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Role of interleukin-1 in the pathogenesis of the infection caused by *Streptococcus suis* serotype 2

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Résumé

Streptococcus suis sérotype 2 est un pathogène important du porc et un agent zoonotique en émergence causant des morts subites (porcs), des chocs septiques (humains) et des méningites (chez les deux espèces), où une réaction inflammatoire sévère est caractéristique de l'infection. Une réponse rapide et efficace du système immunitaire inné contre *S. suis* est critique pour contrôler la croissance bactérienne et pour limiter la propagation du pathogène sans occasionner une inflammation excessive. Bien que l'interleukine (IL)-1 soit considérée comme l'un des médiateurs pro-inflammatoires les plus efficaces et produit le plus rapidement, son rôle dans la pathogénèse de l'infection par *S. suis* n'a pas encore été étudié.

En utilisant un modèle murin d'infection systémique bien standardisé, nous avons démontré que la souche nord-américaine de virulence intermédiaire de « sequence type » (ST) 25, la souche européenne hautement virulente ST1 ainsi que la souche épidémique chinoise ST7, induisent toutes de hauts niveaux d'IL-1 dans des organes de filtration, tels que le foie et la rate. De plus, les cellules dendritiques et les macrophages, deux types de cellules jouant un rôle central dans la pathogénèse de *S. suis*, sont des sources importantes de cette cytokine. Les études des mécanismes impliqués dans la production de cette cytokine ont démontré que la production d'IL-1, indépendamment de la souche bactérienne utilisée, dépendait de MyD88 et impliquait les récepteurs TLR2 et possiblement TLR7 et TLR9. Cela suggère que les composantes bactériennes responsables de l'activation cellulaire sont similaires et conservées entre les différentes souches. Cependant, seuls de très hauts niveaux de suilysine, produite par la souche ST7, provoquaient une maturation importante de proIL-1β. Cette maturation implique l'activation des inflammasomes NLRP3, NLRP1, AIM2 et NLRC4, qui est due à la formation de pores et à un efflux d'ions.

De surcroît, nous avons évalué le rôle global de cette cytokine chez des souris IL-1R$^{-/-}$, démontrant que l'IL-1 pourrait jouer un rôle bénéfique lors d'une infection systémique par *S. suis* en modulant l'inflammation requise pour contrôler et éliminer la charge bactérienne, ce qui favorise la survie de l'hôte. Toutefois, au-delà d'un certain seuil, l'inflammation causée par *S. suis* ne peut plus être contrebalancée par cette signalisation, ce qui complique la détermination exacte du rôle de l'IL-1. Une meilleure compréhension des mécanismes sous-jacents impliqués dans le contrôle de l'inflammation et de la charge bactérienne aiderait à développer de meilleures mesures de contrôle pour ce pathogène important à la fois chez le porc et chez l'Homme.

Mots-clés: *Streptococcus suis* sérotype 2; interleukine-1; cellules dendritiques; inflammation; infection systémique; suilysine.

Abstract

Streptococcus suis serotype 2 is an important porcine bacterial pathogen and an emerging zoonotic agent causing sudden death (pigs), septic shock (humans), and meningitis (both species), with exacerbated inflammation being a hallmark of the infection. A rapid, effective, and balanced innate immune response against *S. suis* is critical to control bacterial growth and limit the spread of the pathogen without causing excessive inflammation. Even though interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced, its role in the *S. suis* pathogenesis has not been studied.

Using a well-standardized mouse model of systemic infection, we showed that an intermediately pathogenic sequence type (ST) 25 North American strain, a highly pathogenic ST1 European strain, and the epidemic ST7 Chinese strain induce high levels of IL-1 in important filter organs such as liver and spleen. Moreover, dendritic cells and macrophages, which are two cell types centrally involved in the *S. suis* pathogenesis, are important sources of this cytokine, with the ST7 strain secreting the highest levels. The study of the underlying mechanisms involved in this production showed that, independently of the strain, IL-1β production required MyD88 and involved recognition via TLR2 and possibly TLR7 and TLR9. This suggests that recognized bacterial components are similar and conserved between *S. suis* strains. However, very high levels of the pore-forming toxin suilysin produced by the ST7 strain only, are required for efficient maturation of proIL-1β. Such maturation involved the activation of the NLRP3, NLRP1, AIM2, and NLRC4 inflammasomes via pore formation and ion efflux.

