, ON, Canada). Oligonucleotide primers (listed in Supplementary Table 3) were from Integrated DNA Technologies (Coralville, IA, USA). Plasmid preparations were performed using the QIAprep Spin Miniprep kit (Qiagen, Toronto, ON, Canada). Restriction enzymes and DNA-modifying enzymes were purchased from ThermoFisher (Waltham, MA, USA) and used according to the manufacturers' recommendations. PCR reactions were carried out with iProof high-fidelity DNA polymerase (BioRad) or with Taq DNA polymerase (Qiagen). Amplification products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer using the ABI PRISM dye terminator cycle sequencing kit v3 (ThermoFisher).

Mutant generation

SNP replacements in gene *cpsK* were performed by allelic exchange. PCR amplicons were generated using specific primers and cloned into plasmid pCR2.1 (ThermoFisher), extracted using EcoRI, and subcloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s [38] previously digested with EcoRI, giving rise to replacement vectors p4cpskG483T and p4cpskT483G. These vectors were then electroporated into recipient *S. suis* strains using a Biorad Gene Pulser Xcell apparatus (BioRad) under specific conditions (12.5 kV/cm, 200 Ω, and 25 µF). Isoallelic mutants were isolated as previously described [39]. Sanger sequencing confirmed adequate replacement of nucleotide 483 of *cpsK* genes. Whole-genome sequencing using Illumina MiSeq technology of all parental and mutant strains, and polymorphism identification were performed as previously described [21].

Serotyping

Serotyping was performed by coagglutination as previously described [10]. Results were deemed positive when a strong reaction was obtained within 1 min or less. Dot blot assays were used to confirm the CPS antigenicity of constructed mutants using highly purified CPS preparations, as previously described [19].

Transmission electron microscopy

TEM was carried as previously described [23]. Unless otherwise indicated, chemicals were from Sigma-Aldrich. Briefly, bacteria were grown to mid-logarithmic phase and washed in 0.1 M cacodylate buffer, pH 7.3. Rabbit antisera (150 µl) directed against the different CPS types

were used for CPS stabilization. Next, cells were immobilized in 4% (w/v) agar in 0.1 M cacodylate buffer pH 7.3 (Canemco & Marivac, Canton de Gore, QC, Canada). Prefixation was performed adding 0.1 M cacodylate buffer, pH 7.3, containing 0.5% (v/v) glutaraldehyde and 0.15% (w/v) ruthenium red for 30 min. Fixation was performed for 2 h at room temperature with 0.1 M cacodylate buffer, pH 7.3, containing 5% (v/v) glutaraldehyde and 0.05% (w/v) ruthenium red. Post-fixation was carried out with 2% (v/v) osmium tetroxide in water at 4˚C for 16 h. Samples were washed with water every 20 min for 2 h to remove osmium tetroxide and dehydrated in increasing graded series of acetone. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope at 80 kV (Hitachi model HT7770, Chiyoda, Tokyo, Japan).

Purification and physicochemical characterization of CPS

S. suis strains were grown in 150 ml of THB at 37°C for 16 h, diluted to 6 l in fresh THB, and grown overnight. The cells were pelleted by centrifugation at 10,000 × *g* for 40 min, suspended by repeated pipetting in 33 mM phosphate-buffered saline (PBS), pH 8.0, and chilled. The CPSs were then purified as previously described [17]. Purified CPSs were characterized by SEC–MALS, and M_w of each CPS was determined as previously described [18, 40].

NMR spectroscopy

CPSs were exchanged in phosphate buffer, pD 8.0, in D_2O (99.9 atom % D), freeze dried, and dissolved in D_2O (99.96 atom % D) to a final concentration of 33 mM. NMR spectra were acquired on polysaccharide samples at concentrations of 0.4–1.3%. ^{1}H chemical shifts δ in ppm were referenced to internal deuterated 2,2-dimethyl-2-silapentane-5-sulfonate at δ 0 as recommended by Wishart *et al.* [41]. Spectra were acquired at 11.75 T on a Bruker Avance 500 spectrometer equipped with a 5-mm triple resonance TBI probe with ^{1}H, ^{13}C, and ^{109}Ag–^{31}P channels at 75–77°C or at 16.45 T on a Bruker Avance 700 spectrometer with a 5-mm cryoprobe with ^{1}H and ^{13}C channels at 42°C using standard Bruker pulse sequences at the Centre régional de résonance magnétique nucléaire, Department of Chemistry, University of Montreal. Conventional 1D ^{1}H spectra were acquired with 30° pulses. The gradient-enhanced two-dimensional (ge-2D) COSY spectrum was acquired in magnitude mode using 45° or 90° pulses with or without purge pulses, respectively. Spectra were processed off-line with the software package SpinWorks 4.2.0.0 available at

http://home.cc.umanitoba.ca/~wolowiec/spinworks/ For 1D spectra, 32–40 K complex data points were acquired and processed by exponential multiplication with a line-broadening factor equal to the digital resolution, zero filling, complex Fourier transform, phase correction, and fifth-order polynomial baseline correction. Zhu-Bax forward–backward linear prediction with 16 coefficients was systematically applied to 2D processing in the f_1 dimension [42].

Modeling methods

Protein similarity searches were carried out with BLASTP https://blast.ncbi.nlm.nih.gov using the *S. suis* serotype 2 CpsK amino acid sequence against the PDB database. Three available 3D structures showed high identity with the catalytic module of CpsK and were identified as (i) a putative glycosyltransferase from *Streptococcus parasanguinis* (GalT1, PDB code 5hea, identity 39.13% covering 98% sequence), (ii) a putative glycosyltransferase from *Bacteroides fragilis* (PDB code 3bcv, identity 35.6% covering 88% sequence), and (iii) the chondroitin polymerase from *E. coli* strain K4 in complex with UDP (PDB code 2z87, identity 28% covering 52% sequence). We selected PDB 5hea and used it as a template to build a structural model for *S. suis* CpsK with either W161 or C161. Structural models for both variants were generated independently using the Swiss-Model server [43]. For modeling the interaction with ligands UDP-Gal and UDP-GalNAc, we used the two available 3D structures complexed with substrates that showed the highest identity with *S. suis* CpsK: (i) chondroitin polymerase from *E. coli* strain K4 (K4CP) complexed with UDP-glucuronic acid and UDP (PDB code 2z86 and 2z87, identity 23.22%) and (ii) the human UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase [44] (pp-GalNAc-T10, PDB code 2D7i, identity 18%). UDP-Gal and UDP-GalNAc PDBs were built using the electronic Ligand Builder eLBOW implemented in Phenix [45]. The generated substrates were structurally superimposed using the UDP-glucuronic acid present in PDB 2z86 as a template.

Experimental animal infection

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and the Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal, and approved by the latter Committee (Protocol RECH-1570). A well-standardized *S. suis* murine model of infection was used [25]. A total of 80 six-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were acclimated to standard laboratory conditions. On the day of the experiment, mice were assigned randomly to 8 groups of 10 mice

each. Each group was inoculated by intraperitoneal injection of 1 ml of bacterial suspensions of either one field strain or its derivative mutant expressing a switched serotype. Bacterial inocula were 5 × 10^7 colony forming units (CFU) for serotypes 2 and 1/2 and corresponding mutants or 1 × 10^8 CFU for serotypes 14 and 1 and corresponding mutants. These inocula were chosen based on preliminary trials carried out with parental strains and a reduced number of animals (data not shown). Mice were monitored at least three times a day for mortality and clinical signs of systemic disease, such as depression, swollen eyes, rough coat hair, and lethargy. To evaluate bacteremia, blood samples were collected from the tail vein at 12, 24, 48, and 72 h post-infection, plated onto THA using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA, USA) and bacterial colonies enumerated after incubation at 37°C for 16h.

Phagocytosis assay

Phagocytosis assays were performed using the murine macrophage cell line J774A.1 (ATCC TIB 67) maintained and cultured as previously described [46]. For bacterial phagocytosis, 48 h cell cultures were scraped, washed twice with phosphate-buffered saline (PBS), pH 7.4, and resuspended in antibiotic-free medium at 1 × 10^5 cells/ml. Cell suspension was then distributed into 24-well tissue culture plates (1 ml/well) and incubated for 3 h to allow cell adhesion. The cell culture medium was removed and cells were infected by adding 250 µl of a 4 × 10^7 CFU/ml bacterial suspension in culture medium (without antibiotics) and 250 µl of mouse serum (from C56BL/6 mice and stored at −80°C), to obtain a ratio of 100 bacteria per cell. The infected cells were incubated for 60 min at 37°C with 5% CO_2 to allow phagocytosis. Assay conditions were chosen based on preliminary studies (data not shown). After incubation, cells were washed with warm PBS and incubated for 1 h in medium containing 5 µg/ml penicillin G (Sigma-Aldrich) and 100 µg/ml gentamicin (ThermoFisher) to kill extracellular bacteria as previously described [46]. After antibiotic treatment, cells were washed and lysed with 1 ml of sterile distilled water. After vigorous pipetting to ensure complete cell lysis, viable intracellular bacterial counts were determined by plating serial dilutions onto THA using an Autoplate 4000 Automated Spiral Plater. Each test was repeated four times in independent experiments, and the number of CFU recovered per well (mean ± SEM) was determined.

Statistical analysis.

All data are expressed as mean ± SEM. *In vitro* data were analyzed for significance using the Student's t-test. Normality was previously verified in order to use Student's t-test. Log-Rank

(Mantel-Cox) test was used to analyze survival rates between parental field strains and derivative serotype switching mutants in animal infection assays. Statistical analyses for bacteremia were calculated using the Mann-Whitney Rank Sum test. A *P* value < 0.05 was used as a threshold for significance.

Results

Exchange of cpsK alleles differing by a single-nucleotide polymorphism between strains of serotypes 2 and 1/2, and between strains of serotypes 14 and 1, results in serotype switching

We hypothesized that the SNP at codon 161 of *cpsK* gene confers CpsK with different substrate predilection and results in the preferential addition of Gal (W161; serotypes 2 and 14) or GalNAc (C161; serotypes 1/2 and 1) residues to the nascent CPS repeating unit. Consequently, replacement of the W161 CpsK variant by the C161 CpsK variant, or vice versa, should result in strain serotype switching. To begin to test this hypothesis, we generated by allelic exchange the following *cpsK* isoallelic mutants: i) strain SS2to1/2 (derived from a serotype 2 field strain, has a W161C substitution in CpsK); ii) strain SS1/2to2 (derived from a serotype 1/2 field strain; has a C161W substitution in CpsK); iii) strain SS14to1 (derived from a serotype 14 field strain; has a W161C substitution in CpsK), and iv) strain SS1to14 (derived from a serotype 1 field strain; has a C161W substitution in CpsK). Whole-genome sequencing of parental and mutant strains confirmed the intended mutation, and did not identify spurious mutations elsewhere in the genome of the mutant strains, with the exception of strain SS1to14, which, compared to the WT serotype 1 strain, presented additional polymorphisms in gene *gatB*, encoding one subunit of a putative aspartyl/glutamyl-tRNA amidotransferase. These additional polymorphisms might impact the pool of arginine and glutamate amino acids of the mutant strain but are unlikely to affect CPS expression.

In all cases, parental and mutant strains expressed CPS of comparable thickness as determined by transmission electron microscopy (TEM) (Supplementary Fig. 1). When examined in the coagglutination test, all mutant strains appeared to have switched serotype (Table 1). However, since the coagglutination test uses polyclonal antibodies that may potentially recognize antigens other than the CPS, we next performed dot blotting with the same antisera and purified CPS from each pair of field and mutant strains. Consistent with the hypothesis of serotype switching, the CPS from the serotype 2 field strain reacted with anti-serotype 2 but not with anti-serotype 1 sera, while the CPS from mutant strain SS2to1/2

(W161C) reacted with both antisera (Fig. 2a, top panel). Essentially similar results were observed for CPS preparations from a serotype 14 field strain and its mutant SS14to1 (W161C) when blotted with anti-serotype 14 and anti-serotype 1 sera (Figure 2b, top panel). As expected, the CPS from the serotype 1/2 field strain reacted with both anti-serotype 1 and anti-serotype 2 sera, while the CPS from mutant strain SS1/2to2 (C161W) reacted with anti-serotype 2 but not with anti-serotype 1 sera (Fig. 2a, bottom panel). Essentially similar results were observed for CPS preparations from the serotype 1 field strain and its mutant SS1to14 (C161W) (Figure 2b, bottom panel), although in this latter mutant, cross-reaction with the anti-serotype 1 serum appeared to be slightly more intense than that observed for the CPS from the field serotype 14 strain. Taken together, these results demonstrate that a single amino acid substitution (W161C or C161W) in the glycosyltransferase CpsK is sufficient to effect serotype switching in each pair of serotypes (2 and 1/2, and 14 and 1).

The W191 CpsK variant adds a Gal residue to the CPS repeating unit; the C191 CpsK variant adds a GalNAc residue instead

To further test the hypothesis that a single amino acid substitution confers CpsK polymorphic variants with different sugar substrate predilection, we next performed nuclear magnetic resonance (NMR) analysis of purified CPSs obtained from each pair of field strains and derivative serotype switching mutants using previously described protocols [17, 18, 19]. The analysis revealed one noticeable additional methyl group signal (δ 2.06) from GalNAc in the one-dimensional (1D) ^{1}H NMR spectrum of the CPS preparation from the SS2to1/2 mutant (Fig. 3a and d), as well as in the spectrum of the CPS preparation of the SS14to1 mutant (Fig 4a and d), compared to CPS preparations from the parental field strains of serotypes 2 (Fig. 3c) and 14 (Fig. 4c). Inversely, this signal was absent from the spectra of CPS preparations from mutants SS1/2to2 (Fig. 3b and f) and SS1to14 (Fig. 4b and f) and present in the spectra of CPS preparations of parental field strains of serotype 1/2 (Fig. 3e) and serotype 1 (Fig. 4e). In the anomeric region, the chemical shift of H-1 of the side-chain 6-substituted residue (GalNAc or Gal) depended on the sugar identity (Fig. 3a and b and Fig. 4a and b).

To determine the position of the H-1–H-2 cross peak, we acquired correlation spectroscopy (COSY) spectra (Supplementary Fig. 2a to d). H-2 resonated at a much higher frequency when an *N*-acetamido moiety instead of a hydroxyl group was present on C-2: for CPSs from SS2to1/2 and SS1/2to2 mutants, the H-1/H-2 signal was found at δ 4.51/3.92 and 4.44/3.54, respectively, as opposed to δ 4.44/3.54 and 4.49/3.94 in the CPSs from parental field strains

of serotypes 2 and 1/2, respectively. Similarly, for CPSs from the SS14to1 and SS1to14 mutants, the signal was found at δ 4.52/3.93 and 4.45/3.54, as opposed to δ 4.45/3.54 and 4.51/3.93 in the CPSs from parental field strains of serotypes 14 and 1, respectively [17, 18, 19]. A small shift of the anomeric proton of GlcNAc, to which GalNAc or Gal is attached, was also observed in all cases (Supplementary Fig. 2a to d). Collectively, ^{1}H and COSY NMR spectra unambiguously demonstrated that the SS2to1/2 and the SS1/2to2 mutants synthesized serotypes 1/2 and 2 CPSs, respectively. Similarly, the data unequivocally demonstrated that the SS14to1 and the SS1to14 mutants synthesized serotypes 1 and 14 CPSs, respectively.

It is apparent from the previous results that CPSs expressed by field strains of serotypes 2, 14, 1/2, and 1 (henceforth defined as "native" CPSs) have the same sugar composition and repeating unit structure as those expressed by mutants SS1/2to2, SS1to14, SS2to1/2, and SS14to 1 (henceforth defined as "mutant" CPSs), respectively. To investigate whether other differences existed between native and mutant CPSs, we next performed size-exclusion chromatography coupled with multi-angle light scattering (SEC–MALS). The predicted molecular mass (M_w) of the different CPS preparations were relatively similar between serotype 2 **"native"** CPS (435 kg/mol) and serotype 2 "mutant" CPS (prepared from strain SS1/2to2) (504 kg/mol), and between serotype 14 "native**"** CPS (421 kg/mol) and serotype 14 "mutant" CPS (prepared from strain SS1to14 (571 kg/mol), suggesting similar chain lengths. However, "mutant" serotype 1/2 and "mutant" serotype 1 CPSs (prepared from strains SS2to1/2 and SS14to1, respectively) appear to have reduced CPS chain lengths when compared to **"**native**"** serotype 1/2 CPS (483 vs. 709 kg/mol) or **"**native**"** serotype 1 CPS (490 vs. 741 kg/mol), respectively (Supplementary Table 1). However, it must be noted that data for "mutant" CPS were acquired by analysis of a single batch of CPS preparation per mutant strain. Further experiments are needed to confirm whether those differences in *M*w actually represent differences in CPS length.

Three-dimensional modeling of polymorphic CpsK with either W161 or C161 is *compatible with substrate predilection for Gal or GalNAc, respectively*

Since the only difference between each pair of parental and mutant strains is one SNP in the *cpsK* gene, we concluded from experiments presented above that the polymorphism in amino acid 161 of CpsK is the sole factor determining which sugar residue this glycosyltransferase adds to the CPS repeating unit. To investigate substrate predilection of both polymorphic forms of CpsK in more detail, we next built a three-dimensional (3D) model for the serotype 2 CpsK

protein variant (bearing W161) (Fig. 5a). CpsK belongs to the glycosyltransferase family 2 (GT2) that is a member of the clan GT-A, all of which present two tightly associated β/α/β domains that form a central eight-strands β-sheet in a Rossman-like fold. As described in the carbohydrate-active enzymes (CAZY) database (www.cazy.org)[21], GT2 enzymes present an inverting mechanism. Members of this family are responsible, generally, for the transfer of nucleotide-diphosphate sugars to substrates such as polysaccharides and lipids. Strict conservation of the nucleotide-binding site and availability of several 3D structures complexed with different substrates allowed us to identify the localization of the saccharide moieties bound to the activated nucleotide sugar in CpsK. Residue 161 is located at the core of the catalytic center at the beginning of the last β-strand of the central β-sheet and close to the nucleotide-biding site (Fig. 5a). Our modeling analysis revealed that recognition of the nucleotide is not affected by replacement of residues at position 161. Indeed, all the residues required for recognition of the uracil group, the ribose moiety, and the pyrophosphate group are conserved in both variants of CpsK (Fig. 5 and Supplementary Fig. 3).

Docking of uridine diphosphate (UDP)-Gal and UDP-GalNAc in the active sites of both CpsK variants provided a clear explanation of the potential role of residue 161 in substrate specificity. C161 in CpsK from serotype 1/2 could stabilize the GalNAc residue in the UDP-GalNAc substrate by establishing a polar interaction with the acetyl group of the sugar (Fig.5b) (in addition to the potential H-bonds created by the conserved residues with the other oxygen atoms from the GalNAc sugar). The distance between the SH group of C161 and the carbonyl oxygen of GalNAc is in the range of a hydrogen bond formation (3.1-3.7 Å, according to the values stored in the Cambridge Structural Database).

The same happens for the interaction of UDP-Gal with W161-bearing CpsK from serotype 2 (Fig. 5c). In this case, the W161 residue shapes the cavity to accommodate this smaller ligand and provides an H-bond with the O2 of Gal through the N atom of the indole ring (Fig. 5c). Interestingly, this interaction is also observed in the 3D structure of one of CpsK closest homologues, the chondroitin polymerase from *Escherichia coli*, in complex with UDP-glucuronic acid (Protein Data Bank database [PDB] code 2Z86) where one of the N atoms of the side chain of H581 makes an H-bond with the O2 of glucuronic acid (Supplementary Fig. 3b). In both cases, the predicted interactions would contribute to the specificity of each CpsK variant for their respective substrates. However, while each polymorphic form can stabilize its natural substrate (UDP-Gal and UDP-GalNAc in CpsK from serotypes 2 and 1/2, respectively),

the exchange of substrates is not possible. With C161, there is enough room to accommodate the *N*-acetyl group bound to the galactosamine moiety at the catalytic groove. However, with W161, the steric hindrance generated between the *N*-acetyl group and the side chain of W161 would prevent the accommodation of UDP-GalNAc at the catalytic groove (Supplementary Fig. 4). Taken together, 3D modeling results provide an explanation to the differential galactosyltransferase and *N*-galactosaminyltransferase activities detected for CpsK W161 and C161 polymorphic variants, respectively.

The CPSs of serotypes 2, 1/2, 14, and 1 have similar antiphagocytic properties, and serotype switching does not alter strain virulence

Although the virulence of *S. suis* is multifactorial, studies with mutant strains impaired in CPS expression have conclusively shown that the CPS plays a key role in the pathogenesis of infection of this pathogen [22, 23, 24]. However, little is known on the effect of serotype switching on the virulence of any given strain. One hypothesis is that inasmuch as the organism expresses a capsule the specific CPS type of any particular strain will not affect virulence. However, the fact that only a few serotypes (notably, serotype 2) predominate among strains isolated from diseased animals and humans (serotype 1/2 strains have never been isolated from human cases so far) might indicate that the specific type of CPS may be important *per se* in defining the virulence of the strain. To begin to differentiate between these hypotheses, we took advantage of the fact that our mutant strains were generated from parental field strains belonging to different STs with known virulence differences. Indeed, the serotype 2 parental field strain belongs to ST1, the parental serotype 1/2 field strain belongs to ST28, the serotype 14 parental field strain belongs to ST6, and the serotype 1 parental field strain belongs to ST1. Thus, with the exception of the spurious mutation in *gatB* noted above in mutant strain SS1to14, each pair of parental and mutant strains are truly serotype variants that differ by only one SNP genome-wide, and the differences in virulence between pairs of parental field and mutant strains may only result from their different CPSs.

Virulence assay results using a validated murine model of infection [25] showed that the virulence of the SS2to1/2 mutant strain (expressing serotype 1/2 CPS) was virtually identical to that of the parental serotype 2 field strain (P = 0.4510). Indeed, both strains caused at least 80% mouse mortality after 3 days post-infection, as expected for highly virulent ST1 strains (Fig. 6a). Mice in both groups had high bacteremia after 24 h post-infection (P = 0.8917) (Fig. 6e). In addition, mice infected with either strain showed severe clinical signs such as depression,

swollen eyes, rough coat hair, and lethargy. On the other hand, the serotype 1/2 parental strain and its derivative mutant strain SS1/2to2 (expressing serotype 2 CPS) were of low virulence, consistent with previously reported low virulence of ST28 strains [26]. No mortality was recorded in either group (P = 1.0000) after 3 days (Fig. 6b), and mice showed only mild clinical signs of infection and low bacteremia in general (P = 0.1116) (Fig. 6f). Similarly, no virulence differences were observed between the serotype 14 field strain (ST6 genetic background) and mutant SS14to1 (P = 0.6273). Indeed, both groups showed more than 80% mortality, high bacteremia (P = 0.6842), and severe clinical signs of infection (Figs. 6c and g). Finally, the virulent serotype 1 ST1 field strain showed high virulence with 70% mortality after 3 days post infection, and mutant SS1to14 induced similar mortality (80%) (P = 0.6419) (Fig. 6d). Similarly to other tested ST1 strains, mice infected with either strain showed high bacteremia (P = 0.5583) (Fig. 6h) and severe clinical signs.

Next, we investigated *in vitro* the antiphagocytic properties of the different four *S. suis* CPSs by means of phagocytosis assays. Results showed that, independently of CPS type and strain genetic background, all strains were similarly internalized by murine macrophages in the presence (Fig. 7) (P = 0.9662 for SS2 and SS2to1/2, P = 0.8873 for SS1/2 and SS1/2to2, P = 0.9874 for SS14 and SS14to1 and P = 0.9639 for SS1 and SS1to14) or absence (data not shown) of serum. Thus, substitution of Gal by GalNAc, or *vice versa*, does not significantly alter the antiphagocytic properties of the tested CPSs. Taking the *in vitro* and *in vivo* virulence assays together, we conclude that the different CPSs possess similar antiphagocytic properties, that serotype switching does not impact the virulence of *S. suis* strains that share a similar genetic background, at least for the four serotypes tested here, and that the virulence arsenal particular to the specific genetic background of a given strain is more likely to influence its virulence.

Discussion

Integrated systems biology approaches combining sequencing of multiple genomes of closely related organisms, in combination with animal infection models and relevant *in vitro* approaches, have been instrumental in recognizing the key contribution of small genetic changes such as SNPs and short insertion/deletions to the virulence, phenotypic characteristics, and other important biological traits of strains of several bacterial species [27, 28, 29]. Here, we show that a single amino acid polymorphism in the glycosyltransferase CpsK leads

to enzyme variants with differential substrate predilection for Gal and GalNAc, defining the sugar residue added to the CPS repeating unit and thus determining four *S. suis* serotypes. Specifically, we demonstrate that for serotype pairs 2 and 1/2, and 14 and 1, a CpsK variant with W161 results in strains that are serotypes 2 and 14, while a CpsK variant with C161 results in strains that are serotypes 1/2 and 1. Our findings provide a definitive molecular explanation to intriguing previous results showing that strains of serotypes 2 and 1/2 and strains of serotypes 14 and 1 have *cps* loci with identical gene content, but their CPS structures differ between members of each pair, namely by the presence of either a Gal or a GalNAc as the CPS side-chain sugar residue bearing sialic acid [16, 17, 18, 19]. However, NMR is our sole source of structural data, and further studies are needed to elucidate the impact, if any, of the polymorphism on other important CPS characteristics such as the number of synthesized CPS chains and their lengths.

Glycosyltransferases are a large family of proteins that are ubiquitous in bacteria and eukaryotes[30]. Despite the large number of sequence families that have been defined, structural analysis has shown that all but a few glycosyltransferases possess GT-A or GT-B folds. The catalytic domain of the GT-A-fold enzymes can be viewed as a single domain composed by two closely abutting β/α/β Rossmann domains. The Rossmann fold is found in proteins that bind nucleotides and is responsible for binding the nucleotide sugar donor substrate. With only one exception, GT-A enzymes have been found to possess a DXD motif and are metal-ion-dependent glycosyltransferases. The GT-B-fold enzymes possess also two Rossmann domains but separated by a cleft that binds the acceptor. The carboxy-terminal domain is primarily responsible for binding the nucleotide sugar donor substrate. Unlike enzymes that contain the GT-A fold, the GT-B glycosyltransferases are metal-ion independent and do not possess a DXD motif. In this study, the targeted SNP corresponding to amino acid 161 of CpsK protein is located within the glycosyltransferase functional domain. 3D modeling using relevant available crystal structures clearly suggests that the amino acid substitution at position 161 of *S. suis* CpsK leads to conformational and functional changes that permit the enzyme to select between either Gal or GalNAc. A SNP in the gene encoding the glycosyltransferase *wcrL* of *Streptococcus pneumoniae* has been shown to be responsible for the CPS differences observed between serotypes 11A and 11D of that species [31]. However, WcrL variants were shown to have bi-specificity for both Gal and GalNAc, and the resulting CPS differences were due to variable capsular Gal/GalNAc repeat unit ratio[31]. In contrast, our data indicate that *S. suis* CpsK variants are monospecific and incorporate either Gal (W161) or GalNAc (C161).

The CPS plays a key role in *S. suis* virulence. TEM showed that all four isolallelic mutant strains generated here were as encapsulated as their respective parental strains of serotypes 2, 1/2, 14 and 1. Most previous studies have only investigated the impact of abolishing CPS expression on the virulence of the organism [23, 32]. These types of studies cannot differentiate whether a specific CPS composition is important for the virulence of a strain. For example, work on *S. pneumoniae* has shown that specific CPS types endow the strains with differential ability to avoid complement deposition and modulate the virulence of the strain in murine infection models [33, 34]. Previous studies that have compared the virulence of *S. suis* strains expressing different CPS types have, for the most part, used strains with dissimilar genetic background or whose genetic backgrounds were not known [35]. Here, the use of isoallelic mutants and both in vitro and in vivo infection models permitted us to conclude that the CPS composition plays an unnoticeable role in the virulence of *S. suis* strains of serotypes 2, 1/2, 14, and 1. Indeed, a highly virulent parental ST1 serotype 2 strain was as virulent in mice as its isoallelic mutant expressing serotype 1/2 CPS, while the low virulence of an ST28 serotype 1/2 remained essentially unchanged in its isoallelic mutant expressing type 2 CPS. Similarly, highly virulent ST1 serotype 1 strain expressing serotype 14 CPS and virulent ST6 serotype 14 strain expressing serotype 1 CPS were as virulent as their parental strains. Moreover, we observed no differences in the antiphagocytic properties of CPS 2, 1/2, 14, and 1. One limitation of our study in comparison with the abovementioned work on *S. pneumoniae* is that we evaluated CPS types that differ only by one sugar, i.e., CPS structural changes are relatively minor and may thus not significantly impact virulence. Additionally, it can be hypothesized that the CPSs tested here may possess similar virulence-related properties. Indeed, the cross-reactions between serotypes 2 and 1/2 and serotypes 14 and 1 CPSs in the coagglutination test[16] support the idea that these different CPSs elicit partially overlapping immune responses from the host [19]. *S. suis* strains of serotypes 2 and 14 have caused human disease, while, to our knowledge, strains of serotypes 1 and 1/2 have not [1]. Our results suggest that this differential ability to cause disease in the human host is unlikely to be related to the different compositions and structures of the CPSs of strains of the two serotype pairs.

Small genetic changes such as short insertion/deletions and, particularly, SNPs are key contributors to the genetic diversity of bacterial pathogens [36]. Their impact on bacterial phenotypic traits, including virulence, is only beginning to be uncovered. Here, we show that a single amino acid polymorphism at position 161 of the glycosyltransferase CpsK defines the enzyme specificity for either Gal or GalNAc, and that incorporation of either sugar residue into

the CPS repeating unit by polymorphic CpsK is the crucial event in the differentiation between *S. suis* serotypes 2 and 1/2 and between serotypes 14 and 1. Our findings solve a 3-decade long dilemma about the nature of serotyping cross-reactions in *S. suis* serotypes 2, 1/2, 14, and 1 and extend our understanding of how small genetic changes influence bacterial traits and pathogenesis of infection

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Supporting Information (Available at the Scientific Reports Website)

Supplementary Table I. Average yields for CPS purification of the different *S. suis* field and mutant strains and CPS molecular weights.

Supplementary Table II. Bacterial strains and plasmids used in this study

Supplementary Table III. Oligonucleotide primers used in this study.

Supplementary Figure 1. Transmission electron micrographs

Supplementary Figure 2. Portion of the 500 MHz ge 2D NMR COSY spectrum of *S. suis* CPSs.

Supplementary Figure 3. Structural and substrate-binding conservation in closest homologues of CpsK from *S. suis*.

Supplementary Figure 4. Steric hindrance between tryptophan 161 and the N-acetyl group of UDP-GalNAc

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Tables

Table I. Results of the coagglutination test.

Strain	Tested antisera			Interpretation
	Anti-serotype 1	Anti-serotype 2	Anti-serotype 14	
SS2	-	+		Serotype 2
SS2to1/2	+	+		Serotype 1/2
SS1/2	+	+		Serotype 1/2
SS1/2to2	-	+		Serotype 2
SS14	-		+	Serotype 14
SS14to1	+		+	Serotype 1
SS1	+		+	Serotype 1
SS1to14	-		+	Serotype 14

Figures

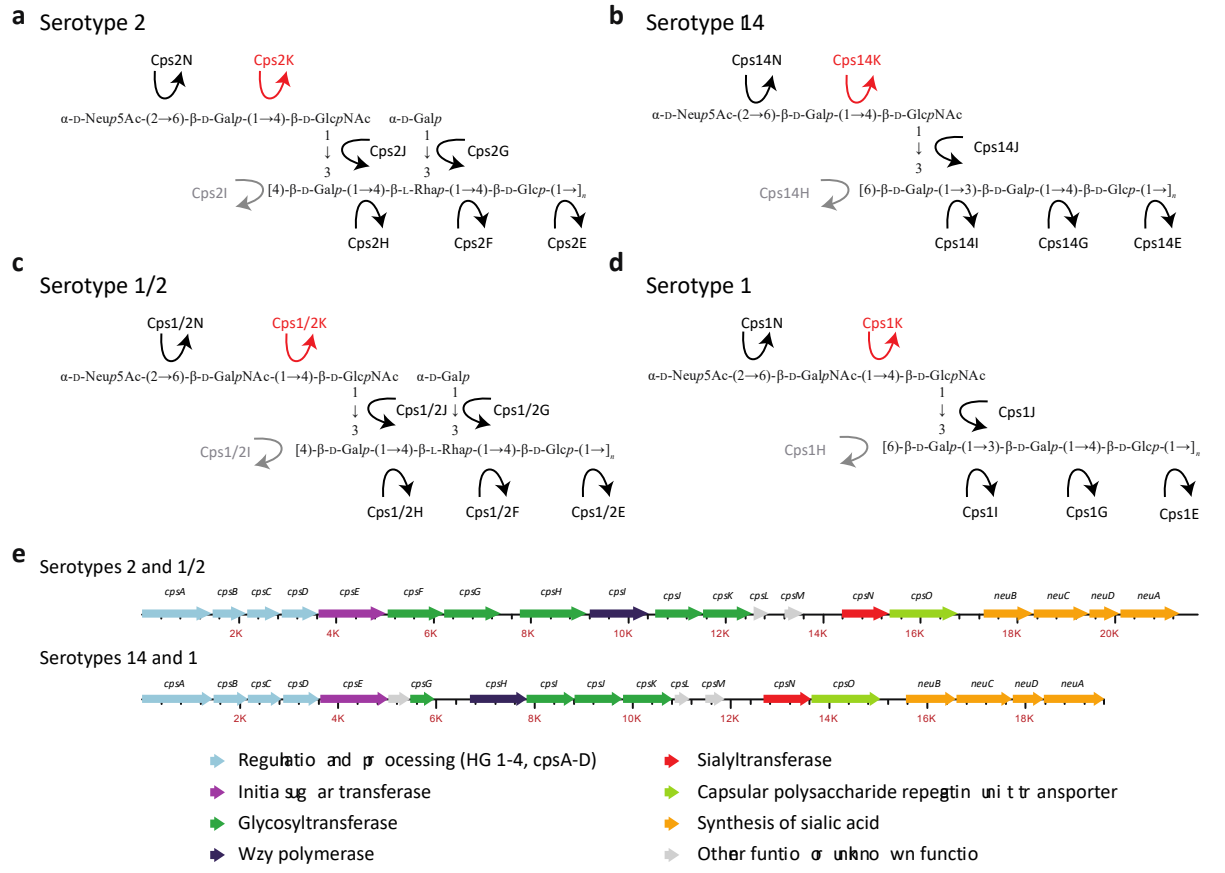

Figure 1. Capsular polysaccharide structures of *S. suis* serotypes 2, 1/2, 14, and 1 capsular polysaccharides, and schematics of the *cps* loci of these serotypes. a–d, CPS structures of serotype 2 (a), serotype 14 (b), serotype 1/2 (c), and serotype 1 (d). In serotypes 1 and 1/2, CpsK is predicted to catalyze the transfer of the side chain *N*-acetylgalactosamine (GalNAc) residue to the CPS repeating unit, while in serotypes 2 and 14, CpsK would catalyze the transfer of the galactose (Gal) residue at the same corresponding side chain. (e), All enzymes involved in CPS biosynthesis, including CpsK, are encoded by genes located in a single *cps* locus. Serotypes 2 and 1/2 and serotypes 14 and 1 have identical CPS gene content and organization. The putative functions of the enzymes encoded by *cps* genes are depicted with different colors. Please note that Cps enzymes (and *cps* genes) have been renamed compared to previous publications [12, 14, 16, 17, 18, 19] to reflect recent developments in actual or predicted function.

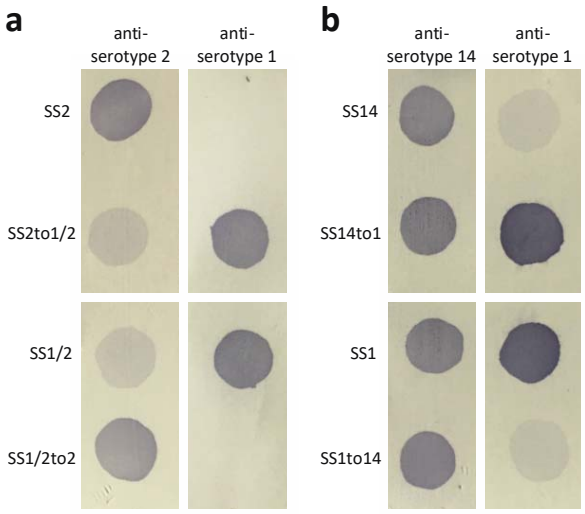

Figure 2. Serotype switching of mutants is confirmed by dot blotting of purified CPS preparations and specific antisera. a, the CPS from a serotype 2 field strain reacts with anti-serotype 2 but not with anti-serotype 1 sera, while the CPS from mutant strain SS2to1/2 (W161C) reacts with both antisera (top panel). The CPS from a serotype 1/2 field strain reacts with both anti-serotype 1 and anti-serotype 2 sera, while the CPS from mutant strain SS1/2to2 (C161W) reacts with anti-serotype 2 but not with anti-serotype 1 sera (bottom panel). b, the CPS from a serotype 14 field strain reacts strongly with anti-serotype 14 and weakly with anti-serotype 1 sera, while the CPS from mutant strain SS14to1 (W161C) reacts strongly with both antisera (top panel). The CPS from a serotype 1 field strain reacts strongly with both anti-serotype 14 and anti-serotype 1 sera, while the CPS from mutant strain SS1to14 (C161W) reacts strongly with anti-serotype 14 but weakly with anti-serotype 1 sera (bottom panel).

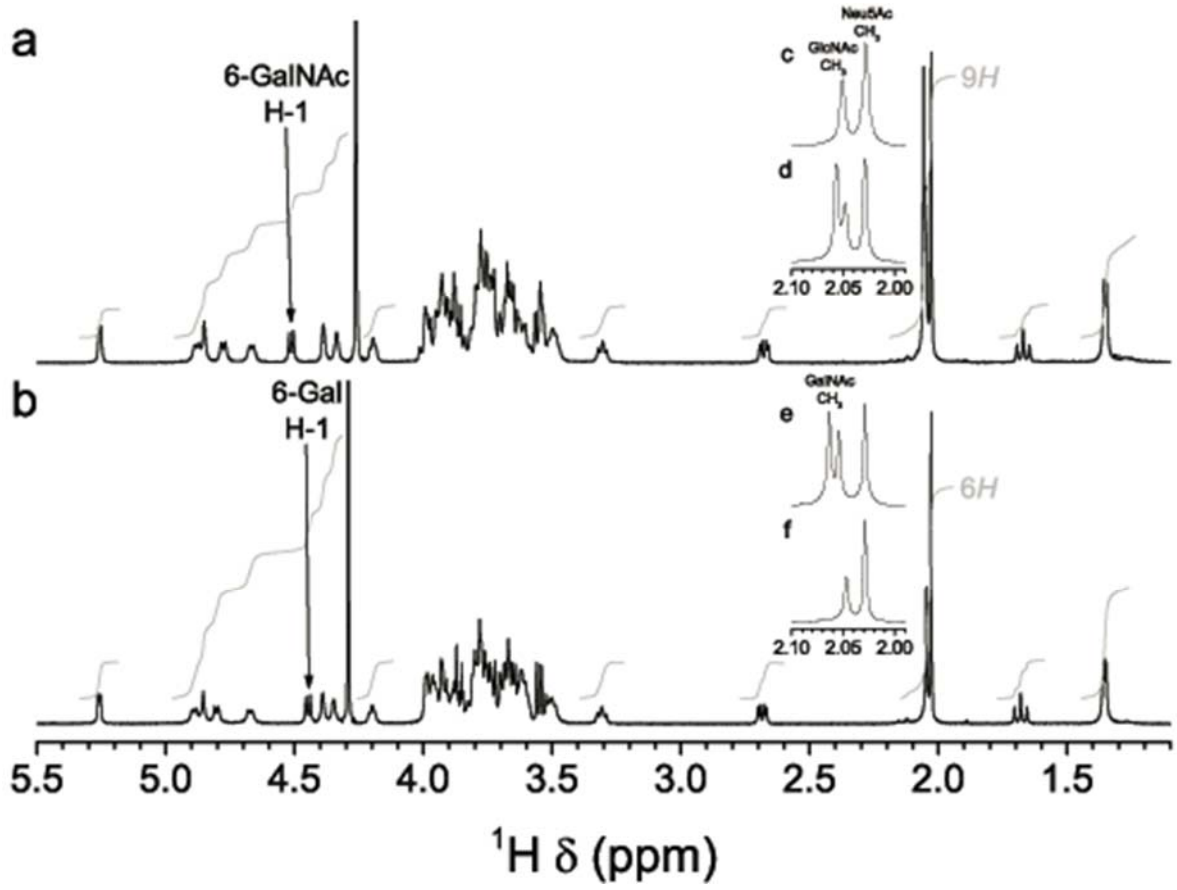

Figure 3. 1D ^{1}H NMR spectra of CPS preparations from serotypes 2 and 1/2 in 33 mM phosphate pD 8.0 in D_2O. a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS2to1/2 mutant, 500 MHz, 77°C. b, f, SS1/2to2 mutant, 500 MHz, 75°C. c, Serotype 2 field strain, 600 MHz, 50°C [17]. e, Serotype 1/2 field strain, 700 MHz, 42°C [19]. Specific characteristics of the ^{1}H NMR spectrum due to the presence of GalNAc in the native serotype 1/2 CPS were also found in the spectrum of the CPS from the corresponding mutant expressing serotype 1/2 CPS. Conversely, the spectrum of the CPS from the mutant expressing serotype 2 CPS, as well as that of the native serotype 2 CPS, lacked the signal attributed to *N*-acetyl of GalNAc.

