Université de Montréal

RÔLE DES CELLULES DENDRITIQUES DANS LA MODULATION DE LA RÉPONSE IMMUNITAIRE DE L'HÔTE CONTRE *STREPTOCOCCUS SUIS*

par

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Résumé

Streptococcus suis est un important pathogène porcin et agent zoonotique responsable de méningites et de septicémies. À ce jour, les mécanismes impliqués dans la réponse immunitaire de l'hôte lors de l'infection par *S. suis* sont peu connus; et il en est de même pour les stratégies utilisées par *S. suis* afin de déjouer cette réponse. L'augmentation de l'incidence et de la sévérité des cas humains souligne le besoin d'une meilleure compréhension des interactions entre *S. suis* et le système immunitaire afin de générer une réponse immunitaire efficace contre ce pathogène. Les cellules dendritiques (DCs) sont de puissantes cellules présentatrices d'antigènes qui stimulent les lymphocytes T et B, assurant la liaison entre l'immunité innée et l'immunité adaptative.

L'objectif principal de ce projet était d'évaluer le rôle joué par différents facteurs de virulence de *S. suis* sur la modulation de la fonction des DCs et de la réponse T-dépendante.

Nous avons examiné l'effet des facteurs clés pour la virulence de *S. suis*, dont la capsule polysaccharidique (CPS), les modifications de la paroi cellulaire (D-alanylation de l'acide lipotéichoïque et N-déacétylation du peptidoglycane) et la toxine suilysine, sur l'activation et la maturation de DCs murines dérivées de la moelle osseuse (bmDCs). Suite à l'infection par *S. suis*, les bmDCs sont activées et subissent un processus de maturation caractérisé par l'augmentation de l'expression de molécules de co-stimulation et la production de cytokines pro-inflammatoires. La CPS est le principal facteur interférant avec la production de cytokines, même si les modifications de la paroi cellulaire et la suilysine peuvent également moduler la production de certaines cytokines. Enfin, la CPS, les modifications de la paroi cellulaire et la suilysine interfèrent avec la déposition du complément à la surface des bactéries et, en conséquence, avec le « killing » dépendant du complément. Les résultats ont été confirmés à l'aide de bmDCs porcines.

Nous avons aussi voulu identifier les récepteurs cellulaires impliqués dans la reconnaissance de *S. suis* par les DCs. Nous avons démontré que la production de cytokines et l'expression des molécules de co-stimulation par les DCs sont fortement dépendantes de la signalisation par MyD88, suggérant que les DCs reconnaissent *S. suis* et deviennent activées

majoritairement via la signalisation par les récepteurs de type Toll (TLRs). En effet, on remarque une diminution de la production de plusieurs cytokines ainsi que de l'expression de certaines molécules de co-stimulation chez les DCs $TLR2^{-/-}$ ou $TLR2^{-/-}$ et $TLR9^{-/-}$ double négatives. Finalement, le récepteur NOD2 semblait jouer un rôle partiel dans l'activation des DCs suite à une infection par *S. suis*.

Enfin, nous avons évalué les conséquences de la modulation des fonctions des DCs sur le développement de la réponse T-dépendante. Les splénocytes totaux produisent plusieurs cytokines en réponse à *S. suis*. Des analyses *in vivo* et *ex vivo* ont permis d'observer l'implication des cellules T $CD4^+$ et le développement d'une réponse de type « T helper » 1 (T_H1) bien que la quantité de cytokines T_H1 produites lors de l'infection *in vivo* par *S. suis* demeure assez basse. La CPS de *S. suis* interfère avec la production de plusieurs cytokines par les cellules T *in vitro*. Expérimentalement, l'infection induite par *S. suis* résulte en de faibles niveaux de production d'anticorps anti-*S. suis*, mais aussi d'anticorps dirigés contre l'ovalbumine utilisée comme antigène rapporteur. Cette interférence est corrélée avec la sévérité des signes cliniques, suggérant que *S. suis* interfère avec le développement d'une réponse immunitaire adaptative appropriée qui serait requise pour contrôler la progression de l'infection. Les résultats de cette étude mèneront à une meilleure compréhension de la réponse immunitaire de l'hôte lors de l'infection par *S. suis*.

Mots-clés: *Streptococcus suis*, cellules dendritiques, cellules T, cytokines, molécules de co-stimulation, phagocytose, récepteurs cellulaires, capsule polysaccharidique, paroi cellulaire, réponse immunitaire

Summary

Streptococcus suis is an important swine pathogen and an emerging zoonotic agent of septicemia and meningitis. Knowledge of host immune responses towards *S. suis,* and strategies used by this pathogen for subversion of these responses is scarce. Increased severity of *S. suis* infections in humans underscores the critical need to better understand the interactions between *S. suis* and the immune system to generate an effective immune response against this pathogen. Dendritic cells (DCs) are powerful antigen-presenting cells. Once activated, they stimulate T cells and B cells, linking innate and adaptive immunity. Thus, the main objective of this project was to evaluate the role of different *S. suis* virulence factors on the modulation of DC functions and the T cell-dependent response.

Initially, we investigated the effect of *S. suis* key virulence factors, including the capsular polysaccharide (CPS), the cell wall modifications (D-alanylation of the lipoteichoic acid and N-deacetylation of the peptidoglycan) and the toxin suilysin, on the activation and maturation of mouse bone-marrow derived DCs (bmDCs). We observed that following *S. suis* infection, bmDCs are activated and go through a complex maturation process characterized by the up-regulation of the surface expression of costimulatory molecules and the production of pro-inflammatory cytokines. The CPS is the main virulence factor interfering with cytokine production, even if cell wall modifications and suilysin can also modulate the production of cytokines. Finally, CPS, cell wall modifications and suilysin were shown to interfere with complement deposition on *S. suis*, and consequently with complement-dependent killing. Results were confirmed using porcine bmDCs.

We also aimed to identify the cellular receptors involved in *S. suis* recognition by DCs. Production of cytokines and expression of co-stimulatory molecules by DCs were shown to strongly rely on MyD88-dependent signaling pathways, suggesting that DCs recognize *S. suis* and become activated mostly through Toll-like receptor (TLR) signaling. Supporting this fact, TLR2$^{-/-}$ or double negative TLR2$^{-/-}$ and TLR9$^{-/-}$ DCs were severely impaired in the release of several cytokines and the surface expression of certain costimulatory molecules. In addition, NOD2 receptor also seems to play a partial role in DC activation by *S. suis*.

Finally, we evaluated the consequences of the modulation of DC functions on T cell activation. In response to *S. suis* infection, total splenocytes readily produced several cytokines *ex vivo*. *Ex vivo* and *in vivo* analysis revealed the involvement of $CD4^{+}$ T cells and development of a T helper 1 (T_H1) response. Nevertheless, levels of T_H1-derived cytokines during *S. suis* infection were very low. The bacterial CPS was shown to interfere with the release of several T cell-derived cytokines *in vitro*. As a consequence, a clinical infection resulted in low levels of not only anti-*S. suis* antibodies but also of those directed against ovalbumin, used as reported antigen. This interference was correlated with the presence of severe clinical signs of *S. suis* disease. These data suggest that *S. suis* impairs the development of an efficient adaptive immune response, which is required to control the infection progress. Overall, these results will permit a better comprehension of the host immune response during *S. suis* infection.

Mots-clés: *Streptococcus suis*, dendritic cells, T cells, cytokines, co-stimulatory molecules, phagocytosis, cellular receptors, capsular polysaccharide, cell wall, immune response

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LISTE DES ABBRÉVIATIONS

ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
BBB	Barrière hémato-encéphalique
BmDC	Cellule dendritique dérivée de la moelle osseuse
BMEC	Cellules endothéliales microvasculaires cérébrales
CMH	Complexe majeur d'histocompatibilité
CPEC	Cellules épithéliales porcines du plexus choroïde
CpG	Cytosine-phosphate-Guanosine
CPS	Capsule polysaccharidique
DAMPs	Motifs moléculaires associés à des signaux de danger
DCs	Cellules dendritiques
FACS	Cytométrie en flux
GAS	Streptocoque du groupe A
GBS	Streptocoque du groupe B
IFN	Interféron
IL	Interleukine
KO	Knock-out
LPS	Lipopolysaccharide
LTA	Acide lipotéichoïque
MAPK	« Mitogen-activated protein kinase »
NLR	Récepteur de type NOD
NOD	« Nucleotide-binding oligomerization domain »
PAMPs	Motifs moléculaires associés aux pathogènes
PBMEC	Cellules endothéliales porcines de microvaisseaux cérébraux
PG	Peptidoglycane
PRR	Récepteur reconnaissant des motifs moléculaires particuliers des pathogènes
Siglecs	« Sialic acid-recognizing Ig superfamily lectins »
SNC	Système nerveux central
ST	Séquence-type

TLR	Récepteur de type Toll
TNF	Facteur de nécrose tumorale
WT	Type sauvage

À ma famille et à mes proches
qui m'ont supportée et encouragée

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I. INTRODUCTION

Streptococcus suis est un important pathogène porcin, mais aussi un agent de zoonose. Chez le porc, *S. suis* est principalement à l'origine de méningites, mais d'autres pathologies ont aussi été décrites telles que la septicémie, l'endocardite, la pneumonie et l'arthrite. Chez l'humain, *S. suis* est responsable de méningites, de septicémies et de chocs septiques. Jusqu'à récemment, les infections humaines causées par *S. suis* étaient considérées comme rares et affectant principalement les personnes en contact avec le porc ou des produits dérivés du porc. Cependant, *S. suis* est maintenant considéré comme un agent de zoonose émergeant, particulièrement en Asie. En effet, *S. suis* a été identifié comme le principal pathogène responsable de méningites chez les adultes au Vietnam, le second en Thaïlande et le troisième à Hong Kong. De plus, en 2005, une importante épidémie en Chine a résulté en plus de 200 cas humains avec un taux de mortalité de 20%. Les patients ont présenté des symptômes associés au syndrome de type choc toxique streptococcal tel que la fièvre, des malaises, des nausées et vomissements, des hémorragies sous-cutanées et le coma dans les cas les plus sévères. Les facteurs de virulence de la bactérie et la pathogenèse de l'infection sont encore peu connus et il n'existe aucun vaccin permettant de contrôler l'infection. Il est donc crucial d'étudier les interactions entre *S. suis* et le système immunitaire de l'hôte afin de mieux comprendre comment générer une réponse protectrice efficace contre ce pathogène en émergence.

À ce jour, plusieurs facteurs de virulence sont proposés pour *S. suis*. La capsule polysaccharidique (CPS) est considérée comme un facteur de virulence critique puisqu'elle protège les bactéries contre la phagocytose. Plusieurs protéines et enzymes produites par *S. suis* ont été identifiées. Parmi celles-ci, une hémolysine (suilysine) a été caractérisée. La suilysine est toxique pour plusieurs types cellulaires, tel que les neutrophiles, les monocytes, les cellules endothéliales et les cellules épithéliales. Des résultats récents ont aussi démontré que des modifications de la paroi cellulaire telle que la D-alanylation de l'acide lipotéichoïque (LTA) et la N-déacétylation du peptidoglycane (PG) contribuent à la virulence de *S. suis*. En effet, des mutants isogéniques déficients pour ces modifications de la paroi étaient atténués dans des modèles murin et porcin d'infection.

Les cellules dendritiques (DCs) sont des cellules présentatrices d'antigènes professionnelles qui initient la réponse immune contre les pathogènes. Elles capturent et apprêtent les antigènes, pour ensuite subir un processus de maturation complexe caractérisé par la production de cytokines et l'expression en surface de molécules de co-stimulation. Ensuite, les DCs migrent au niveau des organes lymphoïdes adjacents où elles vont activer les cellules T. Ainsi, les DCs sont des joueurs clés dans l'orchestration de la réponse immunitaire. Elles détectent les motifs moléculaires associés aux pathogènes (PAMPs) grâce à leurs récepteurs et initient et contrôlent les réponses immunitaires innée et adaptative. Les DCs représentent donc une cible de choix pour les pathogènes voulant éviter la reconnaissance par le système immunitaire et ainsi causer la maladie.

Malgré leur importance, les interactions entre *S. suis* et les DCs ainsi que les conséquences de ces interactions sur le développement de la réponse T-dépendante sont inconnues. Puisqu'à ce jour aucun vaccin ne permet une protection contre l'infection par *S. suis*, nous croyons que *S. suis* possèdent des facteurs de virulence lui permettant de moduler la réponse immunitaire de l'hôte. Plus précisément, nous proposons que *S. suis* possède des facteurs de virulence lui permettant de moduler les fonctions des DCs et, par conséquent, la réponse T-dépendante. L'objectif général du projet est donc d'étudier la modulation de la fonction des DCs et de la réponse T-dépendante par des facteurs de virulence de *S. suis*. Les objectifs spécifiques de cette recherche sont :

1. Étudier le rôle joué par certains facteurs de virulence clés de *S. suis* sur les interactions avec les DCs murines dérivées de la moelle osseuse (bmDCs).
2. Étudier les récepteurs cellulaires impliqués dans la reconnaissance de *S. suis* par les bmDCs murines.
3. Confirmer le rôle joué par les facteurs de virulence de *S. suis* sur les interactions avec les bmDCs porcines, hôte naturel de *S. suis*.
4. Évaluer la capacité des DCs activées par *S. suis* à activer les cellules T.

II. RECENSION DE LA LITTÉRATURE

1. *Streptococcus suis*

1. 1 Aspects généraux de *S. suis*

S. suis est considéré comme l'un des plus importants pathogènes porcins et est responsable de pertes économiques considérables au sein de l'industrie porcine. Les pathologies les plus fréquentes incluent la méningite, la septicémie, l'arthrite, la pneumonie et l'endocardite [1]. *S. suis* est aussi un agent de zoonose à l'origine de méningites, de septicémies et de chocs septiques chez l'homme [2,3]. *S. suis* se présente sous forme de coques Gram-positifs encapsulés possédant des déterminants antigéniques reliés au groupe D de Lancefield, bien qu'il ne soit pas génétiquement relié aux autres membres de ce groupe [1,4].

S. suis apparaît généralement seul, en paires et rarement en courtes chaînes. *S. suis* est une bactérie anaérobie facultative qui forme de petites colonies grises. Bien que certaines souches soient β-hémolytiques et produisent une hémolysine sur agar contenant du sang de cheval, toutes les souches de cette espèce sont α-hémolytiques sur agar contenant du sang de mouton [5]. *S. suis* est caractérisé par une absence de croissance en présence de 6.5% de NaCl, un résultat négatif au test de Voges-Proskauer, une production d'acide en bouillon contenant du tréhalose et de la saliciline ainsi que par la production d'amylase [6,7]. Le test NaCl (6.5%) différencie clairement *S. suis* et *Streptococcus bovis* des *Enterococcus*. Le test Voges-Proskauer est critique et demeure le test le plus fiable afin de différencier *S. suis* de *S. bovis*, puisque ce dernier produit de l'acétoïne à partir de la fermentation du glucose alors que ce n'est pas le cas pour *S. suis* [7].

Au début des années 1950, Jansen et van Dorssen ont décrit pour la première fois des cas de méningo-encéphalites chez des porcelets et des porcs adultes à partir desquels des streptocoques α-hémolytiques ont été isolés [8]. Ces streptocoques ont été classés par de Moor en quatre nouveaux groupes de Lancefield : R, S, RS et T [9]. Cependant, en 1966, Elliot démontra que cette classification était erronée et que *S. suis* faisait plutôt partie du groupe D de Lancefield. L'erreur de classification était due au fait que le LTA, l'antigène

de la paroi cellulaire de la bactérie servant à classer les streptocoques parmi le groupe D, était masqué par les antigènes de la CPS dont de Moor s'était servi [10]. *S. suis* a été officiellement reconnu en 1987 alors que Kilpper-Balz et Schleifer ont fait la démonstration que *S. suis* est génétiquement homogène et diffère suffisamment des autres membres du groupe D de Lancefield pour constituer une nouvelle espèce [11].

À ce jour, la classification de *S. suis* est basée sur les antigènes capsulaires et 35 sérotypes ont été décrits, soit les sérotypes 1, 1/2 et 2-34 [1]. Cependant, des résultats récents suggèrent que les sérotypes 32 et 34 devraient être exclus de l'espèce *S. suis* et désignés comme *Streptococcus orisratti* [12]. De plus, l'analyse de la séquence de l'acide ribonucléique (ARN) ribosomal 16S de 35 souches de références de *S. suis* a démontré que ces deux sérotypes ainsi que le sérotype 33 pourraient appartenir à une espèce différente [13]. Certaines des souches de références étudiées proviennent de porcs malades alors que d'autres ont été isolées de cavités nasales de porcs cliniquement sains [1]. Les souches de références des sérotypes 20 et 31 ont été retrouvés chez des veaux malades, alors que la souche de référence du sérotype 33 a été isolée d'un agneau et la souche de référence du sérotype 14 d'un cas humain de méningite [7,14,15]. La plupart des souches de *S. suis* isolées chez des porcs malades appartiennent à un nombre limité de sérotypes, notamment les sérotypes 1 à 9 [16-19]. Le sérotype 2 est considéré parmi les plus virulents et il est aussi le plus étudié. Bien que les isolats de sérotype 2 prédominent dans la plupart des pays, la distribution peut différer selon la location géographique. En effet, à la différence de ce qui est observé en Europe et en Asie, la prévalence des souches de sérotype 2 isolées d'animaux malades demeure relativement basse en Amérique du Nord (environ 20%) [20-22].

Le nombre d'isolats non-typables est généralement relativement faible. Ces isolats sont le plus souvent récupérés à partir de cas sporadiques d'infection [23,24] et il semble actuellement qu'il n'y ait pas de justification pour la caractérisation de nouveaux types capsulaires [18]. Certains sérotypes montrent des réactions croisées, indiquant que certains déterminants antigéniques sont partagés. Ceci est le cas pour le sérotype 1/2 qui réagit avec les antisérums de sérotypes 1 et 2 [25].

Le séquençage de type multilocus a démontré que les souches de sérotype 2 de *S. suis* peuvent être divisées en au moins 16 séquences-type (ST). Les ST étroitement reliés sont regroupés en complexes ST. Bien que les complexes ST1, ST27 et ST87 dominent, la majorité des isolats invasifs appartiennent au complexe ST1 [26]. Par exemple, la plupart des souches isolées de patients humains au Japon sont ST1 [27], alors que les souches responsables d'épidémies en Chine (voir section1.2.2.1) sont ST7, elles-mêmes incluses dans le complexe ST1 [28,29]. Cependant, Takamatsu *et al.* ont démontré que 80% des isolats provenant du sang ou du fluide cérébro-spinal des humains infectés en Thaïlande appartiennent à des STs du complexe ST27 [30]. Une étude rapporte qu'en Amérique du Nord, la plupart des souches sont ST28 (51%), ST25 (44%) et ST1 (5%) [31].

1. 2 Infection et transmission de *S. suis*

1.2.1 Chez le porc

Le porc est l'hôte naturel de *S. suis* et ce dernier peut affecter les porcs de tous âges. Reams *et al*. ont démontré que 75% des porcs infectés par *S. suis* sont âgés de moins de 16 semaines [17]. En effet, la majorité des cas d'infection survient entre 3 et 12 semaines d'âge, soit suite au sevrage [32]. L'incidence de l'infection varie selon les troupeaux et aussi selon le temps au sein d'un même troupeau. Aucune incidence saisonnière n'a été notée chez le porc [33,34]. Différentes pratiques de production et/ou la présence d'autres pathogènes ont été suggérées comme des facteurs prédisposant à l'infection [35]. En fait, l'infection par *S. suis* est associée avec des pratiques de production stressantes comme des fluctuations excessives de température, une humidité relative élevée, une faible ventilation, un entassement des animaux, une différence d'âge de plus de deux semaines entre les porcs d'un même enclos, le sevrage et le déplacement des animaux [35].

Chez le porc, on retrouve *S. suis* principalement au niveau du tractus respiratoire supérieur (particulièrement au niveau des amygdales et des cavités nasales) [1], ainsi qu'au niveau du tractus génital et du tractus alimentaire [34]. *S. suis* se transmet à la fois de façon horizontale et verticale [1]. Les porcelets nés de truies avec des infections

utérines/vaginales sont déjà infectés ou le deviennent à la naissance [36]. Ils peuvent aussi acquérir la bactérie en étant en contact intime avec la truie, ses fèces ou d'autres porcelets [37]. Dans les cas de transmissions horizontales (par exemple suite à l'introduction de porteurs sains dans un nouveau troupeau), il est généralement accepté que *S. suis* pénètrent dans l'hôte via les voies respiratoires supérieures, et que les bactéries résident ensuite dans les amygdales palatines et pharyngiennes du porc [38,39]. En effet, il a été démontré que *S. suis* se loge au niveau des amygdales des porteurs sains et qu'il peut y survivre même lorsque ces porcs reçoivent des antibiotiques ou développent des anticorps opsonisants [40]. Le taux de porteurs à l'intérieur d'un troupeau est variable et peut atteindre 100% [41,42].

Il a été montré, lors d'infections expérimentales, que des porcs infectés une première fois deviennent résistants à une seconde infection par la même souche ou par une souche du même type [43]. La protection à long terme n'a toutefois pas été évaluée dans cette étude.

S. suis est responsable d'une grande variété de pathologies chez le porc. Il a été isolé de cas de méningite, méningo-encéphalite, septicémie, arthrite, endocardite, péricardite, rhinite, avortement, pneumonie et vaginite [44,45]. Le début de l'infection se caractérise par de l'anorexie, suivie de dépression et de fièvre pouvant atteindre 42°C. Après plusieurs jours, les animaux malades développent des signes neurologiques incluant l'opisthotonos, le décubitus latéral, les mouvements de pédalage, des convulsions et de l'ataxie. Certains animaux peuvent aussi présenter de la boiterie dans un membre lorsque l'arthrite se développe. En Amérique du Nord, *S. suis* est le pathogène le plus souvent isolé lors d'endocardites porcines, et les porcs en souffrant peuvent mourir subitement ou montrer des niveaux variables de dyspnée, de cyanose et de dépérissement [2]. Cependant, la méningite est la manifestation clinique la plus importante et est souvent à la base d'un diagnostic présomptif [18,25]. Des lésions histopathologiques caractéristiques des méningites purulentes apparaissent avec une infiltration diffuse de neutrophiles [46]. On observe également la nécrose des vaisseaux dont l'endothélium est gonflé, et parfois une occlusion de la lumière des vaisseaux par des cellules inflammatoires [47].

1.2.2 Chez l'humain

Les cas d'infections humaines à *S. suis* sont le plus souvent rapportés dans les pays où l'élevage porcin est commun. Pendant longtemps, les infections humaines à *S. suis* ont été considérées comme des infections liées à la profession (maladies occupationnelles). En effet, la majorité des cas mentionnés concerne des personnes travaillant avec le porc ou des produits dérivés du porc [48,49]. Depuis la première infection humaine signalée au Danemark en 1968 [50], plusieurs cas ont été rapportés dans différents pays en Europe (Pays-Bas, Italie, Espagne, Royaume-Uni, Belgique, Croatie, Autriche, Suisse, Irlande, Hongrie, France, Grèce, Portugal) et en Asie (Japon, Chine, Hong Kong, Taiwan, Thaïlande, Singapour), ainsi qu'au Canada, aux États-Unis, en Australie, en Nouvelle-Zélande et en Argentine [51-56]. La plupart des infections a été attribuée à des souches de sérotype 2 [56]. Cependant, des cas liés aux sérotypes 4 [49], 14 [57-59] et 16 [60] ont aussi été rapportés. Deux cas humains ont été attribués au sérotype 1, quoique les isolats n'aient pas été confirmés par réaction sérologique avec un antisérum spécifique [61].

Cependant, suite à deux importantes épidémies en Chine en 1998 et 2005, le portrait classique de l'infection par *S. suis* en tant que maladie occupationnelle a drastiquement changé [56,62]. Actuellement, les infections humaines à *S. suis* représentent un enjeu important en santé publique dans plusieurs pays d'Asie (ANNEXE I). En effet, *S. suis* est la principale cause de méningite adulte au Vietnam, la seconde en Thaïlande et la troisième à Hong Kong [63-66]. Néanmoins, le taux d'infection humaines à *S. suis* demeure imprécis dans les pays en voie de développement possédant une production porcine intense, comme en Asie du Sud-Est, où les infections à *S. suis* ne sont pas rapportées et sont souvent sous-diagnostiquées [63].

Chez l'humain, *S. suis* cause une infection systémique affectant plusieurs organes, mais la méningite demeure la manifestation la plus commune. Le Tableau I décrit les manifestions possibles lors de l'infection par *S. suis* chez l'humain.

Tableau I : Manifestations possibles de l'infection à *S. suis* chez l'humain

Manifestations possibles de l'infection à *S. suis* chez l'humain
Méningite [48]
Endocardite [67]
Péritonite [68]
Arthrite [68]
Spondylodiscite [68]
Rhabdomyolyse [69]
Pneumonie [68]
Uvéite [68]
Endophtalmite [68]
Embolie pulmonaire [67]
Abcès épiduraux lombaires [54]
Anévrisme aortique [70]
Ophthalmoplégie avec diplopie persistante [71]

La surdité et/ou la dysfonction vestibulaire, permanente ou handicapante, sont les séquelles les plus communes de la méningite à *S. suis* et sont retrouvées chez près de la moitié des patients. Cette perte de l'audition peut être le résultat d'un dommage au huitième nerf crânien ou secondaire à la septicémie cochléaire, résultant du passage de l'organisme de l'espace sous-arachnoïdien à la périlymphe via l'aqueduc cochléaire [68,71]. Pour des raisons inconnues, le nombre de patients atteints de surdité suite à l'infection par *S. suis* surpasse les cas rapportés pour d'autres pathogènes responsables de méningite tel que *Streptococcus pneumoniae*, *Neisseria meningitidis* et *Haemophilus influenzae*. Dans le cas de *S. suis*, les pourcentages peuvent atteindre 50% et 65% en Europe et en Asie, respectivement [72]. Cependant, la surdité n'a jamais été rapportée dans des cas d'infection par *S. suis* ne causant pas de méningite [2]. Globalement, le taux de mortalité lors d'infection par *S. suis* est de près de 13% en Europe et 20% en Asie [68]. De façon intéressante, seulement cinq cas humains de méningite (non-fatales) ont été rapporté en Amérique du Nord (trois au Canada et deux aux États-Unis). Cependant, des informations

récentes suggèrent que les infections humaines à *S. suis* seraient plus communes qu'on ne le croit puisque les personnes exposées aux porcs possèdent des titres d'anticorps contre *S. suis* plus élevés que les personnes non-exposées [73]. Comme *S. suis* peut être confondu avec des entérocoques, des streptocoques oraux, *Listeria* spp. ou *S. pneumoniae*, il devrait être pris en considération lors du diagnostic d'une septicémie, spécialement lorsque la méningite apparaît comme complication chez des adultes ayant un historique de contact avec des porcs ou des produits dérivés du porc [2].

Chez l'humain, la route d'infection diffère de celle du porc puisque l'entrée du pathogène se fait généralement via des coupures au niveau de la peau, des muqueuses de la bouche ou de la cavité nasale [2,74]. L'infection via l'ingestion de nourriture contaminée a aussi été proposée [75]. En général, une haute exposition à *S. suis* peut mener à la colonisation des voies respiratoires supérieures sans engendrer de conséquences. Dans certains cas seulement, une infection clinique suivra [2]. La bactérie pourrait aussi coloniser le tractus gastro-intestinal, comme suggéré par la diarrhée en tant que prodrome [76]. La période d'incubation peut aller de quelques heures à deux jours [76]. Presque tous les cas d'infection chez l'humain peuvent être associés à des contacts avec des porcs ou à une haute exposition à de la viande de porc non-préparée qui, dans certains marchés, à été démontrée comme étant contaminée avec *S. suis* [65,77]. Avec quelques exceptions, la plupart des cas (des adultes de sexe masculin) sont retrouvés chez des fermiers, des travailleurs d'abattoirs, des personnes travaillant dans le transport du porc, des inspecteurs de viande, des bouchers et des vétérinaires [3,68]. La splénectomie, l'alcoolisme et le diabète mellitus ont été suggérés comme des facteurs prédisposants importants pour le développement d'une infection sérieuse [68,78]. La plupart des études ont démontré que les souches isolées des cas humains sont phénotypiquement et génotypiquement similaires à celles retrouvées chez les porcs de la même région géographique. De plus, la virulence des souches isolées chez les porcs ou chez les humains semble être similaire [2].

1.2.2.1 Épidémies chez l'humain en Chine

La première épidémie importante de *S. suis* sérotype 2 chez l'humain a eu lieu en 1998 dans la province de Jiangsu en Chine avec 25 personnes infectées et 14 décès [79]. En

2005, un autre épisode important d'infection humaine a eu lieu dans la province de Sichuan en Chine avec 215 cas d'infection aigue et 39 décès enregistrés. Dans l'épidémie de Sichuan, tous les individus infectés étaient des fermiers directement exposés à l'infection durant la manipulation d'animaux morts de causes inconnues ou tués pour la consommation, et ce même si les porc étaient malades. De plus, la route d'infection orale ne peut être exclue [80]. Ces épidémies en Chine n'ont pas suivi les présentations cliniques classiques de l'infection par *S. suis*. En effet, elles étaient caractérisées par une haute incidence d'infection systémique avec un taux de mortalité élevé et un nombre faible de cas de méningites [56]. Aucun des cas n'a montré d'évidence de transmission entre humains. Au départ, les cas ont été faussement diagnostiqués comme étant des syndromes de choc toxique streptococcal puisque les patients présentaient des malaises soudains accompagnés d'une forte fièvre, de diarrhée, d'hypotension, de pétéchies, d'éruptions érythémateuses et d'une dysfonction multiple des organes comme un syndrome de détresse respiratoire aigu, une défaillance du foie et du cœur, une coagulation intravasculaire disséminée et une défaillance rénale aigue [3,80]. Une hypothèse plausible expliquant le développement abrupte de la maladie et la présentation clinique inhabituelle aurait pu être la présence d'une souche hautement virulente ayant la capacité de produire des superantigènes, mais la présence de tels superantigènes n'a pas pu être démontrée chez les souches isolées durant l'épidémie de Sichuan [3,29,80].

Les souches de *S. suis* ST7 ont d'abord émergées à Hong Kong en 1996 et sont responsables des épidémies dans les provinces de Jiangsu et Sichuan en Chine, en 1998 et 2005, respectivement. La souche ST7 SC84, isolée d'un patient atteint de syndrome de choc toxique streptococcal durant l'épidémie de 2005 et la souche ST1 31533, isolée chez un porc atteint de méningite [81], ont démontré des différences au niveau de la production de cytokines. En effet, la souche ST7 SC84 possède une plus grande capacité à stimuler la prolifération des cellules T naïves et des cellules mononucléaires que la souche ST1 31533. La réponse des cellules T aux deux souches est dépendante de la présence de cellules présentatrices d'antigènes autogéniques mais aussi allogéniques, indiquant un possible effet mitogène sur les cellules T [28]. Cet effet était plus marqué avec la souche épidémique

ST7. De plus, plusieurs études ont démontré une capacité stimulatrice plus forte pour cette souche lors de l'activation de l'immunité innée [82-84].

1.2.3 Chez d'autres espèces animales

S. suis a également été isolé chez d'autres espèces animales, incluant les ruminants, les sangliers sauvages, les chevaux, les chats, les chiens, les oiseaux et les hamsters [85-90]. De façon similaire au porc, il semble que *S. suis* colonise aussi les amygdales de ces autres espèces [85,91,92]. Quelques cas de transmission de *S. suis* à l'homme par des sangliers sauvages ont été rapportés [56,93,94]. En effet, il semble que la prévalence de *S. suis* sérotype 2 chez le sanglier sauvage (11%) soit similaire à celle des porcs (14%) en Allemagne [95].

1.3 Facteurs de virulence de *S. suis*

Au cours des dernières années, un intérêt grandissant à été porté à *S. suis* suite à son émergence comme pathogène chez l'humain. Différentes méthodes, telle que la technologie d'expression *in vivo*, la mutagenèse par étiquette signature, la capture sélective de séquences transcrites et l'hybridation soustractive suppressive ont été utilisées afin de découvrir les facteurs responsable de la virulence de ce pathogène (Tableau II) [96-100]. L'identification des facteurs de virulence de *S. suis* demeure toujours difficile, principalement en l'absence d'une définition précise du terme « virulence ». En effet, le concept de virulence de *S. suis* diffère entre les groupes de recherche qui utilisent différents modèles d'infection *in vivo* pouvant mener à des conclusions divergentes. Afin de déterminer la virulence des souches de terrain, différents groupes de recherche ont proposé des critères comme la condition clinique de l'animal à partir duquel la souche a été isolée (animal infecté ou sain); la présence de protéines liées à la virulence; et la virulence chez les porcs provenant de troupeaux dont le statut de santé est élevé, de porcs sans pathogène spécifique, et de porcs ou porcelets de différents âges privés de colostrum et provenant de troupeaux conventionnels ou sans pathogène spécifique [81,101,102]. En fait, les résultats d'infection expérimentales par *S. suis* chez le porc peut dépendre du statut immunologique de l'animal, de la route de l'infection, de la concentration de l'inoculum et de la colonisation des voies respiratoires supérieures chez des porteurs sains [103]. De plus, des

divergences importantes existent dans la littérature concernant la virulence d'une même souche [103,104]. Aussi, certains facteurs de virulence proposés sont présents à la fois chez des souches virulentes et des souches non-virulentes. En plus, le rôle dans la virulence de certains de ces facteurs n'a pas pu être évalué de façon certaine puisque des souches mutantes « knock-out » (KO) ne sont pas disponibles. Finalement, la virulence des souches de sérotype 2 retrouvées en Europe et en Asie chez des porcelets malades semble plus forte que celle observée en Amérique du Nord. La plupart des études concernant les facteurs de virulence de *S. suis* ont été conduites avec des souches de sérotype 2, et il existe peu d'informations concernant des facteurs de virulence présents chez les autres sérotypes. Malgré tout, plusieurs facteurs sont considérés comme importants dans la pathogenèse de *S. suis* sérotype 2.

1.3.1 Capsule polysaccharidique (CPS)

La CPS de *S. suis* sérotype 2 a récemment été purifiée et caractérisée. Elle est composée de cinq sucres différents : galactose, glucose, rhamnose, N-acétylglucosamine et acide N-acétylneuraminique (acide sialique) [105]. Le locus *cps* codant pour la CPS de *S. suis* sérotype 2 a été cloné et caractérisé (Figure 1A). Ce locus contient plusieurs gènes codant pour des activités glucosyl-, galactosyl-, N-acetylglucosaminyl- and rhamnosyltransférase, ainsi que des gènes impliqués dans la synthèse de l'acide sialique [106,107].

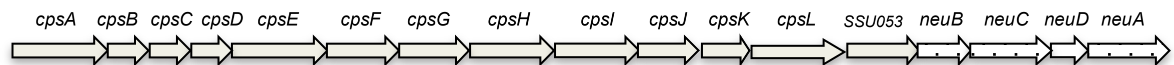

Figure 1A. Locus *cps* de *S. suis* sérotype 2

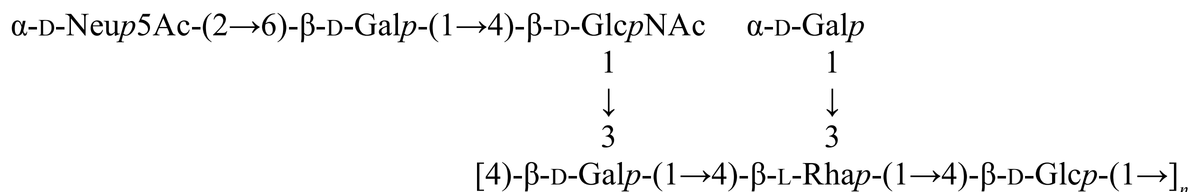

Figure 1B. Structure de la CPS de *S. suis* sérotype 2

Selon des études conduites avec des mutants isogéniques non-encapsulés, la CPS demeure, à ce jour, le seul facteur de virulence démontré comme étant critique pour *S. suis*. L'absence de CPS est corrélée avec une augmentation de l'hydrophobicité et de la phagocytose de *S. suis* par des phagocytes murins et porcins [106,108]. Des résultats comparables ont aussi été obtenus avec des neutrophiles porcins [109]. Il a été démontré que la CPS module l'activation de voies de signalisation intracellulaire impliquées dans la phagocytose par les macrophages murins [110]. Récemment, en utilisant des billes de latex liées de façon covalente à la CPS purifiée de *S. suis*, il a été démontré que la CPS est suffisante pour inhiber à la fois la phagocytose des billes de latex et d'autres billes fluorescentes à l'intérieur des macrophages. Suite au contact avec les macrophages, la CPS de *S. suis* déstabilise les microdomaines lipidiques (radeaux lipidiques) à la surface cellulaire. La CPS bloque aussi la production d'oxyde nitrique suggérant qu'elle a un effet sur les voies de signalisation impliquées dans la formation des radeaux lipidiques, plus particulièrement sur les voies impliquant la kinase Lyn. Effectivement, la production d'oxyde nitrique est contrôlée par la kinase Lyn qui est elle-même activée par le lactosylcéramide, un glycosphingolipide neutre, qui est présent dans les microdomaines lipidiques des cellules immunes. Le lactosylcéramide a la capacité de reconnaître des patrons à la surface des pathogènes. Dans le cas de *S. suis,* il a été démontré que la CPS empêche la reconnaissance de *S. suis* par le lactosylcéramide alors, qu'au contraire, le lactosylcéramide participe à l'internalisation du mutant non-encapsulé. Ainsi, la CPS jouerait un rôle de bouclier, permettant à la bactérie de camoufler les patrons reconnus par le lactosylcéramide et d'empêcher son accumulation à la coupe phagocytique. Enfin, un mutant non-encapsulé était facilement internalisé via les radeaux lipidiques, de façon sensible à la filipine, menant au recrutement du lactosylcéramide et à une forte production d'acide nitrique [111].

La CPS pourrait aussi être impliquée au niveau de la réponse inflammatoire. En effet, il a été démontré que la CPS induit spécifiquement la production de la chimiokine MCP-1/CCL2 de façon MyD88-indépendante chez des macrophages murins [56]. De plus, une étude récente a démontré que la CPS de *S. suis* possède la capacité d'induire la production

de MIP-1α/CCL3 via des mécanismes partiellement dépendants du récepteur de type Toll (TLR)-2 et de MyD88, ainsi que de MCP-1/CCL2 via des voies de synthèse TLR-indépendantes [112].

L'importance de la CPS a aussi été démontrée *in vivo* où des mutants non-encapsulés se sont avérés être non-virulents et rapidement éliminés de la circulation, à la fois dans des modèles d'infection chez le porc et la souris [106,113]. Certaines recherches ont proposé que l'épaisseur de la CPS soit corrélée avec la virulence. Les isolats *de S. suis* sérotype 2 récupérés chez des animaux malades présentent en effet une CPS plus épaisse que ceux isolés chez des animaux sains [114]. Une augmentation de l'épaisseur de la capsule suite à la croissance *in vivo* a aussi été notée pour une souche virulente mais pas pour des souches non-virulentes. Cette augmentation de l'épaisseur de la capsule a aussi été associée avec une résistance au « killing » par des leucocytes polymorphonucléaires porcins [115]. Cependant, d'autres études n'ont pas démontré de corrélation entre l'épaisseur du matériel capsulaire et la virulence [116]. De plus, les bactéries des souches de sérotype 2 ne sont pas couvertes d'un matériel capsulaire plus épais comparativement aux autres sérotypes [113,116].

Bien que le CPS semble un facteur de virulence critique pour *S. suis*, il n'en demeure pas moins que la majorité des souches non-virulentes sont aussi encapsulées, ce qui suggère que d'autres facteurs de virulence sont également essentiels et que la virulence est multifactorielle [1].

1.3.1.1 Acide sialique

Comme mentionné ci-dessus, la structure de la CPS de *S. suis* sérotype 2 et plus récemment de la CPS du sérotype 14 ont été décrites, indiquant la présence d'acide sialique en position terminale de la chaine latérale, formant une liaison α2, 6 avec le galactose adjacent (Figure 1B) [105,117]. L'acide sialique est un important facteur de virulence pour plusieurs bactéries responsables de méningites tel que, les streptocoques du groupe B (GBS ou *Streptococcus agalactiae*), *Escherichia coli* K1 et *N. meningitidis* [118,119]. Les déterminants génétiques responsables de la synthèse et du transport de l'acide sialique chez

S. suis sont retrouvés dans le locus *cps* [107]. Ceci inclut les gènes *neuA*, *neuB*, *neuC* et *neuD*. En plus des sérotypes 2 et 14, la présence de l'acide sialique dans la CPS a aussi été prédite pour les sérotypes 1, 16 et 1/2 [120]. Ainsi, les CPS des sérotypes causant les plus fréquemment des infections humains (sérotypes 2 et 14) possèdent de l'acide sialique. Les sérotypes 1 et 16 ont aussi occasionnellement causé des infections chez l'humain. Chez *S. suis*, il avait été suggéré que l'habileté invasive des souches de *S. suis* sérotype 2 dépendrait de la composition du matériel capsulaire. Cependant, il a été démontré que des souches virulentes et non-virulentes possèdent des CPS de taille semblable avec des concentrations similaires d'acide sialique [113].

L'acide sialique a aussi été suggéré comme étant impliqué dans l'adhérence de *S. suis* sérotype 2 aux monocytes, suggérant un modèle de « cheval de Troie modifié ». Selon ce modèle, le pathogène pourrait circuler dans le sang en s'associant, par adhésion, à ces cellules [1].

Un effet de mimétisme moléculaire a aussi été suggéré basé sur le fait que le groupement terminal de l'acide sialique en liaison α-2, 6 est similaire aux épitopes des sucres présents à la surface des cellules des mammifères [4]. Ce mimétisme moléculaire pourrait mener à l'absence de reconnaissance de l'antigène par le système immunitaire de l'hôte. En effet, la CPS de *S. suis* sérotype 2 a été rapportée comme étant pauvrement immunogène chez le porc et le cheval [43,121].

Chez GBS, l'acide sialique terminal en lien α-2, 3 a été démontré comme jouant un rôle majeur dans la virulence, incluant le mimétisme moléculaire, l'évasion du complément, la prévention de la phagocytose et la modulation de fonctions bactéricides de neutrophiles [122]. La CPS de GBS a aussi été rapportée comme capable de se lier aux « sialic acid-recognizing Ig superfamily lectins » (Siglecs), une famille de récepteurs exprimée sur les leucocytes [123]. De plus, une étude a démontré que l'acide sialique capsulaire de GBS peut être modifié par O-acétylation [124]. La O-acétylation de l'acide sialique modifie (réduit ou augmente) la liaison à différents Siglecs [123]. Ce phénomène, jusqu'à récemment méconnu, pourrait potentiellement favoriser l'hôte ou le pathogène

dépendamment de différentes variables [123]. La présence chez *S. suis* de gènes homologues aux gènes *neuD* (O-acétyltransférase) et *neuA* (O-acétylestérase) de GBS suggère que la CPS de *S. suis* serait aussi O-acétylée [107]. Cependant, de tels rôles n'ont pas encore été démontré dans le cas de *S. suis.* Tel qu'indiqué, la liaison de l'acide sialique à la CPS diffère entre GBS et *S. suis* sérotypes 2 et 14, ces derniers étant en liaison α-2, 6 plutôt que α-2, 3 [105,117]. Ainsi, les différents rôles joués par l'acide sialique dans la virulence de ces deux pathogènes pourraient s'expliquer, du moins en partie, par les différences conformationnelles de la CPS de ces deux espèces.

1.3.2 Paroi cellulaire

Plusieurs études menées avec des mutants non-encapsulés suggèrent que des composantes de la paroi cellulaire joueraient un rôle important dans la réponse inflammatoire induite par *S. suis*, principalement au niveau de la production de cytokines [125-129]. Cependant, les mécanismes demeurent peu décrits.

1.3.2.1 D-alanylation de l'acide lipotéichoïque

L'acide lipotéichoïque (LTA) est une molécule retrouvée dans la paroi cellulaire de la plupart des bactéries Gram-positif. Il est considéré comme l'équivalent du lipopolysaccharide (LPS) des bactéries Gram-négatif [130]. Le LTA est associé avec le développement de choc septique chez l'hôte lors d'infection par des bactéries Gram-positif puisqu'il active les leucocytes, stimule une production exacerbée de cytokines pro-inflammatoires et de molécules cytotoxiques et cause ainsi une réponse inflammatoire systémique pouvant mener au dysfonctionnement de plusieurs organes et à la mort [130]. Le LTA est un polymère de phosphoglycérol substitué avec un ester D-alanyl ou un résidu glycosyl et ancré dans la membrane par un glycolipide terminal. La D-alanylation du LTA permet aux bactéries Gram-positif de moduler leur charge à la surface, de réguler la liaison aux ligands et de contrôler les propriétés électromécaniques de la paroi cellulaire [131]. Des études génétiques concernant la biosynthèse du LTA chez diverses bactéries Gram-positif, comme *Lactococcus caseii* et *Bacillus subtilis*, ont démontré que l'incorporation de résidus D-Ala requiert l'activité de quatre gènes codés par l'opéron *dlt* [132,133]. Peu de données sont disponibles concernant l'importance de la D-alanylation du LTA et le rôle joué dans la

virulence des streptocoques. Des études utilisant des mutants isogéniques ont démontré que la D-alanylation du LTA chez GBS permet la résistance à l'effet bactéricide des défensines et des cellules phagocytaires [134]. Chez les Streptocoques du groupe A (GAS) et *Streptococcus gordonii*, il a été démontré que la D-alanylation du LTA confère une résistance au lysozyme ainsi qu'aux peptides antimicrobiens [135-137]. Le rôle de la D-alanylation dans la résistance au complément a aussi été décrit, entre autres chez *Streptococcus pyogenes* [138,139].

L'importance de la D-alanylation du LTA dans la virulence de *S. suis* sérotype 2 a été évaluée à l'aide d'un mutant isogénique Δ*dltA*. L'absence de D-alanylation du LTA a résulté en une augmentation de la susceptibilité aux peptides cationiques antimicrobiens. De plus, la souche mutante était tuée plus efficacement par des neutrophiles porcins et présentait une adhérence ainsi qu'une invasion diminuée des cellules endothéliales porcines de microvaisseaux cérébraux (PBMEC). De plus, la souche mutante Δ*dltA* a montré une atténuation de la virulence en modèles murin et porcin d'infection, comparativement à la souche de type sauvage. Cette atténuation pourrait être associée à une diminution de l'habileté à échapper aux mécanismes de l'immunité, à une plus faible capacité à activer la réponse inflammatoire et/ou à une diminution de l'habileté à traverser les barrières de l'hôte [140].

1.3.2.2 N-déacétylation du peptidoglycane

Le principal composant de la paroi cellulaire des bactéries Gram-positif est le peptidoglycane (PG) qui assure stabilité et rigidité à la paroi cellulaire. Le PG consiste en des chaînes de glycans faites en alternance de β-1,4 *N*-acétylglucosamine et d'acide *N*-acétylmuramique reliés entre eux par des courtes chaînes peptidiques. La structure des chaînes de sucres est hautement préservée entre les espèces bactériennes alors que la composition des sous-unités peptidiques varie [141]. Le PG agit en synergie avec le LTA pour induire la réponse inflammatoire de l'hôte et causer un choc septique [142]. Cependant, cette structure invariante est aussi exploitée par l'hôte pour la reconnaissance des bactéries via les récepteurs de type NOD (nucleotide-binding oligomerization domain) comme NOD1 et NOD2 (décrits dans la section 2.1.2). Ces récepteurs reconnaissent les

muropeptides relâchés lors du retournement de la paroi cellulaire. Le PG est aussi reconnu par l'hôte pour la destruction des microorganismes via l'activité hydrolytique du lysozyme [143,144]. Néanmoins, certains pathogènes Gram-positif ont développé des moyens efficaces pour déjouer les défenses de l'hôte [145]. Plusieurs bactéries Gram-positif modifient la structure de leur PG afin d'éviter la reconnaissance par le système immunitaire de l'hôte. Par exemple, *S. pneumoniae* et *Listeria monocytogenes* N-déacétylent les résidus *N*-acétylglucosamine ce qui permet aux bactéries d'éviter la reconnaissance par NOD1 et NOD2, en plus d'augmenter la résistance au lysozyme [146,147].

S. suis possède le gène *pgdA* responsable de la N-déacétylation de son PG [148]. Une étude a démontré que l'expression du gène *pgdA* est augmentée suite à l'interaction de la bactérie avec les neutrophiles *in vitro* ainsi qu'*in vivo* dans un modèle murin d'infection, suggérant que *S. suis* augmente la N-déacétylation de son PG dans ces conditions. La virulence d'un mutant isogénique Δ*pgdA* est hautement atténuée comparativement à la souche parentale dans des modèles murin et porcin d'infection, confirmant ainsi un rôle pour la N-déacétylation du PG dans la pathogenèse de l'infection par *S. suis*. De plus, le mutant Δ*pgdA* s'est avéré incapable de persister en circulation sanguine ou d'échapper aux mécanismes de défense de l'hôte assurés par les neutrophiles [148].

1.3.3 Hémolysine (suilysine)

La suilysine, une hémolysine de 54 kDa, est retrouvée chez différents sérotypes de *S. suis*, incluant les sérotypes 1, 1/2, 3, 4, 5, 7, 8, 9, 14, 15, 17, 18, 19, 23 et 28 [92,149,150]. La suilysine fait partie de la famille des toxines cytolytiques liant le cholestérol, incluant aussi la pneumolysine (*S. pneumoniae*) [92], la streptolysine O (GAS), la listériolysine (*L. monocytogenes*) et la perfringolysine (*Clostridium perfringens*) [151]. Les toxines cytolytiques sont produites sous forme de monomères solubles et s'oligomérisent à la surface de la cellule où elles forment un pore, menant ainsi à la lyse cellulaire. La suilysine possède plusieurs caractéristiques communes avec ces toxines tel que la perte d'activité suite à l'oxydation, la réactivation suite à la réduction, l'inhibition par de petites quantités de cholestérol, la formation de pores transmembranaires et un mécanisme d'action « multi-

hit » [152]. Le cholestérol de la membrane des cellules eucaryotes semble être le site d'attache de la toxine [151].

À ce jour, il a été démontré que la suilysine est toxique pour plusieurs types cellulaires comme les cellules endothéliales et épithéliales ainsi que les neutrophiles, macrophages et monocytes [109,127,153-155]. De plus, la suilysine induit la production de cytokines pro-inflammatoires par des cellules endothéliales microvasculaires cérébrales (BMEC) humaines et porcines [110,156,157], par les cellules mononucléaires sanguines porcines [158] et par les macrophages alvéolaires porcins.

Il a aussi été proposé que la suilysine pourrait relâcher l'hémoglobine des globules rouges, contribuant ainsi à augmenter les niveaux de médiateurs pro-inflammatoires en agissant en synergie avec les composantes de la paroi cellulaire [159].

La suilysine augmente aussi l'expression des molécules d'adhésion chez les monocytes humains et est impliquée dans la sécrétion d'acide arachidonique [160], un précurseur de la prostaglandine, par les cellules endothéliales humaines [161]. Ces résultats indiquent que la suilysine semble jouer un rôle important dans la dissémination bactérienne ainsi que dans l'inflammation et l'invasion de différents tissus par *S. suis*. Néanmoins, certains de ces résultats demeurent controversés comme, par exemple, l'absence de mortalité chez la souris suite à l'injection systémique de surnageant de culture d'une souche positive pour la production de la suilysine [162]. D'un autre côté, un mutant obtenu par échange allélique du gène *sly* a été montré comme étant non-toxique pour des macrophages murins et non-virulent dans un modèle murin d'infection, alors que sa virulence était seulement atténuée dans un modèle porcin d'infection systémique [163]. Au contraire, d'autres recherches ont démontré qu'un mutant déficient pour la production de la suilysine et sa souche parentale étaient tous deux résistants aux facteurs bactéricides présents dans le sang complet porcin. De plus, dans un modèle d'infection porcin, la souche mutante a induit l'infection de façon similaire à la souche de type sauvage, avec la présence de signes cliniques associés à l'infection et l'isolation de la bactérie à partir de différents tissus [164]. Finalement, bien que la suilysine soit présente chez la plupart des souches de *S. suis* sérotype 2 asiatiques et

européennes, elle est seulement présente chez un nombre restreint de souches de *S. suis* sérotype 2 nord-américaines [2,21,92,150,165-167].

1.3.4 Autres protéines associées à la virulence

Plusieurs autres facteurs de virulence ont aussi été proposés pour *S. suis*. Pour certains, leur rôle dans la virulence de *S. suis* à été confirmé alors que pour d'autres une fonction spécifique dans le développement de la maladie n'a pas encore été démontrée. Le tableau II résume les autres facteurs de virulence putatifs décrits à ce jour.

Tableau II : Facteurs de virulence putatifs et confirmés de *Streptococcus suis*

Facteur de virulence	**Fonction proposée ou confirmée dans la virulence**	**Pathogénicité de mutants déficients pour le facteur en modèle d'infection *in vivo* chez diverses espèces d'animaux**
Adhésion		
Fbps	Adhésion à la fibronectine et au fibrinogène	Partiellement atténuée (porc)
Énolase	Adhésion au plasminogène et à la fibronectine	Mutant non disponible
Pilus *srtF* (ANNEXE II)	Adhésine putative	Aucun effet (souris)
Pilus *srtG*	Adhésine putative	Non testée
Glycéraldéhyde-3-phosphate-déshydrogénase	Adhésion au plasminogène et aux cellules de la trachée du porc)	Mutant non disponible
Dipeptidylpeptidase IV	Adhésion à la fibronectine	Atténuée (souris)
Glutamine synthétase	Adhésion aux cellules épithéliales humaines Hep-2	Atténuée (souris)
Facteur d'opacité du sérum	Possible adhésion à la fibronectine	Atténuée (porc)
6-phosphogluconate-déshydrogénase	Adhésion aux cellules Hep-2 et HeLa	Mutant non disponible
Amylopullulanase	Adhésion à l'épithélium porcin et au mucus)	Non testée
Adhésine P	Hémagglutinine	Mutant non disponible
Sortase A	Adhésion à la fibronectine plasmatique, fibronectine cellulaire et collagène I	Aucun effet (souris)
Protéases		
Dipeptidylpeptidase IV	Clive après les résidus X-Pro et X-Ala en N-terminal des chaînes polypeptidiques	Atténuée (souris)
IL-8 sérine protéase (ANNEXE III)	Dégradation de la chimiokine IL-8	Non testée

Protéine de type subtilisine (SspA)	Dégradation de la chaîne Aα du fibrinogène	Atténuée (souris)
Protéase IgA1	Clivage d'IgA1	Atténuée (porc)
Métaux		
Dpr	Résistance à la toxicité par le fer	Mutant non disponible
Zur	Résistance à la toxicité par le fer	Non testée
AdcR	Régulateur d'acquisition du zinc	Atténuée (souris)
Fur	Régulateur d'acquisition du fer	Atténuée (souris)
FeoB	Transporteur du fer	Atténuée (souris)
TroA	Acquisition du manganèse	Atténuée (souris)
SSU0308 (Lipoprotéine 103)	Acquisition du zinc	Atténuée (souris)
Lipoprotéines		
Lipoprotéine signal peptidase	Exportation de lipoprotéines	Aucun effet (porc)
Lgt	Prolipoprotéine diacylglycéryl transférase	Non testée
Gène *Ipp*	Lipoprotéine	Atténuée (porc)
Gène homologue à GBS SAG0907	Lipoprotéine	Aucun effet (porc)
Résistance		
Superoxyde dismutase	Résistance à la toxicité	Mutant non disponible
Système arginine déiminase	Résistance à l'acidité	Non testée
Quorum sensing		
LuxS	Quorum sensing	Atténuée (poisson zèbre)
Dégradation		
Endo-β-N-acetylglucosaminidase D	Dégradation des oligosaccharides de surface	Atténuée (porc)
Nucléase SsnA	Dégradation de l'ADN de l'hôte	Non testée
Hyaluronate lyase	Dégradation de l'acide hyaluronique	Non testée
Collagénase	Dégradation du collagène	Partiellement atténuée (porc)
Phospholipase C	Modulation de la production d'acide arachidonique chez l'hôte	Mutant non disponible
SalK/SalR	Système à deux composantes	Atténuée (porc)
CiaRH	Système à deux composantes	Atténuée (porc)
Perméases		
Perméase	Transporteur de type ABC (acides aminés)	Atténuée (porc)
Perméase	Transporteur de type ABC (drogues)	Atténuée (porc)

Régulateurs		
RevSC21	Régulateur de réponse orphelin	Atténuée (souris)
CovR	Régulateur de réponse orphelin	Hypervirulent (porc)
RevS	Régulateur de réponse orphelin	Atténuée (souris)
Rgg-like	Régulateur transcriptionnel	Atténuée (porc)
Gène *treR*	Régulateur transcriptionnel	Atténuée (porc)
Gène *nadR*	Régulateur transcriptionnel	Atténuée (porc)
Gène homologue à SMU_61 de *S. mutans*	Régulateur transcriptionnel	Atténuée (porc)
Métabolisme		
CcpA	Régulateur catabolisme des sucres	Non testé
Gène *scrR*	Répresseur de l'opéron du sucrose	Atténuée (porc)
Protéine de 38 kDa	Phosphoglycérate mutase putative	Mutant non disponible
Glutamate déshydrogénase	Glutamate déshydrogénase	Mutant non disponible
Gène *gtfA*	Sucrose phosphorylase	Atténuée (porc)
Gène *purA*	Adénylosuccinate synthétase	Atténuée (porc)
Gène *purD*	Phosphoribosylamine-glycine-ligase	Atténuée (porc)
Gène *scrB*	Sucrose-6-phosphate hydrolase	Atténuée (porc)
Gène *cdd*	Cytidine déaminase	Atténuée (porc)
Gène *guaA*	GMP synthase	Aucun effet (porc)
Gène *guaB*	Inosinemonophosphatedéshydrogénase	Aucun effet (porc)
Gène *manN*	Mannose-specific transport PTS IID	Atténuée (porc)
Autres		
Protéine membranaire de 44 kDa	Inconnue	Non testée
Gène homologue à *S. pneumoniae srp*1018	Inconnue	Atténuée (porc)
Gène *glnH*	Inconnue	Atténuée (porc)
Sao	Inconnue	Mutant non disponible
VirA	Inconnue	Atténuée (lapin)
Trag	Inconnue	Atténuée (poisson zèbre)
Protéine Htps	Inconnue	Aucun effet (souris)
Protéine relâchée par traitement à la muramidase	Inconnue	Aucun effet (porc)
Facteur extracellulaire	Inconnue	Aucun effet (porc)

Adapté de Fittipaldi *et al*. [168].

1.4 Pathogenèse de l'infection

La plupart des études concernant la pathogenèse de l'infection ont été réalisées avec des souches de *S. suis* sérotype 2, ce sérotype étant considéré comme le plus fréquemment isolé lors d'infections autant chez le porc que chez l'humain [1].

1.4.1 Porte d'entrée et colonisation

Comme mentionné précédemment, chez le porc, il est généralement accepté que *S. suis* pénètre dans l'hôte via les voies respiratoires supérieures. Les amygdales palatines et pharyngiennes seraient des sites de portage importants pour *S. suis* [38,39]. C'est à partir des amygdales que la bactérie pourrait ensuite se disséminer au travers de la circulation sanguine ou du système lymphatique [1]. Les amygdales font partie des tissus lymphoïdes associés à la muqueuse. Il a été démontré que *S. suis* est retrouvé en association avec des cellules myéloïdes à l'intérieur des amygdales [38]. L'expression de CD16 et de CD163 sur ces leucocytes suggère une association avec des macrophages matures des amygdales. Cette association peut mener à l'élimination ou au contrôle des bactéries [38]. D'autres études ont confirmé que la présence de *S. suis* dans les cryptes des amygdales mène à une infiltration locale par des lymphocytes CD4 et CD8. Ceci suggère que l'initiation des réponses immunitaires innée et adaptative contre *S. suis* pourrait avoir lieu au niveau des cryptes des amygdales [169].

Chez l'homme, comme mentionné plus haut, la bactérie pourrait entrer en circulation par l'entremise de blessures ou coupures cutanées ainsi que par la voie orale [1].

1.4.2 Interactions avec les cellules épithéliales

S. suis est retrouvé en très faible quantité au niveau des surfaces muqueuses et les mécanismes utilisés par *S. suis* pour traverser les barrières épithéliales demeurent, à ce jour, peu connus. Afin de se rendre dans la circulation sanguine, *S. suis* doit traverser la muqueuse épithéliale des voies respiratoires supérieures [1].

Il est généralement accepté que *S. suis* adhère aux cellules épithéliales d'origines porcine et humaine [154,155]. Les adhésines impliquées dans cette adhésion semblent être partiellement masquées par la CPS et faire partie de la paroi cellulaire [154].

S. suis pourrait aussi endommager les barrières épithéliales afin de continuer sa progression dans les tissus de l'hôte. À cet égard, il a été démontré que la suilysine produite par *S. suis* est cytotoxique pour les cellules épithéliales [1,154,155]. Cependant, les souches ne produisant pas la suilysine ont aussi la capacité de se disséminer dans la circulation sanguine [168]. Ainsi, la possibilité que *S. suis* soit capable de traverser les barrières épithéliales sans cytotoxicité est toujours sujet à controverse, ainsi que l'invasion des cellules épithéliales qui a été démontré seulement pour les souches non-encapsulées et non-typables de *S. suis* [170].

1.4.3 Dissémination sanguine : interactions avec les cellules phagocytaires

Le développement de la septicémie est une étape critique du développement de la méningite causée par *S. suis* [1]. Les interactions entre *S. suis* et les cellules phagocytaires jouent donc un rôle déterminant dans la pathogénèse de *S. suis.*

S. suis doit d'abord survivre en circulation. C'est la CPS dont est pourvu *S. suis* qui lui permet de résister à la phagocytose par les cellules du système immunitaire comme les neutrophiles, les monocytes et les macrophages [168].

Au cours des dernières années, plusieurs théories ont été suggérées concernant la dissémination sanguine de *S. suis*. Une première hypothèse appelée « cheval de Troie » a été proposée. Selon cette théorie, les bactéries sont internalisées par les monocytes (en l'absence d'anticorps spécifiques) et survivent à l'intérieur de ces cellules, facilitant ainsi l'invasion du système nerveux central (SNC) [171]. D'autres études par cytométrie de flux (FACS) ont démontré l'internalisation de *S. suis* par des phagocytes humains et porcins [172], appuyant l'hypothèse que l'internalisation des bactéries par ces cellules pourrait avoir lieu directement dans les amygdales ou en circulation sanguine [1]. Néanmoins, plusieurs études plus récentes ont plutôt suggéré que *S. suis* utiliserait des stratégies

différentes. En effet, les bactéries semblent voyager de façon libre en circulation sanguine ou adhérées à la surface des monocytes [1]. Donc, au lieu d'être phagocytées, les bactéries demeureraient seulement attachées à la surface des cellules phagocytaires [127,173], phénomène attribué à la présence de la CPS qui régule et inhibe les voies de signalisation impliquées dans la phagocytose par les macrophages, tel que décrit dans la section 1.4.1 [126].

Tel que mentionné précédemment, la suilysine produite par *S. suis* présente une activité cytotoxique pour les monocytes/macrophages et les neutrophiles, ce qui pourrait être un autre moyen pour les bactéries d'éviter la réponse immunitaire innée [109,127]. La suilysine interfère aussi avec la capacité des neutrophiles porcins à tuer les bactéries par la voie du complément en affectant l'opsonisation de *S. suis* et l'activité bactéricide des neutrophiles [109].

1.4.4 Développement de la réponse inflammatoire

Les interactions entre *S. suis* et les cellules phagocytaires sont cruciales pour le développement de la réponse inflammatoire. Il a été démontré chez les monocytes humains que *S. suis* induit l'expression de ICAM-1/CD154, CD11a/CD18 et CD11c/CD18, et des molécules impliquées dans l'inflammation [160]. Ceci peut résulter en une augmentation du recrutement des leucocytes au site d'infection. Une fois en contact avec les monocytes/macrophages humains et murins, il a été démontré que *S. suis* induit la production de différentes cytokines pro-inflammatoires telles que TNF-α, IL-6, IL-1β, IL-8 et MCP-1/CCL2 [128,129]. Segura *et al.* ont aussi démontré que *S. suis* stimule les cellules sanguines du sang complet de porc à produire de hauts niveaux de TNF-α, IL-1β, IL-6 et des niveaux intermédiaires de IL-8 et MCP-1/CCL2 [158]. La paroi cellulaire semble être le principal responsable de la production de ces médiateurs inflammatoires [158]. En effet, d'autres études ont démontré que des macrophages porcins stimulés avec une souche mutante de *S. suis* déficiente pour l'expression de la CPS, et donc exposant la paroi cellulaire, montrent une activation plus grande des gènes pro-inflammatoires incluant ceux codant pour TNF-α, IL-1β et MIP-2/CXCL2 en comparaison avec la souche parentale [174]. De plus, la stimulation de monocytes humains par une souche encapsulée de *S. suis*

ou des composants purifiés de la paroi cellulaire induit la production d'un profil similaire de cytokines et de chimiokines [56]. Chez des porcs infectés expérimentalement, *S. suis* induit une production systémique d'IL-6 et d'IL-8 [175].

La production de médiateurs pro-inflammatoires peut avoir plusieurs effets au niveau systémique, contribuant au recrutement des leucocytes au site d'infection, augmentant l'hématopoïèse et induisant la fièvre [176]. Cependant, bien que les cytokines et chimiokines inflammatoires contribuent au processus anti-infectieux, leur production peut entraîner des effets dommageables pour l'hôte. À cet égard, un modèle murin d'infection a été mis au point afin d'étudier le choc septique et la méningite causée par *S. suis* [177]. Ce modèle a principalement servi à l'étude de la réponse pro-inflammatoire associée à l'infection par *S. suis*, tant au niveau systémique qu'au niveau du SNC. Les résultats ont démontré la production de plusieurs cytokines pro-inflammatoires (TNF-α, IL-6, IL-12p40/p70, IFN-γ) et chimiokines (KC/CXCL1, MCP-1/CCL2, RANTES/CCL5) qui ont entraîné un choc septique et la mort de 20% des animaux [177]. Le rôle de l'inflammation dans la mortalité a été confirmé en comparant deux lignées murines différentes: la lignée C57BL/6 (considérée comme résistante à l'infection par *S. suis*) et la lignée A/J (considérée comme sensible à l'infection par *S. suis*) [178]. Le haut taux de mortalité observé chez la lignée sensible a été attribué à un choc septique non contrôlé. En effet, les souris A/J présentaient des taux exceptionnellement élevés de TNF-α, IL-12p40/p70, IL-1β et IFN-γ, significativement supérieurs à ceux retrouvés dans la lignée C57BL/6. Les souris C57BL/6 présentaient une production plus élevée d'IL-10, une cytokine anti-inflammatoire, ce qui suppose que la cascade pro-inflammatoire est mieux contrôlée chez cette lignée de souris, entraînant un plus haut taux de survie. La survie au choc septique de *S. suis* semble donc impliquer un contrôle précis des mécanismes pro- et anti-inflammatoires.

1.4.5 Atteinte du système nerveux central

Pour causer la méningite, *S. suis* doit atteindre et envahir le SNC. Il est généralement accepté qu'une fois en circulation (libres ou associées aux monocytes), les bactéries traversent la barrière hémato-encéphalique (BBB) et survivent et se multiplient dans l'espace sous-arachnoïdien afin d'initier une réponse inflammatoire chez l'hôte, altérant

ainsi l'intégrité de la BBB. Différents mécanismes ont été suggérés comme étant impliqués dans l'invasion du SNC par *S. suis*. Pour mieux comprendre ces mécanismes, il est essentiel d'étudier les interactions entre *S. suis* et les cellules endothéliales de la BBB. Il est aussi nécessaire d'identifier d'autres voies d'entrée possibles tel que la barrière sang-liquide céphalorachidien. En effet, bien que la barrière sang-liquide céphalorachidien ait une plus petite surface que la BBB, elle pourrait tout de même jouer un rôle critique pour la translocation des bactéries ainsi que pour la migration des leucocytes, puisque le plexus choroïde est une région très vascularisée du SNC. De plus, des dommages au niveau du plexus choroïde ont été observés suite à des méningites causées de façon naturelle par *S. suis* ou induites expérimentalement [47,177,179].

La BBB est une barrière formée par des BMEC et a pour fonction de protéger le cerveau des microbes et toxines pouvant être retrouvés dans la circulation sanguine. La perturbation de la BBB est une caractéristique importante des méningites bactériennes [180]. Plusieurs études ont porté sur les interactions entre *S. suis* et les BMEC d'origine porcines ou humaines. La suilysine a été rapportée toxique pour les BMEC, il a été ainsi suggéré que les souches suilysine-positives utilisent l'adhérence et la cytotoxicité pour atteindre le SNC au lieu de l'invasion directe [110,153]. En réponse à *S. suis*, la production de plusieurs cytokines incluant IL-6, IL-8 et MCP-1/CCL2 a été observée chez les PBMEC ou les BMEC d'origine porcine [156,157]. Il a aussi été démontré que *S. suis* stimule l'expression de molécules d'adhésion comme sICAM-1 [181] ainsi que d'autres médiateurs pro-inflammatoires comme la prostaglandine E2 et la métalloprotéinase 9 [182]. La production de cytokines et autres médiateurs pro-inflammatoires ainsi que l'expression des molécules d'adhésion pourrait jouer un rôle principal dans le changement de perméabilité ou de propriété d'adhésion des BMEC. Une autre étude a aussi révélé que *S. suis* possède la capacité d'envahir les PBMEC [110,183].

Il a aussi été suggéré que *S. suis* rejoigne le SNC en affectant l'intégrité de la barrière formée par les cellules épithéliales du plexus choroïde. Un modèle transwell d'infection *in vitro* a démontré que l'épithélium du plexus choroïde a la capacité de restreindre la croissance de *S. suis*, puisque son activation entraîne la production de cytokines pro-

inflammatoires qui créent un environnement non-favorable pour les bactéries [184]. Cependant, de son côté, *S. suis* affecte l'intégrité de la barrière, en particulier grâce à l'effet cytotoxique de la suilysine. D'autres mécanismes, comme l'apoptose, seraient aussi impliqués [185,186]. Par contre, le développement d'un système de transwell "inversé" de cellules épithéliales porcines du plexus choroïde (CPEC) a permis de démontrer l'invasion et la translocation de *S. suis* au travers des CPEC du côté basolatéral. Ce processus semble influencé par la viabilité bactérienne, la présence de la CPS et les mécanismes de régulation du cytosquelette des CPEC. De façon intéressante et contrastante avec ce qui avait été observé précédemment, aucun dommage pour les cellules et aucune perte de l'intégrité de la barrière n'ont été observés dans cette étude. En effet, les expériences de translocation bactérienne ont démontré que *S. suis* traverse les CPEC en quelques heures, sans changements marqués au niveau des jonctions serrées. Les auteurs n'ont pas observé d'évidence morphologique ou biochimique du passage des bactéries entre les cellules [187].

Il a aussi été démontré que les cellules de la microglie sont impliquées dans l'activation de la réponse inflammatoire contre *S. suis*. De plus, la CPS de *S. suis* contribue à la résistance de ce dernier à la phagocytose par les cellules microgliales, en plus de réguler la réponse inflammatoire en cachant les composants pro-inflammatoires de la paroi bactérienne (ANNEXE IV).

1.4.6 Modèle proposé

Pour résumer, un modèle des différentes étapes impliquées dans la pathogenèse de la méningite causée par *S. suis* a été proposé (Figure 2) [4]. D'abord, *S. suis* interagirait avec les cellules épithéliales du système respiratoire supérieur (colonisation) pour ensuite accéder au système sanguin. Les souches produisant l'hémolysine sont toxiques et pourraient ainsi causer des dommages aux cellules et les envahir afin d'accéder à la circulation sanguine. Les mécanismes utilisés par les souches adhérentes hémolysine-négatives sont encore inconnus. Une hypothèse suggère que *S. suis* pourrait être directement internalisé par des monocytes ou des macrophages et entrer dans la circulation sanguine. Cependant, cette étape semble improbable puisque *S. suis* est hautement résistant à la phagocytose. D'autres voies d'entrée possibles incluent les abrasions/blessures au

niveau de la peau et la contamination orale suivie par une translocation au niveau du tractus intestinal. Une fois en circulation sanguine, les souches encapsulées de *S. suis* peuvent résister à la phagocytose et au « killing » assurés par le complément. En effet, les souches encapsulées de *S. suis* ont été démontrées comme pouvant être largement attachées, mais non phagocytées, par les monocytes/macrophages. Ainsi, le transport des bactéries dans le sang se ferait principalement sous la forme de bactéries libres ou de façon extracellulaire associées aux monocytes. La présence de bactéries en circulation cause une bactériémie ou une septicémie pouvant mener au choc septique. Différents mécanismes sont proposés pour expliquer comment *S. suis* peut traverser la BBB et causer la méningite. Les souches hémolysine-positives peuvent pénétrer au niveau du SNC après avoir augmenté la perméabilité de la BBB par une toxicité directe due à l'effet de la suilysine. Bien que l'apoptose puisse être impliquée, la nécrose des cellules formant la BBB est suggérée comme étant le mécanisme principal utilisé par ces souches. De plus, il a été rapporté que les souches suilysine-positives induisent la production de cytokines, d'acide arachidonique, de prostaglandine E et de métalloprotéinase par les cellules formant la BBB. Cette réponse inflammatoire pourrait aussi contribuer à l'augmentation de la perméabilité de la BBB, permettant l'accès des bactéries au SNC. Il a été suggéré que les bactéries hémolysine-négatives induisent principalement l'apoptose, possiblement par le relâchement de protéases bactériennes ou d'autres facteurs inconnus. La production de cytokines induites par les souches hémolysine-négatives pourrait aussi contribuer à l'augmentation de la perméabilité de la BBB. La production d'acide arachidonique, de prostaglandine E et de métalloprotéinase induites par les souches ne produisant pas l'hémolysine n'a pas encore été rapportée/évaluée. Néanmoins, la réponse inflammatoire induite par les deux types de souches (hémolysine positives et négatives) pourrait aussi résulter en une augmentation dans l'expression de molécules d'adhésion cellulaire et la migration de leucocytes, ce qui « ouvrirait la porte » à la circulation des bactéries libres. L'invasion directe et la translocation de bactéries libres (hémolysine positives ou négatives), au travers des cellules de la BBB ont été rapportées comme d'autres mécanismes permettant à *S. suis* de traverser la BBB. Finalement, des bactéries associées aux monocytes pourraient pénétrer le SNC par un « cheval de Troie » (bactéries à l'intérieur des cellules; peu probable) ou un « cheval de Troie modifié » (bactéries adhérées aux cellules; plus probable).

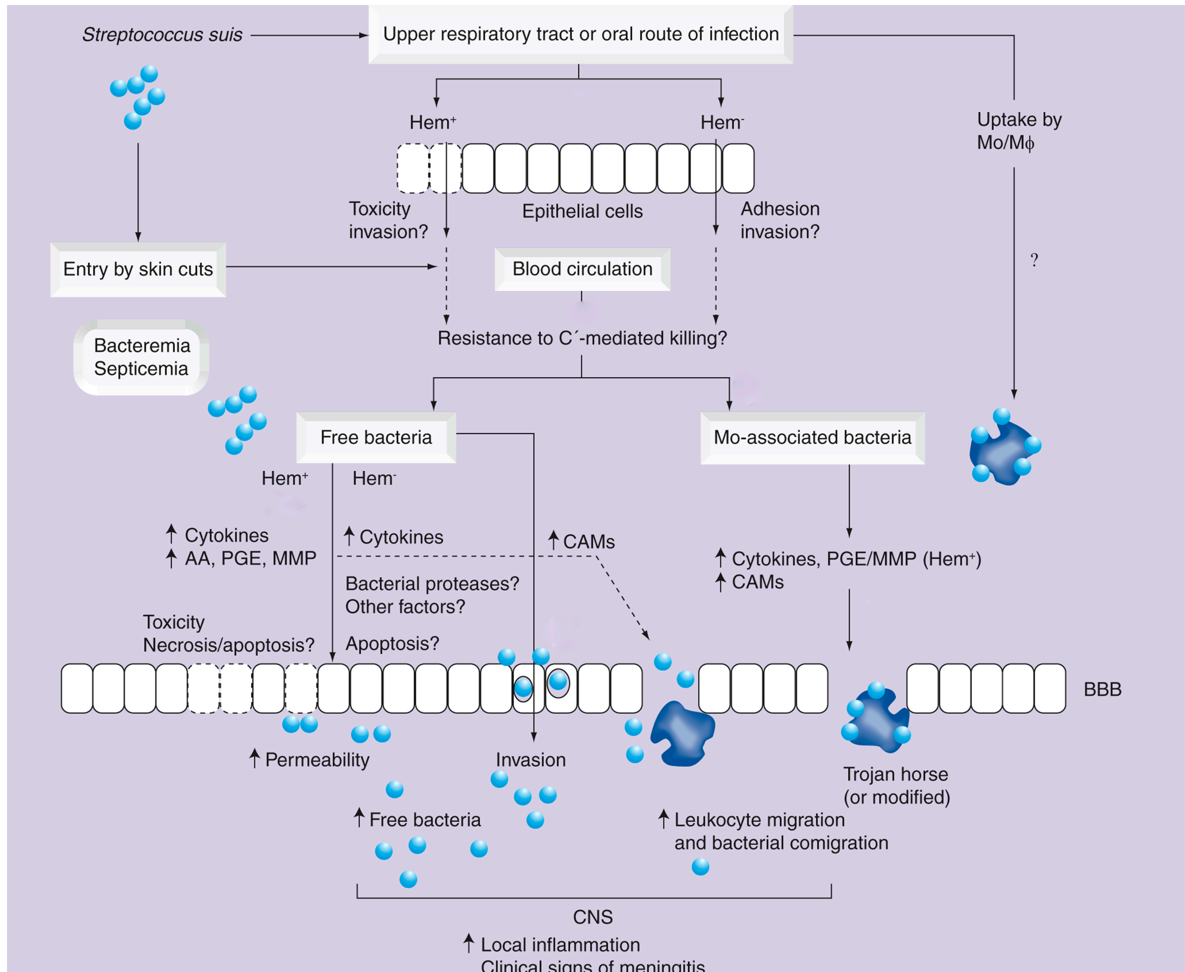

Figure 2. Hypothèses proposées pour les différentes étapes impliquées dans la pathogenèse de la méningite due à *S. suis* sérotype 2. AA : acide arachidonique; BBB : barrière hémato-encéphalée; C' : complément; CAM : molécule d'adhésion cellulaire; CNS : système nerveux central; Hem⁻ : souche hémolysine-négative; Hem⁺ : souche hémolysine-positive; Mo : monocyte; Mϕ : macrophage; MMP : métalloprotéinase; PGE : prostaglandine E. Modifié à partir de Gottschalk *et al.* [4].

2. Réponse immunitaire innée

2.1 Composantes de la réponse immunitaire innée

Chez les vertébrés, le système immunitaire se divise en système immunitaire inné et système immunitaire adaptatif. L'immunité innée est la première ligne de défense vis-à-vis des pathogènes. La reconnaissance des pathogènes par le système immunitaire inné repose principalement sur la reconnaissance de différentes molécules, soit les PAMPs et les DAMPs (motifs moléculaires associés à des signaux de danger), reconnus par différents récepteurs reconnaissant ces patrons (PRRs) [188,189]. Les PAMPs sont considérées comme des molécules exogènes, puisqu'ils sont associés aux pathogènes. Suite à la reconnaissance des PAMPs, les PRRs initient un programme de signalisation cellulaire complexe. De plus, la signalisation par les PRRs induit la maturation des cellules présentatrices d'antigènes, telles que les DCs, qui sont responsables de l'induction de la seconde ligne de défense de l'hôte, soit l'immunité adaptative.

Les cellules de l'immunité innée, dont les cellules présentatrices d'antigènes, peuvent aussi être activées par les DAMPs. Les DAMPs sont des molécules dérivées des cellules qui peuvent initier et perpétuer l'immunité en réponse à un trauma, une ischémie, un cancer ou d'autres situations où des tissus sont endommagés. Les DAMPs peuvent être localisés au niveau du noyau, du cytoplasme, des exosomes, de la matrice extracellulaire et dans des composants du plasma. Suite à l'interaction des DAMPs avec leurs récepteurs, des voies de signalisation cellulaire sont activées, menant à la survie ou à la mort cellulaire [190].

2.1.1 Récepteurs de type Toll (TLRs)

Les TLRs sont les PRRs les plus étudiés (Tableau III). Ils sont considérés comme les premiers senseurs des pathogènes. Les TLRs sont des protéines transmembranaires de type I possédant des ectodomaines contenant des répétitions riches en leucine qui permettent la reconnaissance des PAMPs; des domaines transmembranaires; et des domaines intracellulaires Toll–interleukine 1 (IL-1/TIR) requis pour la transduction du signal en aval. À ce jour, 10 et 12 TLRs fonctionnels ont été identifiés respectivement chez l'humain et la

souris. Les TLR1-TLR9 sont conservés chez les deux espèces. Le TLR10 murin n'est pas fonctionnel dû à l'insertion d'un rétrovirus et les TLR11, TLR12 et TLR13 ont été perdus chez le génome humain [191].

Les TLRs sont principalement divisés en deux sous-groupes dépendamment de leur localisation cellulaire et de leurs ligands respectifs. Un groupe est composé des TLR1, TLR2, TLR4, TLR5, TLR6 et TLR11. Ces derniers sont exprimés à la surface des cellules et reconnaissent principalement des composants de la membrane microbienne comme les lipides, les lipoprotéines et les protéines. L'autre groupe est composé des TLR3, TLR7, TLR8 et TLR9 qui sont exprimés exclusivement dans des vésicules intracellulaires tel que le réticulum endoplasmique, les endosomes, les lysosomes et les endolysosomes. Ces TLRs reconnaissent les acides nucléiques microbiens [191]. La localisation cellulaire des TLRs est importante pour l'accessibilité aux ligands, la maintenance de la tolérance aux molécules de soi comme les acides nucléiques, ainsi que pour la signalisation cellulaire.

Tableau III : TLRs et leurs ligands. Les TLRs reconnaissent une grande variété de patrons moléculaires des pathogènes, incluant les bactéries, les fungi, les protozoaires et les virus.

TLR	**Ligand**
TLR1/2	Lipopeptides triacylés (bactéries)
TLR2	LTA, PG (bactéries Gram positif), lipoprotéines, lipopeptides, LPS atypiques, lipoarabinomannane (mycobactéries) Zymosan, phospholipomannane (fungi) Ancres GPI (protozoaires) Protéines d'enveloppe virales
TLR3	Poly (I :C); ARN double brin (virus)
TLR4	LPS (bactéries Gram-négatif) Protéine de fusion RSV (virus) Glycoinositolphospholipides (protozoaires) Mannane, glucoronoxylomannane (fungi)
TLR5	Flagelline (bactéries)
TLR6/2	Lipopeptides diacylés (bactéries)
TLR7/TLR8	ARN simple brin (virus, bactéries) Molécules synthétiques imidazoquinoline-like
TLR9	ADN CpG (bactéries, virus, protozoaires) Hémozoïne (protozoaires)
TLR10 (pseudogène chez la souris)	Inconnu, peut former un hétérodimère avec TLR1 ou TLR2
TLR11 (souris)	Composants des bactéries uropathogènes
TLR12 (souris)	Inconnu
TLR13 (souris)	Inconnu

Adapté de Takeuchi et Akira [192].

2.1.1.1 PAMPs bactériens reconnus par les TLRs

Les TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 et TLR9 sont principalement dédiés à la reconnaissance de divers composants bactériens. Le LPS, un composant majeur de la paroi cellulaire des bactéries Gram-négatif est reconnu par le TLR4 complexé à MD2 [193]. Un

autre composant majeur essentiel des bactéries Gram-positif initialement identifié comme étant reconnu par le TLR2 est le PG. Par contre, la reconnaissance du PG par le TLR2 est toujours sujet à controverse [194,195]. Les mycobactéries, une classe de bactéries riches en lipoarabinomannan, sont aussi reconnues par le TLR2 [196]. TLR2, en complexe avec TLR1 ou TLR6 reconnait les lipopeptides diacylés ou triacylés à la surface des bactéries, mycobactéries et mycoplasmes [195]. TLR5 et TLR9 reconnaissent respectivement la flagelline exprimée par les bactéries flagellées ainsi que l'ADN génomique bactérien/viral riche en Cytosine-phosphate-Guanosine (CpG) non-méthylé [195]. L'ARN bactérien dérivé de GBS et produit dans les compartiments lysosomaux est reconnu par le TLR7 [197]. La reconnaissance de PAMPs par les TLR1, TLR2, TLR4, TLR5 et TLR6 induit principalement la production de cytokines inflammatoires, alors que les TLR7 et TLR9 induisent principalement des interférons (IFNs) de type I.

2.1.1.2 Signalisation par les TLRs

Après avoir reconnu leur PAMP respectif, les TLRs activent différentes voies de signalisation intracellulaire qui permettront ensuite d'induire des réponses immunologiques spécifiques adaptées aux pathogènes exprimant ce PAMP (Figures 3 et 4). La réponse spécifique initiée par les TLRs dépend du recrutement de différentes protéines adaptatrices (MyD88, TIRAP, TRIF ou TRAM) [191]. La protéine adaptatrice MyD88 est utilisée par tous les TLRs, à l'exception du TLR3. MyD88 transmet ensuite des signaux qui culminent en l'activation de NF-κB et des « mitogen-activated protein kinases » (MAPKs), ainsi qu'à la production de cytokines inflammatoires. TLR3 et TLR4 utilisent TRIF afin d'activer une voie alternative menant à l'activation de NF-κB et IRF3, et à l'induction des IFNs de type I et de cytokines pro-inflammatoires. TLR2 et TLR4 utilisent TIRAP comme protéine adaptatrice supplémentaire pour le recrutement de MyD88.

TLR4 est le seul TLR qui recrute quatre protéines adaptatrices et active deux voies de signalisation distinctes : la voie « MyD88-dépendante » et la voie « TRIF-dépendante » [191]. Ces deux voies de signalisation possèdent des cinétiques d'activation différentes. TLR4 recrute initialement TIRAP et MyD88. La protéine TIRAP est localisée à la membrane plasmique via son interaction avec PIP_2, où elle joue le rôle de pont entre

MyD88 et TLR4 suite à l'engagement par le LPS. MyD88 recrute alors « IL-1 receptor kinase » (IRAK), TRAF6 et le complexe TAK1, menant à l'activation de NF-κB et des MAPKs [191]. TLR4 est ensuite endocyté et transporté vers des vésicules intracellulaires afin de former un complexe avec TRAM et TRIF, qui recrutent ensuite TRAF3 et les protéines kinases TBK1 et IKKi, ce qui catalyse la phosphorylation de IRF3, menant à l'expression des IFNs de type I. TRAM-TRIF recrutent aussi TRAF6 et TAK1 pour induire l'activation tardive de NF-κB et des MAPKs.

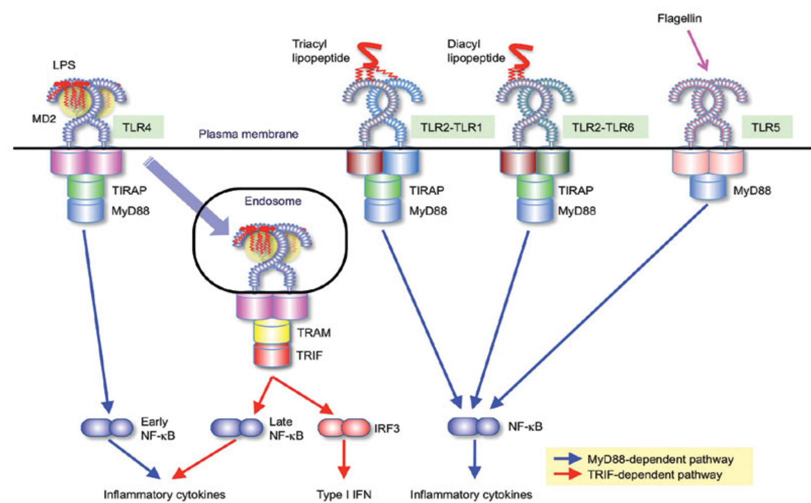

Figure 3. Reconnaissance des PAMPS par les TLRs extracellulaires. Le LPS engage le complexe formé par le TLR4 et MD2. Un premier signal est transmis pour la phase d'activation précoce de NF-κB en recrutant TIRAP et MyD88. Le complexe TLR4-MD2-LPS est alors internalisé et retenu dans le compartiment endosomal où il induit la signalisation en recrutant TRAM et TRIF, menant à l'activation de IRF-3 et de la phase tardive de NF-κB pour la production d'IFN de type I (voie TRIF-dépendante). Les deux phases d'activation (précoce et tardive) sont nécessaires à la production de cytokines inflammatoires. Les hétérodimères TLR2-TLR1 et TLR2-TLR6 induisent l'activation de NF-κB via le recrutement de TIRAP et MyD88. TLR5 active NF-κB via MyD88. Reproduit à partir de Kawai *et al.* [191].

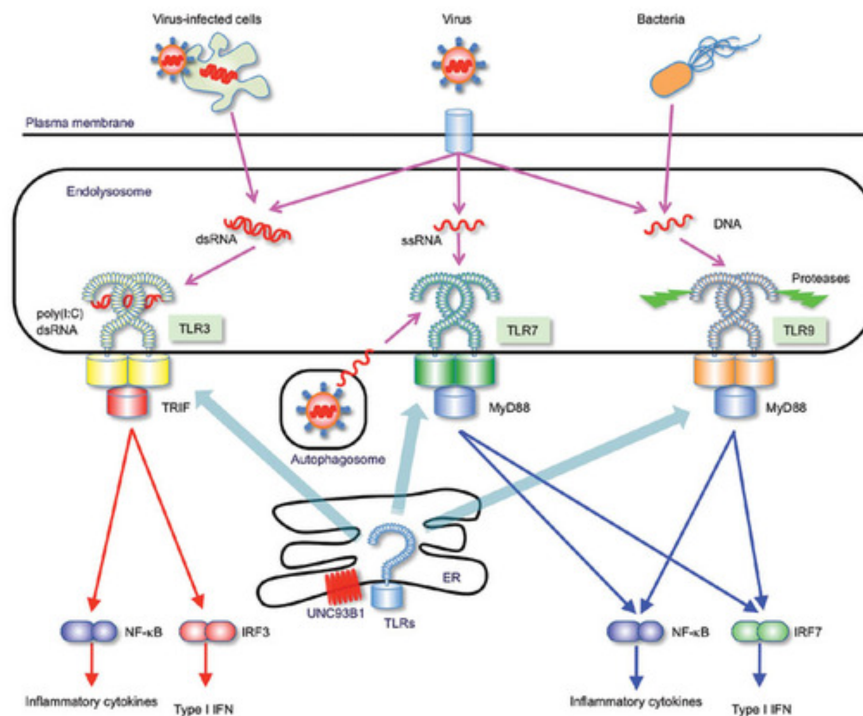

Figure 4. Reconnaissance des PAMPs par les TLRs intracellulaires. TLR3 active la voie TRIF-dépendante, menant à la production d'IFN de type I et de cytokines inflammatoires. TLR7 active NF-κB et IRF7 via MyD88 et induisant respectivement la production de cytokines inflammatoires et d'IFN de type I. TLR9 recrute MyD88 pour activer NF-κB et IRF7. Reproduit à partir de Kawai *et al.* [191].

2.1.1.2.1 Signalisation MyD88-dépendante

Dans la voie de signalisation MyD88-dépendante, suite à la liaison du ligand, il y a dimérisation du TLR/IL-1, ce qui induit un changement de conformation permettant le recrutement de la protéine adaptatrice MyD88. Suite à la stimulation, MyD88 recrute ensuite IRAK au TLR via l'interaction des « death domains » des deux molécules. Deux membres de la famille IRAK (IRAK-1 et IRAK-4) sont activés par phosphorylation, dissociés de MyD88 et associés avec TRAF6. TRAF6 forme un complexe avec l'enzyme de conjugaison d'ubiquitine pour activer TAK1. TAK1 forme ensuite un complexe avec TAB1, TAB2 et TAB3. TAK1 mène à l'activation de NK-κB (IκB) et « activator protein-1 » (AP-1) via la kinase IκB (IKK) et MAPK, respectivement. L'activation de AP-1 est principalement médiée par les MAPKs comme la kinase c-Jun N-terminal, p38 et ERK [198-200] (Figure 5).

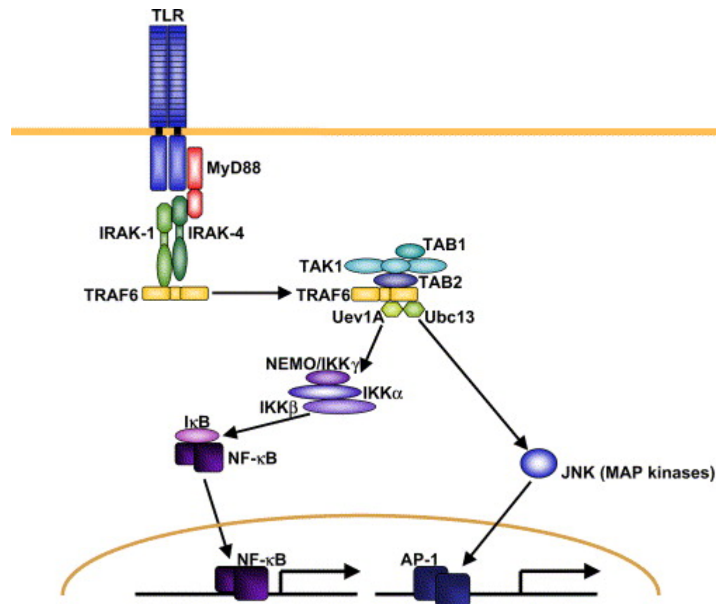

Figure 5. Signalisation MyD88-dépendante. La protéine adaptatrice MyD88 se lie à la portion cytoplasmique des TLRs. Suite à la stimulation, IRAK-4, IRAK-1 et TRAF6 sont recrutés au récepteur, ce qui induit l'association entre IRAK-1 et MyD88 via les « death domains ». IRAK-4 phosphoryle ensuite IRAK-1. IRAK-1 phosphorylé, avec l'aide de TRAF6, se dissocie du récepteur, permettant l'interaction de TRAF6 avec TAK1, TAB1 et TAB2. Le complexe TRAF6, TAK1, TAB1 et TAB2 forme ensuite un second complexe avec Ubc13 et Uev1A, ce qui induit l'activation de TAK1. TAK1 activé phosphoryle ensuite le complexe IKK (formé de IKKα, IKKβ, NEMO/IKKγ ainsi que de kinases MAP comme JNK) induisant l'activation des facteurs de transcription NK-κB et AP-1. Reproduit à partir de Takeda *et al*. [201].

2.1.1.2.2 Signalisation MyD88-indépendante

L'activation d'IL-1R ou TLR recrute IRAK au complexe via la protéine adaptatrice MyD88, suivi de l'activation de TRAF6, ce qui mène ultimement à l'activation de NF-κB et MAPK. Les macrophages MyD88 KO ne répondent pas au LPS ou à d'autres dérivés bactériens tel que le PG ou l'ADN riche en CpG, ce qui démontre le rôle essentiel joué par la protéine adaptatrice MyD88 dans la réponse contre les pathogènes [199]. Cependant, bien que le LPS n'induise pas la production de cytokines, l'activation de NF-κB et MAPK est tout de même observée, mais selon une cinétique différente [199]. Ceci indique que la réponse induite par le LPS pourrait être médiée à la fois par une voie de signalisation MyD88-dépendante et une voie de signalisation MyD88-indépendante, chacune d'elle

menant à l'activation de NF-κB et MAPK. La voie de signalisation MyD88-indépendante implique, via les molécules d'adaptation TRAM et TRIF, l'activation du facteur de transcription IRF-3 ainsi que l'induction d'IFN-β, ce qui active ensuite STAT1 et mène à l'activation de plusieurs gènes inductibles par l'IFN [199]. Les ligands de TLR3 activent aussi IRF-3. Ainsi, TLR3 et TLR4 utilisent la voie MyD88-indépendante [199].

2.1.1.3 Importance des TLRs lors de l'infection par *S. suis*

Les récepteurs impliqués dans l'activation des cellules par *S. suis* ont été partiellement étudiés. Il a été démontré que la stimulation de monocytes humains par *S. suis* encapsulé ou par sa paroi cellulaire purifiée influence l'expression de l'ARNm de TLR2 et CD14 [56]. Récemment, une étude a démontré que TLR2 et TLR6 sont activés par des lipoprotéines de *S. suis* sérotypes 2 et 9 [202]. La stimulation de ces récepteurs induit la sécrétion de cytokines et chimiokines, laquelle est significativement diminuée suite à l'utilisation d'un anticorps neutralisant contre TLR2 mais non contre TLR4 [56]. Des macrophages murins déficients en TLR2 ont aussi démontré une production réduite de cytokines pro-inflammatoires en réponse à *S. suis* encapsulé. Cette réponse est complètement abolie chez des macrophages déficients pour la protéine adaptatrice MyD88. Aussi, d'autres TLRs pourraient également être impliqués dans la production MyD88-dépendante de cytokines par *S. suis* encapsulé. De plus, il a été démontré que la présence de la CPS module les interactions de *S. suis* avec les TLRs. En absence de CPS, les composants de la paroi cellulaire semblent induire la production de cytokines et de chimiokines via des voies de signalisation TLR2-dépendantes et indépendantes. Cependant, la CPS contribue à la production de MCP-1/CCL2 de façon MyD88-indépendante et TLR-indépendante [56,203]. De plus, la CPS induit la production de MIP-1α/CCL3 par les DCs selon des mécanismes partiellement dépendants du TLR2 et du facteur MyD88 [203]. Suite à l'invasion de *S. suis* au niveau du système nerveux central, une activation transcriptionnelle de TLR2, TLR3 et CD14 a été observée [177]. Une étude récente a rapportée que *S. suis* induit une augmentation de l'expression de TLR2 chez des astrocytes murins, et que des astrocytes isolés chez des souris déficientes pour l'expression du TLR2 présente une réduction significative de la production de cytokines pro-inflammatoires en réponse à *S.*

suis [204]. Très récemment, une étude a démontré une implication des TLR2, 6 et 9 en réponse à *S. suis* ST1 et ST7 [205].

Il a récemment été démontré que la reconnaissance de *S. suis* par le TLR2 contribue à l'infection aigue causée par *S. suis* ST1, puisque des souris TLR2$^{-/-}$ se sont avérées plus résistantes à l'infection comparativement aux souris de type sauvage (WT) [206]. La survie n'est pas associée à une charge bactérienne moins élevée. Cependant, une diminution significative dans la production des médiateurs pro-inflammatoires a été observée. D'un autre côté, des souris TLR2$^{-/-}$ infectées avec une souche provenant d'une épidémie de *S. suis* ST7 n'ont présenté aucune différence significative au niveau de la survie et de la production des médiateurs pro-inflammatoires comparativement aux souris WT. Ces résultats suggèrent qu'une réponse immune TLR2-indépendante peut être induite par *S. suis* dépendamment de la souche responsable de l'infection [206].