Using IL-1R$^{-/-}$ mice, we demonstrated that IL-1 signaling may play a beneficial role during *S. suis* systemic infection by modulating the inflammation required to control and clear bacterial burden, thus, promoting host survival. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counter-balanced by this signaling, making it difficult to discriminate its role. A better understanding of the underlying mechanisms involved in the control of inflammation could help to develop control measures for this important porcine and zoonotic agent.

Keywords: *Streptococcus suis* serotype 2; interleukin-1; dendritic cells; inflammation; systemic infection; suilysin.

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Review of the Literature

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List of Acronyms and Abbreviations

AIM2: Absent in melanoma 2

ASC: Apoptosis-associated speck-like protein containing a CARD

ATP: Adenosine triphosphate

BBB: Blood–brain barrier

BMEC: Brain microvascular endothelial cells

CARD: Caspase activation recruitment domain

CCL: Chemokine (C-C motif) ligand

CFU: Colony-forming unit

CNS: Central nervous system

COX: Cyclooxygenase

CPEC: Choroid plexus epithelial cell

CPS: Capsule polysaccharide

CRP: C-reactive protein

CSF: Cerebrospinal fluid

CXCL: Chemokine (C-X-C motif) ligand

DC: Dendritic cell

DP: Dipeptidyl peptidase

EF: Extracellular factor

ERK: Extracellular-signal-regulated kinase

FBP: Fibronectin-fibrinogen binding protein

GAPDH: Glyceraldehyde-3-phosphatase dehydrogenase

GAS: Group A S*treptococcus*

GBS: Group B *Streptococcus*

GDH: Glutamate dehydrogenase

GM-CSF: Granulocyte macrophage colony-stimulating factor

HPA: Hypothalamic pituitary adrenal

i.p.: Intraperitoneal

iE-DAP: γ-D-Glu-meso diaminopimelic acid

IFN: Interferon

IFNAR: Interferon-α/β receptor

IL: Interleukin

IRAK: Interleukin-1 receptor-associated kinase

JNK: c-Jun N-terminal kinase

KC: Keratinocyte chemoattractant

KO: Knock-out

LF: Lethal factor

LPS: Lipopolysaccharide

LRR: Leucine-rich repeat

LTA: Lipoteichoic acid

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein-1

M-CSF: Macrophage colony-stimulating factor

MDP: Muramyl dipeptide

MIP: Macrophage inflammatory proteins

MLST: Multilocus sequence typing

MOI: Multiplicity of infection

MRP: Muramidase-released protein

MyD88: Myeloid differentiation primary response protein-88

Mθ: Macrophages

NCL: Novel capsular polysaccharide locus

NF-κB: Nuclear factor- kappa-light-chain-enhancer of activated B cells

NK: Natural Killer cell

NLR: NOD-like receptor

NLRP1: NACHT, LRR and PYD domains-containing protein 1

NLRP3: NACHT, LRR and PYD domains-containing protein 3

NLS: Nuclear localization signal

NOD: Nucleotide-binding oligomerization domain

p.i.: Post-infection

PA: Protective antigen

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PCR: Polymerase chain reaction

PG: Peptidoglycan

PGE2: Prostaglandin E2

PLY: Pneumolysin

PYD: Pyrin domain

RIG: Retinoic acid-inducible

RLR: Retinoic acid-inducible gene I-like receptors

RTqPCR: Real-time quantitative chain reaction

SLY: Suilysin

SMS: Streptococcal meningitis syndrome

ST: Sequence type

STSLS: Streptococcal toxic shock-like syndrome

T3SS: Type three secretion system

T4SS: Type four secretion system

THA: Todd Hewitt broth agar

THB: Todd Hewitt broth

THP-1: Tamm-Horsfall protein 1

TIR: Toll-interleukin 1 receptor

TIRAP: Toll-interleukin 1 receptor domain containing adaptor protein adaptor protein

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRAF: TNF receptor associated factor

TRIF: TIR-domain-containing adaptor-inducing interferon-β

WT: Wild-type

A mama y papa por su incondicional apoyo.