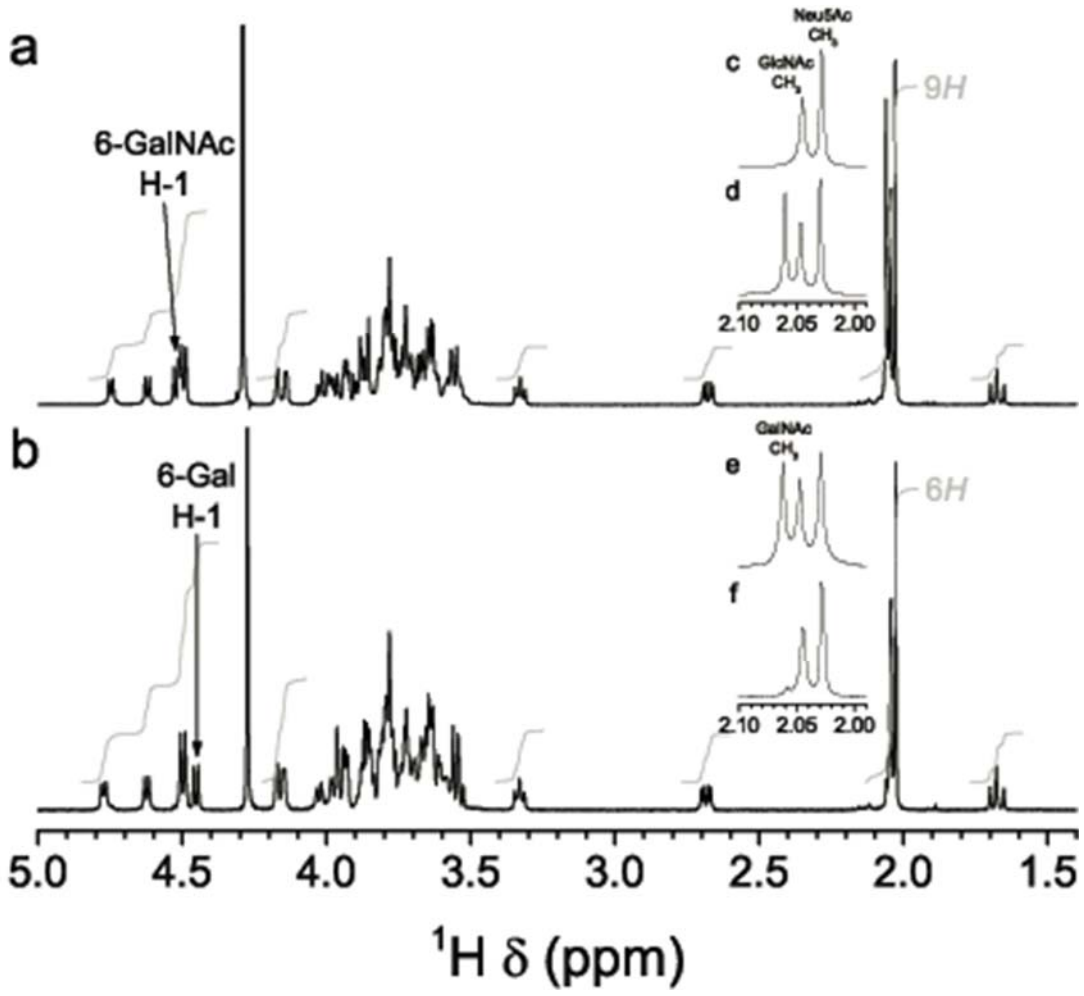

Figure 4. 1D ^{1}H NMR spectra of CPS preparations from serotypes 14 and 1 in 33 mM phosphate pD 8.0 in D_2O. a–b, Full spectrum. c–f, Expansion of the methyl region. a, d, SS14to1 mutant, 500 MHz, 75°C. b, f, SS1to14 mutant, 500 MHz, 77°C. c, Serotype 14 field strain, 500 MHz, 77°C [18]. e, Serotype 1 field strain, 700 MHz, 70°C [19]. Specific characteristics of the ^{1}H NMR spectrum due to the presence of GalNAc in the native serotype 1 CPS were also found in the spectrum of the CPS from the corresponding mutant expressing serotype 1 CPS. Conversely, the spectrum of the CPS from the mutant expressing serotype 14 CPS, as well as that of the native serotype 14 CPS, lacked the signal attributed to *N*-acetyl of GalNAc.

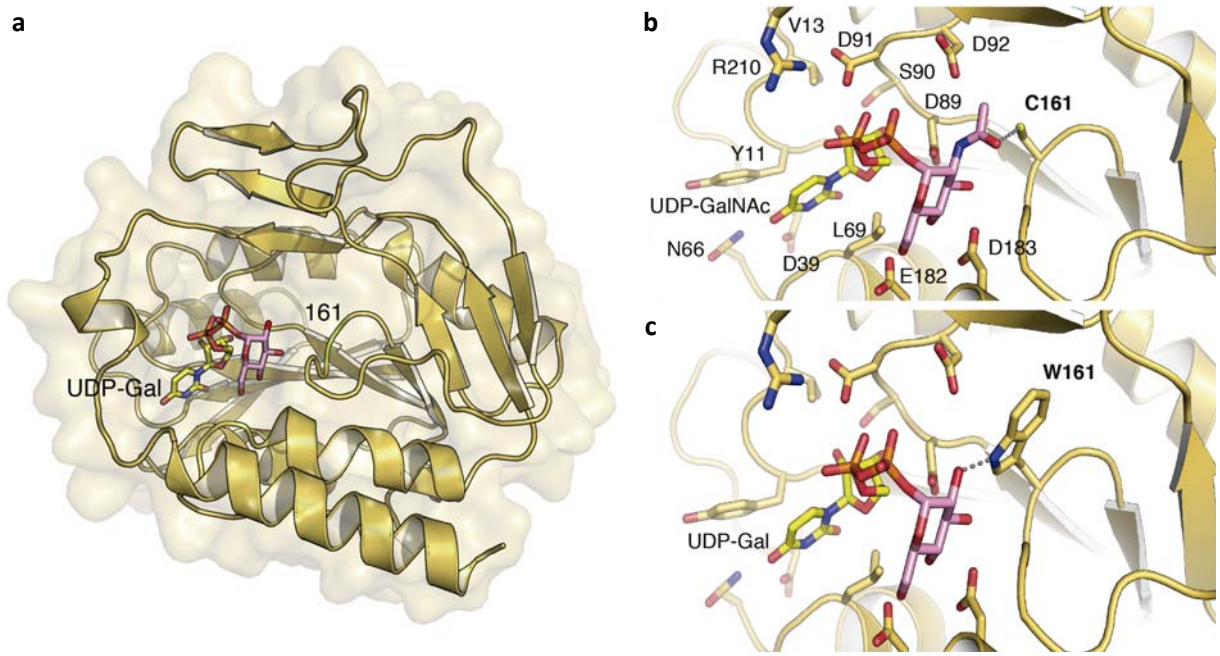

Figure 5. Three-Dimensional modeling of CpsK polymorphic variants. a, The serotype 2 CpsK protein structure is depicted in yellow ribbon, and the position of amino acid residue 161 was colored in green and labeled. The docked substrate UDP-Gal is shown in sticks with the Gal moiety in pink. b, Detailed view of the catalytic center of protein CpsK from serotype 1/2 with a cysteine residue at position 161 (C161) in complex with UDP-GalNAc as a substrate. Residues predicted to play a role in substrate binding and stabilization are depicted as capped sticks and labeled. Potential hydrogen bond between C161 and *N*-acetyl group of GalNAc is represented with a dashed grey line. c, Same view as in panel a for the catalytic center of protein CpsK from serotype 2 with a tryptophan residue at position 161 (W161) in complex with UDP-Gal molecule as a substrate. Dashed grey line represents the potential hydrogen bond between W161 and the hydroxyl group of Gal.

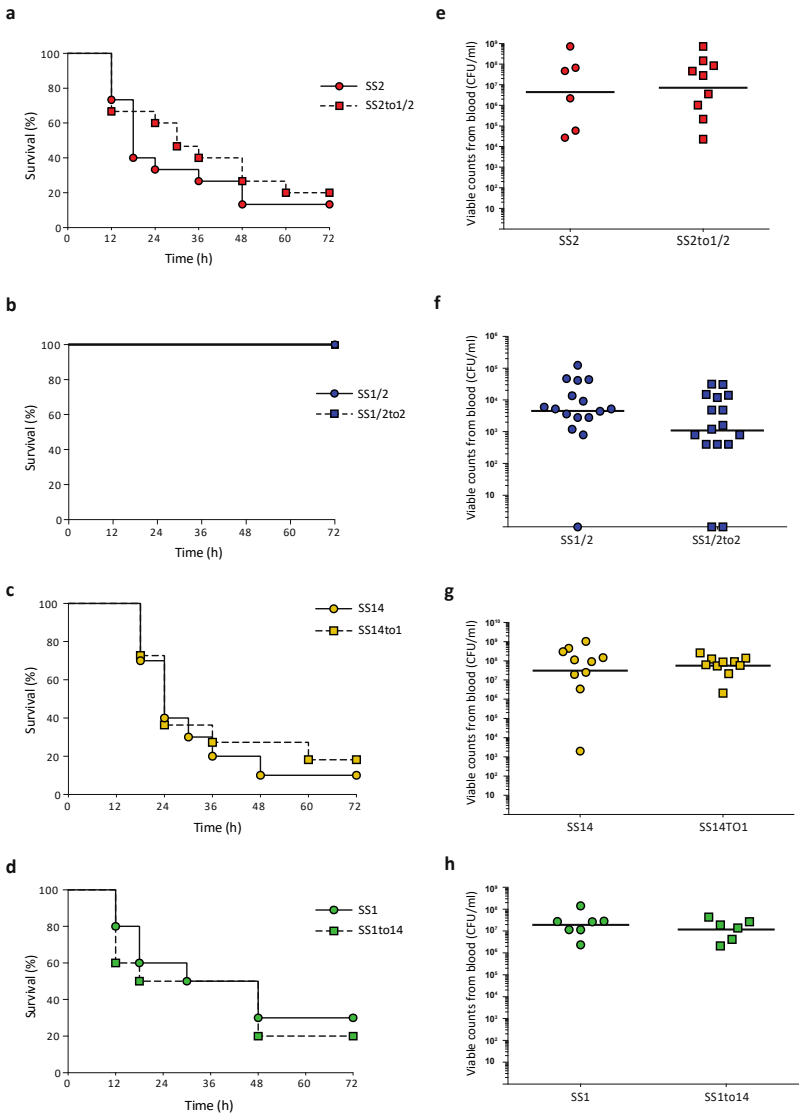

Figure 6. Serotype switching does not impact strain virulence. a–d, Survival of CD1 mice inoculated by intraperitoneal injection with either 5×10^7 CFU of serotype 2 (a) or 1/2 (b) strains or with either 1×10^8 CFU of serotype 14 (c) or 1 (d) strains. In all cases, Log-Rank (Mantel-Cox) test revealed no significant differences in survival rates between the field parental strains and derivative mutants. All control animals injected with vehicle (Todd Hewitt Broth) survived the trial (data not shown for simplicity). e–h, Bacterial load in blood was evaluated in all groups by drawing 5 µl of blood from the tail vein of mice followed by plating and enumeration (see methods). e, Serotype 2 field strain and derived mutant SS2to1/2. f, Serotype 1/2 field strain and derived mutant SS1/2to2. g, Serotype 14 field strain and derived mutant SS14to1. h, Serotype 1 field strain and derived mutant SS1to14. No significant differences in bacterial load were observed between parental strains and their corresponding mutants (Mann-Whitney Rank Sum test, $P<0.05$).

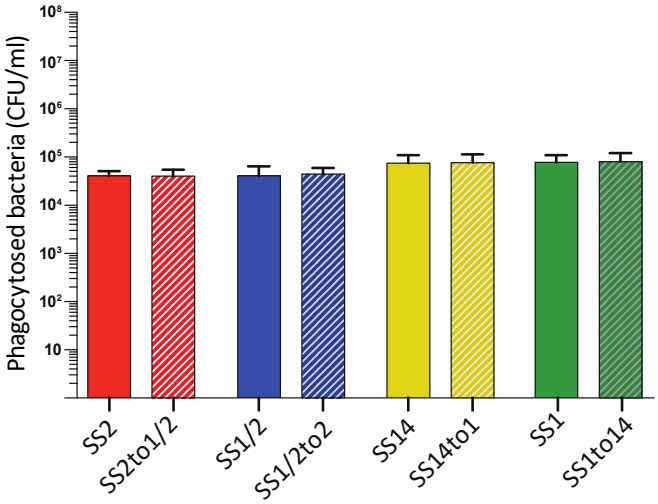

Figure 7. The antiphagocytic properties afforded to *S. suis* by CPS types 2, 1/2, 14, and 1 appear similar under *in vitro* conditions. Parental strains and isogenic mutants (1×10^7 CFU/ml) were incubated for 60 min with J774 macrophages (multiplicity of infection = 100) in the presence of 50% murine serum. Results represent the mean (CFU/ml) + SEM of four independent experiments. Statistical analyses using the Student's t-test showed no significant differences in the number of internalized bacteria between strains.

ANNEXES - ARTICLE VI

Role of the capsular polysaccharide as a virulence factor for *Streptococcus suis* serotype 14

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-auteur de l'article. J'ai participé à la conception des approches méthodologiques et aux expériences *in vitro* impliquant les cellules. J'ai effectué les expériences *in vivo* et j'ai participé à l'analyse des résultats, à l'écriture de la première version du manuscrit et dans la révision de celui-ci.

Abstract

Streptococcus suis is an important swine pathogen and a zoonotic agent causing meningitis and septicemia. Although serotype 2 is the most virulent type, serotype 14 is emerging, and understanding of its pathogenesis is limited. To study the role of the capsular polysaccharide (CPS) of serotype 14 as a virulence factor, we constructed knockout mutants devoid of either *cps14B,* a highly conserved regulatory gene, or *neu14C,* a gene coding for uridine diphospho-N-acetylglucosamine 2-epimerase, which is involved in sialic acid synthesis. The mutants showed total loss of the CPS with coagglutination assays and electron microscopy. Phagocytosis assays showed high susceptibility of mutant Δ*cps14B.* An *in vivo* murine model was used to demonstrate attenuated virulence of this non-encapsulated mutant. Despite the difference in the CPS composition of different serotypes, this study has demonstrated for the first time that the CPS of a serotype other than 2 is also an important antiphagocytic factor and a critical virulence factor.

Introduction

Streptococcus suis, a Gram-positive bacterium, is an important swine pathogen causing meningitis, septicemia, endocarditis, arthritis, and other infections (1). It is responsible for economic losses to the swine industry and is considered an emerging zoonotic agent causing mainly meningitis and septic shock in humans (2). Thirty-five serotypes based on capsular epitopes have been described (1). Serotype 2 is considered the most virulent and has several reported virulence-associated factors implicated in infection (3). Among these factors, the capsular polysaccharide (CPS) is one of the most important, playing a crucial role in the infection (4). In fact, almost all studies in the literature on virulence factors have been done with serotype 2 strains (3).

In addition to serotype 2, serotype 14 is highly virulent and represents serious health and economic problems in several countries, such as Thailand (in humans) and those of the United Kingdom (in swine) (5,6). Swine and human cases due to this serotype have also been described in Canada (7,8). Recent chemical analyses of the CPS of serotypes 2 and 14 revealed differences in sugar composition. For example, no rhamnose residue was found in the serotype 14 CPS (9). A side chain containing sialic acid (coded by the genes *neuA* to *neuD*) and an α-2,6-sialyltransferase are present in the CPS of both serotypes (10). Bacterial sialic acid has been implicated as a virulence factor for several pathogens, such as the closely related group B *Streptococcus* (GBS) (11). However, mutants lacking genes involved in sialic acid synthesis were found to have poorly encapsulated or non-encapsulated phenotypes for GBS and *S. suis* serotype 2, respectively, which complicates the study of the sialic acid moiety as a virulence factor (12).

Since no studies had yet been carried out on the role of CPS as a virulence factor for any *S. suis* serotype other than serotype 2, we constructed a serotype 14 isogenic knockout mutant devoid of a highly conserved regulatory gene, *cps14B.* In addition, we studied the effect of the absence of sialic acid on the expression of the whole CPS of serotype 14 by constructing a mutant deficient in the *neu14C* gene, which codes for uridine diphospho-N-acetylglucosamine 2-epimerase, an enzyme involved in sialic acid synthesis.

Materials and Methods

The well-encapsulated *S. suis* serotype 14 reference strain DAN13730, isolated from a human case in The Netherlands (13), was used as the host strain for in-frame allelic deletion mutagenesis. The well-encapsulated virulent serotype 2 strain P1/7 and its previously obtained isogenic capsule-deficient mutant Δ*cps2F* (14) were used for comparison purposes. The bacterial strains and plasmids used in this study are listed in Table I. The *S. suis* strains were grown in Todd Hewitt broth (THB) or agar (THA) (Becton-Dickinson, Sparks, Maryland, USA) at 37°C. Precise in-frame deletions in *cps14B* and *neu14C (cps14Q)* were achieved by using splicing-by-overlap- extension polymerase chain reaction (PCR), as previously described (16).

Genomic DNA of *S. suis* was purified by InstaGene Matrix solution (BioRad Laboratories, Hercules, California, USA). The primers used for the construction of deletion alleles (Table II) were obtained from Integrated DNA Technologies (Coralville, Iowa, USA). The PCR reactions were carried out with iProof proofreading DNA polymerase (BioRad Laboratories) or with *Taq* DNA polymerase (Qiagen, Valencia, California, USA). Amplification products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer and the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). Overlapping PCR products were cloned into plasmid pCR2.1 (Invitrogen, Burlington, Ontario), extracted with EcoRI, and recloned into the thermosensitive *Escherichia coli–S. suis* shuttle vector pSET4s digested with the same enzyme, which gave rise to the p4Δcps14B and p4Δneu14C mutation vectors (17). The recombinant plasmids were extracted and purified with the QIAprep Spin Miniprep kit (Qiagen). Restriction enzymes and DNA-modifying enzymes were purchased from Fisher Scientific (Ottawa, Ontario) and used according to manufacturer recommendations.

Final constructions of pSET4s, p4Δcps14B, and p4Δneu14C were electroporated into *S. suis*-competent cells with the Biorad Gene PulserXcell apparatus (BioRad Laboratories) under specific conditions: 12.5 kV/cm, 200 Ω, and 25 μF. Transformants were plated on THA supplemented with spectinomycin (THA + Sp) and incubated for 3 d at 28°C. Several Sp-resistant colonies were then subcultured on THA + Sp for 3 d at 28°C. The candidates were next cultured on THA + Sp and incubated at 37°C for 2 successive passages. Temperature- and Sp-resistant clones were successively cultured on THA and THA + Sp to obtain Sp-

sensitive candidates. Deletion of the genes *cps14B* and *neu14C* was confirmed by PCR and sequence analysis.

For complemented mutants, intact *cps14B* and *neu14C* genes were amplified from genomic DNA of the wild-type strain with primers designed with restriction sites (Table II). The PCR products and pMX1 vectors were then digested with the appropriate restriction enzyme before ligation. Final constructions were cloned into *E. coli* MC1061. The *E. coli* strains were grown in Luria–Bertani broth or agar (Becton-Dickinson) at 37°C. When needed, antibiotics (Sigma-Aldrich Canada, Oakville, Ontario) were added to the culture medium at the following concentrations: for *S. suis,* spectinomycin at 100 µg/mL; for *E. coli,* kanamycin and spectinomycin at 50 µg/mL; and for *E. coli,* ampicillin at 100 µg/mL. Complementation of both mutants was achieved by electroporation with pMX14B and pMXNEU14C under the conditions mentioned previously.

Serotyping was carried out by coagglutination tests as described by Gottschalk et al (13), and positive results were recorded when a strong reaction was obtained within 1 min. Transmission electron microscopy (TEM) was carried out as described by Jacques et al (18) with a few modifications. Briefly, bacteria were grown to mid-logarithmic phase and resuspended in 0.1 M cacodylate buffer, pH 7.3, containing 2.5% (v/v) glutaraldehyde and 0.05% (w/v) ruthenium red. Ferritin was then added, to a final concentration of 1 mg/mL, and the suspension incubated for 30 min at room temperature. Afterwards the cells were immobilized in 3% (w/v) agar, washed 5 times in cacodylate buffer containing 0.05% ruthenium red, and fixed with 2% (v/v) osmium tetroxide for 2 h. Samples were washed and dehydrated in graded series of acetone, then washed twice in propylene oxide and embedded in Spurr low-viscosity resin (Sigma-Aldrich Canada). Thin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan) at 80 kV.

The J774A.1 murine macrophage-like cell line (American Type Culture Collection TIB 67; Rockville, Maryland, USA) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin, 25 units/mL (Gibco, Burlington, Ontario). The cells were grown at 37°C with 5% CO_2 until confluent. The cultures were then scraped and the cells washed twice with phosphate-buffered saline (PBS), pH 7.4, resuspended in antibiotic-free medium at 1×10^5 cells per well in a 24-

well tissue culture plate (VWR CanLab, Montreal, Quebec), and incubated for 3 h at 37°C with 5% CO_2 to allow cell adhesion. The cells were infected by removing the medium and adding 1 mL of a bacterial suspension, 1×10^7 colony-forming units (CFU)/mL in antibiotic-free medium; the multiplicity of infection (MOI) was thus 100. The infected cells were incubated for 60 min at 37°C with 5% CO_2 to allow phagocytosis. The optimal incubation time and MOI were chosen according to the results of preliminary studies (data not shown). After incubation, cell monolayers were washed twice with PBS and incubated for 1 h with medium containing penicillin G (Sigma-Aldrich Canada), 5 µg/mL, and gentamicin (Gibco), 100 µg/mL, to kill extracellular bacteria. The cell monolayers were washed 3 times with PBS and lysed with sterile water. The presence of viable intracellular bacteria was determined by plating serial dilutions on THA. Each test was repeated 4 times in independent experiments.

A well-standardized *S. suis* serotype 2 murine model of infection (19) was adapted for the first time to serotype 14. Six-week-old female CD1 mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were used, the experiments involving them being conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and the Use of Laboratory Animals by the Animal Welfare Committee of the University of Montreal. The 45 animals were divided into 3 groups of 15 animals each. On the day of the experiment, each animal was inoculated by intraperitoneal injection with 1.5×10^8 CFU of the *S. suis* serotype 14 wild-type strain DAN13730 or the mutant strain Δ*cps14B* or with a vehicle (THB) as a control. The bacterial concentration was determined from preliminary trials with DAN13730 to establish a high but controlled mortality level (data not shown). The mice were examined at least 3 times daily for clinical signs of septic disease, such as depression, swollen eyes, rough coat hair, and lethargy, for 72 h after infection. Blood samples (5 µL) were collected from the tail vein at 12, 24, 48, and 72 h after infection and plated on THA for evaluation of bacteremia.

All data are expressed as mean ± standard error. For the *in vitro* experiments, data were analyzed for significance with the Mann–Whitney rank-sum test. For the *in vivo* virulence experiments, the Mantel–Cox log-rank test was used to evaluate the difference in mortality rate between the groups, and the Mann–Whitney rank-sum test was used to evaluate the difference in bacteremia between the groups. A *P*-value of less than 0.05 was considered significant.

Results and Discussion

The coagglutination serotyping test is an easy and rapid method to indirectly observe the presence of the capsule in *S. suis* isolates. In this study, both Δ*cps14B* and Δ*neu14C* mutants showed a clear negative result: no agglutination reaction with any typing antiserum. The wild-type strain, DAN13730, showed a strong and fast reaction with serotype 14-specific antiserum. As depicted in Figure 1, TEM showed a thick capsule surrounding the wild-type strain (A) and a complete absence of CPS structure for both mutants (B, C). The gene deletions were complemented in Δ*cps14B/cps14B* and Δ*neu14C/neu14C* to partially restore CPS production, as expected (D, E). Smith et al (20) demonstrated the importance of the glycosyltransferase gene *cps2EF* and a gene involved in chain-length determination *(cps2B)* in the production of CPS for serotype 2, using mutants that resulted in a capsule-deficient phenotype. The non-encapsulated Δ*cps14B* mutant showed that *cps2B* is also required for CPS synthesis in serotype 14. Lack of the sialic acid synthesis gene prevented CPS production in the Δ*neu14C* mutant. This result suggests that, despite differences in CPS composition, sialylation of the *S. suis* serotype 14 CPS repeating units is crucial for CPS exportation or polymerization, as has been reported for serotype 2 (12). Unfortunately, it is still not possible to precisely study the role of sialic acid in the virulence of both serotypes.

Since both mutants were non-encapsulated, further experiments were carried out with Δ*cps14B* alone to investigate the role of CPS in the pathogenesis of *S. suis* serotype 14 infections. Bacterial clearance by phagocytic cells represents an important host mechanism for defending against bacterial infection. To evaluate the susceptibility to phagocytosis of the capsule-deficient mutant Δ*cps14B,* bacteria were incubated with the J774A.1 murine macrophage-like cell line. As expected, the control serotype 2 strain P1/7 and its capsule-deficient mutant Δ*cps2F* were poorly and highly internalized by macrophages, respectively (Figure 2). The wild-type serotype 14 strain DAN13730 was highly resistant to phagocytosis, and only a few bacteria were internalized. In contrast, the serotype 14 non-encapsulated mutant Δ*cps14B* was significantly more internalized than its wild-type strain ($P < 0.05$). The Δ*cps14B/cps14B* complemented strain, in which the CPS was partially restored, was less internalized than the Δ*cps14B* mutant strain but still more phagocytosed than the wild-type strain ($P < 0.05$). These results are similar to those previously reported for serotype 2 strains with use of the RAW264.6 murine macrophage cell line and porcine alveolar macrophages (12,20,21). Overall, the high phagocytosis susceptibility of Δ*cps14B* compared with the wild-

type strain shows the essential role of the CPS in phagocytic resistance to *S. suis* serotype 14. Interestingly, the wild-type serotype 14 strain demonstrated even greater resistance to phagocytosis than the serotype 2 strain in this *in vitro* model ($P < 0.05$).

The role of the CPS in the virulence of a serotype other than 2 was also demonstrated *in vivo.* In the first mouse model for a serotype 14 strain, we investigated the effect of capsule loss using an *in vivo* CD1 murine model of *S. suis* infection. All 15 mice inoculated with the wild-type strain presented severe clinical signs of infection and died within the first 36 h (Figure 3A). The animals died relatively fast and mainly from septicemia and septic shock. Reducing the dose by 1 log resulted in complete absence of clinical signs and death. In contrast to the results with serotype 2, no cases of meningitis were observed, suggesting that the 2 serotypes do not behave identically in this animal model. None of the mice inoculated with the non-encapsulated Δ*cps14B* mutant strain died from the infection ($P < 0.0001$), and few clinical signs were observed. At 12 h after infection the mice inoculated with the wild-type strain had a mean blood bacterial burden of 2×10^8 CFU/mL, significantly greater ($P < 0.001$) than the burden of the mice inoculated with the Δ*cps14B* mutant (Figure 3B). At 24 h after infection the few surviving mice infected with the wild-type strain had a blood bacterial burden similar to that at 12 h and also significantly greater ($P < 0.001$) than that of the mice inoculated with the Δ*cps14B* mutant (data not shown). As Δ*cps14B* is avirulent in our infection model, the *in vivo* results confirm the phagocytosis results and demonstrate the crucial role of CPS in the virulence of *S. suis* serotype 14.

Conclusion

In conclusion, whereas there are differences in CPS composition and structure between serotypes 2 and 14, this study has demonstrated that the CPS of serotype 14 possesses important antiphagocytic properties and is a critical virulence factor. Since serotypes 14 and 1 have not only epitopes in common but also highly similar *cps* clusters and highly similar CPS structures (9; unpublished observations), the serotype 1 CPS may play a role in virulence and phagocytosis similar to that of serotypes 2 and 14. Further studies are needed to confirm this theory. This is the first report on the role of the CPS of an *S. suis* serotype other than 2. As with serotype 2, it has so far been impossible to evaluate the specific role of sialic acid in the virulence of *S. suis* serotype 14 since no CPS is produced in the absence of this sugar. Although the mouse model used in this study may be used to evaluate the systemic virulence

of *S. suis* serotype 14, other models of meningitis with this serotype must be standardized. Further characterization and investigation will be necessary to dissect other virulence factors that would explain why serotype 2 strains are more widespread globally and seem to be more virulent in the field than serotype 14 strains.

Acknowledgments

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Tables

Table I. Bacterial strains and plasmids used in this study

Strains/Plasmid/Primers	General characteristics	Source/Reference
Escherichia coli		
TOP 10	F-mrcA Δ(mrr-hsdRMS-mcrBC)ϕ80 lacZΔM5 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
MC1061	*araD139 Δ*(*ara-leu*)*7697 ΔlacX*74 *galU galK hsdR2*(rK- mK+) *mcrB1 rpsL*	[35]
Streptococcus suis		
P1/7	Wild-type, highly encapsulated serotype 2 strain isolated from a clinical swine case in the United Kingdom	[36]
DAN13730	Wild-type, highly encapsulated serotype 14 strain isolated from a human case in The Netherlands	[22]
Δ*cps2F*	Non-encapsulated isogenic mutant strain derived from strain P1/7. Deletion of the *cps2F* gene	[23]
Δ*cps14B*	Non-encapsulated isogenic mutant strain derived from strain DAN13730. Deletion of the *cps14B* gene	This work
Δ*neu14C*	Non-encapsulated isogenic mutant strain derived from DAN13730. Deletion of the *neu14C* gene.	This work
Δ*cps14B/cps14B*	Mutant Δ*cps14B* complemented with pMX14B complementation vector	This work
Δ*neu14C/neu14C*	Mutant Δ*neu14C* complemented with pMXNEU14C complementation vector	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3 and pUC19, MCS and *lacZ* of pUC19, Sp^R	[27]

pMX1	Replication functions of pSSU1, MCS of pUC19, Sp^R, malX promoter of *S. suis,* derivative of pSET2	[37]
p4Δcps14B	pSET4s carrying the construct for *cps14B* allelic replacement	This work
p4Δneu14C	pSET4s carrying the construct for *neu14C* allelic replacement	This work
pMX14B	pMX1 complementation vector carrying intact *cps14B*	This work
pMXNEU14C	pMX1 complementation vector carrying intact *neu14C*	This work

Table- II. Oligonucleotide primers used in this study

Oligonucleotide primers, sequence (5' – 3').		Constructs
cps14B-ID1	GACCAAGATAACATCACCGC	p4Δcps14B
cps14B-ID2	GGTTGGCATTTGTCTACAGT	p4Δcps14B
cps14B-ID3	ACCACTCCAAATACAAAACG	p4Δcps14B
cps14B-ID4	GCTCGCGCTATATTCTCTTG	p4Δcps14B
cps14B-ID5	CGACTTGGTGGGTGGAATTG	p4Δcps14B
cps14B-ID6	TCTTCGATGTCCTGAGGACGGTCAACA CTGCAGTTAAGAG	p4Δcps14B
cps14B-ID7	CTCTTAACTGCAGTGTTGACCGTCCTCA GGACATCGAAGA	p4Δcps14B
cps14B-ID8	GGTTTCTCCCAACCCTACTG	p4Δcps14B
neu14C-ID1	CGGTGATGTTCATCTAGCACGG	p4Δneu14C
neu14C -ID2	AGCGATCCCCCAGAATCAACAC	p4Δneu14C
neu14C -ID3	CACAGCCGAAGAACAAACGCAG	p4Δneu14C
neu14C -ID4	TGGACGCATGAGGACTTGAACC	p4Δneu14C
neu14C -ID5	TCTCAGCTCGAAATGACTCGTC	p4Δneu14C
neu14C -ID6	CATGGTTGAGGCCTGACGAGAGCCTGT CAC	p4Δneu14C
neu14C -ID7	GTGACAGGCTCTCGTCAGGCCTCAACC ATG	p4Δneu14C
neu14C -ID8	AGGTCCCTGACTCCGTCAAC	p4Δneu14C
pCPS14BF_NcoI[b]	AGCCATGGAGTCCGTACTTGTTTA	pMX14B
pCPS14BR_EcoRI[b]	GTACGTGGAATTCCTAACATTGCC	pMX14B
pNEU14CF_PstI[b]	TGAGCTGCAGCAAAATATTTGCCATAGTG C	pMXNEU14C
pNEU14CR_PstI[b]	CATCTGCAGAGGTACCCGCTCCTAGAAA GG	pMXNEU14C

Figures

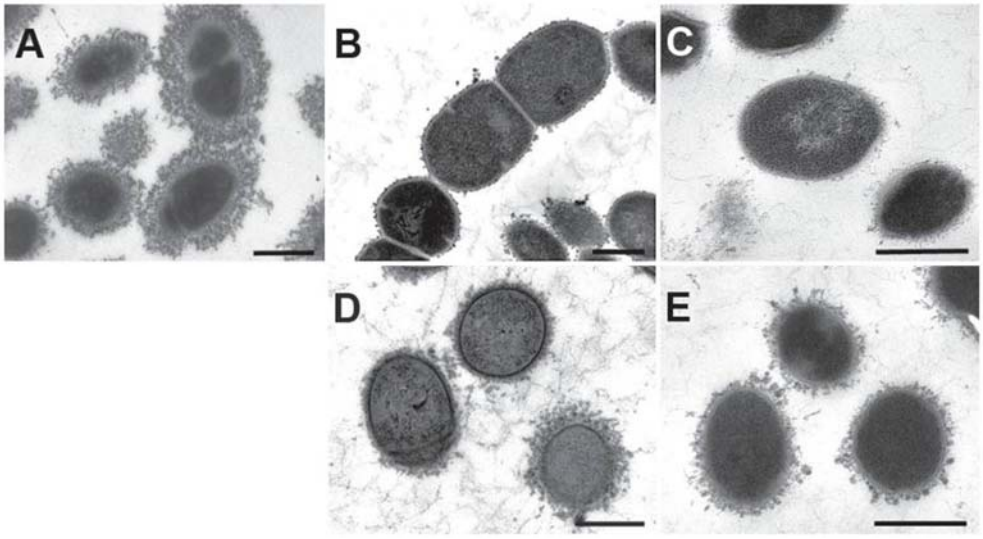

Figure 1. Transmission electron micrographs showing the expression of capsular polysaccharide (CPS) by the wild-type strain of *Streptococcus suis* serotype 14 and its derived mutants. The CPS was labelled with polycationic ferritin. The wild-type strain, DAN13730 (A), is surrounded by a thick capsule, whereas the Δ*cps14B* and Δ*neu14C* mutant strains (B and C, respectively) are non-encapsulated. The complemented strains Δ*cps14B/cps14B* (D) and Δ*neu14C/neu14C* (E) show an intermediate state of CPS production. Bars = 0.5 µm.

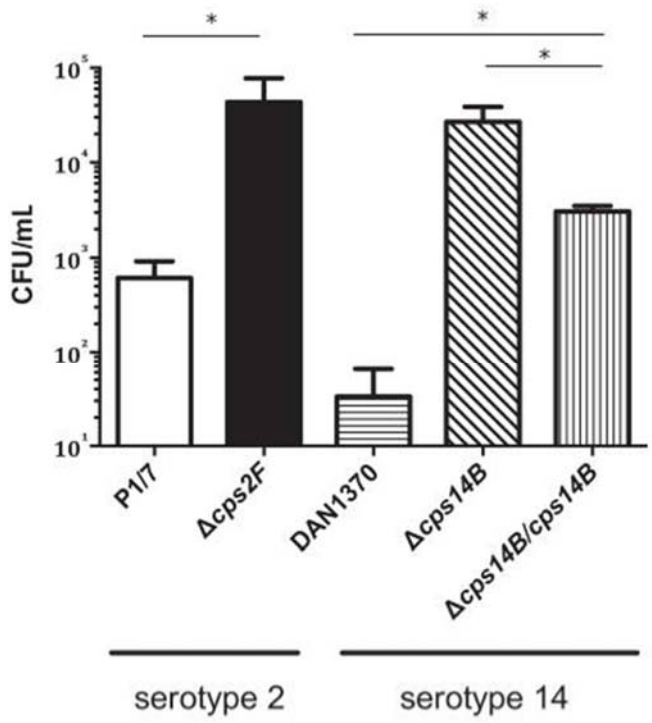

Figure 2. Phagocytosis of *S. suis* serotypes 2 and 14 by murine macrophages. Results for the well-encapsulated virulent serotype 2 strain P1/7 and its previously obtained isogenic capsule-deficient mutant Δ*cps2F* are depicted for comparison only. Bacteria, 1×10^7 colony-forming units (CFU)/mL, were incubated for 60 min with J774A.1 murine macrophage-like cells, at a multiplicity of infection of 100, and gentamicin/penicillin G was used to kill any extracellular bacteria remaining after incubation. Intracellular counts were done after 3 washes and cell lysis with water. Results represent the mean count + the standard error in 4 independent experiments. Each asterisk indicates a significant difference between strains ($P < 0.05$) according to the Mann–Whitney rank-sum test.

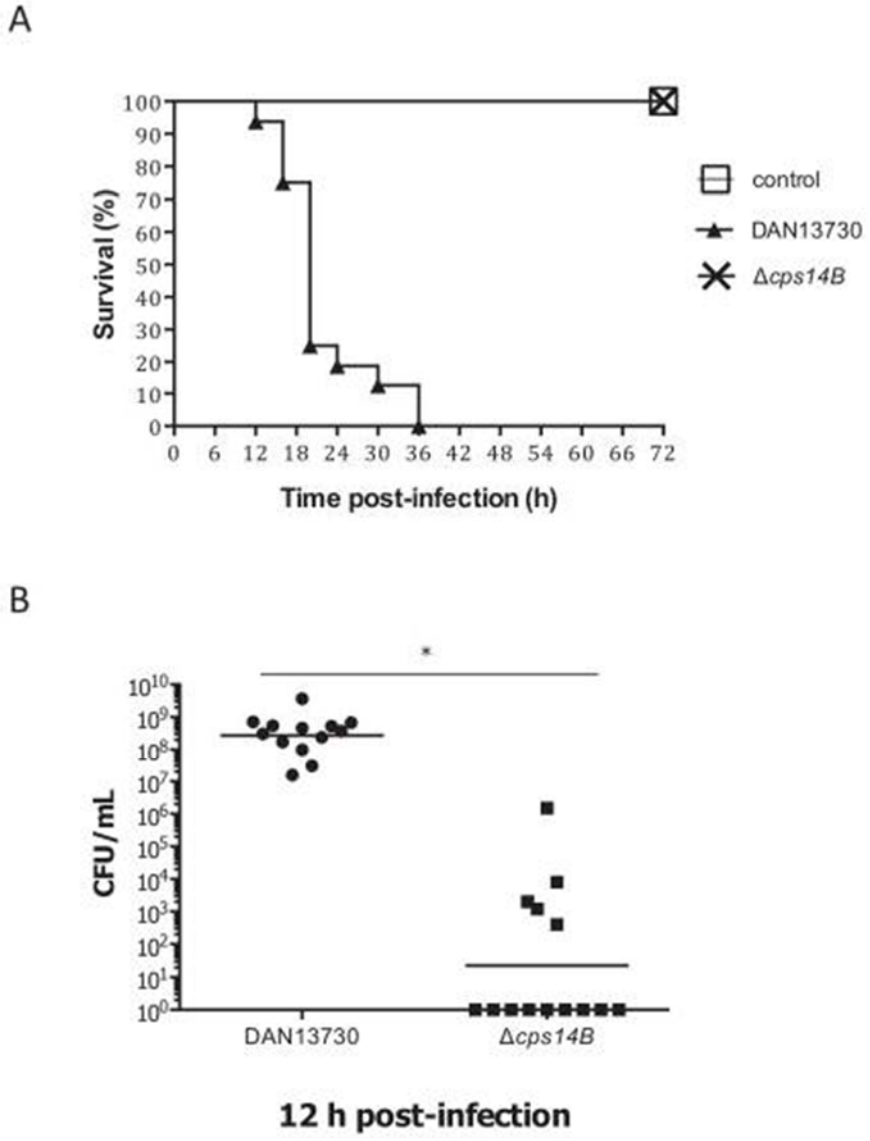

Figure 3. Survival (A) and blood bacterial burden (B) of CD1 mice inoculated by intraperitoneal injection with 1.5 × 10^8 CFU of DAN13730 or Δ*cps14B* or with Todd Hewitt broth as a control. Bacterial counts were determined in serial dilutions of 5 µL of blood obtained from the tail vein 12 h after inoculation. The Mantel–Cox log-rank test revealed a significant difference ($P < 0.0001$) in survival rate between the groups: all mice receiving the wild-type strain died, whereas all those receiving the mutant strain survived, and all the control animals were unaffected. The Mann–Whitney rank-sum test showed a significant difference ($P < 0.001$) in blood bacterial burden between the mice inoculated with the wild-type strain and those inoculated with the mutant strain (asterisk).

ANNEXES - ARTICLE VII

Characterization of the zinc metalloprotease of *Streptococcus suis* serotype 2

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-auteur de l'article. J'ai participé à la réalisation des expériences *in vivo* et à l'analyse des résultats. J'ai été impliqué dans la révision du manuscrit et j'ai participé aux corrections lors des étapes de révision pour publication.

Abstract

Streptococcus suis is an important swine pathogen and zoonotic agent responsible for meningitis and septic shock. Although several putative virulence factors have been described, the initial steps of the pathogenesis of the *S. suis* infection remain poorly understood. While controversial results have been reported for a *S. suis* serotype 2 zinc metalloprotease (Zmp) regarding its IgA protease activity, recent phylogenetic analyses suggested that this protein is homologous to the ZmpC of *Streptococcus pneumonia*e, which is not an IgA protease. Based on the previously described functions of metalloproteases (including IgA protease and ZmpC), different experiments were carried out to study the activities of that of *S. suis* serotype 2. First, results showed that *S. suis*, as well as the recombinant ZmpC, were unable to cleave human IgA_1, confirming lack of IgA protease activity by this pathogen. Similarly, *S. suis* was unable to cleave P-selectin glycoprotein ligand-1 and to activate matrix metalloprotease 9, at least under the conditions tested. However, *S. suis* was able to partially cleave mucine 16 and syndecan-1 ectodomains. Experiments carried out with an isogenic Δ*zmpc* mutant showed that the Zmp protein was partially involved in such activities. The absence of a functional ZmpC protein did not affect the ability of *S. suis* to adhere to porcine bronchial epithelial cells *in vitro*, or to colonize the upper respiratory tract of pigs *in vivo*. The *iga* terminology for the gene coding for the ZmpC of *S. suis* should be avoided and changed by *zmpc*. Taken together, our results show that *S. suis* serotype 2 ZmpC is not a critical virulence factor and highlight the importance of independently confirming results on *S. suis* virulence by different teams of investigators.

Introduction

Streptococcus suis is a swine pathogen responsible for cases of meningitis, arthritis, endocarditis, and sudden death in post-weaned piglets. It is responsible for substantial economic losses to the porcine industry and it also represents a serious problem due to the routine use of antimicrobials in the field in attempts to control the infection [1]. It is also an emerging zoonotic agent causing meningitis and septic shock in individuals associated with the swine/pork industry in Western countries or among the general population in some Asian countries [1]. A total of 35 capsular-based serotypes have been reported, with certain of these having recently been described as belonging to other bacterial species [2]. Of the different serotypes, serotype 2 is the most frequently isolated from diseased pigs and humans worldwide [1]. Serotype 2 strains differ greatly in terms of virulence potential and geographic distributions and it can be further classified into different sequence types (ST) based on the multilocus sequence typing (MLST) scheme. Indeed, most virulent strains isolated in Europe and Asia belong to ST1, whereas ST25 and ST28 strains, considered as less virulent, are mainly present in North America [3]. ST7 serotype 2 strains, responsible for at least two major outbreaks of human *S. suis* infections in China, are considered highly virulent [4].