2.1.2 Récepteurs de type NOD (NLRs): NOD1 et NOD2

Les récepteurs de type NOD (NLRs) reconnaissent une grande variété de ligands à l'intérieur du cytoplasme des cellules. À ce jour, on dénombre 23 membres de cette famille chez l'humain et approximativement 34 chez la souris. Parmi ceux-ci, certains sont très bien caractérisés. Ces récepteurs sont formés de trois domaines. Le domaine C-terminal consiste de plusieurs répétitions riches en leucine et semble être impliqué dans la reconnaissance des PAMPs microbiens, ou molécules endogènes de l'hôte. Le domaine N-terminal est plus variable et peut être constitué d'un domaine DED (« death effector domain »), d'un domaine PYD (« pyrin domain »), d'un domaine CARD (« caspase activation and recruitment domain »), de motifs BIRs (« baculovirus inhibitor repeats ») et/ou d'un domaine « acidic transactivating ». Le domaine N-terminal est requis pour les interactions homotypiques avec les protéines de signalisation. Finalement, le domaine central NACHT (« nucleotide-binding and oligomerization domain ») est requis pour l'oligomérisation de senseurs ATP-dépendant et la formation du complexe activé du récepteur pour l'activation de la cascade de signalisation.

Les membres de la famille des NLRS sont divisés en au moins cinq sous-familles selon la structure de leur domaine N-terminal [207]. Suite à la reconnaissance des PAMPs, les NLRs activent NF-κB ou les MAPKs afin d'induire la production de cytokines inflammatoires ou d'activer un complexe protéique, l'inflammasome, initiant le clivage protéolytique de diverses caspases, résultant en la maturation et la production de cytokines inflammatoires comme IL-1β et IL-18, ou initiant la mort cellulaire [208].

NOD1 et NOD2, de la famille des NLRs, sont très bien caractérisés. Ces récepteurs sont principalement exprimés dans le cytosol de diverses cellules. Néanmoins, leur expression à la membrane plasmique a aussi été rapportée [209,210]. Ils reconnaissent des motifs structuraux distincts du PG. NOD1 reconnaît l'acide g-D-glutamyl-meso-diaminopimelique (iE-DAP), retrouvé dans les structures du PG de toutes les bactéries Gram-négatif ainsi que chez certaines bactéries Gram-positif tel que *B. subtilis* et *L. monocytogenes*. Au contraire, NOD2 reconnaît le muramyl dipeptide, un composant du PG présent chez toutes les bactéries Gram-positif et Gram-négatif [207]. L'importance des NODs dans l'immuno-pathogenèse de l'infection a été démontré chez plusieurs pathogènes bactériens tel que *Staphylococcus aureus, S. pneumoniae*, *S. pyogenes*, *L. monocytogenes*, *H. influenzae* et autres [211-214].

3. Réponse immunitaire adaptative T-dépendante

3.1 Cellules dendritiques : lien entre les réponses immunes innée et adaptative

Steinman et Cohn furent les premiers à identifier et caractériser les DCs dans une série d'études publiée en 1973. Alors qu'ils examinaient des cellules spléniques adhérentes chez la souris, ils ont observé une caractéristique unique chez ces cellules, soit des extensions cellulaires de type « dendritique ». C'est ainsi que ces cellules furent nommées « cellules dendritiques » [215].

Les DCs sont des cellules dérivées de la moelle osseuse et sont retrouvées dans tous les tissus lymphoïdes et la plupart des tissus non-lymphoïdes. Les DCs représentent un groupe hétérogène de cellules présentatrices d'antigènes possédant la capacité d'activer les cellules T naïves [216]. En effet, les DCs sont les principales cellules contrôlant l'activation et la régulation des lymphocytes T. Elles sont aussi impliquées dans la modulation des réponses des cellules B et des cellules « Natural Killer » (NK).

Bien qu'au départ on croyait que les DCs représentaient un groupe homogène de cellules, il s'est rapidement avéré que les DCs sont plutôt très hétérogènes. Elles peuvent être classées en différentes sous-populations selon leur localisation anatomique, leur stade de différenciation, leur phénotype et leurs fonctions.

3.1.1 Cellules dendritiques murines

Les DCs murines sont amplement utilisées par les chercheurs qui étudient leurs rôles dans différents contextes d'infection. Bien qu'il existe des différences entre les DCs humaines et murines, elles représentent tout de même un modèle important et approprié pour l'étude de plusieurs infections.

3.1.1.1 Origine et développement des cellules dendritiques murines

Les DCs murines, dérivées des cellules souches hématopoïétiques $CD34^{+}$, sont classiquement regroupées en DCs myéloïdes et DCs lymphoïdes. Les évidences de

l'existence des DCs myéloïdes proviennent principalement d'études *in vitro* dans lesquelles des précurseurs myéloïdes ont générés à la fois des granulocytes/monocytes et des DCs myéloïdes sous l'influence du GM-CSF [217,218]. Les DCs peuvent aussi provenir de précurseurs lymphoïdes [219]. Cependant, il est maintenant reconnu que les sous-populations de DCs murines ne sont pas aussi facilement séparées en ces deux lignées distinctes. Il reste toujours à savoir si les différentes sous-populations de DCs ont un précurseur commun. Manifestement, les DCs peuvent être dérivées de précurseurs myéloïdes ou lymphoïdes. Elles pourraient être différenciées de précurseurs lymphoïdes ou myéloïdes dépendamment des conditions environnementales telles que leur localisation et la présence d'inflammation ou d'infection [220]. Par exemple, les DCs peuvent aussi être séparées en sous-populations selon l'expression de CD4 et CD8α, des marqueurs qui étaient initialement utilisés seulement pour la différenciation des cellules T [221]. Bien que l'origine lymphoïde des DCs ait été démontrée seulement pour les DCs $CD8\alpha^+$, la similarité du phénotype des DCs originant du thymus aux DCs spléniques $CD8\alpha^+$ et aux DCs des ganglions lymphatiques suggère une origine commune [216]. Cependant, une étude suggère que la présence de CD8α en surface des DCs n'indique pas une origine lymphoïde, mais reflète plutôt le degré de maturation ou de différenciation et prédit la fonction des DCs [222]. Un modèle théorique du développement des DCs murines est suggéré [220] (Figure 6).

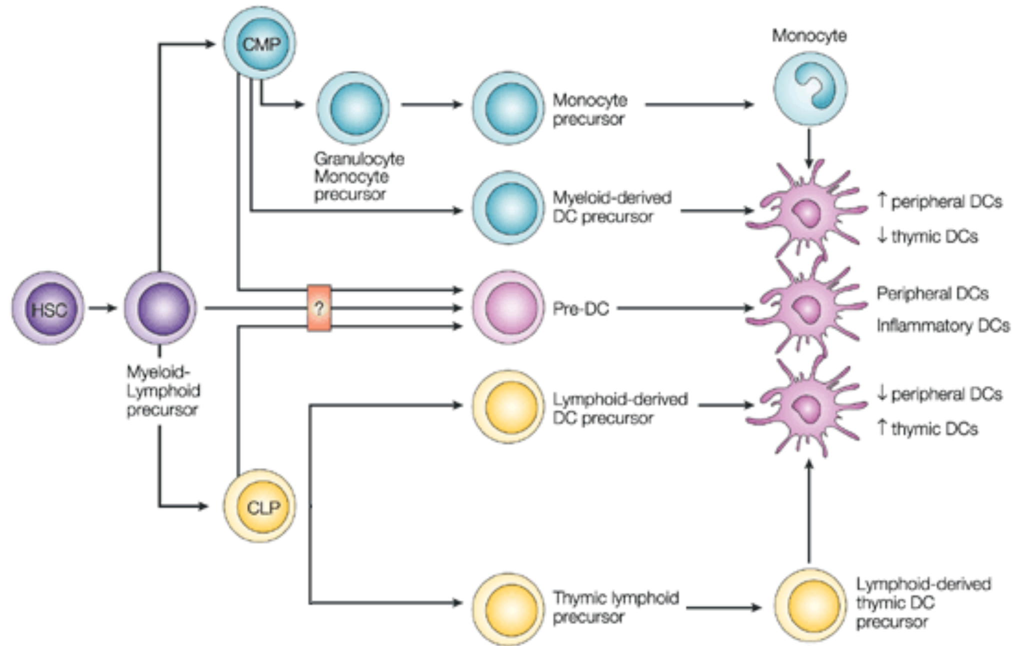

Figure 6. Modèle théorique du développement des DCs murines. La différenciation des DCs, incluant les DCs CD8α$^-$, CD8α$^+$, B220$^+$ et les cellules de Langerhans, été proposée comme découlant directement de précurseurs myéloïde (CMP) et lymphoïde (CLP), ainsi que d'un précurseur commun circulant (Pre-DC). Il a été établit que la différenciation des DCs thymiques résulte d'une contribution égale de ces précurseurs, alors que les DCs périphériques sont principalement dérivées des CMPs. Il est suggéré que les monocytes et pré-DCs sont impliqués dans la génération des DCs après leur recrutement aux sites réactifs. Reproduit à partir d'Ardavin [220].

HSC : Hematopoietic stem cell; CMP : Common myeloid progenitor; CLP : Common lymphoid progenitor;

Sur les bases du nombre absolu de progéniteurs lymphoïdes communs (CLPs) et de progéniteurs myéloïdes communs (CMPs), il a été établi que la différenciation des DCs originant du thymus résulte d'une contribution égale des deux précurseurs, alors que les DCs périphériques sont principalement dérivées des CMPs [223,224]. Les précurseurs des DCs lymphoïdes pourraient être dérivés des CLPs localisés dans la moelle osseuse ou des précurseurs lymphoïdes du thymus. Les pré-DCs et les monocytes ont été proposés comme étant impliqués dans la génération des DCs après leur recrutement au site d'infection [225]. Une étude D'Amico *et al.* propose que la différenciation des précurseurs des DCs est

corrélée avec l'expression du récepteur CD135, aussi nommé Flt3 (« fms-related tyrosine kinase 3 ») [226]. Flt3/CD135 est le récepteur de la cytokine « Flt3 ligand ». De plus, il a été rapporté que les CLPs (qui sont majoritairement $Flt3^+$) sont plus efficaces dans la génération des DCs, spécialement des DCs plasmacytoïdes (pDCs), que la fraction de CMPs $Flt3^+$ [220]. Flt3 a pour cible les progéniteurs hématopoïétiques de la moelle osseuse et induit leur expansion et leur différenciation [227]. Le nombre de DCs myéloïdes et lymphoïdes augmente significativement suite à l'injection de Flt3 [216]. De façon intéressante, le GM-CSF peut augmenter le nombre de DCs matures chez la souris, mais augmente préférentiellement les sous-populations de DCs myéloïdes *in vivo* [216].

3.1.1.2 Sous-populations de cellules dendritiques murines

En général, les DCs peuvent être regroupées en deux familles distinctes. La première famille comprend les DCs résidant dans les organes lymphoïdes secondaires, alors que la deuxième famille est constituée des DCs des tissus périphériques qui migrent vers les ganglions lymphatiques en conditions inflammatoires. Les DCs sont identifiées comme étant $CD11c^{high}CMH\text{-}II^+$, et peuvent être divisées en sous-groupes selon l'expression en surface de molécules appartenant à la lignée myéloïde ou lymphoïde. La rate des souris contient les sous-populations $CD8\alpha^+CD11b^-CD4^-$, $CD8\alpha^-CD11b^+CD4^+$, $CD8\alpha^-CD11b^+CD4^-$. Les deux dernières sous-populations sont fréquemment analysées ensemble en tant que sous-population $CD8\alpha^-$, comptant pour 75% des DCs spléniques chez la souris [220,228]. Ces sous-populations sont aussi retrouvées dans les ganglions lymphatiques périphériques, les plaques de Peyer, les ganglions lymphatiques mésentériques et le foie, à l'exception des DCs $CD4^+$ qui sont rares ou absentes dans les tissus autres que la rate. À ce jour, la pertinence fonctionnelle de la différenciation des DCs selon l'expression de CD4 demeure controversée. Les plaques de Peyer, les ganglions mésentériques et le foie possèdent aussi une sous-population de DCs $CD8\alpha^-CD11b^-$ [229].

Certains auteurs stipulent que les $CD4^-CD8\alpha^-$ et $CD4^+CD8\alpha^-$ originent de deux sous-populations de DCs fonctionnellement indépendantes. Au contraire, une autre étude suggère que les DCs $CD4^-CD8\alpha^-$ constituent une forme plus activées ou différenciées des $CD4^+CD8\alpha^-$ [230]. Il a toutefois été démontré que les DCs $CD8\alpha^+$ sont principalement

localisées dans les zones riches en cellules T des organes lymphatiques, alors que les DCs CD8α$^-$ sont surtout présentes les zones environnantes, principalement dans les zones de captures des antigènes, et semblent se localiser dans les zones riches en cellules T seulement sous conditions inflammatoires (150).

Les DCs migratoires retrouvées dans les organes non-lymphoïdes incluent les cellules de Langerhans (LCs), les DCs de la peau et les DCs interstitielles [215]. Ces DCs migratoires sont aussi détectées dans les ganglions lymphatiques, mais ne sont pas présentes dans la rate puisqu'elle n'a pas d'afférences lymphatiques [231].

Finalement, l'expression de B220 (isoforme de CD45) définit la population murine fonctionnellement équivalente des pDCs humaines. Les pDCs B220$^+$ sont retrouvées dans tous les organes lymphoïdes de la souris et sont caractérisées par leur potentiel à produire de grande quantité d'IFN de type I lors d'infections virales [232]. Il a aussi été proposé que les pDCs B220$^+$ soient impliquées dans le maintien de la tolérance en induisant la différenciation des cellules T régulatrices (Tregs) qui peuvent empêcher l'activation des cellules T naïves selon un mécanisme IL-10-dépendant [233]. Les pDCs B220$^+$ expriment des niveaux différents de CD8α selon leur localisation. En plus des sous-populations de DCs décrites ci-haut, d'autres sous-populations ont aussi été décrites dans des organes spécifiques de la souris tel que les poumons, le cœur et les reins. Par contre, les données obtenues à ce jour ne permettent pas encore d'assigner ces DCs à des sous-populations particulières [220]. Le tableau IV fournit un bref résumé des sous-populations de DCs décrites ci-haut.

Tableau IV: Distribution des sous-populations de DCs murines

Sous-populations de DCs	Thymus	Rate	Ganglion lymphatique	Plaques de Peyer	Peau	Foie
$CD8^-$ DCs	*	+	+	+	-	+
$CD8^+$ DCs	+	+	+	+	-	+
$CD8^{int}$ DCs	-	-	+	-	-	-
Cellules de Langerhans	-	-	-	-	+	-
DCs dermiques	-	-	-	-	+	-
$B220^+$ DCs	+	+	+	+	-	N. D.

*Les $CD8^-$ DCs peuvent être détectées dans le thymus, bien qu'elles constituent une minuscule proportion des DCs thymiques. +, présentes; -. absentes; $CD8^{int}$, niveau intermédiaire d'expression de CD8; DC, cellule dendritique; N. D., non déterminé.
Adapté à partir de Lipscomb *et al.* [221].

Les DCs ne diffèrent pas seulement phénotypiquement, mais aussi fonctionnellement. Par exemple, les DCs $CD8\alpha^+$ et $CD8\alpha^-$ diffèrent dans l'expression des récepteurs « C-type lectin » (CLRs) et expriment différents TLRs [231]. Les sous-populations de DCs diffèrent aussi dans leur capacité à apprêter et présenter certains types d'antigènes. Par exemple, les DCs $CD8\alpha^+$ sont spécialisées pour la présentation croisée des antigènes aux cellules T $CD8^+$ et dans le maintien de la tolérance, alors que les DCs $CD8\alpha^-$ présentent plus efficacement les antigènes sur les molécules CMH-II pour l'activation des cellules T $CD4^+$ [228,234]. De plus, les DCs $CD8\alpha^-$ semblent avoir une meilleure capacité à endocyter et phagocyter que les DCs $CD8\alpha^+$ [235]. Ces caractéristiques spécifiques à chaque sous-population de DCs permettent d'optimiser leurs rôles lors de la mise en place de la réponse immunitaire adaptative [215].

3.1.1.3 Cellules dendritiques murines générées *in vitro*

Traditionnellement, les DCs murines sont générées *in vitro* à partir des cellules souches hématopoïétiques de la moelle osseuse. La technique la plus fréquemment utilisées est décrite par Inaba *et al*. [218]. L'étape clé est d'éliminer la majorité des cellules non-adhérentes (granulocytes) pendant les 2-4 premiers jours de culture *in vitro*. Cela permet la prolifération des colonies de DCs qui sont faiblement adhérentes. Après 5-7 jours de culture, selon la technique utilisée, les DCs peuvent être récupérées. Ces DCs expriment en surface un haut nombre de molécules CMH-II. La génération de ces DCs requiert la présence de GM-CSF lors de la culture [218].

D'autres cytokines ont aussi été suggérées comme améliorant le rendement et la prolifération des DCs *in vitro*. Une étude de Shurin *et al*. rapporte que la culture de cellules spléniques ou de cellules souches de la moelle osseuse en présence de GM-CSF et IL-4 donne un grand nombre de DCs matures et fonctionnellement actives [227]. Il a aussi été démontré que l'ajout du ligand de Flt3 augmente le rendement de DCs générées *in vitro* à partir de précurseurs hématopoïétiques. Par exemple, Siena *et al*. ont démontré que la génération de DCs en présence de GM-CSF et de TNF-α est augmentée de 2.5 fois par la présence du ligand de Flt3 ou du ligand de KIT (« receptor-type protein-tyrosine kinase ligand ») [236]. Le ligand de KIT (aussi nommé « stem cell factor ») est en effet connu pour mobiliser les cellules souches hématopoïétiques de la moelle osseuse vers le sang périphérique et pour favoriser leur prolifération et leur différenciation en présence d'autres facteurs de croissance.

3.1.2 Cellules dendritiques porcines

Les DCs porcines représentent un modèle important pour l'étude du développement de la réponse immunitaire de l'hôte lors de l'infection par *S. suis*.

3.1.2.1 Cellules dendritiques porcines générées *in vitro*

Plusieurs différentes techniques sont décrites dans la littérature concernant la génération de DCs porcines *in vitro*. Les sections suivantes décrivent les principales techniques utilisées,

soit à partir des monocytes sanguins et des cellules souches hématopoïétiques de la moelle osseuse.

3.1.2.1.1 DCs porcines dérivées des monocytes sanguins (moDCs)

De façon similaire aux autres espèces, les DCs porcines peuvent être générées à partir des monocytes sanguins stimulés avec l'IL-4 et le GM-CSF. Après 3 à 7 jours de culture, les cellules non-adhérentes ou faiblement adhérentes présentant des caractéristiques morphologiques typiques des DCs peuvent être récoltées [237,238]. Il a aussi été démontré qu'IL-13 peut être utilisé en remplacement d'IL-4 pour la génération des moDCs [239]. Phénotypiquement, les moDCs porcines sont caractérisées comme étant $CD1^{+}CD14^{+}CD16^{+}CD80/86^{+}CD172a^{+}$ et $CMH\text{-}II^{+}$ [240]. Chez l'humain, CD14 est considéré comme un marqueur typique des monocytes et des macrophages. Néanmoins, les moDCs de différentes espèces, incluant le porc, ont aussi été démontré comme exprimant CD14. Parmi les autres marqueurs, CD172a correspond au SWC3 (« swine workshop cluster 3 ») qui est exprimé chez les cellules de descendance myéloide [241]. Fonctionnellement, ce marqueur représente SIRP-α (« signal regulatory protein alpha »). Ensemble, la co-expression de CD172a et CD1 avec une haute expression de CD80/86 et CMH-II représentent les caractéristiques phénotypiques des moDCs porcines. Par contre, aucun marqueur ne différencie clairement les moDCs porcines des macrophages dérivés des monocytes sanguins [240]. Les moDCs porcines générées en présence de GM-CSF et IL-4 sont considérées immatures et représentent un modèle approprié pour étudier la maturation des DCs.

3.1.2.1.2 DCs porcines dérivées de la moelle osseuse (bmDCs)

Des DCs porcines ressemblant phénotypiquement et fonctionnellement aux moDCs porcines peuvent aussi être générées à partir des cellules hématopoïétiques de la moelle osseuse stimulées en présence de GM-CSF et de TNF-α ou seulement en présence de GM-CSF pour une durée de 7 à 10 jours [237]. Au contraire du GM-CSF, la stimulation avec le ligand de Flt3 induit la différenciation à la fois de DCs conventionnelles (cDCs) et de pDCs. De plus, les cDCs générées en présence du ligand de Flt3 diffèrent à la fois phénotypiquement et fonctionnellement des moDCs et bmDCs obtenues en présence de

GM-CSF. Le ligand de Flt3 induit la différenciation de cDCs CD14$^-$ qui sont plus sensibles à la stimulation par les ligands de TLR2/TLR6, TLR3, TLR4, TLR5 et TLR7 et terme de production de cytokines et de maturation [242].

3.2 Activation et maturation des cellules dendritiques

Typiquement, en conditions stables, les progéniteurs de la moelle osseuse des DCs donnent les précurseurs immatures des DCs qui circulent librement avant de s'installer dans un tissu particulier où ils vont continuellement échantillonner l'environnement afin de détecter des antigènes étrangers avant de retourner vers les ganglions lymphatiques [216]. Sous ces conditions, les DCs présentes de grandes capacités phagocytaires et expriment de faibles niveaux de molécules du CMH et de molécules de co-stimulation. En absence d'inflammation ou de pathogènes, les DCs sont importantes pour l'induction de la tolérance aux antigènes de soi [216]. Cependant, suite à la reconnaissance d'un signal de danger, les DCs doivent subir un processus de maturation afin d'avoir la capacité d'activer les cellules T et autres cellules accessoires à la réponse immune. Ainsi, la maturation des DCs est associée avec plusieurs changements physiologiques [216].

3.2.1 Capture et apprêtement des antigènes

Dans un contexte d'infection, l'activation des DCs est initiée par la reconnaissance des PAMPs par les PRRs, par exemple les TLRs. La capture des antigènes est une étape cruciale qui permet aux DCs d'exercer leurs fonctions, incluant la survie, la prolifération, la production de cytokines et chimiokines ainsi que l'expression en surface de molécules de co-stimulation. Ces fonctions sont influencées par les récepteurs discutés ci-haut.

La présentation des antigènes se fait via deux types différents de molécules du CMH (Figure 7) : les molécules du CMH de classe I (CMH-I) et les molécules du CMH de classe II (CMH-II). La présentation via le CMH-I peut être réalisée par tous les types cellulaires et permet la présentation de peptides aux cellules T CD8$^+$ [189]. La présentation de peptides antigéniques aux cellules T CD4$^+$ se fait via les molécules du CMH-II. Au contraire du CMH-I, la présentation d'antigènes par le CMH-II est réservée aux cellules présentatrices d'antigènes [189]. Les antigènes exogènes sont internalisés par les cellules présentatrices

d'antigènes, dont les DCs, et accèdent aux compartiments endosomaux à l'intérieur desquels des protéases initient la dégradation des antigènes [221]. Cependant, avant que cela se produise, les cellules présentatrices d'antigènes immatures accumulent constamment des molécules CMH-II dans des compartiments reliés aux lysosomes. Ces compartiments sont désignés « compartiments riches en molécules CMH-II » (MIIC). Les chaînes peptidiques α et β des molécules CMH-II sont synthétisées dans le réticulum endoplasmique, où elles sont associées avec la chaîne invariante [243]. La chaîne invariante protège le site de liaison du peptide (« peptide-binding groove ») de l'hétérodimère de CMH-II prématurément occupé par des protéines du soi. Le complexe CMH-II/chaîne invariante est alors transporté du réticulum endoplasmique jusqu'au Golgi où des vésicules apportent les complexes nouvellement synthétisés au MIIC. La chaîne invariante est alors partiellement clivée par la cathepsine, laissant seulement un petit fragment appelé CLIP (« class II-associated invariant-chain peptide ») dans le site de liaison du peptide de la molécule CMH-II [244]. Ensuite, le peptide CLIP est retiré sous l'action de HLA-DM chez l'humain et H-2M chez la souris [221]. L'action de la cathepsine est sous le contrôle de la cystatine, un inhibiteur de la cathepsine. L'activité de la cystatine est diminuée durant le processus de maturation des DCs [245]. Suite à l'internalisation de l'antigène, les polypeptides antigéniques contenus dans le phagolysosome fusionnent vers les MIICs où les peptides vont s'associer avec les molécules CMH-II pré-formées. Finalement, les molécules CMH-II avec leurs peptides antigéniques vont traverser le cytoplasme dans des vacuoles exocytiques pour être exposés en surface des cellules. Chez les DCs immatures, les molécules CMH-II sont rapidement internalisées et ont une courte demi-vie. La maturation des DCs et la présence de signaux inflammatoires augmentent la translocation des molécules CMH-II en surface cellulaire où elles demeurent stables et disponibles pour la reconnaissance par les cellules T $CD4^+$ [246]. Il a récemment été démontré que les molécules de co-stimulation étant membres de la famille B7 sont intégrées aux lipides vésiculaires avec les molécules CMH-II et délivrées à la surface cellulaire en association avec les complexes CMH-II/peptide [247].

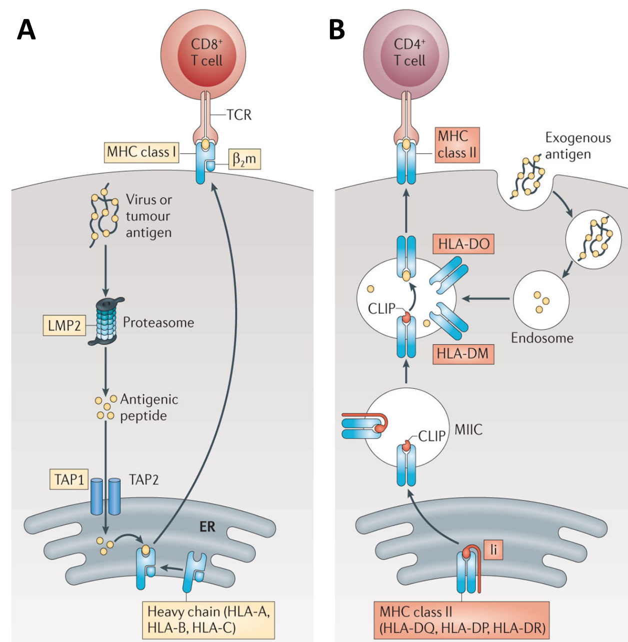

Figure 7 : Mécanisme de présentation des antigènes par les DCs. A) Les antigènes intracellulaires, tels que les virus ou les antigènes tumoraux, sont apprêtés en peptides par l'immunoprotéasome composé de plusieurs sous-unités dont LMP2. Les peptides sont transportés vers le réticulum endoplasmique (ER), où ils sont chargés dans le sillon du complexe CMH-I composé de la chaîne lourde et de la β_2-microglobuline (β_2m). Les complexes CMH-I présentent leurs antigènes aux cellules T CD8$^+$. B) Les antigènes extracellulaires, comme les antigènes bactériens, sont apprêtés en peptides par des enzymes endolysosomales. Les peptides se lient aux molécules CMH-II en déplaçant le peptide CLIP (« class II-associated invariant-chain peptide »), lui-même dérivé de la chaîne invariante Ii. HLA-DO et HLA-DM régulent ce processus. Les complexes CMH-II présentent leurs antigènes aux cellules T CD4$^+$. Reproduit à partir de Koichi *et al*. [248].

3.2.2 Conséquences de la maturation des cellules dendritiques

La maturation des DCs est initiée en périphérie suite à la rencontre avec les antigènes et/ou à la présence de cytokines inflammatoires. Suite à l'internalisation et à la destruction des pathogènes, les DCs migrent vers les ganglions lymphatiques où elles vont présenter les antigènes exposés en surface aux cellules T. Le processus de maturation des DCs est fortement dépendant du type de pathogène rencontré et de la composition de l'environnement qui entoure les DCs. La maturation est complexe et requiert la coordination de plusieurs événements. Ces événements sont la perte des récepteurs d'endocytose et de phagocytose, la diminution de la capacité d'endocytose et de phagocytose, l'augmentation de l'expression en surface des molécules CMH-II ainsi que

des molécules de co-stimulation, la production de cytokines et des changements morphologiques [216].

La capacité des DCs à répondre aux gradients de chimiokines inflammatoires et lymphoïdes est vraisemblablement liée à leur degré de maturation puisqu'au fur et à mesure que les DCs deviennent matures, elles perdent leur réactivité aux chimiokines inflammatoires et augmentent leur réactivité aux chimiokines lymphoïdes. Les DCs immatures humaines dérivées des monocytes et les DCs immatures murines $CD34^{+}$ expriment à la fois les récepteurs de chimiokines CC et CXC (CCR et CXCR) tel que CCR1, CCR2, CCR5 et CXCR1, et répondent aux chimiokines inflammatoires telle que MIP-1α/CCL3, MCP-1/CCL2 et RANTES/CCL5 [221]. Comme les DCs immatures migrent vers des concentrations croissantes de chimiokines inflammatoires, elles sont aussi exposées à des concentrations croissantes de cytokines pro-inflammatoires et aux pathogènes qui initient la réponse inflammatoire [216]. Ainsi, les DCs matures diminuent leur expression de CCR1, CCR5 et CXCR1 pour augmenter leur expression de CXCR4, CCR4 et CCR7 (150, 273). CCR7 est aussi exprimé sur les lymphocytes B et T naïfs, leur permettant de migrer vers les tissus lymphoïdes. CCR7 a été démontré comme étant essentiel puisque, chez des souris $CCR7^{-/-}$, les DCs matures sont incapables de migrer vers les ganglions lymphatiques [249].

La maturation et l'activation des DCs peuvent être influencées par différents facteurs incluant 1) les molécules reliées aux pathogènes comme le LPS; 2) la balance entre les signaux pro-inflammatoires et anti-inflammatoires, incluant TNF-α, IL-1, IL-6, IL-10, TGF-β; et 3) les signaux dérivés des cellules T [216]. Récemment, des études ont démontré que certains signaux sont préférentiellement associés à l'initiation de la polarisation de la réponse des cellules T vers une réponse de type « T helper » (T_H)1 ou T_H2 (décrite dans la section suivante). Souvent, les produits microbiens comme le LPS, la CPS et le CpG sont parmi les premiers PAMPs reconnus par les DCs via les TLRs et les NLRs. Il devient de plus en plus évident que les signaux reçus via l'activation de ces voies de signalisation sont déterminants pour le développement de la réponse immunitaire [221].

3.3 Cellules T

Les cellules T se développent à partir de progéniteurs dérivés des cellules souches hématopoïétiques de la moelle osseuse et migrent vers le thymus où elles deviennent matures [250]. Le développement des cellules T est caractérisé par la maturation du récepteur des cellules T (TCR), qui est composé d'un dimère α:β dans 95% des cas et d'un dimère γ:δ dans 5% des cas. Au niveau du thymus, le développement des cellules T α:β est caractérisé par une étape transitoire où les cellules expriment simultanément CD4 et CD8. Ces lymphocytes T doubles positifs subissent ensuite un processus de sélection positive où seulement les lymphocytes exprimant un TCR reconnaissant une molécule CMH du soi survivront alors que les autres seront éliminées par apoptose. C'est aussi au cours de ce processus qu'émergeront ensuite les lymphocytes simples positifs qui seront soit $CD4^+$ ou $CD8^+$. Enfin, les lymphocytes subissent un processus de sélection négative permettant d'éliminer les lymphocytes T susceptibles de réagir de façon nocive contre les protéines du soi [251,252].

Les cellules T $CD4^+$, aussi appelées cellules T_H, jouent un rôle important dans le développement de la réponse immunitaire adaptative via la production de cytokines et l'expression de molécules de co-stimulation. Lorsque les cellules $T_{H,}$ via le TCR, reconnaissent leur antigène spécifique en surface des molécules CMH-II, elles deviennent activées et acquièrent des fonctions spécifiques caractéristiques des différentes sous-populations de cellules T $CD4^+$ [253].

3.4 Synapse immunologique et différenciation des cellules T $CD4^+$

L'interaction DC-cellule T $CD4^+$ débute lorsque la cellule T naïve reconnaît un peptide présenté par une molécule CMH-II en surface de la DC. Cette interaction constitue le premier signal requis pour l'activation des cellules T. Cependant, ce signal seul n'est pas suffisant pour induire la prolifération, la différenciation et la survie des cellules T. D'autres signaux sont donc essentiels au niveau de la synapse immunologique. Ces signaux proviennent des cytokines et des molécules de co-stimulation (Figure 8). Parmi les protéines importantes de la synapse immunologique, on retrouve principalement : le TCR; les molécules de co-stimulation CD2, CD28, CD40 et CD154; les intégrines CD11a/CD18

(LFA-1) et CD49d/CD69 (VLA4); les molécules d'adhésion CD54 (ICAM-1) et CD50 (ICAM-3); les récepteurs de chimiokines CCR5 et CXCR4; et plusieurs récepteurs de cytokines dont IL-2R et IL-12R [254,255]. Au niveau des DCs, on note principalement l'importance des molécules CD80 et CD86 qui interagissent avec la molécule CD28 en surface des cellules T [256].

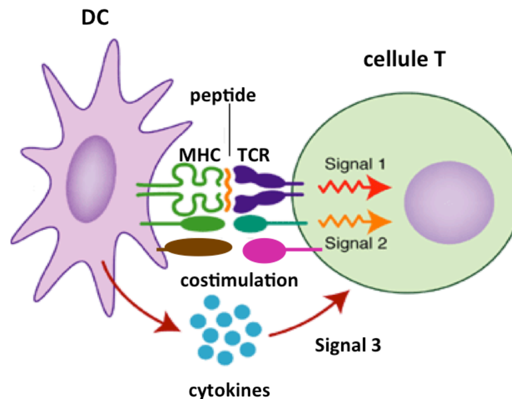

Figure 8. Exemple d'une synapse immunologique créée par le contact entre une DC et une cellule T CD4^{+}. La DC fournit trois signaux à la cellule T CD4+ spécifique à l'antigène présenté. Le premier signal provient de la présentation du peptide antigénique par la molécule CMH-II qui interagit avec le TCR et CD4 en surface de la cellule T. Le second signal est fourni par l'interaction entre les molécules de co-stimulation retrouvées en surface de la DC et de la cellule T. Enfin, le troisième signal découle des cytokines produites par la DC activée. Adapté de Toby *et al*. [257].

Suite à la stimulation antigénique, les cellules T naïves prolifèrent et se différencient en cellules effectrices. Une grande partie de ces cellules quitte ensuite les organes lymphoïdes périphériques pour migrer vers le site d'infection. Certaines cellules T_H différenciées migrent vers les follicules lymphoïdes dans les organes lymphoïdes où elles interagissent avec les cellules B (voir section 3.5).

Les cellules T_H effectrices ont pour rôle principal l'activation des cellules de l'immunité innée, dont les macrophages, ainsi que l'activation des lymphocytes B. Cette activation dépend de l'expression de différentes molécules de co-stimulation ainsi que de la production de cytokines. La protéine CD40L figure parmi les molécules de co-stimulation les plus importantes. En effet, suite à la reconnaissance de l'antigène et autres signaux reçus de part de la DC, le gène codant pour la protéine CD40L est transcrit dans la cellule T. Ceci

permet ensuite la liaison à son récepteur CD40 exprimé en surface des DCs, lymphocytes B et macrophages. L'interaction entre le CD40 des DCs et le CD40L des cellules T stimule l'expression de molécules de co-stimulation sur les DCs ainsi que la production de cytokines activant les lymphocytes T, créant ainsi un mécanisme d'amplification pour l'activation des cellules T.

La différenciation des cellules T $CD4^+$ en T_H1 est principalement induite par la présence d'IL-12 et IFN-γ. Le développement des cellules T_H2 est stimulé par la cytokine IL-4, alors que le développement de la sous-population T_H17 se fait par l'intermédiaire des cytokines IL-1, IL-6, IL-23 et TGF-β (Tableau V). Les conditions menant au développement de chacune de ces sous-populations seront discutées plus en détails dans la section qui suit.

On distingue les différentes sous-populations de cellules T_H $CD4^+$ selon les cytokines qu'elles produisent. Bien qu'à ce jour plusieurs sous-populations de cellules T_H effectrices soient décrites, les principales sont les T_H1, T_H2 et T_H17 (Tableau V). Chacune de ces sous-populations exerce des fonctions immunitaires différentes. Il est toutefois important de noter que de nombreuses cellules T_H $CD4^+$ activées peuvent produire une grande variété de cytokines, ce qui rend difficile la classification de ces sous-populations. De plus, ces sous-populations de cellules sont aussi dotées d'une plasticité considérable leur permettant de se convertir en une autre sous-population en présence de certaines conditions. Les rôles de ces sous-populations de cellules T seront plus précisément discutés dans la section 3.4.

Tableau V : Sous-populations de lymphocytes T CD4$^+$.

Cellules	Induites par	Cytokines produites	Réactions immunitaires	Exemples typiques de pathogènes cibles
T_H1	IL-12 IFN-γ	IFN-γ	Activation macrophages; production d'immunoglobulines (Ig)	Pathogènes intracellulaires
T_H2	IL-4	IL-4 IL-5 IL-13	Activation des mastocytes et éosinophiles; production d'IgE; activation des macrophages	Helminthes
T_H17	IL-1 IL-6 IL-23 TGF-β	IL-17A IL-17F IL-22	Recrutement de neutrophiles et monocytes	Bactéries extracellulaires et champignons

Adapté de Deenick *et al*. [258].

3.4.1 Polarisation vers une réponse T_H1

Les signaux fournis par les cytokines sont les plus importants pour la différenciation des différentes sous-populations de cellules T_H. Le développement des cellules T_H1 débute avec la production d'IFNs de type I et d'IL-12 par les celulles présentatrices d'antigènes. L'activation des cellules présentatrices d'antigènes et la production de cytokines par ces dernières sont largement dépendantes de la reconnaissance des PAMPs par les PRRs dont les TLRs. La production d'IFN-γ par les cellules T_H1 activées agit comme une boucle de rétroaction positive en stimulant la production de cytokines par les cellules présentatrices d'antigènes [259]. L'IFN-γ, les IFNs de type I et l'IL-12 agissent directement sur les cellules T pour induire leur différenciation en cellules T_H1. L'IFN-γ possède, en plus, l'habileté à inhiber la voie de différenciation en cellules T_H2 en empêchant la prolifération des cellules T_H2 [250]. L'attachement d'IFN-γ aux cellules T_H naïves mène à l'activation du facteur de transcription STAT1 via l'activation de JAK1 et JAK2. STAT1 induit ensuite l'expression de T-bet, un facteur de transcription membre de la famille TATAAA-box.

L'activation de T-bet induit le remodelage du gène codant pour l'IFN-γ, la production d'IFN-γ, l'expression du récepteur de l'IL-12 (IL-12R) et la stabilisation de son expression via l'activité autocrine d'IFN-γ [260]. L'augmentation de l'expression de IL-12R mène à une augmentation de son activation suite à sa liaison à IL-12, renforçant ainsi la différenciation des cellules T_H1 [259]. La signalisation via IL-12 active les facteurs de transcription STAT3, STAT4 et NF-κB, promouvant ainsi la production des cytokines associées au phénotype T_H1, ainsi que la liaison de NFAT à ces gènes cibles, amplifiant l'expression de IFN-γ [259,261]. La présence d'IL-12 agit aussi sur l'augmentation de l'expression du récepteur IL-18 (IL-18R). La production d'IL-18 par les DCs potentialise l'action d'IL-12 plus tard dans le développement du phénotype T_H1 [262].

Les signaux reçus par les molécules de co-stimulation jouent aussi un rôle important dans l'activation et dans la polarisation de la réponse des cellules T. Ces signaux sont générés lors de l'interaction entre les DCs et les cellules T dans les organes lymphoïdes ou au site d'infection. Comme mentionné précédemment, l'activation des DCs conduit à l'augmentation de l'expression en surface de plusieurs molécules comme CD40, CD54 et CD80/86. Sur les cellules T, le TCR participe à la synapse immunologique, ainsi que les molécules CD2, CD4 et CD28. Les interactions entre CD28 et les membres de la famille B7 (CD80/86) sont essentielles pour l'initiation de la réponse antigène-spécifique des cellules T, l'augmentation de la production de cytokines, ainsi que pour l'expansion et la prolifération des cellules T. Cependant, cette synapse n'est pas exclusivement réservée à la différenciation du phénotype T_H1 [263]. Il a aussi été proposé que la liaison de CD54 à CD11a/CD18 serait impliqué dans la polarisation vers la réponse T_H1 [263]. De plus, la liaison de CD40 avec CD40L induit une augmentation de l'expression d'autres molécules de co-stimulation, de molécules d'adhésion et d'IL-12 [264].

Les cellules T_H1 stimulent l'ingestion et la lyse des pathogènes par les phagocytes, processus essentiel de l'immunité cellulaire. L'IFN-γ est effectivement un puissant activateur des macrophages. Il stimule également la production d'isotypes d'anticorps favorisant la phagocytose des pathogènes, dans la mesure où ces anticorps se lient directement aux récepteurs Fc des phagocytes et activent le complément. En raison de ces

actions de l'IFN-γ, les cellules T_H1 sont donc particulièrement efficaces pour l'ingestion et la destruction des pathogènes intracellulaires dans les phagocytes. Les cellules T_H1 sont aussi décrites comme importantes pour la défense contre les pathogènes extracellulaires [265,266]. IFN-γ stimule également l'expression des molécules CMH-II et B7 sur les DCs et les macrophages, ce qui pourrait servir à amplifier la réponse des lymphocytes T [267-269].

3.4.2 Polarisation vers une réponse T_H2

Les mécanismes impliqués dans la polarisation de la réponse T_H2 sont moins bien connus que ceux de la réponse T_H1 [253,270]. IL-4, IL-5 et IL-13 sont les cytokines les plus souvent associées au phénotype T_H2. L'engagement du TCR, NFAT et GATA-3 sont les premiers signaux à stimuler une réponse T_H2. Un faible signal du TCR chez les cellules T $CD4^+$ naïves induit la sécrétion d'IL-4. Au contraire, un plus fort signal induit la production d'IFN-γ. En effet, une faible activation du TCR mène à l'activation transitoire d'ERK, à la stabilisation de GATA-3 et induit la production d'IL-4. IL-2 est aussi induit, et agit de façon autocrine, activant STAT5 et fournit ainsi le signal de survie nécessaire pour les cellules T [253]. De façon intéressante, les cellules impliquées dans la production initiale d'IL-4 nécessaire à la polarisation de la réponse T_H2 sont inconnues. En effet, les DCs sont incapables de sécréter cette cytokine. Il a été suggéré que les cellules NK, les éosinophiles, les basophiles ou les mastocytes pourraient être impliqués [253]. La liaison d'IL-4 à son récepteur en surface des cellules T naïves induit la phosphorylation de STAT6, qui active à son tour l'expression de GATA-3 [271]. GATA-3 augmente l'activité promotrice des régions responsables du contrôle de l'expression des gènes responsables de la sécrétion d'IL-4, IL-5, IL-9, IL-10 et IL-13, tout en inhibant l'expression d'IL-12R qui est important pour la réponse T_H1 [259]. Une fois que la production de GATA-3 atteint une certaine limite, son expression génique est auto-activée, stabilisant ainsi le développement du phénotype T_H2 via une boucle de rétroaction positive. De plus, alors que les cellules T_H2 deviennent matures, elles produisent une plus grande quantité d'IL-4, ce qui génère une boucle d'activation paracrine et induit la différenciation des cellules T naïves environnantes en cellules T_H2 [271]. Néanmoins, il est intéressant de noter que, bien qu'IL-4 demeure présentement la molécule considérée la plus importante pour le développement d'un

phénotype T_H2, il a récemment été suggéré que la différenciation d'un phénotype T_H2 *in vivo* pourrait ne pas requérir IL-4 et STAT6, mais passerait plutôt via des voies de signalisation impliquant GATA-3 [272]. Cependant, si une réponse T_H2 peut se développer en absence d'IL-4 et STAT6, quelles sont les cytokines qui pourraient compenser ces voies de signalisation?

Des voies de signalisation indépendantes d'IL-4 ont récemment été identifiées. Ces voies impliquent les lymphocytes de la réponse innée (ICLs). Les ICLs sont des participants importants de la réponse immunitaire innée et jouent principalement un rôle dans le remodelage tissulaire. Cependant, il apparaît que ces cellules pourraient aussi jouer un rôle dans les étapes précoces de l'infection par les microorganismes [273]. Les ICLs peuvent être divisés en trois groupes selon leur propension à produire des cytokines associées avec les cellules T_H1, T_H2 ou T_H17 [273]. Le groupe ICL1 se caractérise par la production d'IFN-γ et inclut, entre autres, les cellules NK. Les cellules du groupe ICL2 produisent IL-5, IL-9 et IL-13 en réponse à IL-25, IL-33 et TSLP (lymphopoiétine stromale thymique). Le groupe ICL3 produit IL-17A et IL-22 [273].

De façon intéressante, les cytokines IL-25, IL-33 et TSLP ont été démontré comme activant directement les cellules ICL2 qui, à leur tour, libèrent des cytokines de type 2 tel qu'IL-5, IL-9 et IL-13. IL-25, IL-33 et TSLP activent aussi directement les DCs qui induisent ensuite une réponse des cellules T_H2 qui participent alors à la production d'IL-5, IL-9 et IL-33 [274,275]. Il a été démontré que TSLP promeut une réponse de type T_H2 via l'augmentation de l'expression d'OX40L chez les DCs et via l'induction directe de la production d'IL-4 chez les cellules T CD4+ naïves [274]. En plus de guider la réponse adaptative, les cellules ICL2 semblent aussi répondre à une variété de signaux produits par les cellules T et les cellules B de manière rétroactive. Les cellules ICL2 expriment les récepteurs pour IL-2 et IL-9, ce qui amène la possibilité que des cytokines libérées par les cellules T influençent le développement, la prolifération, la survie et/ou l'activation des cellules ICL2. Il a d'ailleurs été démontré qu'IL-2 induit la prolifération des cellules ICL2 et augmente leur habileté à sécréter IL-25 et IL-33, des cytokines impliquées dans la

réponse T_H2. Dans certains cas, il a même été démontré qu'IL-2 est essentiel pour l'activation des cellules ICL2 en réponse à IL-33 [275].

IL-6 a aussi été démontré comme étant produite tôt lors du développement de la réponse T_H2. La production d'IL-6 par les mastocytes, macrophages et DCs induit un phénotype T_H2 via l'augmentation de l'expression d'IL-4 et l'inhibition de la phosphorylation de STAT1, empêchant ainsi la production d'IFN-γ [276].

Les DCs activées pourraient aussi induire indirectement la différenciation des cellules T_H2 via la sécrétion d'IL-10 qui inhibe la synthèse d'IL-12 et donc le développement d'une réponse T_H1 [277]. Cette hypothèse demeure controversée puisqu'elle suggère que le phénotype T_H2 résulterait d'une voie de signalisation activée par défaut en absence d'IL-12 [259].

L'induction d'une réponse T_H2 via une faible stimulation du TCR peut contourner le besoin d'une source exogène d'IL-4, mais requiert un second signal via CD28. Les molécules B7 (CD80 et CD86) en surface des DCs s'associent avec CD28 et d'autres membres de la famille CD28, incluant ICOS (« inducible co-stimulator protein ») et CTLA-4 (« cytotoxic T-lymphocyte antigen 4 »), en surface des cellules T. Les signaux induits par la liaison de CD28 avec CD80/86 initient l'augmentation de l'expression de GATA-3. En même temps, le TCR induit le développement des cellules T_H2 selon un mécanisme STAT6 (IL-4)-indépendant [278]. Cependant, comme mentionné précédemment, cette voie de signalisation peut aussi induire une forte réponse T_H1. CTLA-4 interagit aussi avec les molécules B7 en surface des DCs mais, au contraire de CD28 qui fournit aussi un signal de stimulation, CTLA-4 fournit un signal inhibiteur, à la fois pour la réponse T_H1 et T_H2 [259].

D'un autre côté, ICOS, membre de la famille CD28, est exprimé en surface des cellules T $CD4^+$ naïves, et son expression est augmentée sur les cellules T activées. B7RP-1, un autre membre de la famille B7, est exprimé sur les cellules B, les macrophages, les DCs et autres cellules des tissus non-lymphoïdes. B7RP-1 est le ligand pour ICOS [259]. En absence de

CD28, ICOS peut fournir une co-stimulation aux cellules T_H2, bien que cela soit moins efficace. Les études suggèrent une hiérarchie dans la co-stimulation, avec un besoin critique pour CD28 et un rôle moins essentiel pour ICOS [253]. La réponse immunitaire chez des souris $ICOS^{-/-}$ est déficiente, avec l'absence de centres germinatifs et un déficit dans la production d'IL-4 par les cellules T [279]. Cependant, des souris $ICOS^{-/-}$ ont la capacité de développer une réponse cellulaire T_H1, comme observé par la production d'IFN-γ par les cellules T antigène-spécifique. De façon importante, l'addition de CD40 afin d'augmenter l'expression de CD40L compense pour l'absence d'ICOS, ce qui suggère que la liaison entre ICOS et son ligand B7RP-1 en surface des DCs permet surtout de faciliter l'interaction CD40-CD40L en aval [279].

La famille des récepteurs TNF est aussi importante pour le développement de la réponse T_H2. Il a été observé que l'expression de CD134 est augmentée en surface des cellules T peu après l'interaction CD40-CD40L. Les DCs exprimant CD134L fournissent un signal critique pour la survie ou l'expansion des cellules Th2. Cependant, CD134 n'est pas requis pour l'activation initiale des cellules T [253].

3.4.3 Polarisation vers une réponse T_H17

Les cellules T_H17 sont impliquées dans les maladies auto-immunes et dans la défense contre les pathogènes extracellulaires [280]. Elles produisent IL-17, IL-17F et IL-22, induisant ainsi une réaction massive au niveau des tissus qui mène à l'expression des récepteurs pour IL-17 et IL-22. Les cellules T_H17 secrètent aussi IL-21, ce qui permet la communication avec les cellules du système immunitaire. Les facteurs de différenciation (TGF-β et IL-6 ou IL-21), le facteur de croissance et de stabilisation (IL-23) et les facteurs de transcription (STAT3, RORγt et RORα) sont impliqués dans le développement des cellules T_H17. L'activation des cellules T_H17 résulte en l'activation des cellules endothéliales et épithéliales, des fibroblastes et des macrophages qui induisent la production de médiateurs inflammatoires et de chimiokines. Cette cascade d'activation induit au final le recrutement des granulocytes (particulièrement des neutrophiles) et produit un état généralisé d'inflammation tissulaire [281].

La participation de TGF-β à la différenciation des cellules T_H17 place cette lignée en relation avec les cellules Tregs $CD4^+CD25^+Foxp3^+$ (voir plus bas) puisque TGF-β induit aussi la différenciation des cellules T naïves en cellules Tregs $Foxp3^+$ en périphérie. IL-6, un inhibiteur du développement des cellules Tregs joue donc un rôle important en dirigeant la réponse des cellules T vers une réponse inflammatoire plutôt qu'une réponse suppressive [259]. De plus IL-6 agit sur les cellules T naïves pour induire l'expression d'IL-21, ce qui initie une boucle autocrine d'auto-expression [282]. TGF-β agit alors en synergie avec IL-21 pour induire l'expression de RORγt, le facteur de transcription majeur des cellules T_H17, via un mécanisme dépendant de STAT3. L'activation de RORγt induit la transcription des gènes codant pour IL-17 et IL-17F [282]. Enfin, les interactions entre CD40 et CD40L au niveau des DCs et des cellules T naïves sont hautement importantes pour le développement de la réponse T_H17 [283].

3.4.5 Polarisation vers une réponse Tregs

Les Tregs jouent un rôle important dans la régulation des réponses T_H1 et T_H2 via leurs fonctions suppressives. Il existe plusieurs types de Tregs : certaines sont induites en réponse à une infection alors que d'autres sont considérées comme des régulateurs naturels [284]. La famille des Tregs induites comprend trois sous-populations. Les cellules T_H3 sont induites par l'administration orale d'antigènes et exercent leur activité suppressive via la production de TGF-β. Les cellules Tr1 sont induites en présence d'IL-10 et ont une activité suppressive via la production de cette même cytokine. La dernière sous-population de Tregs comprend des cellules $CD4^+CD25^-$ qui, sous certaines conditions, peuvent devenir $CD4^+CD25^+$ et acquérir l'expression de Foxp3 [281]. Les cellules T_H3 et Tr1 n'expriment pas Foxp3 et leurs fonctions suppressives sur les réponses T_H1 et T_H2 ne sont pas restreintes par le CMH ou la spécificité de l'antigène, ce qui suggère une implication plus grande de la réponse innée [285]. Au contraire, les mécanismes impliqués dans l'activation des cellules $CD4^+CD25^+Foxp3^+$ sont toujours controversés puisque l'expression de Foxp3 est différente chez la souris et chez l'humain. Cependant, dans les deux modèles, les cellules fonctionnent en supprimant la prolifération des cellules T effectrices spécifiques à un antigène [281].

De leur côté, les Tregs naturelles proviennent d'un processus de maturation normal dans le thymus et survivent ensuite en périphérie. Les Tregs naturelles expriment CD25 et CTLA-4 de façon constitutive. Foxp3 est nécessaire pour la génération des Tregs naturelles et représente leur marqueur le plus spécifique. Ces cellules régulatrices répondent à une grande variété d'autoantigènes, bien que de plus en plus de données semblent indiquer qu'elles pourraient aussi répondre à des antigènes exprimés par des pathogènes [284].

3.5 Rôle des cellules T CD4$^+$ dans le développement de la réponse humorale

Les réponses humorales dirigées contre différents antigènes sont classées en T-dépendantes ou T-indépendantes selon qu'elles nécessitent ou non la collaboration des cellules T. En effet, les lymphocytes B reconnaissent et sont activés par une grande variété d'antigènes comme des lipides, des protéines, des polysaccharides et des acides nucléiques. Les antigènes protéiques sont décrits comme T-dépendants puisqu'ils doivent d'abord être apprêtés par des cellules présentatrices d'antigènes, tel que les DCs, et sont reconnus par les cellules T. Une fois activées par les DCs, les cellules T_H migrent vers la zone des cellules B des organes lymphoïdes où elles interagissent avec les lymphocytes B stimulés par le même antigène. En effet, les lymphocytes B et les lymphocytes T reconnaissent différents épitopes d'un même antigène protéique. Les cellules B vont donc activer les cellules T effectrices déjà différenciées. Les cellules T_H qui reconnaissent l'antigène présenté par les cellules B activent ces dernières principalement via l'interaction CD40-CD40L ainsi que par la production de différentes cytokines. La commutation isotypique des chaînes lourdes dépend de la combinaison des signaux émis par le CD40L et par les cytokines. Par exemple, la production d'anticorps opsonisants qui se lient aux récepteurs Fc des phagocytes est principalement stimulée par IFN-γ, la cytokine caractéristique des cellules T_H1; alors que la production d'IL-4 par des cellules T_H2 induit plutôt la production d'IgE et d'IgG1. Les lymphocytes T jouent donc un rôle essentiel pour l'activation des lymphocytes B ainsi que pour la commutation isotypique et la maturation d'affinité, contribuant ainsi à la production d'anticorps par les lymphocytes B, ainsi qu'à la génération d'une réponse mémoire [286,287].

3.6 Réponse immunitaire adaptative lors de l'infection par *S. suis*

À ce jour, peu d'études ont porté sur le développement de la réponse immunitaire adaptative lors de l'infection par *S. suis*. La présente étude est la première à s'intéresser aux interactions entre *S. suis* et les cellules dendritiques ainsi qu'aux les conséquences de ces interactions sur le développement de l'immunité adaptative. Quelques études précédentes ont porté sur le développement de la réponse humorale lors de l'infection par *S. suis*, concernant principalement la recherche de potentiels candidats pour la vaccination. En effet, très peu est connu sur le développement de la réponse immunitaire adaptative lors de l'infection par *S. suis*, les types ou sous-populations des cellules impliqués ou les mécanismes moléculaires sous-jacents.

4. Problématique, hypothèse et objectifs

Malgré l'importance des infections à *S. suis*, les facteurs de virulence de la bactérie et la pathogenèse de la maladie demeurent encore peu connus et il n'existe toujours aucun vaccin permettant de contrôler l'infection. Il apparaît donc crucial d'étudier les interactions entre *S. suis* et le système immunitaire de l'hôte afin de mieux comprendre comment générer une réponse protectrice efficace contre ce pathogène en émergence. À ce jour, les interactions entre *S. suis* et les DCs ainsi que les conséquences de ces interactions sur le développement de la réponse T-dépendante sont inconnues. Nous avons émis comme hypothèse que *S. suis* possède plusieurs facteurs de virulence lui permettant de moduler les fonctions des DCs et, par conséquent, la réponse T-dépendante, interférant ainsi avec le développement d'une réponse immunitaire adaptative appropriée qui serait requise pour contrôler la progression de l'infection. L'objectif général du projet est donc d'étudier la modulation de la fonction des DCs et de la réponse T-dépendante par des facteurs de virulence de *S. suis*. Les objectifs spécifiques de cette recherche sont :

1. Étudier le rôle joué par certains facteurs de virulence clés de *S. suis* sur les interactions avec les DCs murines dérivées de la moelle osseuse (bmDCs).
2. Étudier les récepteurs cellulaires impliqués dans la reconnaissance de *S. suis* par les bmDCs murines.

3. Confirmer le rôle joué par les facteurs de virulence de *S. suis* sur les interactions avec les bmDCs porcines, hôte naturel de *S. suis*.
4. Évaluer la capacité des DCs activées par *S. suis* à activer les cellules T.

III. MATÉRIEL, MÉTHODES ET RÉSULTATS

Article I :

Critical role for *Streptococcus suis* cell wall modifications and suilysin in resistance to complement-dependant killing by dendritic cells.

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Rôle de la candidate dans la conception de l'article :

Je suis l'auteure principale de cet article. J'ai participé à la conception et au design des expériences ainsi qu'à la mise au point des techniques. J'ai effectué les manipulations et analysé les résultats. Finalement, j'ai réalisé la conception des graphiques et rédigé le manuscrit.

ABSTRACT

Streptococcus suis is an emerging zoonotic agent of septicemia and meningitis. Knowledge on host immune responses towards *S. suis*, and strategies used by this pathogen for subversion of these responses is scarce. Here, *S. suis* ability to modulate dendritic cell (DC) functions were assessed for the first time. Using *S. suis* knock-out mutants in capsular polysaccharide (CPS) expression it was shown that CPS blocks DC phagocytosis and impairs cytokine release by hindering cell wall components. Mutants impaired in D-alanylation of lipoteichoic acid (LTA) or N-deacetylation of peptidoglycan (PG) further demonstrated the importance of cell wall in modulation of DC activation. Noteworthy, LTA and PG modifications were identified as major players in resistance to complement-dependent killing by DCs. Finally, *S. suis* hemolysin was partially involved in cytokine release and also contributed to bacterial escape of opsono-phagocytosis. Overall, *S. suis* uses its arsenal of virulence factors to modulate DC functions and escape immune surveillance.

INTRODUCTION

Streptococcus suis serotype 2 is a major swine pathogen mainly associated with meningitis and septicemia [1, 2]. Until recently, *S. suis* disease in humans was considered rare, mostly affecting people in contact with swine or pork by-products. However, *S. suis* is now emerging as an important threat to human health, especially in Asia. *S. suis* is identified as the leading cause of adult meningitis in Vietnam, the second in Thailand and the third in Hong Kong. In 2005, an important outbreak in China resulted in more than 200 human cases with a 20% fatality rate. Patients presented symptoms associated with streptococcal toxic shock-like syndrome, including hemorrhage and coma [1].

S. suis pathogenesis of infection is poorly understood, and attempts to control the infection are hampered by lack of effective vaccines. Notably, low specific antibody production is generated during *S. suis* infection [3]. However, mechanisms involved in innate and adaptive immune responses toward *S. suis* remain essentially unknown. Increased severity of *S. suis* infections in humans underscores the critical need to better understand the interactions between *S. suis* and the immune system to generate an effective immune response against this pathogen.

Several virulence factors have been proposed. The capsular polysaccharide (CPS) is considered a critical anti-phagocytic factor [2, 4, 5]. The serotype 2 CPS structure was recently described [6] and is composed of five different sugars including sialic acid. Among several proteins [7], a hemolysin (suilysin) has been characterized as a cholesterol-binding cytolysin [7, 8]. Suilysin is involved in modulation of *S. suis* interactions with different host cells [2, 7]. Modifications of cell wall (CW) components such as the N-deacetylation of peptidoglycan (PG) and the D-alanylation of lipoteichoic acids (LTA) also contribute to *S. suis* virulence as demonstrated in mouse and pig models of infection [9, 10]. As CPS, CW modifications and suilysin seem to be involved in the virulence of *S. suis*, particularly by impairing critical steps in antigen processing such as phagocytosis and killing, we chose to study their role in modulation of dendritic cell (DC) functions.

DCs are powerful antigen presenting cells that capture and process antigens, undergo a maturation process characterized by cytokine production and upregulation of co-stimulatory molecules, and finally migrate to adjacent lymphoid organs to activate T cells [11]. DCs are key elements in induction of innate immunity against pathogens and are crucial for T cell priming and initiation of adaptive responses. Consequently, interactions between DCs and pathogens can strongly influence disease outcome.

Previously, we demonstrated that the mouse model of infection is a valid model to reproduce *S. suis* infection [12]. In this study, we used mouse bone marrow-derived DCs (bmDCs) to investigate *S. suis* capacity to interact with DCs and to induce their maturation and activation. We also examined the contribution of known virulence factors on these interactions.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. *S. suis* strains were grown as previously described [4] and diluted in complete cell culture medium before experiments. The number of CFU/mL in the final suspension was determined using Autoplate® 4000 (Spiral Biotech).

Construction of the Δ*cpsF* mutant

A non-encapsulated mutant was constructed in wild-type strain P1/7 by precise, in-frame deletion of the *cpsF/*SSU_0520 gene using splicing-by-overlap-extension PCR [21]. The Δ*cpsF* deletion allele was cloned into plasmid pCR2.1 (Invitrogen), BamHI and PstI extracted and recloned into thermosensitive *Escherichia coli-S. suis* shuttle plasmid pSET4s [22]. *E. coli* transformation was performed as reported [23]. Procedures for *S. suis* electroporation and Δ*cpsF* mutant isolation were previously described [22]. Allelic replacement was confirmed by PCR and sequencing analysis with a 310 Genetic Analyzer (Applied Biosystems). The non-encapsulated phenotype was confirmed by transmission electron microscopy (TEM) using polycationic ferritin labeling, as reported [24].

BmDC generation

Female C57BL/6 mice (Charles River Laboratories) were used to generate bmDCs as described [25]. Experiments were conducted in accordance with University of Montreal Animal Welfare Committee guidelines and policies. 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). Complete medium was complemented with 20% GM-CSF from mouse transfected Ag8653 cells [25]. On day 8, after a subculture step, cells were collected and used as immature bmDCs. Cell purity was 85-88% $CD11c^+$ cells as reported [25]. For confirmation, bmDCs were further purified to >95% purity by positive selection using anti-CD11c microbeads (Miltenyi Biotec) [26].

Bactericidal assay

Bacteria were not opsonized or opsonized using 20% of either heat-inactivated normal mouse serum or complete normal mouse serum in PBS for 30 min at 37°C with shaking. BmDCs (2×10^6 cells/mL) were mixed with different *S. suis* strains (2×10^4 CFU/mL) in microtubes, and incubated for 6 h at 37°C with 5% CO_2. Assay conditions were chosen based on the kinetics of *S. suis* killing by bmDCs (data not shown). After incubation, cells were lysed and viable bacterial counts performed. Tubes with bacteria alone were treated similarly and used as controls.

BmDCs stimulation assay

BmDCs were stimulated with 1µM CpG (ODN 1826, Invivogen), as positive control, or *S. suis* strains (10^6 CFU/mL; initial infection ratio of 1:1). In selected experiments, bacterial strains were opsonized as described above. At different time post-infection, supernatants were collected for cytokine quantification by sandwich ELISA using pair-matched antibodies from R&D Systems or eBioscience. Cells were analyzed for co-stimulatory molecule expression by FACS. The lactate dehydrogenase (LDH) assay was used to measure cytotoxicity levels (CytoTox96, Promega) as described [5]. Final assay conditions were selected based in kinetics studies and absence of cytotoxicity (data not shown).

FACS analysis

Cells were treated for 30 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab, BD PharMingen), and then incubated with FITC-labeled anti-mouse CD11c mAb for 1 h followed by 1 h-staining with PE-labeled mAb against CD80 (clone 16-10A1), CD86 (clone GL1), CD40 (clone 3/23), CD54 (clone 3E2) or MHC class II (A_{β}^{b}; clone AF6-120.1) from BD PharMingen. Flow cytometry was performed using a FACSCalibur instrument and CellQuest software (BD Biosciences).

Confocal and electron microscopy

For confocal analysis, after 2 h of bacteria-cell contact, cells were washed and fixed with methanol/acetone (80:20) for 20 min at -20°C, blocked, and incubated for 1 h with rabbit anti-*S. suis* serum and rat anti-LAMP1 antibody (Developmental Studies Hybridoma

Bank). Coverslips were then incubated with Alexa-Fluor 488 goat anti-rabbit IgG and Alexa-Fluor 568 goat anti-rat IgG (Invitrogen) for 30 min, and mounted on glass slides with moviol containing DABCO and DAPI. Immunofluorescence studies were also performed to determine levels of complement deposition on bacteria. Either a BSA solution or complete normal mouse serum was added for 30 min at 37°C to a 10^6 bacterial suspension. In some cases, serum was pre-treated with purified suilysin for 30 min at 37°C before opsonization. Bacteria were then washed and incubated with goat IgG fraction to mouse complement C3 (MP Biomedicals) for 1 h, followed by Alexa-Fluor 488 mouse anti-goat IgG for 30 min. Samples were analyzed with a FV-1000 confocal microscope and Fluoview software. For TEM and scanning electron microscopy (SEM), bmDCs incubated with *S. suis* for 4 h were washed, fixed and processed as reported [27].

Statistics

Data are expressed as mean ± SEM, and were analyzed for significance using Student's unpaired *t*-test. A p value < 0.05 was used as a threshold for significance. Experiments were repeated at least three times.

RESULTS

CW modifications and suilysin are important for *S. suis* resistance to killing by bmDCs

Firstly, we confirmed that all mutant strains present similar growth curves as their respective WT parental strains (Fig. 1*A*). As shown in Figure *1B*, the wild-type strains were highly resistant to killing, and the presence of serum slightly, but not significantly, increased killing levels. Conversely, both non-encapsulated mutant strains were largely destroyed in all conditions tested. Thus, killing of non-encapsulated strains is not strictly dependent on serum factors. In contrast, the Δ*dltA*, Δ*pgdA*, and Δ*sly* strains were all resistant to killing in the absence of opsonization or after opsonization with heat-inactivated serum, but significantly more susceptible to killing following complete serum opsonization.

Impairment of complement deposition by CW modifications, suilysin and CPS

C3 deposition on streptococci was examined by immunofluorescence (Figure 1*C*). Wild-type streptococci showed almost complete absence of C3 deposition whereas increased labeling was observed on non-encapsulated strain surface. The Δ*dltA* and Δ*pgdA* strains showed bright, cell contour-labeling, indicating high levels of C3 deposition. As suilysin is a secreted toxin, its role in C3 deposition cannot be analyzed with washed bacteria. To overcome this constrain, we pre-treated complete normal serum with increasing doses of purified suilysin. Suilysin-treated serum was then used to opsonize the Δ*dltA* mutant strain, which should report high levels of C3 deposition. Using this system, we observed that suilysin pre-treatment of serum resulted in markedly reduced levels of C3 deposition on Δ*dltA* bacteria.

Encapsulated wild-type *S. suis* escapes internalization by DCs

Confocal microscopy was performed using serum against *S. suis* and an antibody against LAMP1 (lysosomal-associated membrane protein 1) (Figure 2*A*). Under non-opsonic conditions, few internalized bacteria were noticeable for the wild-type strain 31533 and the mutants *Δsly*, Δ*dltA* and Δ*pgdA*. When these strains were opsonized with complete serum, internalization by bmDCs showed a minor increase for wild-type 31533 strain. This

increase was more marked for Δ*dltA*, Δ*pgdA* and *Δsly* mutants (Figure 2*A*, middle panels). Opsonization with heat-inactivated serum partially restored internalization levels to those observed under non-opsonic conditions (Figure 2*A*, lower panels). In contrast, the non-encapsulated mutant was highly internalized by bmDCs under all conditions tested. This is in agreement with bactericidal test results.

SEM showed that *S. suis* interacts with bmDCs. Cocci were found associated to the cell surface both for the wild-type strain and its non-encapsulated mutant (Figure 2*B*). The CPS might impair *S. suis* interactions with bmDCs, as the non-encapsulated strain seemed to adhere more than the WT to DCs. Compared to the high numbers of streptococci observed intracellularly with the non-encapsulated mutant, TEM analysis showed that few bmDCs contained wild-type strain 31533 cocci despite serum opsonization (Figure 2*C*, II-III). In agreement with aforementioned data, bmDCs incubated with the opsonized Δ*dltA,* Δ*pgdA* or *Δsly* strains showed more bacteria internalized than the wild-type strain (Figure 2*C*, IV-VI).

S. suis virulence factors regulate bmDC release of several cytokines

S. suis stimulation of bmDCs resulted in time-dependent cytokine release (data not shown). At 16 h after stimulation, *S. suis* wild-type strain-stimulated bmDCs produced significant amounts of IL-1β, IL-6, TNF-α, IL-12p70, IL-23, IL-10, CXCL1, CCL2, CXCL9 and CXCL10 (Figure 3*A*-3*J*). Non-encapsulated mutants induced significantly higher levels of all cytokines, except for IL-1β and CCL2 (Figure 3*C*-3*J*). The Δ*dltA* and Δ*pgdA* mutants induced reduced levels of IL-10, IL-12p70 and CXCL10 (Figure 3*D*, 3*E*, and 3*H*), while the Δ*sly* mutant induced lower levels of IL-12p70 and IL-10 (Figure 3*D*-3*E*). Main cytokine results were confirmed with bmDCs purified by magnetic cell sorting (data not shown).

Serum modulates cytokine release by *S. suis*-activated bmDCs

Serum opsonization was shown to significantly modify IL-12p70 and IL-10 production by bmDCs (Figure 4*A*-4*B*). For the Δ*dltA*, Δ*pgdA* and Δ*sly* mutants the complement cascade seems to be, at least in part, involved in the observed down-regulation of IL-10 production

as bmDCs stimulation with these mutants opsonized with heat-inactivated serum significantly reduced its effect. In the case of IL-12p70, when these *S. suis* mutant strains were opsonized with heat-inactivated serum there was a tendency, albeit not significant, to return to normal levels of this cytokine production. Thus, the involvement of other serum components in modulation of cytokine production cannot be excluded. Serum opsonization of the non-encapsulated mutant had no visible effect on cytokine production.

***S. suis* induces bmDC expression of maturation markers**

S. suis ability to increase expression of CD40, CD54, CD80, CD86 and MHC class II (MHC-II) on bmDCs was analyzed. No significant changes in CD54 and CD80 expression were observed (data not shown). However, as shown in Figure 5, *S. suis*-stimulated bmDCs showed higher expression of CD40. Two well segregated sub-populations, a CD86^{high} and MHC-II^{high} subset and a CD86^{low} and MHC-II^{low} subset, were observed among the CD11c+ bmDCs population, as reported [25]. *S. suis* stimulation resulted in higher percentages of CD86^{high} and MHC-II^{high} subpopulations compared to control cells. No differences were observed between the wild-type strain and the mutants (Figure 5, grey filled histograms). Only a slight decrease, albeit reproducible, in the expression of CD40, CD86 and/or MHC-II was discernible when bmDCs were incubated with opsonized *S. suis* strains (Figure 5, black line histograms).

DISCUSSION

Although *S. suis* is a pathogen of increasing importance for human health, mechanisms leading to an efficient immune response against *S. suis* are poorly understood. A typical Type 1 inflammatory response was observed in a *S. suis* mouse model of septicemia and meningitis [12]. However, interactions between *S. suis* and immune mediators have not been characterized. This work describes for the first time *S. suis* interaction with DCs and modulation of their maturation and activation.

Effective bacterial killing by DCs is generally assumed to require internalization, which in turn would have an impact on antigen presentation by DCs. However, we showed here that *S. suis* escapes this immune surveillance. Indeed, under non-opsonic conditions, the CPS is sufficient to protect *S. suis* from bmDC-mediated killing, confirming the role of CPS as an anti-phagocytic factor [4, 5]. Nonetheless, in the presence of serum, as normally occurs during natural infection, *S. suis* uses an arsenal of virulence factors to resist serum-mediated opsono-phagocytosis and killing. Immunofluorescence confirmed that besides CPS, LTA and PG modifications as well as suilysin all impair complement deposition on *S. suis*. Previous studies demonstrated suilysin contribution to *S. suis* resistance to complement-dependent killing by neutrophils [5, 7]. One hypothesis suggested that the toxin might activate complement and reduce complement availability for bacterial opsonization, as previously shown with pneumolysin, a suilysin-related toxin produced by *Streptococcus pneumoniae* [28].

Another major finding is the involvement of CW modifications in resistance to complement-dependent killing. Typically, the CPS is considered the major factor interfering with complement activation, especially for sialic-acid containing capsules, as is the case of *S. suis*. Sialylation of CPS is important for group B *Streptococcus* (GBS) virulence as it has inhibitory effects on bacterial intracellular killing and acts by impairing complement deposition through sialic acid-dependent inhibition of the alternative pathway [29]. This not seems to be the case for *S. suis*, as Δ*dltA*, Δ*pgdA* and Δ*sly* mutants failed to resist opsono-phagocytosis and killing despite the presence of an intact CPS. Generally,

LTA D-alanylation is an important virulence factor for Gram-positive pathogens as it modulates bacterial surface charges, resistance to cationic peptides, and ligand binding [30]. As such, charge compensation by D- alanylation reduces binding of complement component C1q [31]. Recently, LTA D-alanylation was reported as being important for *Streptococcus pyogenes* resistance to complement. The *dltA* inactivation in this pathogen leads to increased C3b deposition concomitant with reduced M protein expression [32]. Albeit no M protein equivalent has been described for *S. suis* [8], increased C3b deposition is clearly observed in *S. suis* Δ*dltA* mutant strain. The mechanism underlying this activity is still unknown.

Besides *dltA*, to the best of our knowledge, this is the first time that N-deacetylation of PG is reported as involved in resistance to complement-dependent phagocytosis and killing. N-deacetylation of PG allows Gram-positive bacteria to circumvent host defenses, mainly by escaping Nod1/2 surveillance and/or increased resistance to lysozyme [33]. A previous study revealed a significant contribution of *pgdA* to *S. suis* virulence traits. *S. suis* Δ*pgdA* mutant was shown to be highly attenuated in murine and porcine models of infection, following a severe impairment in its ability to persist in blood and escape neutrophil-mediated clearance [10]. Modifications of PG, such as O-acetylation, were also shown to regulate ligand binding [34, 35]. Experiments are ongoing to further investigate the importance of LTA D-alanylation and PG N-deacetylation in complement-dependent killing.

All *S. suis* strains triggered bmDC maturation as shown by increased CD40, CD86, and MHC-II expression. No specific role could be attributed to any of the virulence factors tested as all strains induced equally the expression of co-stimulatory molecules by DCs. However, the strains tested differentially modulated the release of pro-inflammatory, Th1 driving and regulatory cytokines. In addition, high levels of the chemokines CXCL1, CXCL10 and CCL2, and low, albeit significant levels of CXCL9 were observed. This is the first report on CXCL9, CXCL10 and IL-23 production following *S. suis* cell stimulation. CXCL9 and CXCL10 are involved in T and NK cell recruitment, while IL-23 is particularly efficient in supporting IFN-γ production and memory T cell proliferation [36,

37]. CPS interfered with the release of several cytokines, but failed to modulate CCL2 and IL-1β production. Previous studies showed that *S. suis* CPS is required for optimal CCL2 production by human monocytes, brain endothelial cells, and blood leukocytes [14, 19, 38]. Increased exposure of CW components due to absence of CPS may account for higher capacity of non-encapsulated mutants to induce most cytokine secretion, and confirm the role of CW components as major cytokine modulators [14, 38, 39]. This is supported by the fact that IL-12p70, IL-10 and CXCL10 release was diminished following bmDC stimulation by the CW mutants. Similarly, a *Streptococcus gordonii* mutant strain defective for LTA D-alanylation induced reduced IL-12p70, IL-10, IL-6, and TNF-α levels [40]. Bacterial derived LTA and PG has already been shown to be responsible for the release of CXCL10 by different cell types [41, 42]. In addition to CW components, several microbial toxins can also modulate inflammatory pathways [43]. In previous work with brain endothelial cells, *S. suis* suilysin was shown to contribute to IL-6 and IL-8 production [19]. In our model, suilysin seems to significantly contribute to the release of the immunomodulatory cytokines IL-12p70 and IL-10. To date, the mechanism by which suilysin induces cytokine release remains unknown. *S. pneumoniae* pneumolysin recognizes TLR4 on DCs [43]. However, previous in vivo studies carried out in our laboratory indicate a strong TLR2 and CD14, but not TLR4, activation in the brains of mice infected with the suilysin-positive *S. suis* strain 31533 [12]. Similar results were obtained *in vitro* with human monocytes [38].

S. suis opsonization with complete serum was found to downregulate IL-12 and upregulate IL-10 release. This modulation seems to be independent of *S. suis* uptake and killing levels by bmDCs since it is also noticeable with the wild-type strain, for which susceptibility to phagocytosis/killing is only slightly increased in the presence of complete serum. *S. suis* activation of cytokine release by phagocytes has been reported as a phagocytosis-independent event [44, 45]. We might hypothesize that increased ligation of receptors specific for serum factors might account for modulation of IL-12/IL-10 production ratio after DC activation with serum-opsonized bacteria. Since the non-encapsulated mutant is highly adherent to DCs (Figure 2*B*), the effect of serum factors is not noticeable for this strain. LPS inhibition of IL-12 production has been reported following ligation of

macrophage Fcγ, complement or scavenger receptors [46]. The inhibitory effect observed was not dependent on particle internalization. Other studies reported that complement C1q regulates LPS-induced cytokine production in bmDCs, and that iC3b is responsible for the induction of IL-10 and the downregulation of IL-12 in monocytes and DCs [47, 48, 49]. Receptor-mediated inhibition of IL-12 production may be exploited by pathogens to suppress or delay cell-mediated immunity development. Serum factors and receptors involved in modulation of IL-12/IL-10 production after bmDCs stimulation with opsonized *S. suis* remain to be characterized. Experiments with heat-inactivated serum showed only a partial role of complement factors in IL-10 up-regulation when *S. suis* is impaired for CW modifications or suilysin production. The role of complement in IL-12 down-regulation could not be clearly demonstrated, and thus other serum factors might be also involved. This is certainly the case for wild-type *S. suis* which is highly resistant to complement deposition. Moreover, IL-10 itself might contribute to the observed down-regulation of IL-12 production. A slight tendency toward a reduction of co-stimulatory molecule expression was also repeatedly observed with bmDCs stimulated with serum opsonized strains. This tendency could also be the result of increased IL-10 production. For instance, a previous study showed that IL-10 induces the intracellular sequestration of MHC-II molecules [50].

To conclude, our results show for the first time that *S. suis* activates bmDCs, and suggest that these cells might play a role in host innate and adaptive immunity during infection. However, *S. suis* possess different virulence factors that modulate these interactions. Based in our results, a model of *S. suis* interactions with DCs is proposed (Figure 6). The CPS acts as a physical barrier that protects bacteria from phagocytosis and reduces cytokine production levels. CW modifications of PG and LTA and suilysin further protect *S. suis* from complement-mediated clearance. Overall, this results in low bacterial up-take which might translate in reduced antigen processing and presentation to T cells. DC activation is additionally modulated by *S. suis* ligation to serum factors which in turn reduced IL-12 production. We are presently evaluating the consequences of *S. suis* modulation of DC functions on the development of adaptive immune responses toward this pathogen.

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Table I: Bacterial strains and plasmids

Strain/Plasmid	General characteristics	Source/Reference
Escherichia coli		
TOP10	F-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Streptococcus suis[a]		
31533	Wild-type, highly virulent strain isolated from a pig with meningitis. Serotype 2	[13]
B218	Non-encapsulated mutant strain derived from 31533	[14]
Δ*dltA*	Mutant deficient for the D-alanylation of LTA. Derived from strain 31533	[9]
Δ*pgdA*	Mutant deficient for the N-deacetylation of PG. Derived from strain 31533	[10]
Δ*sly*	Suilysin negative strain derived from strain 31533	[15]
P1/7	Wild-type, highly virulent strain isolated from a pig with meningitis. Serotype 2	[16]
Δ*cpsF*	Non-encapsulated mutant strain derived from P1/7. In frame deletion of *cpsF*	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement is *S. suis*. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[17]
p4ΔcpsF	pSET4s carrying the construct for *cpsF* allelic replacement	This work

[a] Strain 31533 is a well characterized representative virulent suilysin-positive *S. suis* serotype 2 strain used in previous studies [12, 18, 19]. Strain P1/7 is a virulent *S. suis* serotype 2 strain whose genome was recently sequenced [20].

Δ*dltA*, Δ*pgdA* and Δ*sly* mutants present a normal morphology and are as encapsulated as the WT strain [9, 10].

Δ*dltA*, Δ*pgdA*, Δ*cpsF* and B218 mutants present the same hemolytic activity due to the release of suilysin as the WT strain (data not shown).

LTA, lipoteichoic acid; PG, peptidoglycan.

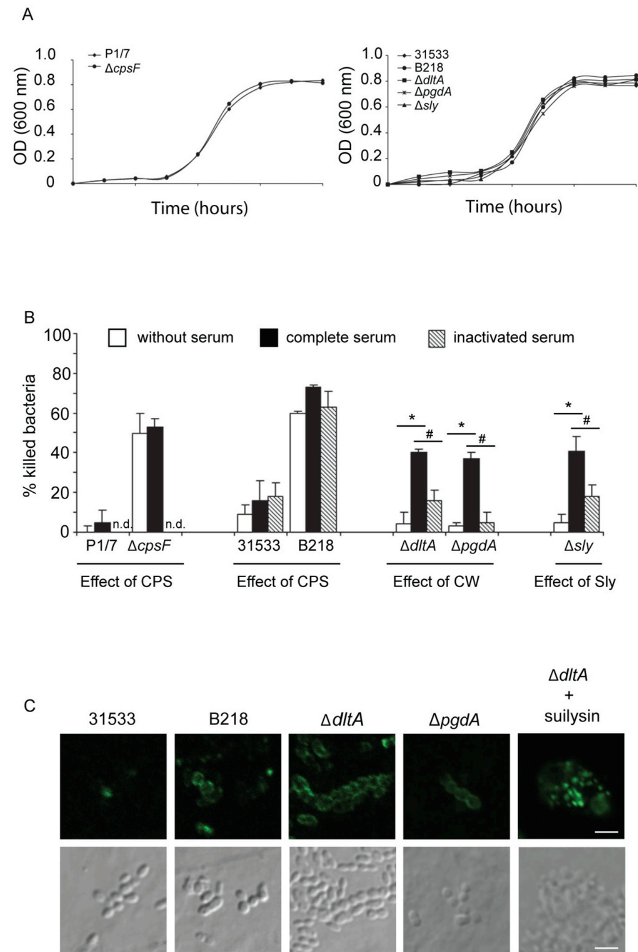

Figure 1. (**A) Growth curves of *S. suis* WT 31533 and mutant strains** (I). Growth curves of S. suis WT P1/7 and its non encapsulated mutant strain (II). **(B) Effect of serum on the capacity of bmDCs to kill different S. suis strains.** Bacteria were either non-opsonized or pre-opsonized with 20% complete serum or 20% heat-inactivated serum for 30 min prior to incubation with bmDCs for 6 h. Data are expressed as mean percentage (± SEM) of killed bacteria. * $p < 0.05$, indicates statistically significant differences between non-opsonized strains and their respective serum-opsonized counterparts. # $p < 0.05$, denotes significant differences between values obtained with serum-opsonized bacteria vs those obtained with heat-inactivated serum. **(C) Confocal microscopy showing complement deposition on S. *suis.*** The wild-type 31533 strain, the non-encapsulated B218 mutant, the Δ*dltA* strain or the Δ*pgdA* strain were pre-opsonized with complete serum and then incubated with an antibody against mouse complement component C3 to observe complement deposition. To assess the role of suilysin in complement deposition, complete serum was pre-treated with 0.2 µg/mL of purified suilysin. Suilysin-treated serum was then used for opsonization of Δ*dltA* strain. Original magnification 150X. Scale bar, 4 µM. CPS, capsular polysaccharide; CW, cell wall; Sly, suilysin.

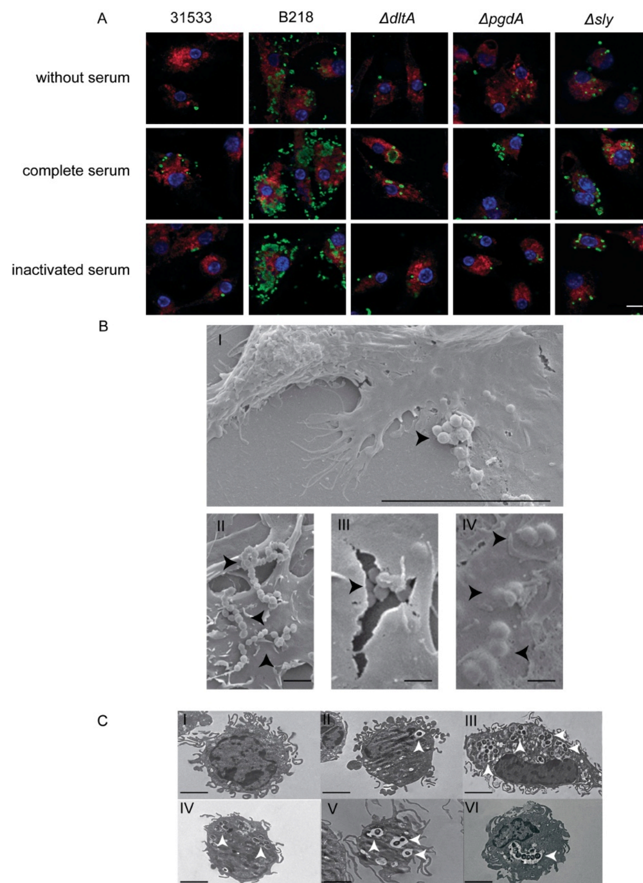

Figure 2. (A) Confocal microscopy showing internalization of S. suis. BmDCs were incubated with the wild-type strain 31533, the non-encapsulated mutant B218, the Δ*dltA* strain, the Δ*pgdA* strain or the Δ*sly* strain, not opsonized (top panels), pre-opsonized with complete serum (middle panels) or with heat-inactivated serum (lower panels). After a bacterial-cell contact of 2 h, cells were fixed and labeled with serum against *S. suis* (Alex-Fluor 488, green) and an antibody specific for LAMP1 (Alex-Fluor 568, red). DAPI was used to stain the nuclei (blue). Original magnification 60X. Scale bar, 10 µM. **(B) Scanning electron micrographs showing interactions between bmDCs and *S. suis* (10^7 CFU/mL) after a bacteria-cell contact time of 4 h.** (I) BmDCs incubated with wild-type strain 31533 show very few cocci on the cell surface. BmDCs incubated with the non-encapsulated strain B218 show several cocci adhering to the cells (II) and underneath the cell surface (III-IV). Arrows show bacterial cells. (I) Scale bar, 10 µm. Original magnification 5000X. (II-IV) Scale bar, 1 µm. Original magnification 5000X. **(C) Transmission electron micrographs showing internalization of *S. suis* by bmDCs (10^7 CFU/mL) after a bacterial-cell contact of 4 h.** (I) Non-infected DC, (II) Most bmDCs were free of *S. suis* or contained very few bacteria when incubated with serum-opsonized wild-type strain 31533, (III) BmDCs incubated with serum-opsonized strain B218 contained high numbers of internalized bacteria. BmDCs incubated with opsonized strain Δ*dltA* (IV), Δ*pgdA* (V), or Δ*sly* (VI) contained more internalized bacteria compared to bmDCs incubated with the wild-type strain. Arrows show internalized bacteria. Scale bar, 2 µM. Original magnification 10000X.

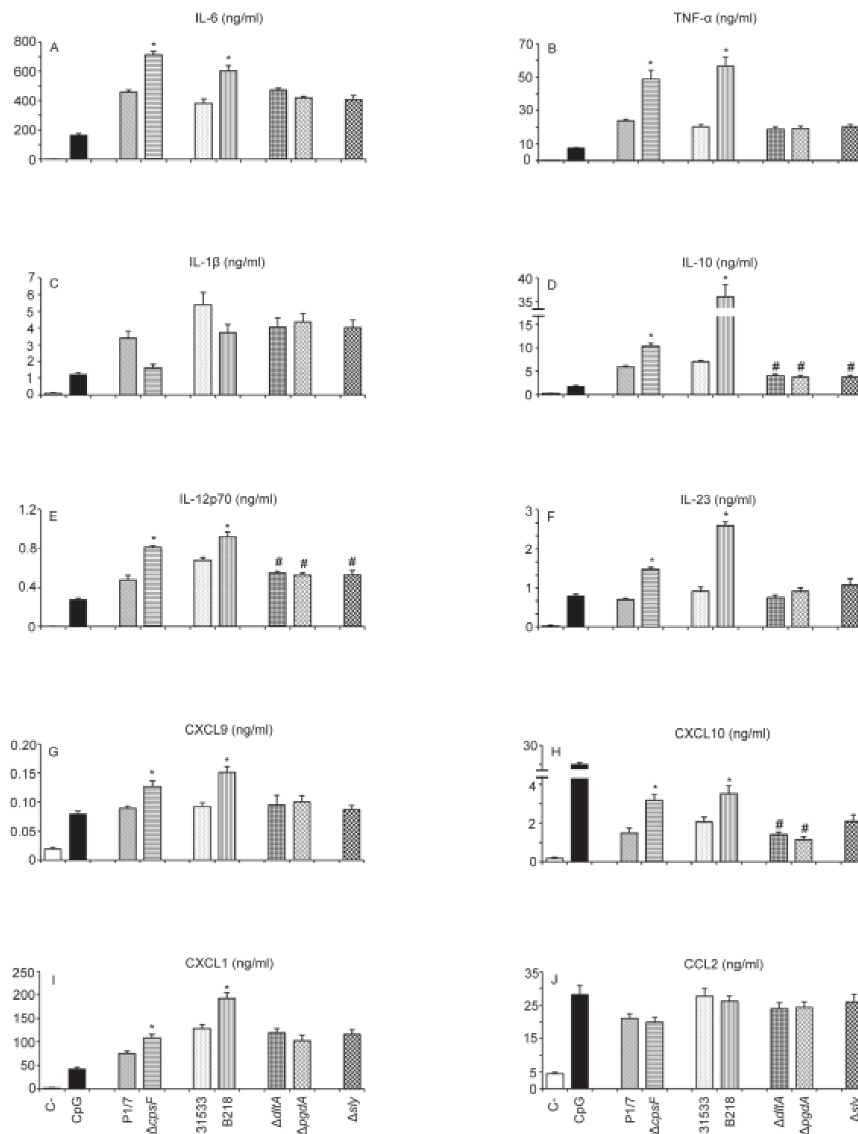

Figure 3. Cytokine production by bmDCs (10^6 cells/mL) in response to stimulation by CpG (1 µM) and different non-opsonized S. suis strains (10^6 CFU/mL) for 16 h. Non-stimulated cells served as negative control (C-) Sample dilutions giving optical density readings in the linear portion of the ELISA standard curves were used to quantify cytokine levels. Data are expressed as mean ± SEM, and were analyzed for significance using Student's unpaired t-test. Experiments were repeated four times with two technical replicates.* $p < 0.05$, denotes values obtained with the non-encapsulated mutants that are significantly higher from those obtained with their respective wild-type strains. $^{\#}$ $p < 0.05$, denotes values which are significantly lower than those obtained with the wild-type strain. Detection limits of cytokines: IL-1β, 0.03 ng/ml; IL-6, 0.125 ng/ml; IL-10, 0.0625 ng/ml; IL-12p70, 0.078 ng/ml; IL-23, 0.0625 ng/ml; TNF-α, 0.125 ng/ml; CXCL1, 0.05 ng/ml; CXCL9, 0.0625 ng/ml; CXCL10, 0.0625 ng/ml; CCL2, 0.0625 ng/ml.

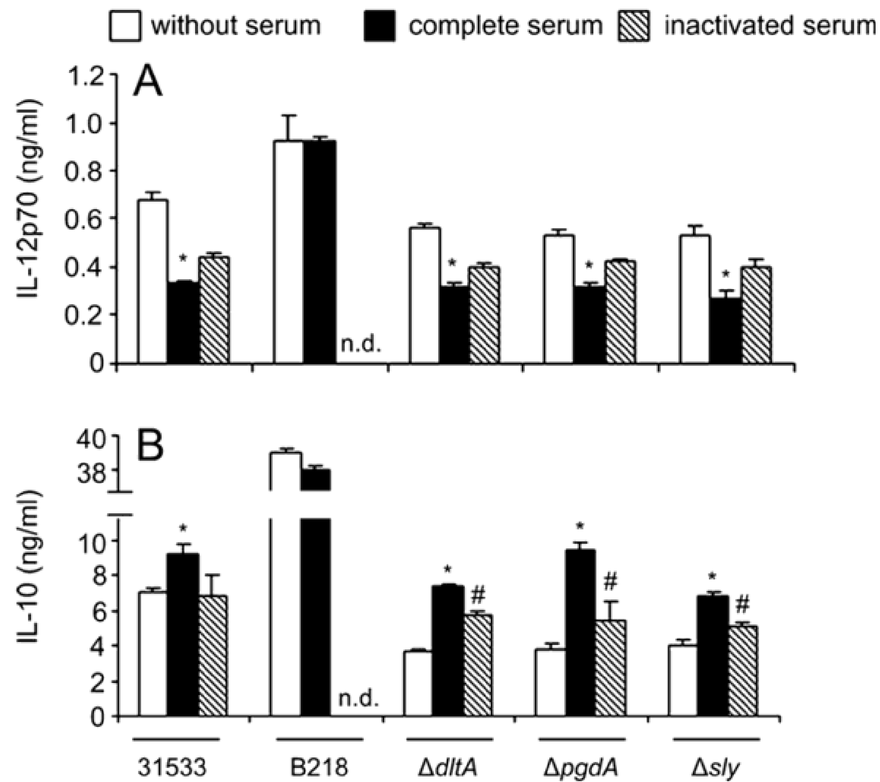

Figure 4. Effect of serum on IL-12p70 and IL-10 production by bmDCs. *S. suis* strains (10^6 CFU/mL) were either non-opsonized or pre-opsonized with 20% complete serum or 20% heat-inactivated serum for 30 min prior to incubation with bmDCs for 16 h. Data are expressed as mean ± SEM, and were analyzed for significance using Student's unpaired t-test. Experiments were repeated four times with two technical replicates. * $p < 0.05$, indicates statistically significant differences between non-opsonized strains and their respective serum-opsonized counterparts. # $p < 0.05$, denotes significant differences between values obtained with serum-opsonized bacteria vs those obtained with heat-inactivated serum.

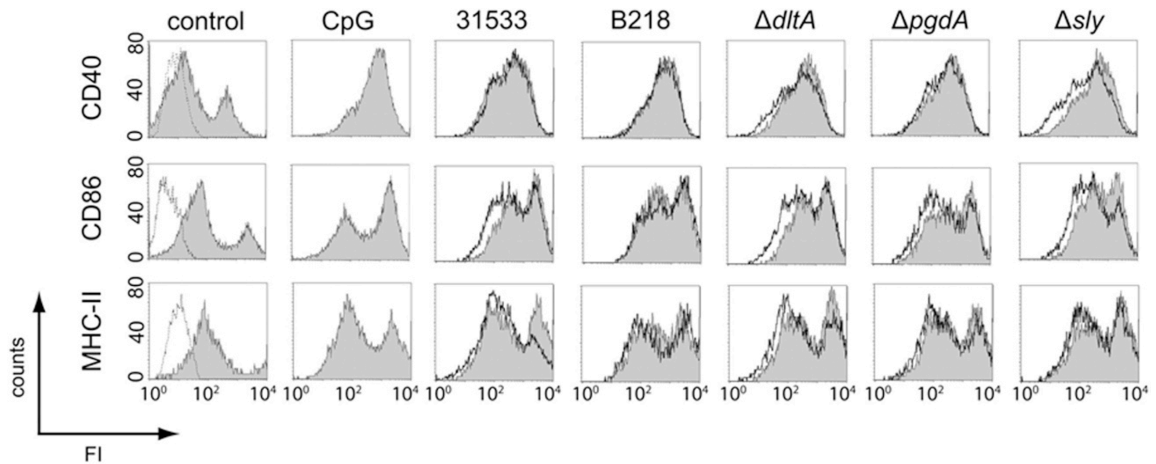

Figure 5. Expression of surface markers by bmDCs stimulated with CpG (1 µM) or *S. suis* (10^6 CFU/mL) for 16 h. S. suis strains were either non-opsonized or pre-opsonized with 20% complete serum for 30 min prior to incubation with bmDCs for 16 h. Unstimulated bmDCs served as control. Twenty thousand gated events were acquired per sample. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales. CD40, CD86 and MHC-II histograms were obtained by gating cells based on positive CD11c staining. FI: fluorescence intensity. Dotted line: isotype control; Grey filled histograms: data obtained with non-opsonized strains; Black line histograms: data obtained with serum-opsonized strains. Data are representative of three independent experiments.