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I- Introduction

Streptococcus suis (*S. suis*) is one of the most important bacterial pathogens affecting the swine industry worldwide (2). In pigs, it has been associated with a variety of pathological processes such as meningitis, septicemia with sudden death, and endocarditis among others (3). In addition, *S. suis* is also an emerging zoonotic agent, responsible for meningitis and septic shock in humans (4, 5). During the last decade, the number of documented cases in humans due to this coccus has dramatically increased worldwide (4, 6). While most of them are due to close occupational contact with pigs/pork products, particularly in Western countries (7), two outbreaks were recorded in China in 1998 and 2005, with more than 200 people affected (8). In Vietnam, Thailand, and Hong-Kong, *S. suis* is one of the most common causes of community-acquired bacterial meningitis (9-11) . In addition, several human cases have also been reported in Canada (12).

Based on the capsular polysaccharides (CPS), 35 serotypes have been identified (5). Among these, serotype 2 is thought to be the most virulent and the most frequently isolated from diseased pigs and humans (5). The predominant *S. suis* serotypes isolated from clinical cases in pigs worldwide, is serotype 2, followed by serotypes 9, 3, ½, and 7, while in humans, serotype 2 is mainly followed by serotype 14 (12). Multilocus sequence typing (MLST) is a technique that allows the identification of sequence types (STs) within a serotype using genomic sequencing (13). In fact, the use of this technique in the recent years has permitted to identify the most important STs within *S. suis* serotype 2. Among these, the epidemic strain ST7 in China, the highly virulent ST1 in Eurasia, and the intermediate and low virulence ST25 and ST28, respectively, in North America are the four predominant sequence types (12).

Even though *S. suis* has been studied for several years, the pathogenesis of its infection as well as the different virulence factors involved, are still not completely understood. Similarly, the mechanisms involved in the host immune response against this pathogen remain poorly characterized. To cause disease, *S. suis* needs to colonize and invade the epithelial surfaces of the host. In this way, bacterial adhesins coupled with the secretion of a pore-forming toxin termed suilysin (SLY) seem to play an important role in this step (14). Once in circulation, its thick CPS will help *S. suis* to survive in blood and disseminate to vital organs such as liver, spleen, kidneys, and lungs (15). At this step, recognition of *S. suis* by host immune cells leads to the production of several pro-inflammatory mediators, which if uncontrolled, could lead to

the development of septic shock. If death by sepsis does not take place, and bacteremia remains high, *S. suis* can reach and invade the central nervous system (CNS) and cause meningitis (14).

Therefore, the severity of the outcome depends on the ability of host immune mechanisms to control bacterial growth and to limit the spread of the pathogen, without causing excessive inflammation (16). The innate immune response is initiated following bacterial recognition by specialized pattern recognition receptors (PRRs), leading to cell activation and synthesis of diverse pro-inflammatory cytokines and chemokines (17). Amongst these, interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced (18). Indeed, IL-1 is a central mediator of immunity and inflammation participating amongst others, in the induction of adhesion molecules, leukocyte recruitment and migration, production of other inflammatory factors such as lipid mediators and acute phase proteins, fever induction, HPA axis regulation, stimulation of effector functions of neutrophils and macrophages, and lymphoid cell-mediated innate and adaptive immunity (19, 20).

The term IL-1 refers to two different cytokines, IL-1α and IL-1β, which are encoded by separate genes and synthesized as precursor peptides (proIL-1α and proIL-1β). While proIL-1α is biologically active and can exert intracellular or extracellular functions, a maturation step via proteolytic cleavage is required for the activation of proIL-1β (20, 21). This activation step is mediated mainly by caspase-1, which also requires activation by multi-protein complexes: the inflammasomes.