The initial mechanisms involved in *S. suis* colonization of the host remain poorly known, with the pathogen being able to survive in the tonsils of swine for long periods of time [5]. *S. suis* has been described to colonize and interact with epithelial cells and mucus of the host upper respiratory tract in order to reach the bloodstream, where it resists phagocytosis and killing [5]. Replication in blood and systemic dissemination allow *S. suis* to subsequently invade the central nervous system and cause meningitis [6]. Over the years, different bacterial components have been suggested to be involved in the *S. suis* pathogenesis, including the capsular polysaccharide, the suilysin, the extracellular protein factor (EF), and the muramidase-released protein [7]. However, controversy continues to persist regarding the role of so-called critical *S. suis* virulence factors [7].

Type A immunoglobulins (IgA) are the predominant immunoglobulin class produced by mucosa-associated lymphoid tissues. They may prevent the adhesion of microorganisms to epithelial cells and consequently facilitate their elimination from the host [5]. In the case of *S. suis*, the secretion of an active human IgA_1 protease, a zinc-dependent metalloprotease (Zmp) encoded by the *iga* gene, has been reported in a serotype 2 ST7 strain [8]. The

decreased lethality in pigs following intranasal inoculation of an Δ*iga* mutant strain suggested that mucosal IgAs play a crucial role in resistance to *S. suis* invasion and host dissemination [8, 9]. However, this conclusion may be questionable based on three main considerations: firstly, porcine specific or cross-reactive IgAs against *S. suis* have never been documented [5]; secondly, no IgA protease activity against human IgAs was detected in any of the *S. suis* strains evaluated in a subsequent study [10], and thirdly, in silico amino acid sequence analysis, as well as structural homology comparisons, do not support the notion that the Zmp protease encoded by gene *iga* can have IgA protease activity. In fact, Zmps have been well described in *Streptococcus pneumoniae* and are classified into four distinct groups: ZmpA (IgA protease), ZmpB, ZmpC, and ZmpD [10]. Similar to *S. pneumoniae* Zmps, that of *S. suis* is a membrane protein attached by a cell-wall LPXTG-anchored motif that possesses G5 tandem repeats and a M26 protease active site. This catalytic site is characterized by a HEMVH motif, which is a key characteristic of the *S. pneumoniae* ZmpC (but not ZmpA) [8, 10]. In accordance, phylogenetic studies have classified the *S. suis* Zmp as an homologue of the *S. pneumoniae* ZmpC based on genomic sequence similarities [10]. To avoid confusion, we will hereafter refer to the factor as ZmpC. Importantly, the gene coding for this protein (*zmp*) was found to be present in several of the genomes of 300 *S. suis* strains recently analyzed [11].

S. pneumoniae ZmpC has been described to possesses different activities, which include activation of matrix metalloproteinase 9 (MMP-9) and cleavage of P-selectin glycoprotein ligand-1 (PSGL-1), mucine 16 (MUC16), and syndecan-1 (SDC-1) ectodomains [10, 12-15]. Consequently, given the genetic sequence similarities between Zmps of *S. suis* and *S. pneumoniae*, it is possible that this protein could have an important impact on the first steps of *S. suis* pathogenesis. Moreover, some of these functions could also play important roles in invasion of the central nervous system [16]. However, the actual role of *S. suis* Zmp in the pathogenesis of the infection has not been completely evaluated. Finally, it is still not clear if Zmp (or *S. suis* in general) is able to present an IgA protease activity.

In the present study, putative functions as well as the role in virulence of the *S. suis* Zmp were studied. We report that *S. suis* not only does not cleave human IgA_1, but it is also unable to cleave PSGL-1 ectodomains or to activate MMP-9. However, Zmp is responsible, at least in part, for the *S. suis* cleavage of MUC16 and SDC-1 ectodomains, though this activity does not

appear to have a critical impact on *S. suis* serotype 2 colonization of the upper respiratory tract nor virulence.

Material and Methods

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in **Table 1**. The virulent *S. suis* serotype 2 strain P1/7 was used for the construction of an isogenic *zmp*-deficient mutant. The *S. pneumoniae* strain TIGR4 (American Type Culture Collection, Manassas, VA, USA) was used as a positive control for most activation and cleavage assays. Streptococci were grown in Todd-Hewitt broth (THB) until exponential growth phase or agar (THA) for 18 h (Becton-Dickinson, Sparks, MD, USA) 37 °C with 5% CO_2, with the exception of the SDC-1 ectodomain cleavage experiment (see below), for which bacteria were used in stationary growth phase as previously described for other pathogens [12, 17, 18]. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or agar (Becton-Dickinson) at 37 °C. When needed, antibiotics were added at the following concentrations: for *S. suis*, spectinomycin at 100 µg/mL; for *E. coli*, kanamycin at 25 µg/mL and ampicillin, spectinomycin and carbenicillin at 50 µg/mL (Sigma, Oakville, ON, Canada).

DNA manipulations

S. suis genomic DNA was extracted using InstaGene Matrix solution (Biorad Laboratories, Hercules, CA, USA). Plasmid minipreparations were performed with the QIAprep Spin Miniprep Kit (Quiagen, Valencia, CA, USA). Restriction and DNA-modifying enzymes were purchased from Fisher Scientific (Ottawa, ON, Canada) and used according to the manufacturer's recommendations. Oligonucleotide primers (listed in **Table 2**) were obtained from Integrated DNA Technologies (Coralville, IA, USA). Polymerase chain reactions (PCR) were carried out using the iProof high-fidelity DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or the Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with an ABI 310 automated DNA sequencer and the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Construction of the* S. suis *zmp-deficient (Δ*zmp*) mutant and complemented strain

Precise in-frame deletion of the *zmp* gene was achieved using splicing-by-overlap-extension PCR [19]. Overlapping PCR-products generated by PCR were cloned into the plasmid pCR2.1 (Invitrogen), extracted using EcoRI, and cloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s, as previously described [20]. The resulting mutation vector p4Δ*zmp* was electroporated into competent *S. suis* recipient cells. Allelic replacement and deletion of the gene was confirmed by PCR and sequence analysis.

Circular polymerase extension cloning (CPEC) was used for the construction of the complementation vector of *zmp* gene, as described previously [21]. Briefly, the complementation vector pMX1, which possesses a *malQ* inducible promoter, was linearized by PCR and the complete *zmp* gene amplified. Overhanging primers were used to ligate the gene into the linearized pMX1. The construction was then purified and transformed into electrocompetent *E. coli* MC1061. The complemented Δ*zmp* (compΔ*zmp*) mutant was obtained by transformation into electrocompetent *S. suis* Δ*zmp*. Obtention of compΔ*zmp* was confirmed by PCR and DNA sequencing analysis.

Absence of Zmp expression in the Δ*zmp* mutant and complementation of *zmp* gene were confirmed by immunoblot as previously described [22], using antisera from rabbits immunized with the recombinant Zmp protein (see below). Expression of a capsular polysaccharide (already described as a major virulence factor [23]) was confirmed by hydrophobicity and coagglutination assays using serotype 2 antiserum, as previously described [24].

Expression and purification of the recombinant Zmp protein

The region corresponding to the *zmp* gene, excluding the region encoding the LPVTG motif, was amplified by PCR and directly cloned into the pET101 vector (Invitrogen), which possesses a C-terminal His-tag, according to the manufacturer's instructions. Protein production was induced in the *E. coli* BL21 (DE3) strain using 0.5 mM IPTG for 4 h, after which cells were lysed by sonication. Cell lysates were used to purify the recombinant Zmp protein by His-Bind Resin Chromatography Kit (Novagen, Madison, WI, USA) according to manufacturer's instructions and dialysed. Protein purity was confirmed by SDS-PAGE and Western blot using an anti-His-tag antibody (R&D Systems, Minneapolis, MN, USA). The purified recombinant protein was concentrated using Amicon Ultra-15 (Millipore, Billerica, MA, USA) and protein quantification

was evaluated using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific). A mono-specific polyclonal hyperimmune serum was produced in rabbits using the purified Zmp protein as previously described [25].

IgA protease cleavage assay

The IgA protease cleavage assay was performed as previously described with some modifications [10, 14]. Briefly, 5 µg of human myeloma IgA_1 (Calbiochem/EMD Millipore, San Diego, CA, USA) were incubated with either purified recombinant Zmp (100 µg), 1 x 10^8 CFU/mL of live washed *S. pneumoniae* (positive control), 1 x 10^9 CFU/mL of live washed *S. suis* P1/7 strain or supernatants (non-concentrated or concentrated up to 10X) of each bacterial species for 16 h at 37 °C. Using similar conditions, the ST7 strain SC84, responsible for the 2005 Chinese human outbreak [26], was also tested. Bacterial supernatants were filtrated and concentrated up to 10X using the Thermo Savant DNA120 Speedvac (Thermo Fisher). Samples were then separated on 7.5% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane (Bio-Rad). The size of uncleaved and cleaved human IgA_1 was detected using Western blot with mouse monoclonal B3506B4 anti-human IgA_1 Fc horseradish peroxidase (HRP)-conjugated antibodies diluted 1:500 (Abcam, Cambridge, MA, USA) and detected using HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ, USA).

Activation of matrix metalloprotease-9

MMP-9 activation assay was performed as previously described with some modifications [10, 14]. Briefly, 10 ng of recombinant human pro-enzyme MMP-9 (Calbiochem/EMD Millipore) were incubated with recombinant Zmp (100 µg), *S. suis* or *S. pneumoniae* (washed bacteria and supernatants as described above) for 1 h at 37 °C. Samples were then separated on 10 % zymogram gels containing gelatin B (Sigma) under non-denaturing conditions. Results were visualized after Coomassie Blue R250 staining (Bio-Rad).

Cleavage of P-selectin glycoprotein ligand-1 ectodomains

The PSGL-1 cleavage assay was performed as previously described with some modifications [15]. Briefly, 10 ng of recombinant human PSGL-1/CD162 Fc (R&D Systems, Minneapolis, MN, USA) were incubated with recombinant Zmp (100 µg), *S. suis* or *S. pneumoniae* (washed bacteria and supernatants, as described above) for 1 h at 37 °C. Samples were then separated

on 7.5% SDS-PAGE under denaturing conditions and transferred to polyvinyl difluoride (PVDF) membrane (EMD Millipore). The size of cleaved and uncleaved human PSGL-1/Fc was detected using Western blot with mouse monoclonal anti-human CD162 antibodies, clones KPL-1 and PL2, diluted 1:1000 (Becton-Dickinson Biosciences; MBL International Corporation, Woburn, MA, USA) and horseradish peroxidase conjugated (HRP) goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Detection was achieved by using HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific).

Cleavage of mucine 16 ectodomains

The MUC16 ectodomain cleavage assay was performed as previously described with some modifications [13, 27]. The HeLa (CCL-2) cell line was obtained from the ATCC and incubated at 37 °C with 5% CO_2. Once confluent, cells were trypsinized, transferred to 24-well tissue culture plates (Costar) at a concentration of 7.5 x 10^4 cells/mL, and further incubated to confluence from 2 to 3 days (approximately 10^5 cells/well) [28]. Cells were then washed twice with phosphate-buffered saline (PBS - pH 7.3) before being treated with recombinant Zmp (100 µg), *S. suis* or *S. pneumoniae* (washed bacteria and supernatants as described above). In a parallel study, *S. suis*Δ*zmp*-concentrated (3X) supernatants supplemented or not with 100 µg of recombinant Zmp and *S. suis* compΔ*zmp* concentrated (3X) supernatants were also tested. Cells were incubated for 4 h at 37 °C, after which supernatants were recovered and concentrated two-fold with Thermo Savant DNA120 Speedvac (Thermo Fisher). Cleaved MUC16 ectodomains were determined using DOT-blot on nitrocellulose membrane with 1:100 mouse anti-human CA125 antibody (M11) (Thermo Fisher) and a 1:4000 HRP conjugated goat anti-mouse antibody (Jackson ImmunoResearch). Detection was achieved by using the HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific) and signals analysed using ImageJ.50i software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Cleavage of syndecan-1 ectodomains

SDC-1 ectodomains cleavage assay was performed as previously described with some modifications [12, 17, 18]. Briefly, NMuMG cell line, derived from a mouse mammary gland, was propagated as previously described [12, 17, 18]. One-day post-confluent NMuMG cells in 96-well tissue culture plates were washed with culture medium and then incubated with 10 % concentrated filtered supernatants (10X) from an overnight culture of *S. suis*, *S. suis* Δ*zmp*,

S. suis comp*Δzmp* for 4 h at 37 °C. Cells were also incubated with recombinant Zmp (100 µg) or 10 % *S. suisΔzmp*-concentrated supernatant (10X) supplemented with 100 µg of recombinant Zmp protein. Phorbol 12-myristate 13-acetate (PMA; Sigma) was used as a positive control at a concentration of 1 µM. After incubation of 4 h, conditioned media were collected and acidified as previously described [12, 17, 18]. Samples were dot-blotted on Immobilon Ny+ (Millipore) and shed ectodomains were quantified by immunoblotting using 281-2 rat anti-mouse CD138 antibodies. The intensity of dots was quantified by ImageJ.50i software.

Adhesion to primary porcine bronchial epithelial cells

Primary porcine bronchial epithelial cells (PBEC) were isolated from lungs obtained from 5-6 month old healthy pigs as previously described [29] and differentiated in air-liquid-interface medium (ALI medium), which consists of 50 % Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) and 50 % Bronchial Epithelial Growth Medium (BEGM; constituted of bronchial epithelial cell basal medium [BEBM; Lonza] supplemented with several required additives [Sigma]), as previously described [30]. After 4 weeks of differentiation, cells were infected from the apical side, as previously described [29], with approximately 10^7 CFU/filter of *S. suis* strain P1/7 (WT) or Δ*zmp* mutant. Adhesion was analyzed using immunofluorescence microscopy and quantified as previously described [29].

Evaluation of the colonization capacity and virulence of the* S. suisΔzmp *in a porcine model of infection

Four-week old piglets (N=12) from a herd with no recent cases of *S. suis* disease were used. Animals were randomly separated into two rooms upon arrival (6 animals per room) and the trachea and tonsils were swabbed to confirm absence of serotype 2 European-like strains using a multiplex PCR targeting the *epf* gene that codes for the EF [31], usually absent in North American serotype 2 strains [3], and *cps2j* gene, coding for the capsular polysaccharide of serotype 2 strains. The *S. suis* wild-type serotype 2 strain P1/7 (which is *epf* and *cps2j* positive) and Δ*zmp*-deficient mutant were cultured as previously described [32] to obtain a final concentration of 2 x 10^{10} CFU/mL. Intranasal infections were carried out as previously described with some modifications [33]. Pigs were inoculated with 2 mL of 1 % acetic acid per nostril 2 h prior to infection with 2 mL per nostril of either the wild-type strain or Δ*zmp* mutant. Trachea and tonsils were swabbed using a catheter (Medline, Waukegan, IL, USA) or a brush

(Medical Packaging Corporation, Camarillo, CA, USA), respectively, immediately prior to infection, 24 h after infection, and every 2 d thereafter. Samples were placed in PBS supplemented with 0.1 % bovine serum albumin and immediately cultured. Serial dilutions (10^0-10^{-6}) were plated on Colombia Agar (Oxoid, Hampshire, UK) supplemented with 5 % defibrinated sheep blood (Cedarlane, Burlington, ON, Canada), and *Streptococcus* Selective Reagent SR0126 (Oxoid). After incubation for 18 h at 37 °C with 5 % CO_2, plates containing between 30 and 300 colonies were selected. Suspected alpha-hemolytic colonies were enumerated and 10 *S. suis*-like colonies/plate were sub-cultured in order to perform the *epf* and *cps2j* multiplex PCR [31].

Clinical signs of pigs were monitored throughout the experiment. Nine days post-infection, remaining animals were euthanized. Liver, spleen, and brain samples were collected upon euthanasia. All samples were evaluated for carriage of *S. suis* serotype 2 *epf*$^+$ as described above. *S. suis* serotype 2 strains recovered from euthanized animals infected with the mutant strain were monitored for absence of the *zmp* gene by PCR.

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). Significant differences were determined using the t-test, Mann-Whitney Rank sum test or one-way ANOVA, where appropriate. Each test was repeated in at least three independent experiments. $p < 0.05$ was considered statistically significant.

Results

Confirmation of the Zmp-deficient and complemented S. suis *mutants*

A Western blot using a rabbit anti-Zmp serum confirmed production of Zmp protein in the *S. suis* wild-type strain P1/7, which was abrogated following in-frame deletion of the *zmp* gene in the mutant strain **(Figure 1)**. Complementation of *zmp* gene (*S. suis* comp*Δzmp*) restored production of Zmp, albeit expression was lower than that of the wild-type parent strain **(Figure 1)**. Moreover, gene deletion and complementation had no impact on growth of the mutant in comparison with the wild-type strain (results not shown). The mutant and complemented strains were shown to be well-encapsulated by the hydrophobicity test and the clear positive reaction by coagglutination using serotype 2 anti-serum (results not shown).

S. suis *is unable to cleave human IgA$_1$*

In order to confirm the ability of *S. suis* and Zmp to cleave IgAs, recombinant Zmp, bacterial suspensions (bact) or bacterial supernatants (sup; either non-concentrated or concentrated) were incubated with human IgA$_1$. For *S. pneumoniae* strain TIGR4 (SP), used as a positive control, an IgA protease activity was clearly observed **(Figure 2)**. On the contrary, recombinant *S. suis* Zmp was unable to cleave human IgAs **(Figure 2).** Indeed, the virulent strain P1/7 of *S. suis* used in this study (both whole live washed bacteria and culture supernatants) was completely unable to cleave human IgAs **(Figure 2)**. Similarly, *S. suis* ST7 strain SC84 did not possess any IgA protease activity **(Additional File 1)**.

S. suis *does not activate MMP-9*

To assess the capacity of *S. suis* and Zmp to activate MMP-9, the enzyme pro-form was incubated with recombinant Zmp, bacteria (bact) or bacterial supernatant (sup; either non-concentrated or concentrated). MMP-9 activation was then visualized using gelatin B zymogram, wherein absence of color after Coomassie staining indicates presence of a gelatinase activity. As observed in **Figure 3**, *S. pneumoniae* strain TIGR4 (SP), used as a positive control, was able to activate MMP-9 since an additional clear band corresponding to the gelatinolytic activity of activated MMP-9 appeared on the gel. However, no activation of MMP-9 was observed for in the presence of recombinant Zmp or with *S. suis* (SS) with either whole live washed bacteria or bacterial supernatants (**Figure 3**).

S. suis *is unable to cleave PSGL-1 ectodomains*

In order to evaluate the ability of *S. suis* and Zmp to cleave PSGL-1 ectodomains, recombianant Zmp, whole bacteria (bact) or bacterial supernatants (sup) were incubated with PSGL-1/fc. To visualize cleavage, two different antibodies were used, whether KPL-1 and PL-2, which recognize the N-terminal epitope of PSGL-1 and a membrane-proximal epitope, respectively [34]. As expected [15], the *S. pneumoniae* strain TIGR4 (SP), used as a positive control, cleaved PSGL-1 ectodomains **(Figure 4**). It was observed that the reactivity of KPL-1 antibody with PSGL-1/fc diminished as a result of the N-terminal epitope of PSGL-1/fc being cleaved **(Figure 4A)**. Meanwhile, PL2 still recognized the degradation products of PSGL-1/Fc **(Figure 4B)**. This was expected since the *S. pneumoniae* ZmpC removes the N-terminus of PSGL-1 required for recognition by KPL1 with PSGL-1/Fc since KPL1 recognizes an N-terminal epitope of PSGL-1 [34]. On the other hand, PL2 can still recognize multiple degradation products

between 150 kDa and 25 kDa since it recognizes a membrane-proximal epitope [34]. Meanwhile, recombinant Zmp, *S. suis* (SS) bacterial cells as well as different concentrations of bacterial supernatants were unable to cleave PSGL-1 ectodomains **(Figure 4)**.

Zmp is partially implicated in the capacity of* S. suis *to cleave MUC16 ectodomains

A treatment of HeLa cells with live washed bacteria or bacterial supernatants concentrated 10X was found to be cytotoxic (results not shown). Thus, *S. pneumoniae* non-concentrated supernatant (positive control) and *S. suis* supernatant (either non-concentrated or concentrated 3X) were used to perform the MUC16 ectodomain cleavage assay. As shown in **Figure 5**, *S. pneumoniae* non-concentrated supernatant cleaved MUC16 ectodomains. However, only after being concentrated 3X was *S. suis* supernatant from wild-type strain P1/7 able to cleave MUC16 ectodomains. As such, Zmp activity of *S. suis* seems to be less strong than that of *S. pneumoniae*. Furthermore, deletion of the *zmp* gene significantly decreased the ability of *S. suis* supernatant to cleave MUC16 ectodomains ($p = 0.017$). However, *S. suis* recombinant Zmp alone is unable to cleave MUC16 ectodomains ($p = 0.304$). Meanwhile, complementation of *zmp* gene in *S. suis* (compΔ*zmp*) partially restored the wild-type phenotype, in comparison with the isogenic mutant ($p = 0.067$),but was still less active than the wild-type strain. However, complementation of the *S. suis*Δ*zmp* supernatant by addition of 100 µg of recombinant Zmp completely restored the wild-type phenotype ($p = 0.907$). Residual MUC16 ectodomain shedding ability when using the *S. suis*Δ*zmp* supernatant suggests that other factors are potentially responsible for cleavage by *S. suis*.

Zmp is partially implicated in the capacity of* S. suis *to cleave SDC-1 ectodomains

NMuMG cells treated with 100 µg of recombinant Zmp or *S. suis* bacterial supernatants were used to assess SDC-1 ectodomain cleavage. Supernatant concentrated 10X (but not non-concentrated or concentrated 3X; data not shown) from *S. suis* wild-type strain P1/7 was able to cleave SDC-1 ectodomains (**Figure 6**). Furthermore, deletion of the *zmp* gene significantly decreased this ability using the same supernatant concentration ($p = 0.024$); the SDC-1 cleavage activity was restored by complementation of *zmp* gene in strain *S. suis* (compΔ*zmp*) ($p = 0.030$), when compared to the activity of the Δ*zmp* mutant strain **(Figure 6)**. In contrast to MUC16 ectodomain shedding experiments, *S. suis* recombinant Zmp was also able to cleave SDC-1 ectodomains when used alone ($p = 0.007$) (Figure 6). Moreover, addition of 100 µg of purified recombinant Zmp protein to *S. suis*Δ*zmp* mutant supernatant completely restored wild-

type phenotype (p = 0.024), suggesting, once again, the presence of co-factor(s) in *S. suis* supernatant. There was also a residual SDC-1 shedding ability in the *zmp*-deficient mutant, suggesting that other factors could potentially be also involved in this activity.

Zmp is not implicated in adhesion to differentiated primary PBEC

The ability of the *S. suis* wild-type strain P1/7 and *S. suis* Δ*zmp* mutant to colonize primary porcine bronchial epithelial cells was analyzed in ALI cultures with primary PBEC. Cells were infected with 10^7 CFU/filter (approximate MOI=25) from the apical side for 4 h and non-adherent bacteria were washed away. Immunofluorescence microscopy showed that the wild-type and Δ*zmp* strains similarly adhered to ciliated epithelial cells **(Figure 7A and 7B)**. Quantification of adherent bacteria revealed no significant differences in the ability of both strains to colonize PBEC **(Figure 7C)**. Bacterial replication was monitored during the experiment and no differences in growth were detectable (**Additional file 2**).

Zmp is not implicated in colonization of the upper respiratory tract of pigs nor in virulence

Considering the presence of MUC16 in the upper respiratory tract of pigs, and given the previously described function of SDC-1 in adhesion to and invasion of epithelial cells [35, 36], the role of Zmp in the pathogenesis of *S. suis* serotype 2 infection, more particularly in colonization, as well as its role as a virulence factor, were evaluated using an intranasal piglet model of *S. suis* infection. Animals were divided into two groups and infected with either wild-type strain P1/7 or its *zmp*-deficient mutant. After infection, PCR tests showed presence of *S. suis* serotype 2 ST1 (*cps*+, *epf*+) in infected animals. Indeed, as illustrated in Figure 8A, tonsil colonization was already detectable 24 h post-infection in both groups. As shown in Figure 8B, *S. suis* serotype 2 ST1 was also present in the trachea of almost all infected animals in both groups and remained present until day 9. Throughout the experiment, no significant differences were observed between wild-type and Δ*zmp*-infected piglets in both the tonsils and trachea **(Figure 8A and 8B)**.

In addition, results showed no differences in virulence, since several animals presented severe clinical signs such as arthritis, depression, and meningitis in both groups **(Table 3)**. Indeed, 33% of piglets in both groups succumbed to infection **(Figure 9)**. Absence of *zmp* gene was

confirmed in all *S. suis* serotype 2 bacteria recovered from the liver, spleen, and brain of ill animals infected with the mutant strain data not shown).

Discussion

Increased research over the past decade has led to the identification of a myriad of novel factors putatively involved in the first steps of the *S. suis* pathogenesis of infection. In many cases, newly identified factors have been described as “critical” for virulence based on little supporting experimental data, and in some cases, controversial results have been reported in subsequent investigations [7]. One example is the Zmp protease encoded by gene *iga* [8, 10, 37]. Zinc-dependent metalloproteases have been studied relatively extensively in *S. pneumoniae*. Four distinct groups have been described: ZmpA, ZmpB, and ZmpC and ZmpD [10]. *S. pneumoniae* ZmpA has been shown to cleave human IgAs, which play an important role in the immunological defense of the respiratory tract and other mucosal surfaces [5], with IgA1 representing 90% of the IgA within the human respiratory tract [38]. Indeed, IgA1 proteases have been regarded as virulence factors in a variety of pathogens [38-40]. In *S. suis*, an IgA protease, with active cleavage effect on human IgA1, has been previously described in a virulent *S. suis* isolate [8]. In addition, an isogenic mutant defective for the gene encoding this IgA protease was shown to be significantly less virulent than the wild-type strain. Although IgA protease activity of the mutant or parent strain was not experimentally tested, the animal data were interpreted to mean that IgA cleavage *in vivo* was crucial for virulence [37]. Later, it was shown that immunization with this IgA protease resulted in 100% protection of mice against challenge with a virulent strain [41].

Surprisingly, in a comparative study using different streptococci, Bek-Thomsen *et al*. reported absence of IgA protease activity in the seven *S. suis* strains tested [10]. Consequently, given the controversy surrounding the activity of this metalloprotease in *S. suis,* the capacity of *S. suis* serotype 2 strain P1/7 (ST1) to degrade human IgA_1 was evaluated. Results obtained in the present study contradict those originally reported [8] and confirm those published later by Bek-Thomsen *et al*. [10], with a total absence of IgA protease activity from both *S. suis* (non-concentrated or concentrated live washed bacteria/bacterial supernatants) and the cloned, expressed, and purified recombinant protein. Both the reference virulent ST1 strain, as well as the highly virulent ST7 strain SC84 (similar to that used in the original report by Zhang et al.

[8]) presented identical results. Indeed, these results also confirm the larger controversy regarding critical *S. suis* virulence factors [7, 42].

The study of Bek-Thomsen *et al*. also suggested that the *S. suis* Zmp homologue belongs to the ZmpC subgroup [10], and comparison of the *S. suis* serotype 2 ZmpC sequence with that of *S. pneumoniae* showed 42% of homology between both proteins (**Additional File 3**). Moreover, the *S. suis* Zmp was also previously described to possess a HEMVH motif in its active site, which corresponds to that of ZmpC [8, 10]. Regardless of these genetic similarities, the specific properties and functions of the *S. suis* Zmp protein remained unknown. Indeed, *S. pneumoniae* ZmpC activates the pro-form of MMP-9 and cleaves PSGL-1, MUC16, and SDC-1 ectodomains [12, 14, 15, 27].

Firstly, the involvement of the *S. suis* Zmp in activation of MMP-9 by pro-form cleavage was evaluated [14]. Of the different MMPs described, MMP-9 has been largely associated with the disruption of both the blood-brain barrier and the blood-cerebrospinal fluid barrier [16]. Given that one of the most important pathologies caused by *S. suis* in diseased pigs is meningitis, MMP-9 could play an important role in the infection [1]. Indeed, previous studies have demonstrated that *S. suis* serotype 2 induces production of MMP-9 from macrophages, and it was suggested that this production might help the pathogen to invade the CNS [43, 44]. Results from this study show that, under the conditions tested, and differently from *S. pneumoniae*, recombinant Zmp does not activate the pro-enzyme form of MMP-9. Moreover, we did not identify this activity in *S. suis* serotype 2. Thus, *S. suis* appears to behave similarly to *Streptococcus sanguinis* and *Streptococcus oralis*, which are also unable to activate MMP-9, despite possessing a *zmpC* gene encoding putative metalloproteases with an HEMTH motif in their active site [10]. We speculate that previously described MMP-9 activation in macrophages during interactions with *S. suis* may instead be due to internal control mechanisms of the host [45, 46].

PSGL-1 is an important molecule expressed at the surface of certain immune cell types and, during stress conditions, it binds the P-selectin at the surface of endothelial cells, which will subsequently allow the diapedesis of cells from the blood vessels to the site of infection [47]. Numerous pathogens, including *S. pneumoniae*, have thus developed strategies to outmaneuver the immune system, including cleavage of PSGL-1 [15, 47]. Results obtained in

this study showed that, unlike that of *S. pneumoniae*, *S. suis* serotype 2 and its Zmp protein are unable to cleave PSGL-1 ectodomains.

Next, the ability of *S. suis* and its Zmp to cleave MUC16 was evaluated. MUC16 is a membrane-associated mucin that is part of the epithelial barrier, and it is found, among other mucosal surfaces, at the respiratory tract levels of mammals [48, 49]. *S. pneumoniae,* via its ZmpC protein, could disrupt the epithelial barrier by cleavage of MUC16 ectodomains, allowing the pathogen to more easily colonize host epithelial cells and underlying basement membranes [13, 27]. In this study, results showed that *S. suis* serotype 2 liberates the MUC16 ectodomains and that this function is Zmp-dependent. This could play a role in *S. suis* adhesion to and invasion of epithelial cells, as was shown for *Staphylococcus aureus* [50]. However, the observed activity seems to be of lower intensity that that of *S. pneumoniae*, as the *S. suis* supernatant induced this effect only after being concentrated. In addition, and differently from what was reported for *S. pneumoniae* [27], recombinant *S. suis* ZmpC alone was not able to induce the cleavage of MUC16 ectodomains. Indeed, recombinant *S. suis* Zmp protein seems to require a co-factor found in the bacterial supernatant in order to be active. This co-factor could be a potential cation or protease: further studies are needed to examine these hypotheses in detail. Indeed, *S. suis* has numerous virulence factors described with redundant functions that could compensate for the loss of another factor [6].

Cleavage of SDC-1 ectodomains was then investigated [12]. SDC-1 is a type 1 transmembrane heparan sulfate proteoglycan mainly expressed by plasma cells and epithelial cells and, to a lesser extent, endothelial cells, macrophages, and fibroblasts [36, 51]. Indeed, SDC1 (CD138) is used as a marker for plasma cells, and myeloma cells and several carcinomas, such as breast cancer cells, express high levels of SDC1. Interactions of bacterial pathogens with heparan sulfate proteoglycans such as SDC-1 have been described as important steps in the pathogenesis of different infections [35, 52]. Indeed, the liberation of SDC-1 ectodomains by α- and β-toxins, allows *S. aureus* to avoid bacterial killing by neutrophils. This could help pathogens to persist in the circulation more easily [53]. Results obtained in the present study confirm that *S. suis* induces SDC-1 shedding, with this activity mainly being Zmp-dependant. Interestingly, a synergic activity was shown when Zmp protein was incubated with *S. suis* Δ*zmp* supernatant, supporting the hypothesis that the activity of Zmp requires a co-factor present in the *S. suis* supernatant. Moreover, as was observed with MUC16, a residual SDC-1

ectodomain shedding activity remains in *S. suis* Δ*zmp* strain, suggesting that other factor(s) could also be implicated in this function. Taken together, these functional assay results demonstrate that the *S. suis* serotype 2 Zmp protein does not possess all of the functions of the *S. pneumoniae* ZmpC.

Given that *S. suis* cleaved MUC16 ectodomains and the fact that the Zmp protein plays an important role in this function, its role in the adhesion to differentiated primary porcine bronchial cells was investigated. ALI culture with PBEC represents an *in vitro* model of well-differentiated ciliated and mucus-producing respiratory epithelial cells [29]. Since MUC16 is a mucin recovered in the mucosal barrier of respiratory epithelial cells, this PBEC are a good model to evaluate the role of *S. suis* serotype 2 Zmp. However, both *S. suis* wild-type and Δ*zmp* strains had a similar capacity to adhere to PBEC under ALI conditions. This lack of difference was confirmed *in vivo*. Indeed, and regardless of its role in MUC16 ectodomain cleavage *in vitro*, no difference was observed between the wild-type and the *zmp*-deficient mutant strains in colonization of tonsils and trachea by *S. suis* after bacterial challenge via the intranasal route. The lack of a critical role in epithelial cell adhesion and colonization might be explained by the concept of bacterial redundancy, which is very common in *S. suis* [7]. In fact, this result is not necessarily surprising given the number of virulence factors expressed by *S. suis* serotype 2 that have been reported to be implicated in colonization of the upper respiratory tracts of pigs, including adhesins and toxins. Indeed, around 40 different factors have been described to be involved in *S. suis* colonization [6]. Animals infected with either the wild-type or the Δ*zmp* strain also presented similar clinical signs and mortality rate. This indicates that a lower liberation of SDC-1 ectodomains by the *S. suis* Δ*zmp* mutant did not increase bacterial killing *in vivo*. As mentioned above, different anti-phagocytic bacterial factors have been described as playing important roles during *S. suis* infection [7].

Finally, and differently from what has been observed in a previous study [37], where the *S. suis* IgA protease encoded by *iga* gene was characterized as a critical virulence factor using an isogenic *iga* mutant (equivalent to the *S. suis* Δ*zmp* strain used herein), the *S. suis* Δ*zmp* strain was as virulent as the wild-type strain in an pig intranasal model of infection. In the case of *S. pneumoniae,* results differed regarding the impact of ZmpC in virulence. Indeed, while a mutant Δ*zmpC* seems to have a reduced virulence in an intranasal mouse model of infection [14, 52], the Δ*zmpC* mutant showed an exacerbate virulence compared to the wild-type strain in an

intravenous model of infection [54]. This does not seem to be the case for *S. suis*, since no significant difference was observed in virulence after intraperitoneal infection of mice with the wild-type and *zmp*-deficient mutant strains (unpublished data).

Conclusions

In conclusion, absence of an IgA_1 protease activity in *S. suis* serotype 2 was confirmed. Indeed, the *S. suis* metalloprotease hypothetically responsible for such activity would rather belong to the ZmpC family reported for *S. pneumoniae*. Indeed, the *iga* terminology for the gene coding for the ZmpC of *S. suis* should be avoided and changed by *zmpc*. Of the different functions previously described for streptococcal ZmpC, that of *S. suis* would be responsible for a partial ability to cleave MUC16 and SDC-1 ectodomains. As such, the present study is the first to show such capacities for *S. suis*. However, the presence of this protein does not appear to be critical for colonization of the porcine upper respiratory tract. In addition, and in disagreement with published results, this protein would not be a critical virulence factor. These results further emphasize, as recently suggested, the need to confirm the critical role of reported candidates in virulence by independent laboratories [7, 42].

Abbreviations

BCA: bicinchoninic acid; BEBM: bronchial epithelial cell basal medium; BEGM: bronchial epithelial growth medium; CPEC: circular polymerase extension cloning; DMEM: Dulbelcco's modified eagle medium; HRP: horseradish peroxidase; IgA : Immunoglobulin A; IPTG: Isopropyl-β-D-thiogalactopyranoside; LB: Luria-Bertani; MMP-9: Matrix metalloproteinase 9; MUC16: mucine 16; PBEC: porcine bronchial epithelial cells; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; pi: post-infection; PMA: phorbol-12-myristate-13-acetate; PSGL-1: P-selectine glycoprotein ligand-1; SDC-1: syndecan-1; SEM: standard error of the mean; ST: sequence type; THA: Todd-Hewitt agar; THB: Todd-Hewitt broth; WT: wild-type; Zmp: zinc metalloprotease.

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Tables

Table 1. Bacterial strains and plasmids used in this study

Strains/plasmids	General characteristics	Source/reference
Escherichia coli		
TOP 10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
BL21	F^-ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm rne131 (DE3)	Invitrogen
Streptococcus suis		
P1/7	Wild-type, virulent European serotype 2 ST1 strain isolated from pig with meningitis	[55]
Δ*zmp*	Isogenic mutant strain derived from P1/7; in frame deletion of *zmp* gene	This work
compΔ*zmp*	Mutant Δ*zmp* complemented with pMX*zmp* complementation vector	This work
SC84	Highly virulent clonal serotype 2 ST7 strain isolated from the 2005 human outbreak in China	[56]
Streptococcus pneumoniae		
TIGR4	Virulent serotype 4 strain isolated from human blood in Norway	ATCC
Plasmids		
PCR2.1	Ap^r, Km^r, oriR(f1) MCS oriR (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement in *S. suis*. Replication functions of pG + host3, MCS oriR pUC19 lacZ Sp^R	[20]
pMX1	Replication functions of pSSU1, MCS pUC19 lacZ SpR, malX promoter of *S. suis*, derivative of pSET2	[20, 57]
pET101	Ap^r, pBR322 *ori*, T7 promotor	Invitrogen
p4Δ*zmp*	pSET4s carrying the construct of *zmp* for allelic replacement	This work
pMX*zmp*	pMX1 complementation vector carrying intact *zmp* gene	This work
pET101*zmp*	pET101 carrying *zmp* gene for protein production	This work

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5' – 3')	Construct
zmp-ID1	CCTTGGTTTAGATGCCC	p4Δ*zmp*
zmp-ID2	CTGAGACAAGTCCCACT	p4Δ*zmp*
zmp-ID3	CAAGCCTTGATGAACTCTAC	p4Δ*zmp*
zmp-ID4	GCTATTCCTAGCTTCTACCT	p4Δ*zmp*
zmp-ID5	TGTTTCTTTCATCCTTGACAG	p4Δ*zmp*
zmp-ID6	CTTGTTAATGATTATTTAATAAGTTGTTACTCCCTAAAATAG	p4Δ*zmp*
zmp-ID7	TAGGGAGTAACAACTTATTAAATAATCATTAACAAGTTGGTC	p4Δ*zmp*
zmp-ID8	ATCTGGCTCATCCATGAC	p4Δ*zmp*
pMX1-cpec-F	AAATAGTATAGAAACGGCATGCAAGCTTGG	pMX*zmp*
pMX1-cpec-R	TCGGAGGTCCTTTAGCCCGGGTACCGAGCT	pMX*zmp*
zmp-cpec-F	CCAAGCTTGCATGCCGTTTCTATACTATTT	pMX*zmp*
zmp-cpec-R	AGCTCGGTACCCGGGCTAAAGGACCTCCGA	pMX*zmp*
pET101-zmp-F	CACCATGGCTCGATATAACCATGCAATC	pET101*zmp*
pET101-zmp-R	TGGGTTAAAAATCGATGTTCTG	pET101*zmp*

Table 3. Clinical signs observed in wild-type (WT)- and Δ*zmp*-infected pigs

Clinical signs	WT	Δ*zmp*
Fever	4/6	4/6
Respiratory problems	0/6	1/6
Arthritis	3/6	5/6
Neurological problems	2/6	2/6

Figures

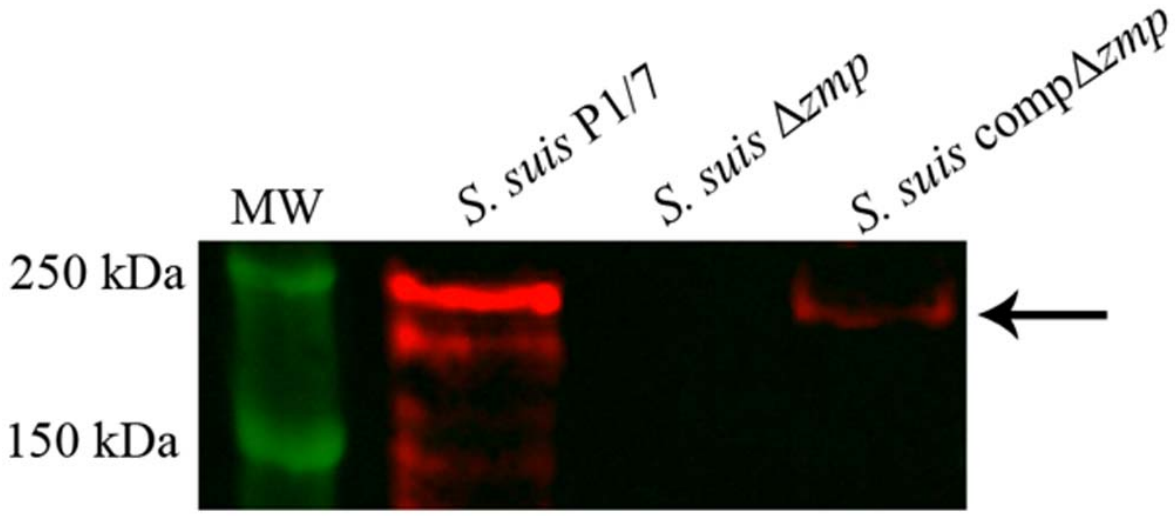


Figure 1. Expression of *S. suis* Zmp protein is abrogated in the *zmp*-deficient mutant. Culture supernatants of the *S. suis* wild-type strain P1/7 (lane 2), *zmp*-deficient mutant (Δ*zmp*) (lane 3), and complemented strain (compΔ*zmp*) (lane 4) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The Zmp protein was detected using a rabbit antiserum against Zmp. The black arrow corresponds to the Zmp protein and MW to molecular weight ladder (lane 1).