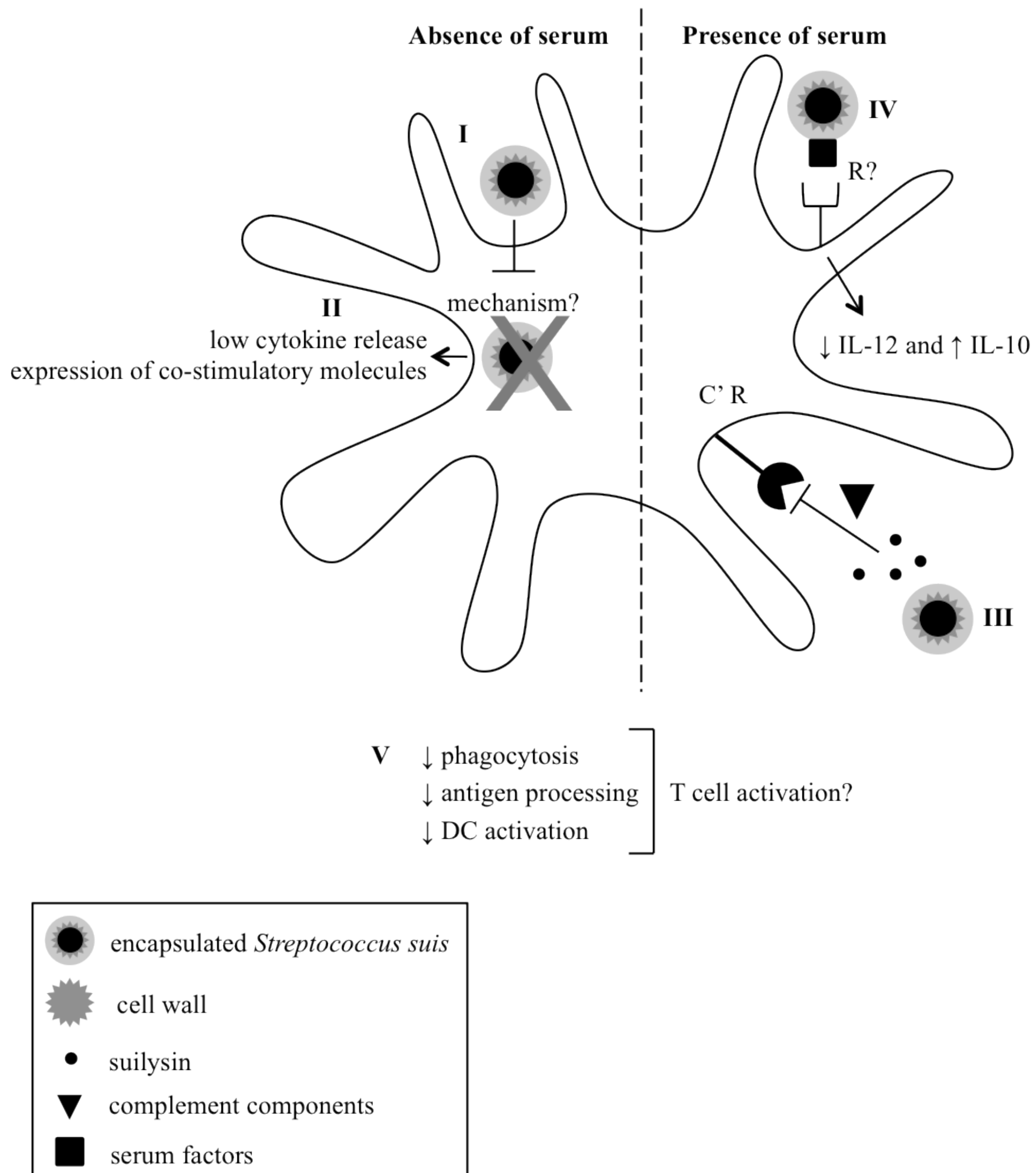

Figure 6. (I) In the absence of serum, the capsular polysaccharide (CPS) is sufficient to block *S. suis* phagocytosis by physical barrier and/or by inhibiting signalling pathways. (II) The presence of CPS further results in low cytokine release, but normal co-stimulatory molecule expression. (III) In the presence of serum, the CPS, the cell wall and the production of suilysin all contribute to reduce complement (C') deposition on *S. suis* and, consequently, the recognition by complement receptors (C' R), thus limiting complement-dependent clearance by dendritic cells (DCs). (IV) In addition, the binding of other serum factors to the surface of S. suis reduces the release of IL-12 and increases the release of IL-10 by DCs. (V) Overall, this results in low bacterial uptake which might translate in reduced antigen processing and DC activation with further consequences on T cell activation.

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Article II:

Characterization of porcine dendritic cell response to *Streptococcus suis*

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Rôle de la candidate dans la conception de l'article :

Je suis l'auteure principale de cet article. J'ai participé à la conception et au design des expériences ainsi qu'à la mise au point des techniques. J'ai effectué les manipulations et analysé les résultats. Finalement, j'ai réalisé la conception des graphiques et rédigé le manuscrit.

ABSTRACT

Streptococcus suis is a major swine pathogen and important zoonotic agent causing mainly septicemia and meningitis. However, the mechanisms involved in host innate and adaptive immune responses toward *S. suis* as well as the mechanisms used by *S. suis* to subvert these responses are unknown. Here, and for the first time, the ability of *S. suis* to interact with bone marrow-derived swine dendritic cells (DCs) was evaluated. In addition, the role of *S. suis* capsular polysaccharide in modulation of DC functions was also assessed. Well encapsulated *S. suis* was relatively resistant to phagocytosis, but it increased the relative expression of Toll-like receptors 2 and 6 and triggered the release of several cytokines by DCs, including IL-1β, IL-6, IL-8, IL-12p40 and TNF-α. The capsular polysaccharide was shown to interfere with DC phagocytosis; however, once internalized, *S. suis* was readily destroyed by DCs independently of the presence of the capsular polysaccharide. Cell wall components were mainly responsible for DC activation, since the capsular polysaccharide-negative mutant induced higher cytokine levels than the wild-type strain. The capsular polysaccharide also interfered with the expression of the co-stimulatory molecules CD80/86 and MHC-II on DCs. To conclude, our results show for the first time that *S. suis* interacts with swine origin DCs and suggest that these cells might play a role in the development of host innate and adaptive immunity during an infection with *S. suis* serotype 2.

INTRODUCTION

Streptococcus suis is a major swine pathogen associated mainly with meningitis, although other pathologies have also been described such as septicemia with sudden death, endocarditis, arthritis, and pneumonia [1]. Among 35 serotypes described, the serotype 2 is considered the most virulent and the most frequently isolated from both diseased pigs and humans. Consequently, most studies on virulence factors and the pathogenesis of infection have been carried out with this serotype [2]. Until recently, *S. suis* disease in humans has been considered as rare and only affecting people working with pigs or pork by-products. However, with a rising incidence in humans over the last years, *S. suis* is now considered as an important emerging zoonotic agent, especially in Asian countries, where *S. suis* has recently been identified as the leading cause of adult meningitis in Vietnam, the second in Thailand, and the third in Hong Kong. In 2005, an important outbreak occurred in China and resulted in 200 human cases with a fatality rate near 20% [1]. In humans, *S. suis* is mainly responsible for meningitis, septicemia and streptococcal toxic shock-like syndrome [1, 3-4].

Despite the increasing number of studies, the pathogenesis of the *S. suis* infection is still not completely understood and, to date, attempts to control the infection are hampered by the lack of an effective vaccine. The mechanisms involved in the host innate and adaptive immune responses toward *S. suis* as well as those used by *S. suis* to subvert these responses are unknown. Several virulence factors have been proposed to be involved in the pathogenesis of *S. suis* infection [5]. Among them, the capsular polysaccharide, which confers to the bacteria antiphagocytic properties, has been demonstrated as a critical virulence factor [2, 6-7] and its structure was recently described [8]. In fact, non-encapsulated mutants were shown to be avirulent in mice and pig models of infection [2]. Among several proteins and enzymes, a hemolysin (suilysin) has been characterized [5, 9]. The suilysin has been described to be involved in the modulation of *S. suis* interactions with host cells, such as endothelial cells, epithelial cells, neutrophils and monocytes/macrophages [2, 5].

Dendritic cells (DCs) are powerful antigen presenting cells that initiate the immune response against pathogens, and interactions between DCs and pathogens can strongly influence the outcome of a disease. After the capture of antigens, DCs undergo a complex maturation process, noticeable by the release of cytokines and the increased expression of co-stimulatory molecules. Mature DCs then migrate to the adjacent lymphoid organs where they activate T cells [10]. Thus, DCs are an essential link between innate and adaptive immunity.

DCs express a wide variety of pattern-recognition receptors (PRRs) that enable them to detect the presence of several pathogens through the recognition of pathogen-associated molecular patterns (PAMPs). Among these PRRs, Toll-like receptors (TLRs) are important for the recognition of pathogens and the initiation of the immune response as well as the shaping of adaptive immunity [11]. Different TLRs recognize different PAMPs of microorganisms. PAMPs recognized specifically by TLR2 include bacterial lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria. However, the recognition of peptidoglycan by TLR2 is still controversial. TLR2 generally forms heterodimers with TLR1 or TLR6 [12]. TLR4 has been reported as important for the recognition of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria [12]. Interestingly, TLR4 was also demonstrated as being involved in the recognition of pneumolysin, a suilysin-related toxin produced by *Streptococcus pneumoniae* [13, 14].

In the present study, we used porcine bone marrow-derived DCs to investigate the capacity of *S. suis* to interact with DCs and to induce their maturation and activation. We also examined the contribution of *S. suis* capsular polysaccharide on these interactions. To our knowledge, this is the first study concerning in vitro cultured porcine DC interactions with a whole live bacterial swine pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *S. suis* serotype 2 virulent suilysin-positive strain 31533, originally isolated from a case of porcine meningitis, and its isogenic non-encapsulated mutant B218 were used. These strains were already used in previous studies [15-17]. *S. suis* strains were grown as previously described [17] using either Todd-Hewitt broth (THB) or agar (THA) (Becton Dickinson, MD, USA) or sheep blood agar plates at 37°C. To perform *S. suis*-DCs interaction studies, isolated colonies were used as inocula for THB, which was incubated 8 h at 37°C with shaking. 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 shaking. Bacteria were washed twice in phosphate-buffered saline (PBS, pH 7.3) and were appropriately diluted in complete cell culture medium for the experiments. The number of CFU/mL in the final suspension was determined by plating samples onto THA using Autoplate® 4000 (Spiral Biotech, Norwood, MA, USA).

Animals

Cells were obtained from 6-8 weeks old SPF piglets. The animals originated from a herd free of major important diseases such as porcine reproductive and respiratory syndrome (PRRS), enzootic pneumonia due to *Mycoplasma hyopneumoniae* and clinical disease related to porcine circovirus. The herd did not have any episode of acute disease related to *S. suis* when the samples were taken. 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 Use of Laboratory Animals by the Animal Welfare Committee of the Université de Montréal.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived DCs were produced according to a technique described elsewhere [18-19]. Briefly, bone marrow was removed from femurs of 9 different animals. After red blood cell lysis, total bone marrow cells (5×10^6 cells/plate) were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine

serum (FBS), 2 mM L-Glutamine, 10 mM HEPES and 100 U/mL Penicillin-Streptomycin. All reagents were from Gibco (Burlington, ON, Canada). Complete medium was complemented with 100 ng/mL of porcine recombinant GM-CSF (Cell Sciences, MA, USA). Cells were cultured for 8 days at 37°C in a 5% CO_2 incubator and were fed on days 3 and 6. On day 8, cells were harvested, washed, and used as immature DCs for the studies. DC phenotype and purity was confirmed by FACS as described **below**.

Phagocytosis assay and intracellular survival

Bacteria were either non-opsonized or pre-opsonized using 20% fresh complete normal pig serum in PBS. Serum was negative for *S. suis* specific antibodies, using a strain-specific ELISA as previously described [20]. Opsonization was performed for 30 min at 37°C with shaking. Phagocytosis (MOI 1:1) was left to proceed for 30 min, 60 min, 90 min, 2 h and 4 h at 37°C with 5% CO_2. After incubation, penicillin G (5 µg/mL) and gentamicin (100 µg/mL) (both from Sigma, Oakville, ON, Canada) were added into 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 3 times, and sterile water was added to lyse the cells. To ensure complete cell lysis, cells were disrupted by scraping the bottom of the well and by vigorous pipetting. Viable intracellular bacteria were determined by quantitative plating of serial dilutions of the lysates onto THB agar. For intracellular survival studies, internalization assay was performed as described above, except that after a 60 min initial bacterial-cell contact, gentamycin-penicillin were added and the treatment was lengthened for different times up to 5 h. Cells were then processed as described above and bacteria counted. Results come from at least three independent experiments.

Confocal microscopy

For confocal microscopy analysis, cells were placed on coverslips and infected with the *S. suis* wild-type or its non-encapsulated mutant strain (MOI:1). After 2 h of bacteria-cell contact, coverslips were washed with PBS to remove non-associated bacteria, and cells fixed with methanol/acetone (80:20) for 20 min at -20°C, washed and blocked for 10 min. Coverslips were incubated 1 h with rabbit anti-*S. suis* serum and with a monoclonal

antibody against swine MHC class II antibody (VMRD, WA, USA). After washing, coverslips were incubated with the secondary antibodies Alex-Fluor 488 goat anti-rabbit IgG (*S. suis*) and Alex-Fluor 568 goat anti-mouse IgG (DC MHC-II) for 30 min, washed and mounted on glass slides with moviol containing DABCO and DAPI to stain the nuclei. Secondary antibodies were from Invitrogen, CA, USA. The polyclonal antiserum against *S. suis* serotype 2 recognizes both wild type and non encapsulated mutants at similar levels and has previously been used in other studies with the same strains (21, 22).

Electron microscopy analysis

For transmission electron microscopy (TEM) and scanning electron microscopy (SEM), *S. suis* strains were incubated with DCs for 4 h or 2 h, respectively. After two washes with PBS, the samples were fixed for 1 h at room temperature with 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and were then postfixed for 45 min at room temperature with 2% osmium tetroxide. Samples were then postfixed in 2% (vol/vol) osmium tetroxide in deionized water. Specimens for TEM were dehydrated in a graded series of ethanol solutions and embedded with LR White resin. Thin sections were cut with a diamond knife and were poststained with uranyl acetate and lead citrate. Samples were observed with an electron microscope model JEOL JEM-1230. Samples for SEM were dehydrated in a graded series of ethanol solutions and covered with gold after critical point drying and were examined with a Hitachi S-3000N microscope.

In vitro DC stimulation assay

DCs were resuspended and stimulated with *S. suis* (MOI: 0.001). Supernatants were collected at 16 h after infection to measure cytokines by ELISA and cells were harvested for analysis of co-stimulatory molecules by FACS. Lactate dehydrogenase (LDH) release measurement assay was used to measure cytotoxicity levels (Promega CytoTox96, Promega Corporation, Madison, WI) as previously described [6]. All experiments were conducted under non-cytotoxic conditions (data not shown). Purified *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) at 1µg/ml (InvivoGen) was used as positive control.

Cytokine quantification by ELISA

Levels of IL-1β, IL-6, IL-8, IL-12p40 and TNF-α in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's recommendations. Twofold dilutions of recombinant porcine 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 each cytokine. Results are from at least three independent experiments with at least two technical replicates.

FACS analysis

DCs were phenotypically characterized for the following markers: SWC3, MHC-I, MHC-II, CD1c, CD4, CD11R1, CD14, CD16, CD80/86 and CD163, and were shown to be composed of $SWC3^{+}/MHC\text{-}I^{+}/MHC\text{-}II^{+}/CD1c^{+}/CD14^{+}/CD16^{+}/CD163^{low}/CD4^{-}/Cd11R1^{-}$ cells, as previously described [18- 19]. Supernatants from hybridomas were used to detect the presence of the following molecules: SWC3, MHC-I, MHC-II, CD1c, CD4, and CD163. Hybridomas specific for these swine molecules were used in previous studies [18, 23-24], and provided by Dr. J. Dominguez (INIA, Madrid, Spain). Commercially available monoclonal antibodies from Serotec (NC, USA) were used to detect CD11R1 (clone MIL4), CD14 (clone MIL-2) and CD16 (clone G7). Antibodies against CD14 and CD16 were respectively conjugated to PE and FITC. A soluble fusion protein was used for detection of CD80/86 (CD152/CTLA-4 muIg, Ancell, MN, USA).

For cell surface staining, 2.5×10^{5} cells were incubated with the appropriate antibody for 1 h on ice followed by washing and staining for 1 h on ice with the secondary antibody goat anti-mouse IgG-PE (Jackson Immunoresearch, PA, USA). After washing, cells were resuspended in sorting buffer for FACS analysis. Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences, USA). A total of 20 000 gated events were acquired per sample and data analysis was performed using CellQuest software. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales. Results are from at least three independent experiments

Analysis of TLR gene expression by real time Reverse Transcriptase-quantitative PCR

DCs were infected with *S. suis* strains 31533 and B218 (MOI: 0.001) for 2 h, 4 h, 10 h and 16 h. Cells stimulated with specific ligands of the TLR family were used as controls. PAM3CSK4 (TLR1/2, final concentration of 500 ng/mL), FSL-1 (TLR2/6, final concentration of 500 ng/mL) and ultra pure LPS (TLR4, final concentration of 1µg/mL) were used and were obtained from InvivoGen (San Diego, CA, USA). Following infection, medium was removed and cells were washed. Total cellular RNA was prepared from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Next, 1 µg of total RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). The cDNA was amplified using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, Hercules, CA, USA). The PCR amplification program for all cDNA consisted of an enzyme activation step of 3 min at 98°C, followed by 40 cycles of a denaturing step for 2 s at 98 °C and an annealing/extension step for 5 s at 58 °C. The primers used for amplification of the different target cDNA are listed in Table I and were all tested to achieve an amplification efficiency between 90% and 110%. The primer sequences were all designed from the NCBI GenBank mRNA sequence using web-based software primerquest from Integrated DNA technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/). The Bio-Rad CFX-96 sequence detector was used for amplification of target cDNA of various TLRs and quantitation of differences between the different groups was calculated using the $2^{-\Delta\Delta Ct}$ method. Peptidylprolyl isomerase A (PPIA) was used as normalizing gene to compensate for potential differences in cDNA amounts. The non-infected DC group was used as the calibrator reference in the analysis. Results are from at least three independent experiments.

Statistical analysis

All data are expressed as mean ± SEM. Data from the phagocytosis assay and ELISA tests were analyzed for significance using Student's unpaired *t*-test. Data from RT-PCR were subjected to ANOVA procedures. A p value <0.05 was used as threshold for significance. All experiments were repeated at least three times.

RESULTS

Capsulated *S. suis* is relatively resistant to phagocytosis by DCs

To determine the ability of DCs to internalize S. suis, pre-opsonized or non-opsonized bacteria were incubated with DCs for different time periods. As shown in Figure 1, the wild-type strain was relatively resistant to phagocytosis and relatively few bacteria were found inside the cells. On the other hand, the non-encapsulated mutant strain was significantly more internalized by DCs under non-opsonic conditions. Thus, the capsular polysaccharide seems to interfere with the phagocytosis of *S. suis* by swine DCs. Serum components did not seem to influence *S. suis* phagocytosis levels by DCs, as no significant differences were noticeable between pre-opsonized and non-opsonized bacteria for either the wild-type strain or the non-encapsulated mutant (Fig. 1).

The ability of DCs to interact and internalize *S. suis* was confirmed by confocal and electron microscopy. Confocal microscopy was performed using serum against *S. suis* and an antibody specific for swine MHC-II. DCs were incubated with either the wild-type strain or the non-encapsulated mutant. Confocal analysis under non-opsonic conditions showed that the average number of internalized bacteria remains very low for the wild-type strain, with only a few bacterial cells present in every DCs. In contrast to the wild-type strain, the non-encapsulated mutant was highly internalized by DCs (Fig. 2). No differences were observed between non-opsonized or pre-opsonized bacteria (data not shown). For further confirmation of these results, SEM and TEM were carried out. Indeed, when DCs where incubated with the wild-type strain, only few cocci were found associated to the cell surface by SEM analysis (Fig. 3A). Following incubation with the non-encapsulated mutant, cocci were largely found adhering to DCs (Fig. 3B-C). TEM analysis also showed that only few DCs contained wild-type strain cocci despite having been opsonized by complete serum (Fig. 4A). In contrast, high numbers of streptococci were observed intracellularly after DC infection with the non-encapsulated mutant pre-opsonized with complete serum (Fig. 4B). Altogether, these results suggest that the non-encapsulated mutant adheres to and is internalized by DCs at markedly higher numbers than the wild-type strain.

***S. suis* is readily destroyed inside DCs**

To analyze the intracellular fate of bacteria once internalized, we modified the phagocytosis assay in order to quantify bacterial intracellular survival over time. Precisely, after 60 min incubation of *S. suis* with DCs, to get optimal internalization, antibiotics were added and the treatment was lengthened for different times up to 5 h. As shown in Figure 5, once internalized, both the wild-type strain and its non-encapsulated mutant were equally destroyed as similar rates and kinetics of reduction in intracellular bacterial numbers were observed. Hence, the capsular polysaccharide interferes with *S. suis* phagocytosis by DCs, but does not protect the bacteria against intracellular killing. No differences in intracellular survival levels were observed between non-opsonized or pre-opsonized bacteria (data not shown).

***S. suis* induces the release of several cytokines by DCs**

The levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, the T cell-activating cytokine IL-12p40, and the chemokine IL-8 in the supernatants of *S. suis*-infected DCs were measured at 16 h after stimulation. Time and bacterial dose for the cytokine stimulation assays were chosen using absence of cytotoxicity and significant activation as selection criteria (data not shown). Our results showed that DCs produced significant amounts of these cytokines after exposure to *S. suis* wild-type strain compared to control, non-activated cells. However, the non-encapsulated strain induced significantly higher levels of all cytokines tested, except for IL-1β, compared to the wild-type strain (Fig. 6A-E).

Involvement of TLR2 and TLR6 in DC activation by *S. suis*

To analyze whether *S. suis* modulates mRNA expression levels of TLR1, 2, 4 and 6, DCs were stimulated with *S. suis* wild-type strain or its non-encapsulated mutant. PAM(3)CSK, FSL-1 and LPS were used as positive controls for TLR2/TLR1, TLR2/TLR6 and TLR4, respectively. As shown in Figure 7, *S. suis* wild-type strain induced significant up-regulation of TLR2 and TLR6 mRNA by DCs at 16 h and 10 h of infection, respectively. Similarly, the non-encapsulated strain activated both TLR2 and TLR6 within 10 h of infection (Fig. 7A-B). As low and variable levels of TLR1 mRNA expression were

observed in *S. suis*-stimulated DCs, no significant differences could be observed compared to control non-infected cells (data not shown). Finally, the expression of TLR4 was not up-regulated in the presence of *S. suis* even though an upregulation was noticeable with the positive control LPS (data not shown).

Encapsulated *S. suis* failed to induce DC surface expression of co-stimulatory molecules

The ability of *S. suis* to induce surface expression of the co-stimulatory molecules MHC-II and CD80/86 by DCs was investigated by FACS 16 h after stimulation (Fig. 8). Interestingly, wild-type *S. suis* failed to induce significant up-regulation of CD80/86 and MHC-II expression by DCs in terms of the percentage of cells expressing these markers compared with non-stimulated, control cells. In contrast, DCs stimulated with the non-encapsulated mutant strain showed significant higher levels of surface expression of CD80/86 compared with non-stimulated cells and cells stimulated with the wild-type strain. Significantly higher levels of surface expression of MHC-II were also observed following DC stimulation with the non-encapsulated mutant strain compared to non-stimulated cells. It should be noted that high variability was observed between animals in terms of MHC-II expression; as such the difference between the wild-type strain and its non-encapsulated mutant was shown not to be significant for this molecule. Altogether, our data suggest that the capsular polysaccharide interferes with co-stimulatory molecule expression by DCs.

DISCUSSION

S. suis is considered as a zoonotic pathogen of increasing importance for human health [1, 3-4]. However, despite the rising number of studies, mechanisms leading to an efficient immune response against *S. suis* are poorly understood. Previously, we have demonstrated that the mouse model of infection is a valid model to reproduce *S. suis* infection [15, 25], and recently, interactions between *S. suis* and mouse bone marrow-derived DCs (mDCs) were described [21]. Results showed that *S. suis* uses an arsenal of different virulence factors to modulate mDC functions and escape immune surveillance, mainly by modulating cytokine release and escaping opsono-phagocytosis. However, it is important to confirm *S. suis* modulation of DC activation in the natural host, the swine. The present work demonstrates for the first time that *S. suis* interacts with porcine DCs and modulates their maturation and activation.

We used a phagocytosis assay, combined with confocal and electron microscopy to measure the ability of DCs to internalize *S. suis* and to evaluate the role of the capsular polysaccharide in this process. We observed that the presence of the capsular polysaccharide protects *S. suis* from DC phagocytosis, both under opsonic and non-opsonic conditions. This confirms the role of the capsular polysaccharide as an anti-phagocytic factor. In agreement, previous studies (using the same wild type and mutant strains included in this study) with monocytes/macrophages, neutrophils and mDCs demonstrated that the capsular polysaccharide reduces *S. suis* phagocytosis [6-7, 21, 26]. Moreover, the capsular polysaccharide was also previously shown to be crucial for the survival of *S. suis* in vivo. Indeed, the non-encapsulated mutant strain used in this study was shown to be avirulent and rapidly eliminated from the bloodstream in a porcine model of infection [27]. Despite the fact that the capsular polysaccharide acts as a physical barrier to block *S. suis* phagocytosis by DCs, the intracellular survival assay showed that once internalized, both encapsulated and non-encapsulated strains are equally destroyed. Our previous data with mDCs showed that both encapsulated and non-encapsulated *S. suis* localizes within LAMP+ vacuoles suggesting phagosome fusion with lysosomes leading to bacterial destruction [21]. Hence, the capsular polysaccharide protects the bacteria against

phagocytosis, but not against intracellular killing. Previous studies with macrophages also showed that neither virulent nor non-virulent encapsulated strains were able to survive inside macrophages [28-29]. Altogether these studies suggest that *S. suis* extracellular localization confers to this pathogen a survival advantage and the capsular polysaccharide is essential to it. Wild-type *S. suis* was shown to trigger DC release of IL-1β, IL-6, IL-8, IL-12p40 and TNF-α. These cytokines, among others, were recently shown to be released by mDCs following stimulation with wild-type *S. suis* strain 31533 [21]. The capsular polysaccharide interfered with the release of IL-6, IL-8, IL-12 and TNF-α by swine DCs, as shown with mDCs and other phagocytic cells [6-7]. Increased exposure of cell wall components due to the absence of a capsule may account for the higher capacity of the non-encapsulated mutant to induce most cytokine secretion, and confirm the role of cell wall components as major cytokine modulators [17, 30-31]. This is further supported by recent results with mDCs where we observed that IL-12p70, IL-10 and CXCL10 release was diminished following mDC stimulation by *S. suis* cell wall mutant strains [21]. Besides *S. suis* cell wall components, studies to date have identified two cytokines for which the capsular polysaccharide is required for optimal induction, MCP-1 and IL-1β [21, 30-31]. Here, *S. suis* capsular polysaccharide was also shown not to interfere with the production of IL-1β production by swine DCs. The molecular pathways underlying the capsular polysaccharide contribution to IL-1β and MCP-1 release are under evaluation. Finally, and accordingly to data from Devriendt *et al*. (2010), porcine DCs were low responsive to LPS (32).

In addition to cytokine production, both the wild-type strain and its non-encapsulated mutant strain increased the expression of TLR2 and TLR6 mRNA. No differences were noticeable between the two strains for the expression of TLR2. However, the expression of TLR6 was increased more rapidly after DC infection with the non-encapsulated strain than that observed for the wild-type strain. This activation pattern is in agreement with that recently reported by Wichgers Schreur *et al*. [33], who showed that human TLR2 and TLR6 are activated by lipoproteins of *S. suis* [33]. However, our results slightly differ from those of these authors who indicate an absence of TLR2 upregulation after culturing human transfected epithelial cells with live or heat-killed whole cells of *S. suis*. It should be noted,

however, that interactions between *S. suis* and epithelial cells can highly differ from those observed with DCs. It has also been demonstrated that stimulation of human monocytes by whole encapsulated *S. suis* or its purified cell wall components influences the relative expression of TLR2 mRNA [30]. Moreover, this stimulation triggered the release of cytokines, which was significantly reduced by neutralizing antibodies against TLR2 but not against TLR4 [30]. Mouse macrophages deficient in TLR2 expression also show reduced cytokine release in response to encapsulated *S. suis*. Since this response was completely inhibited in MyD88-deficient macrophages, other TLRs could be involved in cytokine production induced by *S. suis*. In addition, it was demonstrated that the presence of the capsular polysaccharide modulates interactions between *S. suis* and TLRs, as uncovered cell wall components were shown to induce cytokine production through TLR2-dependent and -independent pathways [30]. Finally, after *S. suis* invasion of the central nervous system, transcriptional activation of TLR2, TLR3 and CD14 has been observed in a mouse model of infection [15]. This study is the first to report TLR activation following *S. suis* stimulation of cells of porcine origin.

The ability of *S. suis* to induce the maturation of DCs was also investigated by evaluating the surface expression of the co-stimulatory molecules CD80/86 and MHC-II on swine DCs. *S. suis* wild-type strain failed to induce the expression of either CD80/86 or MHC-II on DCs. The capsular polysaccharide was shown to be responsible for the impaired expression of CD80/86 on DCs and also seems to interfere, at least in part, with MHC-II expression. This differs with results obtained with mDCs where wild-type *S. suis* induced mDC maturation levels similar to those observed with the non-encapsulated mutant [21]. These differences could be related to the cell origin (swine vs mouse) and also to the fact that mDC are derived from inbred mouse lines while swine DCs are originated from outbred animals. Indeed, high variability was observed in *S. suis*-induced MHC-II expression by DCs derived from different pigs. This could be related to the fact that genes of the MHC complex have high levels of polymorphism [34,35]. Highly polymorphic swine leukocyte antigen (SLA) genes in the porcine MHC have been shown to significantly influence swine immunological traits and vaccine responsiveness [36-39]. The strong influence of the SLA complex is mostly attributable to the antigen-presenting properties of

the MHC proteins in the swine adaptive immune system [40]. The high degree of variability in the ability of DCs to up-regulate surface expression of MHC-II might explain, in part, why *S. suis* would successfully colonize only some piglets and not others, and why some animals will only be healthy carriers and will never develop disease whereas others will develop bacteremia, sometimes septicemia and finally meningitis [2]. As individual variation in responsiveness to vaccine candidates is becoming more of an issue, particularly with non-responders, these observations are crucial for the immunological studies of *S. suis* pathogenesis.

To conclude, our results show for the first time that *S. suis* interacts with swine origin DCs and suggest that these cells might play a role in the development of host innate and adaptive immunity during an infection with *S. suis* serotype 2. *S. suis* resists phagocytosis but is able to activate DC release of pro-inflammatory cytokines mainly through the activation of TLRs 2 and 6. In fact, *S. suis* capsular polysaccharide was shown to modulate most interactions with DCs by protecting bacteria against phagocytosis, reducing the level of cytokine production and preventing the surface expression of co-stimulatory molecules. Overall, capsular polysaccharide-impaired *S. suis* interactions with DCs would result in low bacterial up-take as well as low DC activation and maturation which might translate in reduced antigen processing and T cell activation, although this should be confirmed. The capsular polysaccharide could therefore be considered as an escape mechanism for *S. suis*. It is important to note that since none of the non-encapsulated mutants available in the literature (including the one used in this study) could so far be successfully complemented (showing restoration of capsule production), a certain additional role of other unknown mutation in those mutants cannot be completely ruled out. The importance of DCs on the efficacy of the immune system has been clearly demonstrated in the last years [41-42]. However, to our knowledge, this study is the first to investigate the interactions between a whole live bacterial pathogen and swine DCs.

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Table I: Sequences of porcine-specific real-time PCR primers[a]

Name	Accession Number	Forward	Reverse
TLR1	NM_001031775	CCAGTGTGTTGCCAATCGCTCATT	TCCAGATTTACTGCGGTGCTGACT
TLR2	NM_213761	AGCACTTCCAGCCTCCCTTTAAGT	TACTTGCACCACTCGCTCTTCACA
TLR4	NM_001113039	ACCAGACTTTCTTGCAGTGGGTCA	AATGACGGCCTCGCTTATCTGACA
TLR6	NM_213760	TCCCAGAATAGGATGCAGTGCCTT	ACTCCTTACATATGGGCAGGGCTT
PPIA	NM_214353	AGGATTTATGTGCCAGGGTGGTGA	ATTTGCCATGGACAAGATGCCAGG

[a]Oligonucleotide primers were from Integrated DNA Technologies

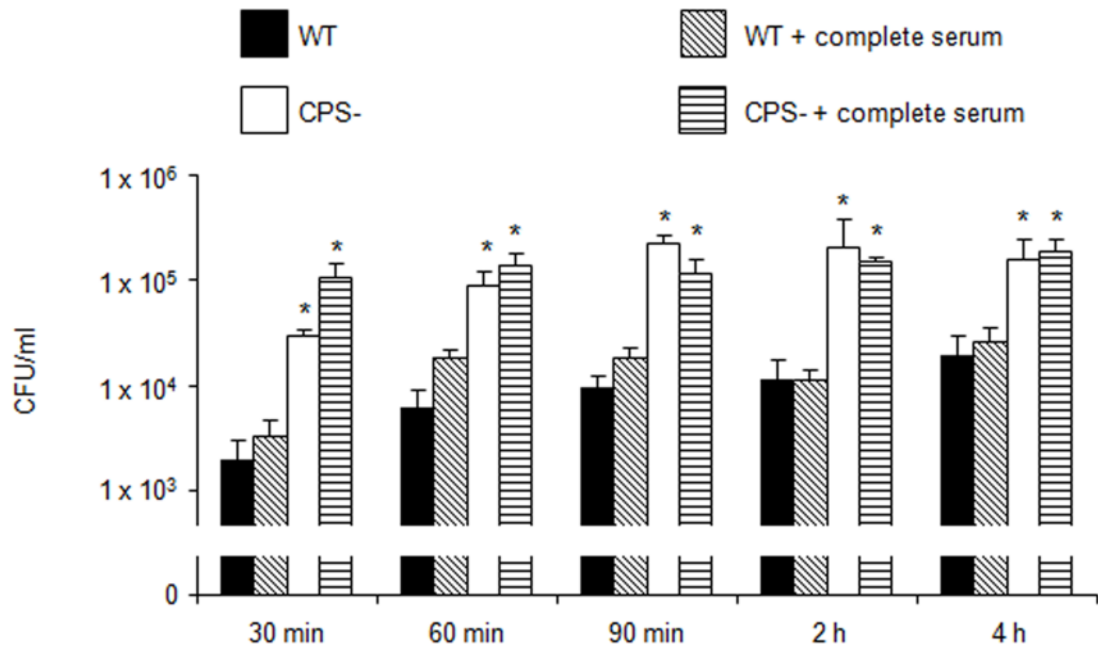

Figure 1. Effect of capsular polysaccharide on the capacity of DCs to internalize *S. suis*. Bacteria (MOI: 1) were either non-opsonized or pre-opsonized with 20% complete normal pig serum for 30 min prior to incubation with DCs for 30 min, 60 min, 90 min, 2 h and 4 h. Numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and results are expressed as CFU recovered bacteria per ml (means ± SEM obtained from independent experiments using DCs derived from 9 different animals. Experiments were repeated at least three independent times.). * $p < 0.05$, indicates statistically significant differences between the wild-type strain 31533 and its isogenic non-encapsulated mutant either non-opsonized or pre-opsonized with complete normal pig serum. WT, wild-type strain. CPS-, non-encapsulated mutant.

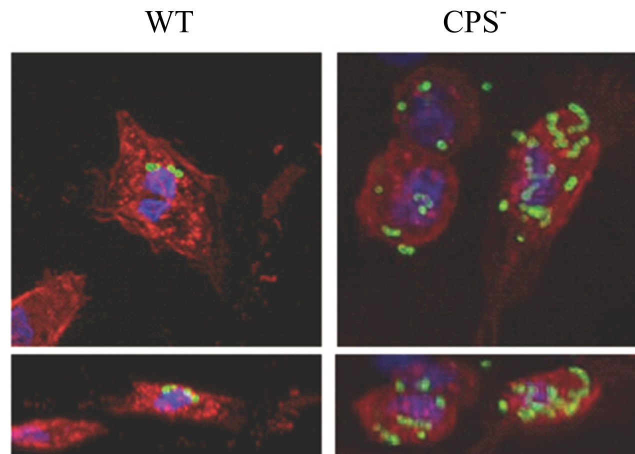

Figure 2. Confocal microscopy showing internalization of *S. suis*. DCs (MOI:1) were incubated with *S. suis* wild-type strain (WT) or the non-encapsulated mutant (CPS-). After a bacterial-cell contact of 2 h, cells were fixed and labelled with serum against *S. suis* (Alex-Fluor 488, green) and a monoclonal antibody specific for swine MHC-II (Alex-Fluor 568, red). DAPI was used to stain the nuclei (blue).

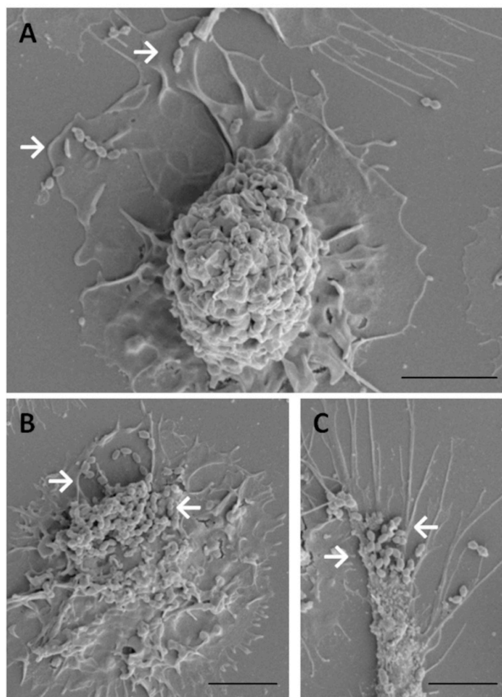

Figure 3. Scanning electron micrographs showing interactions between DCs and *S. suis*. DCs were incubated with *S. suis* (MOI:1) wild-type strain (WT) or the non-encapsulated mutant (CPS-) for 2 h. (A) DCs incubated with *S. suis* WT strain show very few cocci on the cell surface. DCs incubated with the CPS- mutant show several cocci adhering to the cells (B-C). White arrows show bacterial cells. (A) Scale bar, 10 µm. Original magnification 5000X. (B-C) Scale bar, 5 µm. Original magnification 5000X.

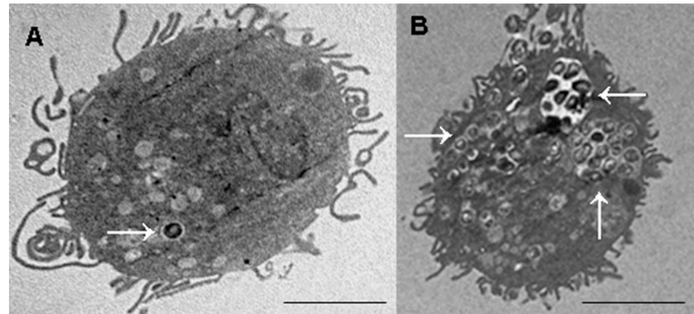

Figure 4. Transmission electron micrographs showing internalization of *S. suis* by DCs. DCs (MOI:1) were incubated with *S. suis* wild-type strain (WT) or the non-encapsulated mutant (CPS-) for 4 h. (A) Most DCs were free of *S. suis* or contained very few bacteria when incubated with serum-opsonized WT strain 31533, (B) DCs incubated with serum-opsonized CPS- mutant contained high numbers of internalized bacteria. White arrows show internalized bacteria, scale bar 2 µm. Original magnification 10000X.

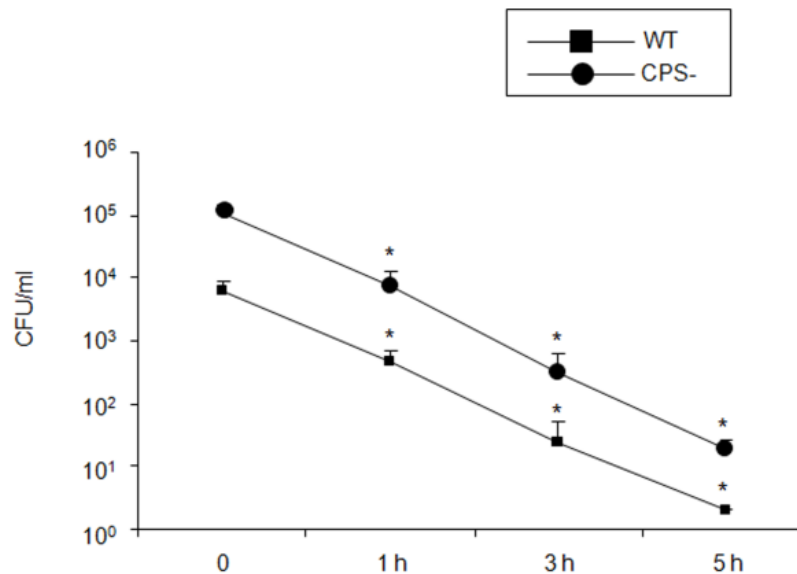

Figure 5. Intracellular survival of *S. suis* within DCs. DCs were infected with *S. suis* (MOI:1) wild-type strain (WT) or the non-encapsulated mutant (CPS-) and phagocytosis was left to proceed for 1 h. Antibiotics were then added for a period time of 1 h (considered here as time 0). This initial antibiotic-treatment was lengthened for different times up to 5 h and cells were lysed to quantified intracellular bacteria by viable plate counting. Results are expressed as CFU recovered intracellular bacteria per ml (means ± SEM obtained from three independent experiments using DCs derived from 9 different animals). An asterisk indicates the incubation time for which the number of intracellular bacteria recovered is significantly different ($p < 0.05$) from number of intracellular bacteria obtained after an initial 1 h antibiotic treatment (considered here as time 0).

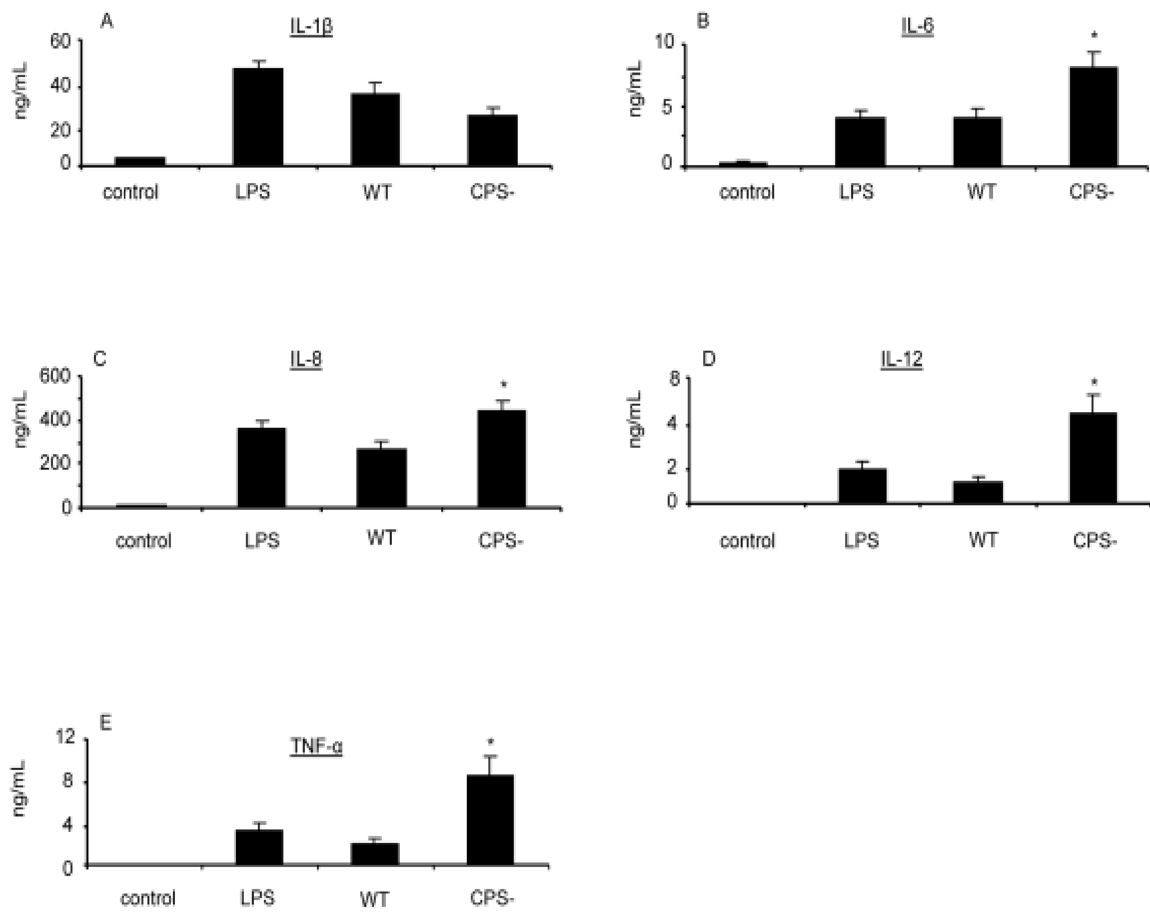

Figure 6. Cytokine production by DCs in response to stimulation by LPS (1 μg/ml) and different *S. suis* strains (MOI: 0.001) for 16 h. Data are expressed as mean ± SEM (in ng/ml) from independent experiments using DCs derived from different 9 animals. Experiments were repeated at least three times with at least two technical replicates. Control, non-infected cells. WT, wild-type strain. CPS-, non-encapsulated mutant. * $p < 0.05$, denotes values obtained with the CPS- mutant that are significantly higher from those obtained with the WT strain.

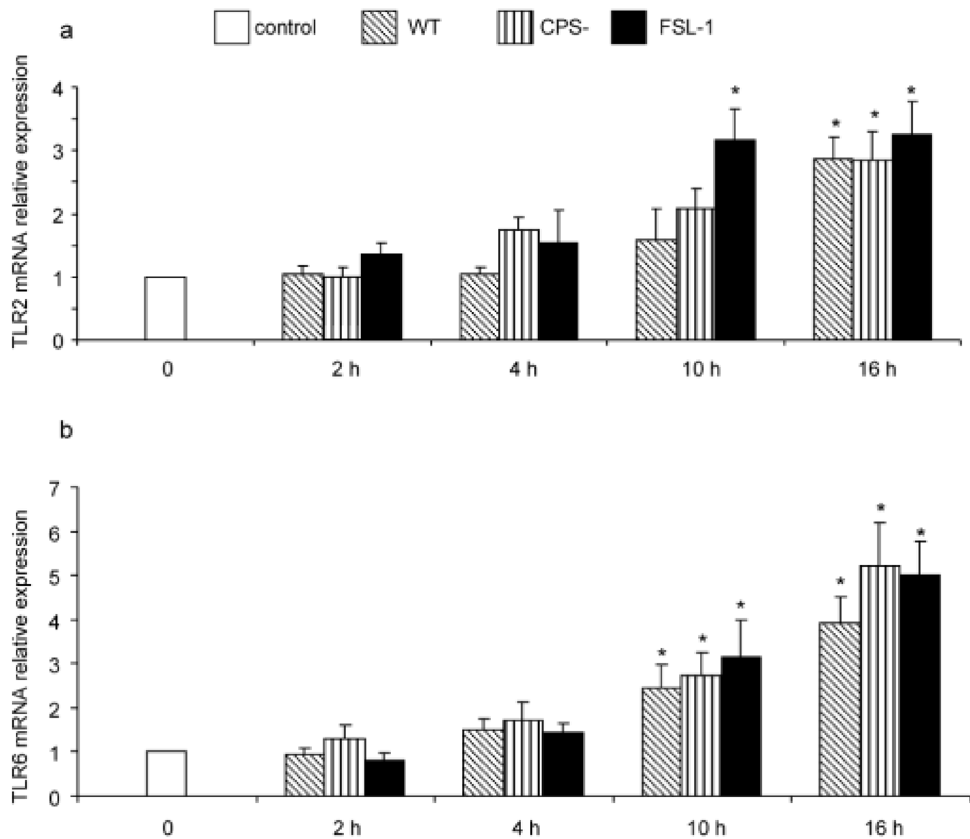

Figure 7. Relative expression of TLR2 (A) and TLR6 (B) mRNA by DCs stimulated with positive control FSL-1 (1 µg/ml) or *S. suis* (MOI: 0.001) wild-type strain (WT) or the non-encapsulated mutant (CPS-) for different incubation times. Unstimulated DCs served as control. Data are expressed as mean ± SEM from independent experiments using DCs derived from 6 different animals. Experiments were repeated at least three independent times. **p* <0.05, indicates that mRNA expression was significantly different compared to control DCs.

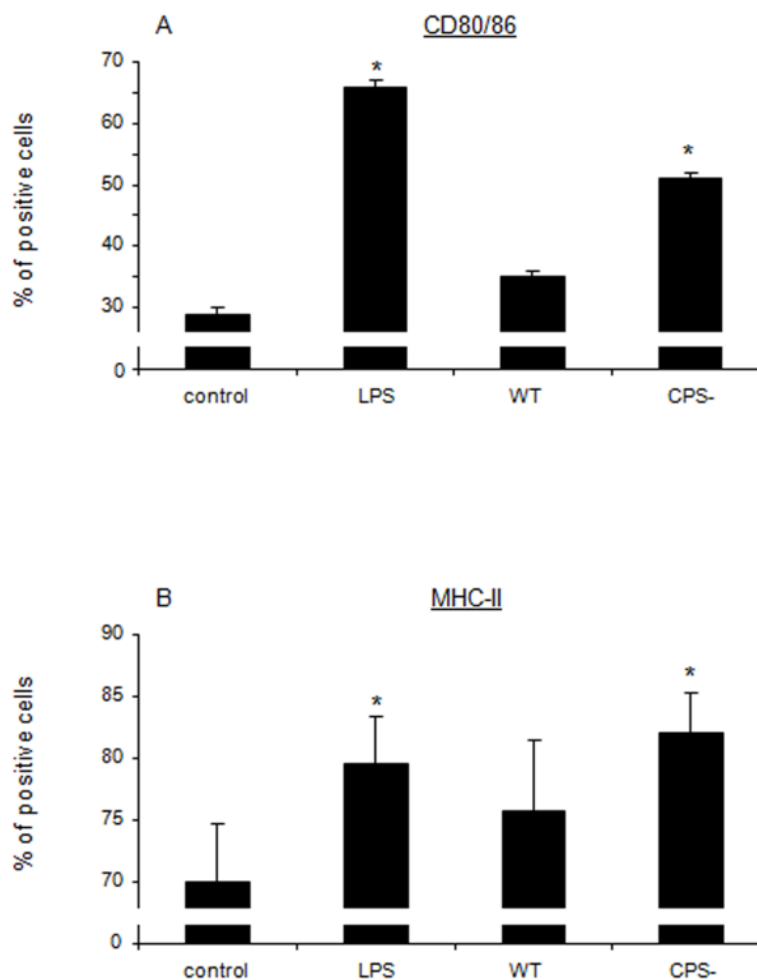

Figure 8. Expression of surface markers MHC-II and CD80/86 by DCs stimulated with LPS (1 µg/ml) or *S. suis* (MOI: 0.001) wild-type strain (WT) or the non-encapsulated mutant (CPS-) for 16 h. Unstimulated DCs served as control. Data are expressed as mean ± SEM (in % of positive cells) from independent experiments using DCs derived from 6 different animals. * $p < 0.05$, denotes values obtained with the CPS- mutant that are significantly higher from those obtained with control cells.

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Article III:

Immune receptors involved in *Streptococcus suis* recognition by dendritic cells.

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Rôle de la candidate dans la conception de l'article :

Je suis l'auteure principale de cet article. J'ai participé à la conception et au design des expériences ainsi qu'à la mise au point des techniques. J'ai effectué les manipulations et analysé les résultats. Finalement, j'ai réalisé la conception des graphiques et rédigé le manuscrit.

ABSTRACT

Streptococcus suis is an important swine pathogen and an emerging zoonotic agent of septicemia and meningitis. Knowledge on host immune responses towards *S. suis*, and strategies used by this pathogen for subversion of these responses is scarce. The objective of this study was to identify the immune receptors involved in *S. suis* recognition by dendritic cells (DCs). Production of cytokines and expression of co-stimulatory molecules by DCs were shown to strongly rely on MyD88-dependent signaling pathways, suggesting that DCs recognize *S. suis* and become activated mostly through Toll-like receptor (TLR) signaling. Supporting this fact, TLR2$^{-/-}$ DCs were severely impaired in the release of several cytokines and the surface expression of CD86 and MHC-II. The release of IL-12p70 and CXC10, and the expression of CD40 were found to depend on signaling by both TLR2 and TLR9. The release of IL-23 and CXCL1 were partially dependent on NOD2. Finally, despite the fact that MyD88 signaling was crucial for DC activation and maturation, MyD88-dependent pathways were not implicated in *S. suis* internalization by DCs. This first study on receptors involved in DC activation by *S. suis* suggests a major involvement of MyD88 signaling pathways, mainly (but not exclusively) through TLR2. A multimodal recognition involving a combination of different receptors seems essential for DC effective response to *S. suis*.

INTRODUCTION

Streptococcus suis serotype 2 is a major swine pathogen mainly associated with meningitis, although other systemic infections have been described [1, 2]. *S. suis* is now emerging as a threat to human health, especially in Asian countries where it has recently been identified as the leading cause of adult meningitis in Vietnam, the second in Thailand, and the third in Hong Kong [2]. Moreover, two important human outbreaks of streptococcal toxic shock-like syndrome (STSLS) due to *S. suis* occurred in China during the last years with a fatality rate near 20% [2].

Several virulence factors have been proposed to be involved in the pathogenesis of the infection. The most important among them is the capsular polysaccharide (CPS), which confers antiphagocytic properties to the pathogen [3]. In addition, the bacterial cell wall and modifications of its components, such as the N-deacetylation of peptidoglycan (PG) and the D-alanylation of lipoteichoic acids (LTA), were shown to also contribute to the virulence of *S. suis* [4-6]. Other virulence factors have also been proposed [3, 7]. Among them, an hemolysin (suilysin), although not considered as a critical virulence factor [8] and being absent in many virulent strains [9], has been shown to play a certain role in *in vitro* interactions between *S. suis* and different host cells [1, 7, 10, 11].

As evidenced by human *S. suis* outbreaks of STSLS as well as by septic shock cases in Europe and Asia, an important release of pro-inflammatory mediators is thought to take place during *S. suis* systemic infections [2]. In fact, *S. suis* is able to induce *in vitro* production of different pro-inflammatory cytokines and chemokines by porcine, murine, and human cells; and *in vivo* upregulation of inflammatory mediators in affected humans as well as in experimental mouse models of infection [12-14].

DCs are powerful antigen-presenting cells that initiate immune responses against pathogens; they capture and process antigens, and then undergo a maturation process characterized by the production of cytokines and upregulation of co-stimulatory molecules. Then, DCs migrate to adjacent lymphoid organs where they activate T cells [15].

Recognition of pathogen-associated molecular patterns (PAMPs) by DCs is mediated by pattern-recognition receptors (PRRs), including the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) families [16]. TLR2 is reported to be specialized for the recognition of lipoproteins by generally forming a heterodimer with TLR1 or TLR6 [17, 18]. Although TLR4 has been shown as important for the recognition of lipopolysaccharide (LPS) [17], it has also been reported to recognize the pneumolysin, a suilysin-related toxin produced by *Streptococcus pneumoniae* [19, 20]. TLR9 is an intracellular receptor involved in the recognition of bacteria-derived DNA [17]. At least two well-characterized NLRs, that is, NOD1 and NOD2, recognize the structures of bacterial PGs, g-D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively [18]. Another group of NLRs participates in the formation of a large multiprotein complex called the inflammasome, whose assembly leads to the activation of caspase 1-mediated innate immune responses [18].

Interactions between TLRs and NODs with their ligands initiate an intracellular signaling cascade that induces the secretion of several pro-inflammatory cytokines and the expression of co-stimulatory cell-surface molecules through the activation of transcription factors including NF-κB [18]. Pathogens can, however, hijack the TLR signaling to evade recognition and elimination by the immune system [21]. TLRs and NODs can synergistically activate proinflammatory cytokine production [16].

Bone marrow-derived DCs (bmDCs) have been shown to be a valid and interesting model to study the host immune response during *S. suis* infection [10]. Using this model, it has been shown that *S. suis* uses an arsenal of different virulence factors to modulate DC functions, particularly cytokine release and complement-dependent opsono-phagocytosis [10], [22]. As such, we hypothesize that *S. suis* activates cells through multiple receptors. In the present study, we used bmDCs to evaluate the importance of specific immune receptors in the recognition of *S. suis* serotype 2.

MATERIALS AND METHODS

Ethics statement

All experiments involving mice 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 Use of Laboratory Animals by the Animal Welfare Committee of the Université de Montréal (Comité d'éthique de l'utilisation des animaux (CÉUA)). The protocols and procedures were approved by the Ethics Committee (CÉUA).

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are described in Table 1. *S. suis* strains were grown on Todd-Hewitt broth (THB) or agar (Becton Dickinson, Mississauga, ON, Canada) or on sheep blood agar plates at 37 °C. *Escherichia coli* was grown on Luria-Bertani broth or agar (Becton Dickinson). When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *E. coli*, kanamycin and spectinomycin at 50 μg/ml; for *S. suis*, spectinomycin at 100 μg/ml. To perform *S. suis*-DCs interaction studies, bacteria suspensions were prepared as previously described [10] and appropriately diluted in complete cell culture medium for the experiments. The number of CFU/ml in the final suspension was determined by plating samples onto THB agar using Autoplate® 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA).

Construction of the knockout vector for gene replacement and generation of *S. suis* Δ*dltaA*/Δ*pgdA* double knockout

Δ*dltaA* and Δ*pgdA* mutants were produced and characterized in our laboratory in the past [4, 5]. In order to evaluate a combined effect of LTA and PG modifications, a Δ*dltaA*/Δ*pgdA* double mutant was generated. Briefly, genomic DNA from parent strain 31533 was prepared using InstaGene Matrix (BioRad Laboratories, Mississauga, ON, Canada). Then, a 1407 bp, precise, in-frame deletion of the *dltA* gene was constructed by using splicing-by-overlap-extension PCR [24] and the primers (Invitrogen, Burlington, ON, Canada) listed in Table 2. The PCR-generated Δ*dltA* deletion allele was subsequently

cloned into plasmid pCR2.1 (Invitrogen), extracted with BamHI and PstI and recloned into the same sites of the thermosensitive *E. coli-S. suis* shuttle plasmid pSET5s, which carries the chloramphenicol resistance gene *cat* [25]. The resulting knock-out vector was named p5ΔdltaA. Restriction enzymes and DNA-modifying enzymes (TaKaRa Bio, Otsu, Shiga, Japan) were used according to the manufacturers' recommendations. PCR reactions were carried out with the iProof proofreading DNA polymerase (BioRad). Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures [26]. To obtain the double mutant, knock-out vector p5ΔdltaA was electroporated into the previously generated *S. suis* Δ*pgdA* mutant strain. Procedures for isolation of mutants were those described previously [27]. Successful allelic replacement of the *dltA* gene in the Δ*pgdA* strain was confirmed by PCR and DNA sequencing analysis using an ABI 3730xl automated DNA sequence and the ABI PRISM dye terminator cycle version 3.1 (Applied Biosystems, Carlsbad, CA).

Generation of mouse bone marrow-derived dendritic cells (bmDCs)

Six to eight week-old mice originated from Jackson Laboratory (Bar Harbor, ME, USA), including wild type (WT) C57BL/6, MyD88$^{-/-}$ (B6.129P2-*Myd88*^{tm1Defr}/J), TLR2$^{-/-}$ (B6.129-*Tlr2*$^{\mathrm{tmlKir}}$/J), TLR4$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ) and NOD2$^{-/-}$(B6.129S1-*Nod2*^{tm1Flv}/J) mice were used. BmDCs were produced according to a technique previously described [10]. Briefly, on day 0, bone marrow was removed from femurs and tibiae. After red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 μg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. All reagents were from Gibco (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 [28]. Cells were cultured for 7 days at 37 °C in a 5% CO_2 incubator and were fed on days 3 and 5. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8, washed, and used as immature DCs for the studies. Cell purity was routinely 86-90% CD11c$^+$ cells as determined by FACS analysis and as previously reported [10].

Phagocytosis assay

Bacteria were pre-opsonized using 20% complete normal mouse serum in PBS for 30 min at 37 °C with agitation, as previously described [10]. DCs (10^6 cells/ml) were infected with pre-opsonized *S. suis* strains (31533, B218 and Δ*dltA*/Δ*pgdA* at a MOI: 1). Phagocytosis was left to proceed for 2 h at 37 °C with 5% CO_2. MOI and assay conditions were chosen based on previous studies on the kinetics of *S. suis* phagocytosis by DCs [22]. After incubation, penicillin G (5 μg/ml) and gentamycin (100 μg/ml) (both from Sigma) were directly added into 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 3 times, and sterile water was added to lyse the cells. To ensure complete cell lysis, cells were disrupted by scraping the bottom of the well and by vigorous pipetting. Each test was repeated at least four times in independent experiments, and the number of CFU recovered per well (mean number ± SEM) was determined by viable intracellular bacterial counting as described above.

In vitro DC stimulation assay

DCs were resuspended at 10^6 cells/ml in complete medium supplemented with 5% GM-CSF supernatant and stimulated with different strains of *S. suis* (10^6 CFU/ml; initial MOI: 1). Conditions used were based on those already published [10]. Bacterial strains were pre-opsonized using 20% complete normal mouse serum as described above. At different time intervals, supernatants were collected for cytokine quantification by ELISA and cells were harvested for analysis of co-stimulatory molecule expression by FACS. Non-stimulated cells served as negative control. Lactate dehydrogenase (LDH) release measurement assay was used to confirm absence of cytotoxicity in bacterial-bmDC cultures (Promega CytoTox96, Promega Corporation, Madison, WI, USA), as previously described [14]. For inhibition of TLR9, DCs were pre-treated with ODN2088 (5 μM) (Invivogen, Burlington, ON, Canada) for 1 h prior to infection with *S. suis*. The TLR9 activator ODN1826 (1 μM) (Invivogen) was used as a positive control to stimulate bmDCs through TLR9 [29]. For neutralization of TLR2, bmDCs were pre-treated for 1 h with 15 μg/ml of anti-TLR2 (clone T2.5, Hycult biotechnology, Plymouth, PA). PAM(3)CSK(4) (TLR1/2 ligand, final

concentration of 500 ng/ml), FSL-1 (TLR2/6 ligand, final concentration of 500 ng/ml) and ultra pure LPS (TLR4 ligand, final concentration of 1 μg/ml) were used as positive controls (Invivogen) (data not shown).

Cytokine quantification by ELISA

Levels of IL-1β, IL-6, IL-10, IL-12p70, IL-23p19, TNF-α, CXCL1 and CXCL10 in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN) or eBioscience (San Diego, CA), according to the manufacturer's recommendations.

FACS analysis

For cell surface staining, 10^6 cells were washed and treated for 30 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab, BD PharMingen, Mississauga, ON, Canada) in sorting buffer (PBS-1% fetal bovine serum). Blocked cells were then incubated with FITC-labeled anti-mouse CD11c mAb clone HL3 (BD PharMingen) for 1 h on ice followed by washing and staining for 1 h with a PE-labeled monoclonal antibody against the following surface molecules: CD86 (clone GL1), CD40 (clone 3/23), and MHC class II (A_{β}^{b}; clone AF6-120.1) from BD PharMingen . After washing, cells were resuspended in sorting buffer for FACS analysis. Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences, Mississauga, ON, Canada). Twenty thousand gated events were acquired per sample and data analysis was performed using CellQuest software. Quadrants were drawn based on FITC- and PE-control and isotype control stains and were plotted on logarithmic scales.

Confocal microscopy

For immunofluorescence studies, DCs (10^6 cells) were placed on coverslips and infected with different strains of *S. suis* (10^6 CFU/ml, MOI: 1). After 8 h of bacteria-cell contact, coverslips were washed with PBS to remove non-associated bacteria, and cells fixed with methanol/acetone (80:20) for 20 min at -20°C, and then washed and blocked for 10 min. Coverslips were incubated for 1 h with rabbit anti-NFκB p65 (Ser 276) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, coverslips were incubated with the

secondary antibodies Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen) for 30 min, washed and mounted on glass slides with moviol containing DABCO and DAPI to stain the nuclei. Samples were observed with an IX-80 confocal microscope integrated into the FV-1000 imagery system and analysed using the fluoview software (Olympus Canada, Richmond Hill, ON, Canada).

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed for significance using ANOVA analysis. A P value < 0.05 was used as a threshold for significance. All experiments were repeated at least three times.

RESULTS

Internalization of *S. suis* is independent on TLR signalization

It has previously been reported that TLRs may be implicated as receptors for bacterial phagocytosis [30]. In order to globally evaluate their implication, we investigated if DC deficiency in MyD88 expression would affect the internalization of *S. suis*. The number of bacteria internalized by MyD88$^{-/-}$ DCs was not significantly different from those obtained with WT DCs for the parental as well as mutant strains (Figure 1). Hence, deficiency in MyD88 signaling does not seem to play a major role in the ability of DCs to internalize *S. suis*. As expected, the non-encapsulated mutant strain was significantly more internalized by DCs than the parental strain 31533 (Figure 1).

Role of different receptors for DC maturation in response to *S. suis* infection

The role of different receptors and signaling pathways in the maturation of DCs by *S. suis* was evaluated by studying the expression of the co-stimulatory molecules CD40, CD86 and MHC-II on DCs from WT or knock-out mice. Compared to control cells, *S. suis*-stimulated WT DCs showed higher surface expression levels of CD40, CD86 and MHC-II mice in terms of the percentage of cells expressing these markers (Figure 2 and Supplemental Figure 1) as well as in MFI levels (data not shown). As expected [10], two well segregated sub-populations, a CD86^{high}/MHC-II^{high} subset and a CD86^{low}/MHC-II^{low} subset, are constantly observed among the CD11c^{+} DC population following *S. suis* infection. As shown in Figure 2, the expression of CD40 and MHC-II was significantly reduced in MyD88$^{-/-}$ DCs following *S. suis* infection, reaching levels similar to those observed in non-activated control cells (Figure 2A, C). Interestingly, the expression of CD86 in MyD88$^{-/-}$ DCs after *S. suis* activation was also significantly reduced but still higher than basal levels, suggesting a partial requirement of MyD88 signaling for CD86 expression (Figure 2B). Therefore, DC expression of surface molecules in response to *S. suis* occurs mainly but not exclusively through a MyD88-dependent pathway.

These results suggested that signaling through TLRs is the main pathway by which DCs sense *S. suis* and become activated. Hence, we investigated the participation of TLR2 in

DC maturation following stimulation with *S. suis*. For all strains tested, no significant differences between the WT DCs and the TLR2$^{-/-}$ DCs were observed for the expression of CD40, suggesting that the expression of this marker is TLR2-independent (Figure 2A). However, analysis of number of cells expressing the CD86^{high} and MHC-II^{high} subsets, revealed that the expression of these molecules were significantly reduced in TLR2$^{-/-}$ DCs infected with *S. suis* (Figure 2B, C). The CPS and cell wall modifications do not seem to play an important role in modulating co-stimulatory molecule expression through TLR2/MyD88 signaling as both mutant strains behaved similarly to the parental strain (Figure 2). No differences were observed between WT DCs and either TLR4$^{-/-}$ or NOD2$^{-/-}$ DCs in the ability to up-regulate expression of the above mentioned co-stimulatory molecules following stimulation with *S. suis*, neither in terms of percentage of cells expressing these molecules or in terms of MFI (data not shown).

Role of different receptors on DC activation in response to *S. suis* infection

The contribution of different receptors in DC cytokine production following stimulation with *S. suis* was investigated. DCs were incubated with different *S. suis* strains for 16 h. Optimal assay conditions were chosen based on previous results [10] and preliminary studies on the kinetics of cytokine release by DCs in response to *S. suis* (data not shown). The levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, the Th1-driving cytokines IL-12p70 and IL-23, the regulatory cytokine IL-10, and the chemokines CXCL1 and CXCL10 in the supernatants of *S. suis*-infected DCs were measured. Production of these mediators was either completely abrogated or dramatically impaired in MyD88$^{-/-}$ DCs for all strains tested (Figure 3). In addition, the nuclear expression of NF-κB was significantly reduced in *S. suis*-stimulated MyD88$^{-/-}$ DCs for all strains tested, confirming participation of MyD88 signaling pathways in DC activation and maturation in response to *S. suis* (Figure 4).

The involvement of TLR2 in DC cytokine production following stimulation with *S. suis* was also investigated using TLR2$^{-/-}$ DCs. The release of IL-1β, IL-6, IL-10, IL-23, TNF-α and CXCL1 was significantly reduced in TLR2$^{-/-}$ DCs infected with *S. suis* parental strain (Figure 3A, B, C, E, F and G). On the other hand, the release of IL-12p70 and CXCL10

was found to be TLR2-independent (Figure 3D and H). Conversely to the parental strain, the non-encapsulated strain B218 maintained its capacity of inducing most cytokines in TLR2$^{-/-}$ DCs, with the exception of CXCL1 (Figure 3G), indicating that high surface expression levels of cell wall components (normally hidden by the CPS) are able to activate cells through other TLRs. The cell wall mutant strain Δ*dltA*/Δ*pgdA* behaved exactly as the parental strain 31533, except for the release of TNF-α, which was found to be TLR2-independent (Figure 3F). Overall these results indicate that the release of most cytokines by *S. suis*-stimulated DCs involves TLR2. However, the fact that the inhibition of cytokine release in TLR2$^{-/-}$ DCs was still significantly different ($P < 0.05$) from the inhibition observed with MyD88$^{-/-}$ DCs, suggests that TLR2-independent pathways would also be involved in DC activation by *S. suis*.

Of all cytokines and chemokines tested, only CXCL1 was reduced following TLR4$^{-/-}$ DC stimulation with *S. suis* parental strain 31533 and its Δ*dltA*/Δ*pgdA* mutant (Supplemental Figure 2). Since TLR4 is known to mediate the recognition of *S. pneumoniae* pneumolysin, a suilysin-related toxin, we evaluated the release of CXCL1 by TLR4$^{-/-}$ and WT DCs following stimulation with a suilysin-deficient mutant strain used in previous studies [6, 10, 14]. No differences were observed between the parental and the suilysin-deficient strain (data not shown), excluding a major role for the suilysin in TLR4-mediated CXCL1 release.

NOD2$^{-/-}$ DCs were also stimulated with *S. suis* parental strain and mutants. The release of IL-23 was significantly impaired in NOD2$^{-/-}$ DCs stimulated with all *S. suis* strains tested (Figure 5A). The release of CXCL1 by NOD2$^{-/-}$ DCs was also significantly reduced except when stimulated with the non-encapsulated strain (Figure 5B). No differences were observed between WT DCs and NOD2$^{-/-}$ DCs in the release of other cytokines (results not shown).

Non-redundant activation of TLR2 and NOD2 contributes to IL-23 production by *S. suis*-stimulated DCs

It has been previously shown that IL-23 has an important role in bacterial infections and NOD2 activation seems to be highly responsible for DC elevated IL-23 production [31]. As in the case of *S. suis*, IL-23 was found to be TLR2- and NOD2-dependant, we investigated if blocking both pathways would further inhibit the release of this cytokine. NOD2$^{-/-}$ DCs were pre-treated with a neutralizing antibody against TLR2. The efficiency and specificity of the neutralizing antibody was evaluated by stimulating DCs with the TLR2-ligand PAM(3)CSK(4) (data not shown). However, as shown in Figure 5C, there was no difference in the production of IL-23 by TLR2$^{-/-}$ DCs, NOD2$^{-/-}$ DCs and NOD2$^{-/-}$ DCs pre-treated with the neutralizing antibody. The inhibition observed in either case was partial, compared to complete abrogation of IL-23 production in MyD88$^{-/-}$ DCs. Hence, the release of IL-23 by *S. suis*-stimulated DCs might involve complex synergies between TLR2, NOD2 and other unknown TLRs. Similar results were obtained for CXCL1 (results not shown).

Dual deficiency in TLR2 and TLR9 results in significant decrease in CD40 expression and in IL-12p70 and CXCL10 production

As mentioned above, the surface expression of CD40 was found to be MyD88-dependent, but TLR2-independent, suggesting a major role played by other TLR-dependent pathways. Since it has very recently been described a potential role of TLR9 in *S. suis* cell activation [32], the involvement of such receptor was investigated by pre-treating WT and TLR2$^{-/-}$ DCs with ODN2088, an inhibitory oligonucleotide for TLR9 [29]. We first confirmed the neutralization specificity and efficacy of ODN2088 by inhibition studies of the TLR9-activator ODN1826 (data not shown). No differences in the expression of CD40 were noticeable with the single inhibition of TLR9. However, dual deficiencies in TLR2 and TLR9 resulted in a reduced expression of CD40 when DCs were stimulated with *S. suis* parental strain 31533 (Figure 6).

As the release of IL-12p70 and CXCL10 was also found to be TLR2-independent but MyD88-dependent, we also investigated the involvement of TLR9 in the release of IL-

12p70 and CXCL10 by *S. suis*-stimulated DCs. No difference in the release of either cytokine was noticeable with the inhibition of TLR9 alone. However, dual deficiencies in TLR2 and TLR9 resulted in a significantly decreased release of both cytokines (Figure 7). Thus, these two receptors might act in a redundant or compensatory manner.

DISCUSSION

The mechanisms involved in the innate and adaptive immune responses toward *S. suis* remain essentially poorly known, and the increase in severity of *S. suis* infections in humans underscores the critical need of a better understanding of the interactions between *S. suis* and the immune system to generate an effective immune response against this pathogen. DCs are activated in the presence of *S. suis,* undergoing a maturation process characterized by the up-regulation of costimulatory molecules and the production of pro-inflammatory mediators [10, 22, 33]. In addition, *S. suis* was previously shown to possess several virulence factors able to modulate such DC functions, potentially leading to a diminished or ineffective host immune response [10, 22, 33]. In the present work, we attempted to further identify receptors involved in the innate immune recognition of *S. suis* serotype 2 by DCs. Murine cells were used since they have been shown to be a highly useful model for *S. suis* infections *in vivo* and *in vitro* [6,12]. In addition, the availability of knock-out mice allows the study of the precise role of some of the receptors. Finally, *S. suis* interactions with murine, porcine and human DCs are similar [10, 22, 33].

The actual role of TLR signaling in bacterial phagocytosis is controversial [21]. It has been reported that activation of the TLR signaling pathways by bacteria regulates phagocytosis at multiple steps including internalization and phagosomes maturation [30, 34]. The absence of TLR2 somehow delayed *S. pneumoniae* phagocytosis and killing by neutrophils [35]. On the other hand, TLRs were shown not to play any significant role in phagocytosis of Group B *Streptococcus* (GBS) by macrophages [36]. There is only one study where the role of TLRs in phagocytosis of a bacterial pathogen (*Streptococcus pyogenes*) by DCs is reported [29], showing an absence of any role of TLRs in the internalization and killing of this pathogen. Results from the present study indicate that, similarly to *S. pyogenes*, TLRs do not seem to be involved in *S. suis* phagocytosis by DCs as being shown to be independent from signaling through MyD88. It should be note that the general phagocytosis rate of a well encapsulated *S. suis* serotype 2 is usually low [10, 22, 33].

TLR/MyD88 pathway was shown to be essential to host defense against several Gram-positive bacteria such as *Staphylococcus aureus*, *S. pneumoniae* and GBS [37-39]. Similar to what has been reported for *S. pyogenes* [29], *S. suis*-induced expression of CD40, MHC-II and CD86 is MyD88-dependant. The production of different cytokines and chemokines by MyD88$^{-/-}$ DCs exposed to *S. suis* was also shown to be dramatically reduced or completely abrogated, hence confirming a central role for TLRs in DC activation by *S. suis*. The impaired expression of NF-κB in MyD88$^{-/-}$ DCs further suggests a pivotal role of MyD88 signaling in DC activation and maturation by *S. suis*. These results are in agreement with a previous study showing that MyD88 is the major downstream mediator of TLR-dependent *S. suis*-induced cytokine production by macrophages [40].

The requirement for the MyD88 signaling pathway suggests that one or several TLRs are involved in DC activation and maturation by *S. suis*. However, MyD88-independent pathways would also be implicated, to a lesser extent, in the release of some mediators, such as CXCL10, and in the expression of CD86, which induction levels were only partially reduced in *S. suis*-infected MyD88$^{-/-}$ DCs. It has been reported that MyD88 deficiency does not alter *Listeria monocytogenes*-induced co-stimulatory molecule up-regulation on DCs in vivo [41]. Since the MyD88-dependent pathway is used by all TLRs except TLR3 [42], a partial role of this receptor might be suggested. Transcription of TLR3 mRNA in brains of *S. suis* infected mice has been described [12]. In addition, a TLR4-mediated but MyD88-independent pathway has been reported to mediate LPS induction of CXCL10 via the TRIF/TRAM arm [43]. The MyD88-independent (TRIF/TRAM) pathway is also activated by TLR3 and is functionally responsible for activation of type I IFN and other IFN-inducible genes, such as CXCL10 [44]. Since TLR4 was not required for *S. suis*-induction of CD86 expression or CXCL10 release by DCs, a partial contribution of TLR3/TRIF pathway in *S. suis*-modulation of DC functions remains to be elucidated.

In order to further study TLRs implicated in the MyD88-dependent arm, DCs lacking TLR2 were infected with *S. suis*. Surface expression of CD86 and MHC-II, as well as the release of most mediators were found to be TLR2-dependent (but TLR4-independent), as previously suggested [40]. An implication of TLR2 and TLR6 in the recognition of *S. suis*

by peripheral blood mononuclear cell (PBMC) and transfected epithelial cells was also reported [32, 45]. A study with swine DCs showed an up-regulation of relative expression of TLR2 and TLR6 mRNA after stimulation with *S. suis* [22]. Interestingly, the induction of three important mediators of T cell activation (CD40, IL-12p70 and CXCL10) was found to be TLR2-independent, which may indicate involvement of different TLR/MyD88 pathways. It has been previously described that TLR9 is also a receptor for the release of IL-12p70 [46]. TLR9 has recently been shown to be involved in *S. suis* activation of PBMC by either heat-killed bacteria or bacterial DNA [32]. In the present study, inhibition of TLR9 did not affect DC maturation and activation; however, deficiency in TLR2 and blocking of TLR9 together significantly affected the surface expression of CD40 as well as the production of both cytokines. A similar cooperation and/or redundancy between TLR2 and TLR9 was shown to be involved in splenic cytokine production by *S. pneumoniae* [47] and in activation of macrophages and DCs infected by *Mycobacterium tuberculosis* [48]. Finally, TLR4 does not seem to play an important role in DCs maturation and activation by *S. suis*. The suilysin, although highly related to the pneumolysin (originally reported to be recognized by this receptor [19]), would play a minor role in DCs activation. Interestingly, it has been recently reported that the pneumolysin can also activate DCs through a TLR4-independent pathway [49].

Another major finding of this work is the involvement of the cytosolic receptor NOD2 in the release of CXCL1 and IL-23 by DCs following stimulation with *S. suis*. IL-23 is a member of the IL-12 family, and is particularly efficient in supporting IFN-γ production and proliferation in memory T cells [50]. CXCL1 is one of the CXCL8 homologs believed to be important in the trafficking and activation of neutrophils in mice [51]. The involvement of NOD2 in cell responses to Gram positive pathogens, such as *S. pneumoniae*, *S. aureus* and *L. monocytogenes*, have also been described [52-56]. Since crosstalk and/or synergy between TLRs and NODs receptors have previously been proposed [57, 58], a possible interaction between TLR2 and NOD2 for *S. suis* DC activation was studied. Our results suggest that a complex non-redundant activation of both receptors seems to be involved in the release of CXCL1 and IL-23. Activation of a cytosolic receptor by a well encapsulated extracellular pathogen was not expected.

Although in low numbers, some bacteria can be found inside DCs [10, 22, 33] which might, in theory, explain such activation. Exact mechanisms used by *S. suis* to activate NOD2 are so far unknown. Nevertheless, it has been proposed that cross-talks between cytosolic NODs and membrane-bound TLRs enhance responses to the multiple antigens simultaneously presented by a microbe [16, 59]. In addition, TLR2 activation has also been reported for some bacterial species to ensure digestion of bacterial cell wall and release of PG, which may activate NOD2 [60].

The presence of CPS in *S. suis* is known to hide cell wall antigens and thus reduce cell activation [6, 10, 22]. However, studies to date have identified cytokines for which the CPS is required for optimal induction, such as IL-1β [10, 13, 22], as also observed in this study. In the absence of CPS, uncovered cell wall components seem to activate DCs through multiple TLRs. However, modifications of cell wall components do not significantly change results of DC maturation and activation by *S. suis*. The presence of deacetylase genes in some pathogenic bacteria indicates that PG N-deacetylation could be a general mechanism used by bacteria to evade the host innate immune system [61]. Interestingly, in the case of *L. monocytogenes*, the N-deacetylation of PG allows the bacteria to avoid recognitions by NLRs, such as NOD [62]. This may be explained by the fact that the latter pathogen is usually found intracellularly. In the case of *S. suis*, cell wall modifications present in the double-mutant (PG/LTA) did not have any influence in modulation of DC activation by this receptor, probably due to the fact that relatively low number of intracellular bacteria are usually found, so low levels of PG are available to interact with NOD receptors.

This study confirms the hypothesis that recognition of *S. suis* by DCs seems to require a multimodal recognition system. Based on our results, a model of *S. suis* recognition by DCs is proposed (Figure 8). MyD88 signaling, mainly through TLR2, would be crucial for DC activation and maturation in response to *S. suis* infection. TLR9 (in conjunction with TLR2) and NOD2 were also involved in cell activation. However, other receptors, including other TLRs (such as TLR3), may mediate activation and maturation of DCs by *S. suis* and participate in the activation of the immune response. A role of NLRs, as recently

described for GBS [63], cannot be ruled out. Further studies on these receptors are warranted.

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Table I: Bacterial strains and plasmids used in this study.

Strains/Plasmids	General characteristics	Source/Reference
Escherichia coli		
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Streptococcus suis		
31533	Wild type, highly virulent strain isolated from a pig with meningitis. Serotype 2.	[23]
B218	Non-encapsulated mutant strain derived from strain 31533.	[13]
Δ*dltA*/Δ*pgdA*	Mutant deficient for the D-alanylation of LTA and the N-deacetylation of PG. Derived from strain 31533.	This work
Δ*sly*	Mutant deficient for the production of suilysin. Derived from strain 31533.	[8]
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET5s	Thermosensitive vector for allelic replacement is *S. suis*. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	
P5ΔdltA	pSET5s carrying the construct for *dltA* allelic replacement	This work

LTA; lipoteichoic acid, PG; peptidoglycan

Table II: Oligonucleotide primers used in this study for construction of in-frame deletion mutants.

Primer name	Sequence (5' – 3')[a]
ID.1_dlTA_left_FWD	CACTCATTACAACTCTCGCAG
ID.2_dlTA_left_REV	TCCAAACTATCAATATGGGCTG
ID.3_dlTA_right_FWD	GCTTATGTTGTCCCTAAAGCAG
ID.4_dlTA_left_REV	GCCCATCAAGAGCATATTTAGC
ID.5_dlTA_left_FWD	AGACCTCACATTTTTTGCG
ID.6_dlTA_left_REV	GTCAAAGGAAGACTGTCTCGGTAGTCAGGATTTTCTGTCG
ID.7_dlTA_right_FWD	CGACAGAAAATCCTGACTACCGAGACAGTCTTCCTTTGAC
ID.8_dlTA_right_REV	TCAATCACCATTCCGACCG

[a]Oligonucleotide primers were from Invitrogen

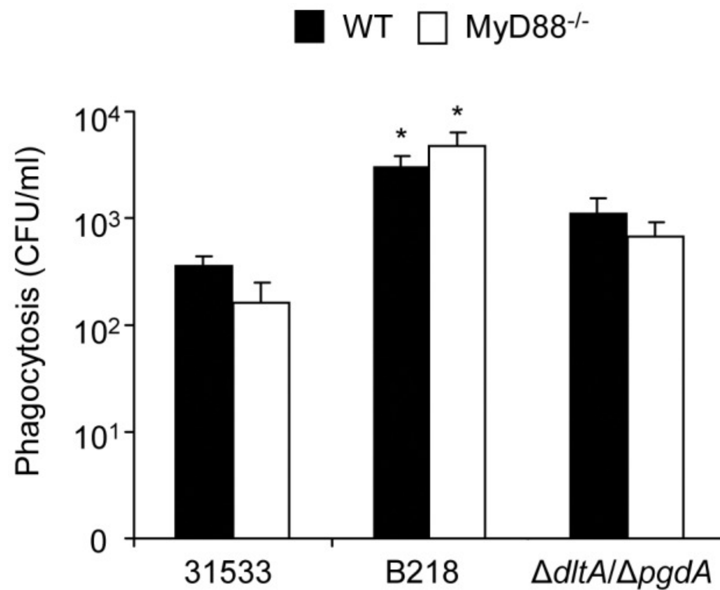

Figure 1. Effect of MyD88 deficiency on the capacity of DCs to internalize *S. suis*. Bacteria (10^6 CFU/ml) were pre-opsonized with 20% complete normal mouse serum for 30 min prior to incubation with DCs (10^6 cells/ml). Phagocytosis was left to proceed for 2 h before antibiotics were directly added into the wells for 1 h to kill extracellular bacteria. Viable intracellular bacteria were determined by quantitative plating of serial dilutions of the lysates onto THB agar. * $P<0.05$ denotes values that are significantly different from those obtained with the *S. suis* parental strain 31533.

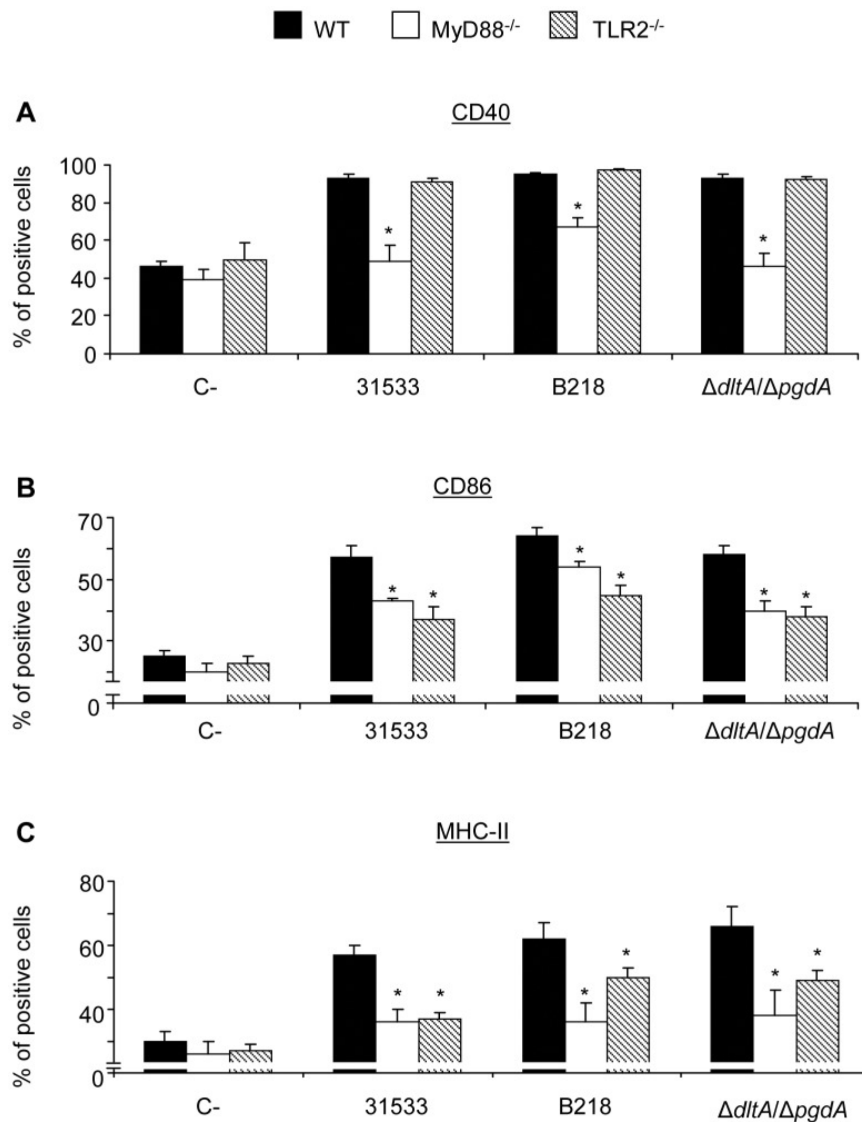

Figure 2. Surface expression of co-stimulatory molecules by DCs in response to *S. suis*. WT, MyD88$^{-/-}$, and TLR2$^{-/-}$ DCs (10^6 cells/ml) were stimulated with S. *suis* (10^6 CFU/ml) for 16 h. Non-stimulated cells served as negative control (C^-). (A) Percentage of CD40 positive cells. (B) Percentage of CD86^{high} positive cells. (C) Percentage of MHC-II^{high} positive cells. Twenty thousand gated events were acquired per sample. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales. CD40, CD86 and MHC-II histograms were obtained by gating cells based on positive CD11c staining. * P<0.05 denotes values that are significantly lower than those obtained with WT DCs.

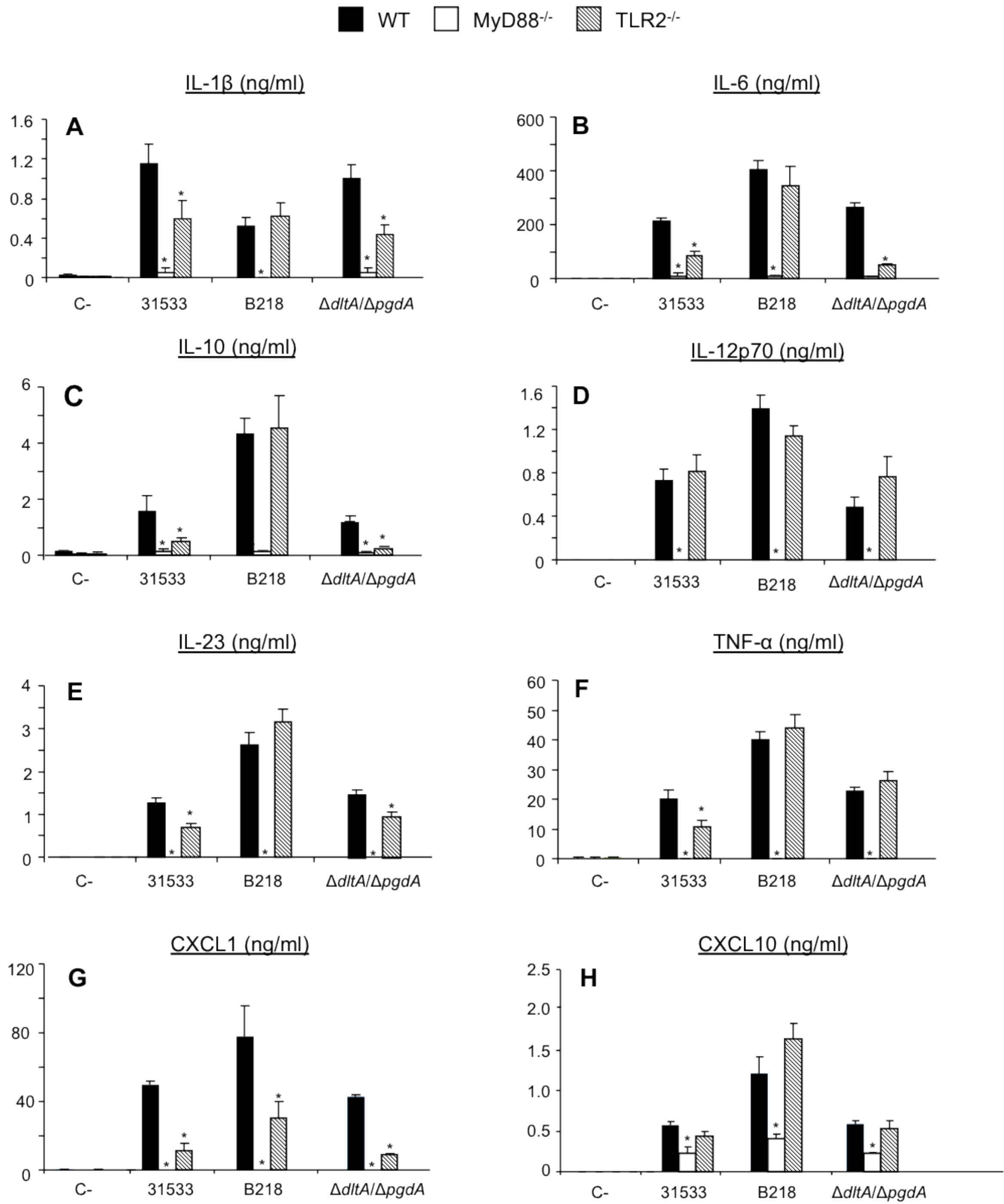

Figure 3. Cytokine production by DCs in response to *S. suis*. WT, MyD88$^{-/-}$, and TLR2$^{-/-}$ DCs (10^6 cells/ml) were stimulated by different *S. suis* strains (10^6 CFU/ml) for 16 h. Non-stimulated cells served as negative control (C^-). Sample dilutions giving optical density readings in the linear portion of the ELISA standard curves were used to quantify cytokine levels. * $P<0.05$ denotes values that are significantly lower than those obtained with WT DCs.

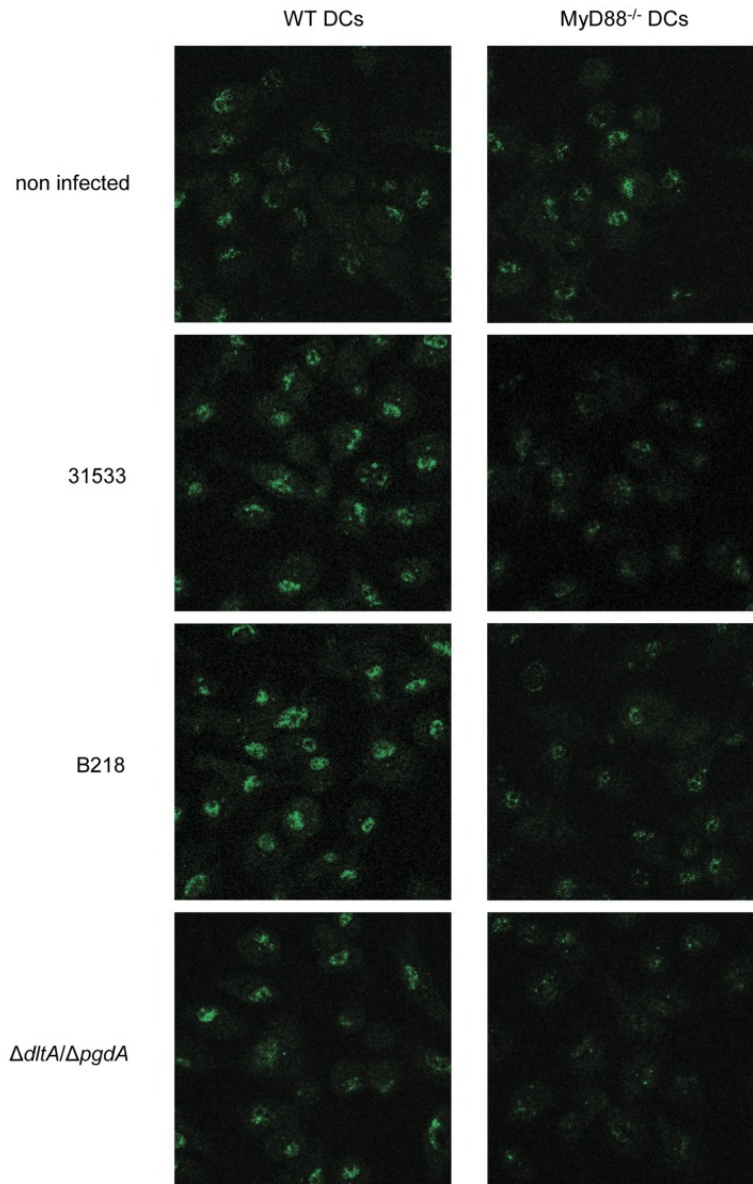

Figure 4. Effect of MyD88 deficiency on NF-κB expression by *S. suis* infected-DCs. WT DCs or MyD88$^{-/-}$ DCs were incubated with the parental strain 31533, the non-encapsulated mutant B218 or the cell wall mutant Δ*dltA*/Δ*pgdA* strain (10^6 CFU/ml). After a bacterial-cell contact of 8 h, cells were fixed and labeled with an antibody specific for NF-κB p65 (Alexa-Fluor 488, green) and analyzed by confocal microscopy.

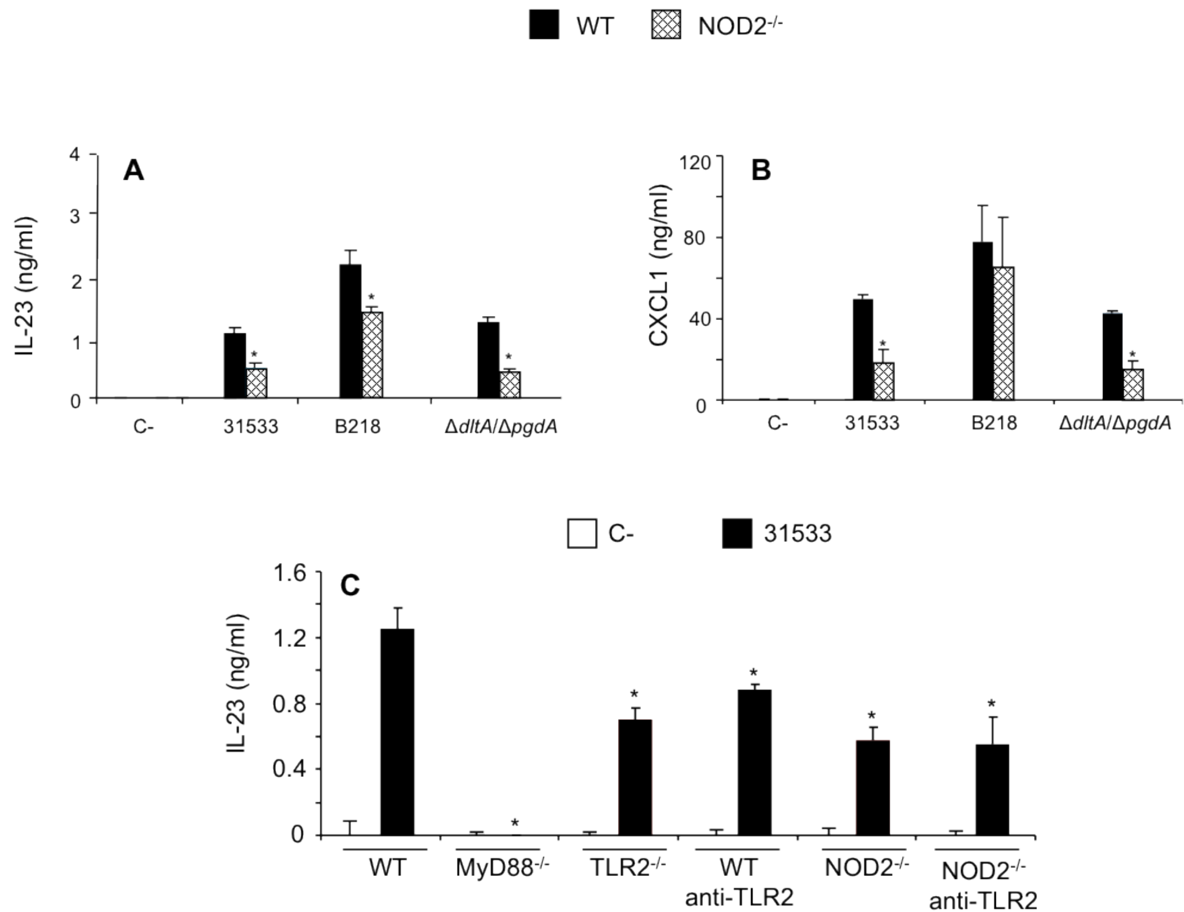

Figure 5. Role of NOD2 receptor in cytokine production by *S. suis*-stimulated DCs. WT and NOD2$^{-/-}$ DCs (10^6 cells/ml) were stimulated by different *S. suis* strains for 16 h. Non-stimulated cells served as negative control (C^-). The production of IL-23 (A) and CXCL1 (B) were measured. (C) WT DCs and NOD2$^{-/-}$ DCs (106 cells/ml) pre-treated or not with a neutralizing anti-TLR2 antibody (clone T2.5; 15 µg/ml) were stimulated by *S. suis* parental strain 31533 (10^6 CFU/ml) for 16 h, and the release of IL-23 was analyzed by ELISA. For comparative purposes, MyD88$^{-/-}$ DCs and TLR2$^{-/-}$ DCs were also included. Sample dilutions giving optical density readings in the linear portion of the ELISA standard curves were used to quantify cytokine levels. * $P<0.05$ denotes values that are significantly lower than those obtained with WT DCs.