Although IL-1 signaling plays essential roles in both immunity and sterile inflammation, uncontrolled production of this cytokine can be detrimental, as observed in cases of rheumatoid arthritis and gout (22). Likewise, during bacterial infections, synthesis of IL-1 is necessary to initiate inflammation, but disproportionate levels of this cytokine can lead to tissue damage and disease. In fact, IL-1 plays a protective role during both pneumococcal and Group B *Streptococcus* (GBS) infections, wherein a lack of IL-1 signaling contributes to a weak inflammatory response and higher bacterial burden (23-26). However, a recent study showed that lack of control of IL-1β production results in a lethal outcome in a mouse model of Group A *Streptococcus* (GAS) infection (27).

Though excessive inflammation is a hallmark of the *S. suis* infection, and IL-1 is a key mediator in this type of process, little is known about IL-1 involvement in the context of *S. suis*

disease. Most studies measure the cytokine as part of a group and do not focus on its mechanisms of secretion, regulation, and most importantly its overall role in the pathogenesis.

That is why, based on the observations mentioned above, we hypothesized that IL-1 plays a crucial role during the pathogenesis of the infection caused by *S. suis* serotype 2. While an immune activation is needed to fight the infection, a high and uncontrolled production of this cytokine significantly enhances the inflammatory reaction, fact that would be detrimental to the host.

Accordingly, the main objective of this research project was to study the production of IL-1 as part of the inflammatory response triggered by *S. suis* serotype 2. More precisely, its role in the pathogenesis of the infection was evaluated to identify whether it is protective and/or detrimental using a mouse model of systemic infection caused by *S. suis* serotype 2.

The specific objectives of this research are:

1. Characterize the production of IL-1 using a well-standardized mouse model of systemic infection (septic shock).
2. Investigate, via *in vitro* studies, the mechanisms implicated in IL-1 production. This objective focuses on the characterization of the different cellular sources and receptors, as well as the intracellular signaling pathways involved in the production.
3. Elucidate the role of IL-1 during *in vivo* infection by *S. suis* serotype 2 with the help of mice deficient in IL-1 receptor.

II- Review of the Literature

1 *Streptococcus suis*: A growing menace

S. suis is an important bacterial pathogen causing sudden death and meningitis in pigs, responsible for important economic losses to the swine industry (2). Additionally, it is also a zoonotic agent causing meningitis and septic shock in humans, and has become a public health concern, particularly in South-East Asia (12). Since being first reported in pigs in 1954, the number of cases, not only in pigs but also in humans, has increased worldwide. Therefore, the study of this pathogen is crucial to guarantee and expedite diagnosis, to develop and implement efficient treatments, and to create efficient control strategies against *S. suis*.

1.1 *S. suis* disease and transmission

1.1.1 In pigs

The natural habitat of *S. suis* is the upper respiratory tract of pigs, more precisely the tonsils and nasal cavities, from where it is frequently isolated (28). Moreover, *S. suis* can also be found in the genital and digestive tracts (29). Pigs carrying *S. suis* can directly transmit the pathogen to other pigs, which represents a major issue in terms of disease spread in herd. In fact, this horizontal transmission through the respiratory route essentially results from nose to nose contact and aerosols (29). In addition, vertical transmission is also possible: piglets born to sows with uterine and vaginal infections are either born infected or become infected while passing through the birth canal. Moreover, they can also acquire the bacterium after birth by close contact with the sow, her feces, and piglets from other litters, as well as from the environment (14, 30).

Several risk factors have been demonstrated to play a key role in the settlement and development of *S. suis* infection. These include the immunity status of the herd and the presence of other infections such as the porcine reproductive and respiratory syndrome virus (31, 32). Additionally, although no seasonal incidence has been noted in pigs, different management practices such as excessive temperature, poor ventilation, and crowding have been suggested as predisposing factors (33).

Of the various manifestations of the disease, septicemia and meningitis are by far the most striking features, but endocarditis, pneumonia, and arthritis can also be observed (14).