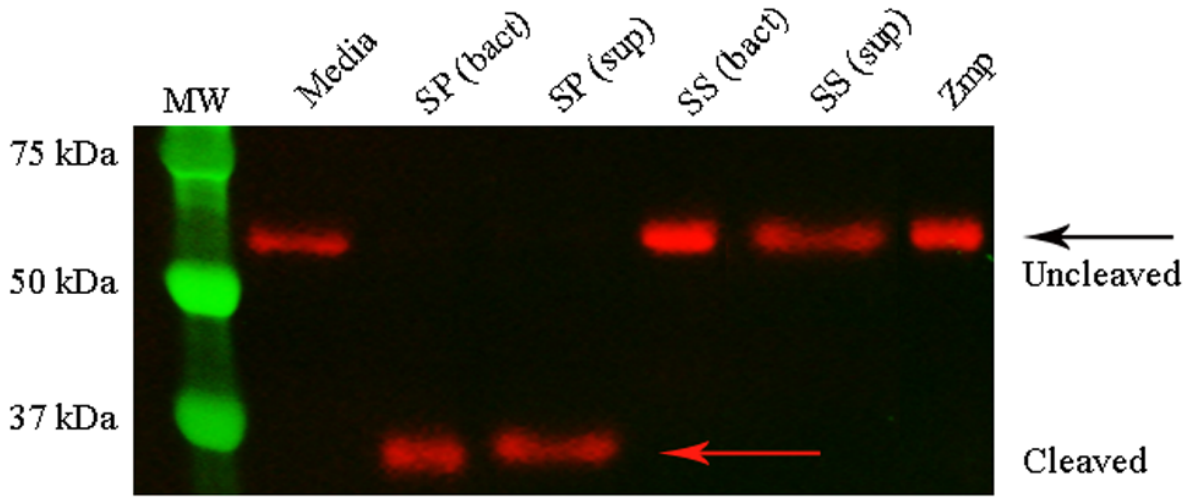


Figure 2. *S. suis* is unable to cleave human IgA_1. Purified recombinant Zmp protein (100 µg), washed bacteria (bact) or bacterial supernatant (sup) from *S. suis* (SS) or *S. pneumoniae* (SP) were incubated with human IgA_1 for 16 h and reactions separated by SDS-PAGE. Cleaved IgA_1 (red arrow) by washed *S. pneumoniae* bacteria or bacterial supernatant (lanes 3 and 4) and uncleaved IgA_1 (black arrow) by washed *S. suis* bacteria (lane 5), bacterial supernatant (10X, lane 6), and purified Zmp protein (lane 7) were visualized using a specific antibody against human IgA_1. MW corresponds to the molecular weight ladder (lane 1) and media correspond to untreated human IgA_1 (lane 2).

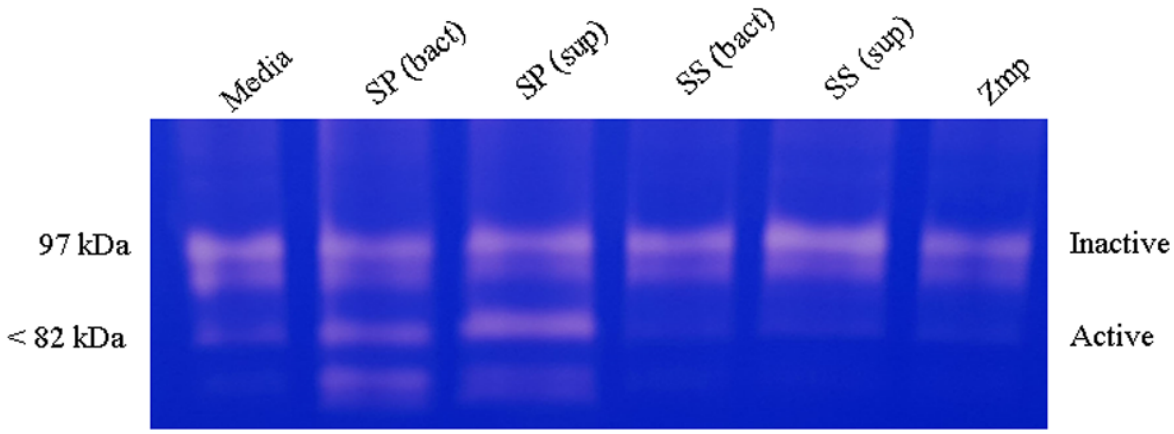


Figure 3. *S. suis* does not activate matrix metalloprotease-9 (MMP-9). Pro-enzyme MMP-9 was incubated for 1 h with purified recombinant Zmp protein (100 µg), washed bacteria (bact) or bacterial supernatant (sup) from *S. suis* or *S. pneumoniae*. Samples were separated in non-denaturing conditions on a gelatin B zymogram. Coomassie coloration shows a second gelatinolytic band for washed *S. pneumoniae* bacteria or bacterial supernatants (lane 2 and 3) corresponding to activated MMP-9, while no second band was observed for media (lane 1) washed *S. suis* bacteria (lane 4), bacterial supernatant (10X, lane 5) or purified Zmp protein (lane 6).

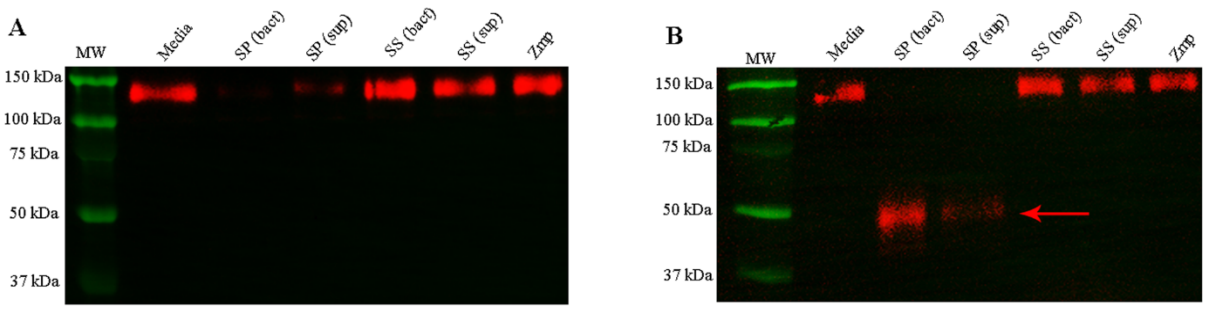


Figure 4. *S. suis* is unable to cleave P-selectin glycoprotein ligand-1 (PSGL-1) ectodomains. Purified recombinant Zmp protein (100 µg), washed bacteria (bact) or bacterial supernatant (sup) from *S. suis* (SS) or *S. pneumoniae* (SP) were incubated with PSGL-1/fc for 1 h. Samples were separated by SDS-PAGE and transferred onto PVDF membranes, which were incubated with KPL-1 (A) or PL2 (B) antibody. No recognition of PSGL-1 by the KPL-1 antibody and visualization of lower molecular weight band with PL2 antibody were observed for washed *S. pneumoniae* bacteria and bacterial supernatant (lanes 3 and 4), which is expected following PSGL-1 ectodomain cleavage. There was no change in the recognition by KPL-1 or in the molecular weight visualized with PL2 for PSGL-1 treated with washed *S. suis* bacteria (lane 5), bacterial supernatant (10X, lane 6) or the purified recombinant Zmp protein (lane 7). MW corresponds to the molecular weight ladder (lane 1) and media correspond to untreated PSGL-1 (lane 2).

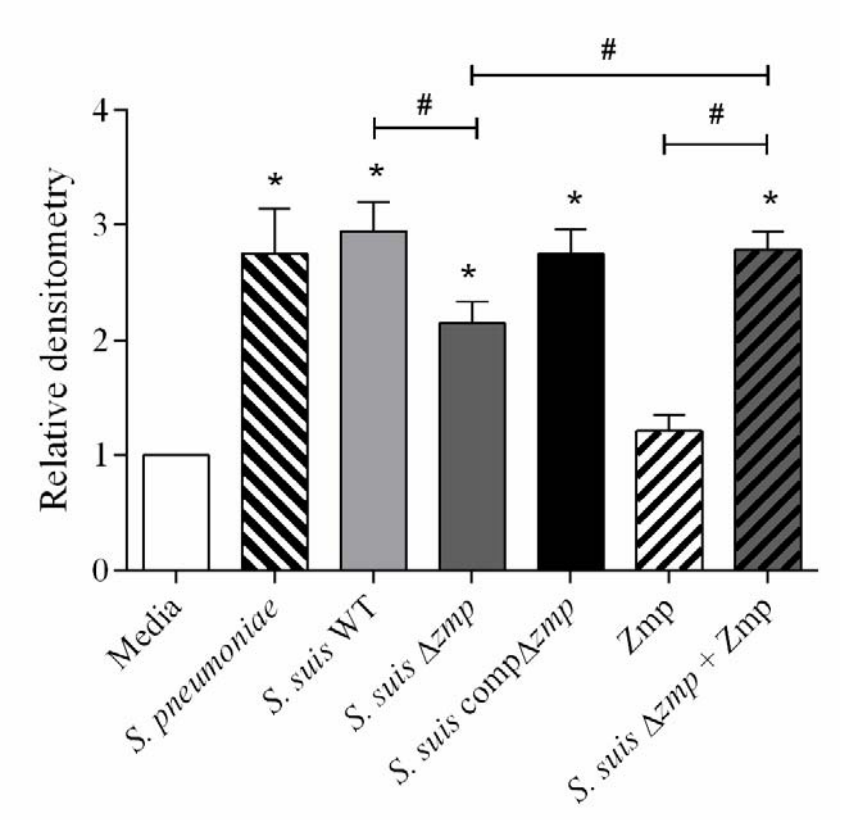


Figure 5. Zmp is implicated in mucine 16 (MUC16) ectodomain cleavage. Relative densitometry of HeLa cell culture medium conditioned with *S. pneumoniae* supernatant, Zmp purified recombinant protein, *S. suis* P1/7 (WT) or *S. suis* Δ*zmp* supernatant (3X) supplemented or not with 100 µg of recombinant Zmp protein. Samples were blotted on a nitrocellulose membrane and incubated with anti-human CA125 antibodies. Compared to the untreated cells, *S. suis* WT strain P1/7 supernatant cleaved MUC16 ectodomains. This activity was significantly reduced when using the supernatant of the Δ*zmp* strain ($p = 0.017$). Supernatant of the compΔ*zmp* significantly increased cleavage when compared to the Δ*zmp* strain. Addition of 100 µg of Zmp protein to the supernatant of the Δ*zmp* strain also restored WT phenotype activity. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between treated groups and the medium; # ($p < 0.05$) between treatment groups.

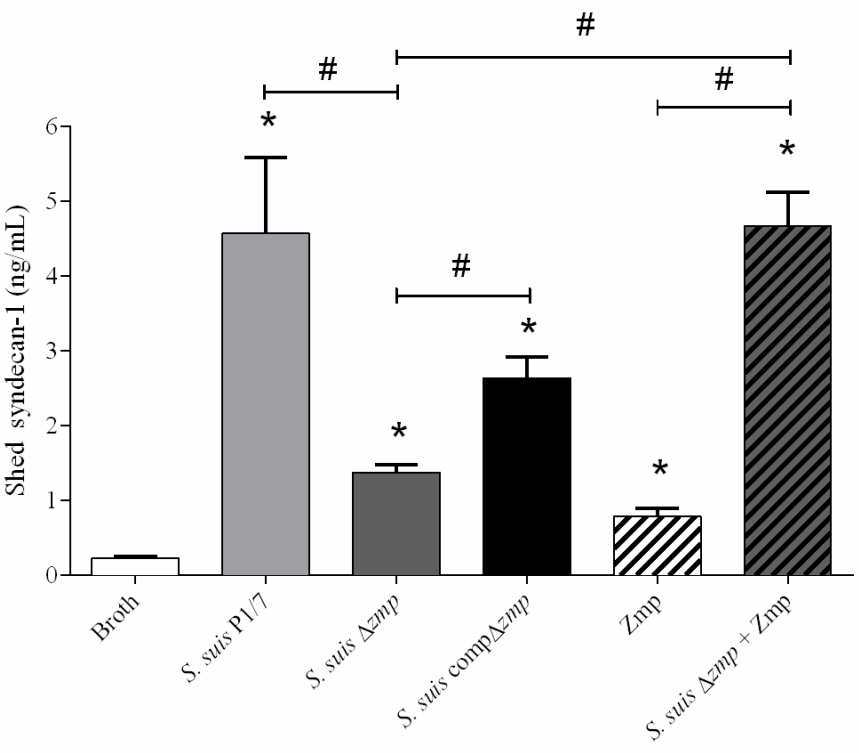


Figure 6. Zmp is implicated in SDC-1 ectodomain cleavage. The concentration of SDC-1-cleaved ectodomains in NMuMG cell culture medium was measured after treatment with 100 µg of Zmp protein, 10X concentrated supernatants of either *S. suis* wild-type P1/7 (WT), *S. suis* Δ*zmp* (supplemented or not with 100 µg of recombinant Zmp) or *S. suis* compΔ*zmp*. Samples were blotted on Immobilon Ny+ and incubated with anti-mouse SDC-1 (281-2) antibodies. The capacity of the Δ*zmp* mutant to cleave SDC-1 ectodomains was significantly reduced in comparison to the WT strain, while the complementation of *zmp* gene restored WT phenotype. Recombinant Zmp protein also cleaved SDC-1 ectodomains. This activity was amplified when Zmp protein was incubated with *zmp*-deficient mutant culture supernatant. The concentration of shed SDC-1 ectodomains was determined using SDC-1 ectodomains purified from NMuMG cells as standards, as previously described [12]. Data represent the mean ± SEM from at least three independent experiments. * ($p < 0.05$) indicates a significant difference between treated groups and the medium; # ($p < 0.05$) between treated groups.

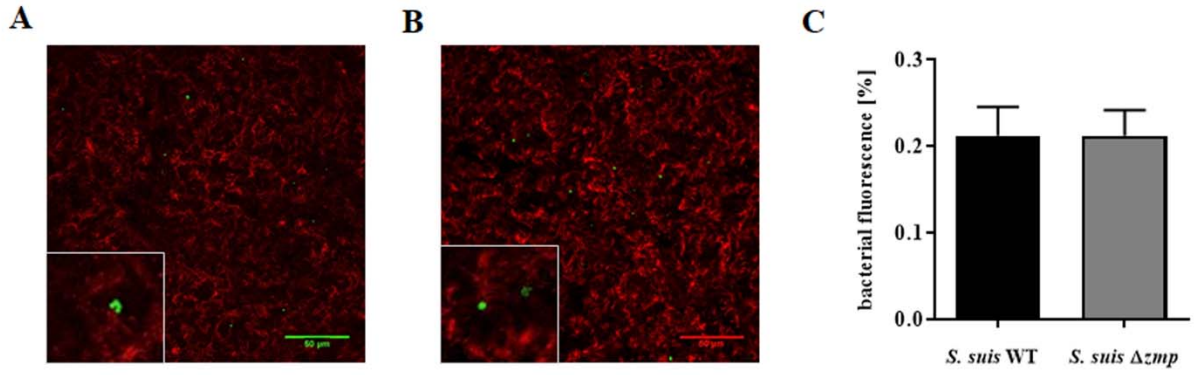


Figure 7. Zmp is not implicated in adhesion to PBEC under ALI conditions. Immunofluorescence analyses of PBEC differentiated under ALI conditions and infected for 4 h with (A) *S. suis* wild-type P1/7 (WT) and (B) *S. suis* Δ*zmp* strain. Ciliated cells were stained for ß-tubulin (red) and streptococci (green). Bars represents 50 µm. (C) Adherent bacteria were quantified by analyzing the epithelial cell surface positive for green fluorescence signal in four randomly chosen areas for each treatment. Results are expressed as mean ± SEM.

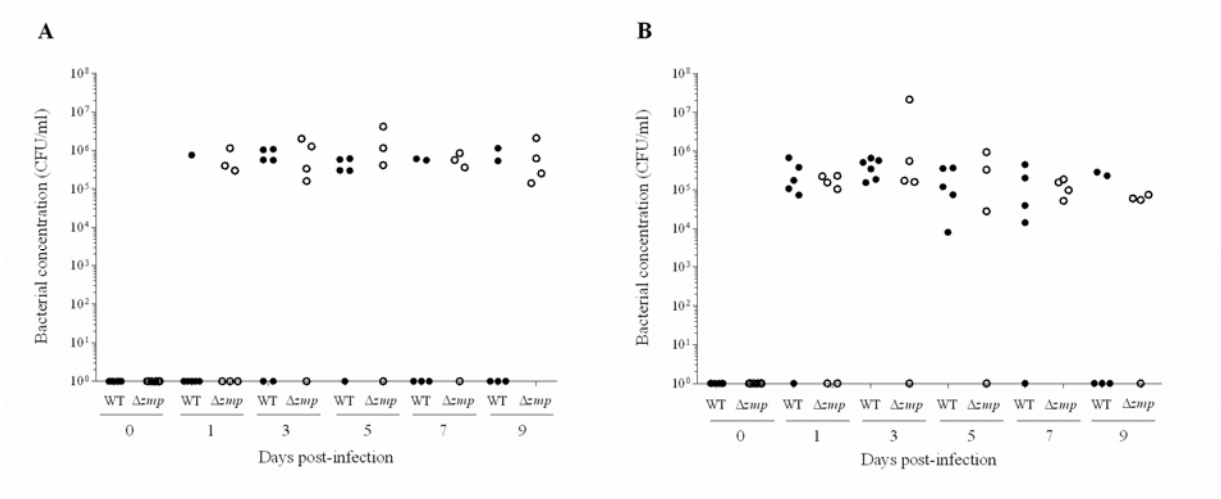


Figure 8. Presence of Zmp does not influence the colonization potential of *S. suis* serotype 2 in a pig model of infection. Bacterial concentrations recovered in the tonsils (A) or trachea (B) of piglets infected with either the wild-type strain P1/7 (WT) or its Δ*zmp* mutant. No differences in colonization of the upper respiratory tract of piglets were observed following infection with the Δ*zmp* mutant. Each point represents an individual animal.

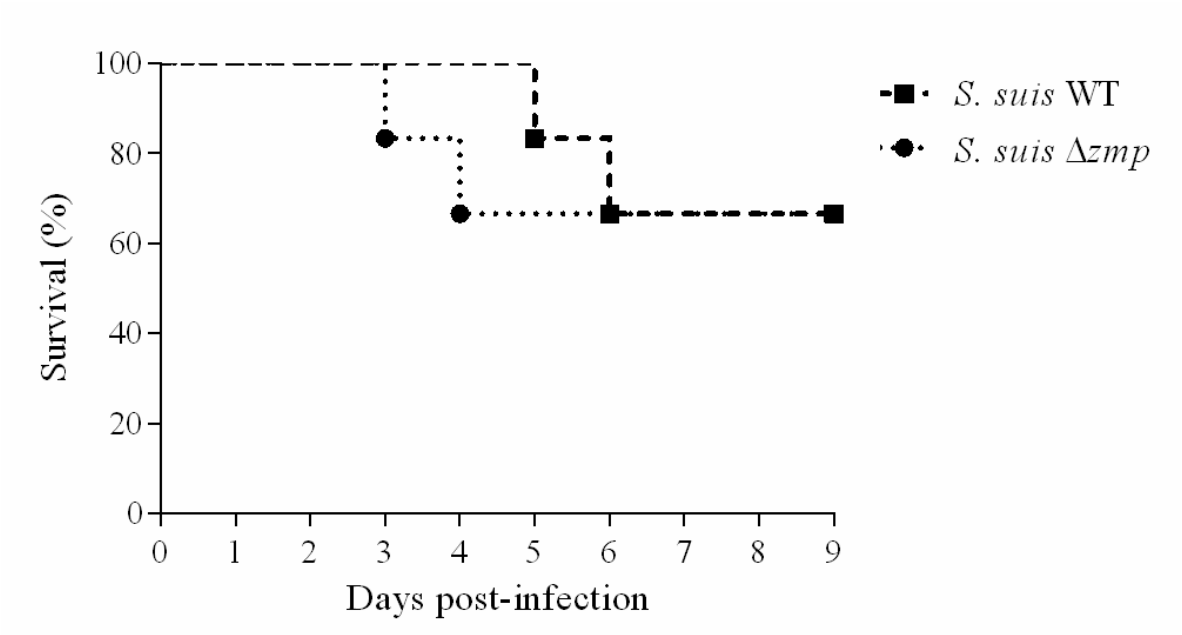
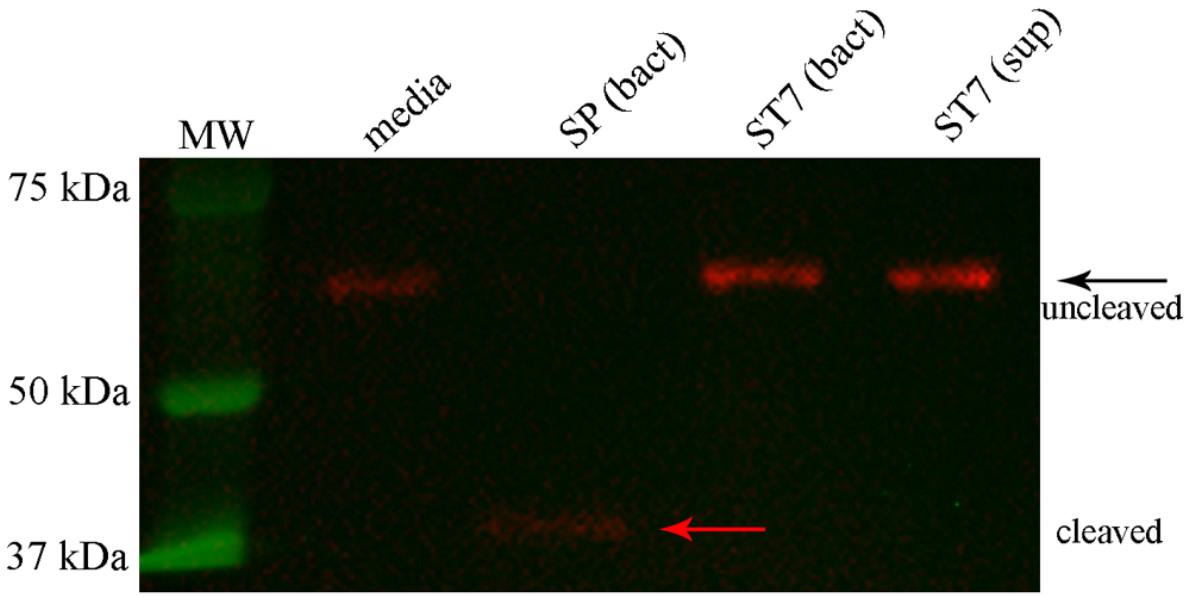
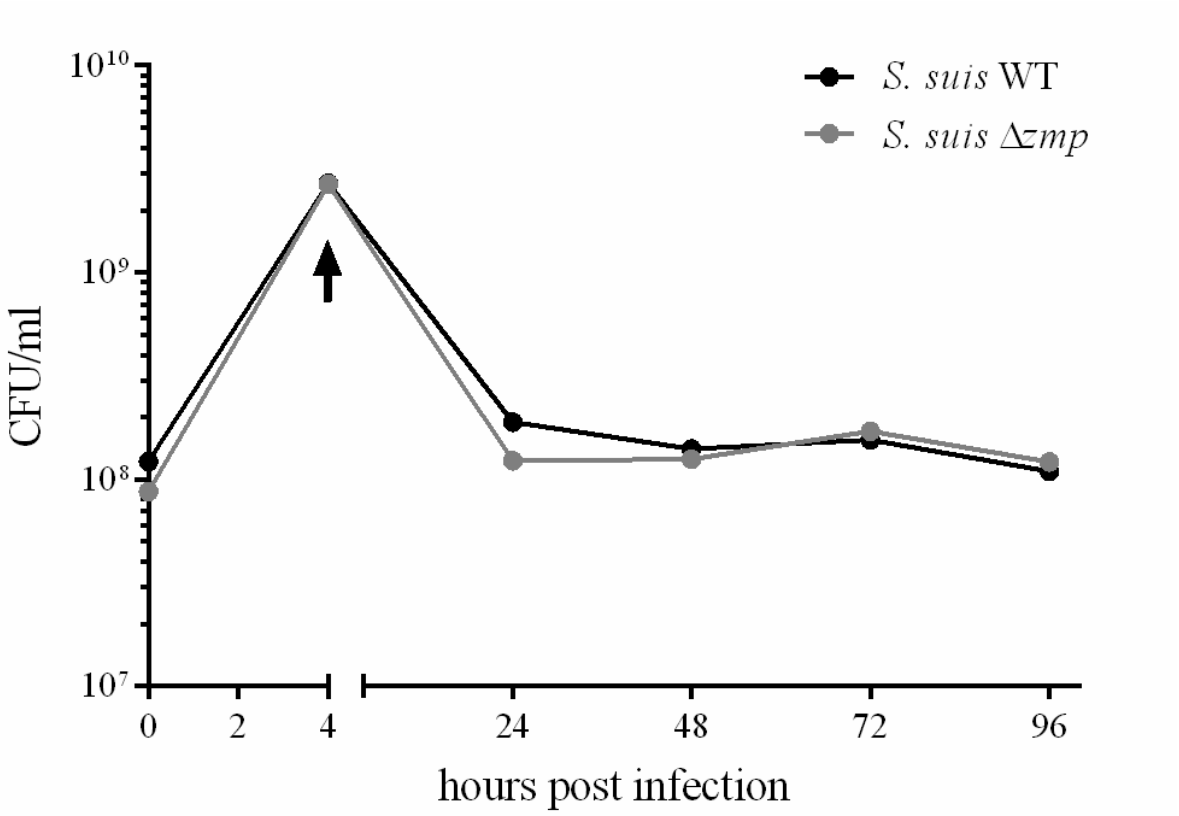


Figure 9. Zmp does not play a role in virulence of *S. suis* serotype 2 using an intranasal pig model of infection. Survival of four-week old piglets infected with the wild-type strain P1/7 (WT) or its Δ*zmp* mutant. No significant differences were observed between groups ($p = 0.839$).



Additional File 1. *S. suis* ST7 is unable to cleave human IgA$_1$. Washed bacteria (bact) or bacterial supernatant (sup) from *S. suis* SC84 (ST7) or *S. pneumoniae* (SP) were incubated with human IgA$_1$ for 16 h and reactions separated by SDS-PAGE. Cleaved IgA$_1$ (red arrow) by washed *S. pneumoniae* bacteria (lane 3) and uncleaved IgA$_1$ (black arrow) by media (lane 2), washed *S. suis* bacteria or supernatant (lanes 4 and 5) were visualized using a specific antibody against human IgA$_1$. MW corresponds to the molecular weight ladder (lane 1).



Additional File 2. Growth kinetics of *S. suis* strains in presence of PBEC. PBEC differentiated under ALI conditions were infected with *S. suis* P1/7 (WT) or *S. suis* Δ*zmp* for 4 h, and non-adherent bacteria were washed away (indicated by arrow). Bacterial replication rates were determined by plating supernatants of infected cells every 24 h.

ANNEXES - ARTICLE VIII

Complex population structure and virulence differences among serotype 2 *Streptococcus suis* strains belonging to sequence type 28

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-auteur de l'article. J'ai effectué les expériences *in vivo* et j'ai participé à l'analyse des résultats, à l'écriture de la première version du manuscrit et à la révision de celui-ci.

Abstract

Streptococcus suis is a major swine pathogen and a zoonotic agent. Serotype 2 strains are the most frequently associated with disease. However, not all serotype 2 lineages are considered virulent. Indeed, sequence type (ST) 28 serotype 2 *S. suis* strains have been described as a homogeneous group of low virulence. However, ST28 strains are often isolated from diseased swine in some countries, and at least four human ST28 cases have been reported. Here, we used whole-genome sequencing and animal infection models to test the hypothesis that the ST28 lineage comprises strains of different genetic backgrounds and different virulence. We used 50 *S. suis* ST28 strains isolated in Canada, the United States and Japan from diseased pigs, and one ST28 strain from a human case isolated in Thailand. We report a complex population structure among the 51 ST28 strains. Diversity resulted from variable gene content, recombination events and numerous genome-wide polymorphisms not attributable to recombination. Phylogenetic analysis using core genome single-nucleotide polymorphisms revealed four discrete clades with strong geographic structure, and a fifth clade formed by US, Thai and Japanese strains. When tested in experimental animal models, strains from this latter clade were significantly more virulent than a Canadian ST28 reference strain, and a closely related Canadian strain. Our results highlight the limitations of MLST for both phylogenetic analysis and virulence prediction and raise concerns about the possible emergence of ST28 strains in human clinical cases.

Introduction

Streptococcus suis is a major swine pathogen responsible for septicemia, meningitis and other diseases in swine that often result in severe economic losses to the porcine industry (1). *S. suis* is also an emerging zoonotic agent (2). Two outbreaks of human *S. suis* disease occurred in China in 1998 and 2005, affecting hundreds of people and killing more than forty (3). Relatively recent reports found that this pathogen is the first and second-most commonly reported cause of adult streptococcal meningitis in Vietnam and Thailand, respectively (4, 5). On the other hand, in European countries, human *S. suis* disease has never been associated with large outbreaks, and has mostly affected workers in the swine industry (6). Relatively very few cases of human *S. suis* disease have been reported in North America (6). Most cases of animal and human *S. suis* infection are caused by serotype 2 strains (7). Interestingly, the percentage of *S. suis* serotype 2 strains recovered from diseased pigs has historically been lower in North America than in other parts of the world (8).

Increased research in recent years has identified a myriad of virulence factors involved in the pathogenesis of infection of *S. suis* serotype 2 (9). *S. suis* strains belonging to serotype 2 can be divided by multilocus sequence typing (MLST), into at least 16 sequence types (STs) with closely related STs grouped into ST clonal complexes (CCs) (7, 10). Most virulence studies have been carried out with a limited number of ST1 and ST7 serotype 2 strains, which were predominately isolated from diseased pigs in the Netherlands, France, Spain, the United Kingdom, and China (7, 9). While ST1 and ST7 strains are more prevalent in these and a few other countries, previous work has shown that in North America ST25 and ST28 strains predominate, accounting for 44% (ST25) and 51% (ST28) of all strains investigated (11). The *S. suis* MLST scheme is based on the sequence of seven housekeeping genes (10). Thus, a significant amount of information such as DNA polymorphisms occurring in other parts of the genome, and gene content variation encoded in mobile genetic elements, is not captured by this typing method. Virulence studies showed that one ST28 strain from Canada was significantly less virulent than ST1 and ST25 strains (11). However, little is known about variation within the ST28 group. Assessing this intra-ST variation is important. For example, earlier work showed that ST28 *S. suis* strain 1330 was avirulent in both mice and swine (12). More recently, it was shown that an ST28 strain isolated from the tonsils of an asymptomatic pig in China had very low virulence in a swine model of infection (13). However, ST28 strains are often isolated from diseased swine in China and Japan (14-16), and at least four human

ST28 cases have been reported in Thailand and Japan (17-19). Moreover, some have speculated that while porcine ST28 *S. suis* infections in North America are most often associated with a concomitant viral infection, in some Asian countries ST28 strains may be the primary pathogen (1). These findings support the hypothesis that not all ST28 strains have the same virulence potential. They also raise doubts about the universal value of previous virulence studies conducted with only one ST28 organism.

Here, we sought to use genomics to analyze the population structure of a collection of 51 *S. suis* serotype 2 ST28 strains isolated in four different countries (Canada, the United States of America, Japan, and Thailand), and to investigate virulence traits of selected ST28 strains. We report a complex population structure among ST28 strains, which were shown to belong to at least 5 different clades following whole-genome-single nucleotide polymorphism (SNP) analysis. We also show important virulence differences between some of these genetic groups.

Materials and Methods

Strains, culture conditions and DNA preparation

A total of 50 *S. suis* serotype 2 ST28 strains isolated from diseased pigs (20 from Canada, 15 from USA, and 15 from Japan) and one strain isolated from a human case in Thailand, collected from 1990 to 2011, were used (S1 Table). These strains had previously been serotyped, and typed by MLST using standard procedures (10, 20). Strains were cultured on Columbia blood agar plates containing 5% sheep blood, and grown at 37°C with 5% CO_2. Liquid cultures were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract. DNA was prepared from 5 ml of overnight *S. suis* cultures using the QIAamp DNA minikit (Qiagen, Toronto, ON, Canada) following the manufacturers' protocol for Gram positive organisms.

Whole-genome sequencing and closure of a reference ST28 genome

Whole genome sequencing libraries were prepared for all 51 isolates using Nextera XT kits (Illumina, San Diego, CA, USA) and sequenced as paired-end reads with either a HiSeq 2500 (101 bp + 101 bp) or a MiSeq (150 bp + 150 bp) instrument. Parsing of the multiplexed sequencing reads and removal of barcode information was done using onboard software. Short-read sequences have been deposited in the Sequence Read Archive under accession number SRP058193. MLST STs were derived directly from the short-read data using SRST2

software (21) and used to confirm previous MLST results. We next sequenced to closure the genome of strain NSUIS002 using SMRT sequencing (Pacific Biosciences, Menlo Park, CA, USA). This strain had previously been named 1088563 and was selected for genome closure because 1) it belonged to the more prevalent Canadian group and 2) virulence data in a murine model of infection had previously been obtained (11). Briefly, two SMRT cells of sequence were run, generating 51,367 reads exceeding 3 kb in length (average read length of 6.4 kb; 112X coverage for reads >3 kb). Next, we used HGAP v2 (22) to correct the long reads and Celera Assembler 7.0 (23) to assemble the corrected reads, followed by two rounds of polishing with Quiver (https://github.com/PacificBiosciences/GenomicConsensus). The coverage of the final assembly in reads >3 kb was 146X. To assess base-calling accuracy in the Pacific Biosciences assembly, Illumina short-reads were aligned to the assembly using BLAT (24). The genome assembly was completely concordant with full length perfectly aligning Illumina short-reads. The genome was formatted to begin at the first nucleotide of the intergenic region immediately preceding gene *dnaA*, encoding a chromosomal replication initiation protein. The finalized genome was annotated using Prokka (25) and deposited in GenBank under Accession number CP011419.

Core-genome, assessment of recombination, phylogenetic analysis, and antibiotic resistance genes

The A5 pipeline was used for *de novo* assembly of Illumina sequenced strains (26). Obtained contigs were ordered relative to the NSUI002 reference genome using Progressive Mauve (27). Then, pseudochromosomes were created for the remaining 50 strains by concatenating the ordered contigs using the sequence NNNNNCATTCCATTCATTAATTAATTAATGAATGAATGNNNNN, which introduces start and stop codons in all 6 reading frames, as a separator. Pseudochromosomes were annotated using Prokka. We next defined a core chromosome following the method of de Been *et al* (28). Briefly, InParanoid (29) and QuickParanoid (http://pl.postech.ac.kr/QuickParanoid) were used to identify ortholog gene clusters between all ST28 strains. Genes encoded in mobile genetic elements and ortholog genes varying in length by more than 9 bp were not considered. Next, for each ortholog group, the sequences were aligned using Muscle v3.7 (30) and gaps were removed using trimAl v1.2 (31). These aligned and trimmed genes were then reassembled for each strain in the order in which the gene appeared in the NSUI002 reference. Recombination occurring in the so defined core genome was assessed using BRATNextGen (32) run with 20

iterations and 100 replicates, using a *p*-value of 0.05 as the significance cutoff. For phylogenetic analysis, SNPs relative to the genome of reference strain NSUI002 were identified for each of the 50 additional ST28 strains using VAAL (33). A matrix file containing the genotype of all strains at each polymorphic locus was then created from the VAAL polymorphism output data using a custom script. Next, all SNPs occurring in areas of the genome not found in the above-defined core genome (i.e. those occurring in mobile genetic elements, intergenic regions, and NSUI002 genes without an ortholog in all 51 ST strains) were discarded. As well, we eliminated SNPs occurring in genes that were deemed to have undergone recombination based on BRATNextGen results. Then, for each individual strain, SNPs were concatenated in order of occurrence relative to the genome of the reference strain and converted to a multiFASTA sequence. Neighbor-joining phylogenetic trees (1,000 bootstrap replications) were generated with SplitsTree4 (34). We used SRST2 and a database listing 1913 variants of genes encoding antimicrobial resistance (21) (https://github.com/katholt/srst2) to test for presence or absence of genetic determinants of antimicrobial resistance in the genomes of the ST28 strains. Genome visualizations were created using BRIG (35) and edited using Adobe Illustrator.

Experimental mouse infections

We used a validated C57BL/6 murine model of infection (36). Briefly, 75 mice (aged 6-10 weeks, Jackson Laboratory) were acclimatized to standard laboratory conditions with a 12-h light/12-h dark cycle and unlimited access to water and food. On the day of the experimental infection, five groups of 15 animals each were defined. Group 1 received a 1-ml injection of the Canadian ST28 strain NSUI002 suspension (at 1 x 10^8 CFU), delivered using the intraperitoneal route. Groups 2, 3, 4, and 5 received the same amount of strains NSUI062, NSUI010, NSUI081, and NSUI036, respectively. Mice were monitored 3 times/day for the first 72 h and then twice daily until 14 days post-infection (pi) for clinical signs and assigned clinical scores as previously described (37). Blood was collected 24 h and 48 h pi from the tail vein (5 µl), appropriately diluted and used to evaluate bacterial load by plating onto sheep blood agar plates and enumeration (37). All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. Humane endpoints approved by the Animal Welfare and Ethics Committee, Université de Montréal, were used. Animals were evaluated every 8 h during the first 72 h and twice daily after, and clinical scores were assigned. Animals presenting a score of 4 or 5 (moderately sick) were evaluated every 4 h. Mice presenting a score of 6 were evaluated every 4 h and euthanized if their score

remained constant after 24 h. Mice presenting a score of 7 were immediately euthanized. Animals were euthanized using inhaled CO_2. Animal suffering was minimized by careful and timed evaluation of animals, by closely following the scoring grid, and by immediate euthanasia, if required.

Statistical analysis

The R package (38) was used for statistical analysis. Differences in survival curves were assessed using log-rank test. Differences in bacteremia were assessed using ANOVA on ranks and Tukey test at 24 h and 48 h post-infection (pi). A *P*-value of less than 0.05 was used as the cutoff for significance.

Results and Discussion

Genome closure of reference strain NSUI002 and comparison to other* S. suis *genomes

We first sequenced to closure the genome of Canadian ST28 strain NSUI002. The genome was a circular chromosome of 2,255,345 bp with a G+C content of 41.1 % (Fig. 1A). The GC content of the NSUI002 genome was similar to that of the 19 *S. suis* genomes previously sequenced to closure (S2 Table). It was also one of the largest *S. suis* genomes sequenced so far, and the largest genome of a serotype 2 strain. One reason for the difference in genome sizes between strains is the presence in NSUI002 of one large mobile genetic element (MGE, ~ 83 kbp) spanning from position 1,105,623 to 1,188,671, which carries gene *tetO* encoding resistance to tetracycline, and several other MGE scattered throughout the genome (Fig. 1A). We identified 2,221 CDSs in NSUI002. This is a slightly higher number than found in the very recently finished genome of avirulent ST28 Chinese strain 05HAS68 (2,009 CDSs) (13). Most other closed *S. suis* genomes belong to either ST1 or ST7 strains, both included in MLST CC1.

Based on previous reports that questioned the annotation quality of some of the *S. suis* closed genomes (39), we chose strain P1/7 as a representative member of CC1 strains to compare the ST28 genomes. Ortholog analysis revealed 1,656 NSUI002 CDSs common between Canadian ST28 strain NSUI002, Chinese ST28 strain O5HAS68 and reference ST1 strain P1/7 (Fig. 1B). NSUI002 had 192 unique CDSs, most of them encoded in MGEs. A total of 34 NSUI002 CDSs had an orthologue in P1/7. When the Illumina reads for NSUI002 were aligned to the P1/7 reference genome using VAAL, there was a total of 42,896 SNPs between them,

as well as 1196 deletions and 738 insertions. Key differences between NSUI002 and P1/7 genomes include the absence in NSUI002 of virulence markers *sly* and *epf* (encoding a hemolysin known as suilysin, and a secreted protein known as extracellular factor, respectively) (40-42). NSUI002 possessed a *srtG* pilus island which was absent from P1/7 (43). The two strains possessed gene *mrp*, encoding a muramidase-released protein that has been described as important but not essential in virulence (44, 45). Using the typing scheme developed by Silva *et al* (46), which amplifies a short region of the *mrp* gene, *in silico* PCR identified that strains P1/7 and NSUI002 both possess the 1148 *mrp* variant. However, when comparing the full predicted translated MRP sequences we identified several amino acid differences between the two strains (S1 Fig.). In addition, although it has been reported that *mrp* was absent from the genome of Chinese ST28 isolate O5HAS68 (13), we did find this gene in that isolate. When we inspected the reported O5HAS68 genome, *in silico* PCR determined it had *mrp*^{S} variant (S1 Fig.).

Consistent with previous findings in ST28 strains (43), NSUI002 genome had a complete *srtF* pilus cluster, but did not possess pilus cluster *srtBCD*. A truncated *srtE* pilus cluster (lacking genes encoding pilin subunits) was also identified. The genome of strain NSUI002 possessed the same two-component systems (TCSs) and global virulence regulators previously identified in strain 05HAS68 (13). Namely, NSUI002 contains *ihk/irr*, *ciaRH*, *covR* and *vicK*. Genes encoding other regulators such as *virR*/*virS* and *revS*, present in strain P1/7 (39), were absent from the NSUI002 genome (Table 1). Other regulators such as *salK*/*salR* and *nisK*/*nisR*, so far only found among Chinese ST7 strains (47), were also not identified in the NSUI002 genome. Homology between NSUI002 and ST1 P1/7 CDSs was lower than between NSUI002 and O5HAS68 (Figs. 1 A and B). Both ST28 strains shared a significant number of orthologous CDSs that were not present in P1/7 (Fig. 1B). Genome alignments using progressiveMauve (27) identified several areas of genome rearrangements, including inversions (Fig. 1C) between the ST28 strains. The majority of these rearrangements occurred at genome areas encoding transposases (76.7%). Other genomic rearrangements occurred at rRNA operons, or sites encoding phage integrases and/or phage related proteins. Finally, we identified that while both ST28 strains possessed gene *tetO*, encoding resistance to tetracycline, only the Canadian ST28 strain NSUI002 possessed gene *ermB*, encoding resistance to macrolides. Resistance to tetracycline and to macrolide and glycosamides is carried in different MGE inserted in different regions of the NSUI002 genome (Figs. 1A and S2).

Sequencing of additional ST28 strains and presence of markers of antimicrobial resistance

We next sequenced the genomes of 50 additional ST28 serotype 2 strains using Illumina technology. The number of short reads obtained for each strain and the calculated coverage are presented in S1 Table. ST28 was confirmed in all 50 strains by extracting MLST information directly from the short-read WGS data using SRST2 (21). We also used SRST2 to identify genes associated with antimicrobial resistance in our strain collection. This information is important to obtain, as *S. suis* is a microorganism that can live in different animal hosts as well as the human host and thus there is potential for possible intersections between animal and human resistomes (48, 49). Gene *tetO* was identified in 49 of the strains, while gene *ermB* was present in 41. Gene *lnuC*, associated with clindamycin and lincomycin resistance, and genes *ant6* and *aph3*', associated with resistance to aminoglycosides, were identified in 8 and 2 of the strains, respectively (S1 Table). Resistance to these antimicrobials has previously been identified in several other diverse *S. suis* isolates (48). We did not notice any clear indication of geographical differences in antimicrobial resistance markers among the strains in this study.