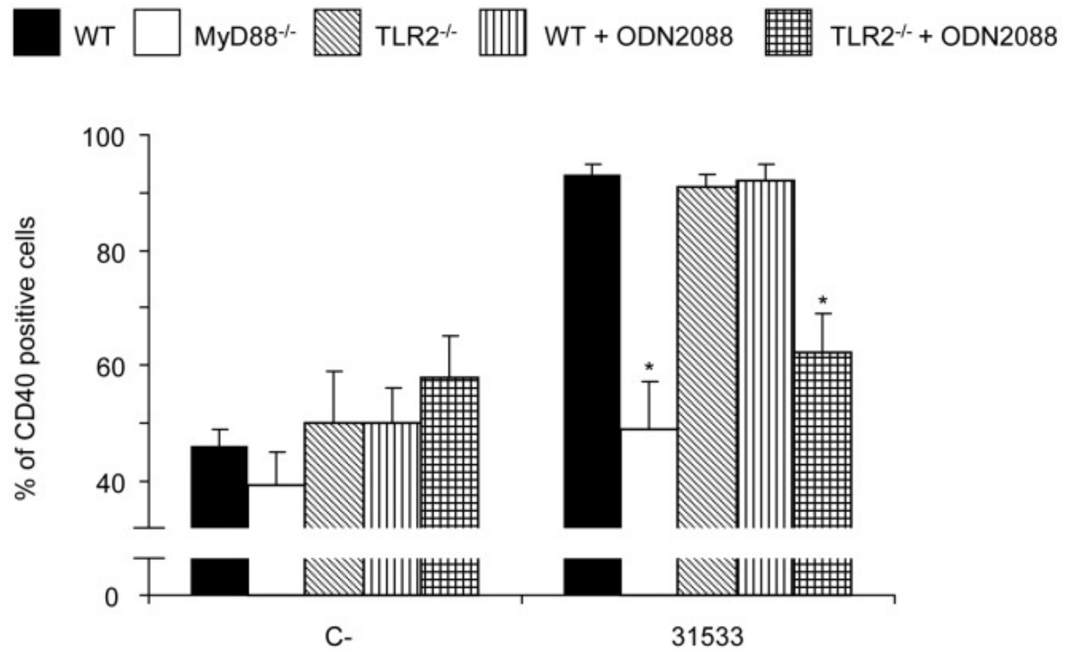

Figure 6. CD40 expression by DCs in response to *S. suis* depends on both TLR2 and TLR9. WT DCs and $TLR2^{-/-}$ DCs (10^6 cells/ml) pre-treated or not with an antagonist for TLR9 (ODN2088; 5 µM), were stimulated with *S. suis* parental strain 31533 (10^6 CFU/ml) for 16 h. Non-stimulated cells served as negative control (C^-). For comparative purposes, $MyD88^{-/-}$ DCs were also included. Twenty thousand gated events were acquired per sample. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales. Histograms were obtained by gating cells based on positive CD11c staining. * P<0.05 denotes values that are significantly lower than those obtained with WT DCs.

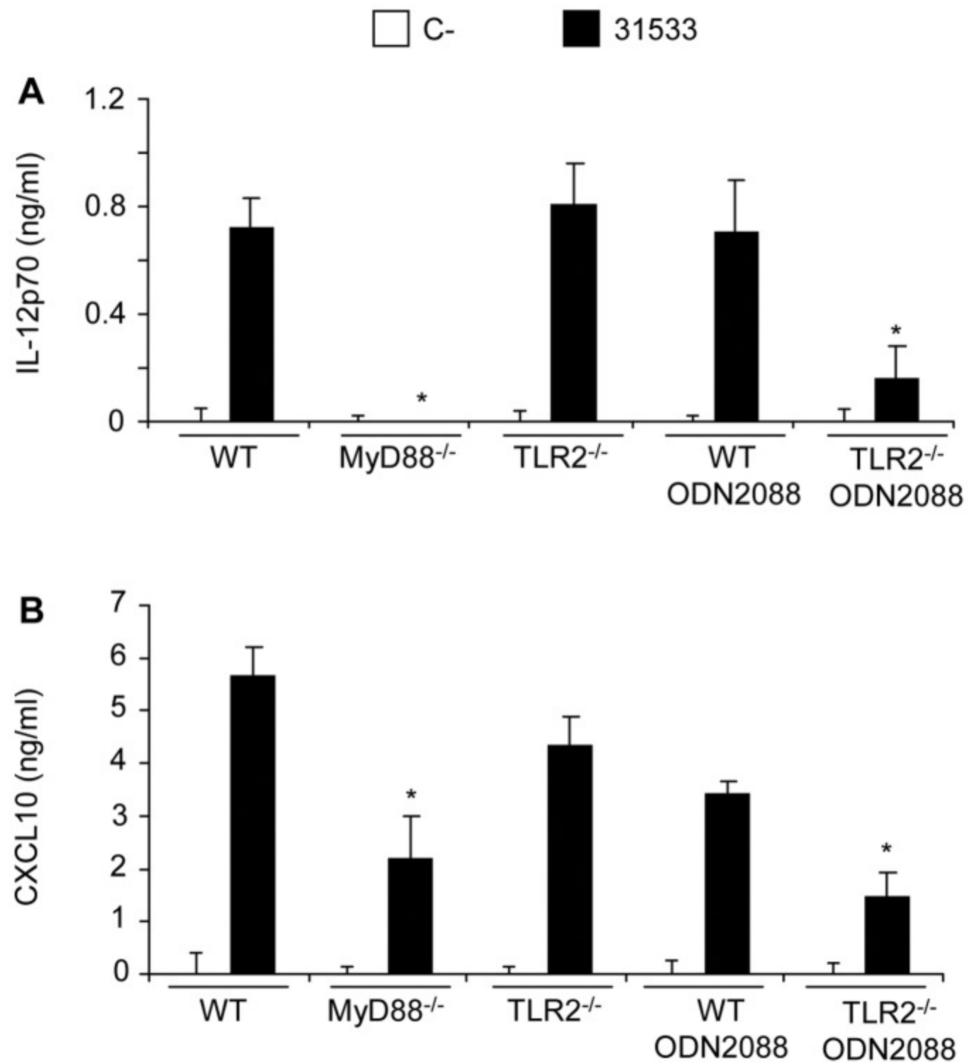

Figure 7. Role of TLR2 and TLR9 in IL-12p70 and CXCL10 production by *S. suis*-stimulated DCs. WT DCs and TLR2$^{-/-}$ DCs (106 cells/ml) pre-treated or not with an antagonist for TLR9 (ODN2088; 5 μM), were stimulated with *S. suis* parental strain 31533 (10^6 CFU/ml) for 16 h, and the release of IL-12p70 (A) and CXCL10 (B) were analyzed by ELISA. Non-stimulated cells served as negative control (C$^-$). For comparative purposes, MyD88$^{-/-}$ DCs were also included. Sample dilutions giving optical density readings in the linear portion of the ELISA standard curves were used to quantify cytokine levels. *P<0.05 denotes values that are significantly lower than those obtained with WT DCs.

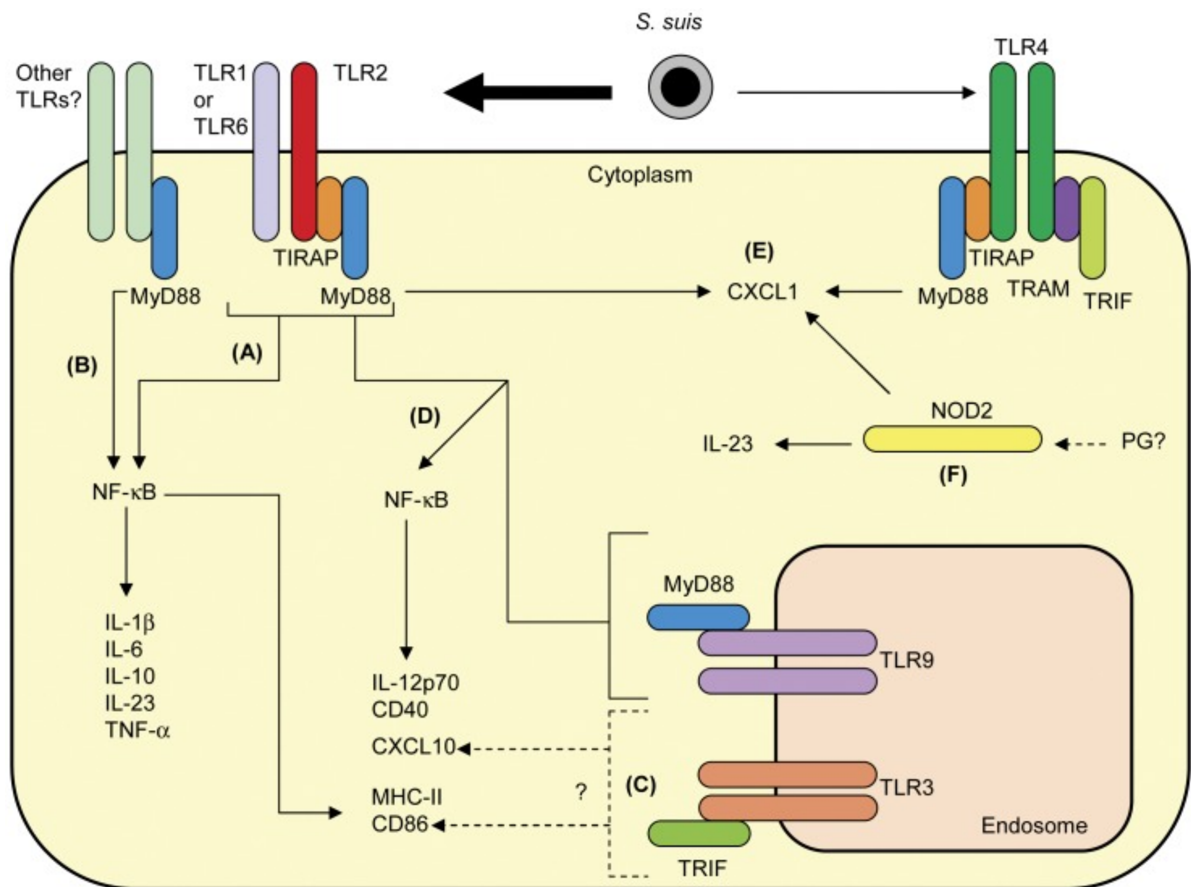

Figure 8. Proposed model of *S. suis* recognition by DCs. (A) The release of IL-1β, IL-6, IL-10, IL-23 and TNF-α is TLR2-dependent. TLR2 is also involved in the surface expression of MHC-II and CD86. (B) Other TLRs would also be implicated in the release of IL-1β, IL-6, IL-10, IL-23 and TNF-α. (C) TLR3 might be involved in the MyD88-independent production of CXCL10 and expression of CD86. (D) Collaboration between TLR2 and TLR9 is involved in the production of IL-12p70 and CXCL10 and the expression of CD40. (E) Collaboration among TLR2 and NOD2, with a minor contribution of TLR4, is involved in the release of CXCL1. (F) NOD2 also contributes to the release of IL-23. Recognition of *S. suis* peptidoglycan (PG) might be involved in NOD2 activation.

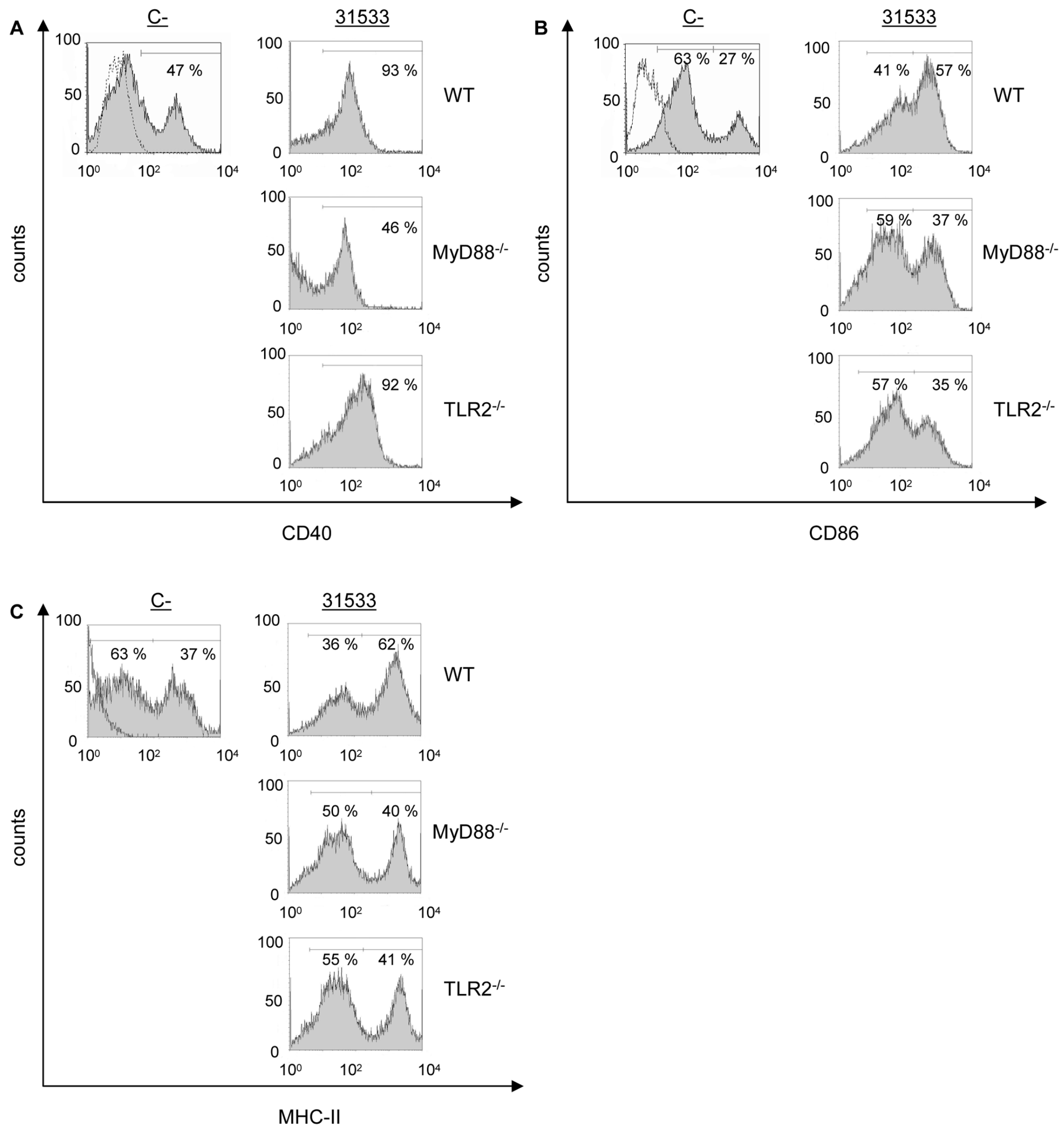

Figure S1. Surface expression of co-stimulatory molecules by DCs in response to *S. suis*. WT and MyD88$^{-/-}$DCs (10^6 cells/ml) were stimulated with S. *suis* WT strain 31533 (10^6 CFU/ml) for 16 h. Non-stimulated cells served as negative control (C-). (A) Percentage of CD40 positive cells. (B) Percentage of CD86 positive cells. (C) Percentage of MHC-II positive cells. Twenty thousand gated events were acquired per sample. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales. CD40, CD86 and MHC-II histograms were obtained by gating cells based on positive CD11c staining.

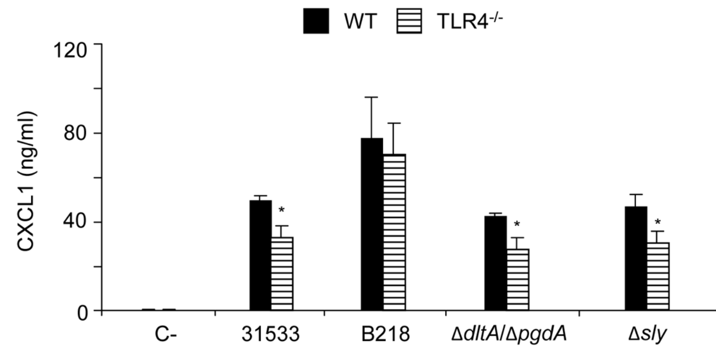

Figure S2. CXCL1 production by DCs stimulated with suilysin-deficient *S. suis* mutant strain. WT and TLR4$^{-/-}$ DCs (10^6 cells/ml) were stimulated by different *S. suis* strains (10^6 CFU/ml) for 16 h. Non-stimulated cells served as negative control (C-). Sample dilutions giving optical density readings in the linear portion of the ELISA standard curves were used to quantify cytokine levels. * $P<0.05$ denotes values that are significantly lower than those obtained with WT DCs.

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Article IV:

Sialylation of *Streptococcus suis* serotype 2 is essential for capsule expression but is not responsible for the main capsular epitope.

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Rôle de la candidate dans la conception de l'article :

Je suis l'une des deux auteurs principaux de cet article. J'ai participé à la conception et au design des expériences ainsi qu'à la mise au point des techniques. J'ai effectué les manipulations et participé à l'analyse des résultats. Finalement, j'ai participé à la conception des figures et à la rédaction du manuscrit.

ABSTRACT

The capsular polysaccharide is a critical virulence factor of the swine and zoonotic pathogen *Streptococcus suis* serotype 2. The capsule of this bacterium is composed of five different sugars, including terminal sialic acid. To evaluate the role of sialic acid in the pathogenesis of the infection, the *neuC* gene, encoding for an enzyme essential for sialic acid biosynthesis, was inactivated in a highly virulent *S. suis* serotype 2 strain. Using transmission electron microscopy, it was shown that inactivation of *neuC* resulted in loss of expression of the whole capsule. Compared to the parent strain, the Δ*neuC* mutant strain was more phagocytosed by macrophages and was also severely impaired in virulence in a mouse infection model. Both native and desialylated *S. suis* serotype 2 purified capsular polysaccharides were recognized by a polyclonal anti-whole cell *S. suis* serotype 2 serum and a monospecific polyclonal anti-capsule serotype 2 serum. In contrast, only the native capsular polysaccharide was recognized by a monoclonal antibody specific for the sialic acid moiety of the serotype 2 capsule. Together, our results infer that sialylation of *S. suis* serotype 2 may be essential for capsule expression, but that this sugar is not the main epitope of this serotype.

INTRODUCTION

Streptococcus suis is a major agent of meningitis and septicemia in swine and is also a zoonotic pathogen [1] and [2]. Although in the past *S. suis* disease in humans was rare and limited to people in close contact with pigs or pork by-products, in recent years *S. suis* has emerged as an important threat to human health, especially in South East and East Asia. This pathogen is now considered as the leading cause of adult meningitis in Vietnam, the second in Thailand and the third in Hong Kong. In the last years, two important outbreaks took place in China with a high fatality rate [1].

Among the 35 *S. suis* serotypes that have been described, serotype 2 is the most virulent for both pigs and humans. The capsular polysaccharide (CPS), which defines the serotype [3], is essential for the virulence of *S. suis* mainly because of its antiphagocytic activity [4]. CPS biosynthesis is driven by enzymatic machinery encoded by genes located in a single, discrete capsular locus (*cps*) [5] and [6]. Inactivation of genes within this locus, such as cps2B, cpsE and cpsF, resulted in severely decreased (cpsB) or completely abrogated capsule production (cpsEF) [5], [6] and [7]. The serotype 2 CPS was recently isolated, purified and characterized [8]. Sugar and absolute configuration analyses of the CPS gave the following composition: d-galactose, d-glucose, d-N-acetylglucosamine, d-Neu5Ac (sialic acid) and l-rhamnose. The CPS structure was also determined. Interestingly, sialic acid was found to be terminal, and the CPS was quantitatively desialylated by mild acid hydrolysis. Consistently with a sialylated CPS, four genes involved in sialic acid biosynthesis (SSU0355, SSU0536, SSU0537 and SSU0538) are found in the *cps* locus of *S. suis* [6] and [9]. These 4 genes are homologous to, and organized in the same way as, the Group B *Streptococcus* (GBS) sialic acid biosynthesis genes *neuBCDA*. High homologies at the amino acid level between *S. suis* and GBS deduced proteins are observed (Supplemental Table 1). In GBS, homologs of the *neuB* and *neuD* genes have been characterized. They encode a sialyl synthetase and a sialyl O-acetyltransferase, respectively [10]. A homolog of the *neuC* gene has been characterized in *Escherichia coli* K1 as an UDP-N-acetylglucosamine 2-epimerase necessary for sialic acid synthesis [11]. The *neuA* gene was recently described as an O-acetylesterase in *S. suis* [12].

Capsular sialic acid is known to be important for the pathogenesis of other meningitis-causing pathogens, such as *E. coli* K1, *Neisseria meningitidis* and GBS type III [13] and [14]. In this latter species, sialic acid is also structurally terminal and thought to be a major virulence factor involved, among others, in complement evasion and prevention of phagocytosis [15]. However, the sialic acid linkage to the CPS backbone differs between GBS type III (α-2,3) and *S. suis* serotype 2 (α-2,6), both linked to galactose [8]. Previous GBS mutagenesis studies reported that mutation of *cpsK* gene, coding for a sialyltransferase, resulted in reduced CPS expression [16]. In addition, the effect of sialic acid on CPS immunogenicity of GBS type III is complex, as it exerts conformational control of the dominant antigenic epitope [17].

The role of sialic acid in the pathogenesis of the infection caused by *S. suis* as well as its contribution to the epitope involved in the capsular serotype recognition are poorly known. In the present study, a *S. suis* mutant strain defective for the production of sialic acid was generated and characterized. Results showed that, similarly to GBS type III, such a mutant was also defective in CPS expression at the bacterial surface. In addition, and differently from the latter pathogen, sialic acid in the CPS is not responsible for the immunodominant capsular epitope of S. suis serotype 2.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Bacterial strains, derived mutants and plasmids used in this study are listed in Table 1. Wild type (WT) strain P1/7 is a virulent *S. suis* serotype 2 strain whose genome has been sequenced [9]. GBS WT strain COH-1 (kindly provided by Dr. C.E. Rubens, Children's Hospital and Medical Center, University of Washington, Seattle, WA, USA) is a type III highly encapsulated strain used and described in several previous studies [16], [25] and [26]. Streptococcal strains were grown on Todd-Hewitt broth (THB) or agar (THA) (Becton Dickinson, Mississauga, ON, Canada) or on sheep blood agar plates at 37 °C. *E. coli strains* were grown on Luria-Bertani broth or agar (Becton Dickinson). When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *E. coli*, kanamycin (Km) and spectinomycin (Sp) were added at 50 µg/ml; for *S. suis* and GBS, Sp was added at 100 µg/ml and 200 µg/ml, respectively. For comparative purposes, a previously characterized CPS-deficient mutant strain derived from *S. suis* P1/7 strain, obtained by precise in-frame deletion of *cpsF* gene coding for CPS biosynthesis, was also included [7]. To perform *S. suis*-macrophage interaction studies, isolated colonies were used as inocula for THB, which was incubated 16 h at 37 °C with agitation. Working cultures for cell stimulation were obtained by inoculating 300 µl of the 16 h-culture into 10 ml of THB that were incubated for 5 h at 37 °C with shaking. Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.3, and appropriately diluted in complete cell culture medium consisting of DMEM (Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) for the experiments. 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, USA). For in vivo infections, isolated colonies were used as inocula for THB, which was incubated 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. Bacteria were washed twice in PBS and were appropriately diluted in THB for the inoculation. To perform dot-ELISA, bacteria were grown overnight

in 10 ml of THB, washed with PBS and resuspended in 1 ml of PBS-0.5% formalin (whole bacteria with a concentration equivalent to 10^9 bacteria/ml).

DNA manipulations

S. suis genomic DNA was prepared by the guanidium thiocyanate method [27]. Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures [28]. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa Bio (Otsu, Shiga, Japan) and used according to the manufacturers' recommendation. PCR reactions were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada). Oligonucleotide primers were from Invitrogen (Burlington, ON, Canada). Amplification products were purified on Sephadex S-400 columns (GE Healthcare, Mississauga, ON, Canada) and sequenced with an ABI 3730xl automated DNA sequencer, using the ABI PRISM dye terminator cycle version 3.1 (Applied Biosystems, Carlsbad, CA, USA).

Construction of the *S. suis* and GBS mutants

In the case of *S. suis*, the *neuC* gene was targeted, since it codes for a protein involved in sialic acid synthesis [29]. For comparative purposes, GBS type III mutants defective in either *cpsE* (1032 bp, gene involved in CPS biosynthesis) or *neuB* (894 bp, gene involved in sialic acid synthesis) were also generated. The DNA genome sequences of *S. suis* serotype 2 strain P1/7 and GBS strain COH-1 were retrieved from GenBank (Accession Numbers AM946016 and AF16383, respectively). In-frame deletions of target genes were constructed by using splicing-by-overlap-extension PCR [30] and the primers listed in Table 2. The PCR-generated deletion alleles were cloned into plasmid pCR2.1 (Invitrogen), extracted with BamHI and PstI and recloned into the thermosensitive *E. coli–S. suis* shuttle plasmid pSET4s [23] digested with the same enzymes, giving rise to the knockout vectors p4ΔneuC for *S. suis* and p4ΔcpsE and p4ΔneuB for GBS. Electroporation of *S. suis* strain P1/7 and GBS strain COH-1 and procedures for isolation of mutants were those described previously [31]. Allelic replacement was confirmed by PCR and DNA sequencing analysis. Serotyping of *S. suis* and GBS mutants was performed by coagglutination test by using

either polyclonal hyperimmune serum as described for S. suis [32] or commercial rabbit antisera against GBS type III capsular material (Denka Seiken, Campbell, CA) [33].

For complementation, the full length *neuC* gene was amplified from genomic DNA of *S. suis* P1/7 using primers described in Table 2, digested with PstI, and cloned into pMX1 via the same restriction site. Plasmid pMX1 is a derivative of the *S. suis–E. coli* shuttle cloning vector pSET2 and possesses the *S. suis* malX promoter for transgene expression in *S. suis* [23]. The resulting complementation vector pCompNeuC was introduced into the *ΔneuC* mutant by electroporation. The presence of CPS was studied for both pathogens and their mutants by electron microscopy, as described below.

Transmission electron microscopy

The presence of capsule in the mutant derivatives was verified by transmission electron microscopy (TEM). Bacterial cells (*S. suis* and GBS) were fixed for 2 h at 20 °C in cacodylate buffer containing 5% glutaraldehyde. Fixed bacteria were suspended in cacodylate buffer and allowed to react with polycationic ferritin for 30 min at 20 °C. The reaction was slowed by 10-fold dilution with buffer, and the organisms were centrifuged and washed three times in cacodylate buffer. Bacterial cells were then immobilized in 4% agar, washed 5 times in cacodylate buffer, and postfixed with 2% osmium tetroxide for 2 h. Washing steps were repeated as above, and the samples were dehydrated in a graded series of ethanol washes. All solutions used in processing the specimens contained 0.05% (wt/vol) ruthenium red. Samples were then washed twice in propylene oxide and embedded with LR White resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope JEOL JEM-1230 (Tokyo, Japan).

***S. suis* phagocytosis assay**

J774-A1 macrophages (ATCC TIB 67, Rockville, MD, USA.) were used for *S. suis* phagocytosis studies as previously described [26]. Macrophages (10^5 cells/ml) were infected with *S. suis* strains (WT, *ΔneuC* mutant and complemented *ΔneuC* (*ΔneuC/neuC*) at a concentration of 1×10^7 CFU/ml (MOI: 100) for 90 min at 37 °C with 5% CO_2. Assay conditions were chosen based on preliminary studies and previous data on the kinetics of *S.*

suis phagocytosis by J774-A1 macrophages ([26] and data not shown). After incubation, penicillin G (5 µg/ml) and gentamycin (100 µg/ml) (both from Sigma) were directly added into 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 3 times, and sterile water was added to lyse the cells. To ensure complete cell lysis, cells were disrupted by scraping the bottom of the well and by vigorous pipetting. All samples were plated using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech). Each test was repeated at least four times in independent experiments, and the number of CFU recovered per well (mean number ± SEM) was determined.

***S. suis* in vivo infection**

A well-standardized model of murine infection was used [34]. Female, 6-week-old CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were acclimated to standard laboratory conditions of 12 h light/12 h dark cycle with free access to rodent chow and water. All experiments involving mice 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 Université de Montréal. A total of 35 animals, divided in 2 groups of 15 animals (P1/7 and Δ*neuC*) and 1 group of 5 animals (non-infected control) were included in the study. On the day of the experiment, a 1-ml volume of the bacterial suspension (5×10^7 CFU/ml) or the vehicle solution (sterile THB) was administrated by i.p. injection. Mice were monitored at least three times daily for mortality and clinical signs of septic disease, such as depression, swollen eyes, rough hair coat, lethargy, and nervous signs of meningitis. Blood samples were collected from the tail at 24, 48 and 72 h post-infection (pi), and plated onto blood agar plates. Blood agar plates were incubated overnight at 37 °C. Colonies were counted as described above and expressed as CFU/ml of blood.

***S. suis* CPS purification and CPS desialylation**

S. suis serotype 2 reference strain S735 was grown in 50 ml of THB at 37 °C for 18 h, diluted to 2 l in fresh THB, and grown to an OD_{540} of 0.8. The cells were pelleted by

centrifugation at 10 000× g for 40 min, suspended in 33 mmol/l PBS pH 8.0 by repeated pipetting, and chilled. The CPS was prepared and purified as previously described [8]. For quality controls, CPS was analyzed by nuclear magnetic resonance (NMR). Lack of protein and RNA/DNA contamination was verified by Lowry method and by spectrophotometry, respectively. When needed, CPS was desialylated by mild acid hydrolysis. CPS (8 mg) was heated in 1 ml of 70 mmol/l HCl at 60 °C for 4 h, neutralized with 2 mol/l NH_4OH, and purified on a Sephadex G10 column (1.5 × 10 cm) (GE Healthcare). Presence (native CPS) or absence (desialylated CPS) of sialic acid was verified by gas chromatography after methanolysis and acetylation and by NMR, and by a reaction with an enzyme linked lectin assay (ELLA) as described below.

Enzyme linked lectin assay (ELLA) to detect the presence of sialic acid

In order to verify the presence or absence of sialic acid in the purified native and desialylated CPS, an ELLA test was carried out with the *Sambucus nigra* lectin (SNA-I, Vector Labs Canada, Burlington, ON, Canada), which specifically recognizes sialic acid as α-Neu5Ac-2,6-D-Galp/GalpNAc [35]. The test was based on a previous described technique [36], adapted to the CPS. Briefly, 200 ng of sample (native CPS, desialylated CPS or skimmed milk as positive control) were added to wells of an ELISA plate (Nunc-Immuno Polysorp, Canadawide Scientific, Toronto, ON, Canada). After being coated, wells were washed and blocked (to avoid non-specific binding) by the addition of Carbo-Free solution 1X (Vector Labs). After washings, biotinylated SNA-I, followed by horseradish peroxidase-labeled Avidin D (Vector Labs) and 3,3′,5,5′-tetramethylbenzidine were added. The enzyme reaction was stopped with the addition of 1N H_2SO_4 and the absorbance was read at 450 nm with an ELISA plate reader.

Dot-ELISA

Ten µl of native or desialylated purified *S. suis* serotype 2 CPS (each at 1 mg/ml), or 10 µl of a formalin-killed whole-bacteria suspension were blotted on a PVDF Western blotting membrane (Roche, Mississauga, ON, Canada). The membrane was blocked for 1 h with a solution of Tris-buffered saline (TBS) containing 2% casein, followed by a 2-h incubation with either monoclonal antibody Z3 supernatant [37], rabbit serum (anti-*S. suis* 735) [38] or

adsorbed rabbit serum (polyclonal anti-*S. suis* capsule, see below). A second polyclonal antibody, produced against the P1/7 strain, was also used. The membrane was washed and appropriate rabbit or mice conjugated antibody was added for 1 h. The membrane was rinsed 3 times with TBS and revealed with a 4-chloro-1-naphthol solution.

Hyperimmune serum was adsorbed with a non-encapsulated mutant (BD101) derived from strain S735 [21] to selectively obtain antibodies against the CPS. Briefly, an overnight culture of *S. suis* strain BD101 was pelleted and resuspended with 10 ml of PBS. The sample was then divided into two 5-ml aliquots, and centrifuged again. One of the two pellets was resuspended in 3 ml of polyclonal serum anti-*S. suis* strain S735, and incubated for 2 h with agitation. The second pellet was kept on ice for the next adsorption. After 2 h, the first suspension was centrifuged, and the supernatant transferred to the second bacterial pellet. After 2 h of incubation, the suspension was centrifuged, and the supernatant recovered and filtered (0.22 µm). A negative reaction with the BD101 mutant, but not with the WT strain S735 was confirmed by dot-ELISA (results not shown). Similarly, antiserum against strain P1/7 was produced and adsorbed with Δ*cpsF* strain as described above.

Statistical analysis

All data are expressed as mean ± SE. For all experiments, except in vivo virulence, data were analyzed for significance using Student's unpaired t-test. For in vivo virulence experiments, survival was analyzed with the LogRank test. A *P* value < 0.05 was used as a threshold for significance. Independent experiments were repeated at least three times.

RESULTS

Inactivation of *neuC* in *S. suis* serotype 2 results in absence of CPS

To verify whether the *S. suis* Δ*neuC* mutant strain was still typable, it was tested by the coagglutination test. Interestingly, both Δ*cpsF* and Δ*neuC* mutants showed absence of reaction with an anti-serotype 2 serum (data not shown). To verify if this negative result was the consequence of the absence of capsular material in the sialic acid negative mutant, both mutants were also tested by TEM. Results showed similar absence of polycationic ferritin marker in both of them, indicating absence of CPS (Fig. 1B and C), when compared to the WT strain (Fig. 1A). The Δ*neuC*/*neuC* strain showed an intermediate expression of CPS, showing that complementation partially restores the phenotype (Fig. 1D).

As previously described for GBS type III Δ*cpsK* and Δ*neuA* mutants [16] and [39], an important reduction of CPS at the cell surface was observed with the GBS Δ*neuB* mutant obtained in this study (Fig. 1G) compared to the WT strain (Fig. 1E); on the other hand, a complete absence of CPS was observed in the GBS mutant defective in CPS biosynthesis (*cpsE*) (Fig. 1F). As expected, results from the coagglutination test revealed a clear positive reaction with the WT strain, a weak but still positive reaction with the Δ*neuB* mutant and a negative reaction with the Δ*cpsE* non-encapsulated mutant.

The Δ*neuC* mutant is highly phagocytosed by macrophages

To determine the ability of J774 macrophages to internalize *S. suis*, bacteria were incubated with cells for 90 min. As shown in Fig. 2A, the WT strain P1/7 was relatively resistant to phagocytosis and relatively few bacteria were found inside the cells. On the other hand, the Δ*neuC* mutant strain was significantly ($P < 0.05$) more internalized by macrophages. The Δ*neuC*/*neuC* strain was significantly less internalized by macrophages than the Δ*neuC* strain, but still more phagocytosed than the WT strain ($P < 0.05$), indicating that the level of phagocytosis would be related to the amount of CPS at the bacterial surface, as shown by TEM.

In vivo infection

Most mice in the WT group presented severe clinical signs associated with septicemia, such as depression, swollen eyes, rough hair coat, prostration and weakness during the first 72 h pi. Twenty-seven percent of animals died or were euthanized for ethical reasons within 72 h (Fig. 2B). *S. suis* could be isolated from blood samples of the WT infected mice within the first 72 h. Still in the WT group, remaining 20% of the animals died or were euthanized for ethical reasons between 3 days and 7 days pi, after the development of nervous signs associated to meningitis. In the Δ*neuC* mutant group, animals did not present any clinical signs associated with septicemia or meningitis, and *S. suis* could not be isolated from blood samples (data not shown).

Sialylation of CPS is not responsible for the conformational immunodominant epitope of *S. suis* serotype 2 capsular polysaccharide

Sialic acid may play a critical role as part of a major conformational epitope of bacterial CPS, as it is the case for GBS type III. On other cases, such as GBS type V, capsular sialic acid is dispensable for the main epitope of this serotype [17], [18], [19], [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33], [34], [35], [36], [37], [38], [39] and [40]. In this study, the role of the capsular sialic acid in the conformational epitope of *S. suis* was studied. Purified native and desialylated *S. suis* serotype 2 CPS were dotted on a membrane and incubated with either a monoclonal antibody against the sialic acid moiety or a polyclonal serum against whole bacteria. As shown in Fig. 3A, the monoclonal antibody recognized the native CPS and whole bacteria, but not the desialylated CPS, as expected. To demonstrate that the monoclonal antibody recognizes an epitope containing the sialic acid moiety, an SNA-I ELLA showed that native CPS, but not desialylated CPS, was clearly recognized by the lectin (Fig. 4). Interestingly, the polyclonal serum recognized native and desialylated CPS as well as whole bacteria (Fig. 3B), indicating that the sialic acid moiety is not essential for CPS recognition by a serotype-specific antibody. Identical results were obtained with monospecific polyclonal antibodies against the CPS of strain S735 (Fig. 3C) or strain P1/7 (results not shown).

DISCUSSION

S. suis is a major swine pathogen and a zoonotic agent of increasing importance [1]. The CPS is considered a critical *S. suis* virulence factor that protects bacteria from killing in the bloodstream. The CPS was previously shown to be sialylated [8]. Capsular sialic acid is a well-known virulence factor for several bacteria causing meningitis. For example, it has been proposed that sialylation of GBS CPS is critical for prevention of opsonophagocytosis through inhibition of alternative complement pathway activation [39]. In the case of *S. suis*, very little is known concerning the role of its capsular sialic acid. Charland *et al*. reported that no differences could be found in sialic acid concentrations between strains of different virulence degrees [41]. In addition, no significant differences could be found in the phagocytosis rate by porcine blood monocytes of *S. suis* treated or not with sialidase or SNA-I [41]. These preliminary results suggested that sialic acid itself might not play an important role in *S. suis* virulence. Although limited knowledge is available concerning the genes involved in sialic acid production in *S. suis*, putative roles can be inferred from other organisms [10], [11] and [12]. In order to further study the role of capsular sialic acid in the pathogenesis of *S. suis* infection, we obtained a nonpolar mutant defective in the production of sialic acid, targeting the *neuC* gene. Based on previous reports [42], the sialic acid biosynthesis pathway begins with NeuC, which present high homology with NeuC from GBS, as it is the case for NeuB and NeuD (Supplemental Table 1) [29].

A major finding of this work is the observation that Δ*neuC* had an important impact on CPS expression. First, the mutant strain was untypable with current antisera used in serotyping. However, this result could also be the consequence of the influence of sialic acid on the immunodominant capsular epitope for *S. suis* serotype 2. However, TEM analysis showed the importance of *neuC* for CPS expression. The WT strain was surrounded by a thick CPS linked to the polycationic ferritin whereas the Δ*neuC* mutant strain showed absence of CPS, similarly to the non-encapsulated Δ*cpsF* mutant. Complementation partially restored the phenotype as an intermediate expression of CPS was noticeable with the complemented Δ*neuC*/*neuC* strain. Results obtained imply that loss of sialylation might be related to the loss of CPS at the bacterial surface in *S. suis*. A *neuB*

mutant of *S. suis* had been previously obtained by transposon mutagenesis and shown to be non-virulent in pigs [43]. Interestingly, we observed that this mutant was untypable with antiserum against *S. suis* serotype 2 and non-encapsulated by electron microscopy (unpublished observations).

S. suis is frequently compared to GBS type III, both being meningitis pathogens and the only *Streptococcus* harboring a sialic acid-rich CPS. However, these pathogens possess different linkages of sialic acid to the respective backbone structures [8]. A previous GBS mutagenesis study reported that mutation of *cpsK* gene, coding for a sialyltransferase, resulted in reduced (80%) CPS expression [16]. It was suggested that the sialyltransferase is an integral part of the glycosyltransferase complex, so its loss may also disrupt the functional integrity of whole CPS synthesis complex [16]. We confirmed, for the first time by TEM, that residual capsular material is still present in GBS Δ*neuB* mutant, defective in the biosynthesis of sialic acid, when compared to GBS Δ*cpsE* mutant, which is completely non-encapsulated. These results were confirmed by a weak reaction of the Δ*neuB* mutant by coagglutination with anti-type III antibodies. The lack of sialic acid seems to differently affect the production of the backbone structure depending on the pathogen, being critical for *S. suis* CPS (this study), partially involved in GBS CPS ([16] and this study) and totally dispensable for *Haemophilus ducreyi* and *N. meningitidis* [44].

In the case of GBS type III, the effect of sialic acid on CPS immunogenicity is complex. For example, sialic acid exerts a conformational control of the dominant antigenic epitope in type III but not in type V [17] and [40]. It had been previously suggested that sialic acid residues in *S. suis* type 2 CPS would be part of the antigen epitope portion of the capsule [45]. However, analysis carried out in this study clearly showed that removal of sialic acid results in loss of reactivity against not only a monoclonal antibody directed to this residue [37] but also a specific lectin [41]. However, it did not prevent the recognition with either a polyclonal antibody routinely used for serotyping or an adsorbed monospecific antibody against the complete CPS. These results indicate that sialic acid in *S. suis* type 2 CPS is not the major or unique determinant of the serotype. Whether a complete CPS would induce different level and isotypes of antibodies than a desialylated CPS, as it is the case of GBS

type V [40], is still unknown and is presently being studied in our laboratory.

Finally, and as expected, the Δ*neuC* behaved as a non-encapsulated mutant being highly susceptible to phagocytosis and non-virulent in a mouse model of infection [26] and [34]. Caution should be used in evaluating the effect of mutations in sialic acid synthesis and its presence on the CPS regarding phagocytosis and/or virulence, since their effects are confounded with an overall decrease in capsule production. Dissection of the molecular pathways underlying sialic acid-control of *S. suis* CPS expression would let the construction of strains that produce normal levels of asialo-CPS, allowing direct evaluation of sialic acid contribution to bacteria–host interactions.

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Table 1 : Bacterial strains and plasmids used in this study.

Strains/Plasmids	General characteristics	Source/Reference
Escherichia coli		
TOP 10	F-*mrcA* *Δ(mrr-hsd*RMS-*mcr*BC*)φ80 lacZΔM5* *Δlac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
MC1061	*araD139 Δ*(*ara-leu*)*7697 Δlac*X74 *galU galK hsdR2*(rK- mK+) *mcrB1 rpsL*	[16]
Streptococcus suis		
P1/7	Wild type, highly virulent serotype 2 strain isolated from a pig with meningitis	[17]
Δ*neuC*	Asialo mutant strain derived from P1/7. In frame deletion of *neuC*	This work
Δ*neuC*/*neuC*	Δ*neuC* complemented with pCompNeuc	This work
Δ*cpsF*	Non-encapsulated mutant strain derived from P1/7. In frame deletion of *cpsF*	[6]
S735	Serotype 2 reference strain isolated from a pig with meningitis	[18]
J119	Non-encapsulated strain derived from S735.	[19]
Group B *Streptococcus*		
COH-1	Wild type, highly encapsulated type III strain isolated from an infant with bacteraemia	[20]
Δ*neuB*	Asialo mutant strain derived from COH-1. In frame deletion of *neuB*	This work
Δ*cpsE*	Non-encapsulated mutant strain derived from COH-1. In frame deletion of *cpsE*	This work

Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET4s	Thermosensitive vector for allelic replacement in *S. suis* and GBS. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[21]
p4ΔneuC	pSET4s carrying the construct for *neuC* allelic replacement	This work
p4ΔcpsE	pSET4s carrying the construct for *cpsE* allelic replacement	This work
p4ΔneuB	pSET4s carrying the construct for *neuB* allelic replacement	This work
pMX1	Replication functions of pSSU1, MCS pUC19 *lacZ* Sp^R, *malX* promoter of *S. suis*, derivative of pSET2	[22]
pCompNeuC	pMX1 carrying the entire *neuC* gene under the control of pMalX	This work

Table 2 : Oligonucleotide primers used in this study[a].

Primer name	Sequence (5' – 3')
For construction of in-frame deletion mutants	
neuC-ID1	TGCCCGTTTATAAGATTCCATC
neuC-ID2	TGAGTTGCTCTGTCAAGGTC
neuC-ID3	TGATTGAAGTGCCCTCATTAC
neuC-ID4	TAAACCTTTTGATCCTGACCG
neuC-ID5	TGAAAAGCACTTTACTCTGGAC
neuC-ID6	CCTTGTAAAGCAGAATCAGGTTGATGCATGGCTGTCACTAC
neuC-ID7	GTAGTGACAGCCATGCATCAACCTGATTCTGCTTTACAAGG
neuC-ID8	ATGTTCCACAATGGCACCC
GBScpsE-ID1	AGAATACTTCAATGCGATCCG
GBScpsE-ID2	TCAAGATAGCCACGACTCC
GBScpsE-ID3	AAGGCGATATGAGTTTAGCAG
GBScpsE-ID4	CGCCATGTGTGATAACAATCTC
GBScpsE-ID5	TGGAACTATTAAAGGCTTGACG
GBScpsE-ID6	CCTGTCCCGAGTAAAACTACTACAACTGTTTGAATCATCGC
GBScpsE-ID7	GCGATGATTCAAACAGTTGTAGTAGTTTTACTCGGGACAGG
GBScpsE-ID8	TCCCCACTGTGACAAAAATC
GBSneuB-ID1	TCTAGGGTTTTTGGAGCTTTTG
GBSneuB-ID2	ACAGCATCAACACCACAAG
GBSneuB-ID3	TGGATCAGAAGTACCTATCGC
GBSneuB-ID4	GGTTGTTCTCCCATCTGAATC
GBSneuB-ID5	GCTCAAATAATGGGAGGAGAC
GBSneuB-ID6	GTCCCAAGATGTCATACCAGTGATTGCAACCAATCTCTGC
GBSneuB-ID7	GCAGAGATTGGTTGCAATCACTGGTATGACATCTTGGGAC
GBSneuB-ID8	TTCGTAACGATCCCCTAAAATG
For construction of complemented mutants[b]	
neuC-CF	TGAG**CTGCAG**CAAAATATTTGCCATAGTGC
neuC-CR	CAT**CTGCAG**AGGTACCCGCTCCTAGAAAGG

[a]Oligonucleotide primers were from Invitrogen
[b]PstI restriction sites indicated in bold

Supplemental Table 1 : Homology between *Streptococcus suis* and GBS proteins encoded by *neu* locus.

Protein	Identities[a]	Positives[a]	Accession number[b]
NeuB	273/338 (80%)	310/338 (91%)	AAK43615.1
NeuC	209/377 (55%)	271/377 (71%)	AAR29929.1
NeuD	119/207 (57%)	161/207 (77%)	ZP_08650487.1
NeuA	198/394 (50%)	276/394 (70%)	EFV97495.1

[a]Amino acid identities and positives were identified by Blastp using the deduced amino acid sequences of the proteins encoded by *neu* genes in *Streptococcus suis* strain P1/7 as query.

[b]GenBank accession numbers are provided for the GBS best match.

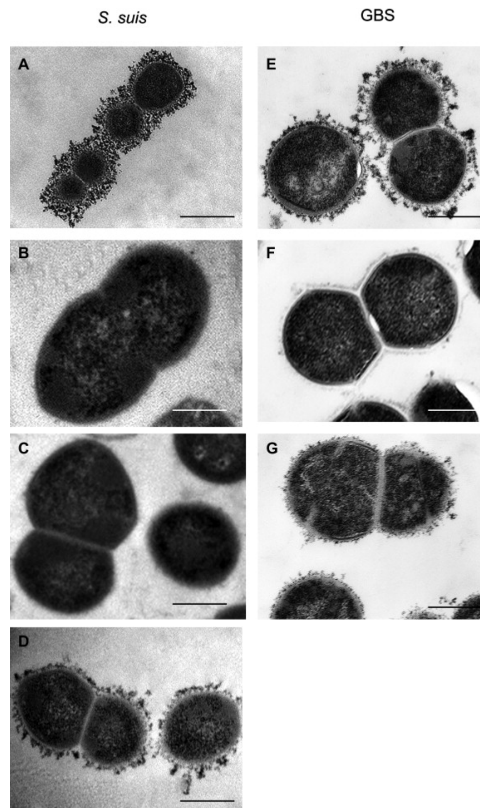

Figure 1. Transmission electron micrographs showing capsular polysaccharide (CPS) expression by different strains of *S. suis* and Group B *Streptococcus* (GBS). (A) *S. suis* wild type strain P1/7 is surrounded by a thick capsule whereas the Δ*cpsF* and the Δ*neuC* mutant strains are non-encapsulated (B–C). The Δ*neuC*/*neuC* strain shows intermediate CPS expression (D). GBS wild type strain COH-1 is surrounded by a thick capsule (E) whereas the Δ*cpsE* strain is non-encapsulated (F). The Δ*neuB* strain presented some residual CPS (G). Bars = 0.5 µm.

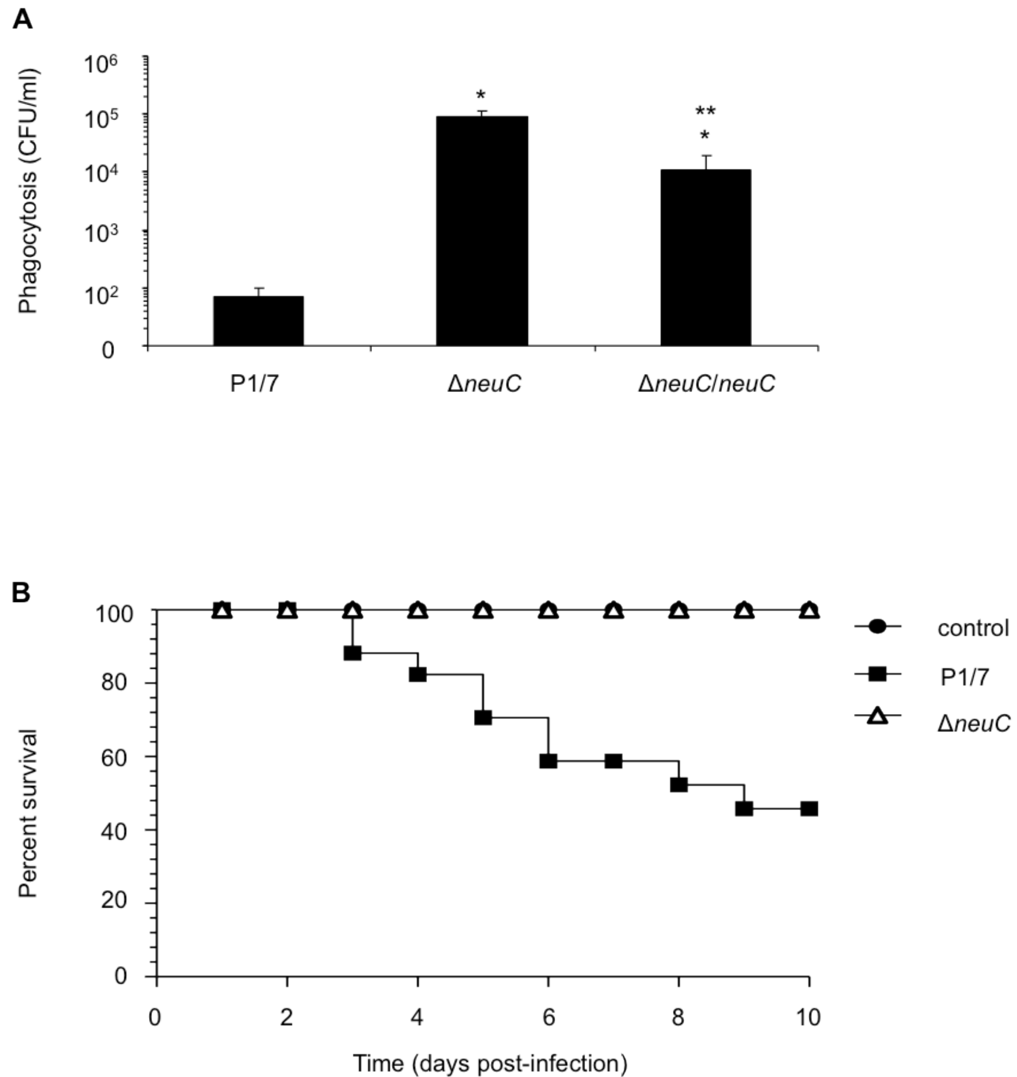

Figure 2. Effect of the deletion of neuC on the resistance of *S. suis* to phagocytosis by macrophages and on virulence. (A) Phagocytosis studies with J774 macrophages and different strains of *S. suis*. Bacteria were incubated with macrophages for 90 min. Numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and the results are expressed as CFU recovered bacteria per ml (means ± SEM obtained from four independent experiments). **P* < 0.05, indicates statistically significant differences between the wild type strain and the Δ*neuC* mutant and Δ*neuC*/*neuC* strains. ***P* < 0.05, indicates statistically significant differences between the Δ*neuC* mutant strain and the Δ*neuC*/*neuC* strain. (B) Survival of mice infected with the wild type P1/7 strain and its Δ*neuC* mutant. All mice in the Δ*neuC* mutant group (n = 15) survived, whereas 53% of the wild type group (n = 15) died from septicemia or meningitis. Significant differences in survival were noted (LogRank test, P < 0.05).

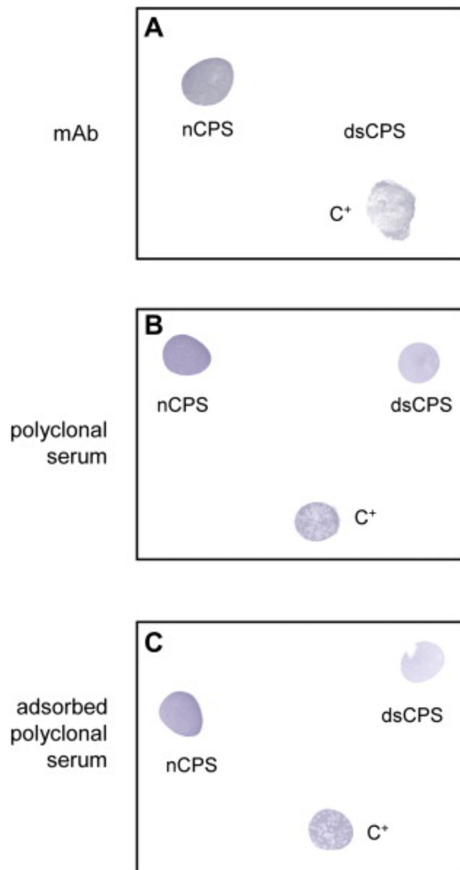

Figure 3. Role of capsular polysaccharide (CPS) sialylation as the main capsular epitope of *S. suis* serotype 2. Whole bacteria (C^{+}) and native (nCPS) or desialylated purified CPS (dsCPS) were incubated with a monoclonal antibody directed against the sialic acid moiety of the CPS, total polyclonal anti-*S. suis* serotype 2 serum or a monospecific adsorbed polyclonal antibody anti-*S. suis* serotype 2 CPS. (A) The monoclonal antibody recognized the native CPS and whole-cell bacteria. (B) The polyclonal anti-*S. suis* serotype 2 serum recognized whole-cell bacteria and both the native and desialylated CPS. (C) The adsorbed monospecific polyclonal anti-*S. suis* serotype 2 CPS serum also recognized whole-cell bacteria and both the native and desialylated CPS.

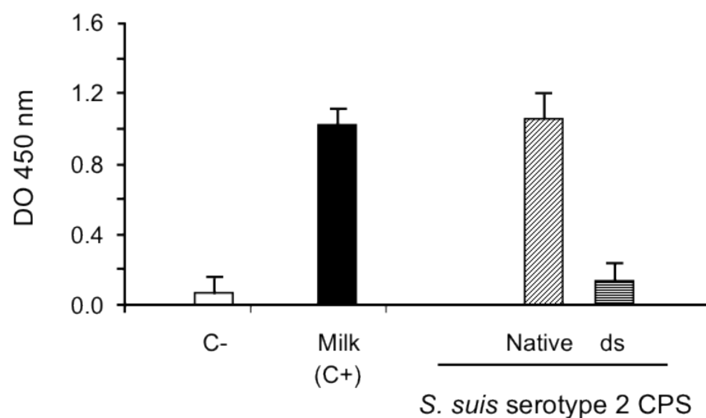

Figure 4. Recognition of *S. suis* sialic acid by *Sambucus nigra* lectin (SNA-I). SNA-I Enzyme Linked Lectin Assay showed that native capsular polysaccharide (CPS), but not desialylated CPS (dsCPS), was clearly recognized by lectin. Dilution buffer was used as negative control (C-) and skimmed milk was used as positive control (C+).

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Article V :

Immune-responsiveness of $CD4^+$ T cells during *Streptococcus suis* serotype 2 infection

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En préparation pour soumission.

Rôle de la candidate dans la conception de l'article :

Je suis l'auteure principale de cet article. J'ai participé à la conception et au design des expériences ainsi qu'à la mise au point des techniques. J'ai effectué les manipulations et participé à l'analyse des résultats, à la conception des figures ainsi qu'à la rédaction du manuscrit.

ABSTRACT

Streptococcus suis is an important swine pathogen and an emerging zoonotic agent of septicemia and meningitis. Knowledge of host immune responses to *S. suis*, and strategies used by this pathogen for subversion of these responses is scarce. Previous results demonstrated that dendritic cells (DCs) are activated after *S. suis* infection, undergoing a maturation process characterized by the up-regulation of co-stimulatory molecules and cytokine production. However, *S. suis* possesses virulence factors, such as the capsular polysaccharide, able to modulate DC functions, and potentially leading to a diminished immune response. The objective of the present study was to evaluate the consequences of the modulation of DC functions on T cell activation. In mice, following *S. suis* serotype 2 infection, total splenocytes readily produced TNF-α, IL-6, IFN-γ, CCL3, CXCL9 as well as IL-10. *Ex vivo* and *in vivo* analysis revealed the involvement of CD4+ T cells and development of a Th1 response. Nevertheless, levels of Th1-derived cytokines TNF-α and IFN-γ during *S. suis* infection were very low. In addition, CD4+ T cells were shown to also secrete IL-10 and failed to up-regulate optimal levels of CD40L and CD69. The bacterial capsular polysaccharide was shown to interfere with the release of several T cell-derived cytokines *in vitro*. As a consequence, low levels of not only anti-*S. suis* antibodies but also of those directed against ovalbumin, used as reported antigen, were observed in animals with clinical infection. This interference was correlated with the presence of severe clinical signs of *S. suis* disease. A transient depletion of CD4+ T cells in the spleen and poor generation of a memory response were also observed. Overall, these data suggest that *S. suis* impairs the development of an efficient adaptive immune response, which is required to control the infection progress.

INTRODUCTION

Streptococcus suis is a major swine pathogen mainly associated with meningitis, although other systemic infections have been described [1,2]. *S. suis* is now emerging as a threat to human health, especially in Asian countries where it has recently been identified as the leading cause of adult meningitis in Vietnam, the second in Thailand, and the third in Hong Kong [2]. Moreover, two important human outbreaks of streptococcal toxic shock-like syndrome (STSLS) due to *S. suis* occurred in China during the last years with a fatality rate near 20% [2]. Among 35 serotypes that have been described, serotype 2 is the most virulent for both pigs and humans, and most of the studies have been performed with this serotype. The capsular polysaccharide (CPS), which defines the serotype, is considered a major virulence factor of *S. suis* serotype 2 [3].

Dendritic cells (DCs) are powerful antigen-presenting cells and are critical for bridging innate and adaptive immune responses [4]. DCs capture and process invading pathogens to present their antigens to corresponding lymphocytes. Following antigen uptake, DCs undergo a maturation process characterized by the expression of different cell surface molecules and the release of cytokines. After DC migration to draining lymph nodes, co-stimulatory molecules bind to naïve T cells, leading to T cell activation [4]. The production of cytokines, such as interleukin (IL)-12, by mature DCs provides additional signals for the acquisition of T cell effector functions.

CD4+ T cells are important for the development of immunity to bacterial infections. After interaction with their cognate antigen presented by activated DCs, naïve CD4+ T cells proliferate and polarize towards different CD4+ lineages, which then shape the immune response. The best characterized CD4+ lineages are T helper type 1 (Th1), which drives the immune response mainly against intracellular pathogens; Th2, which promotes humoral responses; Th17, which contributes to the elimination of extracellular pathogens; and various regulatory T cell (Treg) populations, which prevent the development of autoimmunity [5]. However, there is accumulating evidence that the CD4+ T cell lineages are not as stable as initially thought. Substantial heterogeneity and plasticity, as assessed by

cytokine production patterns, has been observed within these subsets, particularly when generated *in vivo* and during an infection [5]. Hence, it seems more likely that multiple polarized CD4+ T cell subsets are generated. These effector cells secrete large quantities of cytokines and chemokines [6]. For example, the Th1 cells secrete IFN-γ, TNF-α, and IL-2 whereas the Th2 cells secrete high levels of IL-4, IL-5, IL-9 and IL-13 [6].

Despite the increasing number of studies, the pathogenesis of the *S. suis* infection is still not completely understood and, to date, attempts to control the infection are hampered by the lack of an effective vaccine. Mouse bone marrow-derived DCs have been shown to be a valid and interesting model to study the host immune response during *S. suis* infection [7,8]. There is evidence that mouse DCs are activated after *S. suis* infection, undergoing a maturation process characterized by the up-regulation of the co-stimulatory molecules CD40 and CD86 as well as cytokine and chemokine production, including TNF-α, IL-1β, IL-6, IL-12p70, and IL-23 [7,8]. However, *S. suis* possesses virulence factors able to modulate DC functions, particularly cytokine release and opsono-phagocytosis, potentially leading to a diminished immune response [7,8]. In fact, the CPS was shown to strongly reduce DC activation/maturation and *S. suis* internalization and/or to modulate the IL-10/IL-12 and IL-10/TNF-α cytokine production in favor of a more anti-inflammatory profile by either human-, mouse- or swine-derived DCs [7,9,10]. In this study, we hypothesized that encapsulated *S. suis* modulation of DC functions affects the development of T cell-dependent immune responses. Indeed, this study addresses for the first time the role of CD4+ T cells in the host adaptive immune response against *S. suis* and the potential contribution of the bacterial CPS to the modulation of this response.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *S. suis* serotype 2 virulent strain P1/7, originally isolated from a case of porcine meningitis, and its isogenic non-encapsulated mutant strain Δ*cpsF* were used. These strains have already been used used in previous studies [9,11]. *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), which was incubated 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. Bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.3, and appropriately diluted in fresh medium to desired inoculum concentrations. 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).

Mice and experimental infections

All experiments were performed with 5 week-old female, C57BL/6 mice (Charles River Laboratories) and 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 Use of Laboratory Animals by the Animal Welfare Committee of Université de Montréal. Mice were acclimatized to standard laboratory conditions of 12-h light/12-h dark cycle with free access to rodent chow and water. On the day of the experiment, a 1 ml volume of either the bacterial suspension or the vehicle solution (sterile THB) was administrated by intraperitoneal injection (i.p.). Mice were closely monitored daily to record mortality and clinical signs of disease, such as depression, rough appearance of hair coat and swollen eyes [12,13]. Mice exhibiting extreme lethargy were considered moribund and were humanely euthanized. To determine the level of infection, numbers of viable bacteria in blood were quantified at different times post-infection. Blood (5 µl) was collected from the tail vein, serially diluted in PBS and plated using an Automated Spiral Plater. Blood agar plates were incubated overnight at 37°C. Colonies were counted and expressed as CFU/ml.

Generation of mouse bone marrow-derived dendritic cells

DCs were generated from naïve C57BL/6 mice as previously described [7]. Briefly, bone marrow was removed from femurs and tibiae. After red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 μg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol (Gibco, Invitrogen). Complete medium was complemented with 20% GM-CSF from a mouse GM-CSF-transfected cell line (Ag8653) as a source of GM-CSF [14]. Cells were cultured for 7 days at 37 °C in a 5% CO_2 incubator and were fed on days 3 and 5. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8, washed, and used as immature DCs for the studies. Cell purity was routinely 86-90% $CD11c^{high}$ and $F4/80^{-/dim}$ cells as determined by FACS analysis and as previously reported [7].

Isolation of splenic CD4+ T cells

Untouched CD4+ T cells were purified from the spleen of either naïve or infected C57BL/6 mice by negative selection using CD4+ T cell isolation kit II according to the manufacturer's instructions (MACS, Miltenyi Biotec). Briefly, spleens were harvested from naïve or infected mice at the indicated times (see below) and perfused with RPMI complete medium (without antibiotics), teased apart, and pressed gently through a sterile fine wire mesh. The red blood cells were removed by incubation with NH_4Cl lysing buffer (eBioscience). To obtain CD4+ T cells, splenocytes were suspended in sterile PBS containing 2 mM EDTA and separated using Lympholyte-M density gradient (Cedarlane Lab.). Low-density cells at the interphase were collected and further purified by magnetic-activated cell sorting (MACS) negative selection as mentioned above. The enriched CD4+ T cells had > 95% purity as determined by FACS using anti-CD3 and anti-CD4 staining (data not shown).

***In vivo* infection model**

For *in vivo* CD4+ T cell analysis, mice were injected i.p. with 1 x 10^7 CFU of *S. suis* strain P1/7, based on previous work [15]. Surviving animals that had previously displayed clinical symptoms were boosted with a second dose of 1 x 10^7 CFU of *S. suis* strain P1/7 two weeks after initial infection. Bacteremia was monitored during the first 72 h post-primary infection or the first 24 h post-boost infection. Spleens of animals with clinical symptoms and positive bacteremia were harvested 96 h post-primary infection or 48 h post-boost infection (n = 2 per group x 5 individual experimental infections). Before harvesting, mice were injected i.p. with 500 µl of 200 µg of Brefeldin A solution in PBS (eBioscience) 5 h prior to spleen collection. Control (placebo) animals were similarly treated. Spleen CD4+ T cells were purified as described above, in the presence of Brefeldin A solution during all the purification steps, in order to avoid protein secretion. The selected time-points are based in pre-trial analysis using different post-infection times (data not shown). Purified CD4+ T cells were analyzed for cytokine production by intracellular staining followed by by flow cytometry analysis (IC-FACS, see below).

For quantification of total number of splenic CD3+CD4+ T cells or CD3+CD8+ T cells during the infection, spleens (n = 4 per group x 2 individual experimental infections) were collected at different times post-primary infection (2, 4, 6 and 8 d) and post-boost infection (2, 4, 6 and 8 d) and cells quantified by FACS (see below). In selected experiments, for the time point of 8 d post-primary infection, half-spleens (n = 4 per group x 3 individual experimental infections) were preserved in formalin for histopathological analysis in parallel to FACS analysis (see below).

To measure the *S. suis* specific primary antibody response, sera from infected mice were collected 14 days after primary infection (n = 10).

***Ex vivo* analysis of total splenocytes**

C57BL/6 mice were injected i.p with a dose of 5 x 10^7 CFU of *S. suis* strain P1/7. Control mice were injected with the vehicle solution (sterile THB) (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection, perfused with RPMI

complete medium (without antibiotics), teased apart, and pressed gently through a sterile fine wire mesh. After red blood cells lysis and washing, total splenocytes were plated at a concentration of 5 x 10^6 cells/ml in RPMI complete medium (without antibiotics) in 24-well flat bottom plates, and incubated at 37°C with 5% CO_2 for 48 h. However, after the initial 6 h of *ex vivo* incubation, gentamycin (Gibco) was added to the culture to prevent cell toxicity. Total splenocytes from control (placebo) animals were similarly treated. Concanavalin A (ConA, 0.1 μg/ml, Sigma-Aldrich) was used as positive control. Supernatants were harvested at the indicated time point for cytokine analysis by ELISA. In selected experiments, total splenocytes were incubated *ex-vivo* for 14 h or 48 h as described above. However, brefeldin A (3 μg/ml) was added during the last 5 h, and either total splenocytes or CD4+ T cells (MACS-isolated from the culture wells) were analyzed by IC-FACS (see below). The above described final culture conditions for *ex vivo* analysis were selected based on multiple pre-trials using different post-infection times (6 h and 12 h) combined with 14, 24, 48 and 72 h *ex-vivo* incubation times (data not shown).

***In vitro* DC-T cell co-culture model**

For the co-culture model, 1 x 10^5 DCs were plated in 48-well flat bottom plates for 1 h at 37°C with 5% CO_2. Afterwards, 1 x 10^5 CFU of either wild-type (WT) *S. suis* strain P1/7 or Δ*cpsF* mutant strain (MOI: 1) were added to wells for 1 h. Extracellular bacteria were killed using 100 μg/ml of gentamycin and 5 μg/ml of penicillin G (Sigma-Aldrich) as previously described [9]. After 1 h of antibiotic treatment and 3 washing steps, 5 x 10^5 freshly isolated CD4+ T cells from naïve mice (T cell: DC ratio of 5:1) were added to the wells. Co-cultures incubated with medium alone served as negative. Co-cultures treated with either ConA (0.1 μg/ml) or phorbol myristate acetate (PMA, 15 ng/ml) + ionomycine (150 ng/ml) served as positive controls. For FACS analysis of surface marker expression, co-culture plates were incubated for 3, 8, 24 and 48 h at 37 °C, 5% CO_2, prior to cell harvesting and FACS analysis. For T cell cytokine expression, co-culture plates were incubated for 48 h, then centrifuged and replenished with fresh medium containing 10 ng/ml of mouse rIL-2 (Miltenyi Biotec). Plates were incubated at 37°C, 5% CO_2 for 3 days allowing a resting period for activated T cells. After 3 days, T cells were harvested, washed, and seeded into 96 well flat-bottom culture plates coated with 5 μg/ml of anti-

mouse-CD3 mAb (BD Pharmingen) at a final concentration of 1 x 10^5 cells/well. These plates were incubated for 48 h at 37°C, 5% CO_2 prior to supernatant harvesting for ELISA testing. Single cell cultures (either DC alone or T cell alone) were also included as controls. No significant cytokine production was observed in single cell cultures under this protocol conditions (data not shown).

Cytokine quantification by ELISA

Levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, CCL3 (MIP-1α) and CXCL9 (MIG) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems, according to 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 each cytokine. Absorbance was measured at 450 nm. The results are from at least three independent ELISA measurements.

FACS analysis

For cell surface staining of *in vitro* co-cultures, 10^6 cells were washed and treated for 15 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab) in sorting buffer (PBS-1% fetal bovine serum). Blocked cells were then incubated with FITC-conjugated anti-mouse CD4 mAb (clone GK1.5) for 30 min on ice followed by washing and staining for 30 min with PE-conjugated anti-mouse CD69 mAb (clone H1.2F3) or CD40L mAb (clone MR1). All reagents were from BD PharMingen. FACS was performed using a Cell Lab Quanta™ SC MPL MultiPlate Loader instrument (Beckman Coulter). Twenty thousand gated events were acquired per sample and data analysis was performed using Cell Lab Quanta Collection/Analysis software. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales.

For IC-FACS of MACS-purified CD4+ T cells from *in vivo* or *ex vivo* experiments, cells were blocked as described above followed by fixation and permeabilization using IC Fixation/Permeabilization eBioscience kit as per the manufacture's recommendation. Permeabilized cells were then stained for 20 min at room temperature with PE-conjugated

mAbs (eBioscience) directed against the following intracellular molecules: IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), IL-2 (clone JES6-5H4), or IL-10 (clone JES5-16E3). FACS was performed using a FACSCalibur instrument (BD Biosciences). Twenty thousand gated events were acquired per sample and data analysis was performed using CellQuest software. Histograms were drawn based on PE-control stain and were plotted on logarithmic scales.

For multi-parametric IC-FACS of *ex vivo* cultures of total splenocytes, cells were washed and blocked as described above followed by surface staining with PE-conjugated anti-CD19 (clone 6D5), PE-Cy7-conjugated anti-NK-1.1 (clone PK136), FITC-conjugated anti-CD3 (clone 17A2) and/or PE-conjugated CD69 (clone H1.2F3, BD Pharmingen) for 30 min on ice. Following fixation and permeabilization, intracellular staining was performed with APC-conjugated anti-IFN-γ (clone XMG1.2) or APC-conjugated anti-TNF-α (clone MP6-XT22) for 45 min at room temperature. Unless otherwise specified, all mAbs were from Biolegend. FACS was performed using a FACSCanto II instrument (BD Biosciences). Fifty thousand gated events were acquired per sample and data analysis was performed using FACSDiva™ software. Fluorescence Minus One (FMO) control staining was performed for proper analysis and gating of target cells.

For quantification of splenic CD4+ T cells during the infection, spleens collected at different times post-primary infection and post-boost infection were processed as described above and 10^6 cells were washed and treated for 15 min on ice with FcR-blocking reagent in sorting buffer. Blocked cells were then incubated with PE-Cy5-labeled anti-mouse CD3 mAb (clone 145-2C11, BD Pharmingen) for 30 min on ice followed by washing and staining for 30 min with FITC-labeled anti-mouse CD4 mAb (clone GK1.5, BD Pharmingen) and PE-labeled anti-mouse CD8 mAb (clone 53-6.7, BD Pharmingen). FACS was performed using a Cell Lab Quanta™ SC MPL MultiPlate Loader instrument. Twenty thousand gated events were acquired per sample and data analysis was performed using Cell Lab Quanta Collection/Analysis software. Quadrants were drawn based on FITC- and PE-Cy5-control stains and were plotted on logarithmic scales.

ELISA for *S. suis*-specific antibodies

Titers of *S. suis*-specific total Ig and IgG subclasses in mouse sera were determined by ELISA. Polysorp immunoplates (Nunc) were coated with *S. suis* strain P1/7. Briefly, after bacterial growth and washing steps, bacteria were resuspended in sterile water at a concentration of 1 x 10^7 CFU/ml and 100 µl/well were plated. Plates were allowed to dry for 48 h. Then, 50 µl/well of methanol was added for 90 min. Before use, plates were washed three times with PBS containing 0.05% Tween-20. The plates were then incubated with 100 µl of serial dilutions of mouse sera in PBS containing 0.05% Tween-20 for 1 h at room temperature. Bound antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse total Ig [IgG+ IgM] (Jackson Immunoresearch), IgG1, IgG2b or IgG2c antibodies (Southern Biotech) for 1 h at room temperature. The plates were developed with TMB substrate (Invitrogen) and absorbance was measured at 450 nm. The antibody levels in serum were expressed as endpoint titers, the reciprocal of the highest dilution that yielded the background optical density plus 3 times de standard deviation (OD + 3SD).

Ovalbumin (OVA) immunization studies

Mice were infected with *S. suis* WT strain P1/7 (1 x 10^7 CFU/ml) two days prior immunization with 10 µg of OVA (Sigma-Aldrich) formulated with 20 µg of CpG ODN 1826 (Invivogen) as adjuvant. A boost immunization with the same OVA-CpG ODN formulation was given at day 14 post-primary immunization. Serum levels of OVA-specific total Ig, IgG1, IgG2b and IgG2c were measured by ELISA (see below) at 14 and 21 days post-primary immunization (n = 10 per group).

ELISA for OVA specific antibodies

Serum levels of OVA-specific antibodies were determined by ELISA. Briefly, Polysorp Immuno plates were coated overnight at 4 °C with 100 µl of 50 µg/ml of OVA protein in PBS. After blocking for 2 h with 1% casein solution in PBS-0.05% Tween-20, serial dilutions of serum samples were added to the plates and incubated for 1 h at room temperature. After washing, secondary antibodies specific for total Ig and for each IgG

subclass were added as described above. Antibody levels in serum were expressed as endpoint titers.

Statistical analysis

Cytokine and FACS data are expressed as mean ± SEM and analyzed for significance using Student's unpaired *t*-test. Antibody titer levels data were analyzed for significance using ANOVA analysis. All analyses were performed using the Sigma Plot System (v.9; Systat Software). A $P < 0.05$ was considered as statistically significant.

RESULTS

Encapsulated *S. suis* induces a type-1 pro-inflammatory environment in the spleen during systemic infection

In order to characterize the immunological environment during *S. suis* systemic infection, an *ex vivo* approach was first used to measure the production of different cytokines in the spleen. Total splenocytes from mice infected with *S. suis* WT strain P1/7 and presenting clinical signs where collected and incubated in cell cultures plates for 48 h. Splenocytes from control (placebo) animals were used as negative controls and ConA-treated splenocytes were used as positive controls. Under these conditions, a significant production of IL-6 and TNF-α as well as high levels of IFN-γ were observed, indicating the progression of a type-1 pro-inflammatory response (Fig. 1). Infected spleen cells also released significant amounts of IL-10, suggesting a role of this regulatory cytokine in maintaining homeostasis during the inflammatory process. Interestingly, the presence of CCL3 and CXCL9, important chemokines involved in T cell recruitment, was observed (Fig. 1). The production of IL-4 was not detected (data not shown).

CD4+ T cells are involved in the host immune response induced during *S. suis* infection

CD4+ T cells are key players in the development of the host immune responses; however, their activation status and cytokine profile in response to *S. suis* infection has never been investigated. Firstly, we performed a multi-parametric FACS analysis of total splenocyte *ex vivo* production of IFN-γ, a Th1 signature cytokine. As shown in Fig. 2A, IFN-γ production was overall weak and hardly detectable by IC-FACS within the whole spleen cell population. CD3+ T cells contributed to ~ 50% of the IFN-γ response in the spleen of infected mice. However, when considering inter- and intra-experiment variations, this weak IFN-γ production was not statistically significant compared to control mice (Fig. 2B). NKT cells (NK1.1+ CD3+) produced low to negligible levels of IFN-γ (data not shown). Indeed, NK cells (NK1.1+) were the major contributors to IFN-γ production within the CD3- population early during infection (data not shown). As expected, CD19+ cells (B cells) did not produce significant levels of this cytokine (data not shown). Similar findings were

obtained when analyzing TNF-α production (Fig. 2C). Nevertheless, a strong expression of the early leukocyte activation marker CD69 was observed in total splenocytes from infected animals compared to control mice. Around 12.8% ± 2.5% of cells expressed this marker within the CD3+ population, suggesting that a small portion of T cells have been activated during infection (Fig. 2D).

As the frequency of activated CD3+ T cells during *S. suis* infection was very low, and to better evaluate the role of CD3+CD4+ T cells, these target cells were MACS-isolated from *ex-vivo* total splenocyte cultures and analyzed by IC-FACS. As shown in Fig. 3, CD4+ T cells contributed to the release of low, yet significant levels of IFN-γ, TNF-α and IL-2. These data suggest that CD4+ T cells indeed differentiate into Th1 cells, although a low percentage of activated cells are observed relatively to ConA-treated cells, used as positive controls. Interestingly, a significant percentage of IL-10+CD4+ cells was also observed in *ex-vivo* total splenocyte cultures (Fig. 3).

With the aim to measure the frequency and level of activation of CD4+ T cells *in vivo* during infection, mice were injected i.p. with Brefeldin A solution and CD4+ T cells were directly isolated from the spleen 96 h post-primary infection. The percentage of IFN-γ+CD4+ cells was very low after a primary infection, and not significantly different than controls. On the other hand, *in vivo* production of TNF-α and IL-2 by CD4+ T cells was significantly higher than controls cells (Fig. 4). Similarly to *ex vivo* data, a significant production of IL-10 by CD4+ cells was also observed *in vivo* during *S. suis* primary infection. In order to investigate the secondary immune response, surviving mice were challenged with a second infectious dose 2 weeks after primary infection and similarly treated with Brefeldin A. When CD4+ T cells were isolated 48 h post-boost, percentage of IFN-γ+, TNF-α+, IL-2+ and IL-10+ CD4+ cells were similar, or only slightly higher, than those observed 96 h post-primary infection (Fig. 4), suggesting a poor generation of CD4+ memory T cells during *S. suis* infection.

Reduced numbers of CD4+ T cells after *S. suis* infection

To better understand the dynamics of T cell activation *in vivo*, total numbers of CD4+ and CD8+ T cells were quantified during the *S. suis* infection. Spleens from control and infected mice were collected 2, 4, 6, and 8 d post-primary infection and 2, 4, and 6 d post-boost infection. Similar percentage of CD4+ T cells were observed between infected and non-infected controls during the first 6 d post-primary infection (data not shown). However, a significant decrease in the numbers of these cells was observed at 8 d post-primary infection (Fig. 5). This reduction in the number of CD4+ T cells persisted after challenge infection and gradually came back to normal by 6 to 8 d post-boost infection (not shown). Only minor and no significant changes in the CD8+ T cell population were observed (data not shown).

***S. suis* CPS impairs cytokine release by T cells**

Previous experiments showed that albeit CD4+ cells are involved in the immune response induced during *S. suis* infection, their activation status seem to be compromised. To better characterize the effect of *S. suis* on CD4+ T cell functions, we performed *in vitro* DC-T cell co-cultures. As *S. suis* is a well encapsulated bacteria, we also evaluated the impact of CPS on the activation of CD4+ T cells *in vitro*, using non-encapsulated mutant strain. Supernatants from co-culture experiments were collected and tested by ELISA for the presence of CD4+ T cell-derived cytokines. The WT strain P1/7 induced the release of low, but significant levels of TNF-α and IFN-γ. Low levels of IL-10 release by CD4+ T cells were also observed in response to WT *S. suis* activation *in vitro*. (Fig. 6). In contrast to WT *S. suis*, the non-encapsulated mutant strain Δ*cpsF* induced significantly higher levels of TNF-α, IFN-γ and IL-10 by CD4+ T cells. *S. suis*-activated CD4+ T cells released large amounts of IL-2, compared to levels produced of the other cytokines, and this production was not affected in co-cultures stimulated with the Δ*cpsF* mutant strain (Fig. 6). These results suggest that *S. suis* CPS modulates CD4+ T cell activation without effecting autocrine IL-2 secretion.

***S. suis* interferes with T cell expression of co-stimulatory molecules**

In addition to cytokine production, expression of surface molecules on CD4+ T cells is an essential event for proper T cell activation. To measure the ability of *S. suis* to induce optimal activation of CD4+ T cells, we measure surface expression of CD69 and CD40L. Fig. 7A shows that *S. suis* failed to induce a significant increase in surface expression of these molecules *in vitro*, compared to uninfected control cells. Co-cultures infected with the non-encapsulated mutant strain Δ*cpsF* showed a low increase in CD69 expression by T cells; however levels of CD40L expression remained unchanged (Fig. 7B). Similar results were observed independently of the incubation time (3, 8, 24 and 48 h of co-culture incubation, not shown).

***S. suis* induces a weak specific antibody response**

As CD4+ cells play a major role in B cell activation and thus in the generation of a specific humoral response, the anti-*S. suis* antibody response generated during infection was evaluated. Blood from infected mice was collected 14 days after primary infection. As shown in Figure 8, titers of total Ig [IgG+ IgM] directed against the whole bacteria were relatively low, when compared to those obtained after an immunization with OVA (see Figure 9). Nevertheless, isotype switching was observed in infected animals. In agreement with a Th1 profile, levels of *S. suis*-specific antibodies of the type 1 IgG subclasses (IgG2b and IgG2c) were higher than those of the Type 2 IgG1 subclass (Figure 8).

S. suis* interferes with the OVA-specific antibody response *in vivo

To better understand the overall low antibody response generated during a *S. suis* infection, the capacity of *S. suis* to interfere with the development of the antibody response against a bystander antigen was evaluated. C57BL/6 mice were infected with *S. suis* WT strain P1/7 two days prior the injection of OVA formulated with CpG ODN. Two weeks after primary OVA immunization, serum levels of OVA-specific total Ig, IgG1, IgG2b and IgG2c were found significantly lower following mice infection with *S. suis* compared to non-infected mice (Figure 9A). Surviving mice were then boosted with a second dose of OVA to evaluate the effect of *S. suis* infection on the development of anti-OVA memory antibody

response. A significantly lower production of total Ig, IgG1 and IgG2b was also observed after boost (Figure 9B), while no difference was measurable in the case of IgG2c.

Diminished OVA-specific antibody responses correlates with *S. suis*-induction of clinical signs in mice

In spite of similar bacteremia levels, during a *S. suis* infection, some animals displays severe clinical signs whereas others present milder symptoms. A correlation analysis was thus performed between the presence of severe clinical signs in infected animals and the production of OVA-specific antibodies during the primary response. Effectively, as shown in Figure 10, the anti-OVA antibody production was significantly lower when the infected animals displayed severe clinical signs compared to the infected animals with milder clinical signs.

DISCUSSION

The mechanisms involved in the innate and adaptive immune responses toward *S. suis* remain essentially poorly understood, and the increase in severity of *S. suis* infections in humans underscores the critical need of a better understanding of the interactions between *S. suis* and the immune system to generate an effective immune response against this pathogen. DCs are activated in the presence of *S. suis,* undergoing a maturation process characterized by the up-regulation of costimulatory molecules and the production of pro-inflammatory mediators [7-10]. However, *S. suis* was previously shown to possess several virulence factors able to modulate such DC functions, potentially leading to a diminished or ineffective host immune response [7-10]. In the present work, we attempted to further evaluate the consequences of *S. suis* modulation of DC functions on the development of adaptive immune responses toward this pathogen. This study addresses for the first time the contribution of CD4+ T cells in the development of immune functions during *S. suis* serotype 2 infections using *in vivo*, *ex vivo* and *in vitro* analyses.

In general, T cells seem to be essential for the development of the host adaptive immune response. A preliminary experiment conducted in our laboratory with TCRαβ KO mice showed that mice devoid of functional CD4 and CD8 T cells die significantly more rapidly than control mice, suggesting an important role for T cells during *S. suis* infection (unpublished observations). This prompted us to further evaluate the role of CD4+ T cells during *S. suis* infection.

An experimental model of *S. suis* systemic infection has previously been described in our laboratory [12]. During the systemic phase of the infection, among other cytokines, high serum levels of IL-6, TNF-α, IFN-γ and IL-10 were observed [12,15]. In this study, total spleen cells were shown to secrete TNF-α, IFN-γ, IL-6, IL-10, CCL3 and CXCL9, suggesting an activation of splenic cells during the infection and a polarization towards a Th1 response. IL-10 production can be related to immune regulation, while the presence of CCL3 and CXCL9 suggests that T cells might be recruited in the spleen through the release of these chemokines, as is the case for other streptococci [16]. It is interesting to note that

TNF-α and IL-6 have routinely been reported as important mediators of *S. suis* sepsis [12,15]. This observation might also highlight the particular importance of IL-10 in maintaining homeostasis as reported by Dominguez-Punaro *et al* [15]. Similar experiments were conducted in parallel with Group B *Streptococcus* (GBS), a pathogen often compared to *S. suis*. Indeed, GBS is also encapsulated and cause invasive infection leading to sepsis and meningitis as is the case for *S. suis*. Furthermore, GBS and *S. suis* are the only Gram-positive bacteria harbouring terminal sialic acid in their CPSs. In spite of expected similarities, the production of these cytokines and chemokines in the spleens of *S. suis*-infected mice was much lower that that observed in GBS-infected mice under the same experimental conditions (Clarke *et al*., submitted for publication).

CD4+ T cells are expected to be major contributors to cytokine release and to shape the issuing adaptive immune response that follows the initial innate inflammatory response to systemic bacteria [17]. Multiple *ex vivo* and *in vivo* analyses of either total splenocytes, CD3+ T cells or CD3+CD4+ T cells suggested the development of a Th1 response after *S. suis* infection. However, frequency of activated CD4+ T cells and levels of IFN-γ, TNF-α and IL-2 were very low. IL-10 production by CD4+ T cells activated by *S. suis* was also observed. Besides Treg, IL-10 production has been reported by both Th1 and Th2 differentiated T cells [18]. IL-4 production by CD4+ T cells was not detected in our system. The very acute course of the *S. suis* infection suggest the generation of CD4+IL-10+ cells during the type-1 inflammatory process rather than the expansion or generation of a particular Treg population.

The development of immune memory was also evaluated. Two weeks after primary infection, surviving animals were challenged with a second infection. Results showed that CD4+ T cells responded to infection by producing the same pattern of cytokines. However, this response was engaged much more rapidly as CD4+ T cells were isolated and stained 48 h after challenge in comparison to 96 h for the initial infection. Nevertheless, levels of cytokine production were similar to those observed after a primary infection, suggesting a limited development of a memory response.

S. suis possesses a thick CPS known to be its most important virulence factor. The presence of CPS in *S. suis* is known to hide cell wall antigens and thus reduce cell activation [7-9,12]. Previous studies with DCs showed that the CPS modulates DC functions, mostly by interfering with *S. suis* internalization and killing by DCs as well as with DC cytokine release, which could impact T cell activation [7-9]. In order to characterize the role of *S. suis* CPS in the activation of CD4+ T cells, an *in vitro* model was used. *In vivo* studies could not be performed as the mutant strain is rapidly eliminated from the host [19,20]. The CPS was found to interfere with the release of IFN-γ, TNF-α and IL-10 by CD4+ T cells. The reduced internalization of *S. suis* by DCs and the reduced ensuing DC activation could be responsible for the diminished production of cytokines by T cells. However, the CPS has no effect on the release of IL-2, suggesting that the presence of the CPS interferes with CD4+ T cell activation, but not with T cell proliferation. CD69 is the earliest leukocyte maturation marker and is routinely used to evaluate T cell activation [21]. However, it has been demonstrated that CD69-deficient lymphocytes have a normal proliferative response [21]. In the case of *S. suis*, only low expression of CD69 by *in vitro* activated CD4+ T cells was observed. This is in contrast with *Streptococcus pneumoniae* which induces an up-regulation of CD69 on T cells during the infection [22]. *S. suis* also failed to induce significant levels of surface expression of CD40L, an important costimulatory molecule involved in T cell activation. The CPS does not seem to interfere with CD69 or CD40L expression by *S. suis*-stimulated CD4+ T cells. Altogether, these findings suggest that *S. suis* uses multiple virulence factors to reduce either cytokine release or costimulatory molecule expression by CD4+ T cells.

During the infection, we also observed that the production of *S. suis*-specific antibodies is low. This prompted us to evaluate the ability of *S. suis* to interfere with the production of antibodies against a bystander antigen. OVA, in formulation with CpG ODN, was injected in infected mice. The production of both Th1- and Th2-dependent antibody isotypes was markedly reduced during the primary infection, and same results were obtained during the memory response, except for the production of IgG2c, for which no significant difference was noticeable. IgG2c is strongly associated with the development of a Th1 response. As

CpG has a strong Th1 adjuvant activity [23], this could explain why a significant difference during the memory response was difficult to be observed.

Another major finding of this work is the correlation between the presence of clinical signs and the significantly reduced production of antibodies against OVA. After the infection with *S. suis,* animals developed a bacteremia accompanied by either mild or severe clinical signs such as rough hair coat, swollen eyes, depression, prostration and weakness. This suggests that *S. suis* has immunosuppressive properties. The exact mechanisms responsible for the suppression of the immune response during *S. suis* infection will need further investigations. However, such observations have already been described for *S. pneumoniae* [24]. Indeed, it has previously been demonstrated that *S. pneumoniae* inhibits IgG responses to a number of coimmunized soluble antigens. More precisely, *S. pneumoniae* was found to mediate a significant reduction in the formation of Ag-specific splenic germinal center T follicular helper and germinal center B cells and antibody-secreting cells in the spleen and bone marrow in response to OVA [24]. In this regard, a transient depletion of CD4+ T cells in the spleens was observed during a primary *S. suis* infection. It is unknown if this is related to cell death or cell emigration to other target tissues or organs.

To conclude, we observed that total splenocytes are involved in the production of several cytokines following *S. suis* infection. Particularly, we demonstrated the production of TNF-α, IFN-γ, IL-10, and IL-2 by CD4+ T cells, suggesting the development of a Th1 response. However, *S. suis* CPS interferes with CD4+ T cell activation, while having no impact on T cell proliferation. Finally, *S. suis* also hampers the development of specific antibodies during the infection. Animals hampered in antibody production are those displaying the most severe clinical signs. This study is a noteworthy starting point for future research regarding T-cell dependent immunity during *S. suis* infection and the consequences in vaccine development.

ACKNOWLEDGEMENTS

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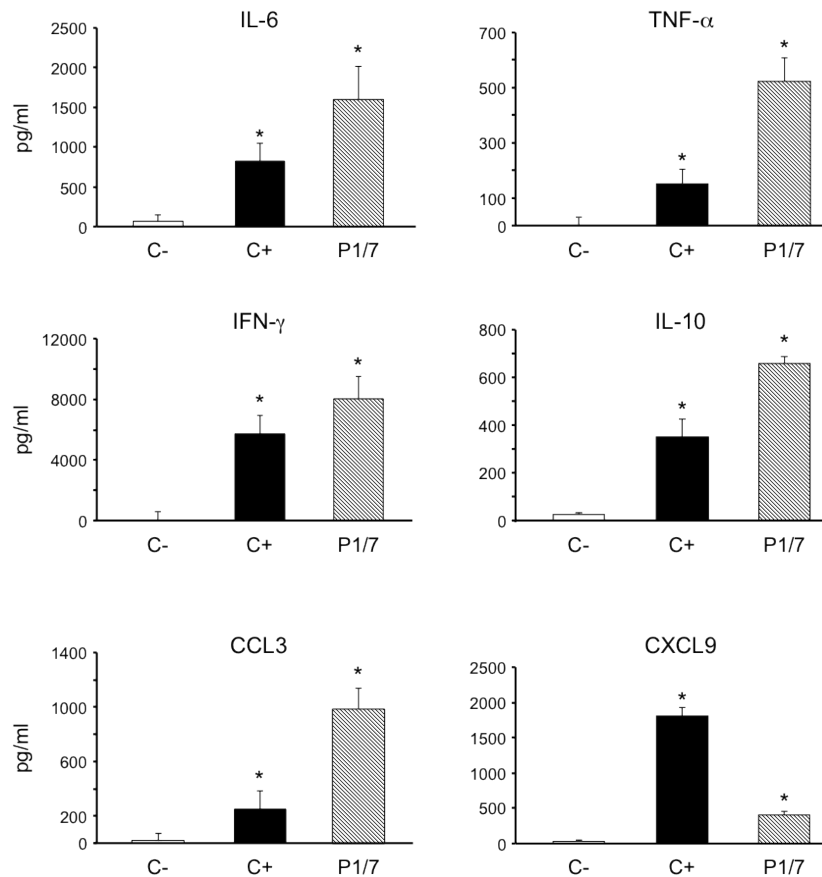

Figure 1. *Ex vivo* cytokine and chemokine production profiles by total splenocytes in response to *S. suis*. Mice were infected intra-peritoneally with a dose of 5 x 10^7 CFU/ml of *S. suis* wild-type strain P1/7 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 6 h of incubation, gentamycin was added to the culture to prevent cell toxicity. Cells were then incubated for 48 h and supernatants were collected for cytokine analysis by ELISA. Non-stimulated cells from mock-infected animals served as negative control for basal expression (C-). Cells stimulated with Concanavalin A (0.1 µg/ml) were used as positive control (C+). Data are expressed as means ± SEM (in pg/ml) from 3 different experimental infections. * $P < 0.05$ denotes values that are significantly higher than those obtained with splenocytes from non-infected mice (C-).

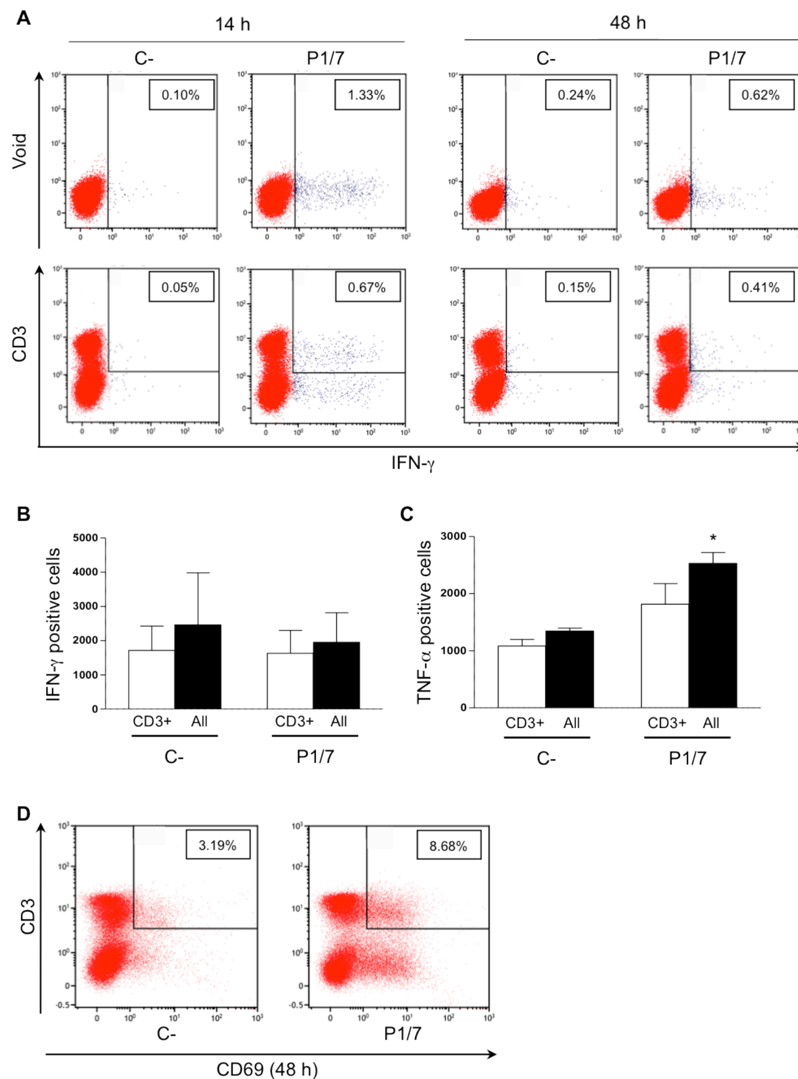

Figure 2. *Ex vivo* analyses of cellular sources of IFN-γ and CD3+ T cell activation during *S. suis* infection. Mice were infected intra-peritoneally with a dose of 5 x 10^7 CFU of *S. suis* wild-type strain P1/7 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 6 h of incubation, gentamycin was added to the culture to prevent cell toxicity. Non-stimulated cells from mock-infected animals served as negative control for basal expression (C-). Total splenocytes were incubated for 14 h or 48 h with brefeldin A (3 μg/ml) added during the last 5 h of incubation. Cells were harvested and intracellularly stained for IFN-γ **(A, B)**, TNF-α **(C)** or surface stained for CD69 **(D)** in combination with several surface markers for multi-parametric FACS analysis. **(A, D)** Representative data from 3 different experimental infections based on CD3+ population or total splenic population (Void). **(B, C)** Number of either IFN-γ+ or TNF-α+ cells within the CD3+ population or within total splenic population (All) at 48 h. Data are expressed as means ± SEM from 3 different experimental infections. *$P < 0.05$, indicates statistically significant difference compared to negative control cells (C-).