Clinical signs can differ between herds depending on the pathogenesis of the disease. Generally, pigs with peracute *S. suis* infection may die within hours of the onset of clinical signs, although it is not unusual for death to occur without any of them (3). The earliest sign is usually an increase in rectal temperature, accompanied by a detectable bacteremia or pronounced septicemia which, if not treated can last up to 3 weeks. During this period there is usually fluctuating fever, and variable degrees of decreased appetite, depression, and shifting lameness (29). After several days, diseased animals develop neurological signs, including opisthotonus, lateral recumbency, ataxia, incoordination, paddling, convulsions, and paralysis (3). Regarding the pathological and histopathological lesions, the most frequently observed in *S. suis* infected pigs are characterized by neutrophilic infiltrates and congestion of the meninges, lymph nodes, and lungs (31).

1.1.2 In humans

The first reported case of *S. suis* in humans was in Denmark in 1968 (34). Since then, over 1600 human cases of infection have been reported worldwide, with probably many more never diagnosed or misdiagnosed (12). *S. suis* infections in humans are most often reported from countries where pig-rearing is common, with the main route of entry being thought to be through contact of cutaneous lesions, most usually on the hands and arms with contaminated animals, carcasses or raw meat (7, 35). However, this situation seems to be different in some Asian countries where the oral route has been proposed, since many infections have been reported after ingestion of contaminated raw pork products (36).

Purulent meningitis is the most frequent manifestation of *S. suis* in humans (37). After an initial incubation period, which ranges from a few hours to days, developed symptoms are, generally, similar to those observed in the case of bacterial pyogenic meningitis which include fever, headache, vomiting, and meningeal signs (36, 38, 39). Interestingly, hearing loss is the most common sequela after recovery from purulent meningitis (35). In addition, although less frequently, *S. suis* can also induce other types of infections including septic shock with multiple organ failure, endocarditis, pneumonia, arthritis, and peritonitis (40-44). Septic shock symptoms include high fever, chills, headache, vomiting, and abdominal pain. Additionally, hypotension, tachycardia, multiple organs dysfunction, subcutaneous hemorrhage, disseminated intravascular coagulation, and death may occur (5).

Current diagnostic procedures are based on the isolation of the bacterium from blood and/or cerebrospinal fluid (CSF) by standard microbiological techniques. However, *S. suis* can be misidentified with other bacterial species such as *Enterococcus faecalis, Aerococcus viridans*, or *Streptococcus pneumoniae* (*S. pneumoniae*) (35). Moreover, bacterial cultures can result in a false-negative result as a consequence of, for example, the use of antibiotics. Nowadays, the implementation of molecular techniques such as the polymerase chain reaction (PCR) has improved the detection of *S. suis* (35).

1.1.2.1 *S. suis* human outbreaks in China

In 1998, in the province of Jiangsu, China, 25 cases of what first was believed to be food poisoning were registered. Later, those cases were categorized as the first outbreak caused by *S. suis* with two main clinical outcomes, streptococcal toxic shock-like syndrome (STSLS) which includes hypotension and multiorgan involvement, and streptococcal meningitis syndrome (SMS) (45). Seven years later, in 2005, another human outbreak was recorded in the province of Sichuan, China, this time with 215 cases: 28% diagnosed as STSLS, 24% sepsis and 48 % meningitis (8). This episode resulted in 39 deaths. Patients presented a sudden onset of disease with high fever, diarrhea, hypotension, petechia, disseminated intravascular coagulation, and dysfunction of multiple organs, such lungs, kidneys, liver, and heart (8, 46).

During the outbreaks, all infections occurred in backyard farmers who were directly exposed to the bacterium during the slaughtering process of pigs that had died of unknown causes or had been killed for food because they were ill. Therefore, the oral route of infection by eating raw pig could not be ruled out (5).