Complex population structure of ST28* S. suis *revealed by phylogenetic analysis

We next defined the ST28 *S. suis* core and pan genomes by performing ortholog analysis between the reference NSUI002 genome and annotated pseudochromosomes of all other 50 ST28 strains. We identified 1,786 core gene clusters and 2,776 pan genome clusters (S3 Fig.). Previously, Zhang *et al*. studied 13 strains of *S. suis* belonging to seven different serotypes and at least six different STs, and defined a core genome size of 1,343 genes and a pan genome of 3,585 genes (50). Since these authors analyzed strains of highly diverse genetic backgrounds, a larger core genome and smaller pan genome was expected in our cohort. We also identified a total of 31,488 non-redundant SNPs between all strains and the NSUI002 reference. To establish phylogenies, we defined a reduced ST28 core genome by first eliminating from the analysis gene clusters encoded in MGEs, and those CDSs present in all strains but whose length differed by > 9bp among the isolates. This left a final number of 1422 core gene clusters (1,269,771 bp) between the 51 ST28 *S. suis* strains under investigation. In this reduced core genome, 11,305 SNPs were identified. However, most of these SNPs were clustered in a few discrete areas of the core genome, which is suggestive of recombination.

Recombination is common among some streptococcal species for which extensive genetic recombination within populations has been observed (51-53). Extensive recombination among

highly diverse *S. suis* isolates has also very recently been described (54). To assess recombination in more detail, we used Bayesian analysis, which revealed 49 regions of recombination containing 441 genes (Fig. 2A and S3 Table). Neighbor joining phylogenetic analysis using the 1,421 informative SNPs remaining after exclusion of areas of the core genome having undergone recombination revealed two singletons (NSUI091, from Canada, and NSUI003 isolated in the USA), and two larger clades (Fig. 2B). One of them, identified here as clade I, comprised most of the Canadian strains in our collection, including the NSUI002 reference strain, and three US strains. The second major clade could be divided into four different subclades, identified here as clades II-V.

Clades II and III had a strong signal of geographical structure: clade II contained solely US isolates, while clade III was formed solely by strains isolated in Japan. Interestingly, while most strains found in clade IV were isolated in Japan, one Canadian and one US strain were also found in this clade. Import into Japan of live pigs from either the US or Canada for the purposes of breeding occurs frequently. Thus, we hypothesize that clade IV may have originated from *S. suis* ST28 strains that were introduced to Japan by import of live hogs from North America. Similarly, clade V had no unambiguous signal of geographic clustering and was formed by three US, one Canadian, one Japanese, and one Thai strain, the latter isolated from a case of human disease (Fig. 2B). We next compared gene content among the five clades defined by phylogenetic analysis. The total number of common genes was 1795 (Fig. 3). As a group, clade I strains did not possess unique gene content, while one unique gene cluster was found among strains of each clades II and IV. A total of 39 genes clusters were specific of clade III strains. Finally, clade V strains had 8 unique gene clusters. S4-S8 Tables list genes found in all strains of each clade.

Significant differences in virulence between ST28* S. suis *strains of clades I and V

Previous studies that each analyzed one ST28 strain have led to the notion that ST28 *S. suis* are typically avirulent or of low virulence (11-13). However, this notion can be challenged based on reports describing frequent isolation of ST28 strains from diseased pigs in some countries, as well as four human cases of *S. suis* ST28 disease (14-19). Inasmuch as our genomics and phylogenetic analysis revealed that rather than being a homogeneous group of organisms, ST28 strains are genetically heterogeneous, we hypothesized that these genetic differences may, in some cases, correlate with dissimilar virulence potential. To begin to test this

hypothesis we compared the virulence of two selected clade I strains (NSUI002 and NSUI062) and three selected clade V strains (NSUI036, NSUI081 and NSUI010) in a murine model of infection. We chose clade I because previous results had demonstrated low virulence of strain NSUI002 in a murine infection model (11); clade V strains were selected because there was no obvious geographic clustering structure and because this clade included a human isolate. Although swine is *S. suis* natural host, mice have frequently been used as a model to study the pathogenesis of *S. suis* diseases. Indeed, several reliable murine models using different mouse strains and routes of infection have been validated for *S. suis* (36, 55-57). Here we used one of these models that uses C57BL/6 mice and the intraperitoneal route of infection (36). Consistent with previous findings (11), no mice in the NSUI002 group died (Fig. 4A) nor showed clinical signs associated with *S. suis* infection, with the exception of slight depression following inoculation which subsided 24 h pi. Bacteria could not be isolated from the blood of most mice in this group at 24 h pi (Fig. 4B). Similar results were observed in the group that received clade I strain NSUI0062, although bacteremia was observed in more animals at 24 and at 48 h pi (Figs. 4B and 4C) in this group than in the NSUI002 group. In strong contrast, mice that received clade V strain NSUI036 showed severe clinical signs associated with septicemia, such as depression, swollen eyes, weakness, and prostration during the first 24 h pi. In fact, several mice died or met standard criteria for euthanasia during the first 4 days pi. There were several cases of meningitis between day 4 and day 6 pi in this group. *S. suis* was isolated in pure cultures at high titers (> 1 x 10^7 CFU/ml in some animals) from blood samples in the NSUI036 group (Figs. 4B and 4C). The other clade V strains evaluated here (NSUI010 and NSUI081) also caused relatively severe clinical signs and induced high bacteremia in inoculated mice (Figs. 4B and 4C). Although mortality was lower than in the NSUI036 group (Fig. 4A), statistical analysis revealed significant differences in survival between NSUI002 and the two clade I strains.

Inspection of unique gene content in clade V strains identified two genes encoding an ABC-type cobalt transport system. This ABC transporter has previously been found to be upregulated *in vivo* by virulent *S. suis* strains (58). Another key difference between clade V and clade I strains was that in the latter group a gene encoding a zinc-dependent IgA protease previously found to be important in *S. suis* virulence (59, 60) was disrupted by a transposon insertion, while the gene was intact in clade V strains (Fig. 5). Interestingly, in clade I strains, an ICE carrying *tetO*, which is absent from clade V strains, lies between another gene encoding a different putative zinc-dependent protease present in strains of both clades (Fig. 5). We also

discovered that NSUI036, the most virulent clade V strain, and the only human case included in our collection, had a 1bp insertion in the *sgp2* gene predicted to result in premature termination of translation of Sgp2, the putative adhesin of the *srtG* pilus (61). Previous reports in *Streptococcus pyogenes* have shown that strains impaired in pilus production are better fit to survive in blood and cause invasive disease (62).

Concluding Remarks

Recent technological advances in whole-genome sequencing now permit the cost-effective and rapid generation of data that can be used to precisely inform us about the population structure of pathogenic or commensal bacteria (63). The characteristics of *S. suis* serotype 2 strains belonging to ST28 (highly prevalent in North America) are poorly known. The use of whole-genome sequencing allowed us to uncover a relatively high level of genetic diversity among a large collection of strains isolated from diseased pigs and humans in different geographies. Experimental animal infections also discovered significant differences in virulence among strains belonging to two of the five different clades identified by whole-genome SNP-based phylogenetic analysis. Our results clearly highlight the limitations of typing *S. suis* strains using the commonly used MLST scheme (10), which failed to reveal the genetically heterogeneous nature of our strain collection. Furthermore, it now seems apparent that using MLST alone as a predictor of *S. suis* strain virulence can be misleading. Indeed, previous reports have proposed that ST28 *S. suis* strains are of low virulence (7, 11, 13), while here we show that at least some ST28 serotype 2 strains can induce severe disease in an experimental infection model. A key difference between these previous studies and this work is that, while the former drew their conclusions from results obtained after evaluation of the virulence of a single ST28 strain, here we used a population-based strain collection. In this regard, our results are consistent with previous findings describing frequent isolation of ST28 strains from diseased swine, and from human cases in China, Japan and Thailand (14-18). Our work is the first step towards better characterization of this diverse group of organisms heretofore considered genetically homogeneous. Further mining of the genome data generated in this study, coupled with mutagenesis of selected virulence factor candidates and animal studies will be instrumental in understanding the genetic basis of virulence differences among serotype 2 ST28 *S. suis* strains.

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Supporting information (Disponibles sur le site web de PLoS One)

S1 Figure. ClustalW alignment of the predicted translated sequences of the different *mrp* gene variants of ST28 strains 05HAS68 and NSUI002, and ST1 strain P1/7.

S2 Figure. Diagram showing the genetic organization of mobile genetic elements carrying genes encoding resistance to antimicrobial agents in ST28 strains NSUI002 and 05HAS68.

S3 Figure. Core and pan-genome of the 51 ST 28 *S. suis* strains.

S1 Table. *Streptococcus suis* strains used in this study.

S2 Table. Characteristics of the NSUI002 and other previously closed *Streptococcus suis* genomes.

S3 Table. Recombination among the 51 ST28 *S. suis* strains as defined by BratNextGen.

S4 Table. Common ortholog gene clusters among clade I ST28 *Streptococcus suis* strains.

S5 Table. Common ortholog gene clusters among clade II ST28 *Streptococcus suis* strains.

S6 Table. Common ortholog gene clusters among clade III ST28 *Streptococcus suis* strains.

S7 Table. Common ortholog gene clusters among clade IV ST28 *Streptococcus suis* strains.

S8 Table. Common ortholog gene clusters among clade V ST28 *Streptococcus suis* strains.

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Tables

Table 1. Presence of two component or standalone global regulators in the different ST28 and ST1 strains.

Regulator	NSUI002 (ST28)	05HAS68 (ST28)	P1/7 (ST1)
ihK/*irr*	+	+	+
ciaRH	+	+	+
vicK	+	+	+
salK/*salR*	-	-	-
nisK/*nisR*	-	-	-
virR/*virS*	-	-	+
covR	+	+	+
revS	-	-	+

Figures

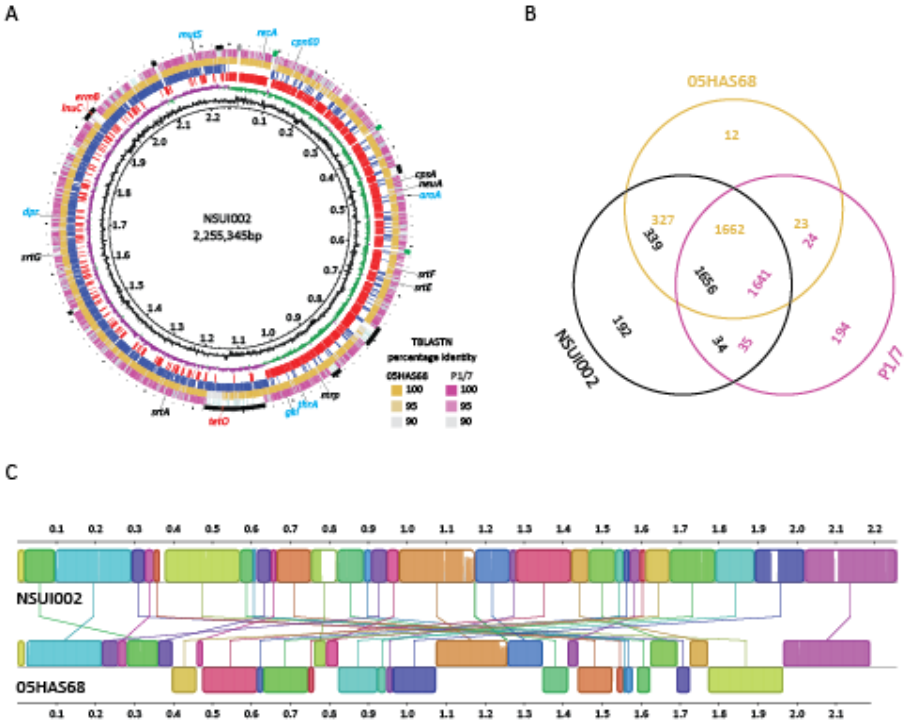

Figure 1. A) Genome atlas of Canadian *S. suis* ST28 strain NSUI002. Depicted data from innermost to outermost circles represent genome size in Mbp (circle 1); percent G+C content (circle 2); GC skew (circle 3), (G-C)/(G+C) averaged over a moving window of 10,000 bp, with excess G and excess C shown in green and purple, respectively; annotated coding sequences (CDSs) encoded on the forward/direct (circle 4, red), and reverse/complementary (circle 5, blue) chromosomal strands; TBLASTN comparisons of the CDSs predicted in ST28 strains NSUI002 and 05HAS68 (circle 6, percent identity defined in the Fig.), TBLASTN comparisons of ST28 strain NSUI002 and ST1 strain P1/7 (circle 7, percent identity defined in the Fig.); reference genome landmarks (circle 8) : ribosomal RNAs are labeled in green; mobile genetic elements are labeled in black, genes used in the *S. suis* MLST scheme are labeled in light blue; genes encoding resistance to antimicrobial agents are labeled in red; other genes are labeled in black. **B) Venn diagram depicting unique and shared CDSs in each of the *S. suis* strains as identified by ortholog analysis**. Each strain is represented by one color, and the number of CDSs are displayed in the same color. Numbers in the intersectional regions indicate CDSs shared by two or three strains. Since there may be more than one CDS in the same ortholog cluster, number of CDSs in the intersections are slightly different between strains **C) Collinearity of the genomes of *S. suis* ST28 strains NSUI002 and 05HAS68**. The genomes of the strains were aligned using progressiveMauve. Sequence alignments that are free of rearrangements are shown as colored local collinear blocks (LCBs). Sequence inversions are denoted by differential positioning of the LCBs relative to a reference axis. Several genome rearrangements between NSUI002 and 05HAS68 are noticeable.

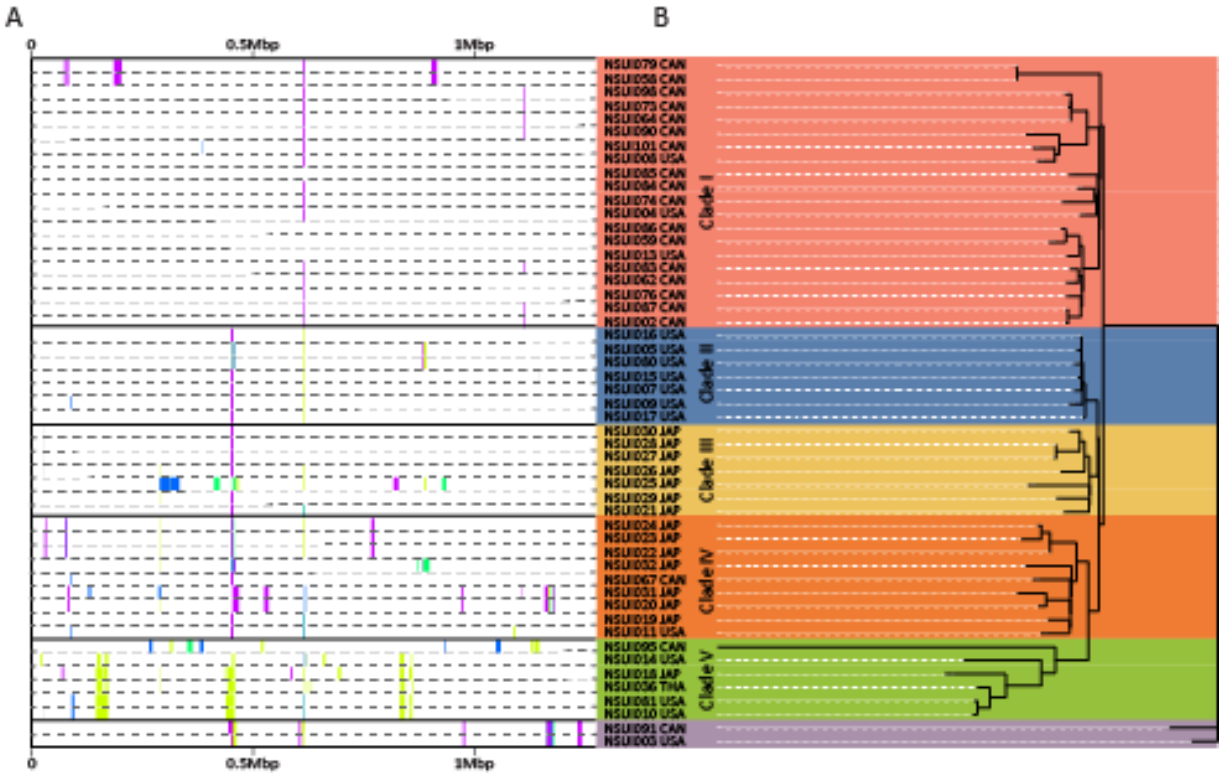

Figure 2. A) Results of Bayesian analysis of recombination for the 51 ST28 *S. suis* strains. The names and countries of isolation of the strains are shown on the right. The colored bars denote the recombination events in the strains along the core genome. The coloring of the bars at a specific genomic location reflects the clustering of the recombination events into groups, and is unrelated to other bars at distant genomic locations. CAN: Canada, USA: United States of America; JAP: Japan; Tha: Thailand. **B) Neighbor-joining phylogenetic tree depicting the relationships between the 51 ST28 *S. suis* strains**. The tree was constructed using 1,421 SNPs identified against the core genome (see text for details). Two singletons and five distinct clades (I to V) were identified.

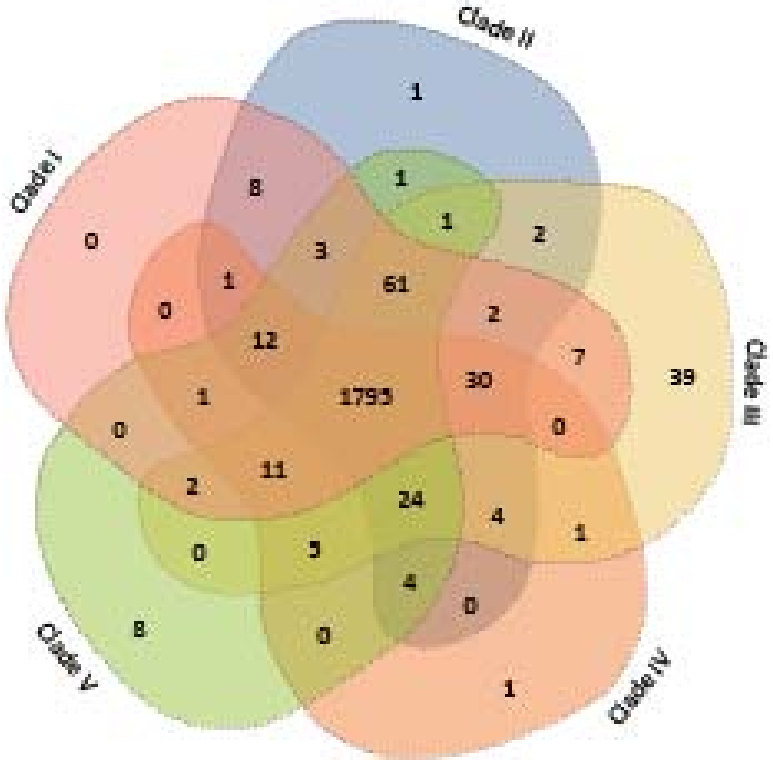

Figure 3. Venn diagram depicting unique and shared ortholog gene clusters in each of the five clades defined among the *S. suis* strains. Numbers shown in the different sections indicate the numbers of ortholog groups. The two ST28 singletons were not included in this analysis

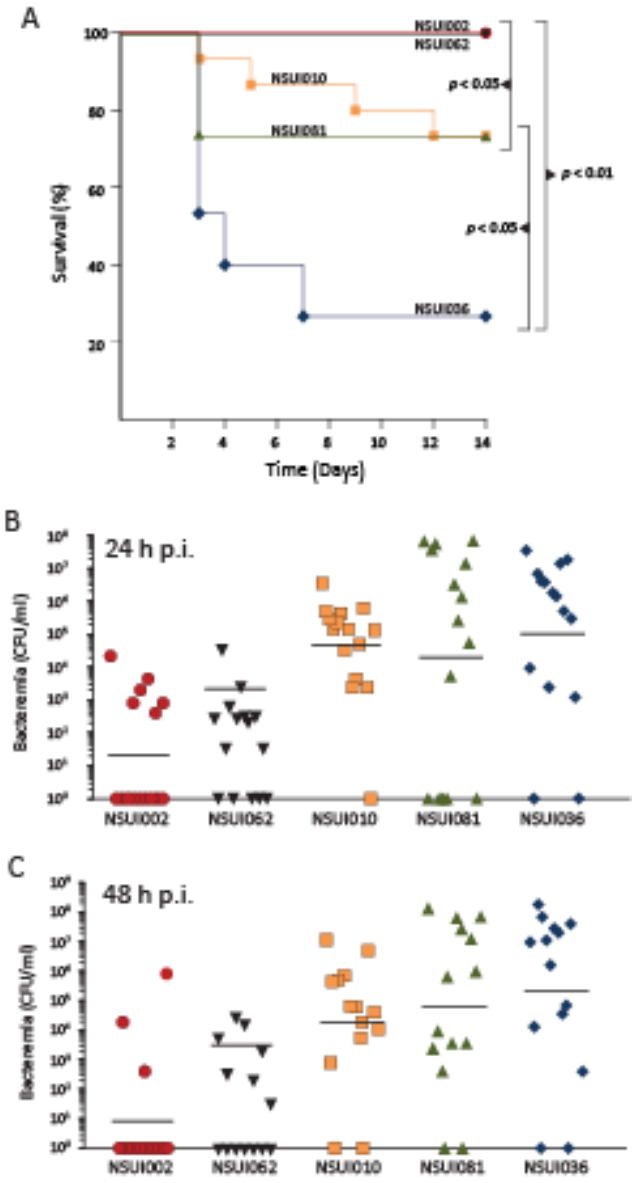

Figure 4. **Results of animal experimental infections**. **A)** Survival of mice inoculated with the different *S. suis* ST28 strains. All the mice in the NSUI002 and NSUI062 groups survived, while approx. 75 % of the animals in the NSUI036 group died from septicemia or meningitis. Animals that received strains NSUI010 or NSUI081 showed reduced mortality compared to NSUI036. Significant differences in survival (LogRank test) are depicted in the Fig. **Bacteremia at 24 h (B) and 48 h (C) post-infection (pi)**. NSUI002 and NSUI062 were isolated at lower titers than the other three strains following inoculation. The different symbols represent values from individual mice. The horizontal lines indicate the geometrical mean for each group. Significant differences in isolation from blood were noted at 24 h between NSUI036 and NSUI002 and NSUI062 and 48 h pi between NSUI002 and NSUI010, NSUI081 and NSUI036 and between NSUI062 and NSUI036 only (ANOVA on ranks, $P < 0.05$).

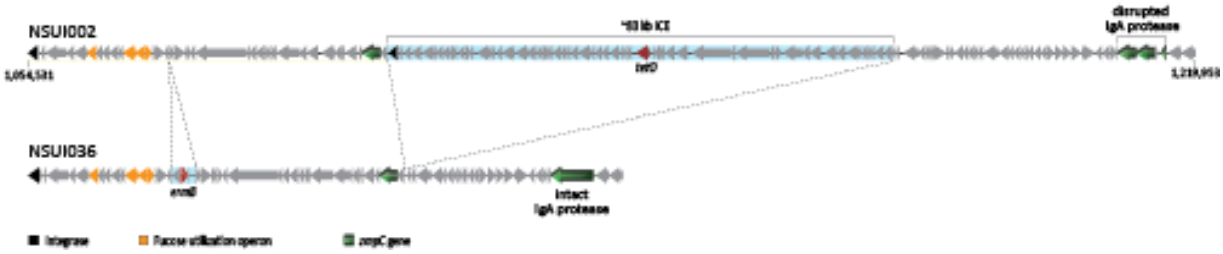

Figure 5. Genetic organization and predicted open reading frames of NSUI002 (clade I) and NSUI036 (clade V) regions containing a fucose utilization operon and *zmpC* genes. The region spans from position 1,054,531 to 1,219,953 in strain NSUI002, and contains 152 CDSs. In NSUI036, the region is notably smaller (83,658 bp). Differences in size are mainly due to the absence in the genome of strain NSUI036 of an approx. 83 kbp mobile genetic element (MGE), highlighted in light blue, that contains gene *tetO*. Other differences include a small MGE (highlighted in light blue) in NUI036 that contains gene *ermB*. In the conserved area, we observed a high degree of gene content conservation, with the exception of a *zmpC* gene also known as *iga*, encoding an IgA protease involved in *S. suis* virulence (59, 60), which is intact in NSUI036 but disrupted by a transposon insertion in strain NSUI002.

ANNEXES - ARTICLE IX

Limited interactions between *Streptococcus suis* and *Haemophilus parasuis* in *in vitro* co-infection studies

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Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-auteur de l'article. J'ai participé activement aux expériences de RT-qPCR et à l'analyse des résultats. J'ai aussi été impliqué dans la révision du manuscrit.

Abstract

Streptococcus suis and *Haemophilus parasuis* are normal inhabitants of the porcine upper respiratory tract, but are also among the most frequent causes of disease in weaned piglets worldwide, causing inflammatory diseases such as septicemia, meningitis, and pneumonia. Using an *in vitro* model of infection with tracheal epithelial cells or primary alveolar macrophages (PAMs), it was possible to determine the interaction between *S. suis* serotype 2 and *H. parasuis* strains with different level of virulence. Within *H. parasuis* strains, the low-virulence F9 strain showed higher adhesion levels to respiratory epithelial cells and greater association levels to PAMs than the high-virulence Nagasaki strain. Accordingly, the low-virulence F9 strain induced, in general, higher levels of pro-inflammatory cytokines than the virulent Nagasaki strain from both cell types. In general, *S. suis* adhesion levels to respiratory epithelial cells were similar to *H. parasuis* Nagasaki strain. Yet, *S. suis* strains induced a significantly lower level of pro-inflammatory cytokine expression from epithelial cells and PAMs than those observed with both *H. parasuis* strains. Finally, this study has shown that, overall and under *in vitro* conditions, *S. suis* and *H. parasuis* have limited interactions between them and use probably different host receptors, regardless to their level of virulence.

Introduction

Streptococcus suis (*S. suis*) and *Haemophilus parasuis* (*H. parasuis*) are among the most frequent causes of disease in weaned piglets worldwide [1]. Both bacterial species, mainly low virulent strains, are also normal inhabitants of the porcine upper respiratory tract and are present in most healthy animals [2, 3]. They are both transmitted by nasal contact from a colonized animal to another, usually from the sow to the piglets, but also among piglets [2, 3]. Both pathogens cause inflammatory infections such as septicemia, polyserositis, meningitis, arthritis and pneumonia [2, 3].

S. suis is classified into 35 serotypes, based on the antigenicity of the capsular polysaccharide (CPS). More recently, some serotypes (20, 22, 26, 32, 33 and 34) have been suggested to belong to different bacterial species [4], whereas strains with new capsular genes have also been described [5]. Serotype 2 has been described as being the most virulent and frequently recovered serotype from diseased animals [6]. However, phenotypic and genotypic differences within serotype 2 strains do exist [7]. The use of multilocus sequence typing has revealed that some strains of serotype 2 belonging to certain sequence types (STs) are more virulent than others. For example, virulent ST1 (as well as other members of the clonal complex 1) strains predominate in most Eurasian countries, whereas ST25 and ST28 strains (intermediate and low virulence, respectively) are widely distributed in North America [7]. The early steps of a *S. suis* infection take place in the upper respiratory tract. Bacteria adhere and, to a certain extent, invade the epithelial cells [8]. Although mechanisms are not completely understood, *S. suis* eventually reaches the bloodstream, remains extracellular by resisting phagocytosis and causes disease [3]. *S. suis* resistance to phagocytosis by phagocytic cells is mainly due to the presence of the CPS [9], which may indeed affect not only its own phagocytosis but also that of an heterologous species, such as Group B *Streptococcus* [10]. Bacteria then induce the production of pro-inflammatory cytokines in the respiratory tract as well as systemically that may compromise the host [11].

H. parasuis is the etiological agent of Glässer's disease, and is classified into 15 serotypes [12]. There are virulent and low-virulent strains, and there is no clear relationship between virulence and serotype [2]. Many of the non-typeable strains identified as such by the use of antibodies can now be serotyped by PCR [13, 14]. Virulent strains of *H. parasuis* are able to colonize and initiate infection by adhesion to and, to a certain extent, invasion of epithelial cells [15]. In the lungs, non-virulent strains of *H. parasuis* can be eliminated through phagocytosis

by alveolar macrophages [16]. In contrast, virulent strains of *H. parasuis* are able to avoid phagocytosis, probably due, among other factors, to the expression of a bacterial capsule, which allows multiplication of bacteria inside the host with a production of a strong inflammatory reaction that results in the characteristic lesions of Glässer's disease [2]. Once virulent strains enter the bloodstream (by still unknown mechanisms), the bacterium is able to avoid complement-mediated killing in an antibody-independent manner [2]. *H. parasuis* is also able to cause bronchopneumonia and virulence of isolates recovered from affected lungs is not completely known, since these isolates may also be the result of aspiration of low virulent colonizers from the upper respiratory tract [17]. Different virulence factors have been suggested to play important roles in the pathogenesis of the Glässer's disease [2]. Among them, the virulence associated trimeric autotransporters (VtaA) are those more characterized and their presence has been used to potentially identify virulent isolates by PCR [18].

Because *S. suis* and *H. parasuis* are present in the upper respiratory tract and both cause inflammatory diseases in young piglets after weaning, interactions between the two species may occur during the early steps of infection. In the present study, the interactions of virulent and intermediate/low-virulent *S. suis* and *H. parasuis* strains with Newborn Pig Tracheal cells (NPTr) and primary porcine alveolar macrophages (PAMs) during single infections and as well as simultaneous or sequential co-infections were studied. Results indicate that, in general, limited interaction occurs between the two bacterial species.

Materials and Methods

Bacterial strains

Two different strains of *S. suis* serotype 2 were used in this study (Table 1); the well characterized high-virulent strain P1/7 (ST1) from Europe, and the intermediate-virulent strain 89-1591 (ST25) from Canada [33]. Bacteria were cultured as previously described with some modifications [34]. Briefly, *S. suis* strains were cultivated on Colombia sheep blood agar plates (Oxoid, Burlington, ON, Canada), which were incubated at 37 °C for 16 h with 5 % CO_2. For cell infections, 5 ml of Todd Hewitt Broth (THB; Difco, Mississauga, ON, Canada) were inoculated with a few colonies of *S. suis* and incubated for 16 h at 37 °C, with agitation. To obtain the final culture, 10 ml of fresh medium were inoculated with 400 µl of the overnight culture and incubated at 37 °C under agitation until reaching the exponential growth phase when an optical density 0.6 (OD_{600nm}) was obtained. Two different strains of *H. parasuis* were also used. The

virulent Nagasaki strain (originally isolated from a case of septicemia with meningitis in Japan) and low-virulent F9 strain, isolated from the nasal cavities of a pig in Spain [27] (Table 1). Bacteria were grown on chocolate agar plates (Oxoid) as previously described [16], incubated 16 h at 37 °C with 5 % CO_2 and harvested with sterile phosphate buffered saline (PBS). After centrifugation, bacteria (*S. suis* and *H. parasuis*) were suspended to the appropriate concentration in cell culture media without antibiotics.

Cell culture

The Newborn Pig Tracheal cells (NPTr) and primary alveolar macrophages (PAMs) were used for co-infection studies. NPTr cells were cultured as described before [11, 24, 39]. Briefly, cells were grown at 37 °C with 5 % CO_2 in Dulbelcco's Minimum Essential Medium (DMEM; Gibco, Burlington, ON, Canada) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), penicillin-streptomycin (100 U/ml; Gibco) and gentamycin (0.04 mg/ml; Gibco). For assays, cells were treated with 0.1 % trypsin in 0.03 % ethylenediaminetetracetic acid (EDTA) solution (Gibco), suspended in fresh culture media and distributed into 24 wells tissue culture plates (Falcon, Mississauga, ON, Canada). The media was replaced after 24 h by cell culture medium without antibiotic, and the cells were incubated until they reached confluence, with a final concentration of 1 x 10^5 cells/ml. The day of the experiment, cells were washed three times with PBS and fresh cell culture medium without antibiotic was added to the wells.

To harvest the PAMs, bronchoalveolar lavages were performed with sterile PBS on lungs from 5 six-week old piglets from a high-health status farm as previously described [40], but without the use of antibiotics. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies (protocol number Rech-1570). Cells were washed twice with DMEM and frozen in liquid nitrogen to a final concentration of 2 x 10^7 cells/ml in DMEM supplemented with 20 % (v/v) FBS use. For the experiments, PAMs were thawed in warm DMEM supplemented with 10% FBS, centrifuged at 800 × *g*, and suspended at a concentration of 1 x 10^5 cells/ml in fresh culture medium. Five milliliters of the suspension were distributed in each well of 6-well tissue Primaria culture plates (Falcon). PAMs were further incubated overnight at 37 °C with 5 % CO_2 in a humid atmosphere. Sterility controls were done in parallel.

***Adhesion and invasion of NPTr by* S. suis *and* H. parasuis**

The so-called adhesion assay (which in fact quantifies total intracellular and surface-adherent bacteria) was performed as described before [19, 24] with some modifications. Briefly, cells were infected with either *S. suis* or *H. parasuis* with a MOI of 5 by removing the cell culture media and by replacing it with 1 ml of 5 x 10^5 of the bacterial suspension. This MOI was established as being optimal during standardization tests. Bacteria were cultured as previously reported [19, 24]. The number of colony forming units (CFU)/ml in the final suspension before each experiment was determined by plating *S. suis* samples onto Todd-Hewitt agar (Difco) or *H. parasuis* samples onto chocolate agar plates (Oxoid) using an Autoplate 4000 automated spiral plater (Spiral Biotech, Norwood, MA). Cell culture plates were then infected with the bacterial suspensions and centrifuged at 800 × *g* for 10 min in order to bring bacteria into close contact with cells [19] and further incubated at different incubation times (from 15 min up to 2 h) for adhesion assays. For simultaneous co-infections, 500 µl of both twice-concentrated bacterial suspensions (total of 1 ml; final MOI of 5 for each bacterial species) were added to the wells. For sequential co-infections, cells were pre-infected with one pathogen (either *S. suis* or *H. parasuis*) at a MOI of 5 (5 x 10^5 bacteria), centrifuged at 800 × *g* and incubated for 15 min at 37 °C with 5 % CO_2. In selected experiments and in order to saturate receptors, an MOI of 500 was also used. Cells were then washed 3 times with sterile PBS and 1 ml of the second bacterial suspension (MOI: 5, 5 x 10^5 bacteria) was added to the wells. The plates were centrifuged again and further incubated up to 2 h. These conditions were established during standardization tests.

For adhesion studies, and after the incubation time, cells were washed five times with PBS and disrupted with sterile ice-cold deionized water followed by cell scraping from the bottom of the well in order to liberate cell-associated bacteria. The cell suspensions were plated and incubated at 37 °C for 24-48 h on THA plates (to count *S. suis* colonies only) or chocolate agar to count either *H. parasuis* or both bacterial species which could be easily differentiated by colony morphology. Levels of adhesion were expressed as the total number of CFU recovered per well. For the invasion assay, a method similar to that of the adhesion assay was followed, except that after 2 h of incubation, the NPTr cell monolayers were washed twice with PBS, and 1 ml of cell culture medium containing 100 µg of gentamicin and 5 µg of penicillin G (Invitrogen, Burlington, ON, Canada) was added to each well. The plates were then further incubated for 1 h at 37°C with 5% CO2 to kill extracellular and surface-adherent bacteria. Cells were washed three times and the last wash was plated to confirm antibiotic activity. Cells were then disrupted

and bacterial CFU numbers were determined as described above. Levels of invasion were expressed as the total number of CFU recovered per well.

Induction of pro-inflammatory cytokine expression

For cell activation, NPTr and PAMs were infected with either *S. suis* or *H. parasuis*, or with both bacterial species simultaneously. All manipulations were carried out on LPS-free conditions. Cell culture media was removed from the wells and replaced by 1000 µl of bacterial suspension (MOI: 10, 1 x 10^6 bacteria) for single infections, or with 500 µl of both twice-concentrated bacterial suspensions for simultaneous co-infections. The plates were centrifuged 10 min at 800 × *g* and incubated 6 h (NPTr) or 12 h (PAMs) at 37 °C with 5 % CO_2. MOI and incubation times were chosen based on cytotoxicity studies, in order to work under non-toxic conditions. The cells were washed twice with warm PBS and homogenized in 1000 µl of QIAzol (Qiagen, Toronto, ON, Canada). The samples were frozen at – 80 °C until RNA extraction.

RNA extraction, cDNA construct and RT-qPCR

The RNA extractions using chloroform were performed according to kit instructions (QIAzol, Qiagen). Purified RNA was suspended in 20 µl of DNAse and RNAse-free water (Fisher, Ottawa, ON, Canada) and was quantified with NanoDrop 1000 (Fisher). Complementary DNA (cDNA) was synthesized from 500 ng of sample RNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The cDNA was diluted seven times in water for qPCR analysis.

Primers (IDT, Coralville, IA) used for quantitative PCR are listed in Table 2. A CFX96 rapid thermal cycler system (Bio-Rad, Mississauga, ON, Canada) showed that primers had a PCR amplification efficiency ranking between 90 and 110 %. The cDNA was amplified by qPCR as described in Lecours *et al* [41]. Briefly, the cDNA was amplified using SsoFast EvaGreen Supermix kit (Bio-Rad). The PCR amplification program for all cDNA consisted of an enzyme activation step of three min at 98 °C, followed by 40 cycles of denaturation for 2 s at 98 °C and an annealing/extension step for 5 s at 58 °C. Two genes, PPIA and Hypox, were used as the normalizing genes to compensate for potential differences in cDNA amounts [11]. Fold changes in gene expression were calculated using the normalized gene expression ($\Delta\Delta$Cq) calculation method of the CFX software manager (v.2.1: Bio-Rad). The non-infected cells group was used

as the calibrator reference in the analysis. The results originated from at least three independent experiments.

Cytotoxicity

The cell supernatants from infected cells under different assay conditions were used for LDH detection using the Cyto Tox 96 Non-Radioactive Cytotoxicity assay kit (Promega, Madison, WI) according to manufacturer's instructions. Medium from non-infected cells and supernatant from non-infected cells lyzed in pure cold water were used as negative and positive controls, respectively.

Effect of* S. suis *on* H. parasuis *association to and phagocytosis by PAMs

Association of* H. parasuis *to PAMs by FACS

In order to evaluate *H. parasuis* association to PAMs by flow cytometry (FACS), bacteria were first stained. Five milliliters of a *H. parasuis* bacterial suspension of an OD_{600nm} of 1.0 were incubated with 2 ng/ml of fluorescein isothiocyanate (FITC; Sigma-Aldrich) for 45 min at 37 °C with agitation [16]. Bacteria were washed three times with PBS supplemented with 1 % (v/v) of FBS. Tenfold dilutions were plated on chocolate agar to determine the final concentration of bacteria. Then, PAMs were infected with 5 ml of stained *H. parasuis* (MOI: 500, which corresponds to 5 x 10^{57} bacteria) suspension. Plates were incubated for 2 h at 37 °C, followed by two washes with warm PBS and cells were harvested with a scraper in PBS supplemented with 1 % (w/v) bovine serum albumin (Difco) [16]. The effect of a pre-infection with *S. suis* on the association of *H. parasuis* to PAMs was evaluated by a pre-treatment of the cells with virulent or intermediate virulent *S. suis* strains (MOI: 50 which corresponds to 5 x 10^6 bacteria) by replacing the cell culture media with 5 ml of the unstained *S. suis* suspension, and by incubating the plates at 37 °C for 1 h, followed by FITC-stained *H. parasuis* infection as described above. Mock-infected and cells infected with unstained *S. suis* only were used as control. Different MOIs were evaluated in pre-standardization tests in order to select the above indicated experimental conditions.

Phagocytosis of* H. parasuis *by PAMs by the antibiotic-protection assay

The viable intracellular count of *H. parasuis* was also assessed to determine the effect of *S. suis* on its phagocytosis by PAMs. Cell culture media was replaced by a suspension of *S. suis* ST1 strain P1/7 (MOI: 100) and the plates were centrifuged at 800 x *g* for 10 min. Non-infected cells

were included as controls. Cells were then further incubated 30 or 60 min, and cell culture medium was replaced with a suspension of *H. parasuis* (MOI :100, 1 x 10^7 bacteria). Plates were centrifuged and phagocytosis was left to proceed for 2 h, to allow optimal phagocytosis, determined during preliminary studies with different incubation times and MOIs. After incubation, cell monolayers were washed twice with PBS and incubated 2 h with medium containing antibiotics to kill extracellular bacteria, as described above. Supernatant controls were taken during every test to confirm the activity of the antibiotics. After antibiotic treatment, cell monolayers were washed three times with PBS, lysed with water and vigorous pipetting, and viable intracellular bacteria determined by plating appropriate dilutions as described above. Each test was repeated at least three times (with cells from 3 different animals) in independent experiments and the number of CFU/mL was determined as described above. To confirm the phagocytosis activity of the cells, a non-encapsulated *S. suis* mutant was used as positive control [41] (Table 1). Lyzed cells and internalized bacteria were plated on chocolate agar (Oxoid) and incubated for 24-48 h at 37 °C. A bacterial count was performed to determine the rate of *H. parasuis* phagocyted by PAMs.

Statistical analysis

All data are expressed as means ± standard errors. SigmaPlot Software (v.11.0) was used for data analysis. Significance was determined with one-way analysis of variance (ANOVA) or Student's unpaired t test where appropriate depending on the experiment. *P* values of < 0.05 were considered significant. Results reflect mean values from at least three independent experiments.

Results and Discussion

Co- or sequential infections of swine tracheal epithelial cells by S. suis *and* H. parasuis *has no impact on their adhesion/invasion capacities*

Both *H. parasuis*, as *S. suis*, first colonize pigs in their upper respiratory tract [2], through adhesion and, to some extent, invasion of epithelial cells [15]. To determine the effect of a co-infection on the early steps of infection in swine, the adhesion to and the invasion of swine epithelial cells from upper respiratory tract (NPTr) by strains of *S. suis* and *H. parasuis* of different virulence was evaluated. For the first time, individual cell adhesion of both bacterial species was compared within the same study and under the same conditions. All studies have

been done under non-toxic conditions (< 15%), as revealed by the lactate dehydrogenase (LDH) test (results not shown).