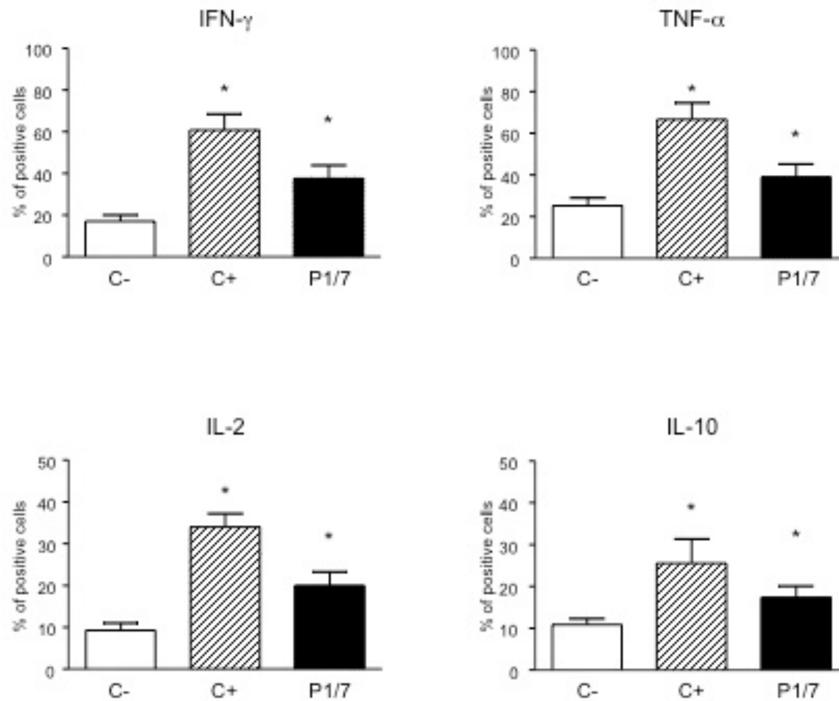

Figure 3. ***Ex vivo*** **analyses of CD4+ T cell contribution to cytokine production.** Mice were infected intra-peritoneally with a dose of 5 x 10^7 CFU of *S. suis* wild-type strain P1/7 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 6 h of incubation, gentamycin was added to the culture to prevent cell toxicity. Non-stimulated cells from mock-infected animals served as negative control for basal expression (C-). Cells stimulated with Concanavalin A (0.1 µg/ml) were used as positive control (C+). Total splenocytes were incubated for 48 h. Brefeldin A (3 µg/ml) was added during the last 5 h of incubation and CD4+ T cells were MACS-isolated from the culture, stained intracellularly for different cytokines and analyzed by FACS. Data are expressed as mean ± SEM (in % of positive cells) from 3 individual experimental infections. *$P < 0.05$, indicates statistically significant difference compared to negative control cells (C-).

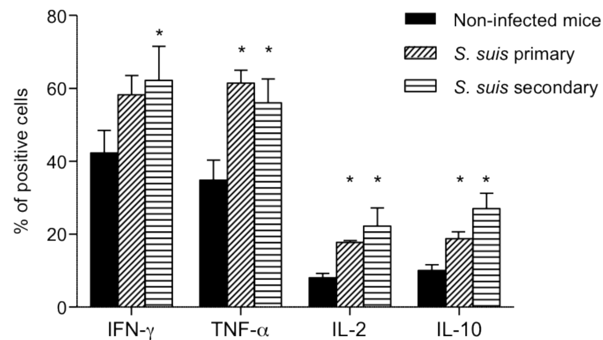

Figure 4. *In vivo* CD4+ T cell contribution to cytokine production during primary and secondary *S. suis* infections. Mice were infected intra-peritoneally with a dose of 1 x 10^7 CFU of *S. suis* wild-type strain P1/7. Surviving animals who had previously displayed clinical symptoms were boosted with a second dose of 1 x 10^7 CFU of *S. suis* wild-type strain P1/7 two weeks after initial infection. Spleens of animals with clinical symptoms and positive bacteremia were harvested 96 h post-primary infection or 48 h post-boost infection (n = 2 per group x 5 individual experimental infections). Five hours prior to spleen collection, mice were injected with Brefeldin A (200 μg). Non-infected control animals were similarly treated. Spleen CD4+ T cells were MACS-purified, stained intracellularly for different cytokines and analyzed by FACS. Cytokine basal expression levels in non-infected animals were similar at 96 h post-primary mock-infection and 48 h post-secondary mock-infection. Data from the latter time point were selected for the figure. Data are expressed as mean ± SEM (in % of positive cells) from 5 individual experimental infections. **P* < 0.05, indicates statistically significant difference compared to non-infected mice.

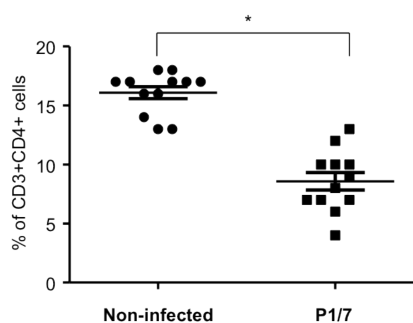

Figure 5. Numbers of splenic CD4+ T cells during *S. suis* primary infection. Mice (n = 4 per group x 3 individual experimental infections) were infected intra-peritoneally with a dose of 1 x 10^7 CFU of *S. suis* wild-type strain P1/7. Non-infected control animals were also included. At 8 days post-infection, % of CD3+CD4+ T cells were evaluated by FACS from the spleens of infected and control animals. **P* < 0.05, indicates statistically significant difference compared to non-infected mice.

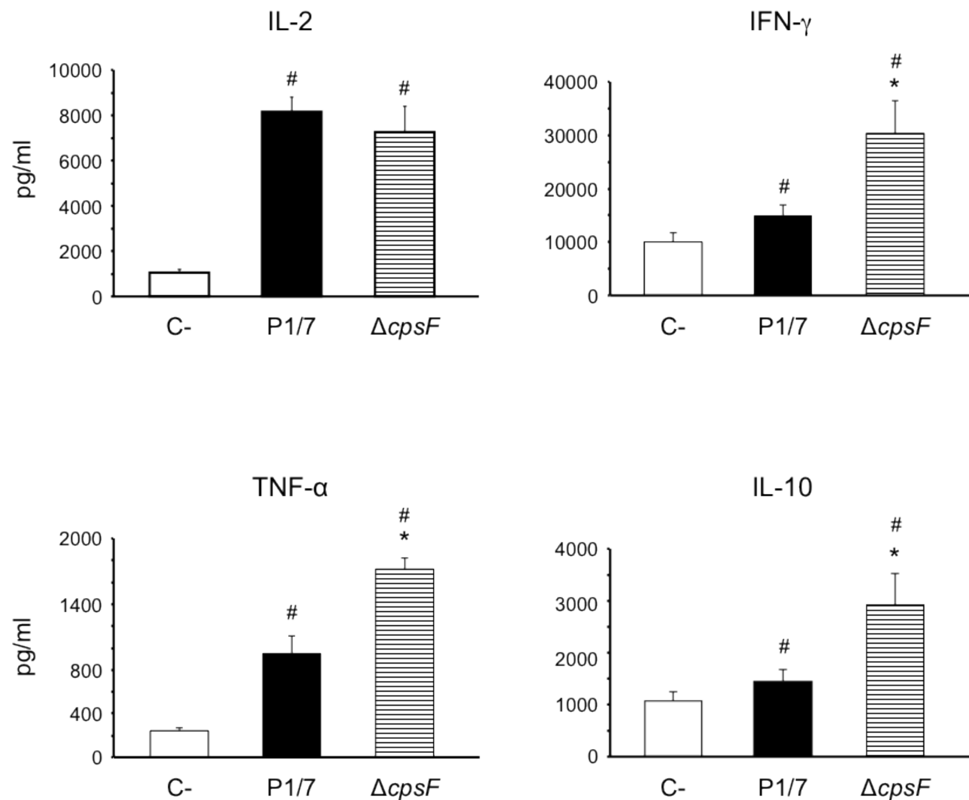

Figure 6. Role of bacterial capsular polysaccharide in the modulation of cytokine production by CD4+ T cells. Dendritic cells (DCs) were infected with either wild-type *S. suis* strain P1/7 or its non-encapsulated isogenic mutant Δ*cpsF* (MOI 1:1) for 1 h. Extracellular bacteria were killed by antibiotic treatment and cultures washed prior to addition of freshly isolated splenic CD4+ T cells from naïve mice (T cell: DC ratio of 5:1). Co-cultures were incubated for 48 h, resuspended in fresh medium containing 10 ng/ml of IL-2 for 72 h (resting period) and then transferred to anti-CD3 coated plates for 48 h. Supernatants were then collected and cytokines quantified by ELISA. Non-stimulated co-cultures served as negative controls (C-) for basal expression. Data are expressed as means ± SEM (in pg/ml) from 5 different experiments. $^{\#}P < 0.05$, indicates statistically significant differences compared to negative controls (C-). $^{*}P < 0.05$, indicates statistically significant differences between co-cultures infected with wild-type strain P1/7 and those infected with the non-encapsulated mutant Δ*cpsF*.

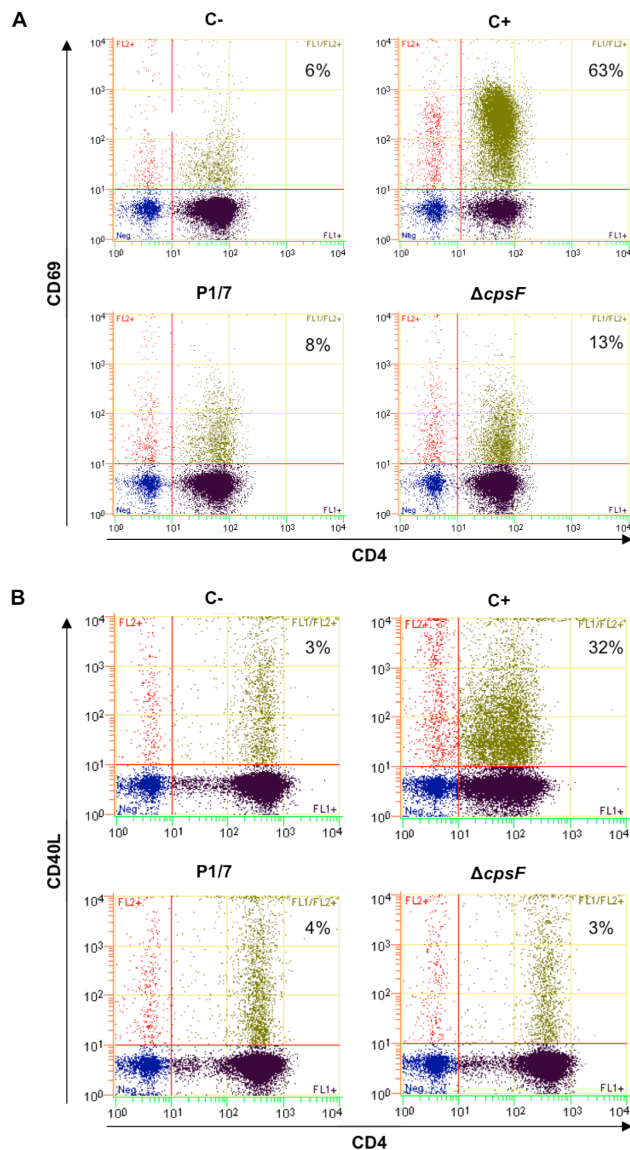

Figure 7. Role of bacterial capsular polysaccharide in the modulation of CD4+ T cell surface expression of CD69 and CD40L. Dendritic cells (DCs) were infected with either wild-type *S. suis* strain P1/7 or its non-encapsulated isogenic mutant Δ*cpsF* (MOI 1:1) for 1 h. Extracellular bacteria were killed by antibiotic treatment and cultures washed prior to addition of freshly isolated splenic CD4+ T cells from naïve mice (T cell: DC ratio of 5:1). Co-cultures were incubated for 8 h, cells harvested and CD69 **(A)** or CD40L **(B)** expression analyzed by FACS. Co-cultures incubated with medium alone served as negative controls (C-). Co-cultures treated with either Concanavalin (0.1 µg/ml) or phorbol myristate acetate (15 ng/ml) + ionomycine (150 ng/ml) served as positive controls (C+) for CD69 and CD40L expression, respectively. Twenty thousand gated events were acquired per sample. Quadrants were drawn based on FITC- and PE-control stains. Representative data from 3 different experiments. Numbers in the upper quadrants indicate the % of CD4+CD69+ or CD4+CD40L+cells.

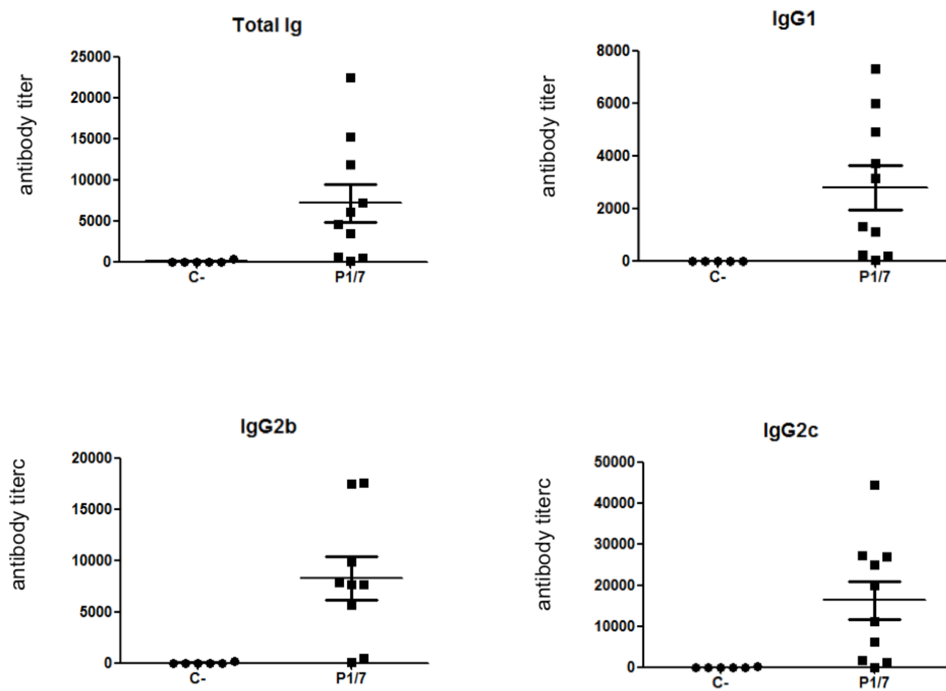

Figure 8. Serum levels of *S.-suis* specific antibodies in infected animals. Mice (n = 10 were infected with *S. suis* wild-type strain P1/7 (1 x 10^7 CFU/ml) and sera collected two weeks post-infection. Total Ig [IgG+IgM], IgG1, IgG2b and IgG2c anti-*S. suis* titers were determined by ELISA. C- represents a pool of control mice (n = 6) injected with vehicle solution.

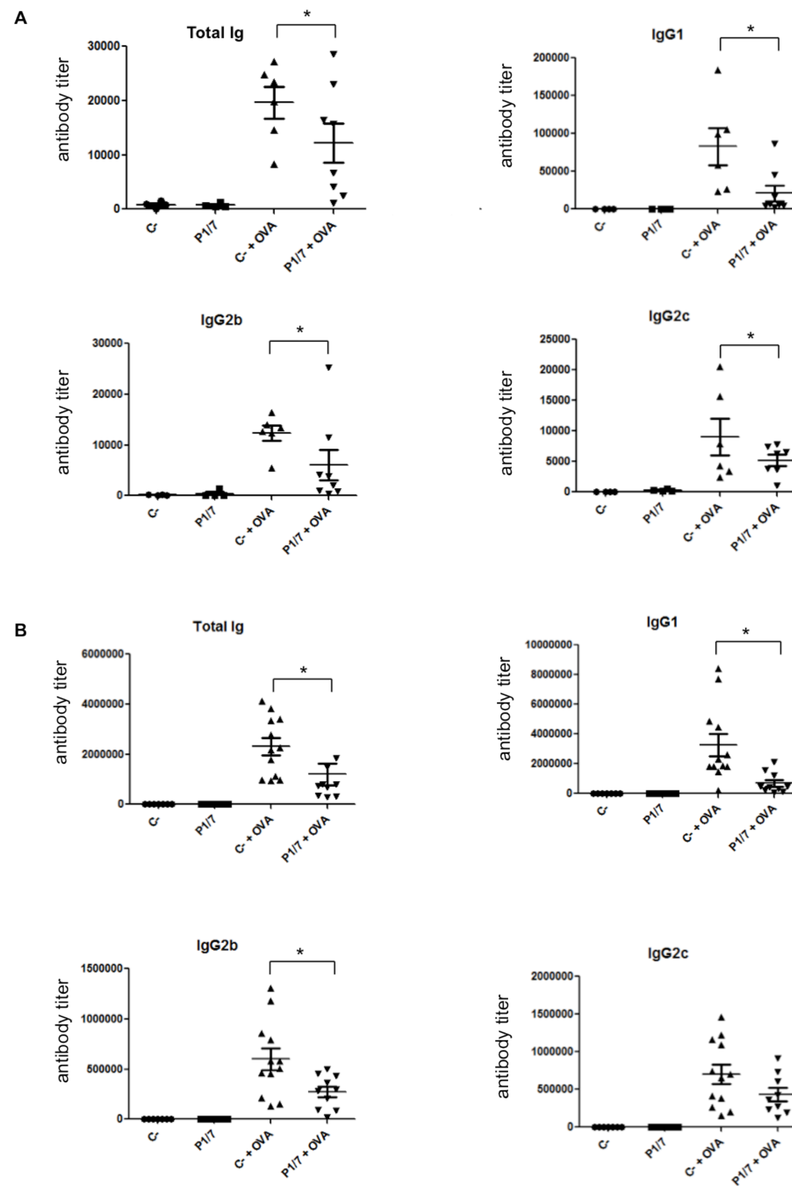

Figure 9. Effect of *S. suis* on the production of ovalbumin (OVA)-specific antibodies. Mice (n = 10) were infected with *S. suis* wild-type strain P1/7 (1 x 10^7 CFU/ml) two days prior immunization with 10 µg of OVA formulated with 20 µg of CpG ODN as adjuvant. A boost immunization with the same OVA-CpG ODN formulation was given at day 14 post-primary immunization. Serum levels of OVA-specific total Ig [IgG+IgM], IgG1, IgG2b and IgG2c were measured by ELISA at 14 **(A)** and 21 **(B)** days post-primary immunization. C- represents control mice injected with vehicle solution only (n = 6). C- + OVA represents control mice injected with vehicle solution followed by OVA immunization under the same protocol as described above (n = 10). A control group infected with *S. suis* wild-type strain P1/7 only was also included (n = 10). * $P < 0.05$ denotes values that are significantly lower in pre-infected animals (P1/7 + OVA) compared to the non-infected, immunized animals (C- + OVA).

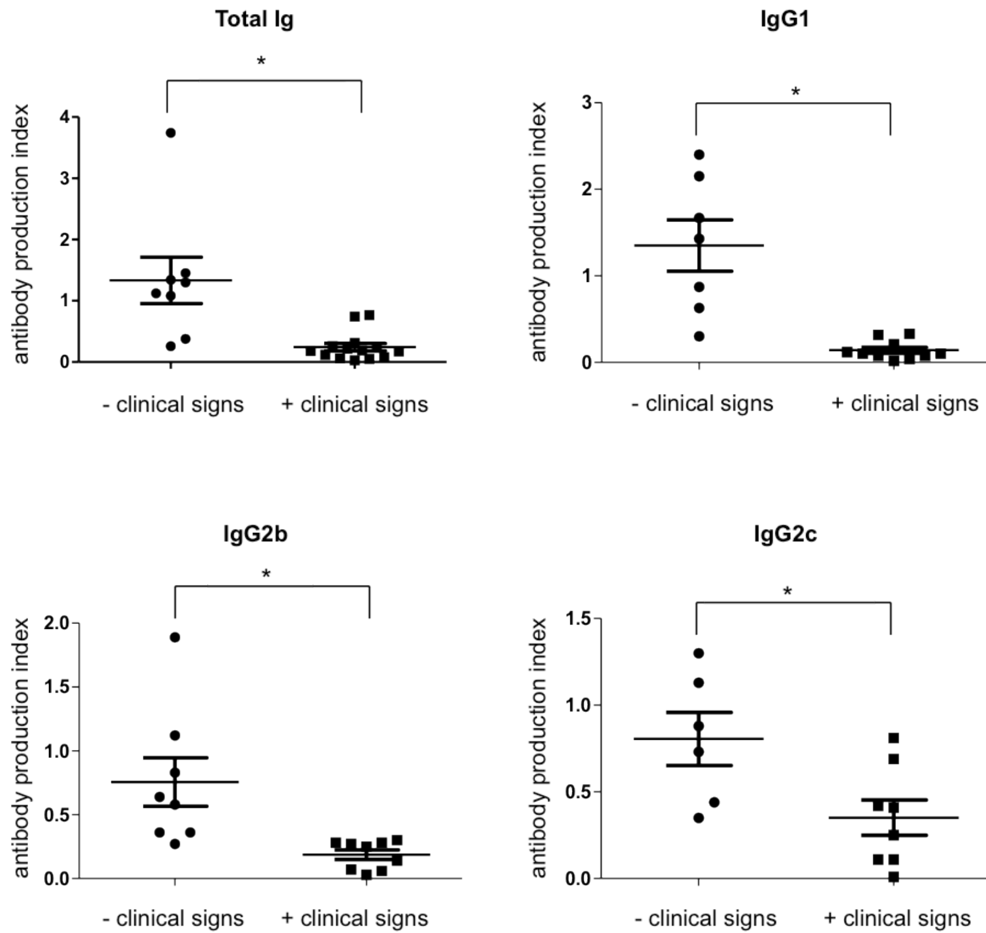

Figure 10. Correlation between serum levels of OVA-specific antibodies and clinical signs developed by *S. suis*-infected mice. Mice (n = 10) were infected with *S. suis* wild-type strain P1/7 (1×10^7 CFU/ml) two days prior immunization with 10 µg of OVA formulated with 20 µg of CpG ODN as adjuvant. Serum levels of OVA-specific total Ig [IgG+IgM], IgG1, IgG2b and IgG2c were measured by ELISA at 14 days post-primary immunization. * $P < 0.05$ denotes values that are significantly lower in infected animals presenting severe clinical signs compared to infected animals presenting mild clinical signs.

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IV. DISCUSSION

1. Modulation des fonctions des DCs par *S. suis*

Les mécanismes menant à une réponse immunitaire efficace lors de l'infection par *S. suis* sont, à ce jour, peu décrits. En effet, les interactions entre *S. suis* et les médiateurs de l'immunité ont été peu caractérisés. La présente étude décrit pour la première fois les interactions entre *S. suis* et les DCs, ainsi que leurs conséquences sur l'activation et la maturation des DCs, puis sur la réponse à médiation cellulaire T-dépendante qui en découle.

1.1 Phagocytose et « killing » de *S. suis* par les DCs

L'internalisation des antigènes est la première étape menant à l'activation et à la maturation des DCs. Nous avons évalué l'internalisation et l'élimination (« killing ») de *S. suis* par les DCs, ainsi que le rôle joué par différents facteurs de virulence.

Il est généralement assumé que le « killing » efficace des bactéries par les DCs requiert au préalable une étape d'internalisation. Dans notre étude, nous avons donc d'abord évalué la capacité des DCs à internaliser *S. suis* et nous avons démontré que *S. suis* semble échapper à l'internalisation par les DCs. En conditions non-opsonisantes, la CPS a été démontrée comme suffisante pour protéger *S. suis* de la phagocytose et, par conséquence, du « killing » médié par les DCs murines et porcines. Ceci confirme le rôle antiphagocytaire attribué à la CPS de *S. suis* suite aux résultats obtenus lors d'études précédentes en modèle d'infection *in vitro* ou *in vivo* [109,126,168]. Cependant, bien que la CPS agisse comme une barrière physique empêchant *S. suis* d'être phagocyté par les DCs, le peu de bactéries phagocytées sont détruites une fois internalisées. Ceci est en accord avec des résultats préalablement décrits chez des macrophages où des souches virulentes ou non-virulentes encapsulées étaient incapables de survivre une fois internalisées [173,288]. La localisation extracellulaire de *S. suis* lui confère donc un avantage de survie et la CPS lui est essentielle. Ceci est contraire à GBS qui possède une capacité de survie intracellulaire importante à l'intérieur des DCs (ANNEXE V). Il est important de préciser que nos études décrivent par la première fois les interactions *in vitro* entre un agent bactérien vivant et des DCs porcines.

Nous avons aussi voulu identifier les récepteurs cellulaires impliqués dans la reconnaissance de *S. suis* par les DCs qui pourraient mener à une éventuelle internalisation de la bactérie, même si cet évènement reste faible. Selon la littérature, les TLRs pourraient être impliqués dans la phagocytose des bactéries [289] bien que leur rôle reste sujet à controverse [195]. Il a été rapporté que l'activation des voies de signalisation TLR-dépendantes par les pathogènes bactériens pourrait réguler plusieurs étapes de la l'internalisation, tel que la maturation des phagosomes [289,290]. Par exemple, l'absence de TLR2 retarde la phagocytose et le killing de *S. pneumoniae* par les neutrophiles [291]. D'un autre côté, les TLRs ne semblent pas jouer un rôle significatif dans la phagocytose de GBS par les macrophages [292]. À ce jour, une seule étude concerne le rôle des TLRs dans la phagocytose d'un pathogène bactérien (*S. pyogenes*) par les DCs [293] et elle démontre que ces récepteurs ne semblent jouer aucun rôle particulier dans l'internalisation ou l'élimination de ce pathogène.

Afin d'évaluer l'implication globale des TLRs dans la phagocytose de *S. suis* par les DCs, des DCs déficientes pour l'expression de la protéine adaptatrice MyD88 ont été utilisées. Nous avons observé que le nombre de bactéries internalisées par les DCs MyD88$^{-/-}$ n'était pas significativement différent du nombre obtenu pour les DCs WT. Ainsi, une déficience au niveau des voies de signalisation MyD88-dépendantes ne semble pas jouer un rôle majeur dans l'habileté des DCs à internaliser *S. suis*. Les résultats obtenus dans la présente étude suggèrent donc que de façon similaire à *S. pyogenes*, les TLRs ne sont pas significativement impliqués dans la faible internalisation de *S. suis* par les DCs.

1.2 Maturation des DCs suite à l'infection par *S. suis*

La maturation des DCs à été évaluée suite à la stimulation des DCs par *S. suis* de type sauvage ou mutants pour différents facteurs de virulence (CPS, suilysine, modifications de la paroi cellulaire). Toutes les souches de *S. suis* testées ont induit la maturation des DCs murines comme démontré par l'augmentation de l'expression en surface de CD40, CD86 et CMH-II. Aucun rôle spécifique n'a pu être attribué aux facteurs de virulence testés puisque l'expression des molécules de co-stimulation induite par les souches mutantes est comparable à la souche de type sauvage.

Nous avons aussi évalué l'habileté de *S. suis* à induire l'expression des molécules de co-stimulation CD80/86 et CMH-II chez des DCs d'origine porcine. La souche de type sauvage n'a induit l'expression ni de CD80/86 ni de CMH-II chez les DCs porcines. La CPS semble responsable de l'expression réduite de CD80/86 sur les DCs et semble aussi partiellement impliquée dans la diminution de l'expression de CMH-II. Ces résultats diffèrent des résultats obtenus avec les DCs d'origine murine où la souche de type sauvage a induit des niveaux d'expression similaires à ceux obtenus avec le mutant non-encapsulé. Ces différences peuvent s'expliquer par l'origine des cellules (murine versus porcine) ou par le fait que les DCs obtenues chez la souris proviennent de lignées *inbred* alors que les DCs porcines proviennent d'animaux *outbred*. Effectivement, une importante variabilité a été observée concernant l'expression de CMH-II sur les DCs provenant de différents porcelets. Ceci pourrait être relié au fait que les gènes du CMH présentent un haut niveau de polymorphisme. Les gènes SLA (« swine leucocyte antigen ») sont hautement polymorphes et ont été démontrés comme influençant la réponse aux vaccins [294-297]. La forte influence du complexe SLA est particulièrement attribuable aux propriétés de présentation antigénique des protéines CMH du système immunitaire adaptatif porcin [298]. La forte variabilité de l'habileté des DCs à augmenter leur expression en surface des molécules CMH-II pourrait expliquer, du moins en partie, pourquoi *S. suis* parvient à coloniser seulement certains porcelets, et pourquoi certains animaux resteront des porteurs sains et ne développeront jamais la maladie alors que d'autres développeront une bactériémie et parfois une septicémie menant finalement à la méningite [1]. La variabilité inter-individuelle dans la réponse de l'hôte suite à la vaccination sont un réel problème, particulièrement pour les non-répondants. Ces observations sont donc cruciales pour les études immunologiques de la pathogenèse de l'infection causée par *S. suis*.

1.3 Activation des DCs suite à l'infection par *S. suis*

Nous avons d'abord évalué la capacité de *S. suis* à moduler la production de cytokines chez les DCs murines. *S. suis* a été démontré comme induisant la production d'IL-1β, IL-6, IL-10, IL-12p70, IL-23, TNF-α, MCP-1/CCL2, KC/CXCL1, MIG/CXCL9 et IP-10/CXCL10. Cette étude est la première rapportant la production de MIG/CXCL9, IP-10/CXCL10 et IL-

23 suite à une stimulation par *S. suis*. Le mutant non-encapsulé a induit des quantités significativement plus importantes pour toutes les cytokines, à l'exception d'IL-1β et MCP-1/CCL2. Des études précédentes avaient préalablement suggérées que la CPS de *S. suis* serait requise pour une production optimale de MCP-1/CCL2 par des monocytes humains, des cellules endothéliales de cerveau et des leucocytes sanguins [56,157,158]. De plus, une étude récente a démontré la capacité de la CPS purifiée à induire la production de MCP-1/CCL2 par les DCs [112]. Chez le mutant non-encapsulé, c'est probablement l'augmentation de l'exposition des composants de la paroi cellulaire dû à l'absence de CPS qui est responsable de la production accrue de la plupart des cytokines. Ceci confirme le rôle des composants de la paroi cellulaire en tant que modulateurs principaux de la sécrétion de cytokines [56,157,158]. Cette hypothèse est renforcée par le fait que la production de IP-10/CXCL10, IL-12p70 et IL-10 soit diminuée en présence des mutants de la paroi cellulaire. De façon intéressante, une souche mutante de *S. gordonii* défectueuse pour la D-alanylation du LTA a été démontrée comme induisant des quantités réduites d'IL-12p70, IL-10, IL-6 et TNF-α [137]. Du LTA et du PG dérivés de bactéries ont aussi déjà été démontré comme responsables de la production de IP-10/CXCL10 par différents types cellulaires [299,300].

En plus des composants de la paroi cellulaire, plusieurs toxines microbiennes peuvent aussi moduler les voies de l'inflammation [301]. Des études précédentes ont démontré que la suilysine de *S. suis* contribue à la production d'IL-6 et IL-8 par des BMEC [157]. Avec les DCs murines, la suilysine contribue à la production des cytokines immunomodulatoires IL-12p70 et IL-10. Les mécanismes impliqués dans la production de cytokines en réponse à la suilysine n'ont pas encore été élucidés. La pneumolysine de *S. pneumoniae* est reconnue par le TLR4 des DCs [301]. Cependant, des études précédentes réalisées en présence de monocytes humains ou directement lors d'infections *in vivo* ne semblent pas indiquer un rôle du TLR4 dans l'inflammation induite par *S. suis* [56,177]. Les résultats de la présente étude concernant le rôle du TLR4 dans la reconnaissance de *S. suis* par les DCs seront discutés plus loin.

Chez le porc, des résultats semblables ont été obtenus. *S. suis* a été démontré comme

induisant la production d'IL-1β, IL-6, IL-8, IL-12p40 et TNF-α par les DCs porcines. La CPS interfère avec la production d'IL-6, IL-8, IL-12p40 et TNF-α. Au contraire, il semble que la présence de la CPS soit essentielle pour la production optimale d'IL-1β par les DCs porcines, tout comme il a aussi été décrit plus haut pour les DCs murines.

1.4 Rôle du complément et/ou composants du sérum dans les interactions *S. suis*-DCs

En présence de sérum complet, comme lors des conditions normales d'infection de l'hôte par *S. suis*, nous avons observé que *S. suis* utilise un arsenal de facteurs de virulence afin de résister à la phagocytose et au « killing » médiés par la présence de sérum. Des études en immunofluorescence ont permis de confirmer que la CPS, la D-alanylation du LTA, la N-déacétylation du PG et la suilysine interfèrent tous avec la déposition du complément à la surface des bactéries. En général, et particulièrement pour les pathogènes possédants des CPS riche en acide sialique, la CPS est considéré comme le facteur majeur interférant avec l'activation de la cascade du complément. Par exemple, la sialylation de la CPS est importante pour la virulence de GBS puisqu'elle inhibe l'élimination intracellulaire des bactéries et empêche la déposition du complément en interférant avec la voie alterne du complément [302]. Ceci ne semble toutefois pas être le cas de *S. suis* puisque les mutants Δ*dltA*, Δ*pgdA* et Δ*sly* ne résistent pas à la phagocytose et au « killing » dépendant du complément malgré la présence d'une CPS intacte. Des études précédentes avaient déjà démontré que la suilysine contribue à la résistance de *S. suis* au « killing » dépendant du complément [109,303]. Une hypothèse avait alors été émise en se basant sur des résultats décrits concernant la pneumolysine de *S. pneumoniae*, une toxine apparentée à la suilysine de *S. suis* [304]. En effet, il avait été suggéré que la toxine pourrait activer le complément et ainsi en réduire la disponibilité pour l'opsonisation des bactéries [109]. D'autre part, l'implication des modifications de la paroi cellulaire dans la résistance au « killing » dépendant du complément peut être considérée comme une découverte majeure de la présente étude. Généralement, la D-alanylation du LTA est un facteur de virulence important pour les bactéries Gram-positif puisqu'elle module les charges en surface, joue un rôle dans la résistance aux peptidiques cationiques et dans la liaison aux ligands, par

exemple à des cations tel que Ca^{2+} et Mg^{2+} [131]. De plus, la D-alanylation du LTA diminue la liaison de la molécule C1q du complément à la surface des bactéries [138]. Récemment, la D-alanylation du LTA a été démontrée comme importante pour la résistance de *S. pyogenes* au complément. L'inactivation du gène *dltA* chez ce pathogène a été rapportée comme augmentant la déposition de C3b en surface des bactéries. Cette augmentation de la déposition du complément était concomitante avec une diminution de l'expression de la protéine M [139]. Cependant, à ce jour, aucun équivalent de la protéine M de *S. pyogenes* n'a été décrit pour *S. suis* [305]. Les mécanismes par lesquels la D-alanylation du LTA interfère avec la déposition du complément restent donc à être étudiés. Cette étude a aussi démontré pour la première fois une implication de la N-déacétylation du PG dans la résistance à la phagocytose et au « killing » dépendant du complément. La N-déacétylation du PG permet aux bactéries Gram-positif de déjouer les défenses immunitaires de l'hôte, principalement en échappant à la surveillance par les récepteurs NOD1 et NOD2 et/ou en augmentant la résistance des bactéries au lysozyme [145]. Il avait précédemment été démontré que la N-déacétylation du PG contribue à la virulence de *S. suis*. En effet, un mutant déficient pour cette modification de la paroi cellulaire est hautement atténué dans des modèles d'infection murin et porcin suite à une moins grande capacité à persister en circulation sanguine ainsi qu'à échapper aux neutrophiles [148]. D'autres modifications du PG, par exemple la O-acétylation, ont déjà été démontré comme jouant un rôle dans la liaison des bactéries aux ligands [306,307].

Nous avons aussi observé que l'opsonisation de *S. suis* par le sérum complet diminue le relâchement d'IL-12p70 et augmente la production d'IL-10. Cette modulation semble indépendante de l'internalisation et du « killing » de *S. suis* par les DCs puisque ces tendances sont aussi observées avec la souche de type sauvage pour qui la susceptibilité à la phagocytose n'est que légèrement augmentée en présence de sérum complet. Des études antérieures portant sur la production de cytokines induites par *S. suis* chez des phagocytes avaient aussi démontré que ce phénomène est indépendant de la phagocytose [128,129]. Nous pourrions émettre l'hypothèse que l'augmentation de la liaison de récepteurs spécifiques pour certains facteurs du sérum serait responsable de la modulation du ratio de production des cytokines IL-12/IL-10 après activation des DCs par les bactéries opsonisées.

Dans la littérature, l'inhibition de la production d'IL-12 a été observée suite à la liaison des récepteurs Fcγ, des récepteurs du complément et des récepteurs de type « scavengers » chez des macrophages [308]. L'effet inhibiteur alors observé était aussi indépendant de l'internalisation de particules. D'autres études ont démontré que C1q régule la production de cytokines induite par le LPS chez les DCs et que iC3b est responsable de la production d'IL-10 et la diminution de la production d'IL-12 chez les monocytes et les DCs [309-311].

L'inhibition de la production d'IL-12 pourrait être un mécanisme exploité par les pathogènes afin de supprimer ou retarder le développement de l'immunité cellulaire. Les facteurs du sérum et les récepteurs impliqués dans la modulation de la production d'IL-12 et IL-10 suite à l'opsonisation de *S. suis* restent à caractériser. Cependant, des expériences réalisées en présence de sérum inactivé par la chaleur ont permis d'observer un rôle partiel des facteurs du complément dans l'augmentation de la production d'IL-10. Le rôle du complément dans la diminution de la production d'IL-12 n'a pu être clairement démontré. D'autres facteurs du sérum pourraient ainsi être impliqués. De plus, la présence d'IL-10 pourrait elle-même contribuer à la diminution de la production d'IL-12.

2. Rôle de l'acide sialique de la CPS de *S. suis* dans la virulence

Les résultats discutés ci-haut nous ont permis d'identifier la CPS comme étant le facteur de virulence principalement responsable de la modulation des fonctions des DCs par *S. suis*. Une des caractéristiques intéressantes de la CPS de *S. suis* est la présence d'acide sialique [105]. Les capsules riches en acide sialique sont décrites comme d'importants facteurs de virulence pour plusieurs bactéries causant la méningite [312]. Par exemple, pour GBS, la sialylation de la CPS est critique pour prévenir l'opsonophagocytose en inhibant l'activation de la voie alterne du complément [312]. Dans le cas de *S. suis*, peu d'informations sont disponibles et les résultats publiés suggèrent que l'acide sialique ne semble pas jouer un rôle important dans la virulence de *S. suis.* En effet, Charland *et al*. ont rapporté qu'il n'y a aucune différence notable au niveau des concentrations en acide sialique entre les souches de différents degrés de virulence [113]. De plus, aucune différence significative n'a été observée dans les niveaux d'internalisation de souches de *S. suis* traités ou non avec la sialidase [113].

Afin d'étudier plus en détails le rôle de l'acide sialique capsulaire lors de l'infection par *S. suis*, nous avons obtenu un mutant déficient pour la production de l'acide sialique en ciblant le gène *neuC* qui serait responsable de débuter la biosynthèse de l'acide sialique [124,313]. La découverte la plus importante de cette étude sur l'acide sialique est que *neuC* a un impact majeur sur l'expression de la CPS de *S. suis*. D'abord, le mutant Δ*neuC* est non-typable avec les antisérums utilisés pour le sérotypage. Cependant, ceci pourrait être la conséquence de l'influence de l'acide sialique sur l'épitope capsulaire immunodominant de *S. suis* sérotype 2. Toutefois, les analyses en TEM ont réellement démontré l'importance de *neuC* pour l'expression de la CPS puisque la souche de type sauvage est entourée par une épaisse CPS alors que le mutant Δ*neuC* montre une absence de CPS semblable à un mutant non-encapsulé (Δ*cpsF*). La complémentation a partiellement restauré le phénotype, nous permettant d'observer une production intermédiaire de CPS. La perte de la sialylation serait donc probablement le résultat de la perte de la CPS en surface de la bactérie. Une étude précédente menée avec un mutant *neuB* de *S. suis* obtenu par mutagenèse par transposon a démontré que ce dernier était non-virulent en modèle porcin [98]. De façon intéressante, nous avons observé que ce mutant est aussi non-typable avec un antisérum contre *S. suis* sérotype 2 et apparaît non-encapsulé en TEM.

Dans la littérature, *S. suis* est fréquemment comparé à GBS de type III puisqu'ils sont deux pathogènes responsables de méningite et qu'ils sont les deux seuls membres des *Streptococcus* à posséder une CPS riche en acide sialique. Cependant, les liaisons de l'acide sialique à la structure principale diffèrent entre ces deux pathogènes [105]. Une étude a démontré que la mutation chez GBS du gène *cpsK* codant pour une sialyltransférase résulte en une diminution de 80% de l'expression de la CPS [314]. Les auteurs ont suggéré que la sialyltransférase fait partie du complexe glycosyltransférase et que sa perte altère l'intégrité fonctionnelle de la synthèse de la CPS [314]. Les résultats ici obtenus par TEM ont permis de confirmer, pour la première fois, que du matériel capsulaire demeure tout de même présent chez le mutant Δ*neuB* de GBS (déficient pour la production de l'acide sialique) lorsqu'il est comparé au mutant Δ*cpsE* de GBS qui est complètement non-encapsulé. Il semble donc que l'absence de l'acide sialique affecte de différentes façons la production de

la CPS dépendamment du pathogène, étant critique chez *S. suis*, comme démontré dans cette étude, partiellement impliqué chez GBS [314] et non-indispensable chez *Haemophilus ducreyi* et *N. meningitidis* [315].

Chez GBS de type III, l'effet de l'acide sialique sur l'immunogénicité de la CPS est complexe. Par exemple, l'acide sialique exerce un contrôle conformationnel de l'épitope antigénique dominant chez le type III mais non chez le type V [316,317]. Il a été suggéré que l'acide sialique de la CPS de *S. suis* sérotype 2 ferait partie de la portion antigénique de la CPS [318]. Cependant, la présente étude démontre clairement que le retrait de l'acide sialique résulte en la perte de la réactivité non seulement envers un anticorps monoclonal mais aussi envers une lectine spécifique [113]. Cependant, la perte de l'acide sialique réduit, mais n'empêche pas la reconnaissance par un anticorps polyclonal utilisé de routine pour le sérotypage ou par un anticorps adsorbé monospécifique contre la CPS. Ces résultats indiquent que l'acide sialique de *S. suis* sérotype 2 n'est pas le seul déterminant du sérotype.

Finalement, le mutant Δ*neuC* se comporte de la même façon qu'un mutant non-encapsulé puisqu'il est fortement phagocyté et non virulent en modèle d'infection murin [173,177]. Nous n'avons donc pas été en mesure d'évaluer le rôle exact de l'acide sialique dans la virulence de *S. suis*. L'étude des effets d'une mutation dans la voie de synthèse de l'acide sialique de *S. suis* doit donc se faire avec attention puisque les effets d'une telle mutation se confondent avec une diminution dans la production de la CPS.

3. Étude des récepteurs impliqués dans la reconnaissance de *S. suis* par les DCs

3.1 Rôle des TLRs dans la maturation et l'activation des DCs

Les voies de signalisation MyD88/TLR ont été démontré comme essentielles pour la défense de l'hôte contre plusieurs pathogènes comme *S. aureus*, *S. pneumoniae* et GBS [319-321]. De façon similaire à ce qui avait été rapporté pour *S. pyogenes* [293], nous avons observé que l'expression des molécules CD40, CD86 et CMH-II induite par *S. suis* est dépendante de la protéine adaptatrice MyD88. La production de différentes cytokines et

chimiokines par les DCs MyD88$^{-/-}$ exposées à *S. suis* a aussi été démontrée comme étant soit réduite ou complètement abolie, confirmant ainsi un rôle important pour les TLRs dans l'activation des DCs par *S. suis*. Nous avons aussi observé une inhibition de l'expression de NF-κB chez les DCs MyD88$^{-/-}$, appuyant davantage l'importance de la signalisation MyD88-dépendante pour la maturation et l'activation des DCs par *S. suis*. Les résultats obtenus concordent avec une étude précédente qui avait démontré que MyD88 est la protéine adaptatrice principale impliquée dans la production de cytokines par les macrophages stimulés avec *S. suis* [56]. Ainsi, l'implication de MyD88 suggère qu'un ou plusieurs TLRs sont impliqués dans l'activation et la maturation des DCs par *S. suis*. Cependant, des voies MyD88-indépendantes pourraient aussi être impliquées, mais de façon moins importante, dans la production de certains médiateurs tel que IP-10/CXCL10 ainsi que dans l'expression de CD86, pour lesquels les niveaux d'induction n'étaient que partiellement réduits chez les DCs MyD88$^{-/-}$ infectées par *S. suis*.

Chez *L. monocytogenes*, une déficience au niveau de la protéine adaptatrice MyD88 n'altère pas l'augmentation des molécules de co-stimulation sur les DCs *in vivo* [322]. Comme la signalisation MyD88-dépendante est utilisée par tous les TLRs à l'exception du TLR3 [208], un rôle partiel de ce récepteur pourrait être suggéré. La transcription d'ARNm du TLR3 a déjà été décrite au niveau de cerveau de souris infectées par *S. suis* [177]. En plus, il a été rapporté que la production d'IP-10/CXCL10 en réponse au LPS pourrait se faire via TLR4 mais de façon MyD88-indépendant, donc via la voie de signalisation TRIF/TRAM [323]. La voie MyD88-indépendante (TRIF/TRAM) est aussi activée par TLR3 et est fonctionnellement responsable de l'activation des gènes induits par les IFNs de type I et autres IFNs, tel que IP-10/CXCL10 [324]. Puisque TLR4 ne semble pas requis pour l'expression de CD86 ou pour la production d'IP-10/CXCL10 par les DCs en réponse à *S. suis*, un rôle de la voie de signalisation TLR3/TRIF pourrait être une hypothèse.

3.2 Rôle des NODs dans la maturation et l'activation des DCs

Une autre découverte intéressante est l'implication du récepteur cytosolique NOD2 dans la production de KC/CXCL1 et IL-23 par les DCs stimulées par *S. suis*. IL-23 est membre de la famille IL-12, et est particulièrement important pour la production d'IFN-γ et la

prolifération des cellules T mémoires [325]. KC/CXCL1 est un des homologues d'IL-8/CXCL8 et semble important pour la circulation et l'activation des neutrophiles chez la souris [326]. L'implication du récepteur NOD2 dans la réponse cellulaire à des pathogènes tel que *S. pneumoniae*, *S. aureus* et *L. monocytogenes*, a été décrite [194,211,327-329]. Comme le *crosstalk* et/ou la synergie entre les TLRs et les NODs ont déjà été proposés [330], nous avons étudié une possible interaction entre TLR2 et NOD2 lors de l'activation des DCs par *S. suis*. Les résultats obtenus dans la présente étude suggèrent qu'une activation complexe et non-redondante de chaque récepteur soit impliquée dans la production de KC/CXCL1 et IL-23. L'activation d'un récepteur cytosolique est surprenant dans le cas d'un pathogène extracellulaire et encapsulé comme *S. suis*. En fait, bien que *S. suis* soit particulièrement résistant à la phagocytose par les DCs, comme discuté ci-haut et aussi récemment démontré dans la littérature [331], un nombre quand même faible de bactéries peut tout de même être retrouvé à l'intérieur des cellules. Ceci pourrait donc expliquer une telle activation de NOD2 par *S. suis*, bien que les mécanismes exacts d'activation de ce type de récepteurs par ce pathogène n'ait pas fait objet de la présente étude. Néanmoins, il a été proposé que le *crosstalk* entre les récepteurs cytosoliques NODs et les récepteurs membranaires TLRs pourrait amplifier la réponse lorsque plusieurs antigènes sont présentés simultanément par un pathogène [192,214]. De plus, l'activation du TLR2 a été rapportée, chez certaines espèces bactériennes, comme assurant la digestion de la paroi cellulaire bactérienne et activant le relâchement de PG, ce qui pourrait contribuer à l'activation de NOD2 [332].

3.3 Rôle des facteurs de virulence dans la reconnaissance par les DCs

Comme discuté précédemment, la CPS de *S. suis* est reconnue pour dissimuler les antigènes de la paroi cellulaire et ainsi réduire l'activation des DCs. Par contre, il a aussi été mentionné que certaines cytokines, tel que IL-1β, semble requérir la présence de la CPS pour leur production optimale. En absence de CPS, les composants de la paroi cellulaire sont exposés et semblent activer les DCs via plusieurs TLRs. Malgré tout, selon les résultats obtenus, des modifications de la paroi cellulaire ne semblent pas significativement altérer la reconnaissance de *S. suis* par les DCs. Chez certains pathogènes bactériens, la N-déacétylation du PG semble être un mécanisme utilisé par les bactéries afin éviter la

réponse immunitaire de l'hôte [145]. Par exemple, chez *L. monocytogenes*, la N-déacétylation du PG permet aux bactéries d'éviter la reconnaissance par les NLRs et particulièrement par les NODs [146]. Ceci peut probablement s'expliquer par le fait que *L. monocytogenes* est un pathogène intracellulaire. Par contre, dans le cas de *S. suis*, les modifications de la paroi cellulaire n'ont eu aucune influence sur la reconnaissance via NOD2, ceci étant probablement lié au fait que peu de bactéries sont retrouvées à l'intérieur des cellules et qu'ainsi seulement une très faible quantité de PG est disponible pour interagir avec les NODs.

3.4 Modèle suggéré pour la reconnaissance de *S. suis* par les DCs

Les résultats obtenus par la présente étude confirment l'hypothèse que la reconnaissance de *S. suis* par les DCs requiert un système complexe. Un modèle de la reconnaissance de *S. suis* par les DCs a été proposé (Figure 9). La signalisation par MyD88, principalement via le TLR2, est cruciale pour l'activation et la maturation des DCs lors de l'infection par *S. suis*. TLR9 (en collaboration avec TLR2) et NOD2 sont aussi impliqués dans l'activation cellulaire. Cependant, d'autres récepteurs, possiblement d'autres TLRs (comme le TLR3), pourraient être impliqués dans l'activation et la maturation des DCs par *S. suis*.

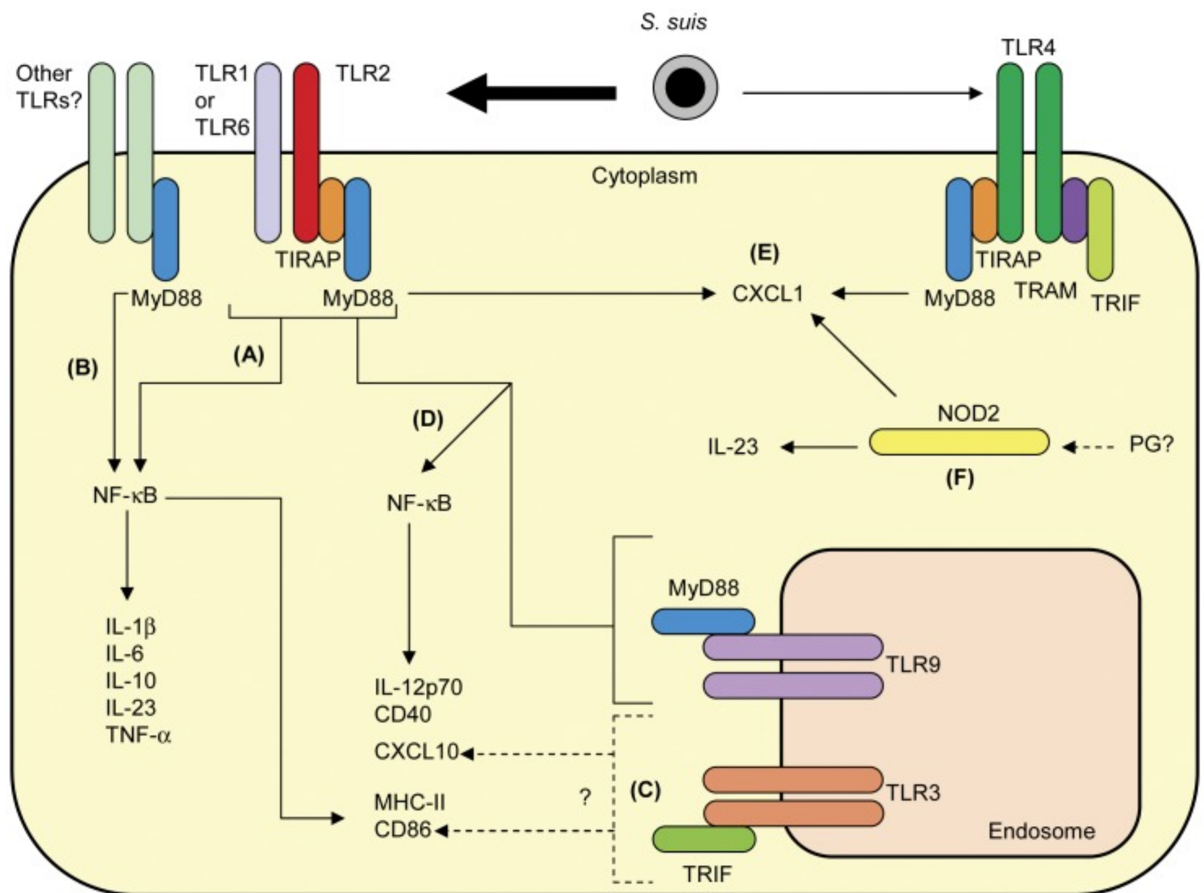

Figure 9. Modèle proposé pour la reconnaissance de *S. suis* par les DCs. (A) La production d'IL-1β, IL-6, IL-10, IL-23 et TNF-α est TLR2-dépendante. TLR2 est aussi impliqué dans l'expression en surface de CMH-II et CD86. (B) D'autres TLRs pourraient aussi être impliqué dans la production d'IL-1β, IL-6, IL-10, IL-23 et TNF-α. (C) TLR3 pourrait être impliqué dans la production MyD88-indépendante d'IP-10/CXCL10 et dans l'expression de CD86. (D) La collaboration entre TLR2 et TLR9 est impliquée dans la production d'IL-12p70 et d'IP-10/CXCL10 ainsi que dans l'expression de CD40. (E) La collaboration entre TLR2 et NOD2, avec une contribution mineure de TLR4, est impliquée dans la production de KC/CXCL1. (F) NOD2 contribue aussi à la production d'IL-23. La reconnaissance du peptidoglycane (PG) de *S. suis* pourrait aussi être impliqué dans l'activation de NOD2.

4. Étude de la réponse T-dépendante induite par *S. suis*

En général, les cellules T sont essentielles pour le développement de la réponse adaptative de l'hôte lors de l'infection. Au sein du laboratoire, une expérience préliminaire chez des souris TCRαβ KO avait déjà démontré que les souris dépourvues de cellules T CD4 et CD8 fonctionnelles meurent plus rapidement que les souris contrôle, suggérant ainsi un rôle important pour les cellules T lors de l'infection par *S. suis* (observations non publiées). Ces

résultats ont suscité l'intérêt d'évaluer plus précisément le rôle des cellules T $CD4^+$ lors de l'infection par *S. suis*.

Un modèle d'infection expérimentale par *S. suis* à préalablement été décrit [177]. Dans la phase systémique d'infection, des niveaux élevés d'IL-6, IL-10, TNF-α et IFN-γ ont été observés [177,178]. Dans la présente étude, les cellules spléniques totales ont été démontré comme produisant TNF-α, IFN-γ, IL-6, IL-10, MIP-1α/CCL3 et MIG/CXCL9, suggérant une activation des cellules spléniques lors de l'infection et une polarisation vers une réponse T_H1. La production d'IL-10 est généralement reliée à la régulation immune, alors que la présence de MIP-1α/CCL3 et MIG/CXCL9 suggère que les cellules T seraient recrutées au niveau de la rate via la sécrétion de ces chimiokines comme il a été décrit chez d'autres streptocoques [333]. Il est intéressant de noter qu'IL-6 et TNF-α ont souvent été rapporté comme d'importants médiateurs de septicémie lors de l'infection par *S. suis* [177,178]. Ceci permet aussi d'illustrer l'importance particulière d'IL-10 dans le maintien de l'homéostasie comme rapporté aussi précédemment lors de l'infection par *S. suis* [178]. Des expériences similaires ont aussi été réalisées en parallèle avec GBS type III, un pathogène souvent comparé à *S. suis* sérotype 2. La quantité de ces cytokines et chimiokines produites par les cellules spléniques était inférieure à celle produites lors de l'infection par GBS dans les mêmes conditions d'expérimentation (ANNEXE VI).

De façon générale, on s'attend à ce que les cellules T $CD4^+$ soient les contributeurs majeurs à la production de cytokine et au développement de la réponse immune adaptative qui s'ensuit [334]. Plusieurs analyses *ex vivo* et *in vivo* des splénocytes totaux, des cellules T $CD3^+$ ou des cellules T $CD3^+CD4^+$ ont suggéré le développement d'une réponse T_H1 suivant l'infection par *S. suis*. Cependant, la quantité de cellules T $CD4^+$ activées ainsi que les niveaux d'IFN-γ, TNF-α et IL-2 observés sont faibles. La production d'IL-10 par les cellules T $CD4^+$ activées a aussi été observée. En plus des cellules Tregs, la production d'IL-10 a aussi été rapportée pour les cellules T_H1 et T_H2 [335]. La production d'IL-4 par les cellules T $CD4^+$ n'a pas été détectée par notre système. L'évolution rapide de l'infection par *S. suis* pourrait suggérer que les cellules $CD4^+IL\text{-}10^+$ seraient générées au cours du processus inflammatoire de type 1 plutôt que par l'expansion ou la génération d'une

population de Tregs. En effet, ici, la production d'IL-10 par ces cellules servirait probablement plutôt de régulateur afin de contrôler une réponse cytokinaire exacerbée.

Le développement de la mémoire immune a aussi été évalué. Deux semaines après l'infection primaire, les animaux survivants ont été challengés par une seconde infection. Les résultats ont démontré que les cellules T $CD4^+$ répondent à l'infection en produisant le même patron de cytokines. Cependant, cette réponse apparaît beaucoup plus rapidement puisque les cellules T $CD4^+$ étaient isolées et marquées 48 heures après le challenge comparativement à 96 heures suivant l'infection primaire. Les niveaux de cytokines produites étaient cependant similaires à ceux de la réponse primaire, suggérant un développement limité de la réponse mémoire.

Comme mentionné précédemment, la CPS de *S. suis* est son principal facteur de virulence. Nous avons donc voulu déterminer l'influence de la présence de la CPS sur l'activation des cellules T $CD4^+$ à l'aide d'un modèle *in vitro*. En effet, un modèle d'infection *in vivo* ne peut être utilisé en présence d'une souche mutante non-encapsulée car elle est éliminée très rapidement de la circulation [108,336]. Nous avons observé que la CPS interfère avec la production d'IFN-γ, TNF-α et IL-10 par les cellules T $CD4^+$. Cependant, la présence de la CPS n'affecte pas la production d'IL-2. Ainsi, ces résultats suggèrent que la CPS interfère avec l'activation des cellules T $CD4^+$ mais non avec la prolifération des cellules T. CD69 est un marqueur de maturation utilisé de routine dans l'évaluation de l'activation des cellules T [337]. Il a cependant été démontré que des lymphocytes déficients pour l'expression de CD69 ont une réponse proliférative normale [337]. Dans le cas de *S. suis*, seulement une faible expression de CD69 a été observée chez les cellules T $CD4^+$ activées *in vitro*. Ceci est en contraste avec *S. pneumoniae* qui induit une forte activation de CD69 sur les cellules T lors de l'infection [338]. *S. suis* n'induit pas non plus l'expression en surface de CD40L, une molécule de co-stimulation importante pour l'activation des cellules T. La CPS ne semble pas interférer avec l'expression de CD69 ou CD40L. Ces résultats suggèrent donc que *S. suis* utilisent plusieurs facteurs de virulence pour réduire la production de cytokines ou l'expression de molécules de co-stimulation par les cellules T $CD4^+$.

Lors de l'infection, nous avons aussi observé que la production d'anticorps spécifiques dirigés contre *S. suis* est faible. Nous avons donc évalué l'habileté de *S. suis* à interférer avec la production d'anticorps contre un antigène rapporteur. De l'ovalbumine (OVA) a été co-injectée avec du CpG ODN chez des souris préalablement infectées par *S. suis*. La production d'isotypes d'anticorps T_H1- et T_H2-dépendants était significativement réduite lors de l'infection primaire. Des résultats semblables ont été obtenus lors de la réponse mémoire. De façon intéressante, nous avons aussi observé une corrélation négative entre la présence de signes cliniques et la production d'anticorps contre OVA. Suite à l'infection par *S. suis*, les animaux développent une bactériémie accompagnée de signes cliniques modérés ou sévères tel que le pelage hérissé, les yeux enflés, de la dépression, de la prostration et de la faiblesse. Au cours de l'infection primaire, les animaux infectés et présentant des signes cliniques sévères produisaient aussi significativement moins d'anticorps OVA-spécifiques que les animaux présentant des signes cliniques faibles. Ces résultats suggèrent que *S. suis* possède des habiletés immunosuppressives. Les mécanismes exacts pour la suppression de la réponse immune lors de l'infection par *S. suis* demeurent toutefois à investiguer. Cependant, de telles observations ont aussi précédemment été décrites chez *S. pneumoniae* [339]. En effet, il a été démontré que *S. pneumoniae* inhibe les réponses IgG dirigées contre plusieurs antigènes solubles co-immunisés. Plus précisément, *S. pneumoniae* induit une réduction significative dans la formation des centres germinatifs spléniques Ag-spécifiques, des cellules T_H folliculaires, des cellules B de centres germinatifs et aussi des cellules B sécrétrices des anticorps dans la rate et la moelle osseuse en réponse à l'OVA [339]. Dans le cas de *S. suis*, une diminution transitoire des cellules T $CD4^+$ dans la rate a aussi été observée lors de l'infection primaire. Cependant, ceci pourrait aussi être relié à de la mort cellulaire ou à l'émigration des cellules vers d'autres organes ou tissus cibles.

Pour conclure, nous avons observé que les splénocytes totaux produisent IL-6, TNF-α, IFN-γ, IL-10, MIP-1α/CCL3 et MIG/CXCL9. Différentes cellules comme les macrophages, les cellules NK et les DCs pourraient être impliquées dans la production de ces cytokines. La présence de MIP-1α/CCL3 et de MIG/CXCL9 pourrait être impliquée

dans le recrutement des cellules T $CD4^+$. En effet, nous avons démontré une production de TNF-α, IFN-γ et IL-2 par les cellules T $CD4^+$, suggérant une réponse T_H1. Cependant, la CPS de *S. suis* interfère avec l'activation des cellules T $CD4^+$. Elle n'a toutefois aucun impact sur leur prolifération. Enfin, *S. suis* interfère aussi avec le développement d'anticorps spécifiques lors de l'infection. Les animaux freinés dans leur production d'anticorps sont aussi ceux montrant les signes cliniques les plus sévères. Les résultats obtenus ont permis d'élaborer un modèle hypothétique pour l'activation des DCs en réponse à *S. suis* (Figure 10). Ces résultats faciliteront la recherche future sur l'immunité dépendante des cellules T lors de l'infection par *S. suis* et les conséquences sur le développement d'un vaccin.

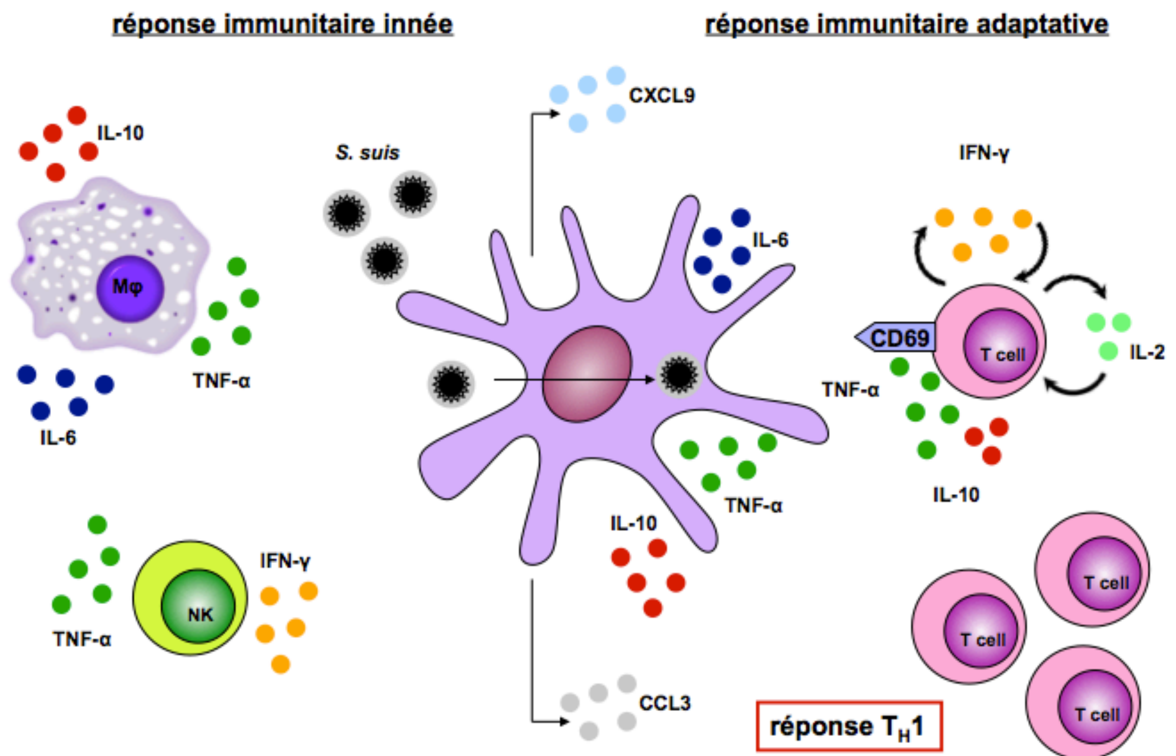

Figure 10. Modèle proposé pour l'activation des cellules immunes lors de l'infection par *S. suis*. La production d'IL-6, TNF-α, IFN-γ et IL-10 a été observée par les splénocytes totaux. Différentes cellules tel que les macrophages (Mϕ), les cellules NK et les DCs peuvent être impliquées dans la production de ces cytokines. Les DCs produisent aussi MIG/CXCL9 et MIP-1α/CCL3, deux chimiokines impliquées dans le recrutement des cellules T. Les cellules T CD4+ produisent à leur tour IFN-γ, TNF-α, IL-2 et IL-10, suggérant le développement d'une réponse T_H1.

V. CONCLUSIONS ET PERSPECTIVES

Conclusions générales

Les travaux réalisés au cours de cette étude représentent un premier pas dans la compréhension des interactions entre *S. suis* et le système immunitaire de l'hôte lors de l'infection. Les résultats obtenus ont permis de démontrer que *S. suis* est résistant à l'internalisation par les DCs. En conditions non-opsonisantes, la CPS est suffisante pour protéger *S. suis* de la phagocytose médiée par les DCs. Comme nous avons démontré que les récepteurs TLRs ne sont pas impliqués dans cette faible internalisation de la bactérie par les DCs, le rôle des autres récepteurs reste à étudier. Par contre, une fois internalisé, la CPS ne permet pas la survie intracellulaire de *S. suis*. En présence de sérum complet, comme lors des conditions normales d'infection de l'hôte par *S. suis*, la CPS, la D-alanylation du LTA, la N-déacétylation du PG et la suilysine interfèrent tous avec la déposition du complément à la surface des bactéries. Cette modulation de l'activation du complément serait importante non seulement pour la survie de la bactérie, mais elle aurait aussi des conséquences dans le développement des réponses immunitaires innées et adaptatives car l'opsonisation de *S. suis* par le sérum complet module le ratio IL-12p70/IL-10, deux cytokines avec des fonctions immuno-régulatrices importantes.

L'interaction de *S. suis* avec les DCs est médiée principalement via la voie de signalisation MyD88-dépendante et le récepteur TLR2. Dans une moindre mesure, TLR9 et d'autres TLRs seraient aussi impliqués dans l'activation et/ou dans la maturation des DCs suite à une infection par *S. suis*. D'autres voies de signalisation, telle que la voie NOD2-dépendante, sont aussi impliquées, suggérant une grande complexité dans la reconnaissance de *S. suis* par les DCs.

Malgré que ces interactions mènent à l'activation des DCs, une fonction optimale de ces cellules serait compromise par la CPS, les modifications de la paroi cellulaire et/ou la production de la suilysine par *S. suis*. Les résultats obtenus lors des études avec les cellules T permettent de suggérer que cette réduction dans la fonctionnalité de DCs entraine une activation sous-optimale des cellules T $CD4^+$, une réduction dans la génération de la mémoire immunologique et un taux réduit d'anticorps spécifiques. Ainsi, globalement, les études réalisées dans le cadre de cette thèse de doctorat suggèrent que *S. suis* possède des

habiletés immunosuppressives et entraine une perte de la capacité de l'hôte à monter une réponse adaptative efficace et à long terme contre ce pathogène.

Perspectives

Des avenues potentielles à la suite de cette thèse seraient :

- l'identification des mécanismes interférant avec la déposition du complément en surface de *S. suis*, plus particulièrement au niveau des modifications de la paroi cellulaire.

- l'identification des autres récepteurs, incluant le TLR3 et les NLRs, pouvant être impliqués dans la maturation et/ou l'activation des DCs par *S. suis*.

- la confirmation des résultats obtenus chez l'hôte naturel.

- l'identification des mécanismes impliqués dans la suppression de la réponse immune lors de l'infection par *S. suis*.

- Des études approfondies sur les stratégies utilisés par *S. suis* qui pourraient entrainer une réduction du développement des cellules T et B mémoires, et donc de la réponse immunitaire adaptative à long terme.

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VII. ANNEXES

Annexe I

Streptococcus suis infections in humans: what is the prognosis for Western countries?

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ABSTRACT

Infections caused by *Streptococcus suis* are an important economic problem in the swine industry. Moreover, *S. suis* (especially serotype 2) is an agent of zoonosis that has the potential to afflict those who are in close contact with infected pigs or pork-derived products. Although sporadic cases of *S. suis* infections in humans have been reported during the last 40 years, a large outbreak emerged in 2005 in China. The severity of the infection in humans during the outbreak, such as clinical signs of streptococcal toxic shock syndrome, attracted much attention from the scientific community and the public press. Many studies have lately shown the importance of the inflammatory properties of *S. suis* in disease outcome. The high prevalence of human infections in Asian countries may be related to a combination of several factors: a) life style; b) prevalence of swine disease; c) levels of virulence of *S. suis* strains; and d) recognition and knowledge of the microorganism by diagnostic laboratories for human disease. The higher prevalence of human disease in Europe than in North America may also be explained by recognition and better knowledge of the pathogen by diagnostic laboratories and possible higher virulence of serotype 2 infecting strains with a consequent significantly higher prevalence of disease caused by this serotype in pigs. In fact, in North America with its highly developed swine production, a relatively low number of human cases are reported each year.

INTRODUCTION

In the swine industry, *Streptococcus suis* infections have been considered a major problem worldwide, especially during the past 20 years. The natural habitat of *S. suis* is the upper respiratory tract of pigs, particularly the tonsils and nasal cavities; and the genital and alimentary tracts (1). The most important clinical feature associated with *S. suis* infection in pigs is meningitis; however, other pathologies have also been described, such as arthritis, endocarditis, pneumonia, and septicemia with sudden death (1). Although *S. suis* is primarily considered a major swine pathogen, it has been increasingly isolated from a wide range of mammalian species and from birds. These findings suggest the existence of complex epidemiological patterns of the infection, since other animal species might also be a source of infection of swine (2-4).

S. suis infections in humans have been usually considered as sporadic infections in people working with pigs or pork-derived products (5). However, the important outbreak in China that occurred in 2005 and that affected more than 200 people with a mortality rate of nearly 20% changed the perspective of the threat of *S. suis* to human health. Although recently reviewed as an "old neglected zoonotic infection" (6), the scientific community still considered *S. suis* as one of the most important emerging infectious diseases in Asian countries, where the majority have regular contact with raw pork meat. Here, we discuss the main characteristics of this infection and its differences in both Asian and Western countries. A complete review of the general aspects of *S. suis* infection in pigs was published by Higgins and Gottschalk in 2006 (1).

S. suis: summary of general characteristics of the microorganism

S. suis is an encapsulated Gram-positive coccus that possesses cell wall antigenic determinants related to Lancefield group D. To date, 35 serotypes of *S. suis* have been described. The composition of the capsule defines the serotype (1). Especially in studies describing human infections, there is confusion about early terminology of Lancefield groups R, S and T, and the relationship of these groups with group D streptococci and the different *S. suis* serotypes that are also referred to as capsular types or serovars (7-9). Various alpha-hemolytic streptococci were ascribed to Lancefield groups R, S, RS and T in

1963 by de Moor (10). Years later, British researchers discovered that de Moor had erroneously worked with antigens extracted from the capsular material rather than from the cell wall, and demonstrated that the lipoteichoic acid present in the cell wall of all these strains reacts with group D antiserum (11). The conventional method for extracting streptococcal group antigens from group D streptococci yields insufficient free teichoic acid to precipitate with potent group D antisera when applied to *S. suis*. The Lancefield groups R and S were later identified as *Streptococcus suis* (Lancefield group D), and reclassified as serotypes 1 (formerly group S) and 2 (formerly group R) (12). Some years later, groups RS and T were also reclassified as serotypes 1/2 and 15, respectively (13, 14). In conclusion, the Lancefield groups R, S, RS and T do not exist and this terminology should be avoided. Although the *S. suis* cell wall antigen shares epitopes with Lancefield group D, studies using *S. suis* reference strains showed that most are very closely interrelated but clearly separated from other Lancefield group D streptococci and enterococci (15, 16).

Most *S. suis* organisms isolated from diseased pigs belong to a limited number of serotypes, often between 1 and 9 (17-20). Although serotype 2 isolates predominate in most countries, the distribution may differ depending on the geographical location. Differing from what has been observed in Europe and Asia, the prevalence of serotype 2 strains recovered from diseased animals in North America remains relatively low (about 20%) (21-23). We hypothesize that Eurasian and North American *S. suis* serotype 2 strains possess different potential for virulence. In addition to serotype 2, many human infections caused by serotype 14, and sporadic infections due to serotypes 4, 14 and 16 have also been described (6).

S. suis infection in humans: clinical characteristics

In humans, *S. suis* usually produces a purulent meningitis (6). Endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis and endopthalmitis have also been reported (24-26). In general, most patients with systemic *S. suis* infection exhibit leukocytosis and neutrophilia and those patients with meningitis have subarachnoid cerebrospinal fluid with greatly increased leukocytes, a high percentage of neutrophils, low sugar and high protein levels (27).

Complete descriptions of the most important clinical characteristics have been recently described (24, 26). The incubation period ranges from a few hours to two days (28). *S. suis* infection in humans generally occurs sporadically without obvious seasonal change. However, a certain tendency for *S. suis* infections to occur during the rainy and hottest months of the year has been suggested (25, 29, 30). These conditions might favor the persistence of biological aerosols in the environment. Mortality rates vary from less than 3% (most Western countries) to 26% (some Asian countries) (6, 24, 26). Deafness and/or vestibular dysfunction are two of the most striking features of *S. suis* meningitis in humans (25, 29, 31). Indeed, the recorded incidence of deafness following infection caused by this pathogen is consistently higher than that reported for other meningitis-causing bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. This incidence can reach 50% and 73% in Europe and Asia, respectively (29, 32). The progression for hearing varies: some patients improve overtime, and others do not (26).

The typical clinical characteristics of acute meningitis in humans caused by *S. suis* changed after the 2005 outbreak in the Chinese province of Sichuan (33). The official report of the National Institute for Communicable Disease Control and Prevention in China reported 215 cases, with 39 leading to death (34). The most important feature of this outbreak was the high incidence of systemic disease with a relatively low number of cases of meningitis. The fatality rate reached 63% among patients with septicemia and septic shock (26). The clinical presentation was characterized as streptococcal toxic shock syndrome (STSS) (34, 35). STSS is a relatively well-defined syndrome usually associated with Group A streptococci and less frequently, with other streptococci (36). It was suggested that the cases in China were STSS based on these symptoms: sudden onset of high fever, diarrhea, hypotension, erythematous blanching rash on the distal part of the extremities, including blood spots and petechia; plus dysfunction of multiple organs, such as acute respiratory distress syndrome, liver and heart failure, disseminated intravascular coagulation, and acute renal failure (34, 35). More pathological characteristics observed in diseased patients have recently been described (37). Since none of the usual superantigens that are typically responsible for the toxic shock syndrome are produced by the strain responsible for the outbreak, it would be more appropriate to classify the Chinese episode as a “streptococcal

toxic shock-like syndrome". However, most Chinese researchers still described the outbreak as STSS (38). Although the 2005 disease outbreak in China is the largest recorded outbreak of *S. suis* infection in humans, another smaller Chinese human outbreak with the same clinical characteristics took place in Jiangsu Province in 1998 that affected 25 people with 14 reported deaths (34, 39).

The 1998 and 2005 Chinese outbreaks are the only reported large human outbreak with many patients presenting acute symptoms related to STSS. However, there is one previous report of STSS caused by *S. suis* (27) and there are many other reports indicating severe cases of *S. suis* septic shock. These are very difficult to differentiate from STSS cases. These outbreak reports also include multiple organ failure, disseminated intravascular coagulation and associated purpura fulminans that lead to death within hours (5, 28, 40-48). Interestingly, of the cases described in Thailand, 10 of 41 presented septic shock in the absence of primary organ infection with a clinical presentation similar to that described in the Chinese outbreaks (49). A serious case of septic shock caused by a non-serotype 2 strain (serotype 14) has also been described (50).

Virulence factors and pathogenesis of the infection

Almost all studies on *S. suis* virulence factors, pathogenesis of the infection and mechanisms of protection have been conducted with serotype 2 strains. Baums *et al*. recently published a complete and comprehensive review on *S. suis* virulence factors (51). The single most critical and validated virulence factor is the capsular polysaccharide (CPS) that protects *S. suis* from immune clearence (52, 53). However, most non-virulent strains are also encapsulated, indicating that other virulence factors are equally essential (54). The bacterial cell wall components have been reported to be surface-exposed (even in the presence of the CPS) and to play an important role in inflammation (54, 55). The N-deacetylation of the peptidoglycan and the D-alanylation of the lipoteichoic acid would also be important for bacterial survival in blood (51). Pili formation has been lately detected in many *Streptococus* species. At least four putative pilus-encoding islands have been identified in *S. suis* serotype 2 strains (56). One of them, named the *srt*F cluster, is a truncated homologue of Group B *Streptococcus* pilus island 2b composed of a signal peptidase-like and a class C sortase-encoding genes, and two genes encoding an ancillary

and the major pilin subunits (56, 57). It has been recently demonstrated that *S. suis* express pili from this cluster, although pili were formed by the major pilin subunit only, due to nonsense mutations at the 5' end of the gene coding for the ancillary subunit, a putative adhesin (58). A recent report indicated, however, that this ancillary subunit might be expressed and could be used as a protective immunogen (59). Studies to explain these discrepencies are underway. Other proteins with a LPXTG-motif have been suggested as putative virulence factors such as the muramidase-released protein (MRP), surface protein one, and a serum opacity-like factor homologous to that of Group A streptococci (54, 55). The mechanisms by which these proteins influence the virulence of a specific strain remain unknown. However, abrogation of the anchoring of LPXTG proteins to the cell wall by deletion of the gene encoding the housekeeping sortase A decreased the virulence of the mutant strain (60-62). In addition, it has been suggested that different enzymes play certain roles in virulence, such as those of the arginine deiminase system, a DNAse, a hyaluronidase, and a glyceraldehyde-3-phophate dehydrogenase (51). We recently identified a *S. suis* gene that codes for a cell surface subtilisin-like protease. It appears to contribute to pathogenicity given that proteinase-deficient mutants are more susceptible to being killed in the bloodstream and are less virulent in mice (63). Two main surface-exposed proteins have been described as fibronectin-binding proteins: the fibronectin- and fibrinogen-binding protein and the enolase (51). Among secreted factors that may play a role in the pathogenesis of the infection, the most important is the suilysin (54, 55). Suilysin has been identified as both a toxic factor for various cell types, and also an interference to complement-mediated phagocytosis and killing (54, 64-69). An extracellular protein factor (EF) has also been associated with virulence, without being considered itself as a virulent factor (70). It is important to note that most of these potential virulence factors are either not essential for virulence, or are found in both virulent and non-virulent strains, or could not be properly studied due to the unavailability of knockout mutants. Knockout mutants of some virulence candidates have been obtained, but in some cases, their virulence has not been tested (55).

Reports give conflicting information about the differentiation between virulence factors and virulence markers. Despite the lack of evidence that some of these putative virulence factors play critical roles in virulence, they nonetheless may serve as virulence markers

and/or for phenotypic comparison of strains. This seems to be the case for the MRP and the EF proteins (71, 72), and suilysin (73, 74). Although it has been shown that they are not critical virulence factors (70, 75, 76), there is still a positive association between the presence of these proteins and virulence of the strains in some European and Asian countries (23, 71, 72, 77). In addition, non-virulent strains possessing MRP, EF and suilysin remain undescribed. The absence of one or both of these proteins is not necessarily associated with a lack of virulence. Indeed, some European, Asian, and most North American virulent isolates do not produce these factors (22, 78-83).

The pathogenesis of *S. suis* infection has been studied mainly using observations of swine and mouse models of infection. The mechanisms that enable *S. suis* to disseminate throughout the animal and the pathogenesis of the infection in humans remain poorly understood. In pigs, evidence shows that the bacterium is able to spread systemically from the nasopharynx, occasionally resulting in septicemia and death (54, 84). The routes of infection in humans seem to be different (see below). It is unknown how *S. suis*, despite its low quantities on swine mucosal surfaces, is able to traverse this first line of host defence to disseminate in the host and initiate disease. Very few studies with contradictory results have investigated the interactions between *S. suis* and epithelial cells (67, 85-87). Survival of the organism once in the bloodstream is facilitated by the CPS, which efficiently hampers phagocytosis. The sialic acid component that is located in a terminal position, as recently reported (88), may be responsible, at least in part, for the anti-phagocytic properties of the CPS. Studies conducted by different laboratories over the past decade clearly indicate that bacteria travel either free in circulation or attached to the surface of monocytes (64, 68). Furthermore, suilysin seems to protect bacteria against complement-mediated uptake and killing by neutrophils, macrophages and dendritic cells (64, 65, 89). *S. suis* can thus be considered a truly extracellular systemic pathogen.

In the event that *S. suis* fails to cause acute fatal septicemia, bacteria are able to reach the central nervous system (CNS) via mechanisms that are only partially elucidated. Brain microvascular endothelial cells (BMEC) and the choroid plexus epithelial cells (CPEC) constitute part of the structural basis of the blood-brain barrier (BBB). It has been shown that *S. suis* affects CPEC barrier function and integrity by cell apoptosis and necrosis. *S.*

suis causes massive rearrangement of the tight junction proteins ZO-1, occludin and claudin-1; loss of actin at the apical cell pole; and induces basolateral stress fiber formation. Studies on BMEC from human or swine origin have shown that adhesion (human cells) or adhesion and invasion (swine cells), with or without toxicity, depending on the strain origin (Eurasian or North American, respectively) might contribute to bacterial crossing of the BBB (66, 90, 91). These and probably other mechanisms facilitate *S. suis* invasion of the CNS (92-95).

Although virulence studies have been performed with strains from swine origin, *S. suis* serotype 2 major virulence factors/markers are present in both human and swine strains, and strain origin-effect was reported to be minor during *in vitro* studies of bacterial-cell interactions (15, 69, 96-98). Interactions between *S. suis* and human leukocytes are similar to those reported with swine or mouse phagocytes (68, 69, 96, 99, 100). Although the route of bacterial entry might differ between human (mainly through skin abrasions and oral mucosa) and pigs (mainly respiratory route), these observations suggest that the pathogenesis of the infection occurs through similar mechanisms.

Is inflammation a major feature of *S. suis* pathology?

The severity of *S. suis* infections in humans has increased. This has been observed in cases of septic shock and STSS: shorter incubation time, more rapid disease progression and a higher rate of mortality. These are indicative of a massive inflammation process that underscores the critical need to better understand the interactions between *S. suis* and cells of the host immune system. Several inflammatory and infectious diseases are associated with the overproduction of pro-inflammatory cytokines and chemokines; the recruitment and activation of different leukocyte populations are hallmarks of acute inflammation. Hence, the ability of *S. suis* to induce cytokine production may have considerable biological relevance. It has been previously demonstrated that this pathogen is able to induce the production of pro-inflammatory cytokines by murine, human and swine cells (69, 96, 99). *S. suis* induces, in general, moderate to very high levels of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8 and monocyte chemotactic protein (MCP)-1. Bacterial cell wall components were described as being the major cytokine-inducing components. In addition, hemoglobin, which may be released *in vivo* by

the action of *S. suis* suilysin on red blood cells, may contribute to raising the levels of pro-inflammatory mediators by acting in synergy with *S. suis* cell wall (101). To study the role of inflammation on *S. suis* infections, we developed a standardized mouse model of early septic shock and late meningitis/encephalitis caused by this pathogen (102). High levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-12 and IFN-γ and the chemoattractants MCP-1, KC and RANTES observed within 24 h post-infection are thought to be responsible for the sudden early death of animals. This was further confirmed by other studies (103, 104). The exaggerated inflammatory reaction was also observed in humans: during the early phase of *S. suis* disease, serum levels of interleukin IL-1β, IL-6, IL-8, IL-12p70, IFN-γ, and TNF-α were more elevated in patients with STSS than in those with meningitis only (103). The anti-inflammatory cytokine IL-10 was up-regulated mainly at 24 h post-infection, following the onset of most pro-inflammatory cytokines. This may indicate a negative feedback mechanism to control the extent of the inflammatory response. Increased or reduced rate of septic shock was observed in *S. suis*-infected mice that were treated either with neutralizing antibodies against IL-10 or with rIL10, respectively (104).

Inflammation also plays an important role during the meningitis phase of the infection. Once the CNS has been invaded by *S. suis*, transcriptional activation of different pro-inflammatory cytokines and chemokines, as well as the cells involved in such activation, at different brain structures have been observed by *in situ* hybridization combined with immunocytochemistry (102). It has also been shown that *S. suis* can induce the release of arachidonic acid by BMEC. It has been suggested that this is a mechanism that facilitates the ability of bacteria to penetrate the CNS and to modulate local inflammation (105). It has been shown that bacterial CPS induces human macrophages to secrete prostaglandin E(2) and matrix metalloproteinase 9 that may be involved in disruption of the BBB (106). Using a co-infection model with both BMEC and human macrophage cells, the levels of inflammatory mediators were even higher (107). Finally, other studies have shown that *S. suis* is able to induce the release of pro-inflammatory cytokines and chemokines by human BMEC and to both up-regulate the expression of adhesion molecules on human monocytes and increase the shedding of soluble intercellular adhesion molecule-1 from BMEC (98, 108, 109). Increased expression and/or shedding of adhesion molecules in endothelial cells

and leukocytes may contribute significantly to the recruitment of leukocytes and to increase local inflammation.

The receptors involved in cell activation by *S. suis* were partially studied. It has been demonstrated that stimulation of human monocytes by whole encapsulated *S. suis* or its purified cell wall components influences the relative expression of Toll-like receptor (TLR) 2 and CD14 mRNA. A very recent study showed that TLR2 and 6 are activated by *S. suis* (110). TLR stimulation triggers the release of cytokines and chemokines, which is significantly reduced by antibody-mediated neutralization of TLR2 but not TLR4. Mouse macrophages deficient in TLR2 also show reduced pro-inflammatory cytokine release in response to whole encapsulated bacteria. Given that this response is completely abrogated in MyD88-deficient macrophages, other TLRs might be involved in MyD88-dependent cytokine production induced by encapsulated *S. suis*. Furthermore, we demonstrated that the presence of CPS modulates *S. suis* interactions with TLRs. In the absence of CPS, uncovered cell wall components induce cytokine and chemokine production via TLR2-dependent and-independent pathways. However, CPS contributes to MCP-1 production in a MyD88-independent manner (100). After invasion of *S. suis* of the CNS, transcriptional activation of TLR2, TLR3, and CD14 have been observed (102).

Since most data seem to indicate that inflammation is mainly responsible for the STSS and it contributes to the meningitis signs caused by *S. suis*, anti-inflammatory medication to treat affected patients should be helpful. Most reported cases in humans concern the use of dexamethasone. Based on individual cases, dexamethasone therapy was either associated or disassociated with protection against severe hearing loss, a consequence of meningitis that is also associated with inflammation (6). So far, there are only two scientifically controlled trials that have studied the effect of dexamethasone on *S. suis* infections. In one, it was shown that dexamethasone treatment during the first 4 days of hospitalization significantly reduced the rate of severe hearing loss due to *S. suis* (111). In the second one, it was reported that dexamethasone does not improve the overall outcome in all adolescents and adults with suspected bacterial meningitis; however, a beneficial effect appears to be confined to patients with microbiologically proven disease, including those who have received prior treatment with antibiotics (112).

Epidemiology of the *S. suis* infection in humans: (a) differences between Asian and Western countries

There are several potential routes of entry of the organism in humans: a small cut in the skin, although in some cases no wound was detected; bacteria may colonize the nasopharynx, as observed in swine; and the gastrointestinal tract, as suggested by diarrhea as a prodromal symptom (28). The incubation period ranges from a few hours to two days (28). Although few facets of the epidemiology of *S. suis* infections in humans have been elucidated, it is apparent that numerous cases of human infection can be ascribed to high exposure to unprocessed pork meat or to close contact with pigs. Some reports indicate a relatively high percentage of patients not recalling any contact with pigs or pork products (26, 49, 111, 113-115). However, since these cases were identified in a retrospective study, data could easily have been missed. Only one report claims to confirm a patient with a complete lack of contact with risk animals or their by-products, but he worked closely with chickens (114).

In Western countries, *S. suis* disease has been considered a rare event in humans. Most cases of human infection are related to employment in the swine industry: pig farmers, abattoir workers, persons transporting pork, meat inspectors, butchers and veterinarian practitioners (25, 32, 35). Two countries consider *S. suis* infections in humans as an Industrial Disease: France and the United Kingdom (6). Handling diseased pigs that would naturally have a high rate of bacterial shedding, increases the risk of human infection (1, 116); however, it has been suggested that manifestation of disease in pigs is not a prerequisite for infection in people who are in contact with pigs, since animals can be healthy carriers, and that does not preclude their being also a source of infection. The preponderance of affected adult males is readily explained, since many acquire the disease following occupational exposure to pigs or pork products (26). In the Netherlands, the Western hemisphere country with the highest number of published reports on *S. suis* infection in humans, the annual risk of *S. suis* infection among abattoir workers and pig breeders was estimated at approximately 3.0/100,000, a rate that is 1500 times higher than that among those not working in the pork industry. Butchers had an annual rate of 1.2/100,000; this rate is even higher in the United Kingdom (32). In Germany, the

nasopharyngeal carriage rate of *S. suis* serotype 2 in the high-risk group (butchers, abattoir workers and meat processing employees) was 5.3%, while those without contact with pigs or pork consistently tested negative (43). Interestingly, some positive individuals were sampled again 3 weeks after the first study, and the presence of *S. suis* serotype 2 could still be confirmed, indicating that the bacteria probably remained in the tonsils of healthy people for a relatively long period of time. This has also been shown in pigs (1); however, since these individuals are continuously exposed to pork products, a repeated re-colonization cannot be ruled out. Other reports confirmed *S. suis* isolation from tonsils in healthy abattoir workers in Western countries (117, 118).

The situation in Asian countries differs greatly from Western countries; these differing characteristics are not just due to the sudden Chinese outbreak. Following *S. pneumoniae* and *Mycobacterium*, human *S. suis* infection has recently been reported as the third most common culture-confirmed cause of community-acquired bacterial meningitis in Hong Kong (119). In Vietnam, *S. suis* is now considered to be the most frequent cause of bacterial meningitis in adults (111). Many characteristics differentiate Asian countries from Western countries: life style (Fig. 1), the common use of backyard systems of production (Fig. 2), and the close contact of different animal species (including humans) with pigs (Fig. 3). Hogs are sometimes illegally home slaughtered and diseased animals are commonly slaughtered for family consumption. In addition, the public often buys raw pork or pork products in the widely available open markets. Thus, we must include indirect exposure to *S. suis* as a route to infection (49). Consumption of fresh and/or raw pork is a common practice in Asia, giving rise to the belief that the oral route accounted for most infection cases of people who are not normally in contact with the swine industry in Asian countries (26, 46). Cases from these countries included not only a significant number of housewives (probably contaminated by raw pork products) but also those who were, as previously mentioned, unaware of their exposure to infected pork (26, 49). It has been shown by direct culture, that more than 6% of pork samples were positive for *S. suis* in 6 wet markets in Hong Kong (120). The presence of *S. suis* in pigs would be confined to the tonsillar region, and other sites such as the head/neck, tonsils, tongue, intestines, bone, and tail that are all readily available in wet markets as ingredients for Asian cuisine. Using a more sensitive technique (Most Probable Number-PCR method), it has recently been

shown that the prevalence of pork products (including meat) contaminated with *S. suis* is probably much higher than previously thought (121).

Studies have been done to understand differences of *S. suis* infection of those in occupations that have direct contact with pigs or pork meat in Asian and Western countries. The annual incidence in Hong Kong for the occupational (direct contact with pigs) group was 32/100,000, 350 times higher than that of general population (0.09/100,000), and 30 times higher than the homologous group in the Netherlands (122). The higher rate in the Hong Kong occupational group compared to that of Netherlands might be explained by the higher prevalence of *S. suis* infections in Asian pigs, although this must be confirmed. On the other hand, the less dramatic difference between the occupational group and the general population (350 times higher in Hong Kong vs 1500 times in the Netherlands) may be due to the general population in Asian countries having greater contact with raw pork meat.

Epidemiology of the *S. suis* infection in humans: (b) differences between Europe and America

Interestingly, a large majority of reported cases of *S. suis* infection in humans are from Europe: The Netherlands, Italy, Spain, United Kingdom, Belgium, Croatia, Austria, Sweden, Germany, Ireland, Hungary, France, Greece, Serbia and Portugal (6). A few cases have been reported in Australia and New Zealand. On the other hand, Canada and United States of America seem to present a special case: together, they are among the most important swine producers worldwide with a livestock of more than 115 million pigs. *S. suis* infection in pigs (different serotypes, with low prevalence of serotype 2) is also one the most important causes of post-weaned piglet mortality in both countries (1). Mysteriously, only two *S. suis* serotype 2 and one case of non-serotype 2 human infection cases (with no mortality) and have been reported in Canada (123-125) and only one in mainland United States (126). An additional US reported case should not be considered truly North American: the patient was infected while travelling in the Philippines and he developed clinical signs upon his return to the United States (127). A recent case in Hawaii has also been reported (128); however, a recent serological survey in mainland USA indicates that swine-exposed persons had higher titers of antibodies to *S. suis* than non-swine-exposed persons (129). The test used detects antibodies not only to serotype 2 but also to other

serotypes. These data suggest that human infections are more common that currently thought and that high exposure to *S. suis* may lead to a colonization of the upper respiratory tract without producing any health consequences. This may be due to the fact that either serotypes not pathogenic for humans or low virulent serotype 2 strains, or both, are involved in human colonization. The capacity to correctly diagnose the infection and the virulence of the strains may also explain differences between Europe and America (see below).

A hypothesis (other than life style) to explain geographical differences: (a) accurate diagnosis of the infection

It is of prime importance that the diagnostic laboratory be aware of this infection. *S. suis* is a pathogen very well known by diagnostic laboratories of human disease in Asia, relatively well known in Europe and generally unknown in America. This may also explain different rates of diagnosis of *S. suis* human disease. A good case example is Thailand; since 2006, the Miscellaneous Bacteriology Laboratory, National Institute of Health, initiated a microbiological service for the identification of *S. suis* in clinical isolates from laboratories of local hospitals (115). It was believed that many infections had been misidentified in the past in Thailand. Coincidentally, an outbreak of *S. suis* was detected in Phayao Province during 2007, with 29 laboratory-confirmed cases transmitted through consumption of raw blood from infected pigs (115). Without the surveillance program, this outbreak would probably have remained undetected. Before 2005, only sporadic cases were reported in Thailand, whereas after 2006, more than 300 cases are mentioned in the website of the Bureau of Epidemiology, Ministry of Public Health (115). In South America, only Argentina has reported two human cases (130, 131) and we have received strains from at least four other human cases from that country (unpublished data). Similar to the situation in North America, its neighbor country Brazil, with a swine population 20 times greater than Argentina, has never reported a human case. Due to the presence in both countries of both very similar swine genetics and similar herd health status, is it likely that serotype 2 strains from Brazil and Argentina are very closely genetically related. In fact, they are phenotypically (MRP, EF and suilysin) very similar (unpublished data). We hypothesize that this discrepancy is best explained by the greater awareness and knowledge of the *S. suis* infection in the Argentinean diagnostic laboratories that in those of Brazil.

From the laboratory point of view, diagnosis should not be more difficult than that of other meningitis- or toxic shock-causing bacteria. In cases of meningitis, the cerebrospinal fluid (CSF) cultures are the most important indicator. However, we need to remember that many patients can present positive CSF cultures, but *S. suis* is not isolated from blood and *vice versa*. Patients with septicemia usually have positive blood cultures, but many of them do not present signs of meningitis and their CSF cultures are negative (122). Field isolates of *S. suis* readily grow on media used for culturing meningitis-causing bacteria, and veterinary diagnostic laboratories easily identify this pathogen. However, many diagnostic laboratories working in human diseases in Western countries (especially in North America) are unaware of this bacteria and usually misidentify it as enterococci, *S. pneumoniae, Streptococcus bovis,* Group D streptococci, viridans group streptococci (*Streptococcus anginosus*, *Streptococcus vestibularis* and others) or even *Listeria* (5, 25, 31, 125, 132, 133). In many cases, the initial Gram stain presumptive diagnosis of the CSF specimen is pneumococcal meningitis. This inaccuracy may have led to misdiagnosis of *S. suis* meningitis in the past. Many cases were diagnosed retrospectively after the isolates were misidentified (28, 123, 134). It has been suggested that when optochin-resistant streptococci are cultured from CSF samples of patients with meningitis, *S. suis* diagnosis needs to be considered (26). However, one should also remember that sporadic cases caused by *S. pneumoniae* strains resistant to optochin and *S. suis* strains susceptible to optochin have been described (135, 136). Many (if not most) diagnostic laboratories working on human diseases propose the use of multi-tests, such as the API Strep System test (BioMérieux, France), the BBL Crystal Gram-positive ID kit (Becton-Dickinson), the Vitek GPI Card (BioMérieux), and the Phoenix System PID (Becton-Dickinson); however, experience shows that some strains of *S. suis* can be still be misidentified using these commercial kits (1, 25, 120, 122, 125, 132, 137, 138). More recently, PCR tests have been used to directly detect *S. suis* DNA from human CSF samples. Those PCR tests have a considerably higher sensitivity than direct culture, especially if antibiotics have been used (26, 111). However, most PCR tests used so far in humans would only detect serotype 2 strains (and 1/2) (26, 111) and they will not detect infections caused by other *S. suis* serotypes. For example, serotype 14 has been isolated from humans many times (50, 124, 139, 140). Recently, Kerdsin *et al*. reported 12 strains of serotype 14 isolated from humans

in Thailand, indicating that serotype 14 is a major zoonotic agent in that country (115). This serotype has been recently isolated in North America for the first time (124). Sporadic cases due to other serotypes, such as serotype 4 (5), serotype 1 (141) and serotype 16 (142) have been reported. In addition to the serotype 2 PCR test, this calls for other validated PCR tests that detect all serotypes of *S. suis* (143).

A hypothesis (other than life style) to explain geographical differences: (b) virulence of the strains

To date, virulence factors/markers have been studied only for serotype 2. The relative low prevalence of serotype 2 among strains isolated from diseased pigs in Canada and USA, compared to Europe and Asia, may also explain the low number of human cases in that part of the world. We propose a hypothesis that lower virulence of serotype 2 strains would lead to less cases of acute disease due to this serotype in pigs with less probabilities of transmission to humans. Since these strains might also be less virulent, infected people would seldom develop disease. North American strains are clearly genotypically and phenotypically different from European and Asian strains (54, 144). Most North American strains do not carry the virulence markers, MRP, EF and suilysin, and are less virulent as demonstrated by standardized comparative experimental infection of piglets (145). Indeed, *S. suis* swine disease in Canada and USA is mainly associated with the presence of an immunosuppressive virus in pigs that is called Porcine Reproductive and Respiratory Syndrome Virus (146-149). This may also explain why (perhaps) lower virulent serotype 2 strains and other serotypes that are not usually transmitted to humans cause important swine outbreaks (1, 54, 150). Multi-locus sequence typing (MLST) is a technique that defines strains by using sequences of different housekeeping loci. MLST has shown that many serotype 2 strains from Canada belong to sequence-type 25 (ST- 25), which differs from European and Asian isolates (see below).

Data suggests a difference in the virulence properties of American and European strains of *S. suis* serotype 2. Are there also differences between Asian and European strains? Many sources suggest that it was a highly virulent Asian strain that caused the Chinese outbreak in 2005 (151). Were there one or more strains at the origin of this outbreak? Independent researchers clearly confirmed the identity of *S. suis* strains recovered from ill patients and

also showed that the strains belong to serotype 2 (34, 44, 152). Using restriction fragment length polymorphisms, ribotyping and pulsed-field gel electrophoresis, strains involved in both the human and swine Chinese outbreaks were shown to be clonal (34, 37, 152). Using MLST, all but two strains from the 2005 outbreak were classified into the single ST-7, included within theST-1 complex (152). The latter has been strongly associated with cases of septicemia and meningitis in swine and humans, mainly in Europe and in some Asian countries (153). However, some ST-25 strains (usually found in North America) from Thailand have recently been described as being responsible for important human diseases (48). These strains should be further studied and compared to Canadian and US strains.

The emerging Chinese clone produces MRP, EF and suilysin proteins, a feature that is typical of most Eurasian strains (34, 152). In the last three years, many studies have used the epidemic ST-7 Chinese strain and two independent groups have sequenced the pathogen (144, 154). One of the Chinese ST-7 strains has been reported to be more toxic for human peripheral blood mononuclear cells (PBMC) than a well-characterized European virulent strain ST-1 (152). The North American ST-25 strain is completely non-toxic. Toxicity would simply be due to the suilysin, and the ST-7 strain is highly hemolytic (our unpublished data). Recent reports indicated that the ST-7 strain also possesses a stronger capacity to stimulate T cells, naive T cells and PBMC proliferation. The ST-7 strain also induces a higher level of pro-inflammatory cytokines in experimentally infected mice than does a well-characterized ST-1 European strain (155, 156). It has been proposed that the ST-7 strain evolved recently from a highly pathogenic ST-1, which in turn, evolved from the intermediately virulent ST-25 that is usually found in North America (103). After genome-wide comparisons, the only large-scale region of difference that could be confirmed was an 89-kb putative pathogenicity island (PI) that is present in the Chinese ST-7 strains but absent in the ST-1 European strain (144). Interestingly, a human ST-1 strain from Vietnam also contains an almost identical PI with some differences that encompass drug-resistance genes, and a two-component regulatory system associated with a bacteriocin cluster. That this PI might be involved in a particular clinical manifestation of *S. suis* infection remains speculative (144). Some other differences in genome content, as well as a large number of single nucleotide polymorphisms and short insertions and

deletions have also been identified between the ST-7, ST-1 and ST-25 strains (144, 154). The contribution of these other discrete genomic regions and polymorphisms to the different virulence traits of the MLST types deserves further study. Previous results indicated that no superantigens could be identified in the Chinese strain (54 and unpublished data). Results from complete genome sequencing confirmed such observations (144, 154). Therefore, although the ST-7 seems to be a highly virulent strain, there is so far no clear proof that its virulence only explains the impact of the disease in China. Future studies to increase knowledge on virulence factors of *S. suis* will be needed to define it as a newly-emerging and dangerous strain.

CONCLUSION

The zoonotic pathogen *S. suis* is the causative agent of serious disease in humans. The high prevalence of human infections in Asian countries may be related to a combination of several factors: a) life style; b) prevalence of swine disease; c) levels of virulence of *S. suis* strains; and d) recognition and knowledge of the microorganism by diagnostic laboratories for human disease. Increased knowledge of the pathogen in diagnostic laboratories in addition to the possible higher virulence of strains (with a significantly higher prevalence of serotype 2 disease in pigs) may explain the higher prevalence of human disease in Europe than in North America. In these regions, a relatively low number of human cases are reported annually despite wide prevalence of the *S. suis* infection (different serotypes) in the pig population. The Chinese outbreak in 2005 attracted strong interest among the scientific community, clearly indicating that this zoonotic bacterium must be given important consideration. Given that many people worldwide are in daily contact with pigs and that *S. suis* is a common cause of disease in swine populations, it is reasonable to suggest that a certain percentage of them harbor this pathogen without presenting clinical signs. Under unusual circumstances, disease may develop. Physicians and microbiologists, especially in North America where very few cases are identified, should be aware of this microorganism and the infections that it causes. More attention should be given to streptococcal meningitis or septic shock in people working with pigs or pork products. Veterinarians should also be aware that there is a low but real risk during handling of *S. suis*-diseased animals that they are probably shedding this zoonotic bacterium. There is some dispute regarding the preventive measures that might be justified due to the high rate of contamination of pigs with *S. suis*. Some advise that prompt first-aid care of injuries in meat handlers might reduce the risk of *S. suis* infections; others consider this recommendation questionable because it is evident that skin lesions have only been reported in some cases and the route of entry of the infection remains unclear. Although it is difficult to recommend effective preventative measures for employees of the food industry, those coming into close occupational contact with pigs or pork products should pay special attention. Health agencies and physicians in Asian countries may have interest in increasing the awareness of the population regarding the risks associated with the habit of eating raw pork products from uncontrolled sources. Increased collaboration between

laboratories with diverse but complementary expertise in different parts of the world is necessary to significantly increase our understanding of this challenging zoonotic pathogen.

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Figure 1. Transporting piglets in Vietnam.

Figure 2. A representative backyard production system in Asian countries. (A) Owner's home; (B) Pig production site.

Figure 3. Intimate contact among different animal species in Asian backyard production systems.

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Annexe II

Mutations in the gene encoding the ancillary pilin subunit of the *Streptococcus suis srtF* cluster result in pili formed by the major subunit only

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ABSTRACT

Pili have been shown to contribute to the virulence of different Gram-positive pathogenic species. Among other critical steps of bacterial pathogenesis, these structures participate in adherence to host cells, colonization and systemic virulence. Recently, the presence of at least four discrete gene clusters encoding putative pili has been revealed in the major swine pathogen and emerging zoonotic agent *Streptococcus suis*. However, pili production by this species has not yet been demonstrated. In this study, we investigated the functionality of one of these pili clusters, known as the *srtF* pilus cluster, by the construction of mutant strains for each of the four genes of the cluster as well as by the generation of antibodies against the putative pilin subunits. Results revealed that the *S. suis* serotype 2 strain P1/7, as well as several other highly virulent invasive *S. suis* serotype 2 isolates express pili from this cluster. However, in most cases tested, and as a result of nonsense mutations at the 5′ end of the gene encoding the minor pilin subunit (a putative adhesin), pili were formed by the major pilin subunit only. We then evaluated the role these pili play in *S. suis* virulence. Abolishment of the expression of *srtF* cluster-encoded pili did not result in impaired interactions of *S. suis* with porcine brain microvascular endothelial cells. Furthermore, non-piliated mutants were as virulent as the wild type strain when evaluated in a murine model of *S. suis* sepsis. Our results show that *srtF* cluster-encoded, *S. suis* pili are atypical compared to other Gram-positive pili. In addition, since the highly virulent strains under investigation are unlikely to produce other pili, our results suggest that pili might be dispensable for critical steps of the *S. suis* pathogenesis of infection.

INTRODUCTION

Streptococcus suis is a major swine pathogen responsible for severe economic losses to the porcine industry [1]. This bacterium is also a zoonotic agent affecting, for the most part, people in close contact with swine or pork by-products [2]. In recent times, however, *S. suis* has strongly emerged as an important public health issue in South East and East Asia. For instance, it has been shown that this pathogen is the primary cause of adult meningitis in Vietnam [3] and the second in Thailand [4]. Moreover, in 2005, more than 200 human *S. suis* cases with a death toll of 39 were reported during a single outbreak in China [5]. In both swine and humans the main clinical manifestations of *S. suis* are meningitis and septicemia [1], [2]. Most cases of *S. suis* disease are caused by strains belonging to the serotype 2 and, therefore, almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype [1]. It has been shown that the polysaccharide capsule is essential for the virulence of *S. suis* by allowing the bacterium to escape phagocyte killing [6]. Modifications of cell wall components such as the N-deacetylation of the peptidoglycan and the D-alanylation of lipoteichoic acids have recently been shown to contribute to the virulence of *S. suis* [7], [8]. As well, an isogenic mutant for a serum opacity-like factor has been found to be attenuated in pigs [9]. In contrast, other factors, such as a hemolysin (suilysin), the so-called extracellular protein factor (EF) and a muramidase-released protein (MRP), have been shown to be linked to, but not essential for, the virulence of *S. suis* [1].

S. suis needs to invade the central nervous system (CNS) in order to cause meningitis in swine. It has been proposed that, among other routes, this pathogen might reach the CNS by crossing the porcine blood-cerebrospinal fluid barrier as well as the blood-brain barrier by transcytosis through porcine choroid plexus epithelial cells and brain microvascular endothelial cells (BMEC), respectively [10], [11], [12]. In previous work using an in vitro model of *S. suis*-porcine BMEC interactions and the selective capture of transcribed sequences (SCOTS), several genes were identified which were highly upregulated by this bacterium upon contact with these host cells [13]. One of these genes was SSU_0424 encoding a putative signal peptidase [13] (the nomenclature used is that of the Sanger Institute for the very recently finished sequencing project of strain P1/7 [14]). Further *in*

silico analysis showed that the three genes downstream the signal peptidase identified by SCOTS, namely SSU_0426, SSU_0427 and SSU_0428, putatively encode two cell wall sorting signal (CWSS)-containing proteins and a class C sortase, respectively [13], [15]. This genetic organization is similar to that of some described Gram positive pilus cluster [16], [17]. In a recent study, the sortase gene was renamed as *srtF* and the signal peptidase gene as *sipF* (for signal peptidase gene in the *srtF* cluster) [18]. The genes encoding the two CWSS-containing proteins were renamed as *sfp2 and sfp1* (for *srtF*-associated pilin subunit), respectively [18]; *sfp2* has been suggested to encode the putative pilin ancillary subunit (a putative adhesin) and *sfp1* the putative main pilin subunit forming the pilus backbone [13]. The full cluster was named *srtF* pilus cluster [18] and found to be highly homologous to the *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) pilus island 2b [13].

Gram-positive pili participate in biofilm formation [19], [20], [21] and have been shown to play important roles in other aspects of the virulence of several invasive human streptococcal pathogens, including GBS, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Group A *Streptococcus*, GAS) [17]. For instance, at least two pneumococcal pili are involved in adherence to epithelial cells and contribute to the virulence of these organisms [22], [23], [24]. In GBS and GAS, besides contributing to phagocyte resistance and systemic survival [25], pili participate in adhesion to extracellular matrix proteins [26] and to human epithelial cells [27]. Furthermore, pili have been shown to be important for GBS adherence to and invasion of human BMEC [28]. We therefore speculated that putative pili encoded by the *srtF* cluster might play a role during the interactions of *S. suis* with porcine BMEC and, also, that they might contribute to the virulence traits of this pathogen. In this work, we characterized the *srtF* pilus cluster in a highly virulent field strain of *S. suis* serotype 2 and investigated the role that pili encoded by this cluster play in some aspects of the pathogenesis of the *S. suis* infection.

MATERIALS AND METHODS

Ethics Statement

All animals used in this study were treated, and trials conducted, in accordance with the guidelines and policies of the Canadian Council on Animal Care (CCAC), enforced locally by the Ethics Committee of the Faculté de médecine vétérinaire of the Université de Montréal. The protocols and procedures were approved by the Ethics Committee.

Bacterial Strains, Plasmids, Media, Culture Conditions and Reagents

Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *S. suis* strains were grown in Todd-Hewitt (Becton Dickinson, Sparks, MD) broth (THB) or agar (THA) at 37°C. *E. coli* strains were grown in Luria-Bertani (LB) broth or agar (Becton Dickinson) at 37°C. When needed, antibiotics (Sigma, Oakville, ON, Canada) were added to the culture media at the following concentrations: for *S. suis*: spectinomycin (Sp) at 100 µg/ml; for *E. coli*: kanamycin (Km) and Sp at 50 µg/ml; chloramphenicol (Cm) at 30 µg/ml and ampicillin at 100 µg/ml. Unless otherwise indicated, all reagents used in this study were purchased from Sigma.

DNA Manipulations

S. suis genomic DNA was prepared by the guanidium thiocyanathe method [46]. Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures [47]. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa Bio (Otsu, Shiga, Japan) and used according to the manufacturers' directions. PCR reactions were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Hercules, CA) or with Taq DNA polymerase (GE Healthcare, Piscataway, NJ). Oligonucleotide primers were from Invitrogen (Burlington, ON, Canada). Amplification products were purified on Sephadex S-400 columns (GE Healthcare) and sequenced with an ABI 310 automated DNA sequencer, using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Construction of Deletion Mutants

Precise, in-frame deletions in *sipF*, *sfp2*, *sfp1 and srtF* were constructed by using splicing-by-overlap-extension PCR [48]. The primers used for the construction of deletion alleles are listed in Table S1. Appropriate deletion alleles generated by PCR were cloned into plasmid pCR2.1 (Invitrogen), extracted with BamHI and PstI and recloned into the thermosensitive *E. coli-S. suis* shuttle vector pSET4s [49] digested with the same enzymes. Plasmid pSAD11 used in this study for deletion of *srtA* was obtained by EcoT22I removal of the *cat* gene from plasmid pSAD1, previously used for insertional inactivation of *srtA* [43]. Complementation of the Δ*srtA* mutant was achieved by electroporation of the mutant with the previously described pSAcomp1 vector [43]. This mutant was also mock-complemented with empty pSET-3 vector from which pSAcomp1 is derived [43], [50]. Electroporation of *S. suis* and procedures for isolation of mutants were those described previously [50]. Deletions of all genes were confirmed by PCR and sequencing analysis.

Expression and Purification of Recombinant 6xHis-Sfp1 and 6xHis-Sfp2 and Antibody Production

DNA fragments intragenic to *sfp1* and *sfp2* were generated by PCR using genomic DNA of *S. suis* strain P1/7 as template and the primer pairs Sfp1-fwd/Sfp1-rev (for *sfp1*) and Sfp2-fwd/Sfp2-rev and Sfp2-fwd2/Sfp2-rev2 (for *sfp2*), respectively (Table S1 and Figure 1). PCR amplicons were digested with NdeI and BamHI and cloned into plasmid pIVEX 2.4d (Roche Applied Science, Laval, QC, Canada), digested with the same enzymes. The resulting recombinant plasmids were introduced into *E. coli* TOP 10 (Invitrogen) for sequence analysis and storage and into *E. coli* BL21λDE3/pDIA17 for protein expression. Induction was carried out with IPTG, as previously described [51]. Recombinant 6xHis-proteins were purified under denaturing conditions by affinity chromatography on Ni-NTA columns (Protino protein purification system, Macherey-Nagel, Düren, Germany) according to the manufacturers' recommendations. Protein purity was checked on SDS-PAGE and accurate protein concentrations were determined by a simplified Lowry test [52]. Rabbit polyclonal antibodies (pAb) against the individual proteins were produced as previously described [53]. The specificity of each antibody was determined by Western blotting against the purified 6xHis-proteins as well as against crude *S. suis* cell extracts prepared from the WT and mutant strains.

Cell Wall, Whole Cell and Culture Supernatant Protein Preparations

S. suis strains were grown in 10 ml of THB at 37°C. Bacteria were harvested by centrifugation during the late exponential phase of culture and resuspended in 220 µl spheroplasting buffer [10 ml spheroplasting buffer: 24 mg Tris, 20 mg $MgCl_26H_2O$, 2.6 g raffinose, 5000 U mutanolysin, one capsule Complete Mini EDTA-free protease inhibitor cocktail (Roche)], as described [54]. The digestion was performed for 1 h at 37°C under gentle agitation. After centrifugation at 13,000×g for 15 min at 4°C, supernatants corresponding to the cell wall fractions were analyzed on SDS-PAGE. Total proteins extracts were prepared as previously described [55]. Supernatants obtained after centrifugation of ON *S. suis* cultures grown in THB were concentrated 10-fold by Ultrafree-MC centrifugal filter devices (Millipore Corp., Bedford MA, USA).

Immunogold Electron Microscopy

S. suis WT and mutant strains were grown overnight at 37°C in 10 ml of THB, harvested by centrifugation, and resuspended in 250 µl of 1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.3. After fixation for 30 min, 20 µl of the bacterial suspensions were placed on nickel-Formvar grids (Canemco, Lakefield, QC, Canada) and allowed to partially air dry. Grids were subsequently blocked for 30 min with 10% normal donkey serum (Jackson ImmunoResearch) in dilution buffer (PBS containing 1% bovine serum albumin and 1% Tween-20, pH 7.3). Thereafter, samples were soaked in 50 µl of anti-Sfp1 or anti-Sfp2 specific pAb or control rabbit normal serum diluted 1/10 in dilution buffer for 2 h. The grids were then washed 5 times with dilution buffer, soaked in 50 µl of 10 nm colloidal gold-goat anti-rabbit IgG (Sigma) diluted 1/20 in dilution buffer, and incubated for 1 h. After three washes with PBS, grids were stained with 2% uranyl acetate for 30 s, and observed with a JEM-1230 electron microscope (JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 80 kV.

Adherence to and Invasion of Porcine BMEC

The porcine BMEC cell line PBMEC/C1-2 [56] was grown in Primaria 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) using IF culture medium (a mixture

of 1:1 Iscove's modified Dulbecco's and Ham's F-12 media, Invitrogen) supplemented as previously described [10]. *S. suis* strains were grown in THB for 16 h at 37°C, harvested by centrifugation, washed twice in PBS, and resuspended in fresh IF culture medium. The invasion assays were performed as described previously [10]. Briefly, confluent monolayers of porcine BMEC at 10^5 cells/well were infected with 1-ml aliquots of bacterial suspensions at 10^5 CFU/ml (multiplicity of infection of 1). The plates were centrifuged at 800×g for 10 min and incubated for 2 h at 37°C under 5% CO_2. The monolayers were then washed twice with PBS, 1 ml of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G was added to each well, and incubation continued for 1 h. After incubation, monolayers were washed three times with PBS, trypsinized and disrupted by repeated pipetting. Serial dilutions of the cell lysates were plated onto THA and incubated overnight at 37°C. Invasion rates were calculated as the number of bacteria remaining after the antibiotic treatment with respect to the total number of inoculated bacteria. Adherence assays were performed essentially as described for invasion, but no antibiotic treatment was performed. After incubation for 2 h, cells were washed five times with PBS, trypsinized, disrupted, and serial dilutions of the cell lysates were plated as described above. Adherence rates were calculated as the number of bacteria remaining attached to cells after the incubation period with respect to the total number of inoculated bacteria.

Experimental Infection of Mice

A validated CD1 murine model of *S. suis* infection was used [11]. In a first experiment, 50 female, 6-week old CD1 mice (Charles River Laboratories, Wilmington, MA) were divided in 5 groups of 10 animals (day 0). Group 1 was inoculated by intraperitoneal injection of 1 ml of *S. suis* strain P1/7 suspension at 5×10^7 CFU/ml, while groups 2, 3, 4 and 5 received the same dose of mutant strains Δ*sipF*, Δ*sfp2*, Δ*sfp1* and Δ*srtF*, respectively. Mice were monitored 3 times/day for 3 days for clinical signs and assigned clinical scores as previously described [11]. Blood samples (5 µl) were collected daily from the tail vein and at necropsy by cardiac puncture and used to evaluate bacterial load by plating onto sheep blood agar plates. Isolated tiny α-hemolytic colonies were counted and assigned to *S. suis* by serotyping as previously described [57]. At necropsy, macroscopic examination was performed. Bacterial colonization of the liver, spleen and brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500

μl of PBS and homogenized. Thereafter, 50 μl of the suspensions were plated as described above. A second experiment was carried out essentially as described above but the mice received a lower dose (1 ml of 1×10^7 CFU/ml) of *S. suis* P1/7 or mutant strains. In this second experiment, the WT and Δ*sipF* groups comprised 11 mice each while the other 3 groups (Δ*sfp2*, Δ*sfp1* and Δ*srtF*) were of 10 mice each. Animals were monitored as described above for 4 days.

RESULTS

The *srtF* Pilus Cluster Encodes Pili Formed by the Major Pilin Subunit Only

The genome of the virulent field strain P1/7 contains a genetic region designated as the *srtF* pilus cluster where four genes encoding a putative signal peptidase (*sipF*), putative ancillary and major pilin subunits (*sfp2* and *sfp1*, respectively) and a putative dedicated class C sortase (*srtF*) are found [13], [14], [18] (Figure 1). To assess whether the *srtF* cluster mediates formation of pili in *S. suis*, we inactivated each of the four genes by precise, in frame, allelic replacement. The resulting mutant strains exhibited growth kinetics equivalent to those of the WT parent strain upon cultivation in standard laboratory media and other media used in our in vitro assays (data not shown). At first, pili production by the WT and mutant strains was studied by Western-blotting of cell wall protein extracts (mutanolysin digests) using a specific antiserum directed against the putative pilin subunit Sfp1. In the WT sample, this antiserum recognized a band of approximately 37 kDa that likely corresponds to a Sfp1 monomer whose N-terminal signal peptide and C-terminal residues after the CWSS motif have been removed (predicted MW of 51 kDa for a native unprocessed monomer). As well, high molecular weight species that likely represent Sfp1-containing polymers were detected in the WT sample (Figure 2, lane 1). The monomer and polymers were also detected in the Δ*sipF* and Δ*sfp2* mutants (lanes 2 and 3), although the intensity of the higher molecular weight polymers was lower in the former mutant. By contrast, neither monomer nor polymers were detected in the Δ*sfp1* mutant (lane 4). On the other hand, the Sfp1 monomer, but not polymerized structures were detected in the cell wall extracts of the Δ*srtF* mutant (lane 5), indicating that polymerization of pili requires the action of the dedicated class C sortase SrtF.

We then performed Western-blotting using two different antisera raised against two different fragments of Sfp2 (named rSfp2 A and B, respectively). Sfp2 is, based on homology to GBS PI-2A (37% of positives, 29% of identity to SAN_1519 of GBS COH1), the putative minor pilin subunit of the *srtF* cluster [13], [18]. Despite the fact that both recombinant proteins were recognized by the respective antisera (Figure 3A, B and C, lanes 1), we failed to detect any reactive protein in both whole cell and cell wall protein preparations, as well as in concentrated culture supernatant preparations of the WT (Figure

3A, B and C, lanes 2) and mutant strains (data not shown). Although these results were at first surprising, while this manuscript was in preparation the genome of strain P1/7 was published [16]. In the released data the gene reported here as *sfp2* (SSU_0426) and a short open reading frame (SSU_0425) upstream of *sfp2* are now annotated as a single pseudogene SSU_0425 [14], [18]. In this pseudogene a premature ochre nonsense mutation is found after codon 49 (Figure 1). Therefore, failure of our two anti-Sfp2 antisera to recognize the native Sfp2 protein confirms this new released sequence data for strain P 1/7.

Electron Microscopy Evidence for Pilus-Like Structures

Antisera raised against recombinant pilin proteins were also used to investigate the protein localization in the cell surface of strain P 1/7 and selected mutants by immunogold electron microscopy (IEM). As expected from the previous Western-blotting results, labeling was not observed with antibodies directed against Sfp2 (data not shown). On the other hand, confirming Western-blotting results, immunogold labeling with the antiserum specific for Sfp1 revealed long and abundant pilus-like structures extending up to 800 nm from the bacterial surface in the WT strain (Figure 4, left panel). Suggesting that Sfp1 monomers form the backbone of the pilus, pili were entirely decorated by the anti Sfp1-specific antiserum and 10 nm colloidal gold-conjugated anti-rabbit IgG antibodies. As expected, no labeling was observed for the Δ*sfp1* mutant (Figure 4, center panel), while labeling of the bacterial surface but not pilus-like structures was observed for the Δ*srtF* mutant (Figure 4, right panel). No immunogold labeling was observed using control rabbit normal antisera (data not shown).

Evaluation of the Role of the Housekeeping Sortase SrtA in Pili Production

The housekeeping sortase SrtA has been shown to mediate anchoring of LPXTG proteins to the *S. suis* cell wall peptidoglycan [29]. We hypothesized that if SrtA was required for the attachment of pili into the cell wall peptidoglycan, then pili produced by a Δ*srtA* mutant should be released into the media in higher amounts than those produced by the WT strain. To investigate the validity of this hypothesis, we constructed a Δ*srtA* mutant by allelic exchange. When we tested production of Sfp1 polymers in the Δ*srtA* mutant by Western-blotting of cell wall proteins, the amount of Sfp1 polymers detected by our antiserum in the Δ*srtA* mutant was similar to that found in the WT strain. This result is in agreement with a

previous report describing the contribution of SrtA to pili production in GBS [29] and conceivably explained by the fact that the pili subunits are assembled by the pilin polymerase SrtF irrespective of the presence of SrtA, remaining transiently associated to the cell wall. However, in contrast to the WT strain, no Sfp1 monomers could be detected in the cell wall fraction of the Δ*srtA* mutant (Figure 5A, lanes 1 and 2). The WT phenotype was restored by complementation in trans of the Δ*srtA* mutant (Figure 5A, lane 4). As expected, more Sfp1 monomers were detected in the culture supernatant of the Δ*srtA* mutant in comparison to the WT strain or the complemented Δ*srtA* mutant. In addition, the amount of Sfp1 polymers released in the culture supernatant fraction by the Δ*srtA* mutant was slightly higher than that of the WT strain or the complemented mutant (Figure 5A). We then analyzed pili production by the Δ*srtA* mutant using IEM. Figure 5B shows a representative Δ*srtA* mutant bacterial cell presenting pilus-like structures much longer than those found in the WT strain, a result that suggests that SrtA might be involved in termination of pilus assembly. Interestingly, and in contrast to the WT strain, pili produced by the Δ*srtA* mutant were scarce in the bacterial surface. Indeed, most cells presented one of these structures and many of them were devoid of pili. Together with the Western-blotting experiments, these IEM results demonstrate that the housekeeping sortase SrtA is not necessary for pilus polymerization but strongly suggest that it is required for anchoring the pilus to the cell wall.

Production of Pili by the *srtF* Cluster in Other Virulent *S. suis* Strains

Absence of the minor subunit in polymerized pili of strain P1/7 seems to be the consequence of a genetic conversion which resulted in inactivation of *sfp2* in this strain. Other strains might, however, present an intact *sfp2* gene. Indeed, by the use of PCR amplification with specific primers a previous study demonstrated the presence of the *srtF* pilus cluster genes in several isolates of *S. suis* serotype 2 from various sources [18]. We therefore analyzed whether the *S. suis* serotype 2 reference strain and other well-characterized highly virulent field strains (Table 1) can express pili that are formed by both Sfp1 and Sfp2 subunits. Western-blotting results showed that most of the investigated strains produced pili formed by the major pilin subunit Sfp1 only and were devoid of Sfp2 (Figures 3A, B and C, lanes 3 to 10 and Figure 6). The exception was strain 89–1591, for which we failed to detect not only Sfp2 but also Sfp1 monomers (Figure 6, lane 9), despite

the fact that it has been reported that this strain contains all four *srtF* cluster genes [18]. However, further analysis of sequence data for this particular strain (available at http://genome.jgi-psf.org/draft_microbes/strsu/strsu.home.html) showed that these four genes are not organized in a typical pilus cluster but, instead, they are found at different locations in the genome (data not shown). Consistently, we failed to amplify the *srtF* pilus cluster in this strain using the primer pair PSF-ID1 and srtF-ID8 (Table S1), which anneal to the region upstream of *sipF* and downstream of *srtF*, respectively, while a fragment of approximately 9.4 kb was amplified from P1/7 and the remaining strains (Fig. S1).

As mentioned above, Sfp2 could neither be detected in extracts of whole cell or cell wall proteins, nor in concentrated culture supernatant fractions of the additional strains under investigation. Consistently, when we sequenced the *srtF* cluster in these strains, the same ochre nonsense mutation found in strain P1/7 was detected in the *sfp2* gene (GenBank Accession numbers GQ279101 to GQ279107.

The *srtF* Pilus is Dispensable for Adhesion to and Invasion of Porcine BMEC

The first gene of the *srtF* pilus cluster, *sipF*, has been found to be highly upregulated by *S. suis* upon contact with cultured porcine BMEC, which are a major type of cells forming the BBB [13]. GBS pili have been shown to be important for adhesion of that pathogen to human BMEC, although in GBS adhesion required the presence of the ancillary subunit [28]. Despite the fact that the homologous putative adhesin Sfp2 is missing from *S. suis* pili encoded by the *srtF* cluster, we investigated the contribution of these pili to the adherence to and invasion of porcine BMEC using in vitro assays. As expected for an adhesin-less pilus, Figure 7A shows that there were no significant differences between the WT and mutant strains regarding *S. suis* adherence to porcine BMEC. A similar absence of differences between the WT and mutant strains was observed when invasion of porcine BMEC by the WT and mutant strains was evaluated (Figure 7B). In contrast, a Δ*srtA* mutant was severely impaired in its interactions with porcine BMEC, as previously reported [30].

Abolishment of Pili Production Does Not Impair *S. suis* Sepsis in the Mouse

Using a GBS mutant that produces adhesin-less pili, it has been shown that the pilus backbone itself promotes phagocyte resistance and systemic virulence [25]. On the basis of these data, we hypothesized that the adhesin-less *srtF* pilus cluster described here might contribute to *S. suis* sepsis. To assess this hypothesis, we performed in vivo trials using a validated CD1 mouse model of *S. suis* infection that uses the intraperitoneal route of inoculation [11]. In a first experiment, mice received 5×10^7 CFU of the WT or mutant strains. Most mice in the WT and the mutant groups presented severe clinical signs associated with septicemia, such as depression, swollen eyes, weakness and prostration during the first 24 h post-inoculation (pi). Most mice died from septicemia in all groups during the first 2 days of the trial and the remaining animals were killed for ethical reasons at day 3 pi (Figure 8A). Moreover, *S. suis* could be isolated at high titers ($>1\times10^7$ CFU/ml) from blood samples and organs such as the liver and spleen of septicemic animals in all the groups ($>1\times10^7$ CFU/0.5 g of tissue in some animals) (data not shown). These results suggest that pili encoded by the *srtF* pilus cluster are not major mediators of *S. suis* sepsis. However, the high dose of inoculation used may not allow for discerning more modest contributions of the pilus structures to the virulence of this pathogen. To address this concern, we performed a second in vivo trial using a lower dose of inoculation (1×10^7 CFU). At this lower dose, animals in the WT and the mutant groups showed, overall, less severe clinical signs than in the previous experiment conducted with the higher dose. Moreover, death was delayed and strongly reduced and several animals in all the groups survived the trial. However, as in the previous trial, no significant differences in the severity of the clinical signs, bacterial isolation from blood and organs (data not shown), nor in mortality (LogRank test, $p = 0.388$) (Figure 8B), were observed between the WT and mutant groups. Taken together, the in vivo trial results strongly argue that pili encoded by the *srtF* cluster may be dispensable for *S. suis* sepsis.

DISCUSSION

The presence of thin pilus-like structures on the surface of *S. suis* was noticed as early as 1990 by ultrastructural studies using electron microscopy [30]. However, only very recently Fittipaldi *et al*. identified a first pilus cluster in *S. suis*, later renamed by Takamatsu *et al*. as the *srtF* pilus cluster [13], [18]. Results presented here show that in the highly virulent *S. suis* strain P1/7 and in other well characterized virulent serotype 2 strains the *srtF* pilus cluster encode pili which are formed by the major pilin subunit Sfp1 only. Indeed, Western-blotting of *S. suis* cell wall proteins with antibodies directed against the Sfp1 subunit clearly showed, for these WT strains, the presence of polymers of the Sfp1 pilin subunit, compatible with a pilus structure in which Sfp1 constitutes the backbone. Moreover, IEM results for strain P 1/7 showed that the gold particles were localized on the *S. suis* surface in pilus-like appendages. Consistently, these structures were absent from a Δ*sfp1* mutant.

It has been shown that in Gram-positive bacteria polymerization of pili is driven by specific, dedicated sortases encoded by genes that are clustered together with the genes encoding the pilin subunits they polymerize, while attachment to the cell wall peptidoglycan is mediated by the housekeeping sortase [16], [31]. In agreement, our results demonstrate that SrtF drives the polymerization of the pilus encoded by the *S. suis srtF* cluster. In fact, Sfp1 monomers, but not Sfp1 polymers, were detected on the cell wall of the corresponding Δ*srtF* mutant. These monomers are likely to be attached to the peptidoglycan through the action of SrtA, as suggested by the fact that they are absent from the cell wall fraction but more abundant in the culture supernatant of the corresponding Δ*srtA* mutant. In this latter mutant, in agreement with the proposed transient, non-covalent anchoring of pili to the cell wall by the dedicated sortases in a housekeeping sortase-less genomic background [16], [27], [29], [32], polymerized pili could be observed in the cell wall fraction. However, Sfp1 polymers were released in higher amounts to the culture supernatant by the Δ*srtA* mutant in comparison to the WT strain. Moreover, pili produced by the Δ*srtA* mutant were, in average, much longer than those observed in the WT parent strain. This observation might imply a role for the housekeeping sortase SrtA in termination of the chain. Additional experiments are needed to ascertain this hypothesis.

To our knowledge, the role in pili biogenesis of signal peptidase-like enzymes found in pilus clusters has only been investigated for the GAS T3 and the *S. pneumoniae* PI-2 pili [22], [33], [34]. It was observed that these signal peptidases (named SipA in the former two species) were essential for pili polymerization and assembly [22], [33], [34]. However, inactivation of the *S. suis sipF* gene did not prevent the polymerization of the pilin backbone subunit Sfp1. It has been suggested that GAS SipA is unlikely to function as a signal peptidase, since it lacks the highly conserved and catalytically important serine and lysine residues of these enzymes [33]. Instead, a putative chaperone function for GAS SipA has been proposed [33]. In contrast, *S. suis* SipF does possess these two conserved residues (data not shown). Therefore, it might be hypothesized that *S. suis* SipF may function as a signal peptidase involved in the removal of the Sfp1 signal peptide leader. However, since the export of Sfp1 was not prevented by deletion of *sipF*, the possibility of other enzymes with signal peptidase activity (most likely the *S. suis* housekeeping signal peptidase) compensating the function of SipF should be envisaged. This hypothetic alternative processing is, however, likely to hamper the efficient polymerization of the pilin subunits, as shown by the fact that the intensity of the higher molecular weight Sfp1-polymers was lower in the Δ*sipF* mutant than it was in the WT strain (Figure 2). Studies are currently being carried out in our laboratories to further understand the participation of SipF in the polymerization of Sfp1.

In this study, with the exception of the Δ*srtA* mutant, we have not complemented the mutant strains with the WT genes that each is missing. However, since the mutants were generated by precise, in frame deletions that were verified by sequencing, we consider very unlikely the possibility of polar effects. In fact, inactivation of *sipF* and *sfp2* (the more upstream genes of the *srtF* cluster) did not result in phenotypic changes, while eventual polar effects in the Δ*sfp1* mutant affecting also *srtF* expression, would have lead to a similar phenotype than that caused by disruption of *sfp1* alone. Further, our results support new genome sequence data indicating that *S. suis* P1/7 *sfp2* is a pseudogene [14], [18]. Importantly, this genetic organization with a non functional *sfp2* gene is shared by strains of *S. suis* serotype 2 belonging to the more virulent multilocus sequence typing ST1 complex [35], such as the recently sequenced Chinese isolates 98HAH12, 05ZYH33 and GZ1 [36], [37]. In fact, in silico analysis suggests that *sfp2* is also a pseudogene in these

strains (data not shown). Furthermore, when we sequenced other well-characterized virulent *S. suis* serotype 2 strains (many of them belonging to the same ST1 complex), they presented the same ochre nonsense mutation in the *sfp2* gene. Consistently, these strains did not produce the Sfp2 pilin subunit. Nevertheless, the possibility of some *S. suis* strains expressing pili formed by both Sfp1 and Sfp2 subunits cannot be excluded. In fact, it has recently been reported that a Spanish *S. suis* isolate may display Sfp2 on its surface [38]. However, it is worth noting that Sfp2 was identified in that Spanish isolate by shaving the surface of bacteria with proteases followed by LC/MS/MS analysis of the resulting peptides and homology comparisons, and not by the use of mutagenesis and specific antibodies directed against that protein [38]. Moreover, that study surprisingly suggested that Sfp2 might form the backbone of the pilus encoded by the *srtF* cluster of that isolate [38]. However, that possibility is not supported by homology comparisons to the pili clusters of other Gram-positive bacteria [17], [22], [23], [24], nor is by data presented in the present study, which clearly show that Sfp1 constitutes the backbone of pili encoded by the *srtF* cluster.

We report here that pili encoded by the *srtF* pilus cluster of *S. suis* strain P1/7 are factors dispensable for adherence to porcine BMEC. They also are of minor importance for invasion of these cells. These results were not unexpected, since the *srtF* pilus lacks the pilin subunit Sfp2, which is, on the basis of homology comparisons, the putative pilin adhesin. To date, all described Gram-positive pili have at least one functional ancillary protein, and several groups of investigators have demonstrated that ancillary proteins play a major role in adhesion to host cells [22], [28], [31], [39]. Indeed, deletion of the putative pilin adhesin in GBS resulted in a mutant strain expressing pili composed of the major subunit only, which showed impaired interactions with human BMEC [28]. Moreover, other studies using streptococcal mutants reported that pili formed only by the major pilin subunit had diminished adhesive capacities compared to their respective parental strains [31], [39]. In this regard, and despite our results for strain P1/7 and other strains analyzed in this study, it should be noted that we cannot rule out the possibility that the *srtF* cluster would contribute to adherence to and/or invasion of porcine BMEC if the putative adhesin Sfp2 were expressed, as it might plausibly be the case in other *S. suis* strains. In addition, although it is an unlikely hypothesis, since we have only tested interactions with porcine

BMEC, we cannot exclude that pili formed only by Sfp1 might play a role in adhesion to other cell types.

The murine model used in this study has proven reliable and reproducible and constitutes an excellent alternative to the use of porcine models of infection [7], [8], [11], [40], [41], [42]. However, this model uses the intraperitoneal route of inoculation [11] and, therefore, it overlooks the initial colonization of the upper respiratory tract by *S. suis*. Consequently, results of the murine trials presented here should be interpreted within these limitations. However, from our in vitro and in vivo results it may be advanced that pili encoded by the *srtF* cluster may not be critical for the full virulence of *S. suis* strain P 1/7. Indeed, the nonpiliated Δ*sfp1* mutant as well as the Δ*srtF* mutant (which expresses Sfp1 monomers but not polymers in its surface) induced as strong sepsis in the mouse as did the WT strain. In addition, since no differences in the interactions with porcine BMEC were observed between the WT and the mutant strains, participation of pili in the first steps of *S. suis* meningitis might be unlikely. Interestingly, besides the *srtF* cluster, only two other putative pili clusters are found in strain P1/7 (Fig. S2) and none of them seem likely to be able to mediate pili formation [18]. Indeed, the first of these additional clusters, designated as the *srtE* cluster, comprises a putative signal peptidase (SSU_0450) as well as the *srtE* gene (SSU_0453). However, this cluster lacks genes encoding the major and the ancillary pilin subunits. Instead, similar to the reported organization of the cluster in the *S. suis* serotype 2 reference strain [43], in strain P1/7 a putative exported protein (SSU_0451) and a transposase fragment (SSU_0452) are found between *sipF* and *srtE* [18 and unpublished data]. The remaining pilus cluster of strain P1/7 is homologous to the *rlrA* pilus island of *S. pneumoniae* TIGR4 [41]. This cluster, named *srtBCD* cluster, contains three sortase-like genes (*srtB*, *srtC*, and *srtD*) and four other genes (designated *sbp1*, *sbp2*, *sbp3*, and *sbp4*), which putatively encode putative cell-wall anchor family proteins containing pilin motifs, E boxes and/or CWSSs [18]. However, *sbp2*, which encodes the putative backbone subunit, is truncated by a nonsense mutation in strain P1/7 [18], leading to the notion that pili cannot be expressed from this cluster. Importantly, further analysis of a large collection of serotype 2 strains indicated that all tested strains of this serotype possessed an incomplete *srtE* cluster similar to that found in strain P1/7, and that they presented the same or other nonsense mutations in the *sbp2* gene encoding the major subunit of the *srtBCD* cluster [18

and unpublished data]. Taken together with our in vivo evaluation in the mouse, these data suggest that pili in general might likely be dispensable for the full virulence of this highly virulent invasive isolate.

Several reports have shown that pili fulfill a myriad of virulence-related functions in different streptococcal pathogens [16], [25], [44]. However, the notion of pili being essential for streptococcal virulence has not been systematically evaluated from an epidemiological perspective. To our knowledge, only a few studies have so far analyzed the correlation between production of pili and virulence using a large number of isolates recovered from the field [22], [44], [45]. In a first study, the pilin-encoding gene *rrgC* (a member of the *rlrA* pilus cluster) was absent from 78% of 484 virulent *S. pneumoniae* strains tested [44]. In addition, the presence of *rrgC* per se did not appear to be associated with increased virulence, since, when present, the gene was found at similar frequencies in nasopharyngeal and septicemic isolates [44]. Similar results (*rlrA* pilus cluster present in only 27% of the virulent strains tested) were obtained when analyzing an essentially different pneumococcal collection of invasive isolates [45]. Finally, a second *S. pneumoniae* pilus (PI-2) was found to be of low prevalence (16%) among clinical isolates [22]. Taken together, these results suggest that despite published data obtained from mouse studies, pili might in fact not represent a central virulence factor for *S. pneumoniae* invasive disease in humans. These *S. pneumoniae* reports [22], [44], [45] sustain therefore findings presented in the present study suggesting that pili may be not critical for the full virulence of some highly invasive *S. suis* isolates. It is to expect that future epidemiological studies carried out with *S. suis* and other streptococci will shed light on the actual contribution of pili to the virulence traits of pathogenic members of this important genus.

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Table I: Bacterial strains and plasmids used in this study.

Strain/Plasmid	General characteristics	Source/Reference
E. coli		
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
BL21λDE3	F^- *omp*T *gal* (*dcm*) (*lon*) *hsd*$S_B(r_B^- m_B^-)$ *endA1* *hsdR17*$(r_K^- m_K^+)$	Invitrogen
S. suis serotype 2		
P1/7	Virulent strain isolated from a pig with meningitis	[58]
31533	Virulent strain isolated from a pig with meningitis	[10]
166'	Virulent strain isolated from a pig with meningitis	[59]
D282	Virulent strain isolated from a pig with meningitis	[60]
S735	Serotype 2 reference strain, isolated from a pig with pneumonia	[61]
24	Virulent strain isolated from a human case of meningitis	[62]
LEF95	Virulent strain isolated from a human case of meningitis	[63]
HUD Limoges	Virulent strain isolated from a human case of septic-shock	[64]
89-1591	Virulent field strain isolated from a pig with septicemia	[65]
Δ*sipF*	Derived from P1/7. In frame deletion of *sipF*	This work
Δ*sfp2*	Derived from P1/7. In frame deletion of *sfp2*	This work
Δ*sfp1*	Derived from P1/7. In frame deletion of *sfp1*	This work
Δ*srtF*	Derived from P1/7. In frame deletion of *srtF*	This work
Δ*srtA*	Derived from P1/7. Deletion of *srtA*	This work
Δ*srtA*comp*srtA*	Δ*srtA* complemented with pSAcomp1	This work
Δ*srtA*compmock	Δ*srtA* transformed with empty pSET-3	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen

pDIA17	Cm^r, *oriR* pACYC184, Tet promoter Δ*lacI*	[66]
pIVEX2.4d	Ap^r, *oriR* pUC, T7 promoter, His-Tag coding sequence	Roche Applied Science
pSET4s	Thermosensitive vector for allelic replacement is *S. suis*. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[49]
p4Δ*sipF*	pSET4s carrying the construct for *sipF* allelic replacement	This work
p4Δ*sfp2*	pSET4s carrying the construct for *sfp2* allelic replacement	This work
p4Δ*sfp1*	pSET4s carrying the construct for *sfp1* allelic replacement	This work
p4Δ*srtF*	pSET4s carrying the construct for *srtF* allelic replacement	This work
pSAD1	pSET4s carrying *cat* flanked by 5′ and 3′ ends of *srtA*	[43]
pSAD11	pSAD1 devoid of *cat*	This work
pSET-3	*S. suis*-*E. coli* shuttle vector	[50]
pSAcomp1	pSET-3 carrying the full length *srtA* gene under the control of *cat* promoter	[43]

Table SI: Oligonucleotide primers used in this study

Primer name	Sequence (5' – 3')	Use
Inter-ID1	CTGGGGATTGGGTGGATAT	Mutant construction
Inter-ID2	TCTTGGTCGGTCTGGGGTTT	Mutant construction
Inter-ID3	CAGAAAACAGACCTGGCCTG	Mutant construction
Inter-ID4	GAACCAAGTATCACCACCAA	Mutant construction
Inter-ID5	TTTGTCATGGGAGATAACCG	Mutant construction
Inter-ID6	TCCATCTCCGCCTGTCGCAGGAGATTGAAC	Mutant construction
Inter-ID7	GTTCAATCTCCTGCGACAGGCGGAGATGGA	Mutant construction
Inter-ID8	ACCACCAAAGTTCGTTGCAG	Mutant construction
SrtF-ID1	GACAGGTGCAGAGTTCAAC	Mutant construction
SrtF-ID2	GTAGGCTTCAATGTCGGATG	Mutant construction
SrtF-ID3	CATAGGCTATTAGTTCGTGG	Mutant construction
SrtF-ID4	TTTTCAAGCCAGAGGACAGA	Mutant construction
SrtF-ID5	CTATGATGCAGCCAACAATG	Mutant construction
SrtF-ID6	ATCATCCAACACTTGGAGCCATTTGATCAT	Mutant construction
SrtF-ID7	ATGATCAAATGGCTCCAAGTGTTGGATGAT	Mutant construction
SrtF-ID8	TCTGCCCGTAGCGCTAGTTC	Mutant construction
Sbp-ID1	AGAATTGGCAGCACCTGATG	Mutant construction
Sbp-ID2	TCCAATCGCACCAGTCAGTACTGCCACAAG	Mutant construction
Sbp-ID3	CTTGTGGCAGTACTGACTGGTGCGATTGGA	Mutant construction
Sbp-ID4	CGGATGCAGTCACATTCCAG	Mutant construction
PSF-ID1	AATTAACTCTGATACATCGCCG	Mutant construction
PSF-ID2	GTATTGCAAATGCTGCAACTAC	Mutant construction
PSF-ID3	TCTCTATAATTGACGGGAGTGG	Mutant construction
PSF-ID4	GACTCTTTCCGGTTATCTCCCATCCTTTCCCTTG AGATTTGAAC	Mutant construction
PSF-ID5	GTTCAAATCTCAAGGGAAAGGATGGGAGATAA CCGGAAAGAGTC	Mutant construction
PSF-ID6	TTGACACTTGCTCAGCAGGG	Mutant construction
Sfp1-fwd	GTGGAGCAGGC**CATATG**ACTGTCTCT [1]	Cloning for protein expression
Sfp1-rev	CACTCCGTCA**GGATCC**TTGACAACCT [2]	Cloning for protein expression
Sfp2-fwd	CCTGCTGAG**CATATG**TCAACAGAGTC [1]	Cloning for protein expression
Sfp2-rev	GGTTGGCGGAGG**GGATCC**AATATTTGA [2]	Cloning for protein expression
Sfp2-fwd2	GGAACTCAAGGA**CATATG**TATCGTTTCT [1]	Cloning for protein expression
Sfp2-rev 2	TTGCTAGC**GGATCC**AGATGTTATGG [2]	Cloning for protein expression

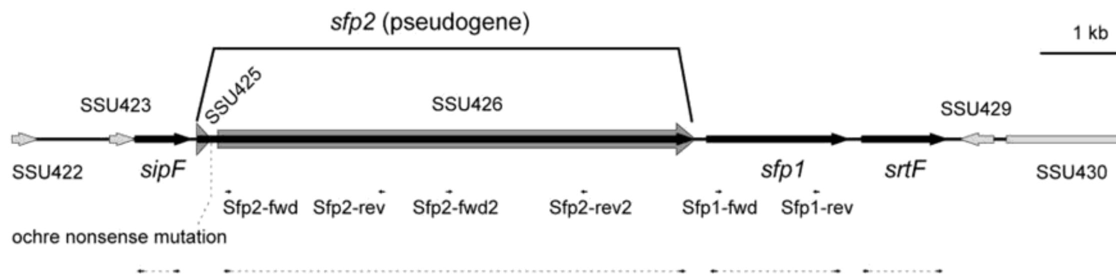

Figure 1. Genetic organization of the *S. suis* strain P1/7 *srtF* pilus cluster.

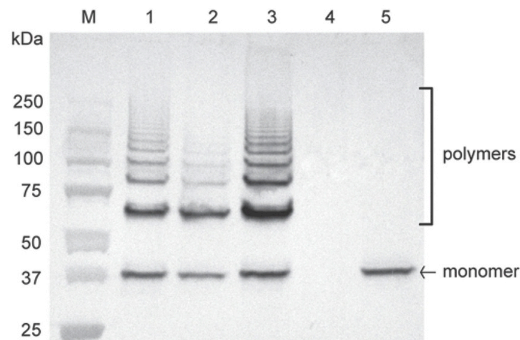

Figure 2. Western-blotting analysis of cell wall-anchored proteins of *S. suis* strain P1/7 and derived mutants with anti-Sfp1 antisera.

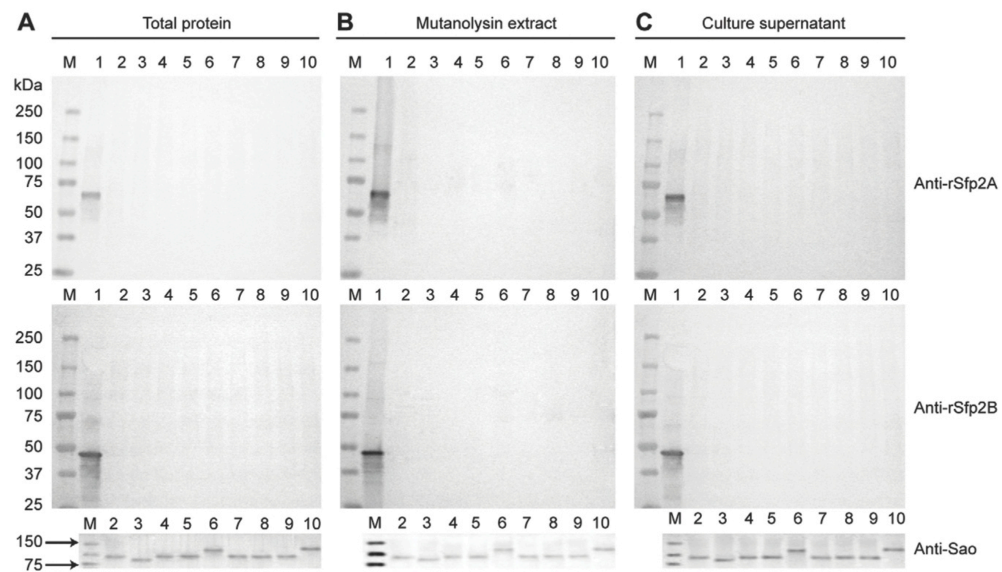

Figure 3. Sfp2 is not produced by *S. suis* strain P1/7 and other highly invasive serotype *S. suis* 2 isolates.

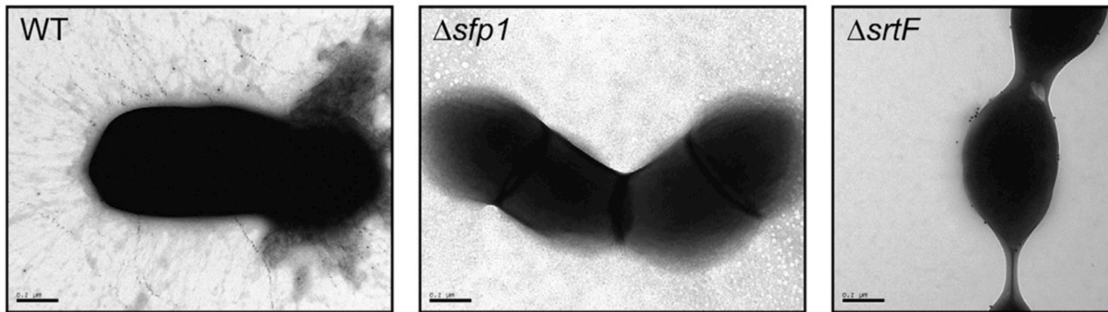

Figure 4. Immunogold labeling and transmission electron microscopy of pilus-like structures on the cell surface of *S. suis* P1/7 and its derived mutants.

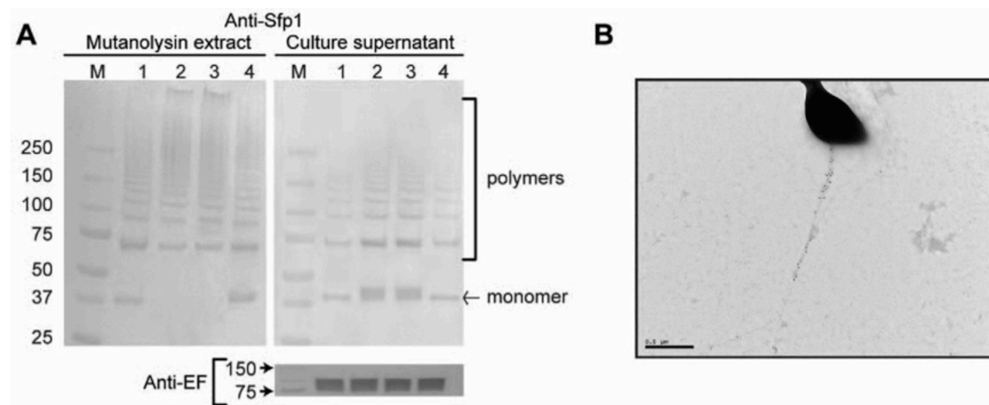

Figure 5. Role of *srtA* in formation of the pilus encoded by the *srtF* cluster.

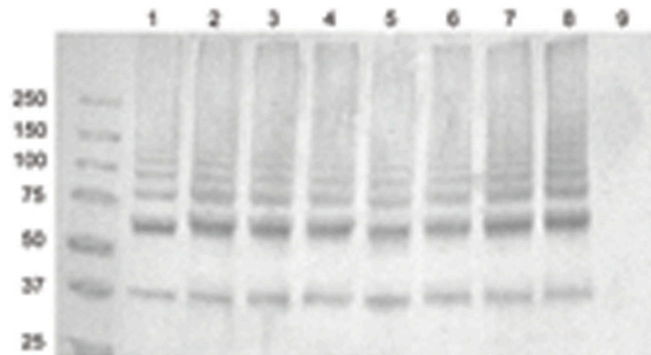

Figure 6. Western-blotting analysis of cell wall-anchored proteins of *S. suis* strain P1/7 and other highly invasive serotype 2 isolates with anti-Sfp1 antisera.

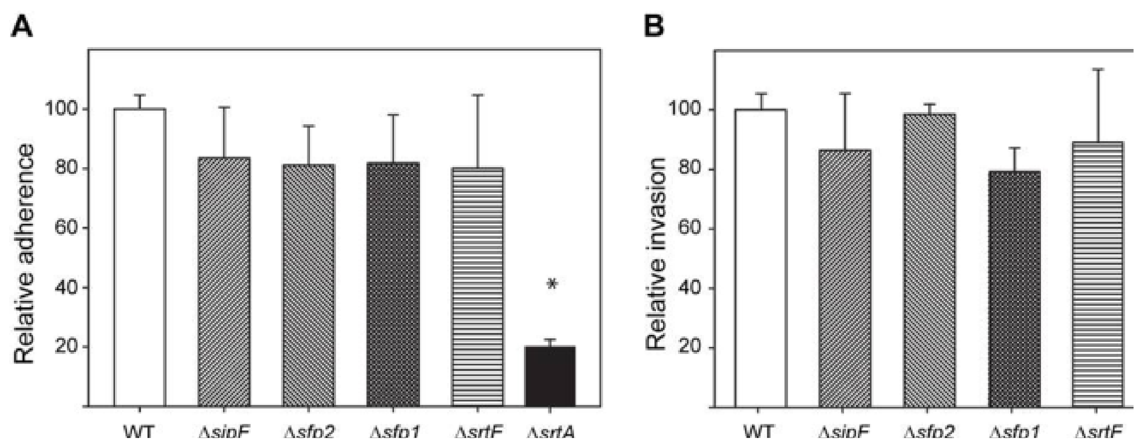

Figure 7. Interactions of the WT piliated strain P1/7 and derived mutants with porcine BMEC.

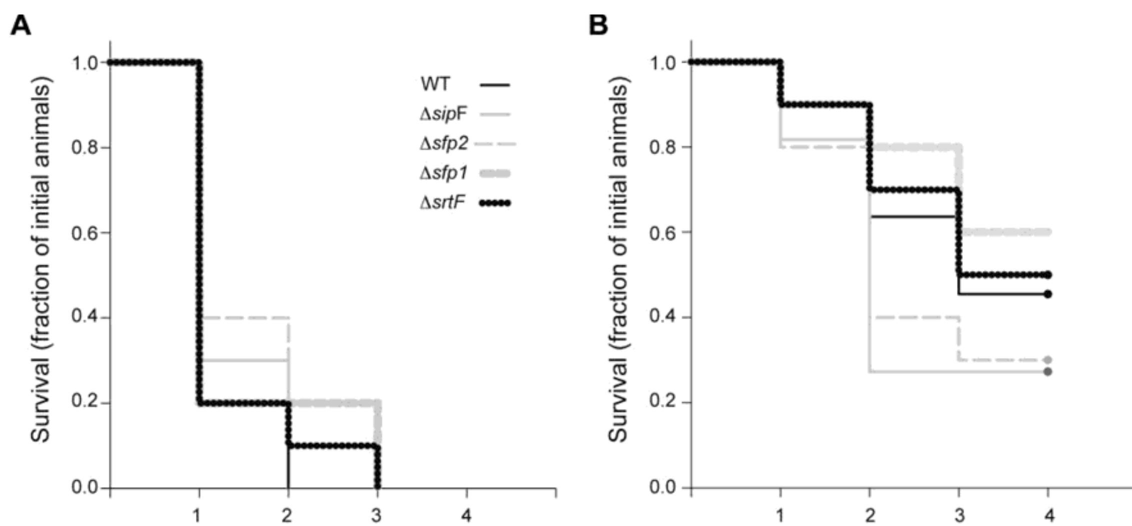

Figure 8. Survival of mice inoculated with the WT piliated strain P1/7 and derived mutant strains.

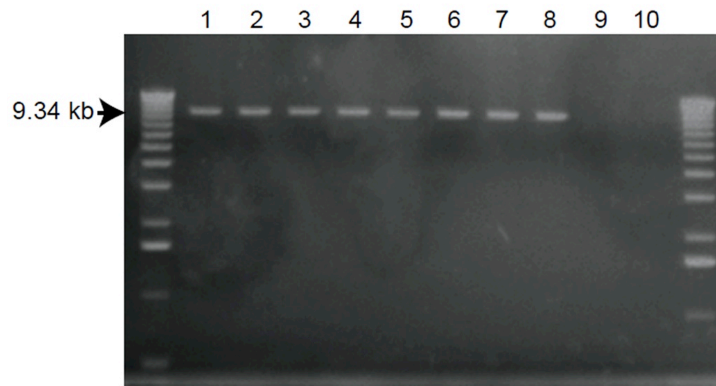

Figure S1. PCR amplification of the *srtF* cluster in different *S. suis* serotype 2 strains using specific primers annealing upstream of *sipF* and downstream of *srtF*. All strains were positive for an 8.34 kb fragment, with the exception of North American strain 89–1591, which had been found not to produce Sfp1 monomers. Lane 1: Strain P1/7. Lane 2: Strain 31533. Lane 3: Strain 166. Lane 4: Strain D24. Lane 5: Strain S735 (serotype 2 reference strain). Lane 6: Strain D282. Lane 7: Strain LEF95. Lane 8: Strain HUD Limoges. Lane 9: Strain 89–1591. Lane 10, no DNA template.

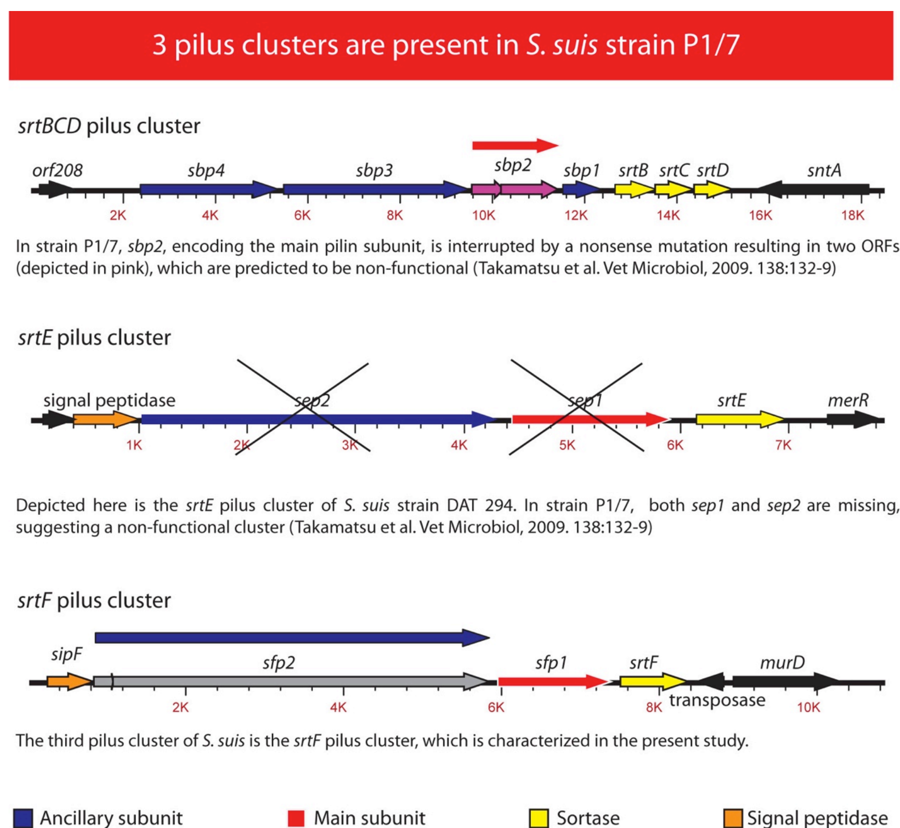

Figure S2. Pilus clusters found in the genome of strain.

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Annexe III

Porcine brain microvascular endothelial cell-derived interleukin-8 is first induced and then degraded by *Streptococcus suis*

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Rôle de la candidate dans la conception de l'article :

Je suis co-auteure de cet article. J'ai participé aux manipulations et aux analyses de résultats.

ABSTRACT

Streptococcus suis is a major pathogen of swine, causing mainly meningitis, and it also represents an emerging zoonotic agent. We investigated its ability to induce the release of pro-inflammatory cytokines and chemokines by porcine brain microvascular endothelial cells (PBMEC). We demonstrated that live *S. suis* induced a strong release of interleukin (IL)-6 and IL-8 by PBMEC. We showed that the suilysin (hemolysin) was largely responsible for such stimulation, although cell wall components also contribute to cell stimulation but to a considerably lower extent. Interestingly, IL-8 production by PBMEC became undetectable by increasing either the incubation time or bacterial concentration of certain live *S. suis* strains. We further demonstrated that this decrease of IL-8 levels was probably linked to the production of a serine protease by *S. suis*. Our results suggest that *S. suis* can induce an exacerbated release of inflammatory mediators by swine endothelial cells that could cause a massive recruitment of leukocytes and subsequent blood–brain barrier breakdown facilitating the pathogenesis of *S. suis*-induced meningitis. In addition, *S. suis* could modulate this response by degrading IL-8 which might delay recruitment of *S. suis* killer-neutrophils to the site of inflammation, allowing this pathogen to survive upon its arrival to central nervous system.

INTRODUCTION

Streptococcus suis serotype 2 is an important swine bacterial pathogen associated mainly with meningitis but also with other infections such as septicemia, endocarditis, and arthritis [1]. Of the 35 serotypes described, serotype 2 is the one most frequently associated with disease [1]. This organism is also recognized as an emerging zoonotic agent with a rising incidence in human over the last years, especially in China, Thailand and Vietnam [2], [3] and [4]. In humans, *S. suis* is mainly associated with cases of meningitis and streptococcal toxic shock-like syndrome (STSLS) [2] and [5]. In 2005, an important outbreak in China resulted in more than 200 human cases (mainly STSLS cases) from which 20% were fatal. This outbreak was directly linked to a concurrent outbreak of *S. suis* infection in pigs [6].

Several virulence factors produced by *S. suis* have been proposed to be involved in the pathogenesis of the infection. A hemolysin, named "suilysin" [7], has been involved in the modulation of *S. suis* interactions with host cells [8], [9] and [10]. The suilysin possesses a multi-hit mechanism of action and belongs to the thiol-activated cholesterol-binding cytolysin family [7] and [11]. In addition to the suilysin, a capsular polysaccharide (CPS) [12], a serum opacity factor (OFS) [13], a fibronectin- and fibrinogen-binding protein (FBPS) [14], adhesins [15], different proteases [16], and other proteins [17] have also been proposed as virulence determinants. However, there is a certain confusion in the literature about the description of *S. suis* virulence and the role of these factors has not been clearly established [18] and [19].

Several questions regarding the pathogenesis of *S. suis* infection remain unanswered. One of these is how the pathogen reaches the central nervous system (CNS) to cause meningitis in swine. It has been suggested that this key step could be achieved by crossing the porcine blood–brain barrier (BBB) by transcytose through porcine brain microvascular endothelial cells (PBMEC) [8] and/or porcine choroid plexus epithelial cells (PCPEC) [20], as well as by disruption of the barrier caused by toxic effects of suilysin on BBB-forming cells [8] and [21]. In addition, inflammation of the CNS seems to play a critical role in the pathogenesis of *S. suis* infection [22]. Hence, up-regulated expression of pro-inflammatory mediators and leukocyte trafficking might also contribute to increased BBB permeability

[19]. Human BMEC have been shown to produce interleukin (IL)-6, IL-8 (CXCL8), and monocyte chemotactic protein (MCP)-1 (CCL2) following activation by *S. suis* [23]. Moreover, *S. suis*-infected human monocytes have been reported to secrete tumour necrosis factor (TNF)-α, IL-1β, IL-6, IL-8 and MCP-1 and to up-regulate the expression of adhesion molecules which increases their adhesion to endothelial cells [24], thus modulating leukocyte infiltration. However, no studies have addressed the capacity of *S. suis* to up-regulate inflammatory mediators by the intermediary of BBB endothelial cells from the natural host, the swine.

In the present study, we used PBMEC to investigate the induction of pro-inflammatory cytokines and chemokines by *S. suis* and to determine the contribution of different bacterial components to cytokine secretion. Our results suggest that *S. suis* serotype 2 is able to induce, at first, the secretion of IL-6 and IL-8 from PBMEC, and that this activation is mainly mediated through the secretion of the suilysin. After this first step that increases inflammation at the CNS, *S. suis* would later produce a protease that is able to degrade IL-8, consequently delaying recruitment of neutrophils to the site of inflammation, allowing this pathogen to survive and to cause damage.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. Strain 31533 is a representative virulent European suilysin-positive *S. suis* serotype 2 strain already used in most of our previous studies [23], [27], [31] and [32]. Two mutants derived from this strain (B218, an unencapsulated mutant [12] and SX911, a suilysin-negative mutant [37]) were also tested. For comparative purposes, several other *S. suis* serotype 2 strains of known origin and phenotype were tested. Bacteria were grown as previously described for 16 h [8], and were washed twice in phosphate-buffered saline (PBS, 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), and were appropriately diluted in cell culture medium before infection of PBMEC. An accurate determination of the number of colony forming units per milliliter (CFU/ml) in the final suspension was made by plating onto Todd Hewitt Broth (THB; Difco Laboratories, Detroit, MI, USA) agar using Autoplate® 4000 (Spiral Biotech, Norwood, MA, USA). In selected experiments, heat-killed bacterial suspensions were used for stimulation assays. Heat-killed bacteria were produced as described previously by incubating organisms at 60 °C for 45 min, which is the minimal condition required to kill *S. suis* cultures [32]. Killed bacterial preparations were stored in PBS at 4 °C, and the required number of bacteria was resuspended in cell culture medium immediately before stimulation assays.

Bacterial components

Purified CPS and purified cell wall of *S. suis* serotype 2 were prepared as previously reported [32] and [53] and were used at 100 µg/ml. Purified *S. suis* lipoteichoic acid was a gift from S. Von Aulock (University of Konstanz, Germany) and was used at 100 µg/ml. Purified suilysin was kindly provided by A. Jacobs (Intervet International, Boxmeer, The Netherlands) and activated by addition of 0.1% 2β-mercaptoethanol before its use at 100 ng/ml. Ultra-purified *Escherichia coli* O55:B5 lipopolysaccharide (LPS) (Apotech Corp, Epalinges, Switzerland) was used at 10 µg/ml as a positive control for stimulation assays.

Cell culture

The PBMEC/C1-2 cell line [54] was cultivated as previously described [8]. Briefly, cells were grown in complete IF medium, which is a mixture of 1:1 Iscove's modified Dulbecco's medium and Ham's F-12 (Invitrogen, Burlington, ON, Canada) supplemented with 7.5% (v/v) heat-inactivated foetal bovine serum, penicillin–streptomycin (Invitrogen), sodium bicarbonate, L-glutamine, human transferrin (MP Biomedicals, Solon, OH, USA), *N*-acetyl-cysteine, hypoxanthine, porcine heparin, human recombinant fibroblast growth factor-basic (Sigma–Aldrich, Oakville, ON, Canada), and β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA). Flasks (Falcon; Becton–Dickinson, Mississauga, ON, Canada) and 24-well tissue culture plates (Primaria, Falcon) were precoated with 1% (w/v) type A gelatin from porcine skin (Sigma) to support the cells. Cells were incubated at 37 °C with 5% CO_2 in a humid atmosphere. For assays, PBMEC were trypsinized by adding a trypsin–EDTA solution (Invitrogen) and diluted in culture medium at 8×10^4 cells/ml. The cell suspension was distributed in tissue culture plates and incubated until confluence. Before the experiments, medium was removed from plates and replaced by medium without antibiotics.

Cell stimulation

Bacteria (at different concentrations) or purified components diluted in complete IF medium supplemented with 25% complete swine serum were added to the cell monolayer. Plates were then centrifuged at $800 \times g$ for 10 min and incubated at 37 °C in 5% CO_2. Stimulated or unstimulated (control) samples were harvested at 6, 12, 24, and 48 h and supernatants were aliquoted and frozen at −20 °C until analysis. Cells incubated in medium alone served as controls for spontaneous cytokine release. In inhibition assays, live strain 31533 was added in combination with ethanol-soluble cholesterol (Sigma–Aldrich) at 2 μg/ml. Similar inhibitory condition was previously established in our laboratory and do not affect either cell or bacterial viability [10] and [21]. At least 3 independent experiments (stimulation assays) were carried out.

Cytokine quantification by ELISA

IL-1β, TNF-α, IL-6, and IL-8 were measured by ELISA using porcine-specific pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations. Twofold dilutions of recombinant porcine IL-1β (100–6000 pg/ml, R&D Systems) TNF-α and IL-6 (78–5000 pg/ml, R&D Systems) and porcine IL-8 (18–1200 pg/ml, R&D Systems) were used to generate standard curves. Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. Standard and sample dilutions were added in duplicate wells to each ELISA plate (Nunc, VWR, Ville Mont Royal, QC, Canada), and all analyses were performed at least four times for each individual stimulation assay. Plates were read in a Molecular Devices UVmax (Molecular Devices Corp, Sunnyvale, CA, USA) microplate reader. For each incubation time, basal cell production of cytokines was corrected from the production level obtained after incubation with *S. suis* or its components to represent the specific induced levels of production only.

Endotoxin contamination

All solutions and bacterial preparations used in this study were tested for the presence of endotoxin by a *Limulus* amebocyte lysate (LAL) gel clot test (Pyrotell STV, Cape Cod, Falmouth, MA, USA) with a sensitivity limit of 0.03 EU ml. In some experiments, endotoxin contamination during stimulation of endothelial cells was controlled by parallel assays with polymyxin B (10 µg/ml) from Sigma–Aldrich. Results from the LAL test and/or data from polymyxin B treatment demonstrated no significant levels of endotoxin contamination from different bacterial preparations (data not shown).

IL-8 degradation assay

Bacteria were grown in THB to reach the exponential growth phase as for the stimulation assay and culture supernatants were obtained by centrifugation at 4000 × *g* for 20 min, and filtration using 0.22 µm filters (Millipore Billerica, MA, USA). Then, supernatants or whole live bacteria were freshly used for IL-8 degradation assays. For this, porcine recombinant IL-8 (R&D Systems) diluted in culture IF medium, to mimic the environmental conditions from the cell stimulation assay, at a final concentration of

10 ng/ml was incubated with bacterial supernatant or THB, as control, for 6 and 9 h at 37 °C at a final ratio of 1:10. For experiments using whole bacteria, this mixture was spun down and samples were frozen at −70 °C for further analysis by ELISA as described above.

To confirm the nature of the *S. suis* protease, the bacterial supernatant was pre-incubated for 30 min at 37 °C with the following serine protease inhibitors: Pefabloc SC (4 mM, Roche Diagnostics, Laval, QC, Canada), or aprotinin (10 µg/ml, Roche). In addition, the assay was performed in the presence of complete Mini protease inhibitor cocktail tablets (Roche), which efficiently inhibit serine, cysteine and metalloproteases, according to the manufacturer's recommendations. Residual IL-8 content was measured by ELISA.

SDS-PAGE and Western blotting

To confirm IL-8 degradation by *S. suis*, a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was also used. Briefly, a 16-h *S. suis* NCTC 10234 strain culture was used to inoculate a fresh THB medium (1/10, v/v) and incubated until it reached an optical density at 600 nm of 0.25. Then, bacteria were washed once with PBS, resuspended in cell culture IF medium containing 0.1% of foetal bovine serum to a quarter of their original volume and incubated for an additional hour at 37 °C to reach the exponential growth phase. Supernatant was obtained after centrifugation at 4000 × *g* for 20 min, and filtrated using 0.22 µm filters (Millipore, Billerica). Then, *S. suis* supernatant or cell culture medium was incubated with 5 µg/ml rIL-8 in the presence of 20 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.2 [50] for 20 h. Thereafter, samples were boiled for 5 min in sample buffer containing SDS before being subjected to 17.5% SDS-PAGE. Protein bands were visualized either by non-oxidative silver staining [55] or by Western blotting. For immunoblotting, gels were electroblotted onto 0.2 µm nitrocellulose membrane (Bio-Rad). After 1 h of blocking in Tris-buffered saline–NaCl (10 mM Tris base, 150 mM NaCl, pH 7.4)–2% non-fat dry milk, membranes were washed and incubated for 2 h at room temperature with polyclonal anti-porcine IL-8 antibody (R&D Systems). After a washing step, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and protein bands were visualized by adding 3.3 mM 4-chloro-1-naphthol in cold methanol with 0.003% (v/v) hydrogen peroxide in Tris-buffered saline–NaCl. The reaction was stopped by washing the nitrocellulose blot in water.

Statistical analysis

Each cell stimulation assay and ELISA was performed at least in triplicate, and each sample was analyzed in duplicate in ELISA assay. All data are expressed as means ± standard deviations (error bars). Data were analyzed by Mann–Whitney *U* test. A $P < 0.05$ was considered as significant.

RESULTS

Kinetics and dose–response of cytokine secretion

The stimulation of PBMEC with live suilysin-positive *S. suis* strain 31533 (10^6 CFU/ml) or LPS (10 μg/ml, positive control) resulted in time-dependent IL-6 and IL-8 release (Fig. 1A and B). For IL-6, stimulation with different concentrations of live (10^3–10^7 CFU/ml) *S. suis* strain 31533 was dose-dependent and reached a plateau at 10^5 CFU/ml (Fig. 1C). However, a bell-shaped response was observed for IL-8 with peak of production at 10^5 CFU/ml of live *S. suis* strain 31533 (Fig. 1D). No production of IL-1β or TNF-α above the limits of detection of the ELISA test was observed, even after stimulation of PBMEC with high doses of *S. suis* or LPS (data not shown). It should be noted that, using Trypan blue staining and lactate dehydrogenase release measurement assay, no cytotoxicity effects were observed at the bacterial doses used in the present study (data not shown). Moreover, pH of the growth medium did not vary significantly and remained around 7.5 over the incubation period (from 0 h to 48 h).

Role of bacterial components in cytokine production

Purified *S. suis* CPS, cell wall (CW) and lipoteichoic acid (LTA) were used to evaluate their relative contribution to IL-6 and IL-8 production at selected doses based on our previous publications [22] and [23]. In addition, the nonencapsulated mutant strain B218, derived from the wild-type strain 31533, was used to stimulate PBMEC. After 24 h of incubation, the nonencapsulated mutant B218 showed a significantly higher ability to induce the expression of IL-6 and IL-8 in comparison to its encapsulated wild-type strain 31533 ($P < 0.0001$; Fig. 2). Moreover, the higher level of PBMEC stimulation induced by mutant B218 was observed at all incubation times tested (6–48 h) (data not shown). Purified CPS was shown to be a weak cytokine inducer (Fig. 2A and B) and probably partially interferes with the stimulation capacity of the bacterial CW in the wild-type strain 31533. Exposed CW in mutant B218 was likely to be partially responsible for this higher activation since purified CW also significantly stimulated PBMEC to release both cytokines (Fig 2A and B). However, purified LTA failed to induce the release of IL-6 or IL-8 by PBMEC (Fig. 2A and B).

The role of *S. suis* suilysin in cytokine secretion was evaluated using the non-hemolytic mutant strain SX911 derived from strain 31533. Surprisingly, and in comparison with its wild-type strain, mutant SX911 induced almost no IL-6 response and a weak IL-8 response by PBMEC ($P < 0.05$, Fig. 2A and B). Accordingly, purified suilysin induced high levels of IL-6 and IL-8 secretion (Fig. 2A and B). To confirm the role of suilysin in the induction of cytokine release, PBMEC were stimulated with strain 31533 in the presence of ethanol-soluble cholesterol, known to bind and block suilysin activity [10], [21] and [25]. Under these conditions, a significantly lower release of both cytokines was observed after 24 h of PBMEC stimulation ($P < 0.05$, Fig. 2A and B).

To further delineate the role of suilysin in the induction of cytokine production, different suilysin-positive and -negative strains, listed in Table 1, were tested for IL-6 (24 h of incubation) and IL-8 (12 h of incubation). Suilysin-positive strains ($n = 10$) induced a significantly stronger IL-6 and IL-8 response than suilysin-negative strains ($n = 11$), with a mean of 1500 pg/ml and 50 pg/ml for IL-6, respectively ($P = 0.0001$), and a mean of 12 500 pg/ml and 2500 pg/ml for IL-8, respectively ($P = 0.001$). A shorter incubation time to study IL-8 response was used because of decreasing IL-8 levels detected after 24 h of incubation as described below.

Evidences suggesting IL-8 degradation during stimulation of PBMEC by *S. suis*

Due to the bell-shaped IL-8 response obtained with different bacterial concentrations (Fig. 1D), the induction of this cytokine by selected representative strains was also studied at 12, 24 and 48 h of incubation. Bacterial growth rates were similar for each tested strain (data not shown). Interestingly, IL-8 release induced by several strains decreased to basal levels (selected strains shown in Fig. 3A). One of the latter was the *S. suis* reference strain of serotype 2, NCTC 10234. In more detail, Fig. 3B shows live NCTC 10234-induced IL-8 level, which reaches its maximum at 12 h of incubation and then showed progressive diminution to reach basal production level at 48 h. Surprisingly, when heat-killed NCTC 10234 was used, an increased production of IL-8 was observed over time with maximal production at 48 h, which was in marked contrast to the kinetics observed with live bacteria (Fig. 3B). Furthermore, when different concentrations of live NCTC 10234 were used to stimulate PBMEC, a strongly accentuated bell-shaped IL-8 response was observed, which

peaked at 10^4 CFU/ml and was almost negative at 10^6 and 10^7 CFU/ml (Fig. 3C). As mentioned above, this effect was neither related to bacterial cytotoxic effects (data not shown) nor to pH variations.

IL-8 degradation

Data mentioned above prompted us to test whether *S. suis* could possess a proteolytic activity that may be responsible for IL-8 degradation. In order to verify this fact, induction of IL-8 by LPS was used as a reporter system. Positive control LPS (10 μg/ml) alone and LPS in combination with live *S. suis* strain NCTC 10234 (10^6 CFU/ml) were used to stimulate PBMEC. Fig. 4 shows a time-dependent decrease in LPS-induced IL-8 levels in the presence of live *S. suis* strain NCTC 10234 after 24 and 48 h ($P < 0.05$) that is consistent with results obtained from kinetics of IL-8 production with live *S. suis* strain NCTC 10234.

Following incubation of swine recombinant IL-8 with supernatant of strain NCTC 10234, a time-dependent decrease of rIL-8 was detected using ELISA (Fig. 5) compared to rIL-8 level observed after incubation with THB alone. In addition, the degradation assay was performed in the absence and presence of class-specific protease inhibitors. Complete Mini protease inhibitor cocktail tablets, aprotinin, and Pefabloc SC significantly inhibited IL-8 degradation (Fig. 5) suggesting that the protease responsible for such degradation is of the serine class. Also, incubation of rIL-8 with whole live bacteria led to bacterial dose-dependent IL-8 degradation (data not shown). Finally, using SDS-PAGE followed by silver staining or Western blotting with polyclonal anti-porcine IL-8 antibody, disappearance of ∼9 kDa porcine rIL-8 was observed following incubation with supernatant of strain NCTC 10234 (Fig. 6). It should be noted that recombinant TNF-α, IL-1β, and IL-6 were also incubated with supernatant from strain NCTC 10234 and no degradation was observed (data not shown).

DISCUSSION

Despite recent advances over the past years, there are still important gaps in the knowledge of the pathogenesis of swine infection caused by *S. suis*. However, the passage throughout the BBB by the bacteria to reach the CNS is considered as a crucial stage in this complex process. We recently demonstrated that *S. suis* is able to invade BBB-forming PBMEC and to induce cellular damage by means of its toxin, suilysin, at high bacterial concentrations only [8]. These findings suggest that BBB may be the portal of entry for the bacteria into the brain. In fact, *S. suis* is commonly recovered from brains of diseased pigs with clinical signs of meningitis [26]. Moreover, microscopic analysis of *S. suis*-infected mouse brain sections showed that cells expressing transcripts for Toll-like receptor 2, CD14, IκBα (an index of NF-κB activation), and MCP-1 were associated with microvascular vessels early after infection, suggesting that endothelial cells play an important role in the pathogenesis of *S. suis*-related meningitis [27].

Inflammation produced by cells from the innate immune system can be considered as a two-sided medal. Then, even if this inflammatory response is necessary to overcome an acute infection, it can potentially contribute to immune-mediated pathology. Well-documented examples for this negative effect are uncontrolled bacteria-induced meningeal inflammation [28] and septic shock with consequent clinical deterioration [29]. Suppurative or fibrinopurulent histopathological lesions in the brain, heart, lungs and serosae have been reported in *S. suis* infections [30]. During meningitis, microglial, monocytic cells, and migrating leukocytes could be the source of pro-inflammatory cytokines in the cerebrospinal fluid. We have previously reported that *S. suis* stimulates the release of pro-inflammatory cytokines by human and murine cells [23], [31] and [32]. More recently, we reported release of inflammatory mediators by cells from swine whole blood, namely monocytes, neutrophils and lymphocytes [22]. Monocytes stimulated with *S. suis* also expressed adhesion molecules, that could facilitate leukocyte transmigration through endothelial cells from BBB [24]. Moreover, it was recently shown that *S. suis* induces the release of arachidonic acid by human BMEC [33]. Nevertheless, no studies have been conducted concerning a putative passage through swine BBB that could be facilitated by uncontrolled inflammation and massive leukocyte recruitment.

As a well-known endogenous pyrogen and an inducer of acute-phase response, IL-6 also displays several pro-inflammatory properties such as maturation and activation of neutrophils and macrophages, and differentiation and maintenance of cytotoxic T cells and natural killer cells [34] and [35]. IL-8 is a CXC chemokine and a potent chemotactic factor for neutrophils (polymorphonuclear cells, PMN) and T lymphocytes, a PMN activator and an adhesion molecule inducer on PMN and endothelial cells [36]. Results obtained in this study showed that *S. suis* does stimulate BBB-forming PBMEC to release these pro-inflammatory mediators. Hence, PBMEC release detectable levels of IL-6 and IL-8 after contact with most of *S. suis* strains used in this study. The time- and dose-dependent release of IL-6 by *S. suis*-stimulated PBMEC was similar to the reported IL-6 production by other cell types stimulated by *S. suis* [23] and [31].

According to the results obtained in this study with non-hemolytic mutant SX911, purified suilysin, cholesterol, and different suilysin-positive or -negative *S. suis* strains, it was shown that the suilysin is an important factor for IL-8 and IL-6 induction by PBMEC stimulated with *S. suis*. Previous studies using swine whole blood cells [22] and human pulmonary alveolar macrophages and monocytes [37] as well as human BMEC [23] showed that, although suilysin does stimulate cells, it only played a limited role in the inflammatory response to *S. suis*. Moreover, Jobin *et al.* [33] reported that suilysin was involved in the release of arachidonic acid, a prostaglandin precursor, from the membrane of human BMEC. Interestingly, other cholesterol-binding cytolysins, such as listeriolysin, pneumolysin, and streptolysin O, are also recognized to stimulate inflammatory response [38], [39] and [40]. Suilysin has been shown to be cytotoxic for many different cell types, but only at very high bacterial concentrations [8] and [21] and it was shown to interfere with the ability of neutrophils to kill *S. suis* [9]. Thus, suilysin has multiple effects toward endothelial cells from BBB and other cells involved in innate immunity, that could help *S. suis* to reach the CNS.

The actual mechanism of action of suilysin leading to the release of cytokines and/or chemokines is unknown and remains to be elucidated. The pneumolysin from *Streptococcus pneumoniae* recognizes Toll-like receptor 4 (TLR4) [41]. Then, it would be

tempting to speculate on a similar mechanism for the suilysin and PBMEC. However, TLR expression on BMEC is poorly known and there is no study on TLR expression on PBMEC. On the other hand, *in vivo* studies in mice carried out in our laboratory indicate a strong TLR2 and CD14, but not TLR4, activation in the brains of mice experimentally infected with the suilysin-positive *S. suis* strain 31533 [27]. These data confirmed our previous *in vitro* studies which also showed that the same suilysin-positive *S. suis* strain stimulates the expression of TLR2, but not TLR4, in human monocytes [42].

Multiple bacterial components seem to be responsible for PBMEC activation. In fact, and in addition to the suilysin, other bacterial components such as those from the cell wall components also seem to be cytokine inducers. This is in agreement with our previous observations with murine macrophages [32] and human BMEC [23]. Surprisingly, LTA from *S. suis* did not induce cytokine secretion by PBMEC. These results diverge from those obtained in others studies demonstrating that LTA of Gram-positive [43] bacteria possesses high inflammatory capacity. However, a recent report from Draing *et al*. [44] showing that LTA stimulation activity depends on its surface presentation could explain our results since we used purified LTA in solution to stimulate PBMEC. *S. suis* cell wall inducing activity could also be due to other components, such as peptidoglycan [45]. The higher stimulatory capacity of the unencapsulated mutant B218 observed in this study could be due to either the better surface exposition of cell wall components or an increased release of suilysin of this mutant compared to its wild-type strain (D. Grenier, unpublished information) or both.

Interestingly, an unexpected pattern of IL-8 production was observed with bell-shaped IL-8 levels depending on the live bacterial concentration used to stimulate the cells. Decreasing patterns of IL-8 levels were observed, especially with several of the suilysin-producing strains. However, this decrease was not observed using killed bacteria. This is in agreement with our previous study in which higher levels of IL-8 using a swine whole blood model were detected after stimulation with killed rather than live *S. suis* [22]. Results also indicated that IL-8 degradation was not related to bacterial growth (and availability of nutrients) or pH variation of the media. In fact, results obtained using a reporter system and specific inhibitors suggest that decrease of IL-8 level was caused by its degradation through a serine protease activity produced by *S. suis*. As virulence factors, bacterial proteases are

involved in several functions, such as iron acquisition [46], immunoglobulin [47] and complement factor degradation [48], and tissue destruction [49]. Recently, production of several proteases by *S. suis* such as Arg-aminopeptidase, chymotrypsin-like, caseinase, and dipeptidyl peptidase IV activities has been reported [16]. To the best of our knowledge, this is the first study reporting the presence of a chemokine-degrading protease in *S. suis*. Recently, *Streptococcus pyogenes* (GAS) was shown to produce ScpC (also known as SpyCEP), a CXC chemokine protease [50] and [51] that is different from the C5a peptidase (ScpA) and the broad-spectrum cysteine protease SpeB produced by that pathogen [50]. ScpC was shown to impair PMN recruitment and was essential for virulence in mouse [52].

Confirming this hypothesis, disappearance of ∼9 kDa porcine IL-8 was observed using SDS-PAGE consistently with previous studies with GAS ScpC which reported disappearance of ∼8 kDa human IL-8 [51] and [52]. ScpC cleaves the CXC human chemokine IL-8 and the functional murine homologs KC and MIP-2 [52]. LPS-induced CXC chemokine (LIX) is also degraded but to a lesser extent, while the CC chemokine RANTES is not degraded [52]. Our results showed degradation of swine CXC chemokine IL-8, but not swine pro-inflammatory cytokines TNF-α, IL-1β and IL-6. However, other chemokines need to be tested to confirm the CXC-specificity of *S. suis* serine protease activity. Sequence comparison between ScpC gene from GAS and complete *S. suis* genomes was also performed using available sequence data for European strain P1/7 produced by the *S. suis* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_suis), for North American strain 89-1591 produced by the Joint Genome Institute Microbial Genomics (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=strsu&advanced=1), and for two Chinese isolates 98HAH33 and 05ZYH33 produced by the Beijing Institute of Genomics, Chinese Academy of Sciences (available through GenBank). An ScpC homologous gene was found in each available complete *S. suis* genome (about 47% of homology). Studies are undergoing in our laboratory to further characterize this protease and its actual role in *S. suis* pathogenesis.

Finally, and since the cells used in this study did not produce IL-1β and TNF-α with the positive control used, no conclusion on a possible induction of any of these cytokines by *S. suis* could be obtained. Interestingly, similar negative results were obtained with human

BMEC [23] and in an *in vivo* study using brain coronal sections of *S. suis*-infected mice that showed a lack of positive transcription signal for TNF-α and IL-1β in microvascular vessels by *in situ* hybridization [27].

Taken together, our results suggest that *S. suis* serotype 2 is able to induce, at first, the production of IL-6 and IL-8 from PBMEC, that may cause an important recruitment of leukocytes and subsequent BBB breakdown facilitating the pathogenesis of *S. suis*-induced meningitis. In a second time, *S. suis* could modulate this response by degrading IL-8 and consequently delaying recruitment of neutrophils to the site of inflammation, allowing this pathogen to survive and to cause meningitis.

ACKNOWLEDGEMENTS

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Table I: *Streptococcus suis* strains used in this study.

S. suis strains	Description		
31533	France	Diseased pig	sly+
NTCT 10234[a]	The Netherlands	Diseased pig	sly+
P1/7[b]	United Kingdom	Diseased pig	sly+
D282	The Netherlands	Diseased pig	sly+
24	France	Diseased pig	sly+
166'	France	Diseased pig	sly+
SX332	United States	Diseased pig	sly+
95-8242	Canada	Diseased pig	sly+
LEF95	France	Human, meningitis	sly+
H11/1	United Kingdom	Human, meningitis	sly+
89-1591[c]	Canada	Diseased pig	sly-
98-8993	Canada	Diseased pig	sly-
98-B099	Canada	Diseased pig	sly-
89-999	Canada	Diseased pig	sly-
AAH4	United States	Diseased pig	sly-
90-1330	Canada	Diseased pig	sly-
TD10	United Kingdom	Healthy pig	sly-
94-623	France	Healthy pig	sly-
T15	The Netherlands	Healthy pig	sly-
3889	The Netherlands	Healthy pig	sly-
AR770353	The Netherlands	Human, meningitis	sly-
B218[d]	Nonencapsulated mutant from 31533		
SX911[e]	Non-hemolytic mutant from 31533		

a ATCC 43765 *S. suis* type 2 reference strain

b *S. suis* Sequencing Group at the Sanger Institute

c Joint Genome Institute Microbial Genomics

d [56]

e [37]

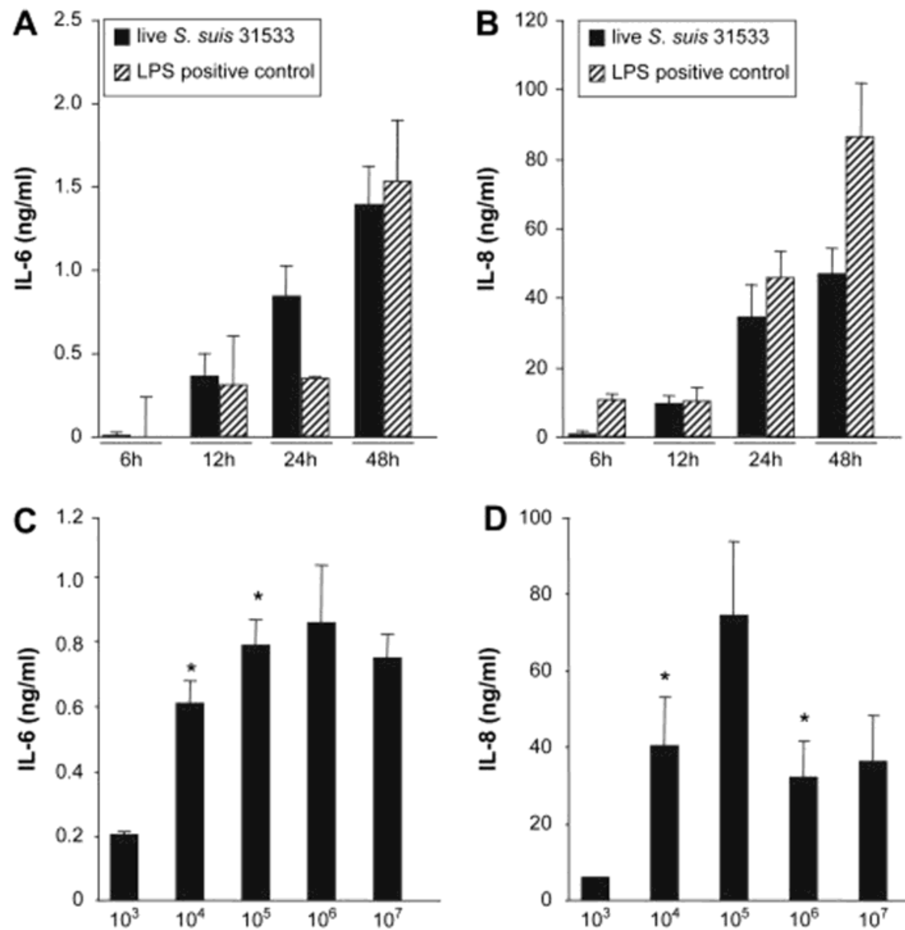


Figure 1. Kinetics of (A) IL-6 and (B) IL-8 release by PBMEC stimulated by live (10^6 CFU/ml), *S. suis* strain 31533 or positive control *E. coli* O55:B5 LPS (10 µg/ml). Effect of live *S. suis* strain 31533 concentration on IL-6 (C) and IL-8 production (D) by PBMEC after 24 h of incubation. * denotes values that are significantly different ($P < 0.05$) from those obtained with the preceding lower concentration of live bacteria. Unstimulated cells were used as control for basal production which was corrected from each experimental data obtained after stimulation. Data are expressed as the mean ± SD in ng/ml of cytokine secretion as measured from supernatants using ELISA.

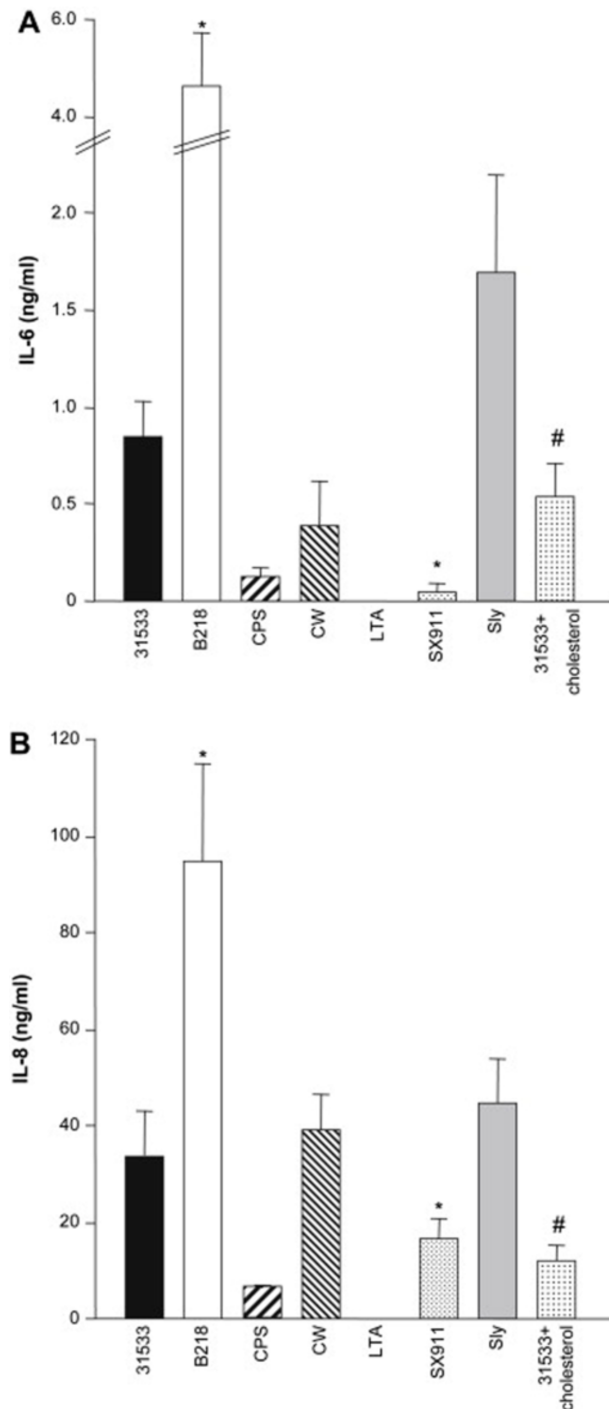


Figure 2. Contribution of different bacterial components to (A) IL-6 and (B) IL-8 production by PBMEC stimulated with live *S. suis* strain 31533 (10^6 CFU/ml), live nonencapsulated mutant B218 (10^6 CFU/ml), CPS (100 µg/ml), CW (100 µg/ml), LTA (100 µg/ml), live suilysin-negative mutant SX911 (10^6 CFU/ml), suilysin (100 ng/ml), or live *S. suis* strain 31533 (10^6 CFU/ml) in the presence of 2 µg/ml of cholesterol. Data are expressed as the mean ± SD in ng/ml of cytokine secretion at 24 h post-stimulation as measured from supernatants using ELISA. * denotes values obtained with the mutant strains that are significantly different ($P < 0.05$) from those obtained with the wild-type strain 31533 alone. # denotes significant differences between cholesterol treated vs non-treated 31533 strain.