As shown in Figure 1A, after 2 h of single infection and an MOI of 5, both high virulent and intermediate virulent S. suis serotype 2 strains presented similar level of adhesion to swine tracheal epithelial cells. Similar results were observed when using different MOIs and incubation times (results not shown). Obtained results were similar to what has previously been described for this pathogen [19]. Most *S. suis* adhesion studies previously done used the low relevant human epithelial cell line HEp-2 [9]. In fact, a few studies have been carried out with swine tracheal epithelial cells and *S. suis*, both also showing similar adhesion levels to those observed in the present study [11, 19-21]. Interestingly, the intermediate virulent North American ST25 strain 89-1591 presented similar adhesion levels than the high virulent ST1 strain P1/7 (Figure 1A). Previous studies with *S. suis* serotype 2 have exclusively used virulent ST1 strains, which produce an hemolysin, called suilysin [22]. In fact, suilysin was shown to have a positive effect on *S. suis* adhesion to epithelial cells in absence of CPS [23]. However, the ST25 North-American strain used in this study (as well as all ST25 strains described so far) does not produce the suilysin, indicating that in the presence of the CPS this hemolysin might not be a critical factor for adhesion. Similar very low invasion rates have been obtained for both strains (Figure 1B), which confirm a previous study using an ST1 strain and the same cells [19]. This may suggest that high and intermediate virulent strains do not differ in these first steps of the pathogenesis of S. suis and differences may probably take place during the systemic phase.

The virulent *H. parasuis* Nagasaki strain adhered to epithelial cells in similar levels than both S. suis strains (Figure 1A). However, the low-virulent *H. parasuis* strain F9 adhered in significantly higher levels when compared to all other strains ($p < 0.05$) (Figure 1A). Similar results were observed when using different MOIs and incubation times (results not shown). Adherence of Nagasaki strain to epithelial cells has been previously shown [15]. Interestingly, in that study, authors showed higher levels of adhesion for this strain to renal epithelial cells, when compared to the low-virulent strain SW114 [15]. These results differ from those obtained in the present study; it is difficult to establish if differences in adhesion between SW114 and F9 strains are really due to their virulence potential or to the cell type (tracheal vs renal) used in the adhesion test and further studies are needed to obtain a definitive conclusion. Two previous

studies also showed similar low levels of adhesion with Nagasaki strain and NPTr cells [24, 25]. In the present study, both strains of *H. parasuis* presented very low levels of invasion (less than 1 bacteria internalized per 2000 cells) (Figure 1B), and no significant differences were observed between the strains. Invasion of *H. parasuis* to epithelial cells is still controversial and, as it is the case for adhesion, there are important differences between results obtained from renal and respiratory epithelial cells [15, 24, 25].

When the two bacterial species were used simultaneously, no differences in the adhesion of *S. suis* or *H. parasuis* could be detected when compared to those observed with individual infections (Figure 2). Similarly, a pre-infection with *S. suis* did not have any effect on *H. parasuis* adhesion to cells and vice-versa (Figure 2). All results were identical independently of the virulence of the strain. Even the use of a first bacterial species at an MOI: 500 to attempt to block cell receptors during the pre-infection did not influence the adhesion of the second bacterial species (results not shown). Similarly, it has not been possible to observe any significant difference in the bacterial invasion of the tracheal cells during co-infections or pre-infections compared to single infections (results not shown). All these results indicate that these bacterial species probably use different cell receptors. Knowledge on epithelial cell host receptors recognized by *S. suis* is limited [8]. A sialic acid-rich carbohydrate receptor (NeuNAca2-3Galb1-4GlcNAcb1-3Gal), host cell surface glycosaminoglycans and the globotriaosylceramide (GbO3) have all been suggested as possible receptors for epithelial cells [8]. On the other hand, receptors involved in *H. parasuis* adhesion/invasion of epithelial cells are unknown.

H. parasuis ***induces a higher expression of pro-inflammatory cytokines by tracheal porcine epithelial cells and primary aleveolar macrophages than*** **S. suis*****, and co-infections only partially modulate this expression***

Inflammation seems to be a hallmark of *S. suis* infections [3]. Although it is not a typical respiratory pathogen, *S. suis* may complicate infections caused by other aetiological agents of the Porcine Respiratory Diseased Complex, such as influenza [19]. It is generally accepted that one of the main role of *S. suis* would be to increase local inflammation [11]. As such, *S. suis* is able to induce inflammatory mediators from respiratory epithelial cells [11, 19]. Interestingly, only one study addressed the inflammatory response of PAMs by *S. suis* [26]. Remarkably, the

latter as well as all other studies of *S. suis* serotype 2 and swine cells have all been performed with virulent ST1 strains, and almost no information is available for North American strains belonging to other STs of lower virulence.

On the other hand, it has also been described that inflammation is an important player in the pathogenesis of the Glässer's disease [2]. It has been previously shown that epithelial cells may highly contribute to local inflammation observed with this pathogen [24]. In addition, if *H. parasuis* reaches the lungs, virulent strains resist to phagocytosis by PAMs and delay the activation of those cells, leading to bacterial multiplication in the lung and, ultimately, the release of inflammatory mediators resulting in pneumonia and polyserositis [16, 27]. For unknown circonstances, strains with a low-virulent profile (based on autotransporters analysis) are, in some cases, isolated from lungs of pigs with pneumonia, which may indicate they are low phagocyted [18]. *H. parasuis* would then induce the production of IL-6 and IL-8 and the apoptosis of respiratory epithelial cells, which may lead to its entry into the bloodstream [24, 28].

In the present study, we aimed to compare the inflammatory response of respiratory epithelial cells and PAMs infected with strains of *H. parasuis* and/or *S. suis* of different virulence. As it was the case with epithelial cells, PAM studies were done under non-toxic conditions (results not shown). First, swine tracheal epithelial cells and PAMs were infected with *S. suis* or *H. parasuis* alone in order to compare the expression of pro-inflammatory cytokines induced by each species and according to the virulence of the strains (Figures 3 & 4, respectively). Cells were infected for 6 h (NPTr) or 12 h (PAMs) and a quantitative RT-PCR was performed to assess the relative expression of IL-6 and IL-8, normalized by using Peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase 1 (Hypox) genes, and compared to the expression in non-infected cells. These incubation times were shown to be optimal for gene expression under non-toxic conditions during preliminary studies (results not shown). In general, *H. parasuis*, whether it is the virulent or the low-virulent strain, induced a higher expression of IL-6 and IL-8 by the epithelial cells during single infections than *S. suis*. Indeed, the latter induced a limited expression of both cytokines (Figure 3). *S. suis* and *H. parasuis* have been described to activate cells through Toll-like receptors (TLR) [29, 30], some of them (such as TLR2 and 6) being common to both species. However, the role of each receptor is

probably different, being *H. parasuis* and *S. suis* a Gram-negative and a Gram–positive microorganism, respectively. Only two studies are available in the literature concerning cytokine expression by epithelial cells infected with *H. parasuis* [24, 31]. Although some differences were observed in one of these studies when field strains of serotypes 4 and 5 were compared [24], this does not seem to be related to the virulence of the strain. In this study, the low-virulent F9 strain induced higher levels of IL-8 expression thant the virulent strain, which might be related to the higher capacity of this strain to adhere to cells. Additional strains should be tested to reach a definitive conclusion. In the case of *S. suis*, expression of both cytokines with high-virulent strains has been previously shown with these cells [11]. Interestingly, the high-virulent P1/7 strain induced a higher expression of IL-6 (4.03 ± 1.28) and IL-8 (4.02 ± 0.80) than the intermediate virulent 89-1591 strain (1.75 ± 0.99 and 2.44 ± 0.63, respectively) (Figure *3)*. Whether or not the capacity to stimulate epithelial cells by strains of different virulence has an influence on the pathogenesis of the infection remains to be confirmed.

When epithelial cells were co-infected with the highly virulent ST1 *S. suis* P1/7 and *H. parasuis* Nagasaki strains, only an additive effect on the expression of IL-6 and IL-8 was observed (Figure 3). However, a synergic effect ($p < 0.05$) on the expression of both cytokines could be observed with a co-infection of the low-virulent strain F9 and the highly virulent strain P1/7 (Figure 3). These results might indicate that a virulent strain of *S. suis* may positively modulate the expression of pro-inflammatory cytokines in the presence of a low-virulent *H. parasuis* strain, which might increase the local virulence in the presence of both bacterial species. However, more strains of known virulence should be tested before this hypothesis is confirmed.

In infected PAMs, *H. parasuis* strains also induced higher levels of IL-6 and IL-8 mRNA than both *S. suis* strains (Figure 4). In addition, the low-virulent F9 strain induced statistically higher levels of IL-6 (Figure 4A) and IL-8 (Figure 4B) than the virulent strain Nagasaki, probably due to higher levels of bacterial association to cells (see below). As observed with epithelial cells, *S. suis* high-virulent P1/7 strain induced higher IL-6 RNA levels (Figure 4A) than the intermediate-virulence 89-1591 strain. When analyzing co-infections, neither a clear additive nor a synergistic effect on the expression of IL-6 was observed when the PAMs were infected with *S. suis* and *H. parasuis* strains (Figure 4A). An additive effect on the expression levels of IL-8 by PAMs co-infected with S. suis intermediate virulent strain (89-1591) and virulent *H.*

parasuis Nagasaki strain was observed (Figure 4B). Other strain combinations did not present any additive or synergistic effect. Overall, these results differ from those observed with co-infected epithelial cells. Indeed, no explanation for such specific interaction with those strains and PAMs could yet be found.

S. suis *has no effect on* H. parasuis *association to or phagocytosis by PAMs*

It has been previously reported that low virulent strains of *H. parasuis* present higher levels of association with PAMs than virulent strains [16]. Accordingly, in the present study, the non-virulent F9 strain presented a significant higher level of association to PAMs than the Nagasaki strain, in terms of % of positive cells as well as in mean fluorescence intensity levels (MFI) (Figure 5).

It has been proposed that the *S. suis* is able to destabilize the lipid rafts on the surface of macrophages which contain lactosylceramide, preventing the interaction and phagocytosis of encapsulated strains [10]. In this way, *S. suis* prevents phagocytosis and remains extracellular [3]. This effect would be attributed to the CPS, since the use of purified CPS was able to inhibit not only the phagocytosis of a non-encapsulated S. suis strain but also that of an heterologous species (Group B *Streptococcus*) [10]. To evaluate a possible role of S. suis on *H. parasuis* association to PAMs, cells were pre-infected with the high-virulent *S. suis* P1/7 strain followed by *H. parasuis* strains. Since the capsular polysaccharide of all *S. suis* serotype 2 strains are chemically and antigenically homogenous [32], strain P1/7 was chosen as a representative strain to test this hypothesis. Interestingly, a pre-infection with S. suis serotype 2 did not change the association levels of any of the *H. parasuis* strains (Figure 5), confirming that these two bacterial species use different receptors.

As mentioned, in the lungs, low-virulent strains of *H. parasuis* can be eliminated through phagocytosis by macrophages [16]. H. parasuis is a ubiquitous bacterium in the upper respiratory tract of conventional pigs, which may be the reason that some strains from the upper respiratory tract may sometimes be found in the lungs. On the other hand, in some cases, strains with a low-virulent profile can be isolated from lungs of affected animals [18]. Since clinical cases of *H. parasuis* can be present in some farms as a consequence of co-infections

(such as Porcine Reproductive and Respiratory Syndrome) [12], it is possible that under those circumstances low-virulent strains are able to induce disease. In the present study, only the low-virulent F9 strain could be recovered intracellularly in the antibiotic-protection assay with PAMs (Figure 6). Indeed, the high-virulent Nagasaki strain could be hardly found inside these cells and could not be recovered in most of the experiments (not shown). Similalry, very low levels of phagocytosis of this strain has previously been reported [16].

As mentioned above, the CPS of *S. suis* serotype 2 was able to inhibit not only the phagocytosis of a non-encapsulated S. suis strain but also that of an heterologous species (Group B *Streptococcus*) [10]. We hypothesized that *S. suis* may prevent phagocytosis of low-virulent H. parasuis which would allow the latter to replicate extracellularly and increase the inflammatory reaction and causing disease. However, our results do not seem to support such hypothesis. A pre-treatment of the cells with encapsulated S. suis for 30 or 60 min did not affect the phagocytosis of the *H. parasuis* low-virulent F9 strain as shown by similar levels of bacteria recovered from the wells (Figure 6). It has been suggested that phagocytosis of *H. parasuis* is probably not dependent on a specific receptor, since phagocytosis of low-virulence strains was not affected by the presence high-virulent strains [16].

Conclusions

This study showed that a low-virulence strain of *H. parasuis* adheres to swine respiratory epithelial cells and PAMs at higher levels than a virulent strain as well as both *S. suis* strains. In addition, *H. parasuis* induce a significant higher level of pro-inflammatory cytokines than *S. suis* from both cell types. Within *H. parasuis* strains, the low-virulent F9 strain induces in general higher levels of pro-inflammatory cytokines than the virulent Nagasaki strain. Finally, this study has shown that, overall and under *in vitro* conditions, *S. suis* and *H. parasuis* have limited interactions between them, regardless to their level of virulence. Although it has been previously described that the CPS of *S. suis* serotype 2 has anti-phagocytic properties against an heterologous bacterial species, pre-treatment of PAMs did not have a clear effect on the phagocytosis of a low-virulence strain of *H. parasuis*. Although not clear interactions could be observed between the two bacterial species in the present study, further *in vitro* experiments (such as biofilm formation), a higher number of tested strains as well as *in vivo* studies should be carried out to reach more definitive conclusions.

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Abbreviations

cDNA: Complementary DNA; CFU: Colony forming unit; CPS: Capsular polysaccharide; DMEM: Dulbecco's modified Eagle's Medium; EDTA: Ethylenediaminetetraacetic acid; FACS: Fluorescence associated cell sorting; FBS: Fetal bovine serum; FITC: Fluoroscein isothiocyanate; GbO3: globotriaosylceramide; Hypox: hypoxanthine phosphoribosyl transferase 1; IL: Interleukin; LDH: lactate dehydrogenase; LPS: Lipopolysaccharide; MFI: Mean fluorescence intensities; MOI: Multiplicity of infection; NPTr: Newborn Pig Tracheal cells; PAMs: Primary alveolar macrophages; PBS: Phosphate-buffered saline; PCR: Polymerization chain reaction; PPIA: Peptidylprolyl isomerase A; RT-PCR: Reverse transcription polymerization chain reaction; ST: Sequence type; THA: Todd-Hewitt broth agar; THB: Todd-Hewitt broth ; VtaA: Trimeric autotransporter

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Tables

Table 1. *Streptococcus suis* serotype 2 and *Haemophilus parasuis* strains used in this study

Strain	ST or serovar	Country	Host	Virulence/Sly	Reference
S. suis					
serotype 2	1	UK	Pig	High virulent/Yes	[35]
P1/7	25	Canada	Pig	Intermediate	[36, 37]
89-1591				virulent/No	
H. parasuis					
F9	6	Spain	Pig	Low-virulent	[27]
Nagasaki	5	Japan	Pig	Virulent	[38]

ST, sequence type as describe by multilocus sequence typing; **Sly,** presence of suilysin.

Table 2. Porcine-specific primer sequences used in the study for pro-inflammatory cytokine detection by real-time quantitative RT-qPCR

Gene name	Forward	Reverse
IL-6	ACTCCCTCTCCACAAGCGCCTT	TGGCATCTTCTTCCAGGCGTCCC
IL-8	TGTGAGGCTGCAGTTCTGGCAAG	GGGTGGAAAGGTGTGGAATGCGT
Hypox	GCAGCCCCAGCGTCGTGATT	CGAGCAAGCCGTTCAGTCCTGT
PPIA	TGCAGACAAAGTTCCAAAGACAG	GCCACCAGTGCCATTATGG

Hypox, hypoxanthine phosphoribosyltransferase 1; **PPIA,** Peptidylprolyl isomerase A.

Figures

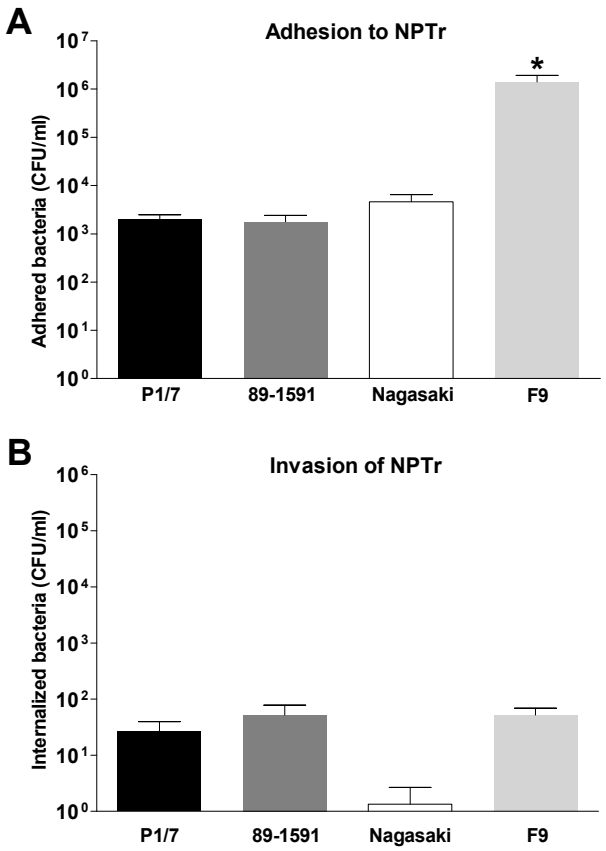

Figure 1. Adhesion to and invasion of Newborn Pig Tracheal cells (NPTr) epithelial cells by *S. suis* or *H. parasuis* strains of different virulence. NPTr cells were infected with *S. suis* serotype 2 strains P1/7 (high virulence) or 89-1591 (intermediate virulence), or with *H. parasuis* strains Nagasaki (high virulence) or F9 (low virulence) with a MOI of 5 for 2 h. (A) After the incubation time, cells were washed to remove non-adherent bacteria and lyzed in pure water to determine the number of adherent bacteria per well. (B) Infected cells were washed and cell culture media was replaced by fresh media with antibiotics and further incubated for 2 h in order to kill extracellular bacteria. Cells were washed to remove the antibiotics and lyzed in pure cold water to release internalized bacteria. Levels of adhesion/invasion are expressed as the total number of colony forming units (CFU) recovered per well. Data are expressed as means ± standard errors from at least four independent experiments. An asterisk indicates significant differences between samples ($p < 0.05$).

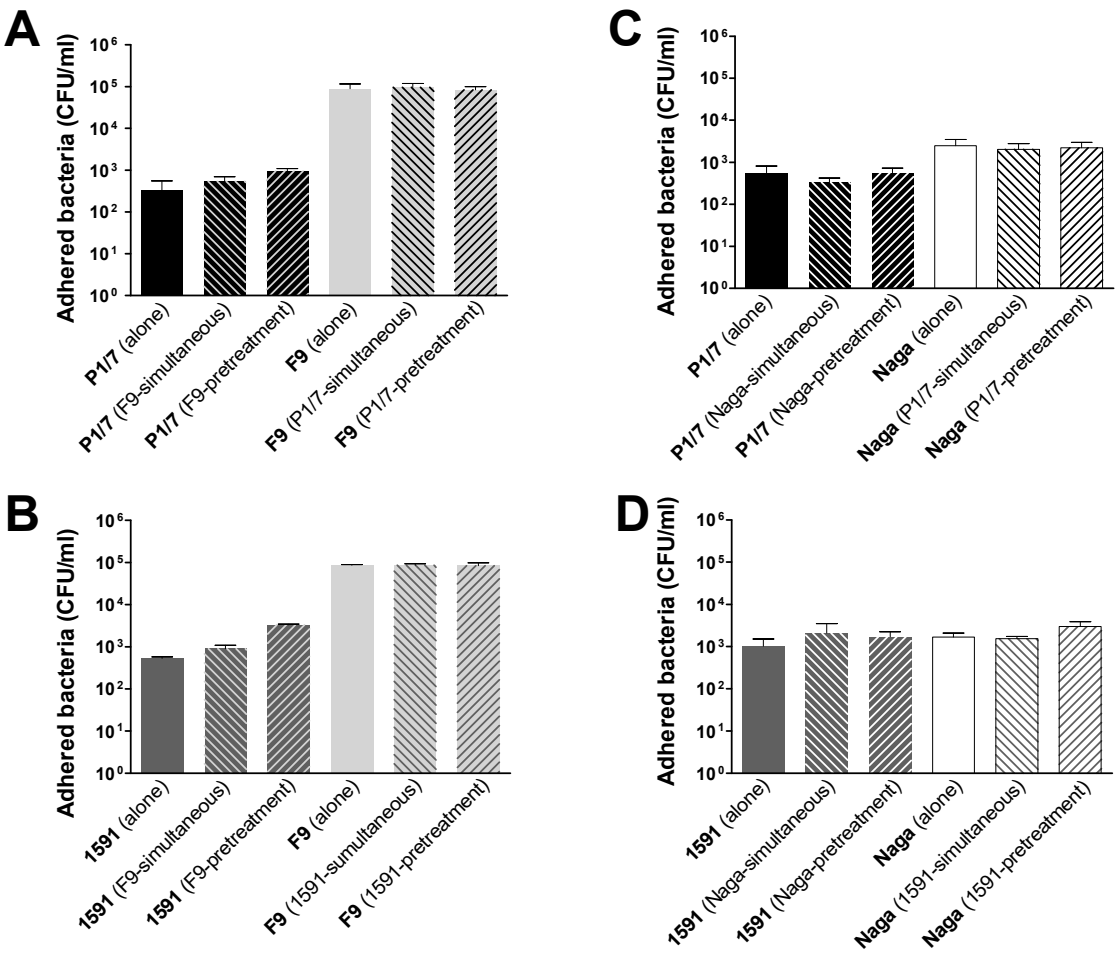

Figure 2. Adhesion of *S. suis* and *H. parasuis* strains of different virulence to Newborn Pig Tracheal cells (NPTr) epithelial cells either alone or in simultaneous or sequential co-infections. NPTr cells were pre-infected with *S. suis* or with *H. parasuis* strains (MOI of 5) for 15 min. Cells were then washed and infected with *H. parasuis* or *S. suis* strains, respectively (MOI of 5) and further incubated for 2 h. Separate wells were also kept with cell culture media (mock infected) as controls. In parallel experiments, cells were infected with both bacterial species simultaneously and also incubated for 2 h. Cells were washed to remove non-adherent bacteria and lyzed in pure water to liberate adherent bacteria. Levels of adhesion are expressed as the total number of colony forming units (CFU) recovered per well. Full bars match single infections. Hatched bars match co-infections. A) Adhesion of high-virulent P1/7 and low-virulent F9 in co-infection. B) Adhesion of intermediate-virulent 89-1591 and low-virulent F9 in co-infection. C) Adhesion of high-virulence P1/7 and high-virulent Nagasaki (Naga) during co-infection. D) Adhesion of intermediate virulent 89-1591 and high-virulent Nagasaki in co-infection. Data are expressed as means ± standard errors from at least four independent experiments.

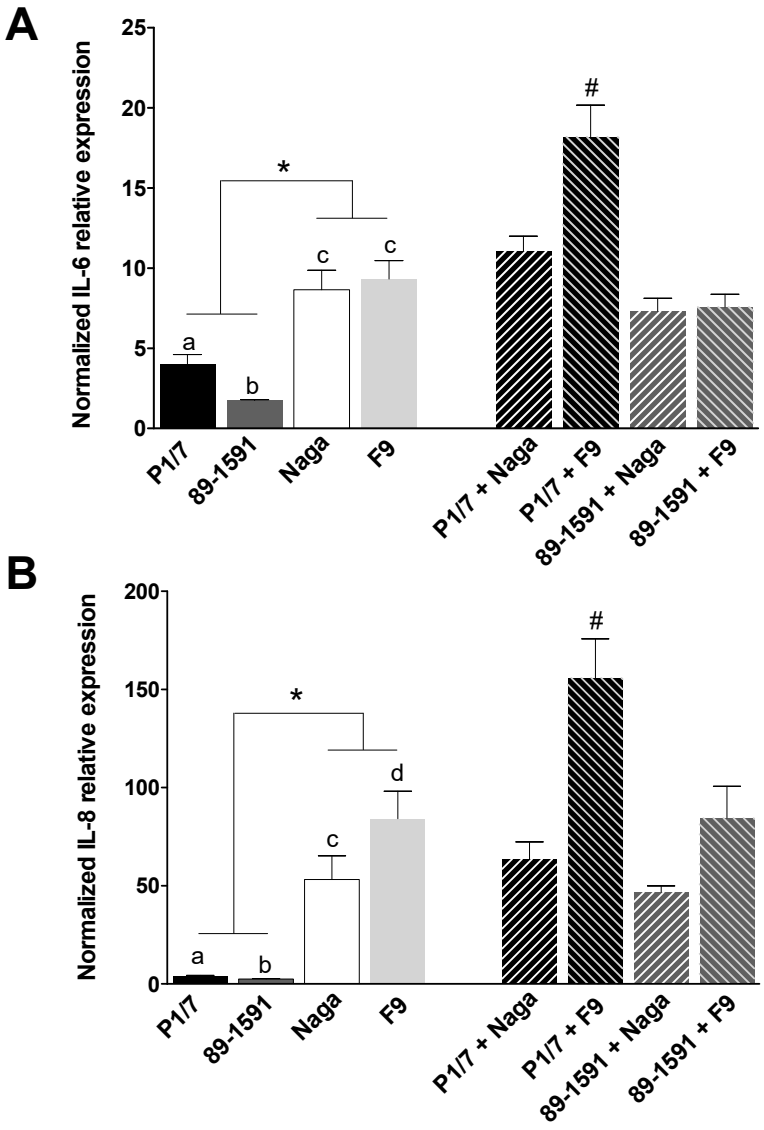

Figure 3. Normalized relative expression of IL-6 (A) and IL-8 (B) by Newborn Pig Tracheal cells (NPTr) epithelial cells during single and simultaneous co-infections with *S. suis* and *H. parasuis*. Cells were infected with *S. suis* (high-virulent P1/7 or intermediate virulent 89-1591 strains) or with *H. parasuis* (low-virulent F9 or high-virulent Nagasaki [Naga] strains) alone or in simultaneous co-infections for 6 h. Gene expression levels were analysed by RT-qPCR and normalized with the expression of PPIA and Hypox. Relative fold differences were calculated compared to non-infected cells. Data represent mean values ± standard errors of the mean from at least four independent experiments. Full bars show results from single infections. Hatched bars show results from simultaneous co-infections. Different letters indicate significant differences between two strains of the same bacterial species ($p < 0.05$). *, indicates significant differences between *S. suis* and *H. parasuis* strains ($p < 0.05$). #, indicates significant synergistic effect on cytokine production when compared respective single infections ($p < 0.05$).

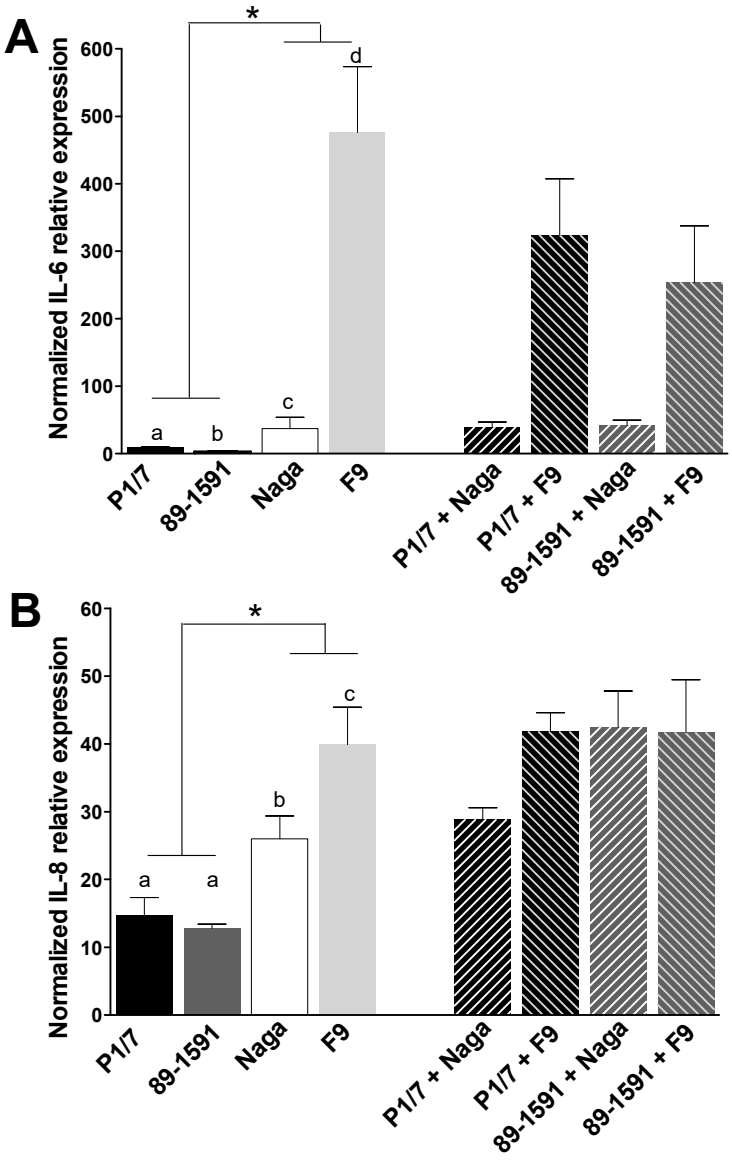

Figure 4. Normalized IL-6 (A) and IL-8 (B) relative expression by primary alveolar macrophages (PAMs) after single and simultaneous co-infections with *S. suis* and *H. parasuis*. PAMs were infected with either *S. suis* (high and intermediate virulent P1/7 and 89-1591 strains, respectively) or with *H. parasuis* (high and low virulent Nagasaki [Naga] and F9 strains, respectively) alone or in simultaneous co-infection for 12 h. Expression of genes was analysed by RT-qPCR and normalized with the expression of PPIA and Hypox genes. Relative fold differences were calculated compared to the non-infected cells. Data represent mean values ± standard errors of the mean from at least four independent experiments. Full bars show results from single infections. Hatched bars show results from simultaneous co-infections. Different letters indicate significant differences between two strains of the same bacterial species ($p < 0.05$). *, indicates significant differences between *S. suis* and *H. parasuis* strains ($p < 0.05$).

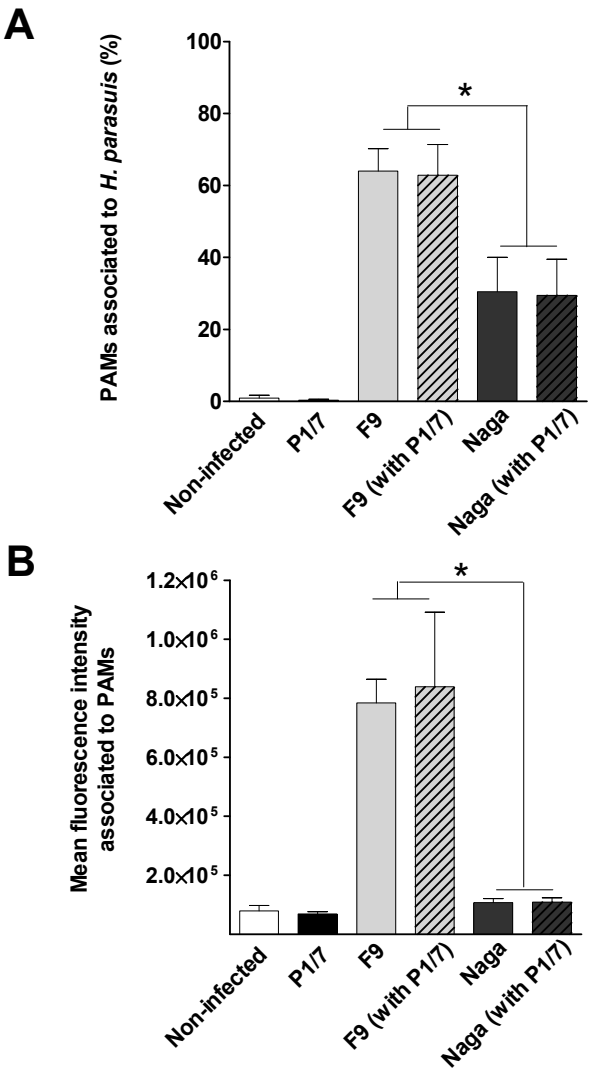

Figure 5. Association of *H. parasuis* to primary alveolar macrophages (PAMs) assessed by FACS. PAMs were incubated with a FITC-stained *H. parasuis* bacterial suspension at a MOI of 500 for 2 h. Cells were then washed and harvested in PBS with bovine serum albumine. The percentage of cells associated with *H. parasuis* (A) and the quantity of *H. parasuis* associated per cells, given by the mean fluorescence intensity (MFI) values (B), were determined by FACS. The effect of a pre-infection with *S. suis* on the association of *H. parasuis* to PAMs was evaluated through a pre-treatment of the cells with unstained *S. suis* P1/7 strain (MOI: 50) for 1 h. Mock infected and cells infected with unstained *S. suis* (P1/7) alone were used as negative controls. Data are expressed as means ± standard errors from at least four independent experiments. An asterisk indicates significant differences between samples ($p < 0.05$). Naga = Nagasaki

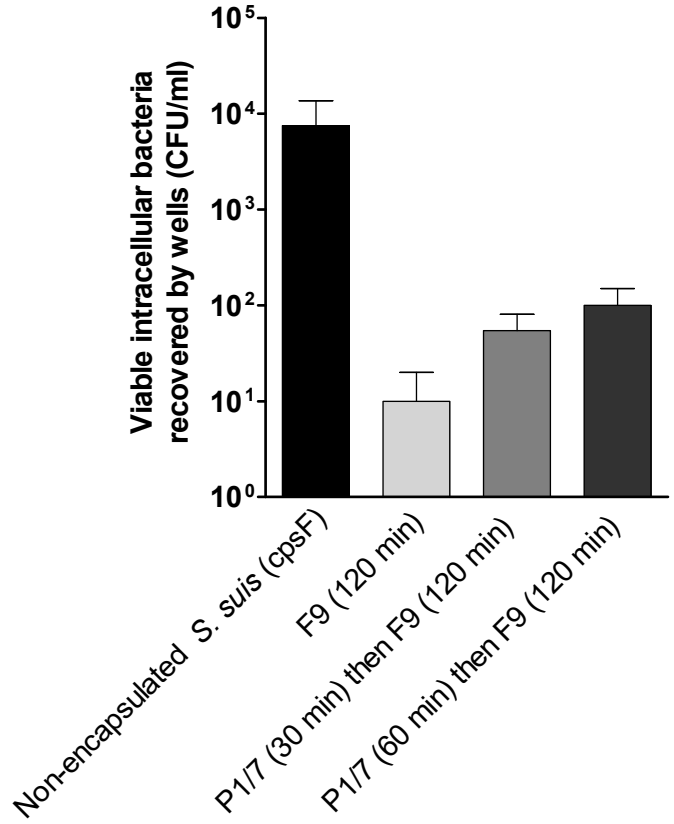

Figure 6. Phagocytosis of *H. parasuis* by primary alveolar macrophages (PAMs) as assessed by the antibiotic-protection assay. PAMs were incubated in presence of low-virulent *H. parasuis* F9 strain with a MOI: 100 for 120 min, washed and antibiotics were added to cell culture media and incubated for additional 120 min. PAMs were washed and lyzed in cold water to disrupt cell membranes in order to release intracellular bacteria. The suspension was plated on chocolate agar and colony forming units (CFU) were counted after an incubation time of 24-48 h. The effect of a pre-infection with *S suis* P1/7 strain on the phagocytosis of *H. parasuis* to PAMs was evaluated after a pre-treatment of cells with *S. suis* (MOI: 100) for 30 or 60 min. Cells infected with non-encapsulated *S. suis* were used as positive control to confirm the phagocytosis capacity of the cells. Data are expressed as means ± standard errors from at least three independent experiments.

ANNEXES - ARTICLE X

Role of interleukin-1 signaling induced by *Streptococcus suis* serotype 2 is strain-dependent and contributes to bacterial clearance and inflammation during systemic infection

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Soumis à *Frontiers in Immunology*. **Décembre 2018**.

Détails sur le rôle du candidat dans la conception de l'article :

Je suis co-auteur du manuscrit. J'ai participé à la conception de l'étude et des approches méthodologiques et aux expériences *in vitro*, aux ELISA et à la RT-qPCR. J'ai effectué les expériences *in vivo* et j'ai participé à l'analyse des résultats, à l'écriture de la première version du manuscrit et dans la révision de celui-ci.

Abstract

Streptococcus suis serotype 2 is an important porcine pathogen and an emerging zoonotic agent causing sudden death, septic shock, and meningitis, with exacerbated inflammation being a hallmark of the infection. A rapid, effective, and balanced innate immune response against *S. suis* is critical to control bacterial growth and limit the spread of the pathogen without causing excessive inflammation. Even though interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced, its role in the *S. suis* pathogenesis has not been studied. We demonstrate that a classical virulent European sequence type (ST) 1 strain and the highly virulent ST7 strain induce important levels of IL-1 in systemic organs. Moreover, dendritic cells and macrophages, which are involved in the *S. suis* pathogenesis, are important sources of this cytokine, with the ST7 strain inducing higher levels. To better understand the underlying mechanisms involved in this production, different cellular pathways were studied. Independently of the strain, IL-1β production required MyD88 and involved recognition via TLR2 and possibly TLR7 and TLR9. This suggests that the bacterial components recognized are similar and conserved between *S. suis* strains. However, very high levels of the pore-forming toxin suilysin, only produced by the ST7 strain, are required for efficient maturation of pro-IL-1β via activation of the NLRP3, NLRP1, AIM2, and NLRC4 inflammasomes via pore formation and ion efflux. Using IL-1R$^{-/-}$ mice, we demonstrate that IL-1 signaling may play a beneficial role during *S. suis* systemic infection by modulating the inflammation required to control and clear bacterial burden, thus promoting host survival. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counter-balanced by this signaling, making it difficult to discriminate its role. A better understanding of the underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

Introduction

Streptococcus suis causes sudden death and meningitis in pigs and is responsible for important economic losses to the swine industry. Additionally, *S. suis* is also a zoonotic agent causing meningitis and septic shock in humans and has become a public health concern, particularly in South-East Asia [1, 2]. Of the thirty-five described serotypes, serotype 2 is considered the most virulent and the most frequently isolated from both pigs and humans worldwide [3]. Using multilocus sequence typing, at least four predominant sequence types (STs) have been identified within serotype 2 strains: the virulent ST1 in Europe and Asia, the highly virulent ST7, responsible for two human outbreaks in China, and the intermediate and low virulent ST25 and ST28, respectively, in North America [4]. Isolates with variable virulence belonging to the latter two STs have also been reported in Asia [3, 5].

Mechanisms used by *S. suis* to infect the host and induce disease remain poorly known. A variety of virulence factors have been proposed, including the capsular polysaccharide, which confers anti-phagocytic properties helping *S. suis* to persist in blood, multiply, and disseminate throughout the host, leading to sepsis and/or meningitis [6]. Furthermore, many virulent strains of *S. suis*, including ST1 and ST7 strains, also produce an hemolysin similar to the pneumolysin of *Streptococcus pneumoniae*, called suilysin (SLY). This toxin may play an important role in bacterial dissemination and host inflammation as it is responsible for cell toxicity and inducing pro-inflammatory cytokines in many cell types [7, 8]. Moreover, bacterial components such as lipoproteins (LPs) and lipoteichoic acid (LTA) modifications have also been suggested to be involved in the *S. suis* pathogenesis [6].

A rapid and effective innate immune response against *S. suis* is critical to control bacterial growth and limit the spread of the pathogen [9]. Initial recognition by specialized membrane-associated or cytoplasmic receptors (pattern recognition receptors [PRRs]) mediates host immune responses by inducing the synthesis of diverse cytokines and chemokines through activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [10]. Previous studies have shown that *S. suis* activates dendritic cells and macrophages through the Toll-like receptor (TLR) pathway [11, 12]. In fact, absence of the adaptor protein myeloid differentiation primary response 88 (MyD88), central to this pathway, results in abrogation of pro-inflammatory mediator production *in vitro* [12]. Moreover, being mostly an extracellular bacterium, recognition of *S. suis* occurs via surface-associated TLR2 and,

possibly, TLR4 [13], although the latter remains to be confirmed. In case of internalization, however, *S. suis* may also activate the endosomal TLR7 and TLR9 [14].

Amongst the mediators induced during inflammation, interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced [15]. It is involved in the recruitment of inflammatory cells and their activation, induces production of other inflammatory factors such as lipid mediators and other cytokines, and participates in adaptive immunity and metabolism [16-18]. In fact, there are two forms of IL-1, IL-1α and IL-1β, which are encoded by separate genes and synthesized as precursor peptides (pro-IL-1α and pro-IL-1β). While pro-IL-1α is biologically active and can exert intracellular or extracellular functions, a two steps mechanism is required for the complete maturation of IL-1β [17, 19]. Firstly, activation of PRRs, including TLRs, leads to transcription and translation of pro-IL-1β. Next, the precursor is cleaved and activated mainly by caspase-1-dependent mechanisms or, less efficiently, by other proteolytic enzymes, such as neutrophil proteinase-3 [20]. Similar to proIL-1β, caspase-1 requires proteolytic processing, which is mediated by inflammasomes in response to a wide variety of stimuli. Though several inflammasomes have been described, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3), the NLRP1, the NLR family CARD domain-containing protein 4 (NLRC4), and the absent in melanoma 2 (AIM2) are the best characterized [21, 22]. Once secreted, IL-1α and IL-1β bind their shared receptor, IL-1 receptor (IL-1R), which is ubiquitously expressed, which leads to the synthesis of cytokines, chemokines, adhesion molecules, and acute phase proteins.

Although IL-1 signaling plays an essential role in immunity by participating in inflammatory response initiation, an uncontrolled production of IL-1 can lead to tissue damage and disease. In fact, IL-1 plays a protective role during both pneumococcal and Group B *Streptococcus* (GBS) infections, wherein a lack of IL-1 signaling contributes to a weak inflammatory response and higher bacterial burden [18, 23-25]. However, a recent study showed that lack of control of IL-1β production results in a lethal outcome in a mouse model of Group A *Streptococcus* (GAS) infection [26, 27]

During *S. suis* infection, host outcome depends on the ability of innate immune mechanisms to control initial bacterial growth and limit spread of the pathogen without causing excessive inflammation, a hallmark of the disease. However, regardless of the numerous studies on the

S. suis pathogenesis, none have focused on the production and role of IL-1. Consequently, we assessed its implication during *S. suis* serotype 2 pathogenesis. Herein, we demonstrate that a classical virulent European ST1 strain and the highly virulent ST7 strain induce important levels of IL-1 in systemic organs. In fact, dendritic cells and macrophages, which are centrally involved in the *S. suis* pathogenesis, are important sources of this cytokine. Production of IL-1 by both strains involved recognition via TLR2 and, possibly, TLR7 and TLR9, suggesting that cell activation is not influenced by the virulence level of the strain. By contrast, pro-IL-1β maturation mechanisms were strain-dependent, with the elevated levels of the pore-forming toxin SLY, only produced by the ST7 strain, participating in this maturation via activation of the NLRP3, NLRP1, AIM2, and NLRC4 inflammasomes. Globally, we demonstrate that IL-1 plays a beneficial role during *S. suis* systemic infection caused by classical strains (ST1) by modulating the inflammation required to control and clear bacterial burden. Beyond a certain threshold, however, such as in the case of the highly virulent ST7, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations of the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The protocols and procedures were approved by the Animal Welfare Committee of the University of Montreal (protocol number rech-1570).