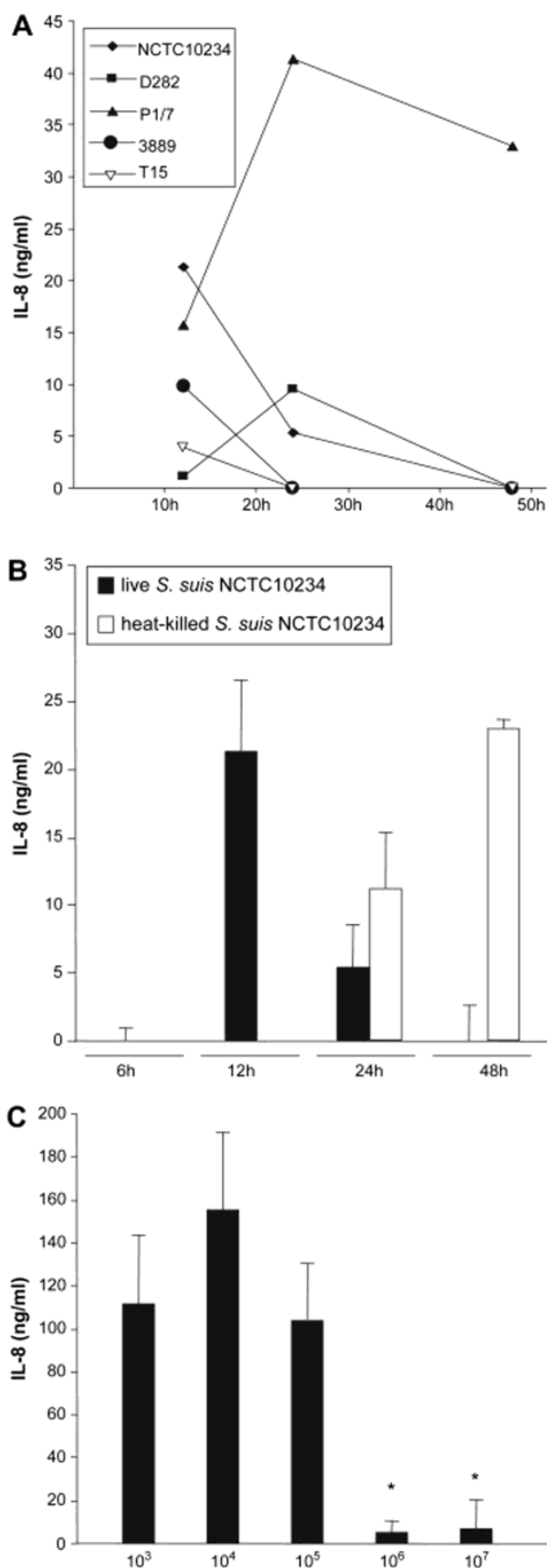


Figure 3. (A) Kinetics of IL-8 release by PBMEC stimulated with live (10^6 CFU/ml) selected *S. suis* serotype 2 strains. (B) Kinetics of IL-8 release by PBMEC stimulated with live (10^6 CFU/ml) (black bars), and heat-killed (10^9 CFU/ml) (white bars) *S. suis* strain NCTC 10234. (C) Effect of live *S. suis* strain NCTC 10234 concentration on IL-8 production by PBMEC after 24 h of incubation. IL-8 production was measured by ELISA titration of stimulated cell supernatants. * denotes values that are significantly different ($P < 0.05$) from those obtained with the 10^4 concentration of live bacteria. Data are expressed as the mean ± SD in ng/ml.

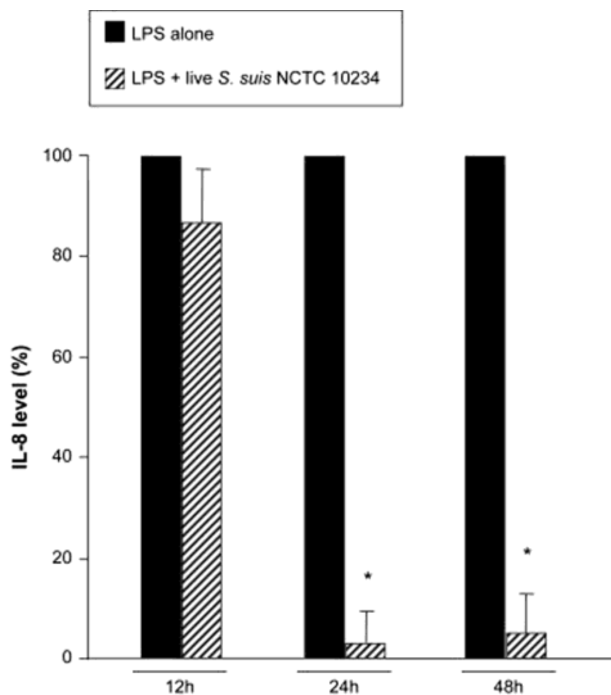
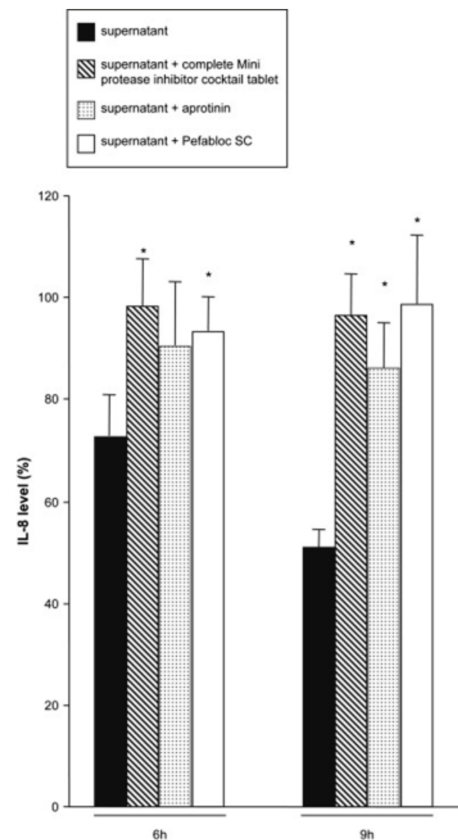


Figure 4. Kinetics of IL-8 release by PBMEC stimulated with positive control ultra-purified *E. coli* O55:B5 LPS alone (10 µg/ml) (black bars) and LPS along with live *S. suis* strain NCTC 10234 (10^6 CFU/ml) (hatched bars), from a representative experiment. * denotes IL-8 levels that are significantly different ($P < 0.05$) from those obtained using ultra-purified *E. coli* O55:B5 LPS alone (considered as 100%).

Figure 5. Swine recombinant IL-8 degradation following incubation with supernatant from *S. suis* NCTC 10234 grown for 16 h (black bars), or with supernatant together with complete Mini protease inhibitor cocktail tablets (hatched barrs), aprotinin (dotted bars), or Pefabloc SC (white bars). Data are expressed as percentage of residual IL-8 where 100% is the amount of IL-8 detected by ELISA following 6 and 9 h of incubation in THB (control). * denotes values that are significantly different ($P < 0.05$) from residual IL-8 detected following incubation with supernatant from *S. suis* strain NCTC 10234 alone.



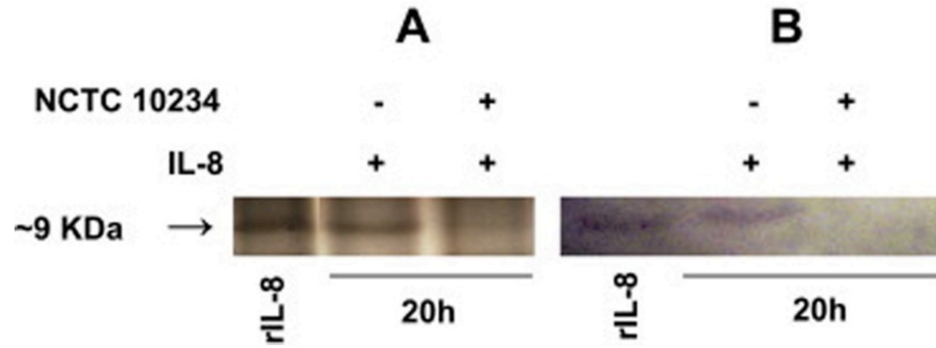


Figure 6. Porcine recombinant IL-8 (~9 kDa) cleavage by supernatant from *S. suis* strain NCTC 10234 after 20 h of incubation. (A) Silver stained SDS-PAGE. (B) Western blotting. + or −, presence or absence of supernatant from *S. suis* prepared in IF culture medium or rIL-8 diluted in cell culture medium and incubated for 20 h. rIL-8 indicates non-incubated rIL-8 directly from the manufacturer.

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Annexe IV

In vitro characterization of the microglial inflammatory response to *Streptococcus suis*, an important emerging zoonotic agent of meningitis.

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Rôle de la candidate dans la conception de l'article :
Je suis co-auteure de cet article. J'ai participé aux manipulations ainsi qu'à la mise au point de la culture cellulaire.

ABSTRACT

Streptococcus suis is an important swine and human pathogen responsible for septicemia and meningitis. *In vivo* research in mice suggested that in the brain, microglia might be involved in activating the inflammatory response against *S. suis*. The aim of this study was to better understand the interactions between *S. suis* and microglia. Murine microglial cells were infected with a virulent wild-type strain of *S. suis*. Two isogenic mutants deficient at either capsular polysaccharide (CPS) or hemolysin production were also included. CPS contributed to *S. suis* resistance to phagocytosis and regulated the inflammatory response by hiding proinflammatory components from the bacterial cell wall, while the absence of hemolysin, a potential cytotoxic factor, did not have a major impact on *S. suis* interactions with microglia. Wild-type *S. suis* induced enhanced expression of Toll-like receptor 2 by microglial cells, as well as phophotyrosine, protein kinase C, and different mitogen-activated protein kinase signaling events. However, cells infected with the CPS-deficient mutant showed overall stronger and more sustained phosphorylation profiles. CPS also modulated inducible nitric oxide synthase expression and further nitric oxide production from *S. suis*-infected microglia. Finally, *S. suis*-induced NF-κB translocation was faster for cells stimulated with the CPS-deficient mutant, suggesting that bacterial cell wall components are potent inducers of NF-κB. These results contribute to increase the knowledge of mechanisms underlying *S. suis* inflammation in the brain and will be useful in designing more efficient anti-inflammatory strategies for meningitis.

INTRODUCTION

Streptococcus suis is one of the most important swine pathogens worldwide, as well as an important agent of zoonosis. So far, 35 serotypes have been described, although serotype 2 is still the most frequently isolated from both swine and humans. In swine, meningitis is the most striking feature of the infection, although other pathologies, such as septicemia, endocarditis, pneumonia, and arthritis, have been described (35). Although the most common pathology associated with *S. suis* infection in humans is also meningitis, cases of septicemia with septic shock, endocarditis, and several other clinical manifestations have been reported (69, 71). As a zoonosis, *S. suis* infection has been traditionally considered an occupational hazard, since most cases described in Western countries have occurred in people working in close contact with pigs or raw pork products. The situation in Asian countries is completely different, as the common population is affected. In addition to an important human outbreak in China caused by *S. suis* in 2005 (71), the pathogen has recently been reported to be the most frequent cause of bacterial meningitis in adults in Vietnam (29) and the third most common culture-confirmed cause of community-acquired bacterial meningitis in Hong Kong. People who survive *S. suis* infection may be handicapped, as severe postinfection sequels, such as deafness, may develop (67, 68).

In recent years, a number of important studies describing the proposed virulence factors of *S. suis* serotype 2 have been published (4, 29). However, few candidates have been shown to be critical for virulence. Among them, the capsular polysaccharide (CPS) is considered an important antiphagocytic factor (13). Although not essential for virulence, a hemolysin (suilysin) produced by most virulent strains in Eurasia has also been shown to be toxic for cells of murine, human, and swine origin (12, 14, 55, 66).

The pathogenesis of *S. suis* infection has been partially elucidated. In swine, infection occurs through the respiratory route with subsequent colonization of the tonsils, while in humans, access is mainly through skin cuts and/or the oral route (3, 28, 29). Once *S. suis* reaches the bloodstream, it travels either free or associated with monocytes (27), with invasion of different tissues and organs. The high mortality observed at this stage of the disease may be associated with septic shock with an exacerbated release of

proinflammatory cytokines (18, 19). However, if the host overcomes septicemia, *S. suis* may still invade the central nervous system (CNS) and cause meningitis and, in some cases, encephalitis. The mechanisms used by the pathogen to gain access to the brain and induce local inflammation are still under debate. It is likely that access is by transcytosis and/or toxicity to brain microvascular endothelial cells (66) and/or choroid plexus epithelial cells that are part of the blood brain barrier (BBB) (64). Increase of BBB permeability due to inflammation cannot be ruled out (27).

Recently, our laboratory developed an *in vivo* mouse model of meningitis/encephalitis after *S. suis* infection via the intraperitoneal route (18). Using this model, an important inflammatory response in the CNS with expression of different proinflammatory genes, including Toll-like receptor 2 (TLR2), CD14, IκBα (an index of NF-κB expression), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein 1 (MCP-1), was observed. Interestingly, the expression of these genes and bacterial antigens was found to probably be associated with microglia and, to a lesser extent, with astrocytes (18). Microglia, the macrophage-like population within the CNS, represent the first line of defense against invading pathogens and have proinflammatory effector functions (49). Although previous findings draw attention to the implication of these cells in the development of meningitis and encephalitis, it is critical to dissect how the cells initiate key proinflammatory mechanisms in order to respond to *S. suis* infection. Hence, the goal of this study was to explore the murine microglial response to a virulent strain of *S. suis*, as well as isogenic mutants defective in either CPS or suilysin production. The ability of microglia to internalize *S. suis*, to activate TLRs, and to secrete different proinflammatory mediators, as well as to activate important inflammatory intracellular signaling pathways, was evaluated.

MATERIALS AND METHODS

Cell culture

The murine microglial cell line BV-2 was kindly provided by S. Rivest (Université Laval, Quebec, Canada). This cell line exhibits morphological and functional characteristics of microglia (7, 8). It has recently been shown that the cell line is a valid substitute for primary microglial cells (34). BV-2 microglia were maintained *in vitro* in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Wisent Inc., St-Jean Baptiste, Quebec, Canada) containing 10% heat-inactivated fetal bovine serum and 2% penicillin-streptomycin (both from Invitrogen, Carlsbad, CA). The cells were kept in 75-cm^2 Falcon flasks at 37°C in a humidified atmosphere containing 5% CO_2. Experiments were performed in 24-well plates unless otherwise specified, with a concentration of 5×10^5 cells/well. Absence of cell toxicity with the different *S. suis* strains and concentrations tested was evaluated by measuring the release of lactate dehydrogenase enzyme with the Cyto Tox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), as previously described (66).

Bacterial strains and growth conditions

Three strains of *S. suis* capsular serotype 2 were used, the virulent wild-type (WT) suilysin-positive strain 31533, originally isolated from a case of porcine meningitis and widely used in previous studies (18, 19, 42, 58, 59), as well as two isogenic mutants obtained by allelic exchange, the suilysin-negative (SLY^-) mutant SX911 (45) and the nonencapsulated (CPS^-) mutant B218 (23). Bacteria were grown overnight on sheep blood agar plates at 37°C, and isolated colonies were inoculated into 5 ml of Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI), which was incubated for 8 h at 37°C with agitation. Working cultures were prepared by transferring 10 µl of 1/1,000 dilutions of 8-h cultures into 30 ml of THB, which was incubated for 16 h at 37°C with agitation. Stationary-phase bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.3. For experiments, the bacterial pellet was resuspended and diluted in cell culture medium to a final concentration of 1×10^6 CFU/ml, unless otherwise specified. When necessary, the WT and CPS^- *S. suis* strains were heat killed, as previously described, at 60°C for 45 min (the minimal experimental conditions required for *S. suis* killing) (57).

Phagocytosis assays

Phagocytosis assays were performed as previously described (60) with some modifications. Briefly, microglial cells were infected by removing the culture medium and adding different *S. suis* strains resuspended in cell culture media at a multiplicity of infection (MOI) of 2:1. In selected experiments, bacterial opsonization was performed by incubating bacteria in the presence of 20% (vol/vol) complete normal or heat-inactivated mouse serum in PBS for 30 min at 37°C with shaking prior to cell infection. Serum from C57BL/6 mice was inactivated by incubation for 30 min at 56°C. After 15, 30, and 60 min of infection, the cell monolayers were washed twice with warm culture medium and reincubated for 1 h with medium containing penicillin G (5 μg/ml) and gentamicin (100 μg/ml) to kill extracellular bacteria (both antibiotics were from Sigma-Aldrich, Oakville, Ontario, Canada). Previous studies with *S. suis* showed that this concentration of antibiotics is able to kill any remaining extracellular bacteria (56, 60). In addition, supernatant controls were used in every test to confirm antibiotic efficacy. After antibiotic treatment, the cell monolayers were washed three times, and the medium was replaced with 1 ml of sterile distilled water to lyse microglial cells. After vigorous pipetting to ensure complete cell lysis, viable intracellular streptococci were determined by quantitative plating of serial dilutions of the lysates on THB agar. All samples were plated using an Autoplate 400 Automated Spiral Plater (SpiralBiotech Inc., Norwood, MA). Each test was repeated four times in independent experiments, and the number of CFU recovered per well (mean number ± standard error of the mean [SEM]) was determined.

Phagocytosis was confirmed by confocal microscopy. Microglial cells were plated on coverslips and infected with WT and CPS^{-} *S. suis* strains. After 2 h of bacterium-cell contact, the coverslips were washed with culture medium to remove nonassociated bacteria, and the cells were fixed with methanol/acetone (80:20) for 20 min at −20°C, washed, and blocked with PBS containing 2% fetal bovine serum for 10 min. The coverslips were incubated for 1 h with optimal dilutions of rabbit hyperimmune anti-*S. suis* serum, produced as described previously (36), and with rat anti-LAMP1 antibody (Developmental Studies Hybridoma Bank, IA). After being washed, the coverslips were incubated with the secondary antibodies Alexa Fluor 488-goat anti-rabbit IgG and Alexa Fluor 568-goat anti-

rat IgG (Invitrogen) for 30 min, washed, and mounted on glass slides with Mowiol containing DABCO (1,4-diazabicyclo-[2,2,2]-octane) and DAPI (4′,6-diamidino-2-phenylindole) to stain the nuclei. Samples were observed with an IX-80 confocal microscope integrated into the FV-1000 imagery system and analyzed using Fluoview software (Olympus, Markham, Ontario, Canada).

Cytokine and chemokine detection by ELISA

Microglial cells were infected with the *S. suis* WT and mutant strains included in the study at an MOI of 2:1. Purified *Escherichia coli* lipopolysaccharide (LPS) at 1 μg/ml (Sigma-Aldrich) was used as a positive control. After 12 h of incubation (the optimal incubation time as observed in kinetic studies [data not shown]), the supernatant was recovered to measure levels of IL-1β, IL-6, TNF-α, MCP-1, and the chemokine CXC chemokine ligand 10/interferon-inducible protein of 10 kDa (CXCL10/IP-10) by a sandwich enzyme-linked immunosorbent assay (ELISA), using commercially available antibody pairs (R&D Systems, Minneapolis, MN), as previously described (58). Standard curves were included in each ELISA plate (Nunc, Ville Mont Royal, Quebec, Canada) as 2-fold dilutions of recombinant mouse IL-1β (1,000 to 39 pg/ml), IL-6 (1,250 to 39 pg/ml), TNF-α (1,000 to 8 pg/ml), MCP-1 (500 to 8 pg/ml), or IP-10 (4,000 to 31 pg/ml) (R&D Systems). Supernatant dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to determine the level of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate.

Analysis of TLR gene expression by real-time reverse transcriptase-quantitative PCR

Microglial cells were infected with WT and CPS^{-} *S. suis* strains at an MOI of 2:1 for 0, 1, 2, 4, and 8 h. Following infection, the medium was removed and the cells were washed with cell culture medium. Total cellular RNA was prepared from the cells using Trizol (Invitrogen) following standard procedures. Next, 1 μg of total RNA was reverse transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, Ontario, Canada), and the resulting cDNA was amplified using the SsoFast EvaGreen Supermix kit (Bio-Rad, Hercules, CA). The PCR amplification program for all cDNA samples consisted of an enzyme activation step of 3 min at 98°C, followed by 40 cycles of a denaturing step for 3 s at 98°C and an annealing/extension step for 5 s at 57°C. Each 10-μl reaction mixture

contained 250 nM (each) forward and reverse primers. The primers used for amplification of the different target cDNAs are listed in Table Table11 and were all tested to achieve an amplification efficiency between 90% and 110%. The primer sequences were all designed from NCBI GenBank mRNA sequences using the Web-based software Primerquest from Integrated DNA Technologies. The Bio-Rad CFX-96 sequence detector was used for amplification of target cDNAs of various TLRs, and quantitation of differences between the different groups was calculated using the $2^{-\Delta\Delta Ct}$ method. β-Actin and β_2 microglobulin (β2m) genes were used as normalizing genes to compensate for potential differences in cDNA amounts between the various samples. These two genes were selected from candidate normalizing genes using the Normfinder V19 algorithm (2), as their expression was found to be the most stable under the experimental conditions. The noninfected BV-2 microglial group was used as the calibrator reference in the analysis.

Measurement of nitric oxide production

Microglial cells were seeded and stimulated with killed bacteria as previously described (56), with some modifications. Briefly, heat-killed *S. suis* WT and CPS^- strains (a concentration equivalent to 1×10^9 CFU/ml) were incubated with cells (5×10^5 cells/well) for 6, 12, 24, and 48 h. LPS (1 µg/ml) was used as a positive control. Nitric oxide (NO) production was determined by measuring nitrite, a stable end product of NO. The microglial culture supernatant was assayed by mixing it with Griess reagent (Promega, Madison, WI). Sulfonamide (1%) and N-1 [(1-naphthyl)-ethylenediamine dihydrochloride] (0.1%) were added to the supernatant. After 30 min of incubation at room temperature, the absorbance was read at 540 nm. The nitrite concentration (expressed as µM/ml) was calculated using standard solutions of sodium nitrite prepared in culture medium.

Signaling pathway analysis by immunoblotting

Western blotting was performed to analyze the phosphorylation states of the protein kinase C (PKC) pathway and several mitogen-activated protein kinases (MAPKs), including stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), extracellular signal-regulated kinase (ERK), and p38, as well as total tyrosine phosphorylation and inducible nitric oxide synthase (iNOS) expression, as previously described (38, 50). Briefly, cells were plated in a 6-well plate and stimulated with *S. suis* WT and CPS^- strains

at an MOI of 2:1. LPS (1 μg/ml) was used as a positive control. After different incubation times (see Results), the cells were lysed in cold buffer containing 0.5 M Tris (pH 6.8), 0.5 M EDTA, 1% (vol/vol) β-mercaptoethanol (Bio-Rad, Mississauga, Ontario, Canada), 10 mM EGTA, 10% (vol/vol) IGEPAL [(octylphenoxy)polyethoxyethanol], 1 mM sodium orthovanadate (Na_3VO_4) (all from Sigma-Aldrich), and the protease inhibitors aprotinin (10 μg/ml) (Sigma-Aldrich) and leupeptin (5 μg/ml) (Roche, Mississauga, Ontario, Canada). The lysates (60 μg/lane) were separated by SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in Tris-buffered saline-0.1% Tween containing 1% bovine serum albumin (or 5% skim milk for anti-α-actin) for 1 h at room temperature. The membranes were then washed and incubated overnight at 4°C with one of the following antibodies: anti-phospho-ERK (p-ERK) 1/2 (Thr^{202}/Tyr^{204}), anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-phospho-(Ser) PKC substrate antibody (all from Cell Signaling, Danvers, MA), anti-phospho SAPK/JNK 1/2 (Thr^{183}/Tyr^{185}) (Invitrogen), anti-phospho-Tyr (clone 4G10; Upstate, Lake Placid, NY), or anti-iNOS (BD Systems, San Jose, CA). After being washed, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (Sigma-Aldrich) for 1 h, and the proteins were visualized with ECL Plus Western blotting reagent (Amersham, Arlington Heights, IL). The membranes were then stripped using the Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), and blotting was repeated using anti-protein antibodies (according to the instructions of the manufacturer of the anti-phospho-protein). Blotting with anti-α-actin antibody (Sigma-Aldrich) was used as a loading contol for p-Tyr, p-PKC, and iNOS blots.

MAPK inhibition assays

In selected experiments, microglial cells were pretreated with the ERK inhibitor apigenin (50 μM), the SAPK-JNK inhibitor SP600125 (50 μM), or the p38 inhibitor SB203580 (75 μM) (all from Biomol Research Laboratories, Plymouth Meeting, PA) 1 h prior to infection. Western blot analysis of the corresponding phosphorylated proteins was done as described above after 2 h or 4 h of incubation in the presence of bacteria. Cytokine measurement was performed as described above after 12 h of incubation. The inhibitors were used at maximal subcytotoxic concentrations as determined by the Cyto Tox 96 Non-

Radioactive Cytotoxicity Assay. An inhibitor was considered cytotoxic if viability was <90% of that of the untreated control after 12 h.

NF-κB binding

An electrophoretic mobility shift assay (EMSA) was performed as previously described (50) with some modifications. Briefly, microglial cells were placed in a 25-cm^2 flask and allowed to adhere overnight prior to infection with either an *S. suis* WT or CPS^- strain at an MOI of 2:1. LPS (1 µg/ml) was used as a positive control. The cells were washed with ice-cold culture medium and scraped in 1 ml of culture medium. After centrifugation, the cells were resuspended in 400 µl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated on ice for 15 min. Twenty-five microliters of 10% IGEPAL was then added. The tubes were vortexed for 10 s and centrifuged at maximum speed for 30 s. Nuclear fractions were resuspended in 50 µl of ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and incubated at 4°C on a shaking platform for 15 min. After centrifugation at 12,000 × *g* for 5 min at 4°C, the supernatants were stored at −70°C until further use. Then, 7 µg of these nuclear protein extracts was mixed with a γ-^{32}P-labeled oligonucleotide containing a consensus binding sequence for NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA). Complexes were resolved by electrophoresis on a 4% nondenaturing olyacrylamide gel. The gel was dried and visualized by autoradiography. The consensus sequence for NF-κB was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′.

Statistical analysis

Each test was done at least in triplicate biological repetitions. Differences were analyzed for significance by using the Student unpaired *t* test (two-tailed *P* value). A *P* value of <0.05 was considered significant. All data are presented as means ± standard errors of the mean (SEM). All statistical analyses were performed using Sigma Plot software (v.11; Systat Software, San Jose, CA).

RESULTS

The ability of murine microglia to phagocytose *S. suis* is modulated by CPS

Figure 1A shows the kinetics of *S. suis* phagocytosis by murine microglia. The WT strain and its SLY^{-} mutant were relatively poorly phagocytosed throughout the experiment, and no statistically significant differences were found between them ($P > 0.05$). This indicates that under nonopsonic conditions, the absence of suilysin, which is considered a putative *S. suis* virulence factor, does not have a major impact on phagocytosis by microglia. On the other hand, the CPS^{-} mutant was quickly phagocytosed, and levels of ingested bacteria in comparison to the WT strain significantly increased with incubation time from 15 ($P = 0.01$) to 60 min ($P = 0.002$) (Fig.1A). The possible effect of opsonization on *S. suis* phagocytosis by murine microglia was also assessed. All strains were preopsonized by incubation in the presence of 20% (vol/vol) complete normal or heat-inactivated serum in PBS for 30 min prior to microglial-cell infection for 30 min. As shown in Fig.1B, *S. suis* opsonization with mouse serum increased the phagocytosis levels of all three strains in comparison to nonopsonizing conditions ($P < 0.05$). However, it is likely that factors present in the serum other than complement were responsible for this phenomenon, as the levels of phagocytosis for each strain opsonized with inactivated mouse serum were similar to those obtained with normal complete serum ($P > 0.05$).

To confirm the intracellular location of bacteria, confocal microscopy was performed using hyperimmune serum against *S. suis* and an antibody specific for LAMP1, a protein enriched in phagolysosomes. Confocal analysis showed that only small numbers of WT *S. suis* bacteria were internalized by microglia (Fig.2). However, the CPS^{-} mutant was found in higher numbers, not only attached to the cell membrane, forming small chains, but also inside the cytoplasm and associated with numerous phagolysosomes of microglial cells (Fig.2).

S. suis induces production of proinflammatory cytokines and chemokines by murine microglia

In *S. suis* meningitis, microglia have been suggested to be, at least in part, responsible for the high inflammatory reaction in the CNS (18). Therefore, microglial cells were stimulated with the *S. suis* WT strain and its isogenic mutants for 12 h, and levels of TNF-α, IL-1β, IL-6, MCP-1, and IP-10 production were analyzed by ELISA. Cell culture medium alone was used as a negative control, and purified *E. coli* LPS was used as a positive control. LPS induced high production levels of the aforementioned proinflammatory cytokines and chemokines (data not shown). *S. suis* induced different patterns of cytokine release by microglial cells (Fig.3). The *S. suis* WT strain induced significantly higher levels of TNF-α and MCP-1 than in noninfected cells but failed to induce IP-10 production over basal levels (Fig.3). The presence of suilysin was not involved in modulation of TNF-α, MCP-1, and IP-10 release, as the SLY^- mutant induced levels of cytokines comparable to those observed for the WT strain ($P > 0.05$). On the other hand, the CPS^- mutant induced significantly higher secretion of all three cytokines than the *S. suis* WT strain ($P > 0.01$).

Different results were obtained for IL-1β, as microglial cells produced low levels of the cytokine after infection with all *S. suis* strains tested (Fig.3), although these levels were significantly higher than those observed with noninfected cells ($P < 0.001$). Interestingly, the production of IL-1β seemed not to be influenced by the absence of the capsule, as levels observed for the CPS^- mutant were not significantly different from those found with the WT strain ($P > 0.05$). In the case of IL-6 (Fig.3), microglial cells did not produce the cytokine after infection with the WT strain or the SLY^- mutant. Only the CPS^- mutant (and the positive-control LPS [data not shown]) was able to induce the release of IL-6 from these cells ($P < 0.001$).

As a whole, the results obtained with microglial cells for both phagocytosis and cytokine production were similar between the WT strain and the SLY^- mutant. Important differences were observed only between the parental strain and its CPS^- mutant. Therefore, we used only these two strains for further characterization of receptor activation and intracellular signaling pathways related to the *S. suis*-induced inflammatory response by murine microglia.

S. suis infection induces TLR2 gene expression

TLRs are receptors that play a key role in the innate immune response. Having observed differences in expression of cytokine levels following infection of microglia by the WT strain and the CPS$^-$ mutant, we were interested in examining whether gene expression of different TLRs was modified. To this end, microglial cells were infected with WT and CPS$^-$ strains of *S. suis* for different times, and TLR1, TLR2, TLR4, TLR6, and TLR9 gene expression was analyzed by real-time PCR. Microglial-cell infection by both *S. suis* strains failed to increase TLR1, TLR4, TLR6, and TLR9 gene expression over basal levels (data not shown). However, TLR2 gene expression was significantly increased in microglia between 4 and 8 h of incubation by either the WT strain or its CPS$^-$ mutant compared to noninfected cells ($P < 0.05$) (Fig.4). Interestingly, the CPS$^-$ mutant was able to induce TLR2 gene expression in microglial cells at a higher level than the WT *S. suis* strain ($P < 0.001$). This finding is in agreement with the fact that the CPS$^-$ mutant was also associated with generally higher cytokine production by these cells.

S. suis induces iNOS expression and NO production in mouse microglia

In the brain, following induction of iNOS, activated microglia release NO, which might lead to tissue destruction and degeneration (15, 46). Therefore, we sought to determine whether *S. suis* induces iNOS expression and NO release from murine microglia over time. Western blot analysis showed strong iNOS expression in microglial cells stimulated with both *S. suis* strains tested. iNOS expression was observed after as little as 6 h of bacterium-cell contact and reached a plateau at 24 h of incubation. The activation of iNOS was higher after infection of microglia with the CPS$^-$ mutant strain at all incubation times (Fig.5). Accordingly, NO production increased over time and reached a plateau at 24 h of incubation. In agreement with iNOS results, NO production was significantly higher with the CPS$^-$ mutant strain (Fig.5) ($P < 0.05$).

The microglial-cell profile of tyrosine phosphorylation is modulated during _S. suis_ infection

Studies with different pathogens have demonstrated that bacterial attachment can lead to activation of host signal transduction cascades, predominantly through Tyr phosphorylation

of proteins that contribute to different signal transduction mechanisms, including internalization of pathogens in host cells (22). Thus, modulation of protein phosphotyrosine residues in response to *S. suis* infection and the effect of CPS on this process were assessed. As shown in Fig.6A, the Tyr phosphorylation response to *S. suis* is considerably downmodulated by the presence of the capsule. Indeed, the WT strain induces a modest Tyr phosphorylation of some proteins, which is more apparent at 2 h postinfection, while the CPS^{-} mutant leads to dramatic changes in the phosphorylation states of numerous proteins. Not only do these changes occur earlier than in the WT strain (15 min), but the level of phosphorylation is also stronger and generally increases over time. It is interesting that during the course of infection the phosphorylation levels of some proteins of ~110, 70 to 75, and 25 to 30 kDa temporarily decreased, suggesting that either different bacterial components or different steps of the infection (adhesion versus internalization) may influence Tyr phosphorylation patterns (Fig.6A).

Murine microglial cells display PKC activity after *S. suis* infection

PKC represents a family of serine/threonine kinases that play central roles in multiple signaling events, such as regulation of the immune response by MAPKs and gene transcription activation (62). Using an antibody specific for PKC substrates containing phosphoserine, the phosphorylation levels of several proteins in microglial cells were shown to be different after infection with the WT strain than after infection with the CPS^{-} mutant (Fig.6B). In fact, the WT strain showed discrete phosphorylation of several proteins, ranging from ~25 to 60 kDa, that occurred at different times postinfection depending on the protein. In contrast, the phosphorylation pattern of PKC substrates observed for the CPS^{-} mutant was stronger and more stable, reaching maximal phosphorylation at 2 h postinfection for most of the proteins. Similarly to that observed for phosphotyrosine (Fig.6A), PKC-dependent phosphorylation of some proteins showed a shift between the dephosphorylated and the nonphosphorylated states during the infection period.

***S. suis* infection activates MAPK phosphorylation in murine microglia**

It is known that MAPK signal transduction pathways are involved in microglial activation, leading to production of different proinflammatory mediators that play essential roles in the

host response to pathogens (6, 51). Therefore, the time course of phosphorylation of the three MAPK signaling pathways, ERK 1/2, SAPK/JNK, and p38, in microglial cells was investigated after stimulation with the *S. suis* WT strain and the CPS⁻ mutant (Fig.7). A specific phosphorylation pattern was observed for each MAPK evaluated, which seemed to also be influenced by the strain tested. ERK 1/2 activation in response to the WT strain was observed as early as 15 min postincubation; however, this phosphorylation was downregulated between 30 min and 1 h postincubation, though a second phase of phosphorylation was found at 2 h. No activation of this MAPK was found with longer incubation times (data not shown). A similar ERK 1/2 activation pattern was detected with the CPS⁻ mutant. Similarly to the WT strain, there was dephosphorylation of ERK 1/2 at 1 h and a second and final phase of phosphorylation at 2 h. When cells were stimulated with WT *S. suis*, the phosphorylated form of JNK was also found at 15 min poststimulation, but similarly to ERK 1/2, phosphorylated JNK was downregulated from 30 min to 1 h. A second phase of activation was found at 2 h and extended up to 4 h of bacterium-cell contact. Interestingly, when the CPS⁻ mutant was used, prompt and lasting JNK phosphorylation was detected from 15 min to 4 h of stimulation. This phosphorylation seemed to be slightly downregulated from 30 min to 1 h. In the case of p38, a very slight activation was found in WT strain-infected microglial cells between 30 min and 2 h postinfection. However, when microglial cells were stimulated with the CPS⁻ mutant, p38 phosphorylation was more marked and extended from 1 h to 4 h postinfection. These results not only show that *S. suis* is capable of inducing activation of different MAPK signaling pathways, but also that cell wall components seem to be mainly implicated in this phenomenon.

The involvement of ERK 1/2, JNK, and p38 in regulation of microglial production of proinflammatory cytokines and chemokines in response to *S. suis* infection was confirmed using specific inhibitors. For this purpose, microglial cells were treated with subcytotoxic doses of ERK 1/2 (Apigenin), JNK (SP600125), or p38 (SB203580) inhibitor and then infected with either the *S. suis* WT strain or its CPS⁻ mutant. Figure 8A shows the results of Western blotting of this selective MAPK inhibition, with an evident abrogation in phosphorylation of all three MAPKs. In parallel, it was possible to confirm that MAPK activity was involved in cytokine and chemokine production, as microglia treated with

MAPK inhibitors prior to *S. suis* infection showed a strong diminution of TNF-α and MCP-1 production (Fig. 8B and C) for either the WT strain or its CPS⁻ mutant.

S. suis infection of murine microglia induces NF-κB activation

NF-κB is one of the most prominent transcription factors involved in the inflammatory response. As our previous *in vivo* research suggested NF-κB activation in the microglia of mice infected with *S. suis* (18), the ability of the pathogen to stimulate NF-κB in microglial cells was studied. Cells were incubated with either the WT or CPS⁻ *S. suis* strain, and the time course of NF-κB translocation and its DNA binding activity were studied by EMSA. LPS, a potent inducer of NF-κB activity, served as a positive control. A basal DNA binding activity of NF-κB was observed in noninfected cells; however, in response to WT *S. suis* infection, an important induction of NF-κB binding activity was recorded at 4 h and increased over time, reaching its maximum at 12 h postinfection (Fig.9). Although the *S. suis* CPS⁻ mutant induced NF-κB translocation and DNA binding activity with a similar time course, this activity was overall stronger than the one recorded for the WT strain. The specificity of NF-κB DNA binding was confirmed by competition analysis with an excess of unlabeled specific or nonspecific oligonucleotides.

DISCUSSION

S. suis is an important agent of swine and human meningitis. Although research has significantly increased in recent years (29), knowledge of the pathogenesis of the infection is still scarce. Once *S. suis* arrives in the CNS, it encounters microglia (as well as meningeal and perivascular macrophages), major brain-resident innate immune effector cells. In fact, microglia play an ambiguous role, since they may protect neurons by preventing the entry of pathogens into the brain, but they can also be toxic to surrounding neurons by releasing NO, glutamate, and proinflammatory cytokines (10, 31, 72). In addition, activated microglia have been implicated in neurodegeneration resulting from bacterial meningitis (47). Using a well-standardized mouse model, it was shown that most *S. suis*-infected mice that survived septicemia later developed CNS clinical signs, such as locomotion problems, episthotonus, opisthotonus, lateral bending of the head, and walking in circles, which could be considered characteristic of brain inflammation. *S. suis* infection clearly induced inflammation and suppurative and necrotizing lesions in different anatomical sites of the brain parenchyma (18). Results from immunohistochemistry studies showed the presence of high bacterial antigen loads in association with cells that morphologically resembled microglia (18). It was hypothesized that these cells would be critically implicated in the CNS inflammatory response induced by *S. suis* (18).

Microglial cells have recently been shown to be able to phagocytose and kill Gram-positive bacteria, including well-encapsulated pathogenic *Streptococcus pneumoniae* (52). In the case of *S. suis*, previous studies carried out with murine, porcine, and human phagocytes indicated that the *S. suis* capsule is critical for bacterial resistance to phagocytosis (12, 56, 61). Similarly, phagocytosis and confocal microscopy results from the present study show that microglial cells hardly ingest well-encapsulated *S. suis*, whereas the CPS^{-} mutant was significantly more often ingested than the WT strain. The absence of suilysin, a virulence factor present mainly in Eurasian strains (54), has been associated with partial susceptibility of encapsulated *S. suis* to killing by neutrophils and dendritic cells in the presence of complement (5, 12; M. P. Lecours, M. Gottschalk, M. Houde, P. Lemire, N. Fittipaldi, and M. Segura, submitted for publication). Since components of the complement cascade can be found in the brain (26), their possible effects on phagocytosis of *S. suis* by microglia

were evaluated. Although the presence of serum significantly increased the phagocytosis rate of *S. suis*, complement components do not seem to be implicated in such a process. In addition, the encapsulated *S. suis* SLY^{-} mutant behaved similarly to the WT strain, indicating a particular behavior of the microglial cells different from that of other phagocytes (5, 12). The observed increased rate of phagocytosis in the presence of serum might be due to other proteins, such as albumin and fibronectin (9).

The results of cell activation by *S. suis* provide support for the relevance of microglia in the development of the inflammatory response against the pathogen, as shown by the production of proinflammatory cytokines and chemokines. This confirms previous *in vivo* findings in the brains of mice, where high mRNA expression levels of different proinflammatory mediators were observed in cells suspected to be microglia (18). Indeed, high levels of TNF-α and MCP-1, but relatively low levels of IL-1β, were observed after *in vitro S. suis* activation of microglial cells. Interestingly, the WT encapsulated strain did not induce IL-6 production. It has previously been shown that, although highly secreted in the bloodstream during the septicemic phase, IL-6 mRNA was not expressed in the brains of *S. suis*-infected mice (18). The results obtained in this study confirm this observation. For so far unknown reasons, the lack of IL-6 production by microglia differs from what was observed with other phagocytic cells and *S. suis* (30, 57, 58), as well as with other streptococci and microglial cells (44).

The pneumolysin produced by *S. pneumoniae*, which shows high homology with suilysin, was shown to play an active role in inflammation (37). This does not seem to be the case for the suilysin, since the *S. suis* SLY^{-} mutant induced cytokine levels similar to those for the WT strain. On the other hand, the capsule seems to be critical for modulating production of proinflammatory mediators, as the CPS^{-} mutant induced the release of significantly higher levels of all proinflammatory mediators. These findings support the assumption that several cell wall components, such as lipoteichoic acid (LTA), peptidoglycan (PG), and lipoproteins, partially masked by the capsule are potent proinflammatory inducers, as recently suggested (24, 25, 70). Finally, as low production of IP-10 for capsulated *S. suis* strains in comparison to the CPS^{-} mutant was also noted, we hypothesize that the CPS might influence the onset of the adaptive inflammatory response,

as recently shown for dendritic cells (Lecours *et al*., submitted). As a consequence, fewer T lymphocytes would be attracted to the site of infection.

It has recently been demonstrated that TLRs, which are crucial pattern recognition receptors in innate immunity, are expressed in microglia (49). TLR activation sets in motion a broad spectrum of intracellular events to initiate the inflammatory response, including MAPK signaling pathways, activation of NF-κB, and cytokine production (1). In the present work, it was observed that *S. suis* induces significant microglial TLR2 mRNA upregulation in a time-dependent fashion. As expected, and in agreement with the cytokine results, upregulation of TLR2 is influenced by direct exposure of cell wall components, as a significantly higher level of TLR2 expression was observed with the CPS^- mutant. These findings confirm previous studies from our laboratory, where *in vitro* recognition of the pathogen by professional macrophages was shown to occur through TLR2 (30). *In vivo*, *S. suis*-infected mice showed clear upregulation of TLR2 in specific parts of the brain where microglial cells were present (18). Interestingly, our results differ slightly from those recently reported by Wichgers Schreur *et al*. (70), since those authors observed TLR2 upregulation after culturing transfected human epithelial cells with extracted lipoproteins but not with live or heat-killed *S. suis*. It should be noted, however, that interactions between *S. suis* and epithelial cells can differ greatly from those observed with phagocytic cells. The fact that no upregulation of TLR1, TLR4, TLR6, and TLR9 was observed should be regarded with caution, since relatively high constitutive expression levels of these mRNAs were observed, and no further upregulation could be observed using the respective positive controls (data not shown).

Sustained and therefore uncontrolled production of toxic products released from microglia may cause irreversible damage to neurons. NO plays a significant role in macrophage bactericidal functions; however, it is also involved in a variety of brain insults, including neurotoxicity, increase of intracranial pressure, and meningeal inflammation (41). It was observed that both *S. suis* WT and CPS^- strains efficiently enhanced the expression of iNOS in a time-dependent manner, which was accompanied by the release of NO from microglia, although levels were again higher with the CPS^- mutant. Cell wall components

of other Gram-positive bacteria, such as LTA, have been reported to be involved in NO production by microglial cells (15).

In the present study, the modulation of classical PKC and Tyr phosphorylation events, which are involved in different processes of macrophage activation, such as phagocytosis, NO production, and cytokine production (11, 21, 32, 48) was examined. The results demonstrated a pattern of low and biphasic phosphorylation of PKC substrates and tyrosyl residues in microglial cells infected with the *S. suis* WT strain, while cells infected with the CPS^{-} strain showed a stronger pattern of phosphorylation. This may indicate that once in contact with microglia, virulent encapsulated *S. suis* is able to modulate intracellular signaling events, most likely to avoid phagocytosis and delay the activation of the inflammatory response. These findings support previous research on *S. suis* modulation of murine macrophage functions, in which it was concluded that the capsule was responsible for weak activation of Akt and PKCα kinases, as well as activation of protein tyrosine phosphatases, which correlated with low levels of phagocytosis (56).

We also examined whether *S. suis* activates the three classical MAPK intracellular signaling pathways and if their phosphorylation was involved in the production of proinflammatory mediators. As expected, the CPS^{-} mutant proved to be particularly potent in MAPK activation, as phosphorylation patterns were stronger and more sustained than those obtained with the WT strain. MAPK phosphorylation levels, in particular those of p-ERK 1/2 and p-JNK, followed a biphasic pattern. This noticeable dephosphorylation of MAPK proteins highlights the intimate cross talk between these signaling pathways and pathogen-derived components and likely has an impact on microglial proliferation and/or activation (20). Interestingly, the phosphorylation levels of p38, a MAPK that plays an important role in activation of inflammatory responses (53), were subtly increased by WT *S. suis* but more noticeably with the CPS^{-} mutant, emphasizing the relevance of CPS in the regulation of proinflammatory events. It is likely that, again, hidden cell wall components are the principal mediators of MAPK pathway activation. In fact, recent studies revealed that a capsule-deficient mutant of *S. suis* was able to induce higher levels of transcriptional expression of different putative genes from the MAPK pathway than the parental strain (17). Furthermore, purified cell wall preparations from *S. suis* and other meningitis-causing

bacteria were shown to trigger the phosphorylation of the MAPK signal transduction pathway (15, 63). The EstA cell surface protein is a recently described *S. pneumoniae* virulence factor that induces MAPK phosphorylation and NF-κB translocation (39). The *estA* gene is also found in *S. suis* (40), so we may hypothesize that activation of the MAPK pathway and other intracellular signaling pathways does not depend solely on a few *S. suis* constituents but that many of them participate in the activation of the proinflammatory machinery. The use of pharmacological MAPK inhibitors revealed almost complete abrogation of cytokine release from microglia infected with either the *S. suis* WT or CPS$^-$ strain, confirming the importance of ERK 1/2, JNK, and p38 in the inflammatory response against the pathogen. MAPK pathways are molecular targets for drug development, and their inhibitors will undoubtedly be one of the next groups of drugs developed for the treatment of human diseases (53), so these results may open the door to future studies using animal models of *S. suis* meningitis to evaluate the *in vivo* efficacy of such drugs.

NF-κB is a central mediator that is critical for driving the innate immune response against many pathogens that infect the brain (43). Both strains of *S. suis* tested were able to increase this DNA binding activity in a time-dependent fashion, yet the activity was faster and more apparent when cells were infected with the *S. suis* CPS$^-$ mutant. Similar to other reports, it is likely that cell wall components, in particular LTA, influence the activation of NF-κB (15). Moreover, these findings support previous research stating that the *in vivo* inflammatory response to *S. suis* in the brains of infected mice, as well as *in vitro* infection of porcine alveolar macrophages, involves NF-κB activation (17, 18). Activation of NF-κB in murine microglial cells infected with *S. suis* would lead to the production of different cytokines and chemokines, as well as production of neurotoxic products, such as NO, as previously demonstrated for other brain pathogens (39, 43).

Finally, it might also be argued that activation of microglial cells may be a direct consequence of phagocytosis. In fact, cytochalasin treatment significantly reduced cytokine release by *S. suis*-infected cells (data not shown). However, levels of cytokines produced by treated cells infected with the nonphagocytosed, well-encapsulated WT strain were also significantly reduced, suggesting that phagocytosis alone was not responsible for cell activation. Rearrangement of the actin cytoskeleton may be necessary for the formation of a

fully active receptor (16) complex, which may indeed be affected by cytochalasin treatment, as reported in other systems (16, 33, 65). In fact, the regulation of inflammatory cytokine production is very complex and is controlled at transcriptional, posttranscriptional, and posttranslational levels. Alteration of cell receptors and/or actin networks could conceivably affect most of these levels of regulation by altering cell surface-mediated events. However, it is also possible that additional distinct mechanisms exist that are not related to alterations in receptor complexes and are probably mediated by phagolysosome "in-out" signals. Further studies are needed to address this issue.

In conclusion, the results obtained in the present study demonstrate that *S. suis* phagocytosis by microglia and consequent activation of these cells is highly influenced by the presence of the capsule and probably involves recognition of cell wall components that requires participation of a TLR2-dependent pathway, activation of different signaling pathways, translocation of NF-κB, and production of different proinflammatory mediators and neurotoxic metabolites. The results obtained may contribute to an understanding of the participation of microglia in the meningitis caused by *S. suis* and the genesis of brain injury associated with the pathogen.

ACKNOWLEDGEMENTS

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Table 1: Primers used in real-time quantitative PCR for TLR amplification and detection.

Gene	Primer		Amplicon size (bp)
	Forward	**Reverse**	
TLR1	CACAGCTCCTTGGTTTTAATG	TGGGTATAGGACGTTTCTGTAG	102
TLR2	TGGAGCATCCGAATTGCATCACCG	GAGCGGCCATCACACACCCC	193
TLR3	CGGGGGTCCAACTGGAGAACCT	GGGCGTTGTTCAAGAGGAGGGC	198
TLR4	GCCTCCCTGGCTCCTGGCTA	AGGGACTTTGCTGAGTTTCTGATCCA	139
TLR6	CCGTCAGTGCTGGAAATAG	CGATGGGTTTTCTGTCTTGG	108
TLR9	CAGTTTGTCAGAGGGAGC	ACTTCAGGAACAGCCAATTG	198
β-Actin	CCAACCGTGAAAAGATGACC	AGCATAGCCCTCGTAGATG	170
β2 m	ATGGCTCGCTCGGTGACCCT	TTCTCCGGTGGGTGGCGTGA	110

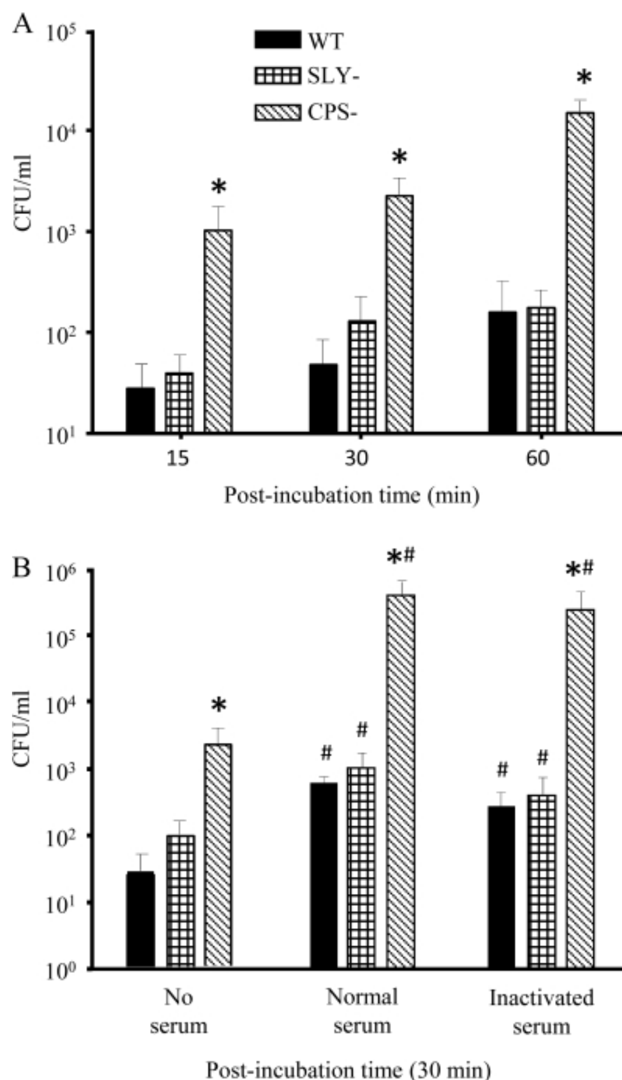

Figure 1. Phagocytosis of *S. suis* by murine microglial cells. (A) Kinetics of phagocytosis of *S. suis* strains (1×10^6) by murine microglia after 15-, 30-, and 60-min infection times. *, $P < 0.05$ compared to phagocytosis levels obtained with the wild-type strain. (B) Effect of opsonization on phagocytosis at 30 min postinfection. Bacteria were nonopsonized (no serum) or preopsonized with 20% either normal or inactivated mouse serum. *, $P < 0.05$ compared to phagocytosis levels obtained with the wild-type strain; #, $P < 0.05$, indicating statistically significant differences between nonopsonized strains and their respective normal-serum- or inactivated-serum-opsonized counterparts. The numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and the results are expressed as CFU of recovered bacteria per ml (means plus SEM obtained from three independent experiments).

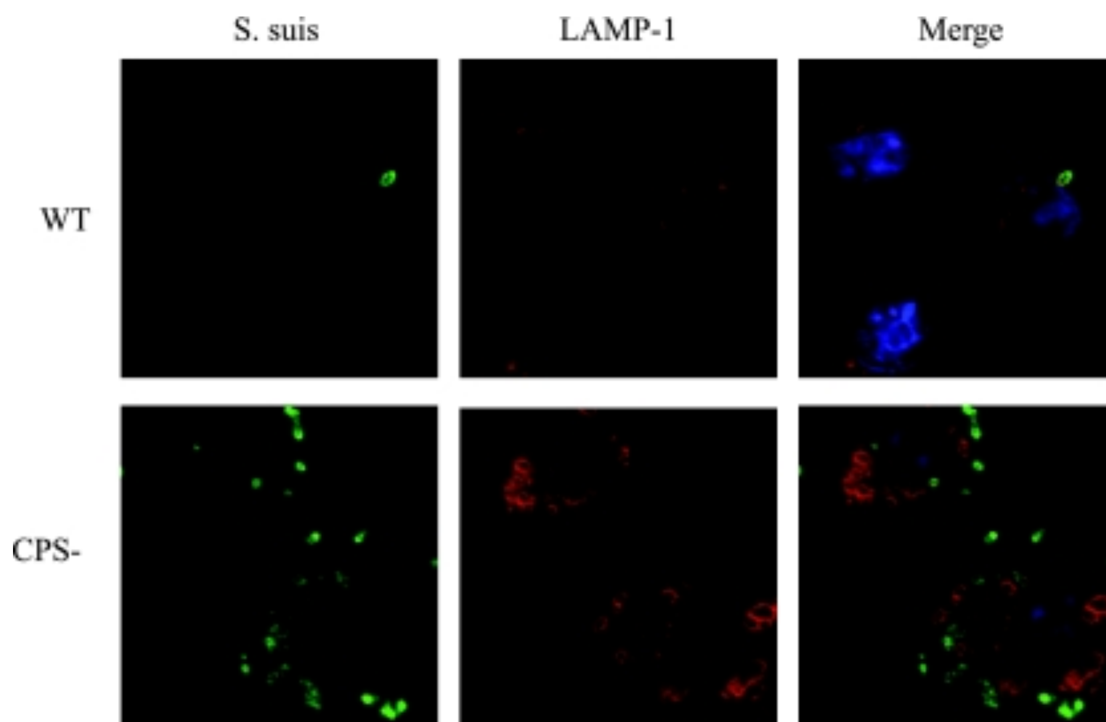

Figure 2. Interaction of murine microglial cells with *S. suis*. Microglia were infected with either the *S. suis* WT strain or the CPS^{-} mutant for 2 h. The cells were then washed, and bacteria were visualized with rabbit anti-*S. suis* serum and Alexa Fluor 488-conjugated goat anti-rabbit IgG (green), while phagolysosomes from microglial cells were evidenced with rat anti-LAMP1 antibody and Alexa Fluor 568-conjugated goat anti-rat IgG (red). The cell nuclei were stained with DAPI (blue). The images were examined with a confocal laser scanning microscope.

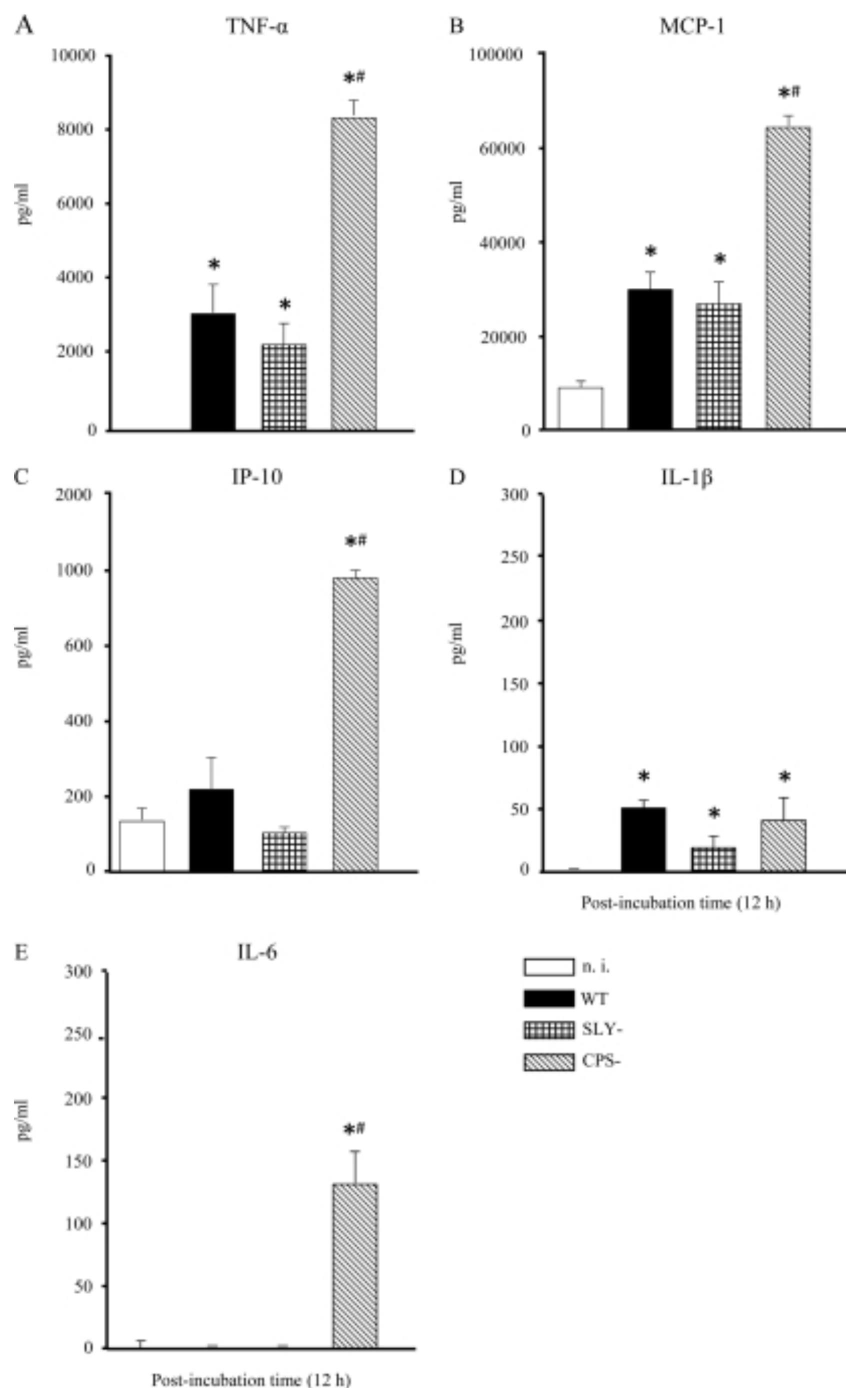

Figure 3. Comparative study of cytokine production: TNF-α (A), MCP-1 (B), IP-10 (C), IL-1β (D), and IL-6 (E). Murine microglial cells were incubated with the different *S. suis* strains (1×10^6). The culture supernatants were harvested at 12 h poststimulation and analyzed for cytokine production by ELISA. The data are expressed as means plus SEM from at least three independent experiments. n.i., noninfected cells. *, $P < 0.05$, indicating significant differences from n.i. cells; #, $P < 0.05$, indicating significant differences from the WT *S. suis* strain.

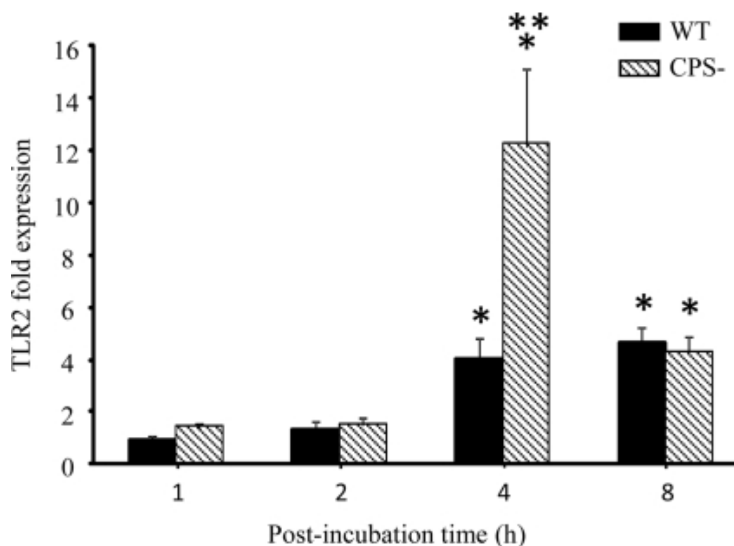

Figure 4. Increase of TLR2 mRNA expression in murine microglial cells following *S. suis* infection. Microglia were stimulated for 1, 2, 4, and 8 h with 1×10^6 *S. suis* bacteria. Total RNA was isolated from the microglia at the indicated time points and analyzed for TLR2 mRNA expression by real-time quantitative PCR as described in Materials and Methods. The levels of TLR2 gene expression following *S. suis* infection were calculated after the cycle thresholds against the β-actin and β_2 microglobulin housekeeping genes were normalized, using the $2^{-\Delta\Delta Ct}$ method. The results are presented as fold induction relative to noninfected microglia. *, $P < 0.05$, indicating significant differences between infected and noninfected cells; **, $P < 0.001$, indicating significant differences between microglia stimulated with WT *S. suis* and cells infected with the *S. suis* CPS^- mutant. The results are means plus SEM of three independent experiments.

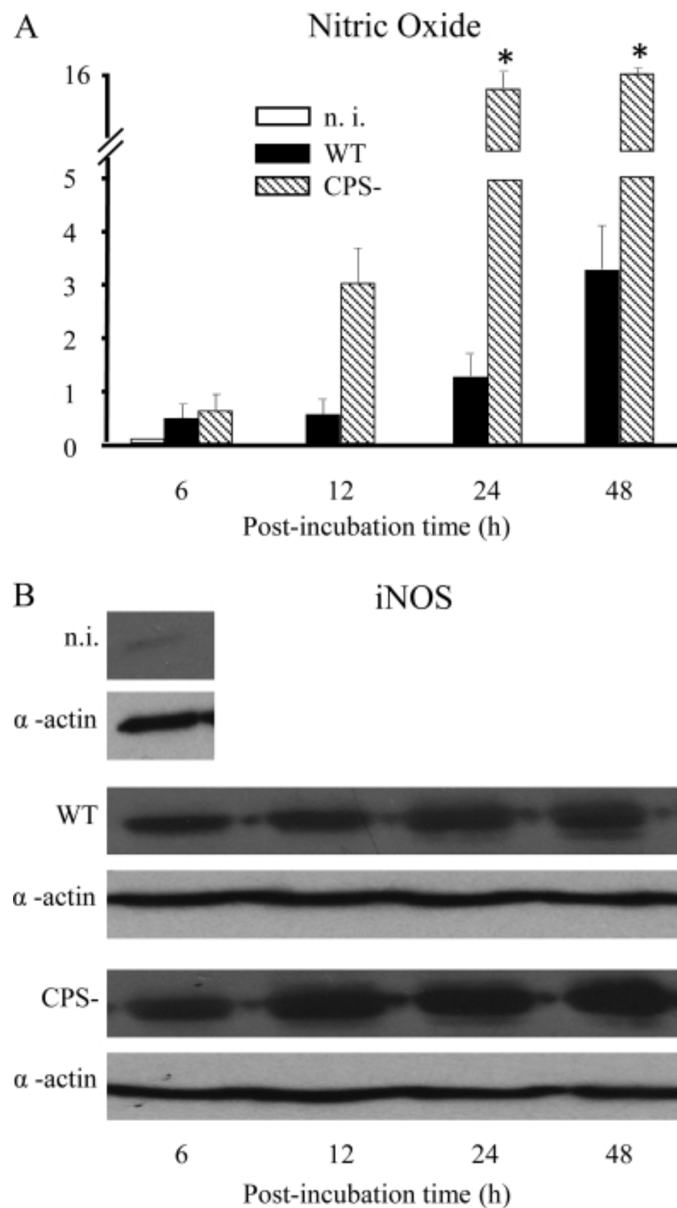

Figure 5. Time course of increase in nitric oxide production (A) and iNOS expression (B) by murine microglial cells treated with *S. suis*. The heat-killed *S. suis* WT or CPS^{-} strain (1×10^{9}) was incubated with microglia for 6, 12, 24, and 48 h. (A) Microglial supernatants were collected to measure nitric oxide production by the Griess reaction method. The data are expressed as the means plus SEM (in μM/ml) of three independent experiments. *, $P < 0.05$, indicating significant differences versus WT *S. sui*s. n. i., noninfected cells. (B) Representative Western blot analysis of murine microglial extracts using an iNOS-specific antibody. Blotting with anti-α-actin antibody was used as a loading control.

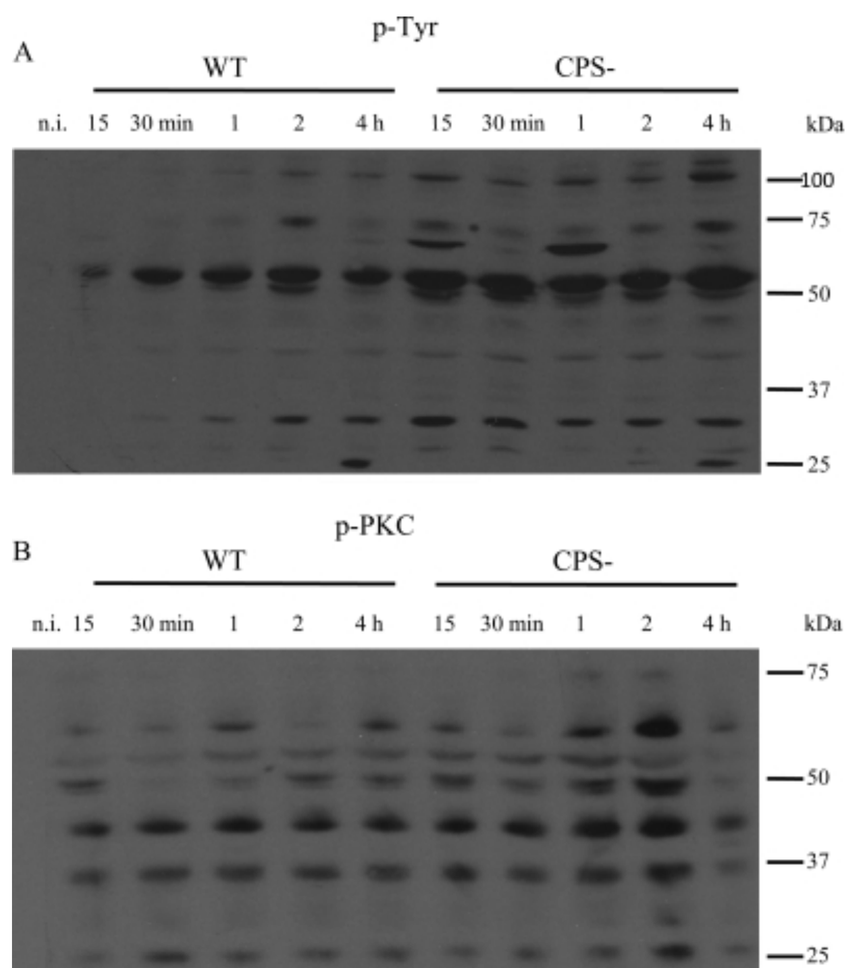

Figure 6. *S. suis*-induced levels of tyrosine phosphorylation (p-Tyr) (A) and serine phosphorylation (p-PKC) (B). Murine microglial cells were infected for 15 or 30 min or 1, 2, or 4 h with either the WT strain or its CPS^{-} mutant (1×10^6 bacteria). Cell lysates (total proteins) from noninfected cells and infected cells were subjected to Western blotting. p-Tyr and p-PKC protein levels were revealed by using anti-p-Tyr (clone 4G10) monoclonal antibody or anti-phospho-(Ser) PKC substrate antibody, respectively. The results are representative of three individual experiments.

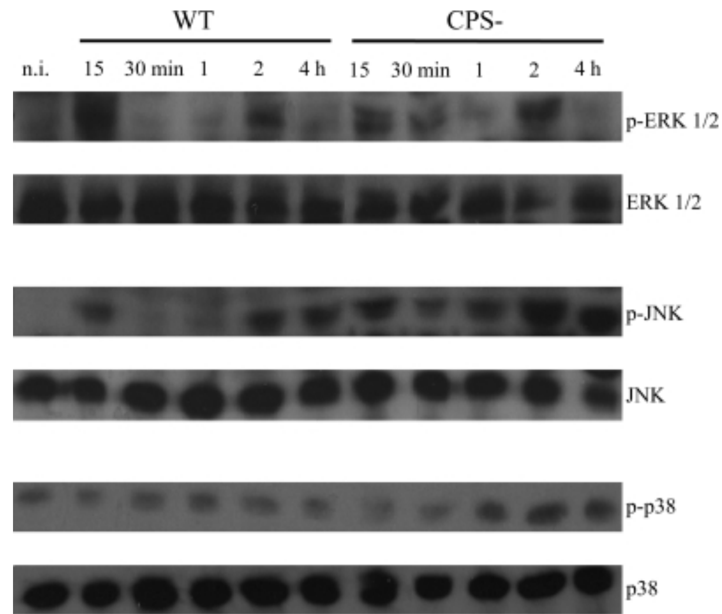

Figure 7. Time course of phosphorylation of MAPKs in murine microglial cells. The cells were infected with either the *S. suis* WT strain or its CPS^{-} mutant (1×10^6 bacteria). Cell extracts were recovered at 15 and 30 min and 1, 2, and 4 h postincubation and were subjected to Western blot analysis using antibodies specific for phospho-MAPKs (p-ERK, p-JNK, and p-p38). Following analysis, the blots were stripped and reprobed with an antibody specific for ERK, JNK, or p38 to verify uniformity in gel loading. The results are representative of three independent experiments. n. i., noninfected cells.

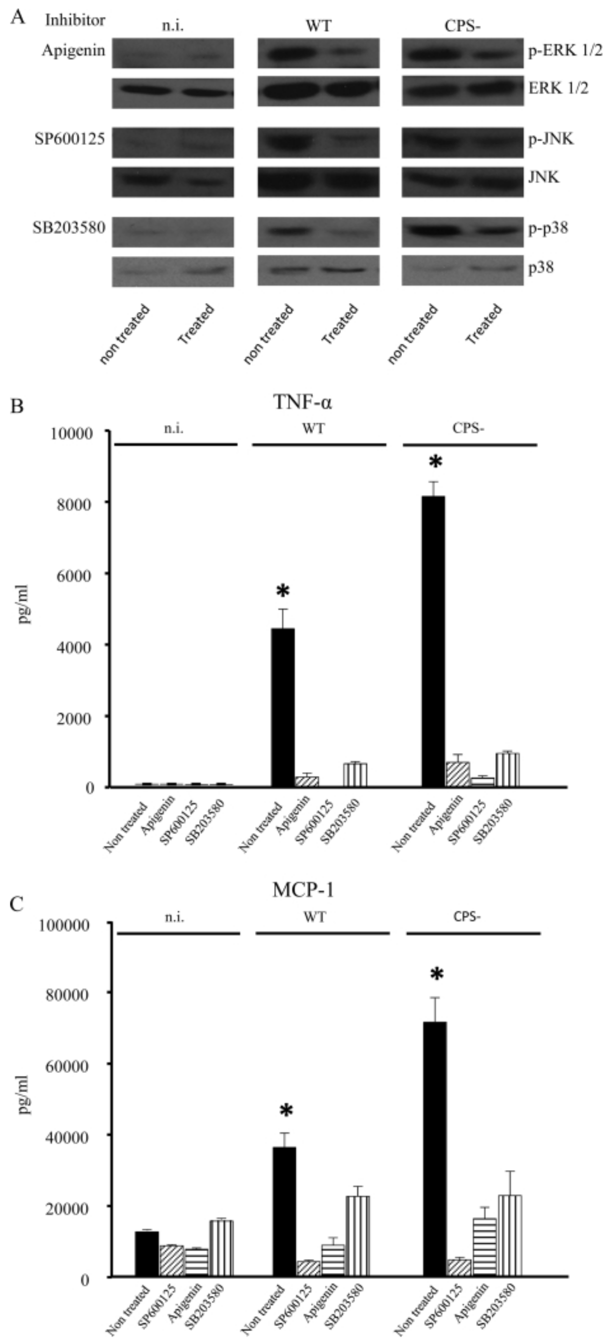

Figure 8. Pharmacologic inhibition of MAPKs. Murine microglial cells were treated with various inhibitors 1 h prior to infection with the *S. suis* WT strain or its CPS^- mutant (1×10^6 bacteria). Apigenin (50 μM), SP600125 (50 μM), and SB203580 (75 μM) inhibit ERK 1/2, JNK, and p38, respectively. The inhibitors were all used at maximal subcytotoxic doses for a total of 13 h. (A) To confirm inhibition of MAPK phosphorylation, cell extracts were recovered after 2 h (p-ERK and p-JNK) or 4 h (p-p38) of bacterium-cell contact and then analyzed by Western blotting using specific antibodies for each of the proteins tested. The results are representative of three independent experiments. (B and C) To evidence inhibition in cytokine production, cells were infected for 12 h, and the supernatant was recovered for detection of TNF-α (B) and MCP-1 (C) production by ELISA. The data are expressed as means plus SEM from three independent experiments. *, $P < 0.05$, indicating significant differences from cells treated with MAPK inhibitors.

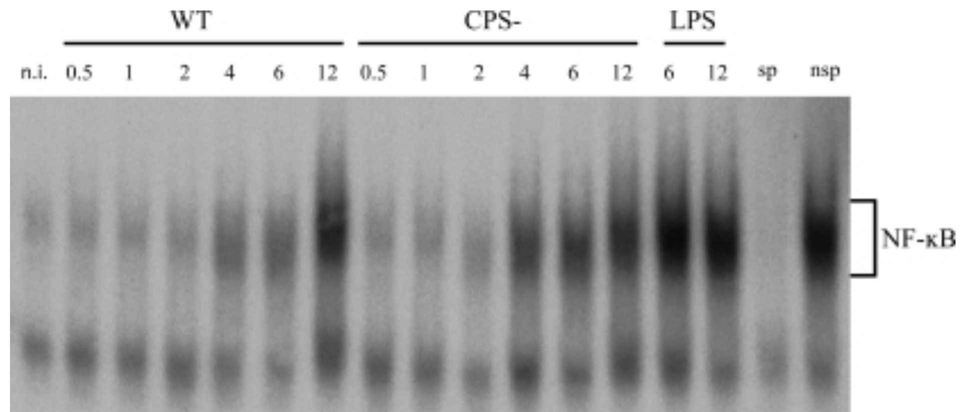

Figure 9. *S. suis* activates nuclear factor NF-κB in murine microglial cells. The cells were infected with either the *S. suis* WT strain or its CPS^{-} mutant (1×10^6 cells) for 0.5 to 12 h. Noninfected cells were used as negative controls. LPS (1 μg/ml) served as a positive control. The cells were lysed, and nuclear extracts were subjected to EMSA. The presence of NF-κB-activated proteins in the cell nuclei was demonstrated by binding to oligonucleotide probes containing a single copy of the NF-κB motif 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ end labeled with [γ-^{32}P]ATP. The binding reaction mixtures were electrophoresed on native 4% polyacrylamide gels to separate bound and unbound DNA probe. sp, specific probe; nsp, nonspecific probe.

Annexe V

Role of capsular polysaccharide in Group B *Streptococcus* interactions with dendritic cells.

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Rôle de la candidate dans la conception de l'article :

Je suis co-auteure de cet article. J'ai participé à la mise au point des techniques.

ABSTRACT

Group B *Streptococcus* (GBS) type III is an important agent of life-threatening invasive infections. Albeit the immune system plays a dual role in development and protection against disease, mechanisms leading to an efficient immune response against GBS remain obscure. Mouse bone marrow-derived dendritic cells (DCs) and primary spleen DCs were used to evaluate GBS capacity to modulate the functions of these important antigen-presenting cells. The role of capsular polysaccharide (CPS), one of the most important GBS virulence factors, in bacterial-DC interactions was evaluated by using a non-encapsulated mutant. Phagocytosis assays, confocal and electron microscopy showed that DCs efficiently internalize encapsulated GBS, but the latter possesses strong intracellular survival capacity. GBS devoid of CPS was internalized and killed at higher and faster rates than encapsulated GBS early after infection. Among several cytokines tested, GBS internalization was required for modulation of IL-12, IL-10 and CXCL10 pathways. In contrast, GBS induced DC expression of co-stimulatory molecules in a phagocytosis-independent manner. Finally, the production of pro-inflammatory and Th1 cytokines by GBS-stimulated DCs was differentially modulated by CPS expression, depending on DC origin. Our data suggest multiple mechanisms involved in GBS modulation of DC functions, which were selectively regulated by the presence of CPS.

INTRODUCTION

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is the main cause of life-threatening invasive bacterial infections in pregnant women and newborns in North America and Western Europe [1]. Clinical manifestations of GBS infection are mainly associated with pneumonia, septicemia, and meningitis. GBS is also increasingly associated with invasive disease in nonpregnant adults, especially among the elderly and individuals with underlying chronic illnesses [2]. Like many pathogenic bacteria, clinical isolates of GBS are covered by a capsular polysaccharide (CPS) recognized as the most important factor for bacterial survival within the host [3]. Among ten GBS capsular types that have been characterized [1] and [2], type III GBS is the most common type in GBS meningitis [1]. A cohort study suggested that the high invasiveness of this serotype may be related, at least in part, to inadequate maternal and infant levels of type III CPS-specific antibodies [4].

The structures of most GBS CPS types are formed by different arrangements of the monosaccharides glucose, galactose and N-acetylglucosamine into a unique repeating unit which contains a terminal sialic acid (Neu5Ac). The GBS terminal sialic acid capsular component mimics carbohydrate epitopes widely displayed on the surface of mammalian cells [3]. It was thus suggested that the sialic acid containing-CPS might be involved in immune evasion by decreasing immune recognition as a result of molecular mimicry of host epitopes and/or decreasing access of host pattern recognition receptors to cell wall components hidden beneath the CPS [5]. Nevertheless, conflicting data exist on the relative ability of encapsulated vs non-encapsulated strains to induce pro-inflammatory events by monocytes/macrophages or neutrophils [5], [6], [7] and [8]. Furthermore, it has been reported that encapsulated and non-encapsulated GBS are equally susceptible to macrophage uptake. As such, the role of CPS in phagocytosis resistance versus evasion of immune cell killing remains uncertain [8]. Effective uptake and killing of encapsulated GBS by these cells might require opsonization of the bacterium by specific antibodies and/or complement. Most studies have heretofore focused on opsonic and non-opsonic phagocytosis or killing of GBS by macrophages and neutrophils [8]. By contrast, the interactions of GBS with dendritic cells (DCs) and the consequences of such interactions in

the development of a specific immune response, including DC maturation and activation, have not been explored in detail.

Different types of leukocytes fulfill specialized tasks in antigen presentation and killing of pathogens [9]. DCs primary function is to alert the immune system, not to clear invading microorganisms. In fact, DCs are recognized as the most powerful antigen-presenting cells (APCs) that initiate immune responses against pathogens and are considered an essential link between innate and adaptive immunity. DCs capture and process antigens, and then undergo a maturation process characterized by the production of cytokines and up-regulation of co-stimulatory molecules. Afterwards, DCs migrate to adjacent lymphoid organs where they activate T cells [10]. In lymphoid tissues, conventional CD11c+ DCs comprise $CD8\alpha^+$ and $CD8\alpha^-$ subsets, which have distinct efficiencies of various antigen presentation pathways [11]. The $CD8^+$ DC subset has in common with other DCs the ability to take up exogenous antigens and to process these for presentation on MHC class II. However, the $CD8^+$ DCs also have antigen handling specializations that distinguish them from their $CD8^-$ DC neighbors. Whereas this major DC subpopulation of $CD8^-$ cells are more efficient at capturing particulate antigens, the CD8+ DCs appear more efficient in loading exogenously-derived protein antigens onto MHC class I molecules, a pathway called cross-presentation [11]. Consequently, the interactions between DCs and pathogens can strongly influence the outcome of a disease, and more importantly the magnitude and phenotype of the ensuing adaptive immune response to the invading pathogen. Thus, in this study, we used C57BL/6 mouse bone marrow-derived DCs (bmDCs) and spleen DCs to investigate their interactions with GBS type III in order to compare bacterial internalization and intracellular survival and the capacity of GBS to induce DC maturation and activation. The role of CPS in these processes was evaluated using an isogenic non-encapsulated mutant. Our results suggest that GBS-stimulated DCs were differentially modulated by the presence of CPS depending on DC origin.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplemental Table 1. Strain COH-1 is a highly encapsulated type III GBS isolate extensively described in previous work [12], [13] and [14]. This strain was kindly provided by Dr. C.E. Rubens, University of Washington, Seattle, WA. An isogenic non-encapsulated (Δ*cpsE*) mutant was constructed (see below). GBS strains were grown in Todd-Hewitt Broth (THB) or agar (THA) (Becton Dickinson, Mississauga, Ontario, Canada) or on sheep blood agar plates at 37 °C for 18 h. Escherichia coli strains were cultured in Luria-Bertani broth or agar (Becton Dickinson) at 37 °C for 18 h. When necessary, antibiotics (Sigma–Aldrich, Oakville, Ontario, Canada) were added to culture media at the following concentrations: kanamycin and spectinomycin at 50 µg/ml for *E. coli*, and spectinomycin at 200 µg/ml for GBS. To perform GBS-bmDCs interaction studies, isolated GBS colonies were inoculated in THB, incubated for 16 h at 37 °C with shaking. Working cultures were obtained by inoculating 100 µl of the 16 h-culture into 10 ml of THB followed by incubation for 5 h at 37 °C with shaking. Bacteria were washed twice in PBS, pH 7.3 and appropriately diluted in complete cell culture antibiotic-free medium. The number of CFU/ml in the final suspension was determined by plating samples onto THA using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA).

Construction of GBS non-encapsulated mutant

GBS genomic DNA was prepared by the guanidium thiocyanate method. Minipreparations of recombinant plasmids and transformation of *E. coli* were performed by standard procedures. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa Bio (Otsu, Shiga, Japan) and used according to the manufacturers' recommendation. PCR reactions were carried out with iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, Ontario, Canada). Oligonucleotide primers were purchased from Invitrogen (Burlington, Ontario, Canada). Amplification products were purified on Sephadex S-400 columns (GE Healthcare, Piscataway, NJ) and sequenced with an ABI 3730xl automated DNA sequencer, using the Bigdye terminator sequencing kit version 3.1 (Applied Biosystems, Streetsville, Ontario, Canada). The DNA sequence of

GBS strain COH-1 capsular (*cps*) locus was retrieved from GenBank (Accession number AF16383). Precise, in-frame deletion of the *cpsE* (1032 bp) gene was constructed by using splicing-by-overlap-extension PCR and the primers listed in Supplemental Table 1. The PCR-generated Δ*cpsE* deletion allele was cloned into plasmid pCR2.1 (Invitrogen), extracted with BamHI and PstI and recloned into the thermosensitive shuttle plasmid pSET-4s [15] digested with the same enzymes, giving rise to the knockout vector p4ΔcpsE. Electroporation of GBS with the recombinant plasmid and procedures for isolation of mutants were those described previously [16]. Allelic replacement was confirmed by PCR and sequencing analysis. The non-encapsulated (Δ*cpsE*) phenotype of the mutant was confirmed by absence of reaction in the coagglutination test using rabbit antisera against GBS type III capsular material (Denka Seiken, Campbell, CA), and by transmission electron microscopy (TEM) using polycationic ferritin labeling as previously described [16] (Supplemental Fig. S1). Growth rates were not significantly affected in the mutant strain compared to wild-type (WT) GBS (Supplemental Fig. S1).

Generation of bone marrow-derived dendritic cells (bmDCs) and purification of primary spleen DCs

BmDCs were generated from female C57BL/6 mice (Charles River Laboratories, Wilmington, MA). All experiments involving mice 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 Use of Laboratory Animals by the Animal Welfare Committee of Université de Montréal. BmDCs were produced according to a technique described elsewhere [17]. Briefly, after red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 μg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM l-glutamine and 50 μM 2-ME. All reagents were from Gibco (Invitrogen). Complete medium was complemented with 20% GM-CSF from a mouse GM-CSF transfected cell line (Ag8653) as a source of GM-CSF [17]. Cells were cultured for 7 days at 37 °C with 5% CO_2. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8 and used as immature bmDCs for the studies. Cell purity was routinely ≥ 88% $CD11c^{+high}$ and $F4/80^{-/dim}$ cells as determined by FACS analysis (Supplemental

Fig. S2), values that were in agreement with those reported in other studies [18], [19] and [20]. Staining for CD3 and granulocyte differentiation antigen 1 (Gr-1; also known as Ly-6 G) were also included as purity controls (Supplemental Fig. S2). For confirmation, bmDCs were further purified by positive selection using anti-CD11c microbeads by MACS (Miltenyi Biotec, Auburn, CA). The resulting bmDCs were routinely > 90–95% $CD11c^+$, in agreement with previously published data [21]. For comparative purposes, in selected experiments primary spleen DCs purified by MACS as previously described [22], were included.

Cell toxicity test

Cell toxicity induced by different GBS strains and concentrations was evaluated by measuring the release of lactate dehydrogenase enzyme (LDH) with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) as previously described [16]. LDH results showed that GBS strains induced significant cytotoxicity (>10–15%) after 6 h of incubation when used at concentrations higher that 10^6 CFU/ml (Supplemental Fig. S2). No differences in toxicity levels were observed between the mutant strain and the WT strain (data not shown).

Bacterial internalization assay

BmDCs or spleen DCs at a concentration of 10^6 cells/ml were infected with 10^6 CFU/ml of different GBS strains (initial MOI:1), and incubated for 0.5–6 h at 37 °C with 5% CO_2. After incubation, 100 μg/ml of gentamycin and 5 μg/ml of penicillin G (Sigma–Aldrich) were added to kill extracellular bacteria. It has been demonstrated that these antibiotics do not penetrate eukaryotic cells under these conditions [23], and preliminary studies have shown that these concentrations of antibiotics were able to kill any remaining extracellular bacteria (data not shown). After 1 h-antibiotic treatment, cells were washed 3 times with PBS, lysed with sterile water and viable intracellular streptococci enumerated by quantitative plating of serial dilutions of the lysates on THA. In selected experiments, cytochalasin D at 0.1, 0.5, 1.0, and 2.0 μg/ml was added to bmDCs for 30 min prior to bacterial infection (initial MOI:1) for 60 min and phagocytosis was evaluated as described above.

For intracellular survival studies, internalization assays were performed as described above, except that after a 60 min initial bacterial-cell contact, gentamycin-penicillin were added and the treatment was lengthened for different times up to 5 h. The results were expressed as CFU/ml of recovered intracellular viable bacteria.

In vitro DC stimulation assay

BmDCs or spleen DCs were resuspended at 10^6 cells/ml in complete medium and stimulated with 1 μM CpG (ODN 1826, Coley Pharmaceutical Inc., Düsseldorf, Germany), used as positive control, or different GBS strains (10^6 CFU/ml; initial MOI:1). After 2 h of DC-GBS infection, the bacteriostatic agent chloramphenicol (CM, 12 μg/ml, Sigma–Aldrich) was added to the culture to prevent cell toxicity. CM is a bacteriostatic agent, keeping a low and controlled level of bacteria through the incubation period ($\sim 5 \times 10^4$ CFU/ml). Time of bacteria-cell contact prior to CM treatment and minimal CM dose required were selected based on preliminary studies on cytokine production combined with LDH test (Supplemental Fig. S2, and data not shown). The chosen dose of CM had no effect of cytokine profiles induced by either the positive or the negative control (data not shown). To evaluate the role of bacterial phagocytosis in bmDC activation, cytochalasin D at 2 μg/ml was added to bmDCs for 30 min prior to bacterial infection. At different time intervals, supernatants were collected for cytokine quantification by ELISA and cells were harvested for analysis of co-stimulatory molecule expression by FACS. Non-stimulated cells served as negative control. In selected experiments, 10^8 latex beads (2 μm, Molecular Probes, Invitrogen) were used as an additional control. All solutions and bacterial preparations used in these experiments were tested for the absence of endotoxin using a Limulus amebocyte lysate test (Pyrotell, STV, Cape Cod, MA) with a sensitivity limit of 0.03 EU/ml.

Cytokine quantification by ELISA

Levels of IL-6, IL-10, IL-12p70, TNF-α, CXCL1 (KC), CXCL9 (MIG) and CXCL10 (IP-10) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN) or eBioscience (San Diego, CA), according to the manufacturer's recommendations. Twofold dilutions of recombinant mouse cytokines were used to generate standard curves. Sample dilutions giving OD

readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine.

FACS analysis

For cell surface staining, 10^6 cells were washed and treated for 30 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab, BD PharMingen, BD Biosciences, Mississauga, Ontario, Canada) in sorting buffer (PBS-1% fetal bovine serum). Blocked cells were then incubated with FITC-labeled anti-mouse CD11c mAb (BD PharMingen) for 1 h on ice followed by washing and staining for 1 h with PE-labeled mAbs against the following surface molecules: CD80 (clone 16-10A1), CD86 (clone GL1), CD40 (clone 3/23), CD54 (clone 3E2) and MHC class II (A_{β}^{b}; clone AF6-120.1), all from BD PharMingen. For cell culture purity tests, PE-labeled anti-mouse CD11c mAb (clone HL3), FITC-labeled anti-mouse F4/80 mAb (clone C1:A3-1), FITC-labeled anti-mouse Gr-1 mAb (clone RB6-8C5), and PE-labeled anti-mouse CD3 mAb (clone 145-2C11) were used (Supplemental Fig. S2). After washing, cells were resuspended in sorting buffer for FACS analysis. Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). Twenty thousand gated events were acquired per sample and data analysis was performed using CellQuest software. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales.

Confocal microscopy

For immunofluorescence studies, bmDCs, treated or not with cytochalasin D as described above, were placed on coverslips and infected with 10^6 CFU/ml of different GBS strains (initial MOI:1). Unless specified, after 2 h of bacteria-cell contact, coverslips were washed with PBS three times to remove non-associated bacteria. Cells were fixed with methanol/acetone (80:20) for 20 min at −20 °C, washed and blocked for 10 min. Coverslips were incubated for 1 h with rabbit anti-GBS serum and with rat anti-mouse lysosomal-associated membrane protein-1 (LAMP-1) mAb (Clone 1D4B, Developmental Studies Hybridoma Bank, Iowa City, IA). After washing, coverslips were incubated with different combination of secondary antibodies Alexa-Fluor 488 (green) or Alexa-Fluor 568 (red) goat anti-rabbit IgG or goat anti-rat IgG (Invitrogen) for 30 min, washed and mounted on glass slides with moviol containing DABCO and DAPI to stain the nuclei. Samples were

observed with an Olympus FluoView™ FV1000 confocal laser scanning microscope and analyzed using Fluoview software (Markham, ON, Canada).

Electron microscopy analysis

For TEM and scanning electron microscopy (SEM), GBS strains were incubated with bmDCs for 4 h. After two washes with PBS, samples were fixed for 1 h at room temperature with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and postfixed for 45 min at room temperature with 2% osmium tetroxide in deionized water. Specimens for TEM were dehydrated in a graded series of ethanol and embedded with LR White resin. Thin sections were poststained with uranyl acetate and lead citrate. Samples were observed with an electron microscope model JEOL JEM-1230 (Tokyo, Japan). Samples for SEM were dehydrated in a graded series of ethanol solutions and covered with gold after critical point drying and were examined with a Hitachi S-3000 N microscope.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed for significance using Student's unpaired t-test. A P value < 0.05 was used as a threshold for significance. All experiments were repeated at least three times. Confocal microscopy data analysis was performed using the Fluoview software. Integrated fluorescence intensities for each fluorescence channel were analyzed. Briefly, 60 cells were randomly selected for each condition. Average integrated fluorescence was calculated by the software and the obtained ratio was normalized according to DAPI/Nuclei channel variance between samples.

RESULTS

DCs efficiently internalize both the non-encapsulated and the encapsulated GBS strains, but the latter shows stronger capacity to survive intracellularly

As antigen uptake is the first step in APC activation we performed an antibiotic-protection phagocytosis assay to evaluate GBS internalization by DCs. Compared to the classical bactericidal test, in this assay extracellular bacteria are eliminated by an antibiotic-treatment step and only intracellular bacteria are thus precisely quantified. As shown in Fig. 1A, under non-opsonic conditions, the encapsulated WT strain was rapidly internalized by bmDCs. Phagocytosis levels increased over time reaching levels as high as 10^7 CFU/ml of total recovered intracellular bacteria. At short incubation times the Δ*cpsE* mutant was significantly more internalized than the WT strain, though differences were lost after longer incubation times. Extracellular bacterial replication over the incubation time was similar between the two strains (Supplemental Fig. S1). Thus differences in the levels of bacterial internalization between strains likely reflect the capacity of bmDCs to more easily and faster internalize the mutant strain at early time points.

As antigen capture capacity might differ depending on the DC subset, we next evaluated GBS internalization by spleen DCs, which contains the two major DC subsets, $CD8^-$ and $CD8^+$. Phagocytosis levels were similar to those observed with bmDC ($P > 0.05$), albeit the differences between the WT and the Δ*cpsE* mutant in terms of internalization were more marked (Fig. 1B).

To analyze the fate of bacteria once internalized, we modified the phagocytosis assay in order to quantify bacterial intracellular survival over time. After optimal internalization following 60 min incubation of GBS with bmDCs, antibiotics were added and incubation extended for different periods of time up to 5 h. A slow decrease in WT GBS intracellular survival was observed (Fig. 1C), which was significant only after 3 h of GBS post-internalization, with $\sim 2 \times 10^3$ CFU of remaining live intracellular bacteria at the end of the survival test. At early time points, the decrease in intracellular survival of the Δ*cpsE* mutant was faster than that observed for WT GBS, and significant differences were observed as soon as 1 h of mutant strain post-internalization (Fig. 1C). Compared to initial numbers of

phagocytosed bacteria (time 0), there was a 25-fold reduction in intracellular numbers of the Δ*cpsE* mutant bacteria, while only an 8-fold decrease in WT GBS intracellular numbers was observed at the end of the survival test.

We next evaluated if GBS intracellular fate might vary upon DC origin (Fig. 1D). Using spleen DCs we observed a reduction in intracellular numbers of WT GBS compared to that observed with bmDCs (8-fold decrease vs. 15-fold decrease, $P < 0.05$). The difference between bmDCs and spleen DCs in their capacity to destroy intracellular bacteria was even more marked in the case of the Δ*cpsE* mutant, which showed 56-fold reduction in intracellular numbers (Fig. 1D).

The phagocytosis and the intracellular survival assays, altogether, showed that encapsulated GBS is easily internalized by DCs, but efficiently survives inside these cells for at least 6 h. The CPS plays a role in modulating both processes as encapsulated GBS showed overall better survival rates than the mutant strain under all conditions tested.

Intracellular GBS locates within LAMP-1$^+$ phagosomes in bmDCs

Internalized bacteria are contained within phagosomes that might mature into phagolysosomes. During maturation, phagosomes sequentially fuse with and acquire membrane markers of early endosomes and late endosomes, including LAMP-1 [24]. Thus, to confirm the intracellular location of the bacteria, confocal microscopy was performed using serum against GBS and an antibody specific for LAMP-1. Confocal microscopy analysis under non-opsonic conditions showed that the number of intracellular GBS within LAMP-1$^+$ vacuoles varied from few cocci per cell to more than 30 within the same bmDC. A tendency of the mutant strain to be more internalized than WT GBS was noticeable (Fig. 2). Quantification of average integrated fluorescence intensities (FI) showed a FI value of 183144 ± 23236 for WT GBS-infected bmDCs compared to a FI value of 223049 ± 14344 for Δ*cpsE* mutant strain-infected cells.

We also performed the phagocytosis assay in presence of cytochalasin D, an inhibitor known to disrupt actin filament polymerization. GBS internalization was dramatically decreased after bmDC pre-treatment with cytochalasin D in a dose-dependent fashion.

Quantitative phagocytosis and confocal microscopy (Supplemental Fig. S3) demonstrated that almost complete inhibition is achieved at 2 µg/ml of cytochalasin D (also see Fig. 1A). Both WT and Δ*cpsE* strains were affected similarly by this reagent, confirming phagocytosis of both GBS strains.

Electron microscopy shows GBS localization inside bmDC phagosomes

As electron microscopy is the ultimate technique to verify the intracellular location of organisms, SEM and TEM examination were performed to confirm confocal microscopy results. SEM showed that after 4 h-infection of bmDCs with either the WT strain or the non-encapsulated Δ*cpsE* mutant, numerous cocci were found associated to the cell surface, underneath as well inside the cells (Fig. 3A). TEM analysis confirmed the intracellular location of bacteria and showed that bmDCs contained high numbers of intracellular WT strain and non-encapsulated Δ*cpsE* mutant (Fig. 3B). The uptake process was accompanied by the formation of phagosomes in which streptococci seemed to reside. These phagosomes appeared to contain one or more bacteria.

GBS induces bmDC maturation and activation

A critical determinant of DC function is their level of maturation. Induction of DC maturation increases their potential to stimulate T cells. Phenotypically, immature DCs are characterized by low surface expression of MHC class I and II proteins, and co-stimulatory molecules (CD40, CD80, and CD86) [25], data in agreement to values observed in our bmDC cultures under steady conditions (Fig. 4A). In contrast, mature DCs normally posses high surface expression of these markers. Maturation is a terminal and critical event in DC development. DCs require maturation before exhibiting their full immunostimulatory potential [25] and [26]. To evaluate GBS capacity to induce bmDC maturation, we investigated expression levels of the co-stimulatory molecules CD40, CD54, CD80, CD86 and MHC-II on bmDCs. The phenotypes of bmDCs were evaluated by FACS at 16 h post-infection. No significant changes in CD54 and CD80 expression in response to GBS stimulation were observed compared to control cells (data not shown). However, GBS-stimulated bmDCs showed higher expression levels of CD40 in terms of the percentage of cells expressing this marker and in mean fluorescence intensity. Two well segregated sub-populations, a $CD86^{high}$ and $MHC\text{-}II^{high}$ subset and a $CD86^{low}$ and $MHC\text{-}II^{low}$ subset, were

observed among the CD11c^{+} bmDC population, as already reported [16]. GBS stimulation resulted in higher percentages of CD86^{high} and MHC-II^{high} sub-populations compared to control cells. No differences were observed between the WT strain and the mutant strain (Fig. 4A).

Production of cytokines by DCs plays a major role not only in modulating the innate immune response, but also in shaping the ensuing adaptive response to a pathogen. Thus, to evaluate GBS capacity to induce DC activation, the levels of selected cytokines and chemokines in the supernatants of GBS-infected bmDCs were quantified (Fig. 4B). The pro-inflammatory cytokines IL-6 and TNF-α have been shown to be major mediators of GBS sepsis [5] and [7]. As shown in Fig. 4B, GBS-activated bmDCs produced very high levels of these two pro-inflammatory cytokines. In addition, bmDCs also released significant amounts of the Th1-driving cytokine IL-12p70 and of the regulatory IL-10 after exposure to GBS strains. Maximal production of all these cytokines was observed at 16 h post-infection compared to control, non-activated cells ($P < 0.001$). Kinetics of cytokine production were similar between encapsulated WT GBS and the Δ*cpsE* mutant strain. The mutant strain showed slightly higher stimulatory capacity at shorter incubation times (6 h post-infection) compared to the WT strain. However, with the exception of IL-10, the observed differences disappeared at longer incubation times (16 h).

As anti-bacterial polymorphonuclear leukocyte activity has been shown to play an important role in the innate immune response to GBS [7], we also evaluated the production of CXCL1, a neutrophil chemoattractant factor. Similar high levels of CXCL1 were induced by both GBS strains (Fig. 4B), indicating that CPS does not interfere or modulate the production of this chemokine. Considering the central role of DCs as APC able to activate NK and T cells, the levels of the chemokines CXCL9 and CXCL10 were also evaluated in GBS-activated bmDCs. GBS strains induced low, but significant, levels of CXCL10 production by bmDCs and failed to induce significant levels of CXCL9 as compared to control, non-infected cells ($P > 0.05$). It should be noted that CpG, used as positive control, induced the release of high levels of these two CXC chemokines, indicating optimal cell culture conditions. Results from cytokine and FACS analysis were confirmed with bmDCs purified by MACS (data not shown).

The presence of CPS differentially regulates cytokine production by spleen DCs

Pattern of cytokine production might vary upon DC phenotype and origin [25] and [27], thus we decided to evaluate the capacity of GBS to modulate cytokine production by freshly isolated spleen DCs (Fig. 5). Overall, cytokine production by GBS-infected spleen DCs was lower than that of infected bmDCs (pg/ml range vs. ng/ml range). Production of CXCL9 was the only exception, as significant levels of secretion of this chemokine was observed in GBS-infected spleen DCs (Fig. 5F), but not in bmDC counterparts (Fig. 4B).

We next evaluated the role of CPS in modulating cytokine production by spleen DCs. Similarly to that observed with bmDCs, production of IL-10 was significantly higher in spleen DCs activated by the Δ*cpsE* mutant strain compared to those activated by WT GBS (Fig. 5D). However, at 16 h post-infection the production of all other cytokines was significantly reduced in Δ*cpsE* mutant-infected spleen DCs compared to WT GBS-infected counterparts (Fig. 5), suggesting differences in the kinetics of cytokine production between bmDCs and spleen DCs.

Effect of bacterial phagocytosis on bmDC activation levels

DC activation can be either surface receptor-mediated or intracellular receptor-mediated or both. As the initial levels of GBS internalization were similar between bmDCs and spleen DCs (Fig. 1), we used bmDCs as a model to evaluate the role of phagocytosis as signal for triggering cytokine release. To this aim, bmDC were pre-treated with cytochalasin D prior to GBS stimulation, based on our previous finding that this inhibitor almost completely abrogated bacterial internalization (Supplemental Fig. 3). As a control, we used 2 μm-latex beads. Latex beads failed to induce significant release of cytokines compared to non-stimulated control cells throughout a period of 16 h (data not shown). As shown in Fig. 6, IL-12p70, IL-10 and CXCL10 were the only three cytokines whose production was almost completely abrogated by cytochalasin D treatment of GBS-infected cells (>90% of inhibition, Fig. 6A–C). For all other cytokines, cytochalasin D-mediated inhibition of GBS-induced cytokine production ranged from 53% to 76% (Fig. 6D–F). These data suggest that both strains use phagocytosis-dependent and -independent pathways for cytokine production.

We also evaluated the effect of bacterial phagocytosis in modulation of bmDC maturation. In contrast with our observations for cytokine production, phagocytosis was not significantly required for GBS-induction of co-stimulatory molecule expression for either strain. Cytochalasin D-mediated inhibition of GBS-induced expression of CD40 was 21 ± 9%; of CD86, 11 ± 6% and of MHC-II, 29 ± 10% ($P > 0.05$). Similarly, surface expression of co-stimulatory molecules was not affected by cytochalasin D in *Streptococcus pyogenes*-infected macrophages [9]. These results suggest multiple mechanisms involved in GBS modulation of DC activation.

DISCUSSION

Despite the importance of DCs in shaping the immune response against pathogens, the interactions between GBS and DCs have not thoroughly been characterized, and only two additional studies are available on GBS activation of bmDC [13] and [28]. In fact, albeit extensive research on GBS interaction with professional phagocytes, such as macrophages and neutrophils, the strategies used by GBS to manipulate the functions of DCs, known as the most potent APCs, have not been investigated. This study addresses for the first time the role of GBS CPS in bacterial internalization by bmDCs and primary spleen DCs and the underlying consequences on their maturation and activation.

It has been well established that GBS is able to invade a variety of host-cell types, including epithelial cells, endothelial cells and macrophages [5], [7], [8], [14] and [23]. We used phagocytosis assays in combination with confocal and electron microscopy to measure the ability of DCs to internalize GBS. We observed that both bmDCs and spleen DCs efficiently internalize encapsulated GBS. Our results are in agreement with previous studies using macrophage/monocyte cell lines or primary macrophages or neutrophils of either human or mouse origin [14], [23], [29], [30] and [31]. Overall, the capacity of DCs to internalize encapsulated GBS seems similar or lower to that reported for these cells. It is generally accepted that different types of leukocytes differentially perform their specialized tasks. As such, it has been shown that neutrophils and monocytes exhibit a much higher capacity to kill ingested bacteria than DCs [9] and [32]. When we evaluated the capacity of bmDCs to eliminate encapsulated GBS, we observed that intracellular survival lasted at least 6 h. In a recent study, Mancuso *et al*. showed that encapsulated GBS survived longer in bmDCs compared to mouse macrophages [13]. However, when we compared GBS survival rates within primary spleen DCs, a reduction in intracellular survival was observed, suggesting that spleen DCs possess stronger bactericidal capacity than bmDCs. In parallel studies we observed that Streptococcus suis, another well-encapsulated invasive meningeal pathogen, was readily and rapidly eliminated by bmDCs [16] and [33]. We thus speculated that the enhanced ability of encapsulated GBS to survive within bmDCs, and at least for few hours in spleen DCs, is not merely a reflection of the inherent capacity of these APCs to kill pathogens, but rather represents intrinsic GBS properties that allow this

organism to resist or delay intracellular bactericidal DC mechanisms. Mancuso *et al*. studied the spatial relationships among GBS and bmDC endosomal markers with structured illumination fluorescence microscopy. They found GBS antigens in two distinct bmDC intracellular compartments: bacterial DNA^+ phagosomes and DNA^- phagolysosomes containing partially digested GBS material [13]. The potential capacity of GBS to avoid or delay phagosome maturation and/or fusion with lysosomes warrants future investigation.

As the role of CPS, a major GBS virulence factor, in the interactions of this pathogen with DCs has never been addressed, we constructed a non-encapsulated isogenic mutant. GBS devoid of CPS was internalized at higher and faster rates than encapsulated GBS early after infection, although the differences were less marked after longer incubation times. The survival of the non-encapsulated mutant was significantly impaired in both bmDCs and spleen DCs compared to WT GBS. As aforementioned, spleen DCs were particularly more efficient in destroying non-encapsulated GBS than bmDCs. The enhanced bactericidal capacity of spleen DCs might suggest that both $CD8^+$ and $CD8^−$ DC sub-populations contributes to bacteria clearance. These data suggest that CPS facilitates, at least in part, GBS survival within DCs. In previous studies, it was shown that under non-opsonic conditions, non-encapsulated mutants were equally phagocytosed and survived at similar levels within macrophages than WT GBS [14] and [30]. Thus, the contribution of the CPS to the modulation of professional phagocyte functions vs. DC functions might be different. In this regard, it is important to notice that GBS CPS harbors terminal sialic acid residues. Sialic acid features prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates and confers important properties at the cell surface. It is therefore not surprising that many pathogenic bacteria have evolved to express sialic acid to resist or modulate host immune responses and to interact specifically with different host cells [34]. For example, it was reported that GBS sialic acid binds to Sia-binding immunoglobin superfamily lectins (Siglecs) and impairs bactericidal functions of neutrophils [6]. It has been suggested that expression of the sialylated CPS by *Neisseria meningitidis* protects bacteria against phagosomal killing by human DCs and monocytes [35] and [36].

One of the consequences of bacterial interaction with DCs is the induction of their maturation and activation. Encapsulated WT GBS was shown to induce increased

expression of the co-stimulatory molecules CD40, CD86, as well as MHC-II; and to trigger DC release of several cytokines and chemokines. The production of these immune mediators was in general higher in GBS-infected bmDCs than in spleen DC counterparts. Similarly, it has been reported that primary spleen DCs infected in vitro with *Listeria monocytogenes* secrete lower levels of cytokines than bmDCs [27]. In agreement with these observations, DCs generated by endogenous GM-CSF overexpression in vivo show a high level cytokine-profile compare to control DCs, suggesting an important immuno-modulatory role of GM-CSF during DC development [25].

We next evaluated the role of CPS in GBS modulation of DC functions. In the absence of the CPS shield, exposition of highly immunogenic cell wall components might induce increased DC activation, as already observed for other encapsulated pathogens [16], [33] and [36]. A transient increase in the release of several cytokines was observed in bmDCs infected with the non-encapsulated mutant; however, these differences were lost at longer incubation times. The only exception was IL-10, which production was consistently higher in bmDCs infected with the mutant strain. In the absence of CPS, the production of IL-10 in infected spleen DCs was even more marked; reaching almost a 3 fold increase compared to WT GBS-infected counterparts. Another striking feature is that the production of all other cytokines by spleen DCs was indeed reduced or almost completely inhibited (as for IL-12p70) after infection with the non-encapsulated mutant. Two interrelated hypothesis might explain these observations, a) increased IL-10 production in the absence of CPS erases differences in cytokine production (in the case of bmDCs) and/or reduce cytokine production (in the case of spleen DCs; b) higher and faster levels of non-encapsulated bacteria killing reduce or impair cytokine production, especially in the case of spleen DCs. The observed CPS regulatory role of DC functions might have important consequences in the immunopathogenesis of GBS infection. In vivo studies demonstrated that IL-12/IL-10 balance is important in controlling the cytokine production that leads to the evolution of GBS-induced pathology and have a major role in restricting bacterial growth during infection [37], [38] and [39]. In this regard, GBS internalization is largely required for modulation of both, the IL-12 and IL-10 pathways. *Staphylococcus aureus* induces IL-10 in macrophages after recognition of peptidoglycan-embedded lipopeptides in the bacterial-cell wall. It has been proposed that APC-peptidoglycan interaction may down-

regulate inflammation when present in large quantities [40] and [41]. Thus, DC signaling triggered by GBS internalization may has an inherent plasticity to induce pro-inflammatory and anti-inflammatory responses depending on the level of cell wall exposition and degree of intracellular bacterial degradation.

Chemokines are also important in regulating innate and adaptive immune responses. GBS-exposed bmDCs and spleen DCs secreted high levels of the neutrophil chemoattractant CXCL1, as already reported with mouse macrophages [42]. High levels of CXCL1 secretion might contribute to host innate defense against GBS but might also results in increased pathology. On the other hand, bmDC stimulated with GBS failed to produce significant levels of CXCL9 and produced low and delayed levels of the CXCL10 chemokine. In contrast, GBS-infected spleen DCs produced significant levels of both, CXCL9 and CXCL10, which might reflects the already reported diverse property of chemokine production among DC subsets [43]. These two chemokines share the ability to signal through CXCR3, which is present on T cells and NK cells. Although up-regulation of Cxcl10 gene expression was observed by DNA microarray analyses of mouse peritoneal macrophages [44], GBS was reported to be unable to induce CXCL10 secretion by these cells [42]. A critical role of type I IFN-dependent CXCL10 has been identified in host defense during microbial sepsis by increasing neutrophil recruitment and function [45]. Mancuso *et al*. reported that GBS induces type I IFN by DCs via a lysosomal TLR7-dependent pathway [13]. This novel bacterial-recognition system operates in conventional DCs but not in macrophages. Thus, GBS modulation of this pathway in DCs might have important consequences for both innate and adaptive immune responses against this pathogen. Mancuso *et al*. suggested that by disrupting phagosomal maturation and/or integrity, bacterial pathogens may avoid not only direct killing but also immune recognition in lysosomal compartments and the subsequent establishment host-protective responses dependent on interferon-regulatory transcription factor-1 [13]. In agreement with these results, CXCL10 production was almost completely abrogated by cytochalasin D treatment of GBS-infected cells, confirming that intracellular bacteria is required for activation of this pathway.

To conclude, our results show that DCs efficiently internalize GBS and might play a role in

the development of host innate and adaptive immunity during an infection with this pathogen. However, the CPS of GBS differently modulates DC activation and function and contributes to bacterial intracellular survival. Finally, bmDCs and primary spleen DCs differ in some aspects of their responses upon infection, illustrating the importance of comparing different DC sub-populations when studying host–pathogen interactions.

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Supplemental Table 1 : Bacterial strains, plasmids and oligonucleotide primers used in this study.

Strains/Plasmid/Primes	General characteristics	Source/Reference
Escherichia coli		
TOP 10	F-*mrcA* *Δ(mrr-hsd*RMS-*mcr*BC*)φ80 lacZΔM5 Δlac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Group B *Streptococcus*		
COH-1	Wild-type, highly encapsulated strain isolated from an infant with bacteremia. Serotype III	[12]
Δ*cpsE*	Non-encapsulated strain derived from strain COH-1. Deletion of the *cpsE* gene	This work
Plasmids		
pCR2.1	Ap^r, Km^r, *oriR*(f1) MCS *oriR* (ColE1)	Invitrogen
pSET-4s	Thermosensitive vector for allelic replacement. Replication functions of pG+host3, MCS *oriR* pUC19 *lacZ* Sp^R	[15]
p4ΔcpsE	pSET-4s carrying the construct for *cpsE* allelic replacement	This work
Oligonucleotide primers, sequence (5' – 3')		
GBScpsE-ID1	AGAATACTTCAATGCGATCCG	
GBScpsE-ID2	TCAAGATAGCCACGACTCC	
GBScpsE-ID3	AAGGCGATATGAGTTTAGCAG	
GBScpsE-ID4	CGCCATGTGTGATAACAATCTC	
GBScpsE-ID5	TGGAACTATTAAAGGCTTGACG	
GBScpsE-ID6	CCTGTCCCGAGTAAAACTACTACAACTGTTTGAATCATCGC	
GBScpsE-ID7	GCGATGATTCAAACAGTTGTAGTAGTTTTACTCGGGACAGG	
GBScpsE-ID8	TCCCCACTGTGACAAAAATC	

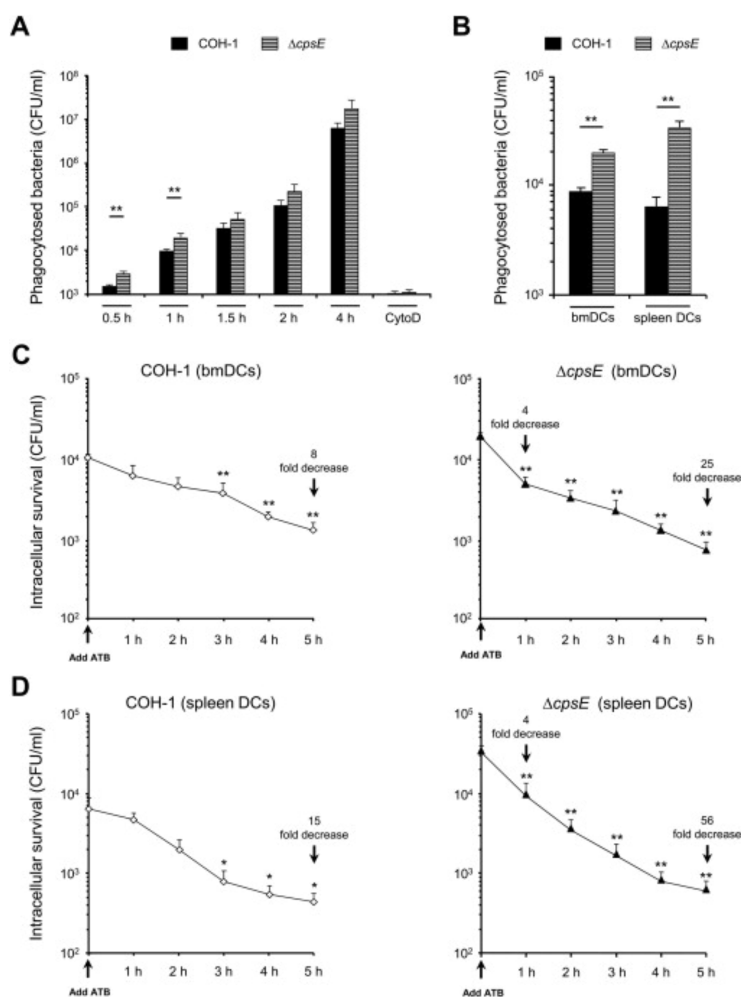

Figure 1. GBS phagocytosis by and intracellular survival within DCs: role of bacterial capsular polysaccharide. (A) Wild-type GBS strain COH-1 or the Δ*cpsE* mutant (10^6 CFU/ml, initial MOI:1) were incubated with bmDCs for different time periods. Internalized bacteria were enumerated by quantitative plating after 1 h of antibiotic treatment to kill extracellular bacteria. At a selected time point of 60 min, bmDC were pre-treated with 2 µg/ml cytochalasin D (CytoD) and phagocytosis evaluated as described. **$P < 0.01$, indicates statistically significant differences between the wild-type strain and the Δ*cpsE* mutant, n = 5. (B) MACS purified-spleen DCs were infected with GBS strains for 60 min (10^6 CFU/ml, initial MOI:1). Internalized bacteria were enumerated as indicated above. **$P < 0.01$, indicates statistically significant differences between the wild-type strain and the ΔcpsE mutant, n = 4. (C and D) For intracellular survival assays, bmDCs or spleen DCs were infected with GBS strains (MOI:1) and phagocytosis was left to proceed for 60 min. Antibiotics (ATB) were then added for 1 h (defined as time 0). This initial antibiotic-treatment was extended up to 5 h and cells lysed to quantify intracellular bacteria by viable plate counting. *$P < 0.05$ or **$P < 0.01$, indicates incubation times for which significantly differences in the numbers of recovered intracellular bacteria were observed compared to time 0, n = 8 (bmDCs) and n = 4 (spleen DCs). All results are expressed as CFU recovered bacteria per ml (means ± SEM).

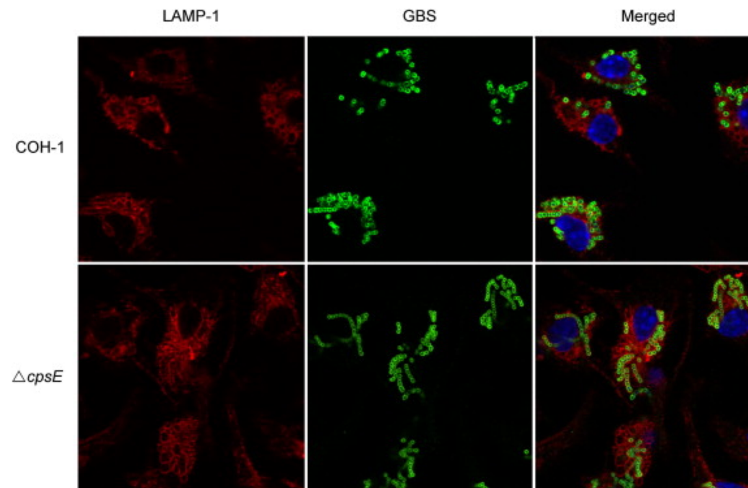

Figure 2. Confocal laser scanning microscopy of GBS internalization by DCs. BmDCs were incubated with the wild-type strain COH-1 or the Δ*cpsE* mutant (10^6 CFU/ml, initial MOI:1). After bacterial-cell contact for 2 h, cells were fixed and labeled with serum against GBS (Alexa-Fluor 488, green) and a mAb specific for LAMP-1 (Alexa-Fluor 568, red). DAPI was used to stain the nuclei (blue). Left and middle panels, red and green optical sections; right, merged sections. Merge of optical sections shows intracellular localization of GBS strains inside LAMP-1+ vacuoles.

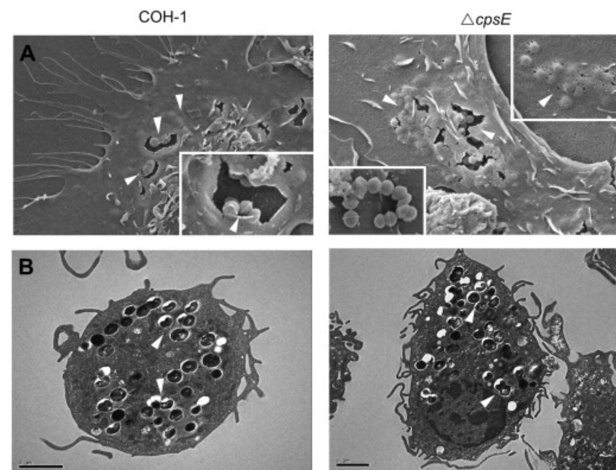

Figure 3. Electron microscopy studies of GBS interactions with DCs. BmDCs were incubated with wild-type GBS strain COH-1 or the ΔcpsE mutant (10^6 CFU/ml, initial MOI:1) for 4 h. (A) Scanning electron micrographs show large quantity of cocci (white arrows) associated to or underneath the cell surface (inserts). BmDCs incubated with the non-encapsulated strain ΔcpsE show several chains of cocci adhering to the cells (insert). Original magnification 5000X. (B) Transmission electron micrographs confirm internalization of GBS by bmDCs as most cells contained high numbers of intracellular cocci (white arrows). Original magnification 10000X. Images are representative of two independent experiments.

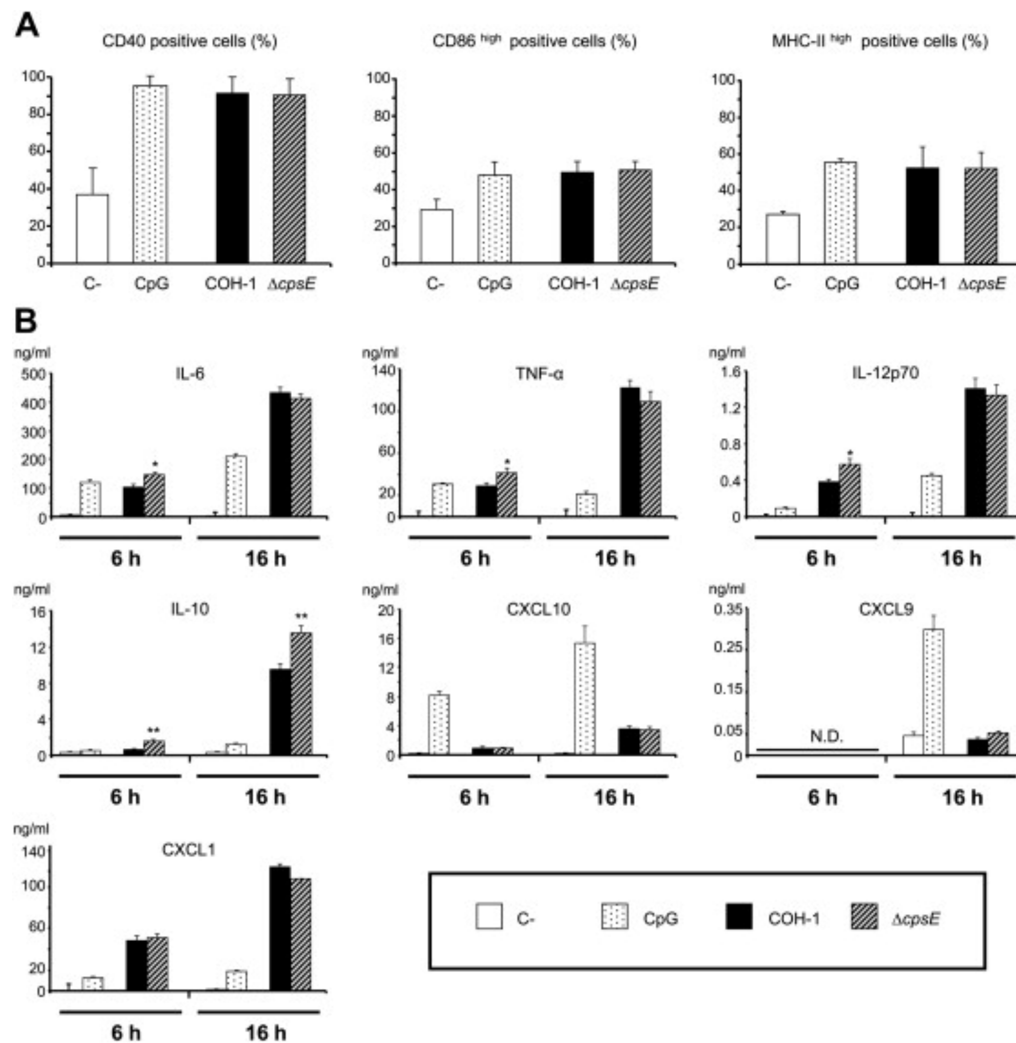

Figure 4. GBS induces DC surface expression of co-stimulatory molecules and cytokine release. BmDCs were stimulated with CpG (1 μM), wild-type GBS strain COH-1 or Δ*cpsE* mutant strain (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 μg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells served as negative control (C^-) for basal expression levels. (A) Cells were harvested at 16 h of incubation and co-stimulatory molecule expression analyzed by FACS. Histograms were obtained by gating cells based on positive CD11c staining. Data are expressed as mean ± SEM (in %) from five independent experiments. (B) Supernatants were harvested at 6 h and 16 h of incubation and cytokine production quantified by ELISA. Data are expressed as mean ± SEM (in ng/ml) from eight independent experiments. *$P < 0.05$ or **$P < 0.01$, indicates statistically significant differences between wild-type strain and the Δ*cpsE* mutant strain.

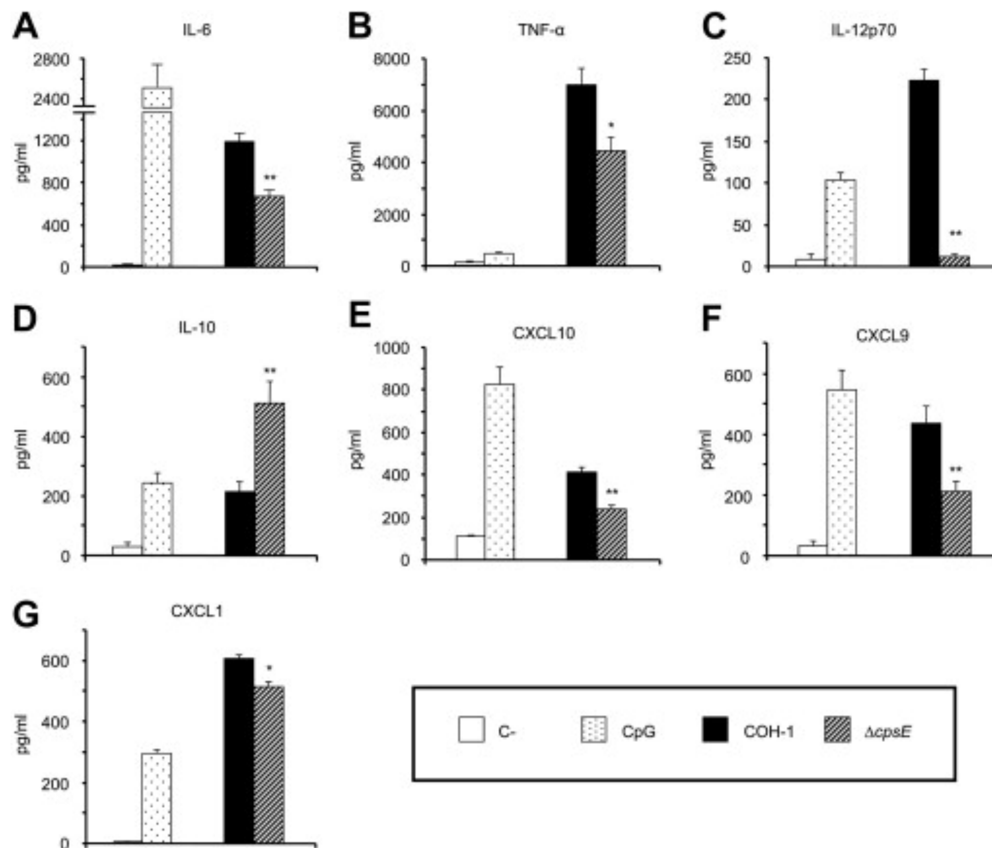

Figure 5. Encapsulated GBS modulates cytokine production by primary spleen DCs. Spleen DCs were stimulated with CpG (1 μM), wild-type GBS strain COH-1 or Δ*cpsE* mutant strain (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 μg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells served as negative control (C^-) for basal expression levels. Supernatants were harvested at 16 h of incubation and cytokine production quantified by ELISA. Data are expressed as mean ± SEM (in pg/ml) from five independent experiments. *P < 0.05 or **P < 0.01, indicates statistically significant differences between wild-type strain and the Δ*cpsE* mutant strain.

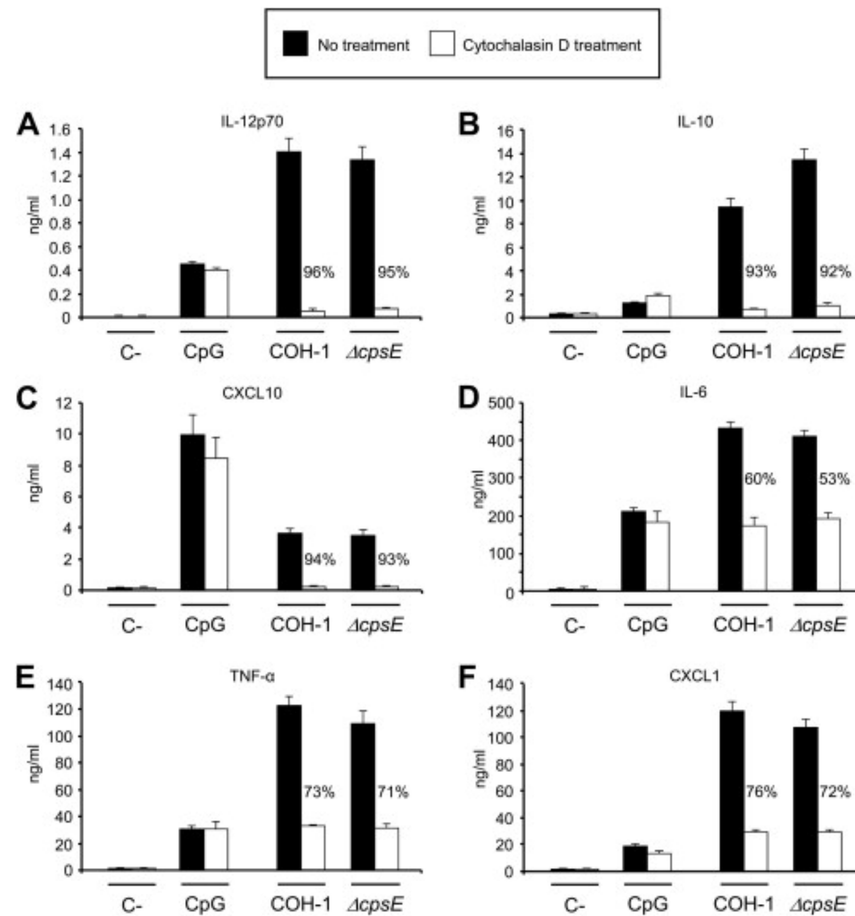

Figure 6. Effect of bacterial phagocytosis on cytokine production by GBS-stimulated DCs. BmDCs were either non-treated or pre-treated with cytochalasin D (2 μg/ml) for 30 min prior to stimulation with CpG (1 μM), wild-type GBS strain COH-1 or Δ*cpsE* mutant strain (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 μg/ml) was added to the culture to prevent cell toxicity. Supernatants were then harvested at 16 h of incubation and cytokine production quantified by ELISA. Non-stimulated cells served as negative control (C-). Data are expressed as mean ± SEM (in ng/ml) from four independent experiments. The percentages of cytochalasin D-mediated inhibition of cytokine production are indicated.

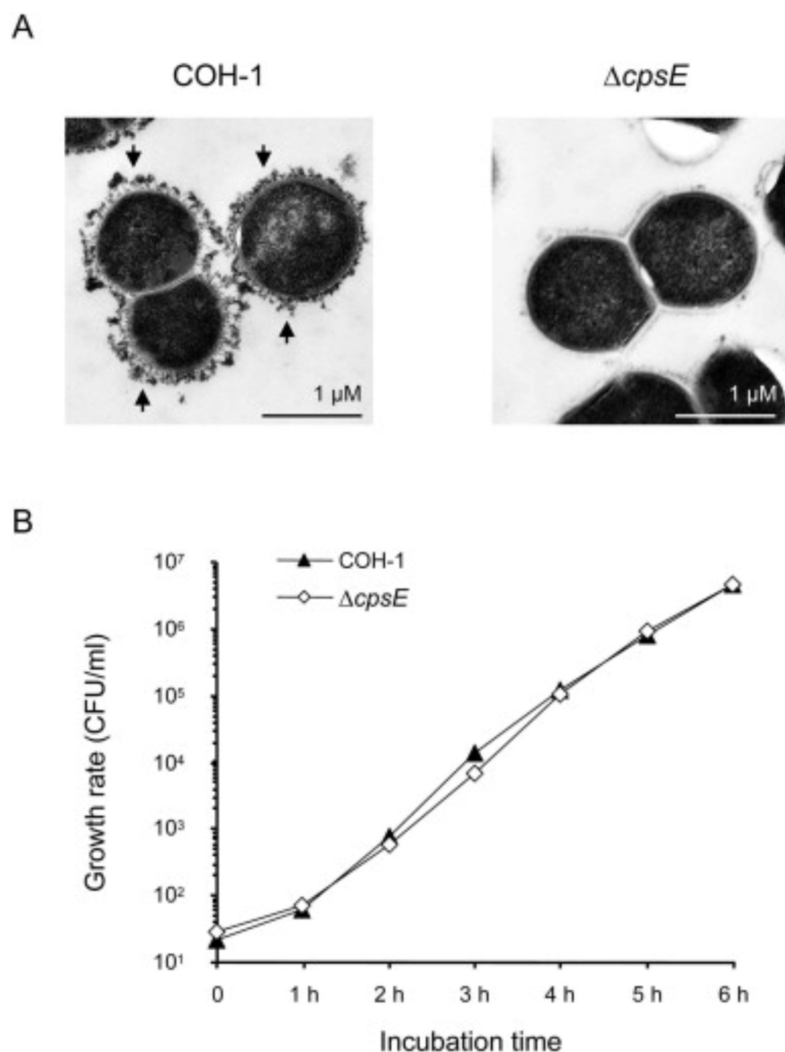

Figure S1. Phenotypic characterization of the GBS mutant strain. (A) Transmission electron micrographs of GBS strains labeled with polycationic ferritin show GBS wild-type strain COH-1 with a thick capsule (indicated by arrows) whereas no capsular material is observed in Δ*cpsE* mutant strain. For ferritin labeling, bacterial cells were fixed in cacodylate-glutaraldehyde buffer prior to polycationic ferritin staining for 30 min at 20 °C. Bacterial cells were then immobilized in 4% agar, postfixed with 2% osmium tetroxide, and samples were dehydrated in a graded series of ethanol. All the solutions used in processing the specimens contained 0.05% ruthenium red. Thin sections of resin embedded samples were poststained with uranyl acetate and lead citrate and examined with a Philips CM 100 electron microscope. (B) Growth curves of GBS wild-type strain COH-1 and Δ*cpsE* mutant strain.

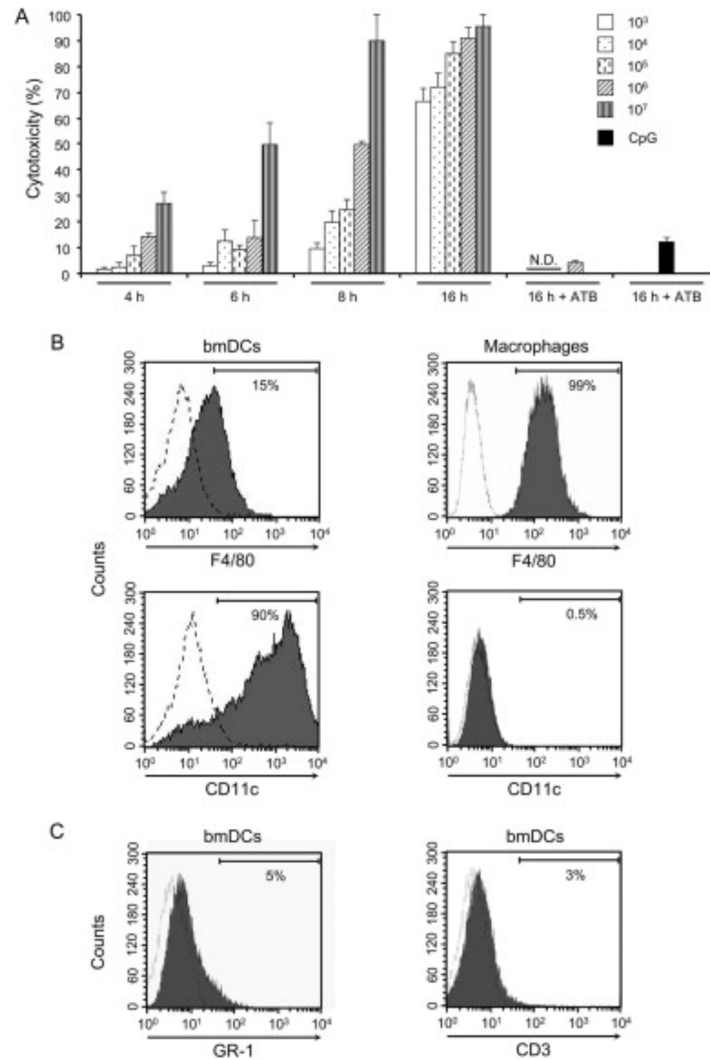

Figure S2. Cell culture quality controls. (A) GBS dose and time dependent cytotoxicity to dendritic cells (DCs). Bone marrow-derived DCs (bmDCs) were infected by wild-type GBS strain COH-1 at different concentrations (CFU/ml) for 4, 6, 8, and 16 h. Cytotoxicity (%) was evaluated by measuring the release of lactate dehydrogenase enzyme (LDH) with the CytoTox 96 Non-Radioactive Cytotoxicity Assay. In selected experiments, bmDCs were stimulated with CpG (1 μM), or wild-type GBS strain COH-1 (10^6 CFU/ml, initial MOI:1). After 2 h of bmDC-GBS infection, a bacteriostatic agent (chloramphenicol, 12 μg/ml) was added to the culture. Cytotoxicity (%) was evaluated as described above after 16 h of incubation (referred here as 16 h + ATB). (B) FACS analysis of expression levels of CD11c and F4/80 markers on bmDCs compared to C57BL/6 mouse bone marrow-derived macrophages (produced as reported, reference [46]). (C) FACS analysis of bmDC purity using PE-labeled anti-mouse CD3 mAb (clone 145-2C11) and anti-mouse Ly-6 G and Ly-6C mAb (clone RB6-8C5). The RB6-8C5 mAb reacts with a common epitope on Ly-6 G and Ly-6C, previously known as the myeloid differentiation antigen Gr-1, which is expressed by granulocytes.

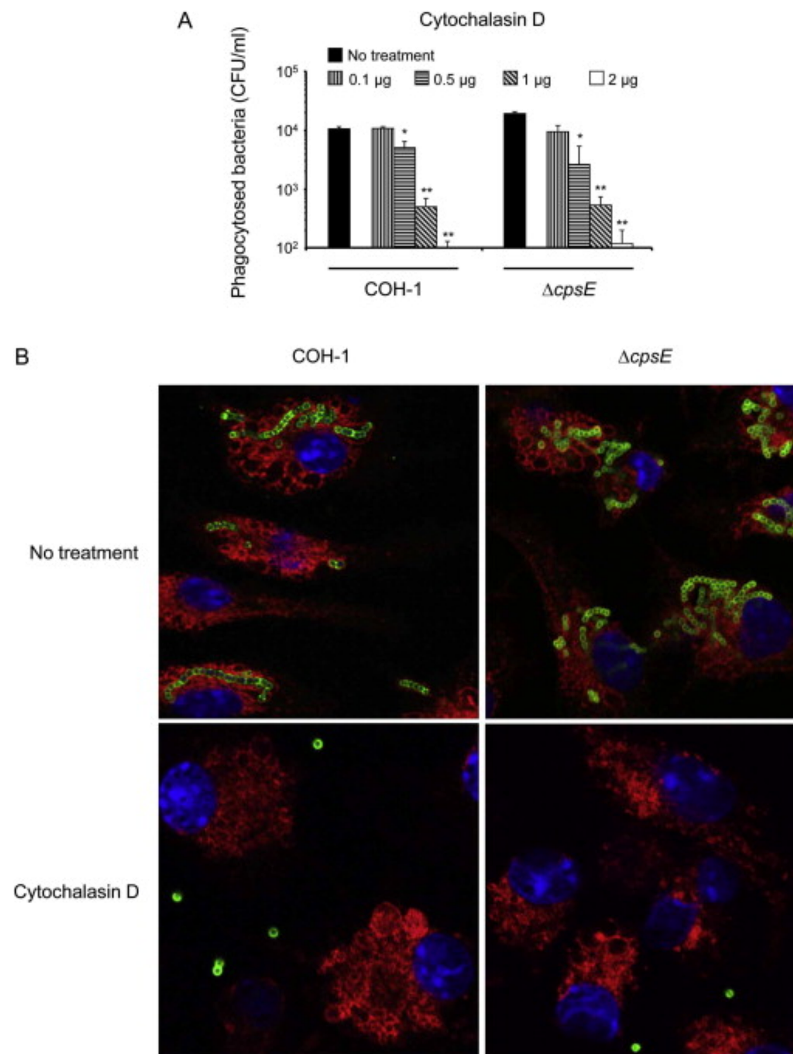

Figure S3. Blockade of actin filaments confirms GBS internalization by DCs. (A) BmDCs were pre-treated with cytochalasin D, a disruptor of actin filaments, at different doses 30 min prior to bmDC infection with wild-type GBS strain COH-1 or the Δ*cpsE* mutant (10^6 CFU/ml, initial MOI:1) for 60 min. Numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment to kill extracellular bacteria and expressed as CFU recovered bacteria per ml (means ± SEM) obtained from four independent experiments. *P < 0.05 or **P < 0.01, indicates statistically significant differences between non-treated cells and treated cells. (B) Confocal laser scanning microscopy studies of GBS internalization by non-treated or cytochalasin D (2 µg)-treated bmDCs. After a bacterial-cell contact of 2 h (initial MOI:1), cells were fixed and labeled with serum against GBS (Alexa-Fluor 488, green) and a mAb specific for LAMP-1 (Alexa-Fluor 568, red). DAPI was used to stain the nuclei (blue). Images are representative of three independent experiments.

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Annexe VI

Group B *Streptococcus* Induces a Robust IFN-γ Response by CD4+ T Cells in an *In Vitro* and *In Vivo* Model

Damian Characterization of porcine dendritic cell response to *Streptococcus suis* [a], Marie-Pier Lecours[a], Paul Lemire[a], Tristan Galbas[b], Jacques Thibodeau[b], Mariela Segura[a].

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Soumis à: Infection and Immunity

Rôle de la candidate dans la conception de l’article :

Je suis co-auteure de cet article. J’ai participé à la mise au point des techniques ainsi qu’aux expérimentations.

ABSTRACT

Group B *Streptococcus* (GBS) serotype III is an important agent of life-threatening invasive infections and meningitis. Over the years, cytokines have emerged as important players for the control of disease, in particular IFN-γ. Although potential sources of this cytokine have been proposed, no specific cell line has ever been described as a leading contributor. In this study, *in vivo*, *ex vivo* and *in vitro* approaches were developed to evaluate CD4+ T cell activation profiles in response to GBS. Results showed that total splenocytes readily produce a type 1 pro-inflammatory response by releasing IFN-γ, TNF-α and IL-6. Splenocytes also actively recruit T cells by producing chemokines such as CXCL9, CXCL10, and CCL3. Specifically, responding CD4+ T cells differentiate into Th1 cells producing large amounts of IFN-γ and TNF-α, as well as IL-2. The effect of GBS capsular polysaccharide (CPS), considered one of the major bacterial virulence factors, on T cell activation was evaluated *in vitro* using co-cultures of dendritic cells and isolated splenic CD4+ T cells infected either with wild-type GBS or a non-encapsulated mutant. Results suggest that GBS CPS differentially modulates surface expression of CD69 and IFN-γ production by CD4+ T cells. Globally, our results further highlight the importance of IFN-γ for the control of GBS infection, which is produced by responding CD4+ T cells.

INTRODUCTION

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is the main cause of life-threatening invasive infections in pregnant women and newborns worldwide (1, 2). GBS is also becoming increasingly associated with invasive disease in non-pregnant adults, in particular the elderly and individuals with underlying chronic illnesses (3). Clinical manifestations of GBS infection are mainly associated with the development of pneumonia, septicemia and meningitis. Type III GBS is one of the three major capsular types associated with invasive neonatal infection and is the most common type in GBS meningitis (1, 2). Cytokines appear to play a particularly important role for the control of disease, although exaggerated responses have also been described as potentially dangerous (4, 5). For instance, TNF-α has been demonstrated to be an important agent in driving GBS-induced sepsis (6, 7). In contrast, other cytokines have been shown to mediate beneficial effects, such as IL-10, IL-12, and IL-18 (6, 8-10).

In particular, IFN-γ has gained much interest over the years for its important beneficial role in controlling GBS infections. In fact, it was shown that IL-12 and IL-18 mediate their therapeutic effects by increasing IFN-γ production by responding immune cells (8-10). Furthermore, IFN-γ production is severely impaired during early life and might partly explain the susceptibility of neonates to GBS infection (9, 11, 12). Although certain immune cells have been proposed to secrete IFN-γ in response to GBS, such as NK and NKT cells (13, 14), no specific cell line has been clearly identified as a major source of this particular cytokine *in vivo* during GBS infection.

Activated CD4+ T cells can differentiate into different types of T helper (Th) cells depending on the signals received from their environment. Th1 CD4+ T cells readily produce IFN-γ upon activation and could therefore represent an interesting potential source of this cytokine during the development of GBS infection. Some evidence already shows that GBS induces a pro-inflammatory response that could potentially and ultimately lead to activation of T cells and production of IFN-γ. For example, GBS-infected dendritic cells (DCs) have been shown to produce large amounts of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (15). Furthermore, GBS-activated DCs also produce and release

chemokines involved in T cell recruitment, such as CXCL9 and CXCL10 (15). Although these evidences strongly suggest a potential involvement of T cells in the production of IFN-γ (16, 17), the specific participation of CD4+ T cells in the immuno-pathogenesis of GBS-induced disease has yet to be evaluated.

As aforementioned, GBS is a well-encapsulated bacterium that possesses a thick capsular polysaccharide (CPS) shell. The structure of type III GBS CPS is formed by the monosaccharides glucose, galactose and *N*-acetylglucosamine into an unique repeating unit that contains a side chain terminated by sialic acid α2,3 linked to galactose (18). The sialylated CPS is recognized as the most important factor for GBS survival within the host and has been suggested to inhibit the activation of the alternative complement pathway and to impair the bactericidal functions of neutrophils (4, 19, 20). Experiments using mouse-derived DCs infected with either encapsulated GBS type III or a non-encapsulated isogenic mutant showed that encapsulated GBS is highly internalized by DCs, but survives better inside these cells than its non-encapsulated counterpart. Furthermore, the bacterial CPS selectively drives GBS internalization via specific endocytic pathways which cannot be engaged by the non-encapsulated bacteria (15, 21). It has been hypothesized that the route of entry might provide GBS a mechanism for the avoidance of intracellular degrading mechanisms (21, 22). Bacterial internalization and the presence of CPS were also related to modulation of several cytokine and chemokine release by GBS-infected DCs (15, 21, 23).

Based on these observations and previous findings on GBS interactions with DCs, the hypothesis of this study is that GBS drives CD4+ T cells to differentiate into IFN-γ producing Th1 cells and, that the bacterial CPS has the potential to modify this response. To this aim, we investigated the role of CD4+ T cells in the development of an immune response against GBS type III using an *in vivo* mouse model as well as *ex vivo* and *in vitro* experimental approaches. A non-encapsulated GBS mutant was included to dissect the role of this important virulence factor in T cell activation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strain COH-1, a highly encapsulated type III GBS isolate extensively described (15, 21, 22), was used in this study as the wild-type (WT) strain. An isogenic non-encapsulated (Δ*cpsE*) mutant, constructed and characterized in our previous work was also included (15, 21). GBS strains were grown on blood agar plates at 37°C for 18 h. Isolated colonies were inoculated in 5 ml of Todd–Hewitt Broth (THB; Becton Dickinson) and incubated for 8 h at 37°C with shaking. Working cultures were prepared by transferring 10 µl of 1/1,000 dilutions of 8 h-cultures into 30 ml of THB, which was incubated with shaking for an additional 12 h. Bacteria were washed twice with PBS pH 7.3 before being appropriately diluted in fresh medium to desired inoculum concentrations. The number of CFU/ml in the final suspension was determined by plating serial dilutions of working cultures on blood agar before enumerating individual colonies after overnight incubation at 37°C.

Mice and experimental infections

C57BL/6 female mice 5-week-old (Charles River Laboratories) were acclimatized to standard laboratory conditions of a 12 h-light/12 h-dark cycle with unlimited access to water and rodent chow. All experiments involving mice 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 Use of Laboratory Animals by the Animal Welfare Committee of the University of Montréal. On the day of the experiment, a 0.5 ml volume of either the bacterial suspension (see below for selected doses) or the vehicle solution (sterile THB) was administrated by intraperitoneal injection (i.p.). Mice were closely monitored daily to record mortality and clinical signs of disease, such as depression, rough appearance of hair coat and swollen eyes (24). Mice exhibiting extreme lethargy were considered moribund and were humanely euthanized. To determine the level of infection, numbers of viable bacteria in blood were quantified at different times post-infection. Blood (5 µl) was collected from the tail vein, serially diluted in PBS and plated using an Automated Spiral Plater (Spiral Biotech). Blood agar plates were incubated overnight at 37°C. Colonies were counted and data expressed as CFU/ml.

Generation of bone marrow-derived DCs

Cells were generated from naïve C57BL/6 mice as previously described (15). Briefly, after red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 µg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. All reagents were from Gibco (Invitrogen). Complete medium was complemented with 20% GM-CSF from a mouse GM-CSF transfected cell line (Ag8653). Cells were cultured for 7 days at 37°C with 5% CO_2. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8 and used as antigen-presenting cells for the studies. Cell purity was routinely 86-90% $CD11c^{high}$ and $F4/80^{-/dim}$ cells as determined by FACS analysis and as previously reported (15).

Isolation of splenic $CD4^+$ T cells

Untouched CD4+ T cells were purified from the spleen of either naïve or infected C57BL/6 mice by negative selection using CD4+ T cell isolation kit II according to the manufacturer's instructions (Miltenyi Biotec). Briefly, spleens were harvested from naïve or infected mice at the indicated times (see below) and perfused with RPMI complete medium (without antibiotics), teased apart, and pressed gently through a sterile fine wire mesh. The red blood cells were removed by incubation with NH_4Cl lysis buffer (eBioscience). To obtain CD4+ T cells, splenocytes were suspended in sterile PBS containing 2 mM EDTA and separated using Lympholyte-M density gradient (Cedarlane Lab.). Low-density cells at the interphase were collected and further purified by magnetic-activated cell sorting (MACS) negative selection as mentioned above. The enriched CD4+ T cells had > 96% purity as determined by FACS using anti-CD3 and anti-CD4 staining (data not shown).

***In vivo* infection model**

For survival curves and selection of the infectious dose, C57BL/6 mice (n =16) were injected i.p. with either 1×10^6, 1×10^7 or 1×10^8 CFU of GBS strain COH-1 and clinical signs monitored daily as described above. Based on the obtained data (see Fig. 1A), for *in vivo* CD4+ T cell analysis, mice were injected i.p. with 1×10^6 CFU. Surviving animals

who had previously displayed clinical symptoms were boosted with a second dose of 1×10^6 CFU of GBS strain COH-1 two weeks after initial infection. Bacteremia was monitored during the first 72 h post-primary infection or the first 24 h post-boost infection. Spleens of animals with clinical symptoms and positive bacteremia were harvested 96 h post-primary infection or 48 h post-boost infection (n = 2 per group x 5 individual experimental infections). Before harvesting, mice were injected i.p. with 500 µl of 200 µg of Brefeldin A solution in PBS (eBiosciences) 5 h prior to spleen collection. Control (placebo) animals were similarly treated. Spleen CD4+ T cells were purified as described above, in the presence of Brefeldin A solution during all the purification steps. The selected time-points are based in pre-trials analysis using different post-infection times (data not shown). Purified CD4+ T cells were analyzed for cytokine production by intracellular staining followed by flow cytometry analysis (IC-FACS, see below).

***Ex vivo* analysis of total splenocytes**

C57BL/6 mice were injected i.p with a dose of 1×10^7 CFU of GBS strain COH-1. Control mice were injected with the vehicle solution (sterile THB) (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection, perfused with RPMI complete medium (without antibiotics), teased apart, and pressed gently through a sterile fine wire mesh. After red blood cells lysis and washing, total splenocytes were counted and plated at a concentration of 5×10^6 cells/ml in RPMI complete medium without antibiotics in 24-well flat bottom plates, and incubated at 37°C with 5% CO_2 for 48 h and 72 h. However, after the initial 4 h of *ex-vivo* incubation, chloramphenicol (CM, 12 µg/ml, Sigma-Aldrich) was added to the culture to prevent cell toxicity. CM is a bacteriostatic agent, keeping a low and controlled level of bacteria through the incubation period as previously reported (15). Total splenocytes from control (placebo) animals were similarly treated. Concanavalin A (ConA, 0.1 µg/ml, Sigma-Aldrich) was used as positive control. Supernatants were harvested at the indicated time points for cytokine analysis by ELISA. In selected experiments, total splenocytes were incubated *ex-vivo* for 48 h as described above. However, brefeldin A (3 µg/ml) was added during the last 5 h, and either total splenocytes or CD4+ T cells (MACS-isolated from the culture wells) were analyzed by IC-FACS (see below). The above described final culture conditions for *ex vivo* analysis were selected

based on multiple pre-trials using different post-infection times (6 h and 12 h) combined with 14, 24, 48 and 72 h *ex-vivo* incubation times (data not shown).

In vitro DC-T cell co-culture model

For the co-culture model, 1 x 10^5 DCs were plated in 48-well flat bottom plates for 1 h at 37°C with 5% CO_2. Afterwards, 1 x 10^5 CFU of either WT COH-1 strain or Δ*cpsE* mutant strain (MOI:1) were added to wells for 1 h. Extracellular bacteria were killed using 100 µg/ml of gentamycin and 5 µg/ml of penicillin G (Sigma-Aldrich) as previously described (15). After 1 h of antibiotic treatment and 3 washing steps, 5 x 10^5 freshly isolated CD4+ T cells from naïve mice (T cell: DC ratio of 5:1) were added to the wells. Co-cultures incubated with medium alone or ConA (0.1 µg/ml) served as negative and positive controls, respectively. For FACS analysis of surface marker expression, co-culture plates were incubated for 8 h and 24 h at 37°C, 5% CO_2, prior to cell harvesting and FACS analysis. For T cell cytokine expression, co-culture plates were incubated for 48 h, then centrifuged and replenished with fresh medium containing 10 ng/ml of mouse rIL-2 (Miltenyi Biotec). Plates were incubated at 37°C, 5% CO_2 for 3 days allowing a resting period for activated T cells. After 3 days, T cells were harvested, washed, and seeded into 96 well flat-bottom culture plates coated with 5 µg/ml of anti-mouse-CD3 mAb (BD PharMingen) at a final concentration of 1 x 10^5 cells/well. These plates were incubated for 48 h at 37°C, 5% CO_2 prior to supernatant harvesting for ELISA testing. Single cell cultures (either DC alone or T cell alone) were also included as controls. No significant cytokine production was observed in single cell cultures under this protocol conditions (data not shown).

Cytokine quantification by ELISA

Levels of IL-6, IL-10, IFN-γ, TNF-α, CCL3 (MIP-1α), CXCL9 (MIG), and CXCL10 (IP-10) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems or eBioscience, according to the manufacturer's recommendations. Twofold dilutions of recombinant mouse cytokines were used to generate standard curves. Sample dilutions giving OD readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. The results are from at least three independent ELISA measurements.

FACS analysis

For cell surface staining of *in vitro* co-cultures, 10^6 cells were washed and treated for 15 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab) in sorting buffer (PBS-1% fetal bovine serum). Blocked cells were then incubated with FITC-conjugated anti-mouse CD4 mAb (clone GK1.5) for 30 min on ice followed by washing and staining for 30 min with PE-conjugated anti-mouse CD69 mAb (clone H1.2F3). All reagents were from BD PharMingen. FACS was performed using a Cell Lab Quanta™ SC MPL MultiPlate Loader instrument (Beckman Coulter). Twenty thousand gated events were acquired per sample and data analysis was performed using Cell Lab Quanta Collection/Analysis software. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales.

For IC-FACS of MACS-purified CD4+ T cells from *in vivo* or *ex-vivo* experiments, cells were blocked as described above followed by fixation and permeabilization using IC Fixation/Permeabilization eBioscience kit as per the manufacture's recommendation. Permeabilized cells were then stained for 20 min at room temperature with PE-conjugated mAbs (eBioscience) directed against the following intracellular molecules: IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), and IL-2 (clone JES6-5H4). FACS was performed using a FACSCalibur instrument (BD Biosciences). Twenty thousand gated events were acquired per sample and data analysis was performed using CellQuest software. Histograms were drawn based on PE-control stain and were plotted on logarithmic scales.

For multi-parametric IC-FACS of total splenocytes, cells were washed and blocked as described above followed by surface staining with PE-conjugated anti-CD19 (clone 6D5), PE-Cy7-conjugated anti-NK-1.1 (clone PK136), FITC-conjugated anti-CD3 (clone 17A2) and/or PE-conjugated CD69 (clone H1.2F3, BD Pharmingen) for 30 min on ice. Following fixation and permeabilization, intracellular staining was performed with APC-conjugated anti-IFN-γ (clone XMG1.2) or APC-conjugated anti-TNF-α (clone MP6-XT22) for 45 min at room temperature. Unless otherwise specified, all mAbs were from Biolegend. FACS was performed using a FACSCanto II instrument (BD Biosciences). Fifty thousand gated events were acquired per sample and data analysis was performed using FACSDiva™

software. Fluorescence Minus One (FMO) control staining was performed for proper analysis and gating of target cells.

Statistical analysis

Survival curves of infected mice were generated using Kaplan-Meier plots. Log-rank (Mantel-Cox) tests were used to compare the survival curves of the different studied groups. Values for bacterial loads in blood were compared using the Mann-Whitney test. Cytokine data are expressed as mean ± SEM and analyzed for significance using Student's unpaired *t*-test. All analyses were performed using the Sigma Plot System (v.9; Systat Software). A $P < 0.05$ was considered as statistically significant.

RESULTS

Survival of GBS-infected C57BL/6 mice is dose-dependent

C57BL/6 mice were injected i.p. with GBS COH-1 strain at 3 different infectious doses: 1 x 10^6, 1 x 10^7, and 1 x 10^8 CFU. At 18 h post-infection, mice infected with 10^7 CFU or 10^8 CFU doses resulted in 75% and 69% mortality ($P > 0.05$), respectively (Fig. 1A). Mouse mortality continued to increase until 24 h post-infection to 82% and 94% ($P > 0.05$), respectively, and was maintained until 60 h post-infection when the experiment was terminated. Mice infected with a dose of 10^6 CFU were significantly less prone to mortality as compared to mice from the other two groups. After 18 h of infection, only a 6% mortality rate was observed in mice infected with 10^6 CFU, which was significantly lower than the other two groups ($P < 0.05$) (Fig. 1A). Mortality continued to increase at 24 h and 36 h post-infection, yet remained significantly lower than mice infected with higher doses ($P < 0.05$). Indeed, groups of mice infected with 10^7 or 10^8 CFU doses quickly manifested intense clinical signs such as tousled hair, prostration, depression, and lethargy as early as 8 h post-infection. In mice infected with 10^6 CFU, appearance of clinical symptoms was delayed, usually to 12 h post-infection, and the observed clinical manifestations were less severe.

Bacteremia of infected mice was also evaluated. Infection of C57BL/6 mice with GBS COH-1 strain induced strong bacteremia. In agreement to survival curves, bacteremia was very high in groups of mice infected with 10^7 or 10^8 CFU at 18 h post-infection and reached an average of 2.6 x 10^8 CFU/ml and 1.3 x 10^9 CFU/ml, respectively (Fig. 1B). In contrast, groups of mice infected with a dose of 10^6 CFU showed significantly lower bacteremia and reached an average of 5.7 x 10^5 CFU/ml at 18 h post-infection (Fig. 1B). Due to high levels of mortality, bacteremia levels could not be followed through time in mice infected with high bacterial doses. However, in mice infected with 10^6 CFU, bacteremia slowly decreased, reaching an average of 7.2 x 10^4 CFU/ml at 72 h post-infection (Fig. 1B).

Splenocytes produce type-1 pro-inflammatory cytokines in response to encapsulated GBS infection

Before understanding T cell activation in response to GBS, we were interested in characterizing the immunological environment produced by total splenocytes. To this aim we used the *ex vivo* approach where total splenocytes from mice infected with encapsulated GBS strain were incubated in culture plates for 48 h and 72 h. Supernatants were then collected and levels of cytokines and chemokines were measured (Fig. 2). Splenocytes from control (placebo) animals were used as negative controls and ConA-treated splenocytes were used as positive controls. ELISA tests revealed the presence of high amounts of IFN-γ, TNF-α and IL-6, suggesting the progression of a type-1 pro-inflammatory response. In agreement with its role in maintaining homeostasis, IL-10 was also upregulated in infected spleens (Fig. 2). No significant differences were observed in the levels of these cytokines between 48 h and 72 h of incubation. In addition to cytokines, it is well known that spleen cells produce chemokines for the recruitment of immune cells, such as T cells. Supernatants tested by ELISA revealed the presence of three important chemokines known to recruit T cells: CXCL9, CXCL10 and CCL3 (Fig. 2). Noteworthy, CXCL9 and CXCL10 are mainly released in response to IFN-γ activation (25), and thus, in agreement to the observed high levels of IFN-γ produced by GBS-infected splenocytes. Kinetics of chemokine production was similar to that of aforementioned cytokines, with the exception of CXCL9, which maximal production was delayed to 72 h of incubation.

Activated CD4+ T cells contribute to IFN-g production during encapsulated GBS infection

With current understanding of the splenic environment produced in response to GBS, we were interested in determining the contribution of activated T cells in the production of cytokines. Firstly, we performed a multi-parametric IC-FACS analysis of IFN-g production by total splenocytes. As shown in Fig. 3A, CD3+ T cells markedly contributed to the IFN-g response in the spleen of infected mice. NKT cells (NK1.1+ CD3+) produced low to negligible levels of IFN-g (data not shown). NK cells (NK1.1+) were the major contributors to IFN-g production within the CD3- population (data not shown). As expected, CD19+ cells (B cells) did not produce significant levels of this cytokine (data not shown). Activated CD3+ T cells also contributed to approximately half the production of

TNF-a by splenic cells (Fig. 3C). Compared to control mice, splenocytes from infected animals showed a significant increase in surface expression of the early activation marker CD69. High expression of this marker was also observed within the CD3+ population (Fig. 3B).

To specifically evaluate the role of CD4+ T cells during GBS infection, these target cells were isolated from *ex-vivo* total splenocyte cultures and analyzed by IC-FACS (Fig. 4). Experiments showed that activated CD4+ T cells readily contributed to the production of IFN-γ and TNF-α. Low levels of intracellular IL-2 were also observed. These data suggest that CD4+ T cells differentiate into Th1 type T cells and further support initial findings of the development of a type-1 pro-inflammatory immune response.

These results were further confirmed using an *in vivo* model of infection. Results obtained from CD4+ T cells directly isolated from the spleen of infected mice 96 h post-primary infection showed that CD4+ T cells contribute to the production of IFN-γ and TNF-α *in vivo*. Intracellular levels of IL-2 were hardly detected during the course of a primary infection (Fig. 5, black histograms). Surviving mice were challenged with a second infectious dose 2 weeks after primary infection. IC-FACS analysis of CD4+ T cells isolated 48 h post-boost displayed an enhanced contribution to IFN-γ, TNF-α and IL-2 production, suggesting that CD4+ memory T cells have been probably generated during the primary infection (Fig. 5, dark grey histograms).

The CPS of GBS modulates cytokine release by CD4+ T cells

As GBS COH-1 strain is a well encapsulated bacteria, we evaluated the impact of CPS on CD4+ T cell activation by using a non-encapsulated isogenic mutant, Δ*cpsE* strain. Non-encapsulated GBS mutants are non-virulent and rapidly cleared from circulation (19). Thus, to be able to directly compared WT GBS and the mutant strain, an *in vitro* DC-T cell co-culture system was established. Supernatants collected from co-cultures experiments were tested for the presence of CD4+ T cell-derived cytokines by ELISA. Results from WT GBS activated co-cultures revealed the presence of extremely high levels of IFN-γ (~ 45000 pg/ml), and significant levels of TNF-α (~ 1500 pg/ml). The loss of CPS was accompanied by a significant reduction in IFN-g production (Fig. 6). DC-T cell co-cultures

stimulated with the non-encapsulated mutant also showed reduced TNF-a production, however, this difference did not reach statistical significance ($P = 0.053$). Overall, these results suggest that DCs pulsed with non-encapsulated GBS induce a reduced cytokine response by CD4+ T cells compared to WT GBS-pulsed DCs.

The CPS of GBS affects surface expression of CD69 on activated CD4+ T cells

In addition to cytokine production, expression of surface molecules on CD4+ T cells is an essential event for proper T cell activation. We therefore also investigated how the CPS of GBS may affect expression of co-stimulatory molecules on activated CD4+ T cells. We selected CD69, as is recognized to be an important marker of T cell activation (26). In DC-T cell co-cultures, maximal CD69 expression was observed at 8 h of incubation, when an average 21.54% (+/- 1.36%) CD4+ T cells responding to WT GBS were stained positive for CD69 expression compared to 37.57% (+/- 2.77%) ($P < 0.01$) in co-cultures infected with the non-encapsulated mutant (Fig. 7). This difference in CD69 expression was gradually lost. At 24 h of co-culture incubation, an average of 15.89% (+/- 2.97%) CD4+ T cells responding to WT GBS stained positive for CD69 compared to 25.32% (+/- 5.28%) in co-cultures infected with the non-encapsulated strain ($P < 0.05$) (Fig. 7). At 48 h of co-culture incubation no significant differences in CD69 surface expression were observed between strains (data not shown).

DISCUSSION

Although information is becoming increasingly available on the interactions between GBS and cells of innate immunity, such as DCs, macrophages and neutrophils, no work to date has focused on activation profiles of cells of adaptive immunity during GBS infection. This study addresses for the first time how CD4+ T cells contribute to the development of immune functions during GBS type III infection using *in vivo*, *ex vivo*, and *in vitro* analysis.

In the case of GBS, cytokines are not only important for the appropriate development of host defences but are also involved in induction of severe pathologies, such as sepsis and meningitis. Initial e*x vivo* analysis of cytokine production by total splenocytes derived from encapsulated GBS infected mice revealed the presence of IFN-γ, TNF-α, IL-6 and IL-10. Production of IFN-γ, TNF-α and IL-6 is suggestive of a type 1 pro-inflammatory response being developed shortly after infection, while IL-10 production can be related to immune-regulation. It is interesting to note that TNF-α and IL-6 have routinely been reported as important mediators of GBS sepsis (6, 7). This observation might also highlight the particular importance of IL-10 in maintaining homeostasis. Indeed, a role of this cytokine in protecting neonatal mice from developing GBS sepsis by reducing TNF-α production has been reported (6).

Several cell types have been reported to secrete TNF-α, IL-6 and/or IL-10, including DCs, monocytes and macrophages when responding to GBS (15, 16, 27-29). On the other hand, sources of IFN-γ remain poorly identified. Indeed, early works reporting IFN-γ production were mostly focused on GBS-infected total splenocyte cultures or mixed mononuclear cells, without identifying the cellular source of this cytokine (7, 9, 10, 16). In the present study we defined the potential role of T cells in IFN-γ production. *Ex vivo* and *in vivo* analysis showed that CD4+ T cells are important producers of IFN-γ and TNF-α during GBS infection. Activated CD4+ T cells also produce low, but still significant levels of IL-2, overall suggesting the development of a Th1 response. Results from surviving animals that were challenged with a booster infectious dose two weeks later showed that CD4+ T cells respond to infection by producing the same pattern of cytokines. This response was faster

and more efficient, suggesting generation of memory cells (30). An important contribution of NK cells to the IFN-γ response was also evidenced *in vivo*, as previously suggested by *in vitro* studies using cultured splenocytes from severe combined immunodeficiency mice (14). Purified GBS glycolipids have been shown to activate NKT cells (13). However, the contribution of these cells to IFN-γ production in the course of GBS infection was very limited, even at earlier time points (unpublished observations).

Early chemokine release by cells of the innate immunity plays an important role in attracting various accessory immune cells to the site of infection, such as T cells. *Ex vivo* analysis of chemokine production by total splenocytes suggested that T cells are actively recruited via the expression of three important chemokines: CCL3, CXCL9 and CXCL10. It is interesting to note that CXCL9 and CXCL10 are two chemokines that bind to CXCR3 receptor, and more importantly, are induced by IFN-γ. CXCR3 is rapidly induced on naive T cells following activation and preferentially remains highly expressed on Th1-type CD4+ T cells (25). In the context of a systemic infection with GBS, several cell types in the spleen are potential sources of these three chemokines. GBS-stimulated spleen DCs were reported to produce CXCL9 and CXCL10 (15). Although up-regulation of *Cxcl10* gene expression was observed by DNA microarray analyses of mouse peritoneal macrophages (29), GBS was reported to be unable to induce neither CXCL10 nor CXCL9 secretion by these cells (31). On the other hand, both macrophages and DCs seem to contribute to CCL3 production (29, 31)(unpublished observations).

As GBS possesses a thick CPS, known to be the most important virulence factor, we were interested in evaluating whether the presence of CPS has the potential to modulate CD4+ T cell activation. To this aim, type III encapsulated GBS strain and its non-encapsulated isogenic mutant were used for *in vitro* infections. Similarly to *ex vivo* and *in vivo* results, DCs pulsed with WT GBS induced the release of high levels of IFN-γ and TNF-α by CD4+ T cells. The production of IFN-γ was significantly decreased when using DCs pulsed with non-encapsulated GBS. Production of TNF-α was also slightly reduced. It is surprising that the loss of capsule is not associated with an exaggerated immune response or increased IFN-γ production by T cells, as reported for other encapsulated pathogens (32-34). However, studies on DC activation by GBS have shown similar trends. Indeed, it has been

reported that WT GBS induces similar or even stronger cytokine production by infected DCs than non-encapsulated mutant-infected counterparts (15). The only exception was IL-10, which production was significantly higher in DCs infected with the non-encapsulated mutant than those infected with the WT strain (15). In this study two interrelated hypothesis were suggested by the authors to explain these observations, a) increased IL-10 production by DCs in the absence of CPS reduce the production of other cytokines; or b) higher and faster levels of non-encapsulated bacterial killing reduce or impair cytokine production by DCs (15, 21). In addition, it was reported that the presence of CPS modulates the endocytic pathways used by DCs for GBS up-take (21). It was demonstrated with murine macrophages that the route of entry exploited by a particular pathogen influences the repertoires of epitopes presented to CD4+ T cells and thus, by extension, may affect the ensuing immune response (35). Thus, in our DC-T cell co-culture system, the consequence of DC modulation by the non-encapsulated strain may be translated into lower levels of IFN-γ production by CD4+ T cells. In contrast to cytokine production, the surface expression of the T cell activation marker CD69 was higher (early time points) or similar (late time points) in CD4+ T cells co-cultured with non-encapsulated mutant-pulsed DCs compared to WT GBS-infected co-cultures. The observed differences could just be related to a delayed kinetics of CD69 expression in mutant-infected co-cultures. In fact, attempting to explain modulation of CD69 expression on CD4+ T cells is quite difficult, due to the lack of information available on this particular surface marker. Although it is well known that CD69 is one of the earliest markers induced upon activation of T cells and acts as a signal-transmitting receptor for immune-regulatory events, its cognate ligand is still unknown (26). Of the few studies available on CD69 expression by T cells upon streptococcal infection, Harimaya *et al.* demonstrated a dose-dependent up-regulation of CD69 on CD3+ T cells from peripheral blood lymphocytes infected with *Streptococcus pneumoniae*. Yet, authors failed to correlate CD69 surface expression and IFN-γ production by these target cells (36). In a more recent study using a *S. pneumoniae* mouse model of infection, CD4+ T cells exhibited significant up-regulation of CD69 in the spleen at 48 h post-infection. As this response was MHC-II unrestricted, authors suggested that this increased CD69 expression on T cells is not a consequence of their direct interaction with pneumococci or presented antigen but is due to secondary factors, e.g., cytokines released by other host cells (37). Likely, a possible polyclonal (indirect)

activation of T cells in our system cannot be ruled out. Nevertheless, it should be highlighted that in our co-culture system, GBS failed to directly activate T cells in the absence of accessory cells (data not shown), similarly to that reported for *S. pneumoniae* (34, 37). Finally, it has been recently suggested that CD69 controls the cross-talk between innate components and lymphocytes and plays an immuno-regulatory role by preventing infection-induced immunopathology (38). Enhanced expression by CD69 may result in reduced IFN-γ production by CD4+ T cells (39).

Undoubtedly, IFN-γ production by CD4+ T cells during GBS infection is crucial for host defence (9), but might also result in disease pathology, as suggested in the mouse model of pneumococcal sepsis (37). Although this study characterized for the first time IFN-γ production by CD4+ T cells, a definitive understanding of all mechanisms regulating IFN-γ production during GBS infection requires further research. For instance, as the CPS confers a survival advantage to GBS (15, 21), persistence of GBS within antigen-presenting cells may affect their activation and thus the ensuing T cell immune response, including altered IFN-γ and CD69 expression balance early during infection.

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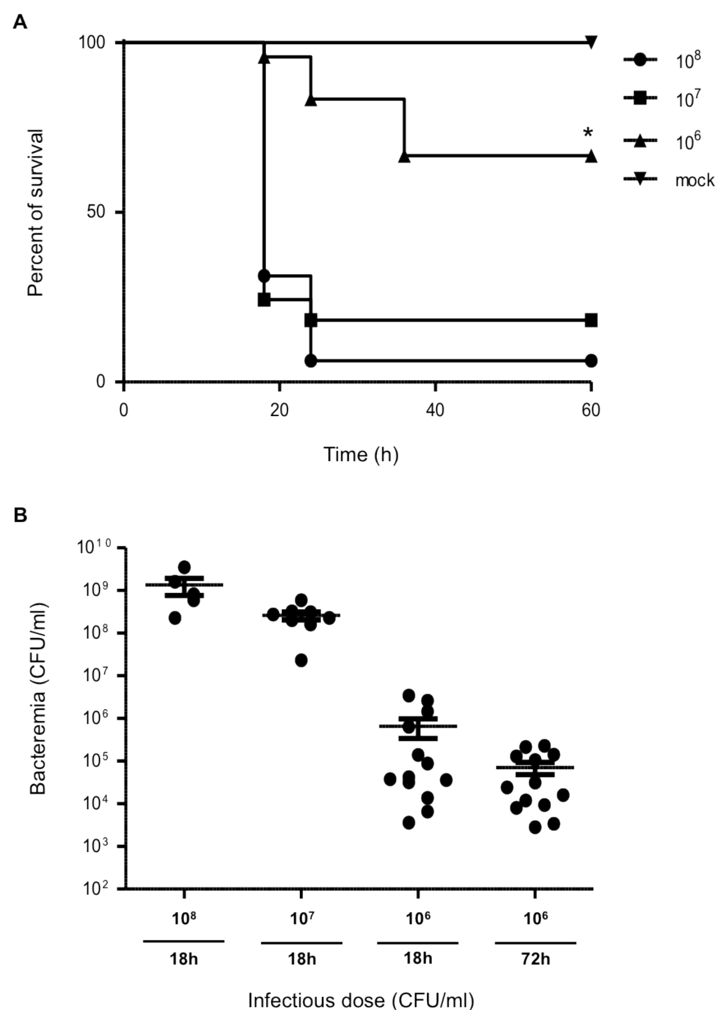

Figure 1. Survival curves and bacteremia levels of GBS-infected C57BL/6 mice. (A) Mice (n =16) were injected intra-peritoneally with different doses of wild-type GBS serotype III strain COH-1 and survival levels recorded. Mock-infected animal (injected with the vehicle solution) were used as controls. (B) Systemic bacteremia levels of infected mice was monitored at 18 h post-infection (for mice infected with 10^6, 10^7 and 10^8 infectious doses) and at 72 h post-infection (for mice infected with 10^6 infectious dose). Blood was drawn by tail puncture and serially diluted in PBS prior to plating on blood agar dishes. Individual colonies were counted and data expressed as CFU/ml of blood. * $P < 0.05$, compared to higher infectious doses.

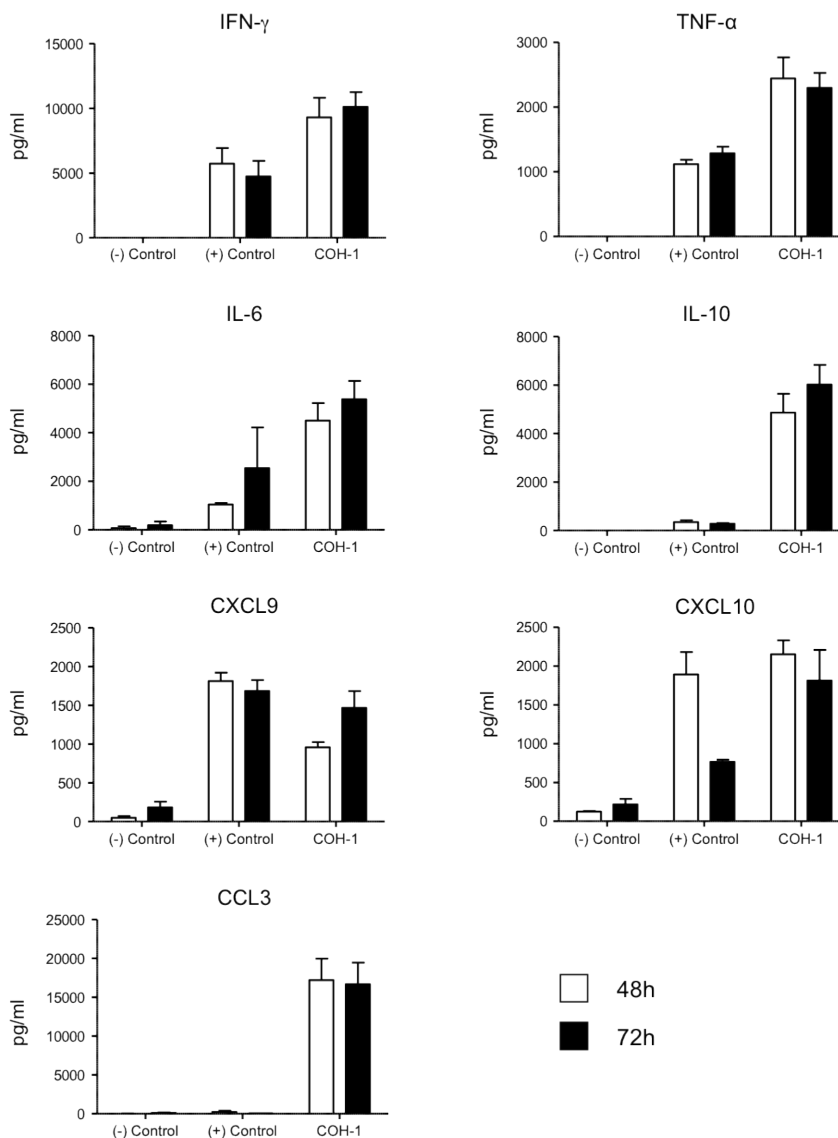

Figure 2. *Ex vivo* cytokine and chemokine production profile by total splenocytes. C57BL/6 mice were injected intra-peritoneally with a dose of 10^7 CFU of wild-type GBS serotype III strain COH-1 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 4 h of incubation, the bacteriostatic agent chloramphenicol (12 µg/ml) was added to the culture to prevent cell toxicity. Cells were then incubated for 48 h and 72 h and supernatants were collected for cytokine analysis by ELISA. Non-stimulated cells from mock-infected animals served as negative (-) control for basal expression. Cells stimulated with Concanavalin A (0.1 µg/ml) were used as positive (+) control. Data are expressed as means ± SEM (in pg/ml) from 3 different experimental infections.

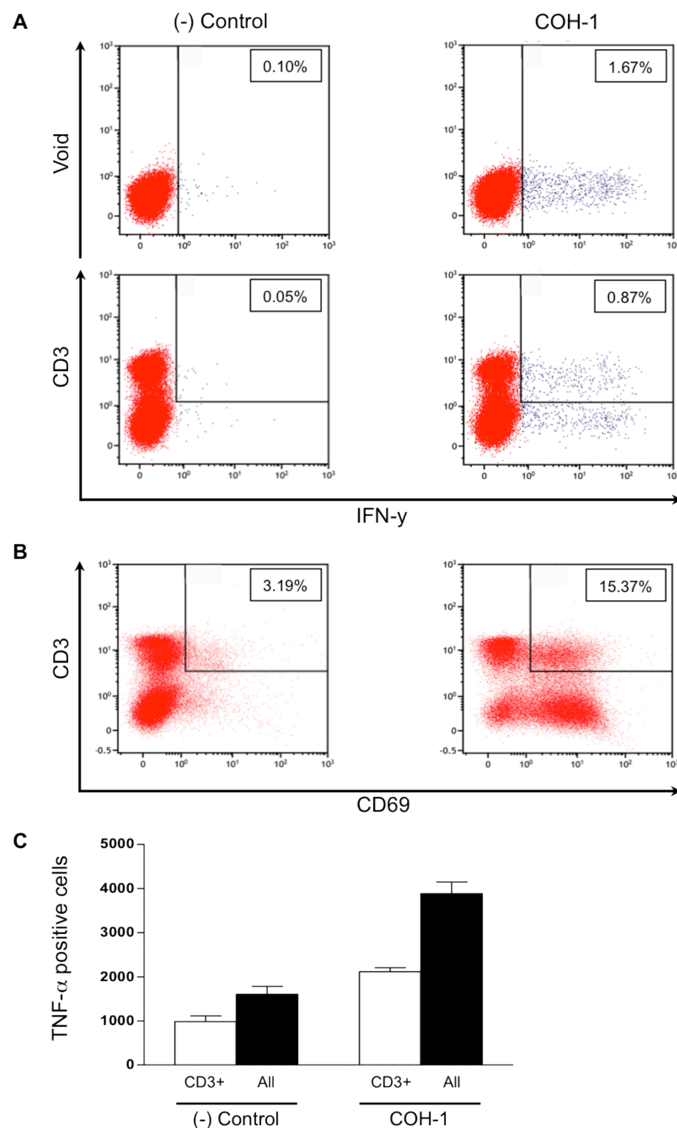

Figure 3. *Ex vivo* analyses of cellular sources of IFN-γ during GBS infection. C57BL/6 mice were injected intra-peritoneally with a dose of 10^7 CFU of wild-type GBS serotype III strain COH-1 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 4 h of incubation, the bacteriostatic agent chloramphenicol (12 µg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells from mock-infected animals served as negative (-) control for basal expression. Total splenocytes were incubated for 48 h with brefeldin A (3 µg/ml) added during the last 5 h of incubation. Cells were harvested and intracellularly stained for IFN-γ **(A)** or surface stained for CD69 **(B)** in combination with several surface markers for multi-parametric FACS analysis. Representative data from 3 different experimental infections based on CD3+ population or total splenic population (Void). **(C)** Number of TNF-α+ cells within the CD3+ population or total splenic population (All). Data are expressed as means ± SEM from 3 different experimental infections.

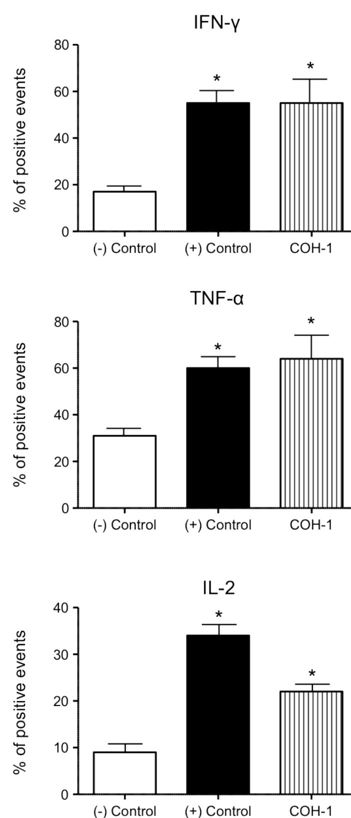

Figure 4. *Ex vivo* analyses of CD4+ T cell contribution to cytokine production. C57BL/6 mice were injected intra-peritoneally with a dose of 10^7 CFU of wild-type GBS serotype III strain COH-1 (n = 3 per group x 3 individual experimental infections). Spleens were harvested 6 h post-infection and total splenocytes plated at 5 x 10^6 cells/well. After 4 h of incubation, the bacteriostatic agent chloramphenicol (12 μg/ml) was added to the culture to prevent cell toxicity. Non-stimulated cells from mock-infected animals served as negative (-) control for basal expression. Cells stimulated with Concanavalin A (0.1 μg/ml) were used as positive (+) control. Total splenocytes were incubated for 48 h. Brefeldin A (3 μg/ml) was added during the last 5 h of incubation and CD4+ T cells were MACS-isolated from the culture, stained intracellularly for different cytokines and analyzed by FACS. Data are expressed as mean ± SEM (in % of positive cells) from 3 individual experimental infections. *$P < 0.05$, indicates statistically significant difference compared to (-) control cells.

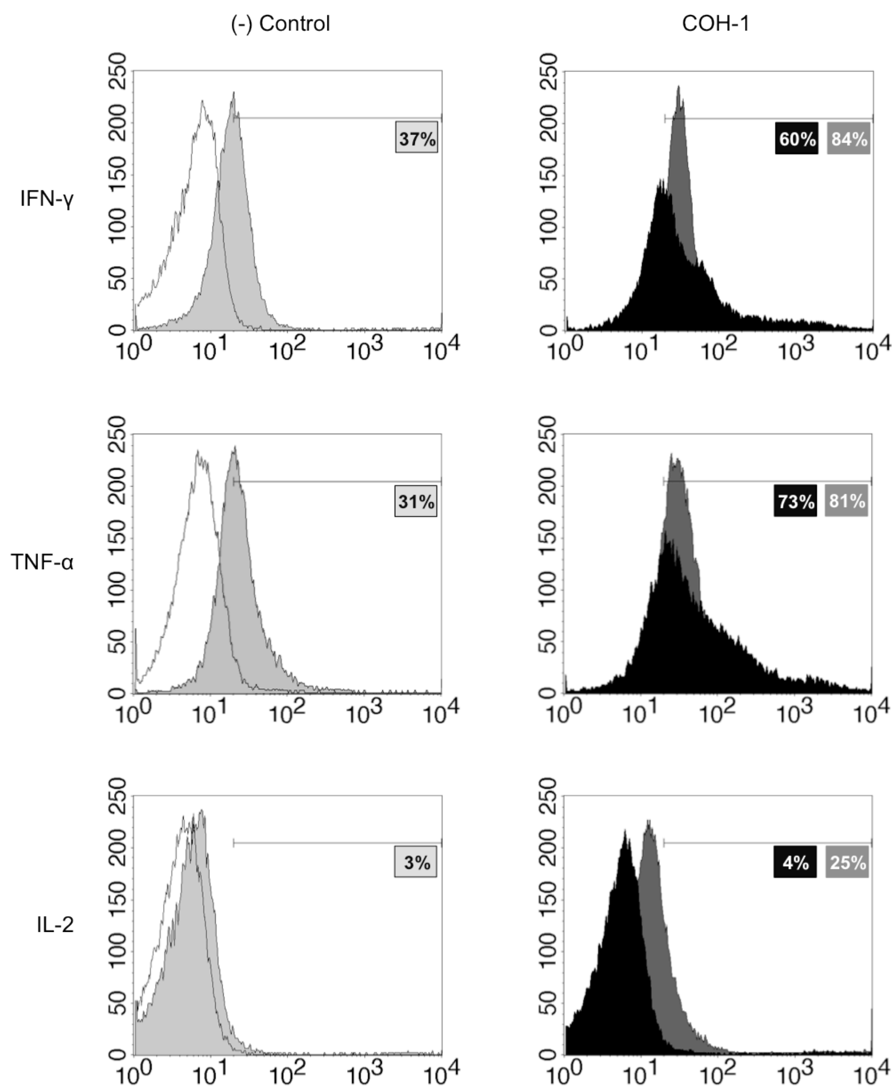

Figure 5. *In vivo* CD4+ T cell contribution to cytokine production during primary and secondary GBS infections. C57BL/6 mice were injected intra-peritoneally with a dose of 10^6 CFU of wild-type GBS serotype III strain COH-1. Surviving animals who had previously displayed clinical symptoms were boosted with a second dose of 1 x 10^6 CFU of GBS strain COH-1 two weeks after initial infection. Spleens of animals with clinical symptoms and positive bacteremia were harvested 96 h post-primary infection or 48 h post-boost infection (n = 2 per group x 5 individual experimental infections). Five hours prior to spleen collection, mice were injected with Brefeldin A (200 μg). Control (-) animals were similarly treated. Spleen CD4+ T cells were MACS-purified, stained intracellularly for different cytokines and analyzed by FACS. Representative data from 5 different experimental infections. Cytokine basal expression levels in (-) control animals were similar at 96 h post-primary mock-infection and 48 h post-secondary mock-infection. Representative histograms from the latter time point were selected for the figure.

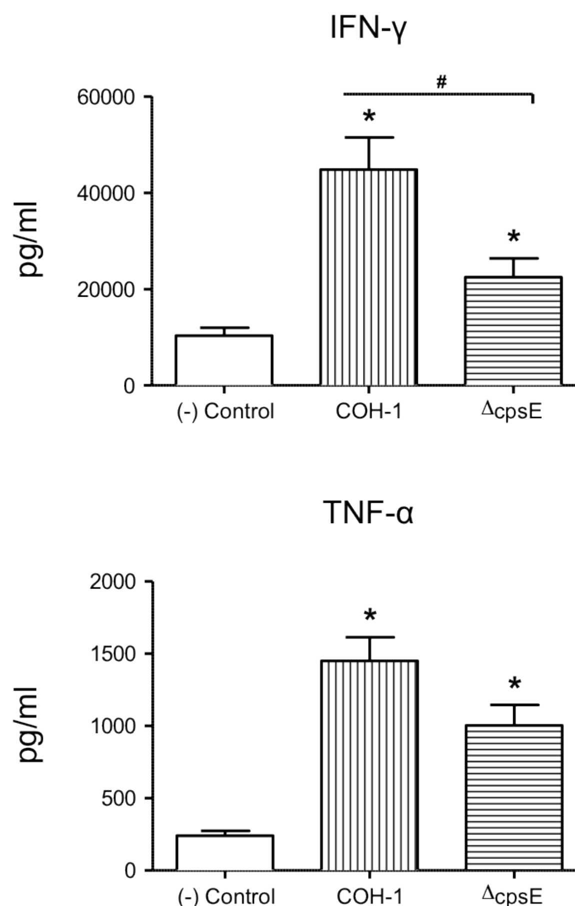

Figure 6. Role of bacterial capsular polysaccharide in the modulation of cytokine production by CD4+ T cells. Dendritic cells (DCs) were infected with either wild-type GBS strain COH-1 or its non-encapsulated isogenic mutant Δ*cpsE* (MOI 1:1) for 1 h. Extracellular bacteria were killed by antibiotic treatment and cultures washed prior to addition of freshly isolated splenic CD4+ T cells from naïve mice (T cell: DC ratio of 5:1). Co-cultures were incubated for 48 h, resuspended in fresh medium containing 10 ng/ml of IL-2 for 72 h (resting period) and then transferred to anti-CD3 coated plates for 48 h. Supernatants were then collected and cytokines quantified by ELISA. Non-stimulated co-cultures served as negative (-) controls for basal expression. Data are expressed as means ± SEM (in pg/ml) from 5 different experiments. $^{*}P < 0.05$, indicates statistically significant differences compared to (-) control. $^{\#}P < 0.05$, indicates statistically significant differences between co-cultures infected with wild-type strain COH-1 and those infected with the non-encapsulated mutant Δ*cpsE*.

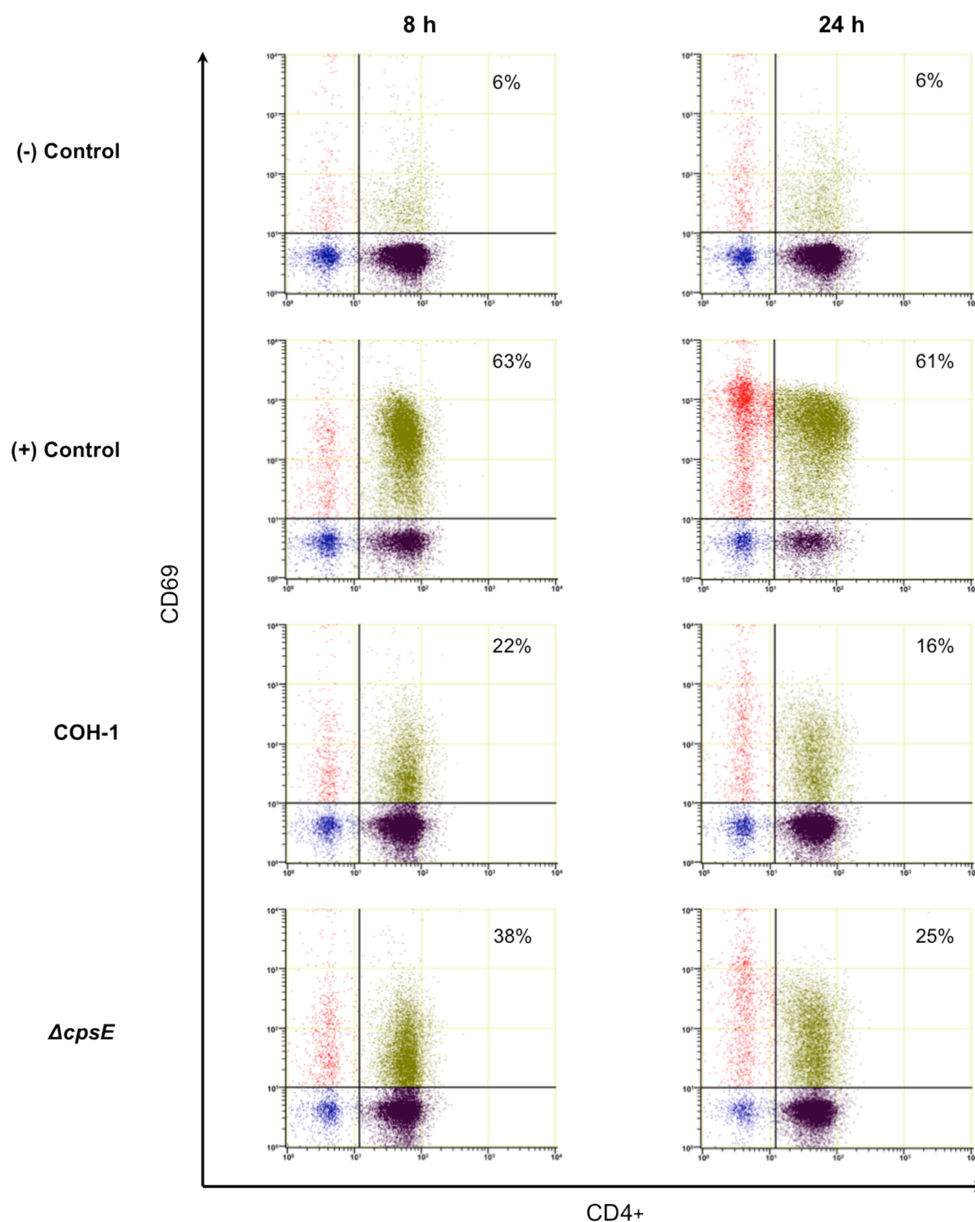

Figure 7. Role of bacterial capsular polysaccharide in the modulation of CD4+ T cell surface expression of CD69. Dendritic cells (DCs) were infected with either wild-type GBS strain COH-1 or its non-encapsulated isogenic mutant Δ*cpsE* (MOI 1:1) for 1 h. Extracellular bacteria were killed by antibiotic treatment and cultures washed prior to addition of freshly isolated splenic CD4+ T cells from naïve mice (T cell: DC ratio of 5:1). Co-cultures were incubated for 8 h and 24 h, cells harvested and CD69 expression analyzed by FACS. Co-cultures incubated with medium alone or Concanavalin (0.1 μg/ml) served as negative (-) and positive controls (+), respectively. Representative data from 3 different experiments. Numbers in the upper quadrants indicate the % of CD4+CD69+ cells.

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