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in **Table 1**. The classical virulent European reference ST1 P1/7 strain and the highly virulent Chinese ST7 SC84 strain were used throughout this study, including for construction of isogenic *sly*-deficient mutants. All *S. suis* strains were grown in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) as previously described [28], diluted in culture medium before experiments with cells, and the final concentration (colony-forming units [CFU]/mL) determined by plating on THB agar (THA). For experimental mouse infections, bacteria were resuspended in THB. *Escherichia coli* was grown in Luria-Bertani broth or agar (Becton Dickinson). When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to culture media at the following concentrations: for *E. coli*,

ampicillin at 100 mg/mL, kanamycin and spectinomycin at 50 µg/mL; for *S. suis*, spectinomycin at 100 µg/mL.

Construction of the isogenic sly-deficient mutants

Precise in-frame deletion of the *sly* gene from *S. suis* strains P1/7 and SC84 was achieved using splicing-by-overlap-extension polymerase chain reaction (PCR) [29]. Oligonucleotide primers (**Table S1 in Supplementary Material**) were obtained from Integrated DNA Technologies (Coralville, IA, USA) and PCRs were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or with the Taq DNA polymerase (Qiagen, Valencia, CA, USA). Overlapping PCR-products were cloned into the plasmid pCR2.1 (Invitrogen, Burlington, ON, Canada), extracted using EcoRI, and cloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s [30]. Final constructions of pSET4s vectors were electroporated into competent *S. suis* cells as previously described [30]. Deletion of the *sly* gene was confirmed by PCR and sequencing.

Cloning, expression, and purification of recombinant suilysin (rSLY)

The region corresponding to the *sly* gene, excluding the signal peptide, was amplified by PCR. PCR amplicons were digested with NdeI and BamHI and cloned into the pIVEX2.4d vector (Roche, Mississauga, ON, Canada), which possesses a N-terminal His-tag, previously digested with the same enzyme. Primers used are listed in **Table S1 in Supplementary Material**. Protein synthesis was induced in the *E. coli* BL21 (DE3) strain using 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h, after which cells were lysed by sonication. The resulting recombinant His-tag suilysin, henceforth called rSLY, was purified by affinity chromatography using the HisPur Ni-NTA Spin Column Kit (Thermo Scientific, Rochelle, IL, USA) according to manufacturer's instructions. rSLY kept its hemolytic activity as evaluated using red blood cells (see below). Protein quantification was measured using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific).

Titration of hemolytic activity

Hemolytic titration was performed by preparing two-fold serial dilutions of bacterial culture supernatant in a solution of 0.145 M NaCl and 7 mM Na_2HPO, pH 7.2, using horse red blood cells as previously described [31]. The titer was defined as the reciprocal of the highest dilution

with observed hemolysis. Results were expressed as the mean of at least three independent experiments.

Lipoteichoic acid preparation

Extraction and purification of LTA was performed as recently described [32, 33]. Yields of LTA preparations from 6 L of bacterial culture were 21.2 mg for strain P1/7, 20.9 mg for strain P1/7Δ*lgt*, 20.2 mg for strain SC84, and 30.9 mg for strain SC84Δ*lgt*.

Mice

MyD88$^{-/-}$ [B6.129P2(SJL)-MyD88$^{tm1.Defr}$/J], TRIF$^{-/-}$ [C57BL/6J-Ticam1^{Lps2}/J], TLR2$^{-/-}$ [B6.129-Tlr2^{tmKir}/J], TLR4$^{-/-}$ [B6.B10ScN-Tlr4$^{lps-del}$/JthJ], caspase-1$^{-/-}$ [B6N.129S2-*Casp1*^{tm1Flv}/J], NLRP3$^{-/-}$ [B6.129S6-*Nlrp3*^{tm1Bhk}/J], NLRP1$^{-/-}$ [B6.129S6-*Nlrp1b*^{tm1Bhk}/J], AIM2$^{-/-}$ [B6.129P2-*Aim2*$^{Gt(CSG445)Byg}$/J], NLRC4$^{-/-}$ [34], and IL-1R$^{-/-}$ [B6.129S7-*Il1r1*^{tm1Imx}/J] mice on C57BL/6 background were housed under specific pathogen-free conditions alongside their wild-type counterparts. Mice were purchased from Jackson Research Laboratories (Bar Harbor, ME, USA), with the exception of NLRC4$^{-/-}$ mice, which were originally produced by Dr. G. Núñez (University of Michigan, USA) [35].

Generation of bone marrow-derived dendritic cells and macrophages

Hematopoietic stem cells from femurs and tibias of wild-type and knockout mice were used to generate bone marrow-derived dendritic cells (DCs) as previously described [12, 14, 36] in complete culture medium, which was composed of RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (all from Gibco, Burlington, ON, Canada) and complemented with 10% granulocyte-macrophages colony-stimulating factor. For macrophages (MΦ), cells (5 x 10^5 cells/mL) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and complemented with 30% L929 cell-derived macrophage colony-stimulating factor supernatant [37]. Cells were cultured for 8 days at 37 °C with 5% CO_2 and trypsinized using 0.05% trypsin-0.03% EDTA (Gibco) prior to infection. Cell purity, evaluated as previously described [12, 36], was at least 85% CD11c$^+$ and F4/80$^+$ for DCs and MΦ, respectively.

Streptococcus suis *infection of dendritic cells and macrophages*

All activation studies were done in the absence of endotoxin contamination and under non-toxic conditions, the latter being evaluated by the lactate dehydrogenase (LDH) release with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different *S. suis* serotype 2 strains listed in **Table 1** (1 x 10^6 CFU/mL; initial multiplicity of infection=1). Conditions used were based on those previously published [7, 12]. At indicated times intervals, supernatants were collected for cytokine measurements. For mRNA expression, cells were harvested in TRIzol (Invitrogen) 6 h following infection. Mock-infected cells served as negative controls. Activation of cells with LTA was performed using 30 µg/mL and supernatants collected 24 h later for IL-1β quantification. For signaling pathway studies, cells were pretreated for 30 min with 10 µM NF-κB inhibitor JSH-23, 10 µM p38 inhibitor SB0203580, 25 µM MEK1/2 inhibitor U0126 or 10 µM JNK inhibitor SP600125 (all from Calbiochem/EMD Millipore, San Diego, CA, USA). For assays with rSLY, a non-toxic concentration of 5 µg/mL was used. For cholesterol inhibition assays, 40 µg/mL of cholesterol (Sigma-Aldrich) was added to wells. When needed 2.5% of 10 mg/mL alhydrogel (Brenntag, Mülheim, Germany) was added as a NLRP3 activator. Finally, for experiments involving extracellular K^+, a stock solution of KCl (Laboratoire Mat. Inc., Quebec City, QC, Canada) was prepared and appropriately diluted.

Streptococcus suis *DNA and RNA préparation and transfection of cells*

For bacterial DNA and RNA isolation, bacteria were grown to mid-log phase. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions, including treatment with DNase I. For DNA preparation, bacteria were harvested in 10 mM Tris, 1 mM EDTA, pH 8.0, and treated with 10% SDS and 20 mg/mL proteinase K (Sigma-Aldrich) for 1 h at 37 °C. DNA was isolated using phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) [38]. After isolation, bacterial DNA was treated with 10 mg/mL RNase A (Roche) for 30 min at 37 °C. Cells were transfected with 1 µg of RNA or DNA complexed with DOTAP liposomal transfection agent (Sigma-Aldrich) as previously described [14, 38, 39] .

Cytokine and chemokine quantification in cell culture supernatants

Levels of IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF) in cell culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using pair-matched

antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations.

Determination of cell mRNA expression by RT-qPCR

Cell mRNA was extracted according to the manufacturer's instructions (TRIzol) and cDNA generated using the Quantitect cDNA Synthesis Kit (Qiagen, Mississauga, ON, Canada). Real-time qPCR was performed on the CFX-96 Touch Rapid Thermal Cycler System (Bio-Rad) using 250 nM of primers (Integrated DNA technologies) and the SsoFast Evagreen Supermix Kit (Bio-Rad). The cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 s and 57°C for 5 s. Melting curves were generated after each run to confirm the presence of a single PCR product. The sequences of primers used in this study are shown in **Table S1 in Supplementary Material** and were verified to have reaction efficiencies between 90% and 110%. The reference genes *Atp5b* and *Gapdh*, determined to be the most stably expressed using the algorithm geNorm, were used to normalize data. Fold changes in gene expression were calculated using the quantification cycle threshold (Cq) method using the CFX software man-ager v.3.0 (Bio-Rad). Samples from mock-infected cells served as calibrators.

Streptococcus suis *serotype 2 mouse model of infection*

Six-week-old male and female wild-type C57BL/6 and IL-1R$^{-/-}$ mice were used. Animals were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow [40]. These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an end point measurement. The different *S. suis* serotype 2 strains, or the vehicle solution (sterile THB), were administered at a dose of 1 x 10^7 CFU by intraperitoneal inoculation. Survival was evaluated and mice were monitored at least three times daily until 7 days post-infection (p.i).

Measurement of plasma, liver, and spleen pro-inflammatory mediators

For kinetics of IL-1α and IL-1β production, wild-type mice were infected with each strain as described above. At various times p.i., blood was collected by intracardiac puncture following euthanasia and anti-coagulated with EDTA (Sigma-Aldrich) as previously described [41, 42]. Plasma supernatants were collected following centrifugation at 10 000 x *g* for 10 min, 4 °C. For

liver and spleen, extraction buffer was prepared using complete Mini, EDTA-free, protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and organs homogenized using a POLYTRON PT 1200E system bundle (Kinematica, Lucerne, Switzerland). Homogenate supernatants were collected following centrifugation at 10 000 x *g* for 10 min, 4 °C, and stored at −80 °C. Levels of IL-1α and IL-1β were determined by ELISA as described, while IL-6, IL-12p70, interferon (IFN)-γ, C-C motif chemokine ligand (CCL) 2, CCL3, and C-X-C motif chemokine ligand (CXCL) 9 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

Measurement of blood, spleen, and liver bacterial burden

Wild-type and IL-1R$^{-/-}$ were inoculated with *S. suis* as described above and blood bacterial burden was assessed 12 h and 48 h p.i. by collecting 5 µL of blood from the caudal vein. For liver and spleen, organs were collected and homogenized as described above. Bacterial burden was determined by plating appropriate dilutions on THA.

Statistical analyses

Normality of data was verified using the Shapiro–Wilk test. Accordingly, parametric (unpaired t-test) or non-parametric tests (Mann–Whitney rank sum test), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel–Cox) tests were used to compare survival between wild-type and IL-1R$^{-/-}$ mice. Each test was repeated in at least three independent experiments. $p < 0.05$ was considered as statistically significant.

Results

Streptococcus suis *serotype 2 induces elevated levels of IL-1 in systemic organs but not in plasma*

Since IL-1 plays a key role in initiating the inflammatory cascade, systemic levels of both IL-1α and IL-1β were measured in plasma, liver, and spleen following infection with the classical European ST1 strain P1/7 and the highly virulent ST7 strain SC84. Levels of IL-1α and IL-1β were barely detectable in mock-infected animals and did not change between 6 h to 48 h. Similarly, IL-1β levels were scarcely detectable in plasma throughout the course of infection,

including upon presentation of severe clinical signs of systemic disease, with no significant differences between strains (**Fig. 1A-B**). By contrast, levels of IL-1β in liver and spleen were high and reached maximum values during the first 12 h p.i., rapidly decreasing thereafter, with similar production patterns in both organs and between strains (**Fig. 1C-F**). IL-1α production patterns in plasma, liver, and spleen were similar to those of IL-1β (**Fig. S1 in Supplementary Material**). Collectively, these data demonstrate that strains P1/7 and SC84 induce elevated levels of IL-1 in organs and that this production remains locally.

Streptococcus suis *induces IL-1 release from dendritic cells and macrophages in a strain-dependent manner*

Given the elevated levels of IL-1 produced in liver and spleen and the fact that numerous cell types can produce IL-1, of which DCs and MΦ are highly important during *S. suis* infection [12, 14], the capacity of these cells to produce IL-1 following *S. suis* infection was evaluated. Strain P1/7 induced modest levels of IL-1β in a time-dependent manner, with production by DCs being slightly greater than that by MΦ (**Fig. 2A-B**). On the other hand, the highly virulent ST7 strain SC84 induced significantly higher levels of IL-1β (**Fig. 2C-D**) at 6 h, 8 h, 12 h, and 16 h p.i. ($p < 0.05$). In fact, levels induced by strain SC84 16 h p.i. were approximately 40 times greater than those induced by strain P1/7. Interestingly, a slight delay in IL-1β production was observed by SC84-stimulated MΦ in comparison to DCs. Moreover, IL-1α kinetics by DCs and MΦ were similar to those obtained for IL-1β (**Fig. S2 in Supplementary Material**), suggesting that *S. suis* induces comparable production of these two cytokines from DCs and MΦ. Importantly, production was not the result of cell death, since toxicity levels remained low (data not shown). Given that IL-1α and IL-1β production kinetics were similar and that both cell types responded similarly, subsequent experiments were only performed for IL-1β using DCs following 16 h of infection.

Role of Toll-like receptors and associated signaling pathways in **Streptococcus suis-***induced IL-1β production*

To better comprehend the differential IL-1β production induced by strains P1/7 and SC84 from DCs, the role of different receptors and signaling pathways involved in this production was evaluated. The TLR pathway has been previously described to be activated by *S. suis* and involved in pro-inflammatory mediator production [12, 14]. As shown in **Fig. 3A**, production of IL-1β was almost completely abrogated in the absence of MyD88 following infection with both

S. suis strains ($p < 0.01$). By contrast, production of IL-1β by both strains was independent of the TIR-domain-containing adapter-inducing IFN-β (TRIF) (**Fig. 3A**). Since *S. suis* is mostly considered an extracellular pathogen, its recognition by surface-associated receptors is crucial. While IL-1β production was significantly (but not totally) reduced in TLR2$^{-/-}$ DCs stimulated with P1/7 or SC84 ($p < 0.01$), no difference was observed with TLR4$^{-/-}$ DCs (**Fig. 3A**).

Given the implication of TLR2 in *S. suis*-induced IL-1β production, potential activators were investigated. Though somewhat controversial, LTA and LPs have been suggested to be activators of TLR2 in Gram-positive bacteria [43-45]. Consequently, LTA was extracted from both strains and used to activate DCs. As shown in **Fig. 3B**, LTA from strains P1/7 and SC84 induced important levels of IL-1β, with no differences between strains. As previously described, however, current methods are unable to completely eliminate co-purified LPs from LTA preparations [44]. As such, LTA was also extracted from *lgt*-deficient mutants (Δ*lgt*), in which absence of the lipoprotein diacylglyceryl transferase, a key enzyme in LP synthesis, renders LPs biological inactive and unrecognizable by TLR2 [46, 47]. In accordance, not only did LTA preparations from *lgt*-deficient mutants induce significantly less IL-1β than those from wild-type strains ($p < 0.01$), but levels were in fact undetectable (**Fig. 3B**). In agreement, IL-1β production was completely abolished in TLR2$^{-/-}$ DCs following activation with LTA preparations from wild-type strains ($p < 0.01$) (**Fig. 3B**). Taken together, these results indicate that co-purified LPs, but not *S. suis* LTA, are important inducers of IL-1β by DCs via recognition by TLR2.

Though considered mainly an extracellular bacterium, dependence of *S. suis*-induced IL-1β production on MyD88, but only partially on TLR2 (and not at all on TLR4), suggested a potential participation of endosomal TLRs. In fact, it was previously demonstrated that in the case of internalization*, S. suis* nucleic acids can induce DC activation [14]. As such, DNA and RNA were extracted from strains P1/7 and SC84 and complexed or not with DOTAP liposomal transfection agent, which allows phagosomal delivery. *S. suis* DNA and RNA induced significant IL-1β production from DCs only when complexed with DOTAP ($p < 0.05$), with similar levels obtained for both strains (**Fig. 3C**). Interestingly, at the same concentration, *S. suis* DNA was significantly more stimulating with regards to IL-1β production than RNA ($p < 0.01$). When Alum was added, a known activator of the NLRP3 inflammasome, a significantly higher production was observed for both genetic materials (**Fig. 3C**) ($p < 0.05$), probably due to

additional processing of proIL-1β into its mature form [48]. This recognition of RNA and DNA suggests a certain involvement of TLR7 and TLR9, respectively, in *S. suis*-induced IL-1β production.

TLR activation triggers a variety of intracellular signalling pathways of which the NF-kB pathway and MAPK p38, Jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK) are amongst the most important. These pathways are implicated in transcriptional control of many pro-inflammatory genes including cytokines and chemokines [49, 50]. To determine which of these pathways were involved in *S. suis*-induced IL-1β production, DCs were pre-treated with different inhibitors (NF-κB inhibitor [i] JSH-23, p38i SB203580, MEK1/2i U0126 or JNKi SP600125) or their vehicle as a negative control. As shown in **Fig. 3D**, treatment with NF-κBi significantly reduced secretion of IL-1β induced by both strains by approximately 50% ($p < 0.01$). Interestingly, differential inhibition by strains P1/7 and SC84 was observed for p38: while p38i had no effect on P1/7-induced IL-1β production, it significantly reduced SC84-induced IL-1β ($p < 0.01$) (**Fig. 3D**). Finally, while treatment with MEK1/2i reduced IL-1β production by 90% for both strains ($p < 0.01$), JNKi had no effect on *S. suis*-induced IL-1β production from DCs (**Fig. 3D**). Consequently, these results indicate that, with the exception of p38 activation, *S. suis* strains P1/7 and SC84 use similar receptors and signaling pathways in the induction of IL-1β.

Inflammasome activation required for IL-1β maturation induced by Streptococcus suis *is strain-dependent*

Processing of pro-IL-1β into its mature form requires cleavage by proteolytic enzymes, the most important of which is caspase-1 [19, 51]. To investigate whether maturation of *S. suis*-induced IL-1β requires this enzyme, caspase-1-deficient DCs were used. As shown in **Fig. 4A**, IL-1β production was reduced by more than 75% in caspase-$1^{-/-}$ DCs ($p < 0.01$), indicating that it is required for maturation of IL-1β following infection by both strains P1/7 and SC84. To determine the mechanisms by which *S. suis* might activate caspase-1, the role of different inflammasomes in IL-1β production was investigated. Presently, several inflammasomes have been described, with an even greater range of identified molecules that can trigger their activation [21]. Of these, NLRP1, NLRP3, AIM2, and NLRC4 are the best characterized inflammasomes [21]. Interestingly, a distinct pattern of inflammasome activation was observed for the two *S. suis* strains tested (**Fig. 4A**). While NLRP3- or AIM2-deficiency resulted in a partial decrease of

IL-1β release following stimulation with P1/7 ($p < 0.05$), there was no significant involvement of NLRP1 or NLRC4 (**Fig. 4A**). Unexpectedly, IL-1β maturation induced by strain SC84 involved all four inflammasomes tested ($p < 0.05$), with a major implication of NLRP3 (**Fig. 4A**). IL-1β-specific activation by these inflammasomes was confirmed by lack of activity on the secretion of TNF, which is inflammasome-independent (**Fig. S3 in Supplementary Material**).

Since inflammasome activation was different between the two strains, we hypothesized that maturation, but not induction, of IL-1β could be responsible for the different levels of IL-1 β induced by both strains *in vitro*. To confirm this hypothesis, IL-1β mRNA levels induced by both strains were evaluated. In accordance, levels of IL-1β expression induced by strains P1/7 and SC84 were similar (**Fig. 4B**). This confirms that while both strains similarly activate DCs to induce proIL-1β, the differential levels of IL-1β produced are the consequence of differences in maturation capacity.

Streptococcus suis *induced-IL-1β production is blocked by additional extracellular potassium*

Potassium (K^+) efflux has been described as a common denominator in the assembly and activation of inflammasomes [21, 52]. Consequently, we evaluated production of IL-1β by *S. suis*-infected DCs exposed to increased concentrations of extracellular K^+ to inhibit its efflux. Interestingly, only 10 mM of extracellular K^+ was required to significantly inhibit IL-1β induced by both strains ($p < 0.05$) (**Fig. 5**). However, the effect was significantly greater for strain SC84 than for strain P1/7 ($p < 0.01$) (**Fig. 5**). Moreover, inhibition appeared to be dependent on K^+ concentration since increasing extracellular levels lead to a greater inhibition, regardless of strain. Importantly though, at concentrations equal to or greater than 40 mM, the decreased levels of IL-1β observed were non-specific due to cell death as determined by LDH release (data not shown). Unspecific inhibition of K^+ efflux at concentrations between 10 mM and 30 mM was discarded by measuring TNF and IL-6 production, which were not inhibited (**Fig. S4 in Supplementary Material**).

The elevated suilysin production by the ST7 strain is required for efficient proIL-1β maturation by dendritic cells

Given that pore-forming toxins have the ability to induce K^+ efflux [21, 52] and that *S. suis* produces SLY, a secreted cytolysin similar to the pneumolysin of *S. pneumoniae*, its role in IL-

1β production was evaluated. It was previously suggested that the highly virulent *S. suis* ST7 strain responsible for the human outbreaks in China has an increased SLY production compared to other serotype 2 strains [53]. We confirmed this by measuring the hemolytic activity in P1/7 and SC84 supernatants, with strain SC84 displaying a two-log greater titer than that of strain P1/7 (results not shown). To evaluate if SLY is implicated in *S. suis*-induced IL-1β release, *sly*-deficient isogenic mutants were constructed and used in parallel with their respective wild-type strains. As shown in **Fig. 6A**, no difference in IL-1β production was observed in the absence of SLY from strain P1/7, suggesting that components other than the SLY (such as LPs) could be responsible for IL-1β production. By contrast, absence of SLY from strain SC84 resulted in a significant decrease of secreted IL-1β ($p < 0.001$), suggesting that the higher levels of SLY produced by this strain are implicated in IL-1β production (**Fig. 6A**). Moreover, IL-1β levels produced by SC84Δ*sly* were similar to those obtained with both wild-type P1/7 and P1/7Δ*sly*, suggesting that common bacterial components are probably responsible for the basal production of IL-1β by both strains (**Fig. 6A**). In addition, the lack of differences in IL-1β mRNA expression between the wild-type and *sly*-deficient mutants confirms that SLY participates in proIL-1β maturation rather than in IL-1β induction (**Fig. 6B**).

Since SLY is a cholesterol-dependent cytolysin, cholesterol inhibits its effects [31]. To evaluate the effect of cholesterol on *S. suis*-produced SLY and its consequences on IL-1β production, cholesterol was added to DCs infected with the different *S. suis* wild-type and *sly*-deficient strains. Importantly, cholesterol itself did not induce IL-1β production (**Fig. 7A**). While addition of cholesterol had no effect on IL-1β production by strains P1/7, P1/7Δ*sly* or SC84Δ*sly*, it significantly decreased production induced by the wild-type strain SC84 ($p < 0.001$), with detected levels similar to those measured for P1/7, P1/7Δ*sly*, and SC84Δ*sly*, confirming the role of SLY in the hyper-producing strain SC84 (**Fig. 7A**).

Further activation experiments using rSLY alone showed the production of moderate levels of IL-1β from DCs (**Fig. 7B**). Since SLY has been previously described to produce TNF following recognition by TLR4 [13], we activated $TLR4^{-/-}$ DCs with rSLY. However, IL-1β production was TLR4-independent **(Fig. S5 in Supplementary Material**), confirming results obtained with live bacteria. Meanwhile, addition of a non-toxic concentration of 5 µg/mL of rSLY resulted in a synergistic and similar increase of IL-1β for strains P1/7, P1/7Δ*sly*, and SC84Δ*sly* ($p < 0.01$)

(**Fig. 7B**). Importantly, this effect was abolished following treatment with cholesterol, confirming an exclusive effect of rSLY (**Fig. 7B**).

Strain-dependent role of IL-1 signaling in host survival during* Streptococcus suis *systemic infection

Given the capacity of *S. suis* to induce IL-1 *in vitro* and *in vivo*, its implication in the balance and/or exacerbation of systemic inflammation induced by this pathogen, and subsequently, host survival was evaluated. Survival of IL-1R$^{-/-}$ mice was significantly decreased in comparison to wild-type counterparts following infection with strain P1/7 ($p < 0.01$), suggesting a beneficial role of IL-1 (**Fig. 8A**). Following infection with strain SC84, however, no statistical difference was observed between survival of wild-type and IL-1R$^{-/-}$ mice (**Fig. 8B**) indicating, that in the case of infection with a highly virulent strain no role can be distinguished.

To better understand this difference between strains, and since IL-1 plays a crucial role in both the initiation and amplification of inflammation, production of other pro-inflammatory mediators in plasma, spleen, and liver was evaluated 12 h p.i. Interestingly, IL-1α and IL-1β liver and spleen levels in WT and IL-1R$^{-/-}$ mice did not show any significant differences, indicating that IL-1 does not autoregulate after 12 h of infection with *S. suis* (**Fig. S6 in Supplementary Material**). As shown in **Fig. 9** and **Fig. 10**, significantly lower levels of IL-6, IL-12p70, IFN-γ, CCL2, CCL3, and CXCL9 were measured in the plasma, spleen, and liver of IL-1R$^{-/-}$ mice compared with wild-type mice following infection with P1/7 ($p < 0.05$), confirming that IL-1 is necessary for pro-inflammatory mediator modulation. However, following infection with strain SC84, levels of mediators where exacerbated in both wild-type and IL-1R$^{-/-}$ mice, indicating that IL-1 signaling cannot counterbalance the exacerbated inflammation induced by this highly virulent strain (**Fig. 9-10**).

During *S. suis* systemic infection, inflammation is required for clearance of bacteria, which, if uncontrolled, can lead to host death. Consequently, we evaluated bacterial load in blood, spleen and liver, 12 h and 48 h following infection with strains P1/7 and SC84. No differences were observed in bacterial burden of wild-type and IL-1R$^{-/-}$ mice 12 h p.i. in blood or organs, this regardless of strain (**Fig. 11**). Interestingly, 48 h p.i. following infection with P1/7 strain, bacterial burden was significantly higher in plasma, liver, and spleen of IL-1R$^{-/-}$ mice, in comparison to wild-type counterparts ($p < 0.01$) (**Fig. 11**). By contrast, no differences were

observed following infection with strain SC84, and this for all organs (**Fig. 11**). Notably, bacterial load of wild-type mice infected with strain P1/7 or SC84 were similar 12 h and 48 h p.i, indicating that the higher virulence of the latter is responsible for the elevated inflammation and detrimental host outcome.

Discussion

Though IL-1 is a key cytokine implicated in host defense response modulation, no studies have focused on *S. suis*-induced IL-1 production. In fact, a few previous studies showed low levels of IL-1 in plasma in comparison with other important pro-inflammatory cytokines such as TNF or IL-6, underestimating the potential role of this cytokine in *S. suis* infection [9, 40, 54, 55] As such, the low levels of IL-1 observed in plasma after infection with both strains were not surprising and not related to the virulence levels of the strain. Several factors could explain this near lack of IL-1, including its short half-life in plasma [56] and its association with other plasmatic proteins [57, 58]. To our knowledge, however, there has been no other study regarding plasmatic levels of IL-1 in similar model of infection (systemic infection). On the other hand, high levels of IL-1α and IL-1β were found in liver and spleen, which are two important filter organs. Previous studies with GBS also reported elevated levels of IL-1β in kidneys [18]. Therefore, although IL-1 cannot be found in plasma, activation of immune cells by infiltrated bacteria in liver and spleen could be responsible for the induction of IL-1, which remains locally.

To further analyze differences in IL-1 signaling between strains, *in vitro* studies with DCs and MΦs were performed. Interestingly, production by MΦ was somewhat delayed compared to that by DCs following infection with both *S. suis* strains. This appears to be a characteristic of extracellular pathogens, as it was previously reported for GBS, GAS, and *S. pneumoniae* [59-61], and might be due to a less efficient capacity of macrophages to process proIL-1β into its mature form.

Unlike with most other cytokines, IL-1β production is controlled by a two-step signaling system. Firstly, activation of PRRs such as TLRs leads to the transcription of proIL-1β. Subsequently, a second signal induces cleavage of the precursor into active IL-1β through caspase-1- and inflammasome-dependent maturation. The higher levels of IL-1 induced by strain SC84, in comparison to strain P1/7, suggested differential cell activation or processing mechanisms. However, the cellular activation leading to IL-1β production was similar for strains P1/7 and

SC84, indicating that the recognized components are relatively well-conserved between these two strains, which might be the case for most *S. suis* serotype 2 strains. Indeed, production of IL-1β was MyD88-dependent and partially involved recognition of surface lipoproteins by TLR2. Comparable results were reported for GBS and *S. pneumoniae*, suggesting that recognized bacterial motifs might even be conserved amongst streptococci [59, 62]. Meanwhile, early studies suggested that certain toxins such as pneumolysin [63, 64], listeriolysin O [65] and, more recently, SLY [13], may activate cells trough TLR4, an extracellular receptor which can signal via MyD88. However, the capacity of these toxins to activate TLR4 remains controversial. More recent studies with *S. pneumoniae* (including recombinant pneumolysin) showed that production of IL-1 was TLR4-independent [61, 66]. In our study, IL-1β production by DCs induced by SLY-positive P1/7 and SC84 strains as well as rSLY was TLR4-independent, confirming preliminary results [12]. Moreover, IL-1β production was TRIF-independent, confirming the lack of TLR4 implication, since this adaptor protein is engaged by the latter and TLR3. Interestingly, to our knowledge, this is the first study evaluating the role of TRIF during *S. suis* infection. Finally, although considered a classical extracellular pathogen, *S. suis* strains P1/7 and SC84 can be internalized, albeit at low rates. When internalized, however, TLR7 and TLR9, which detect nucleic acids, were recently shown to recognize *S. suis*, resulting in IFN-β production [14]. In this study, we demonstrated that RNA and DNA from both strains also have the capacity to induce IL-1β, and equally so for both strains. Importantly, production was only observed when DNA and RNA were complexed with DOTAP, suggesting that recognition occurs in a process similar to that of IFN-β, following internalization and degradation [14].

Following engagement of TLRs, activation of the MAPK and NF-κB signaling pathways results in the initiation of an inflammatory response resulting in cytokine production. IL-1β production by DCs induced by both strains P1/7 and SC84 was dependent on the NF-κB and ERK pathways but independent of JNK, similar to what has previously described for other cytokines induced by *S. suis* [67] and for other streptococci [68-70]. Meanwhile, IL-1β production induced by strain SC84, but not strain P1/7, was also p38-dependent, suggesting differential mechanisms, possibly due to differences in bacterial components or virulence. Indeed, pore-forming toxin secretion and its induced osmotic stress were observed to modulate MAPK phosphorylation for listeriolysin O and streptolysin O [68, 71]. As such, the higher production of SLY by strain SC84 could be involved in p38 activation, which remains to be confirmed.

Since receptors and pathways engaged by this pathogen and involved in IL-1β production could not explain the differences observed in IL-1β production between strains, confirmed by IL-1β gene induction, the steps involved in IL-1β maturation were evaluated. Though IL-1β production induced by both strains depended on caspase-1, inflammasome activation was different between strains: while maturation of proIL-1β induced by strain P1/7 was only partially dependent on NLRP3 and AIM2, strain SC84 activated NLRP3 and, to a lesser extent AIM2, NLRP1 and, surprisingly, NLRC4. These differences in inflammasome activation between strains, both in the different inflammasomes activated and their implication levels, could explain the differential IL-1β levels produced by DCs. Although NLRP3 and AIM2 participate in IL-1β release by DCs and MΦ following infection by GBS and *S. pneumoniae* [59, 66], the implication of NLRP1 and NLRC4 have not yet been described following streptococcal infection [72]. The specific factors responsible for *S. suis*-dependent inflammasome activation are difficult to determine since all four inflammasomes can be activated by a wide range of molecules. Previous studies showed that NLRP1 could directly sense the protease activity of the *Bacillus anthracis* lethal toxin [73]. Although activation of NLRP1 by streptococcal pore-forming toxins has not yet been evaluated, elevated levels of the *S. suis* SLY might be involved in a similar process. Moreover, strain SC84, unlike strain P1/7, also possesses a type IV secretion system encoded by its 89 K pathogenicity island [74], which might be responsible for NLRC4 activation [75], although this remains only an hypothesis. Regarding the AIM2 inflammasome, it has been previously shown to be activated by DNA [76]. In accordance, we observed that levels of IL-1β induced by DNA were higher than those induced by RNA.

Given the activation of a wide range of inflammasomes by strain SC84, it was hypothesized that ion fluxes might be involved since K^+ efflux is a common denominator in the assembly of these four inflammasomes [21, 52]. Indeed, bacterial pore-forming toxins can play a key role in IL-1β processing by generating K^+ efflux as previously described for pneumolysin and the β-hemolysin of GBS [18, 64]. In this study, we showed that SLY plays an important role for the SC84 strain, but not for P1/7, and that this role is associated only with IL-1β maturation. The reduction of IL-1β observed after addition of cholesterol, which is an inhibitor of SLY, further supports these results. The fact that levels of SLY produced by strain SC84 are much higher than those produced by strain P1/7 could explain these differences. Indeed, the fact that a role of SLY was observed for strain SC84, but not P1/7, when using the SLY-deficient mutant confirms that a minimal level of SLY (threshold) is required. In other words, although cell

activation by strains P1/7 and SC84 leads to similar levels of proIL-1β, the high levels of SLY produced by strain SC84 result in a more efficient maturation of IL-1β via pore formation, depletion of K^+ efflux, and a wider inflammasome activation. In accordance, the addition of rSLY to strain P1/7 or the SLY-deficient mutants synergistically increased IL-1β production by DCs. Interestingly, though rSLY itself induced some IL-1β secretion from DCs, levels were similar to those observed when cells were stimulated with Alum alone. Since Alum is a known activator of NLRP3, but not an inducer of IL-1β mRNA, it has been suggested to cause maturation and release of the IL-1β naturally synthetized by the cell in the absence of prior stimulation [48]. In fact, addition of Alum to DNA and RNA increased the production of IL-1β, probably through activation of NLRP3 inflammasome. Noteworthy, the *sly*-deficient SC84 mutant produced similar IL-1β levels as those by strain P1/7, suggesting that both strains possess mechanisms other than SLY that participate in IL-1β maturation, which should be dissected in future studies using SLY-negative *S. suis* strains. Finally, the addition of extracellular K^+ inhibited *S. suis*-induced IL-1β production. Importantly, although only 10 mM of K^+ was sufficient to reduce IL-1β production, the effect was accentuated for strain SC84, which goes along with results showing a broad inflammasome activation by this strain. For strain P1/7, the K^+ efflux generated could be due to other yet unknown bacterial mechanisms that probably shared by other classical *S. suis* strains and responsible for "normal" inflammasome activation by this pathogen. Moreover, though inhibition was concentration-dependent, addition of more than 40 mM had a cytotoxic effect on DCs, with 100% cell death when using 130 mM.

Following secretion, IL-1α and IL-1β bind their shared receptor, IL-1R, leading to cell activation, stimulation and secretion of diverse pro-inflammatory cytokines (positive feedback loop), recruitment of neutrophils and macrophages, and activation of killing mechanisms, amongst other effects [77]. Previous studies with GBS and *S. pneumoniae* showed a protective role of this cytokine during infection: absence of IL-1 signaling alters bacterial clearance and survival [18, 23-25]. In the case of *S. suis*, IL-1 signaling also plays a central and beneficial role following infection with the ST1 strain P1/7, which represents classical strains. Indeed, IL-1 signaling induced by strain P1/7 modulates the host innate immune response by increasing production of other pro-inflammatory cytokines and chemokines required for control of bacterial burden in blood and organs, which if unrestricted, causes host death (i.e. $IL\text{-}1R^{-/-}$ mice). However, and similarly to what has been described for type I IFN, the “protective effect” of IL-1 was not observed following infection with the highly virulent ST7 strain SC84 [14]. In fact, the levels of

IL-1 induced by this strain were unable to modulate overall inflammation and host outcome since levels of inflammatory mediators were exacerbated. However, the similar bacterial load in P1/7- and SC84-infected wild-type mice indicates that the difference in the role of IL-1 signaling observed is not due to excessive bacterial burden by SC84, but rather by the exacerbated inflammation due to the virulence level of the strain. These results suggest that during *S. suis* systemic infection, the levels of induced inflammation play a critical role, and this regardless of the strain. In accordance, while IL-1 signaling initiates the cascade of inflammation, generating a positive loop, and stimulating the synthesis of more mediators necessary to fight the pathogen, IL-1 signaling itself cannot counterbalance exacerbated inflammation, resulting in host death. The latter is exemplified by the highly virulent ST7 strain SC84, which, possessing additional virulence factors such as the 89 K pathogenicity island [78], is responsible for a greater innate immune system activation, resulting in a cytokine storm. In fact, this cytokine storm is associated with the streptococcal toxic shock-like syndrome caused by this strain [79]. It should be noted, however, that even in the presence of IL-1 (i.e. wild-type mice), mice still succumb to *S. suis* infection, though to a significantly lower degree and rate, demonstrating the need in a balanced and controlled inflammation. Interestingly, *S. suis*-induced IL-1 did not autoregulate itself, suggesting that levels induced in the first hours of infection are sufficient to activate the immune system. This is in accordance with results obtained during systemic infection with GBS, during which levels of IL-1β are similar between wild-type and IL-1R$^{-/-}$ mice in kidneys, peritoneal lavage, and brain [18].

In conclusion, this study demonstrates that a classical (P1/7) and highly virulent (SC84) *S. suis* strain induce IL-1 *in vivo*, but only in organs. While both strains similarly activate innate immune cells due to conserved bacterial components such as LPs, the pore-forming toxin SLY, which is highly produced by strain SC84 only, plays an important role in IL-1β maturation via activation of the NLRP1, NLRP3, AIM2, and NLRC4 inflammasomes. Based on these results, a model of the mechanisms involved in *S. suis*-induced IL-1β production by DCs is proposed (**Fig. 12**). Globally, *S. suis*-induced IL-1 plays a beneficial role during systemic infection by initiating the inflammatory cascade. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role. A better understanding of the underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

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Abbreviations

AIM2, absent in melanoma 2; CCL, C-C motif chemokine ligand; CFU, colony-forming unit; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; GAS, Group A *Streptococcus*; GBS, Group B *Streptococcus*; IFN, interferon; IL, interleukin; IL-1R interleukin-1 receptor; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LP, lipoprotein; LTA, lipoteichoic acid; MΦ, macrophage; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK 1/2; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; NLR, NOD-like receptor; NLRC, NLR family CARD domain-containing protein; NLRP, NLR family pyrin domain-containing; NOD, nucleotide oligomerization domain; PCR, polymerase chain reaction; p.i., post-infection; PRR, pattern recognition receptor; p38, p38 mitogen-activated protein kinase; rSLY, recombinant SLY; SLY, suilysin; ST, sequence type; THA, Todd Hewitt broth agar; THB, Todd Hewitt broth; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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Tables

Table. 1. Bacterial strains and plasmids used in this study

Strain/plasmid	General characteristics	Source/reference
Streptococcus suis		
P1/7	Classical virulent serotype 2 ST1 strain isolated from a pig with meningitis in the UK	[22]
P1/7Δ*sly*	Isogenic mutant strain derived from P1/7; in frame deletion of *sly* gene	This work
P1/7Δ*lgt*	Isogenic mutant strain derived from P1/7; in frame deletion of *lgt* gene	[33]
SC84	Highly virulent serotype 2 ST7 strain isolated from a human case of streptococcal toxic shock-like syndrome during the 2005 human outbreak in China	[61]
SC84Δ*sly*	Isogenic mutant strain derived from SC84; in frame deletion of *sly* gene	This work
SC84Δ*lgt*	Isogenic mutant strain derived from SC84; in frame deletion of *lgt* gene	[33]
Escherichia coli		
TOP 10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
BL21	F^-ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm rne131 (DE3)	Invitrogen
Plasmids		
pIVEX2.4d	Ap^r, pUC ori, T7 promoter, His tag-coding sequence	Roche Bioscience
pSET4Δ*sly*	pSET4s carrying the construct of *sly* gene for allelic replacement	This work
pIVEX*sly*	pET101 carrying *sly* gene for protein production	This work

Figures

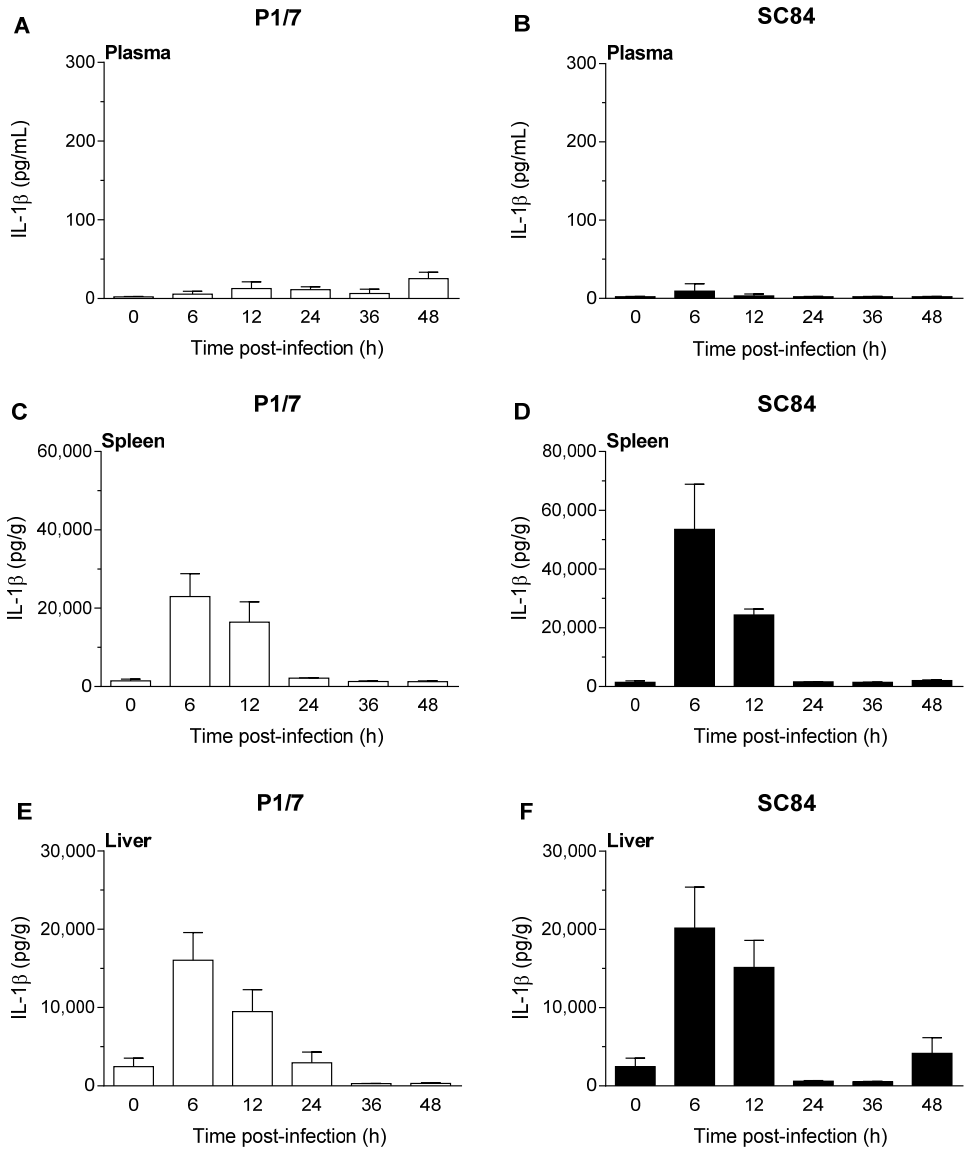

Figure 1. *Streptococcus suis* serotype 2 induces elevated levels of IL-1β in liver and spleen but not in plasma. C57BL/6 mice were intraperitoneally inoculated with the *S. suis* strain P1/7 (white bars) or SC84 (black bars). Plasma (**A** & **B**), spleen (**C** & **D**), and liver (**E** & **F**) were collected at different times post-infection and levels of IL-1β were quantified by ELISA. Values for mock-infected controls did not change between 6 h and 48 h. As such, 0 h represents results for mock-infected mice throughout the experiment. Data are expressed as mean ± SEM of at least 3 independent experiments

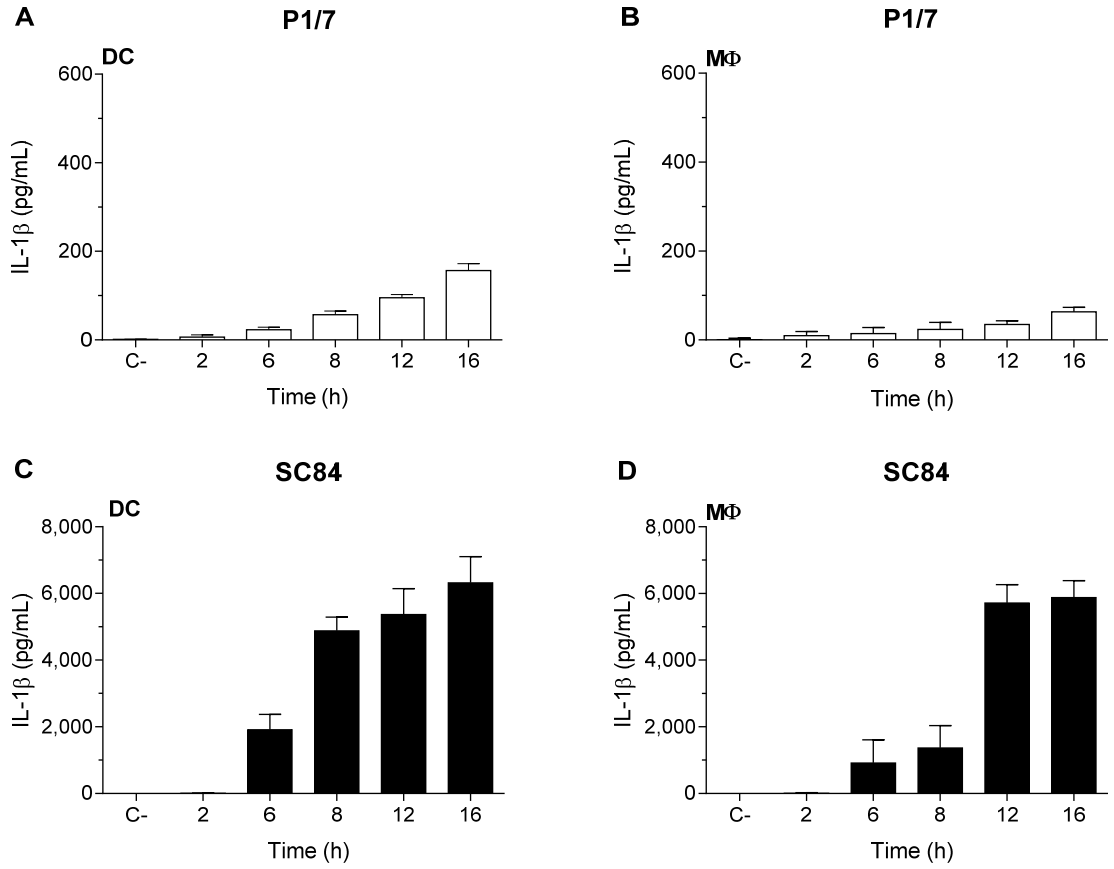

Figure 2. IL-1β released from dendritic cells (DCs) and macrophages (MΦ) stimulated with *Streptococcus suis* is strain-dependent. IL-1β kinetics as measured by ELISA following infection of DCs (**A** & **C**) or MΦ (**B** & **D**) with strain P1/7 (white bars) or SC84 (black bars). Non-stimulated cells served as negative control (C-). Data represent the mean ± SEM of at least 3 independent experiments.

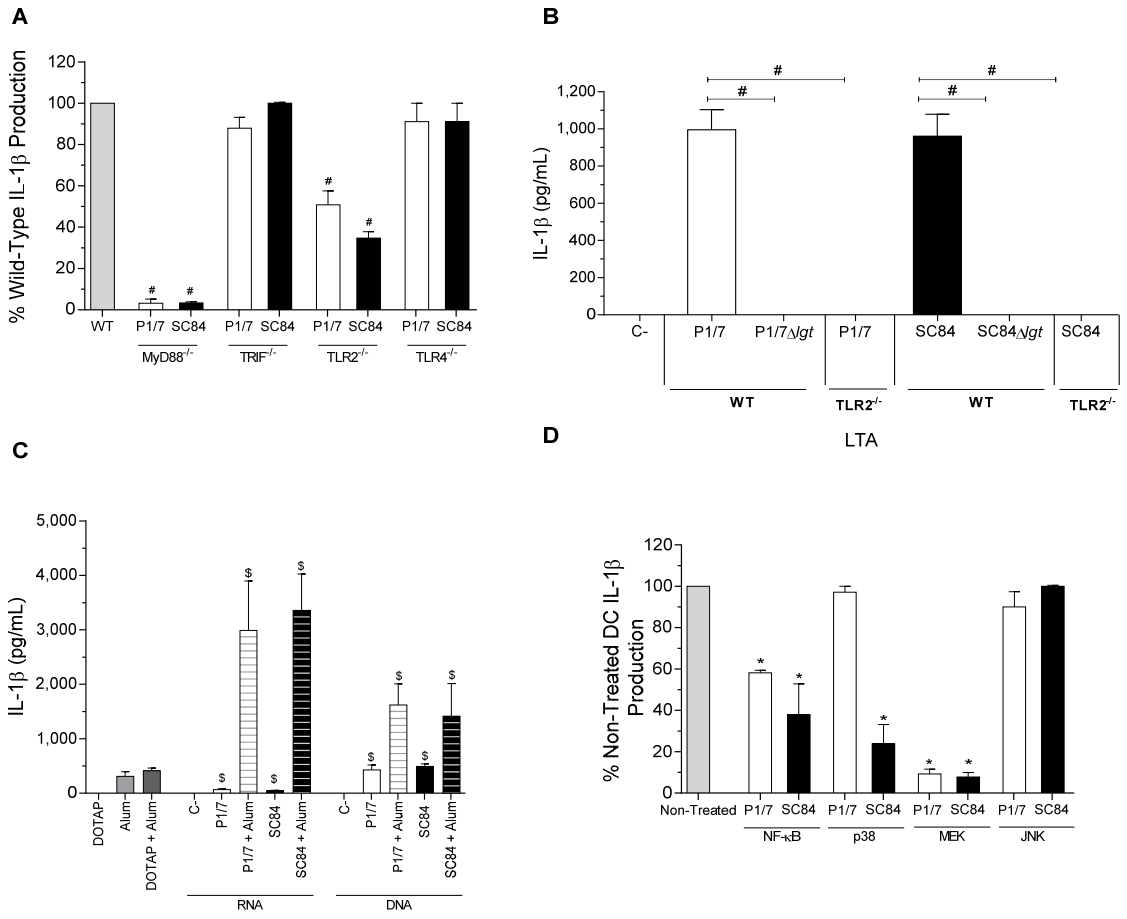

Figure 3. Role of Toll-like receptors (TLRs) and associated signaling pathways in *Streptococcus suis*-induced IL-1β production by dendritic cells (DCs). (**A**) Percentage of IL-1β production induced by *S. suis* strain P1/7 (white bars) or SC84 (black bars) 16 h following infection of DCs deficient for MyD88, TRIF, TLR2 or TLR4, with regards to wild-type (WT; normalized to 100%) counterparts (gray bar); (**B**) IL-1β production following activation of wild-type (WT) or TLR2-/- DCs with 30 µg/mL of LTA extracts from strains P1/7 or SC84 or their *lgt*-deficient mutants (Δ*lgt*); (**C**) IL-1β production by DCs following phagosomal delivery of 1 ug of *S. suis* RNA or DNA in the presence or absence of Alum; (**D**) Percentage of IL-1β production from DCs following pre-treatment with either NF-kB inhibitor (i), p38i, MEKi or JNKi and infection with *S. suis*, with regards to non-treated DCs. Data represent the mean ± SEM of at least 3 independent experiments. # ($p < 0.01$) indicates a significant difference with wild-type DCs; $ ($p < 0.01$) indicates a significant difference with negative control (elution buffer); * ($p < 0.05$) indicates a significant difference with non-treated DCs

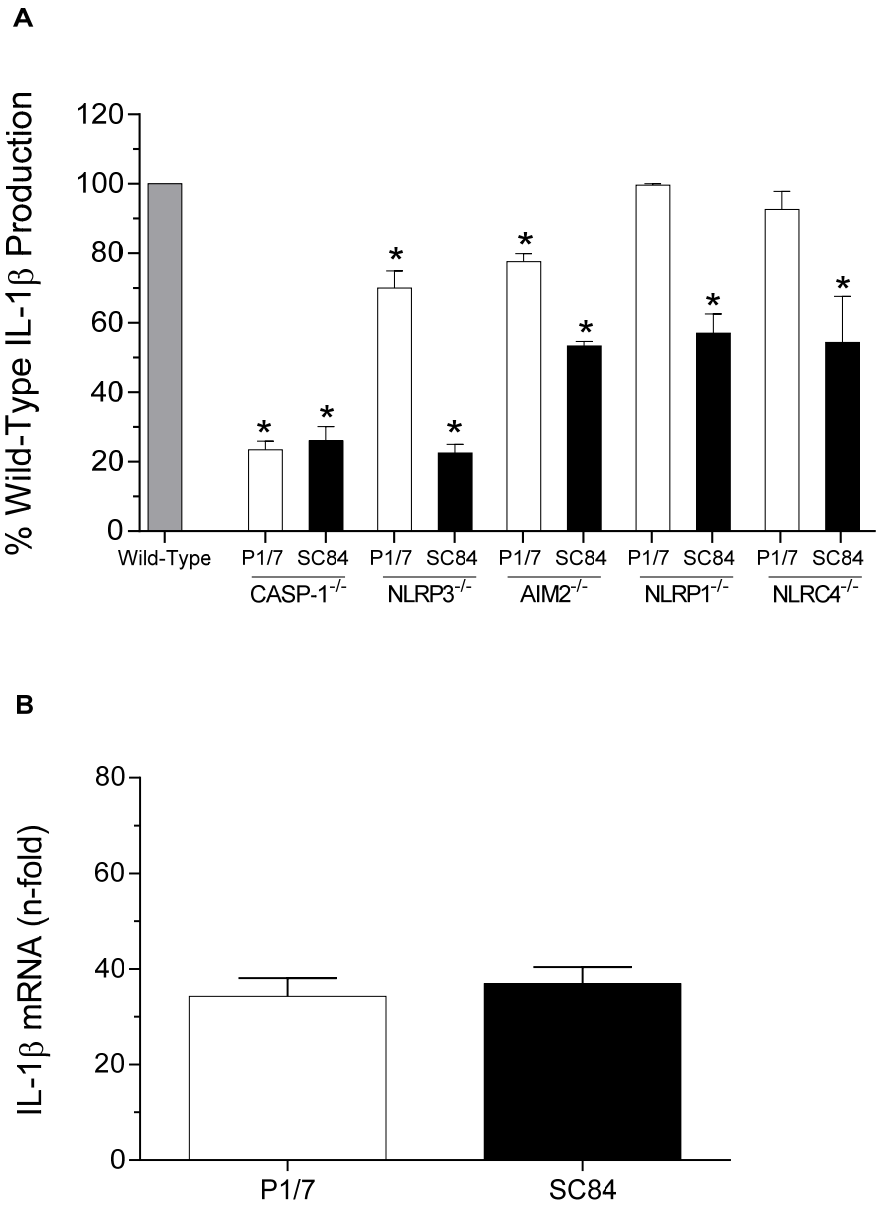

Figure 4. Inflammasome implication in *Streptococcus suis*-induced IL-1β dendritic cell (DC) production is strain-dependent. Percentage of IL-1β secretion by caspase-1 (CASP-1), NLRP3, AIM2, NLRP1 or NLRC4-deficient DCs induced by P1/7 (white bars) or SC84 (black bars) after 16 h of incubation, in comparison to wild-type counterparts; (**B**) DCs were infected with P1/7 and SC84 strains for 6 h and IL-1β mRNA expression was measured by RT-qPCR. Data are presented as 'fold' increase in mRNA expression relative to non-infected cells. Data represent the mean ± SEM of at least 3 independent experiments. * ($p < 0.05$) indicates a significantly difference with wild-type DCs

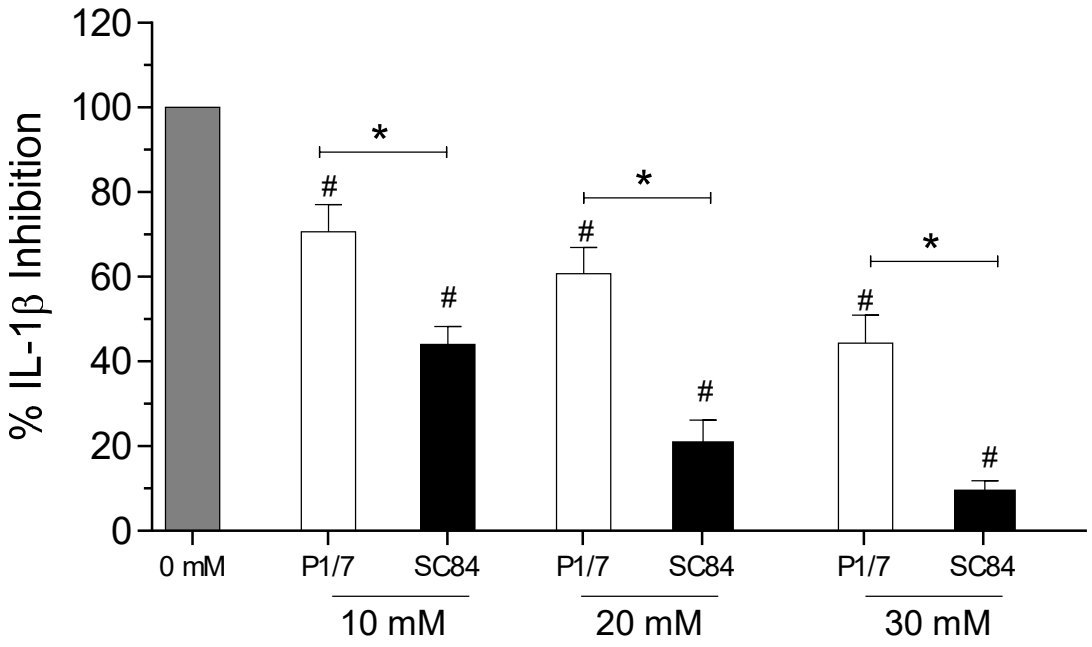

Figure 5. IL-1β secretion by dendritic cells (DCs) activated by *S. suis* is blocked by additional extracellular potassium (K^+) levels. DCs were infected with either strain P1/7 or SC84 in the presence of different concentrations of KCl and IL-1β production was measured after 16 h by ELISA. Data represent the mean ± SEM of at least 3 independent experiments. # ($p < 0.05$) indicates a significant difference with non-treated DCs and * ($p < 0.05$) indicates a significant difference between P1/7 and SC84.

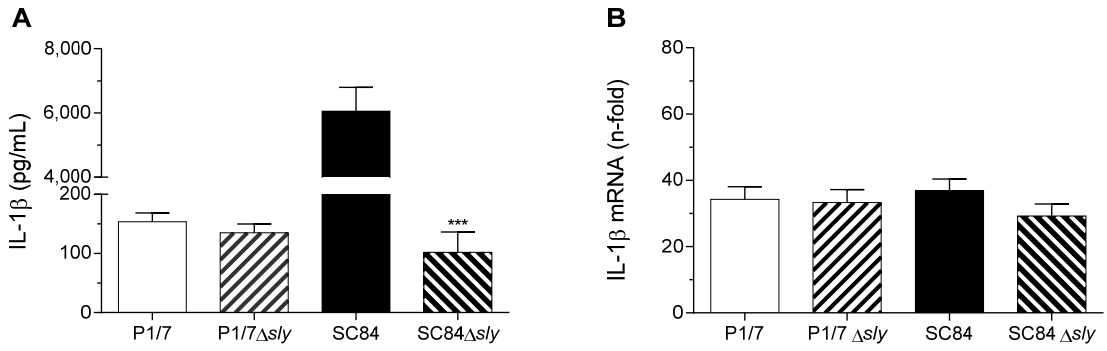

Figure 6. Suilysin (SLY) is involved in the maturation of *Streptococcus suis*-induced IL-1β by dendritic cells (DCs). (**A**) DCs were infected with the *S. suis* wild-type strains P1/7 and SC84 or their SLY-deficient mutants (Δ*sly*) for 16 h and IL-1β release was measured by ELISA; (**B**) DCs were infected with the different wild-type and mutant strains for 6 h and IL-1β mRNA expression was measured by RT-qPCR. Data are presented as 'fold' increase in mRNA expression relative to non-infected cells. Data represent the mean ± SEM of at least 3 independent experiments. *** ($p < 0.001$) indicates a significant difference between SC84 and SC84Δ*sly*.

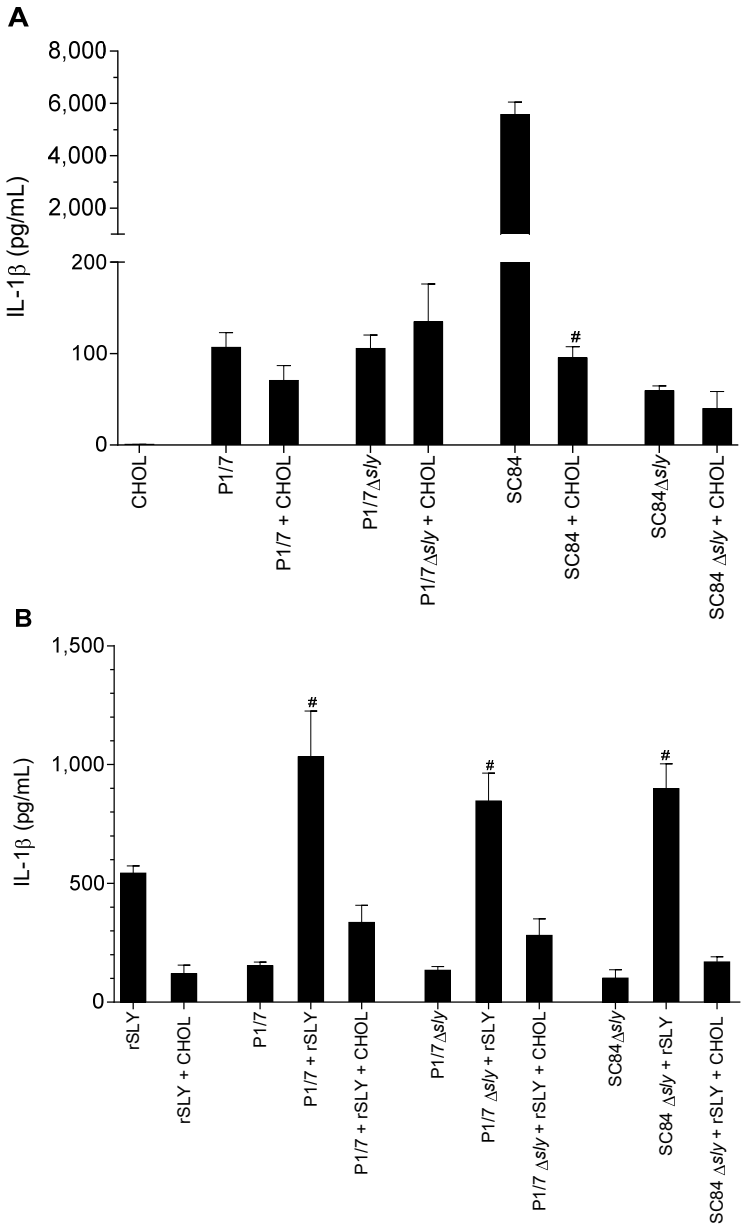

Figure 7. Co-stimulation with recombinant suilysin (rSLY) enhances *Streptococcus suis*-induced IL-1β production by dendritic cells (DCs), which is inhibited by cholesterol (CHOL). (**A**) DCs were stimulated with the different strains of *S. suis* in the presence or absence of CHOL for 16 h and IL-1β production was measured by ELISA; (**B**) DCs were infected with the different strains of *S. suis* alone, in combination with 5 µg/mL of rSLY or with 5 µg/mL of rSLY and CHOL. Data represent the mean ± SEM of at least 3 independent experiments. # ($p < 0.01$) indicates a significant difference with bacteria alone.

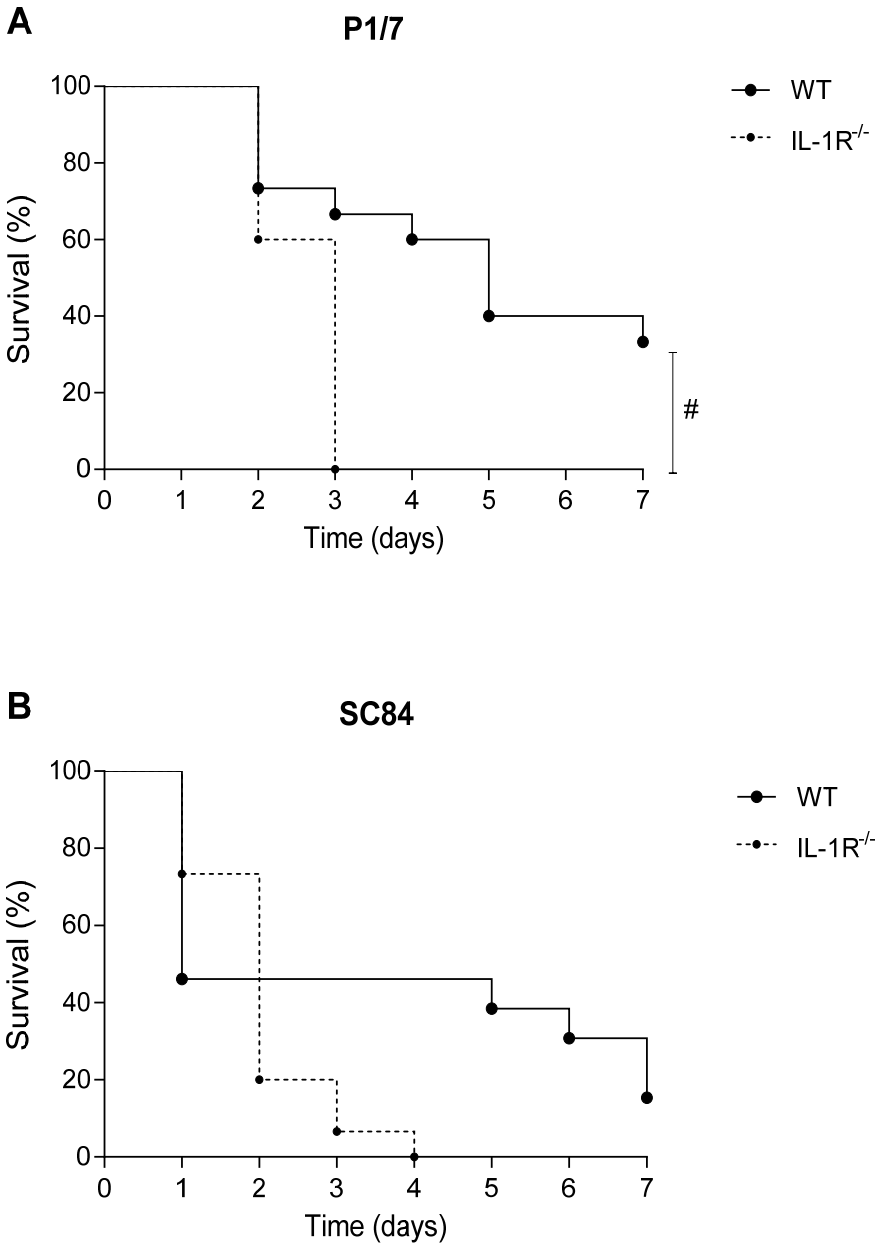

Figure 8. Survival of wild-type (WT) and IL-1 receptor-deficient (IL-1R$^{-/-}$) mice following *Streptococcus suis* systemic infection. WT and IL-1R$^{-/-}$ mice were inoculated with strain P1/7 (**A**) or SC84 (**B**) and survival was monitored. Data represent survival curves (n=15). # ($p < 0.01$) indicates a significant difference between survival of WT and IL-1R$^{-/-}$ mice.

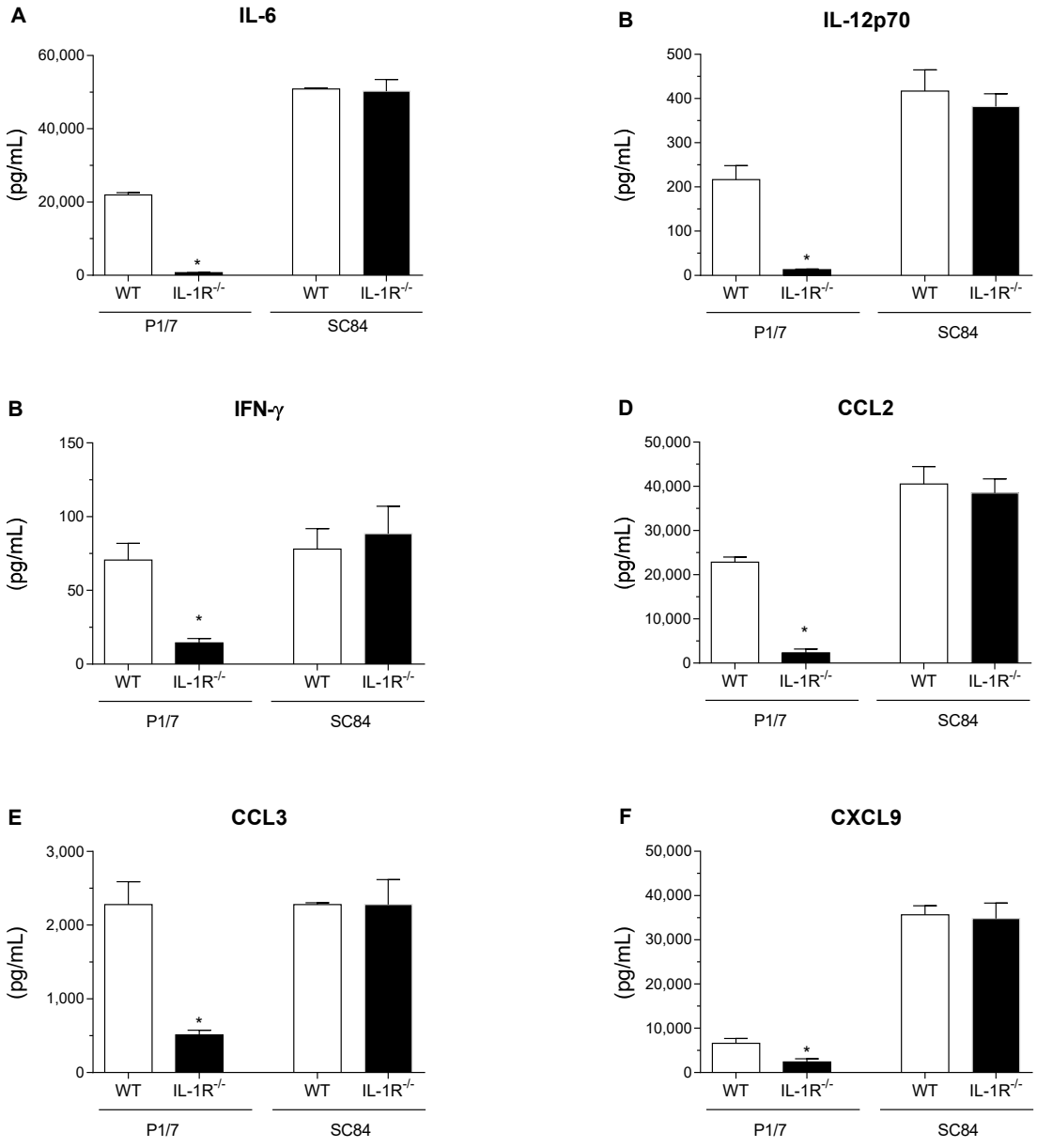

Figure 9. Plasma pro-inflammatory mediator production during *Streptococcus suis* systemic infection. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R-/- mice 12 h following infection with strain P1/7 or SC84. Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

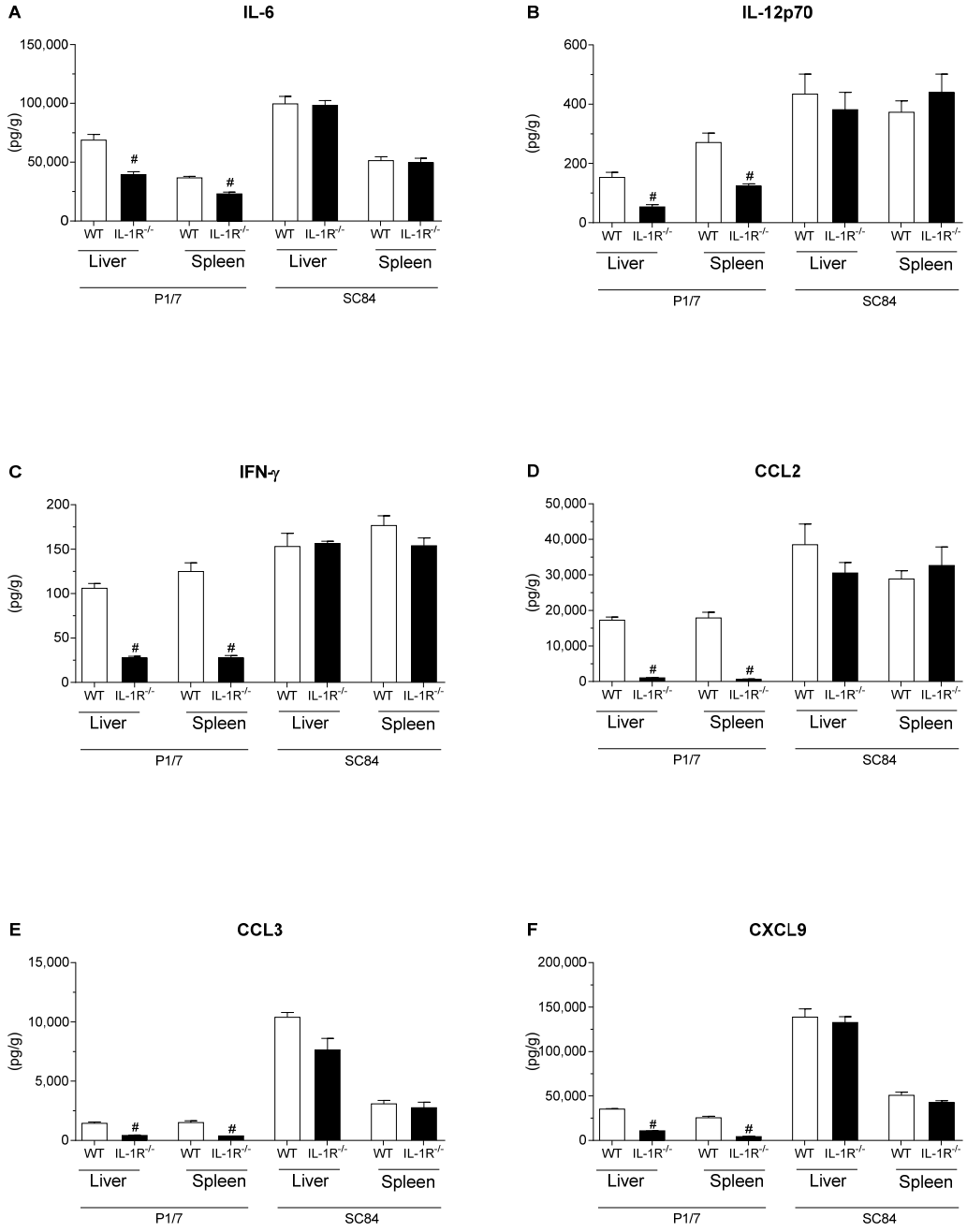

Figure 10. Pro-inflammatory mediator production in spleen and liver during *Streptococcus suis* systemic infection. Spleen and liver levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following infection with strain P1/7 or SC84 strain. Data represent the mean ± SEM of at least four individuals. # ($p < 0.01$) indicates a significant difference between WT and IL-1R-/- mice.

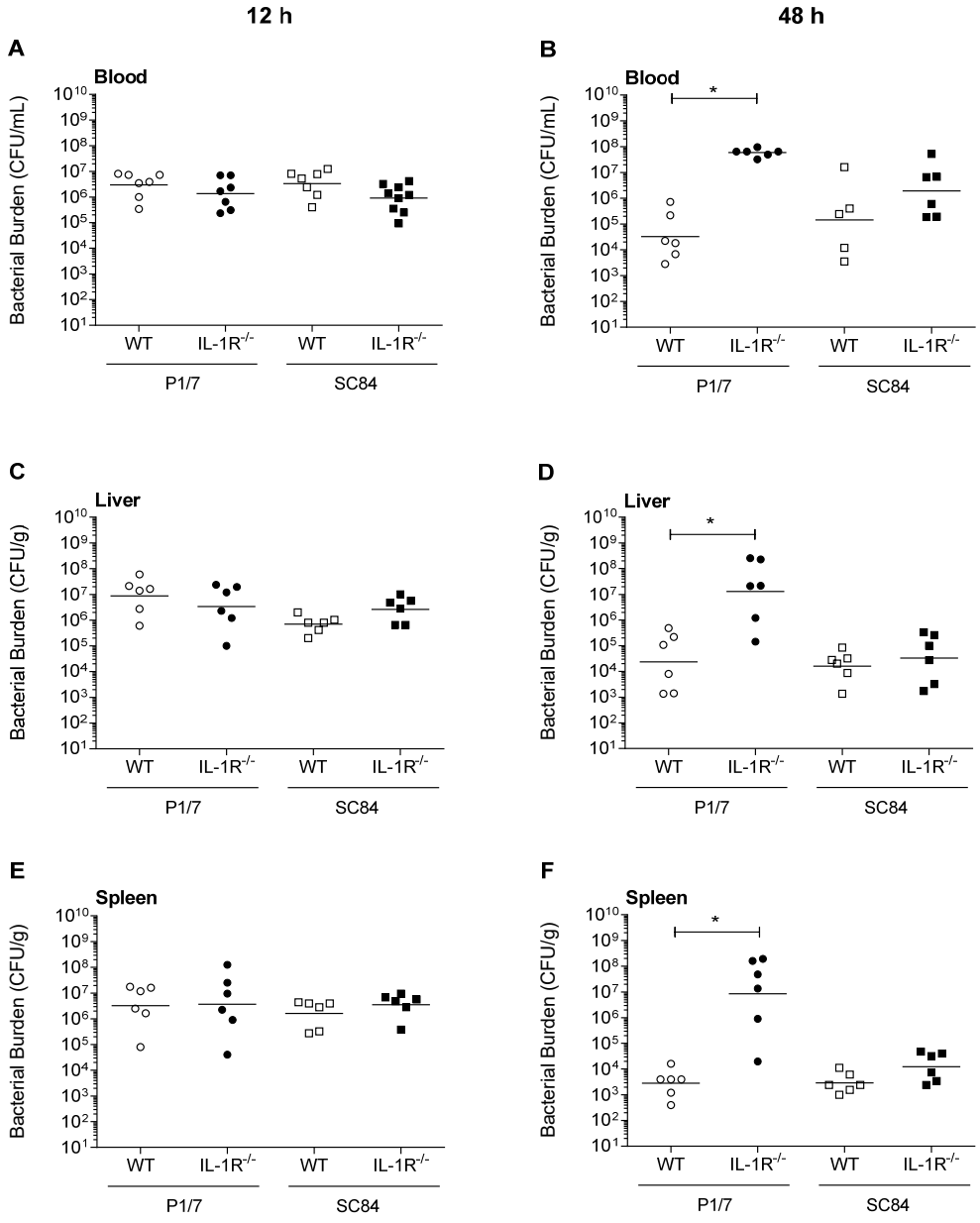

Figure 11. IL-1 signaling is required for control of bacterial burden in blood, liver, and spleen. Bacterial burden in blood (**A** & **B**), liver **(C** & **D),** and spleen (**E** & **F**) of wild-type (WT) and IL-1R$^{-/-}$ mice infected with strain P1/7 or SC84 12 h (left panel) or 48 h (right panel) post-infection. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. Data represent the geometric mean of at least six individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

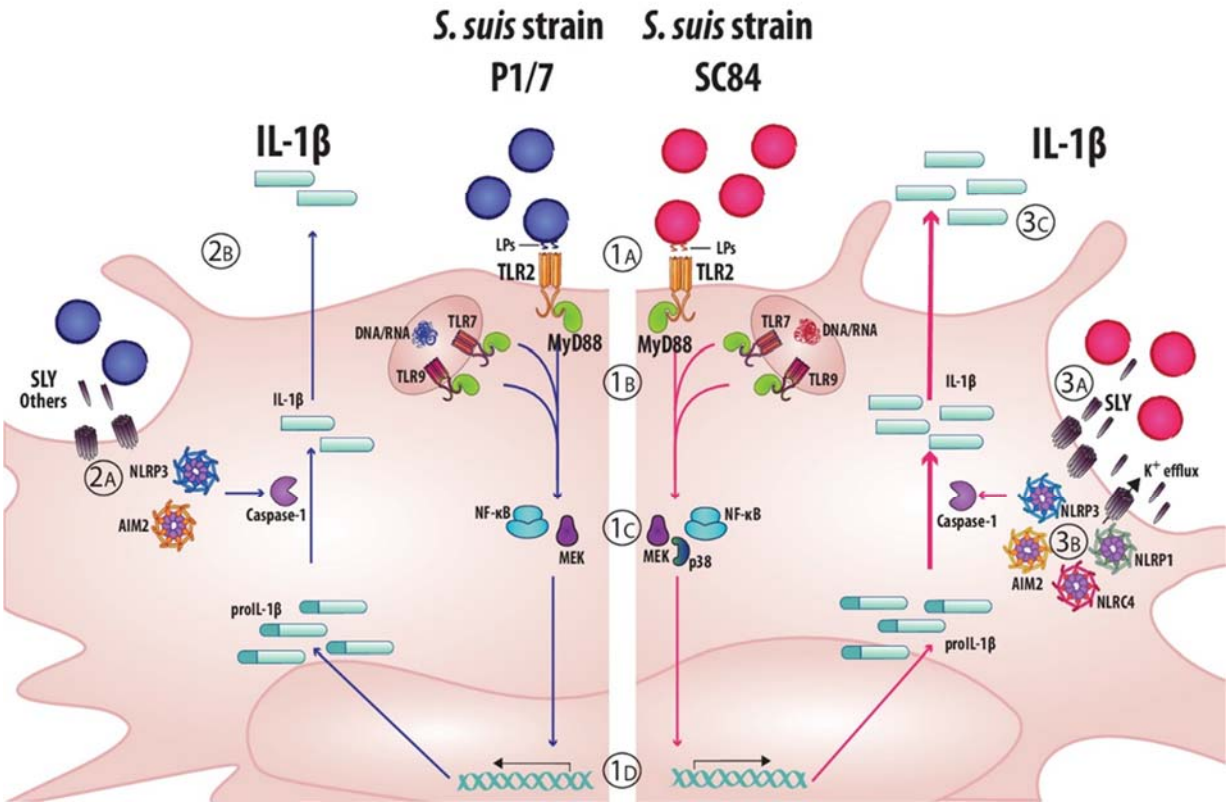

Figure 12. Model of the mechanisms involved in *Streptococcus suis*-induced IL-1β production by dendritic cells (DCs). 1A: Strain-independent recognition of *S. suis* by DCs requires MyD88-dependent signaling and partially involves TLR2 activation via recognition of surface lipoproteins (LPs); **1B:** If internalized, *S. suis* DNA and RNA can induce the production of IL-1β, possibly via recognition by endosomal receptors TLR7 and TLR9; **1C:** Recognition of *S. suis* leads to activation of the NF-κB and MEK pathways for both strains, alongside p38 for SC84; **1D:** Strains P1/7 and SC84 induce comparable transcription of IL-1β mRNA; **2A:** For strain P1/7, low levels of suilysin (SLY) and other not yet identified bacterial components lead to partial NLRP3 and AIM2 inflammasome activation; **2B:** Caspase-1 cleavage leads to maturation of moderate levels of IL-1β that are then secreted; **3A & 3B:** For strain SC84, secretion of high levels of SLY induces an important K^+ efflux that results in a activation of multiple inflammasomes, including NLRP3, NLRP1, AIM2, and NLRC4; however, other bacterial components could also influence this activation. **3C:** Increased caspase-1 cleavage leads to a more efficient maturation of the proIL-1β, resulting in the secretion of high levels of IL-1β.