The strains isolated from both outbreaks were characterized as *S. suis* serotype 2 ST7 (47). Evidence indicated that the virulence of *S. suis* ST7 was high, but the particular mechanism of infection was unknown at the time and is still not completely understood. A study comparing the strain SC84, isolated from a patient with STSLS during the 2005 outbreak, and a typical highly pathogenic strain isolated from a diseased pig, 31533, showed that the strains display some differences in cytokine production. Indeed, strain ST7 showed a stronger capacity to stimulate T cells, naïve T cells, and peripheral blood mononuclear cell proliferation than strain 31533, which might help explain the high virulence of SC84 (48). Moreover, the presence of a

pathogenicity island probably played an important role in the rapid adaptation and increased virulence of SC84 strain (49). In addition, the production of a pore-forming toxin, suilysin (SLY) could also have contributed during *S. suis* invasive infections (50).

1.2 General features of *S. suis*

S. suis is an encapsulated Gram-positive coccus that occurs singly, frequently in pairs or occasionally, in short chain. Though it grows well in aerobic conditions, it is considered a facultative anaerobic. All strains are α-hemolytic when grown on sheep blood agar plates and some are β-hemolytic on plates containing horse blood (3). Biochemically, *S. suis* is characterized by absence of growth in 6.5% NaCl agar, a negative Voges-Proskauer test, production of acid in trehalose and salicin broth, and production of amylase (51).

1.3 Serotype identification and geographical distribution

To identify the different serotypes of *S. suis*, serological and molecular techniques can be used. Tests such as co-agglutination, capillary precipitation, or Neufeld's capsular reaction can be performed using reference antisera (52). In addition, PCR, which directly targets genes of the capsular polysaccharide (CPS), is frequently used for its simplicity and effectiveness (53). Nevertheless, despite the numerous techniques, certain strains are difficult to serotype possibly due to a novel capsular type or an acapsular phenotype. To overcome this problem, novel *cps* loci (NCL) have been recently under study (54, 55).

Consequently, based on the CPS, 35 serotypes have been described for the moment (types 1–34 and $^{1}/_{2}$) (12, 56). However, within the last years, and with the appearance of new techniques, 6 of these serotypes were suggested to belong to species other than *S. suis*. As an example, serotypes 32 and 34 have been reclassified as *Streptococcus orisratti* (57). Yet, the classification of these strains is still controversial and there is no actual consensus. More consideration is needed on the subject since a proper classification is central to diagnostic, treatment and control of the disease (12).

Among all capsular types, serotype 2 is the most commonly associated with disease in pigs and humans. As an example, in cases of infections in pigs, serotype 2 is the most predominant in Asia (44.2%) and in North America (24.3%) (58). In Europe, however, serotype

2 takes the second place while serotype 9 prevails, being responsible for 61% of the cases. Concerning human cases, serotype 2 is responsible for 74,7 % of them worldwide. Since it is considered the most virulent serotype and the main cause of economic loses to the swine industry, serotype 2 became the subject of numerous studies.

Additional phylogenetic studies on *S. suis* can be carried out with a technique termed multilocus sequence typing (MLST). Based on the genetic diversity, this technique can further classify *S. suis* according to allelic types or sequence types (STs). MLST is based on the nucleotide sequencing of fragments of 6 to 7 well-conserved housekeeping genes within the bacterial genome. Allelic variation at each locus is then compared with isolate profiles in an international database. In 2002, King *et al.* (13) established a model of MLST for *S. suis* using seven different house-keeping genes: *cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS*).

More than 100 different STs of SS2 have been described up to date. As shown in the representative map in **Figure A**, there is a distinct geographic distribution of 6 predominant STs in different regions of the world. The ST1, associated with disease in pigs and humans, is mainly found in Asia, Europe, and South America (12). In North America, the STs most frequently isolated are ST25 and ST28, which can be also found in Thailand and Japan, respectively (59). In Europe, particularly in the Netherlands, ST20 strains have been isolated as the cause of disease in pigs and humans. Remarkably, ST7, responsible for the two human outbreaks in China, is endemic to this region (60). In the case of ST104, this ST is endemic to Thailand and appears to be more and more commonly isolated from human cases (12).

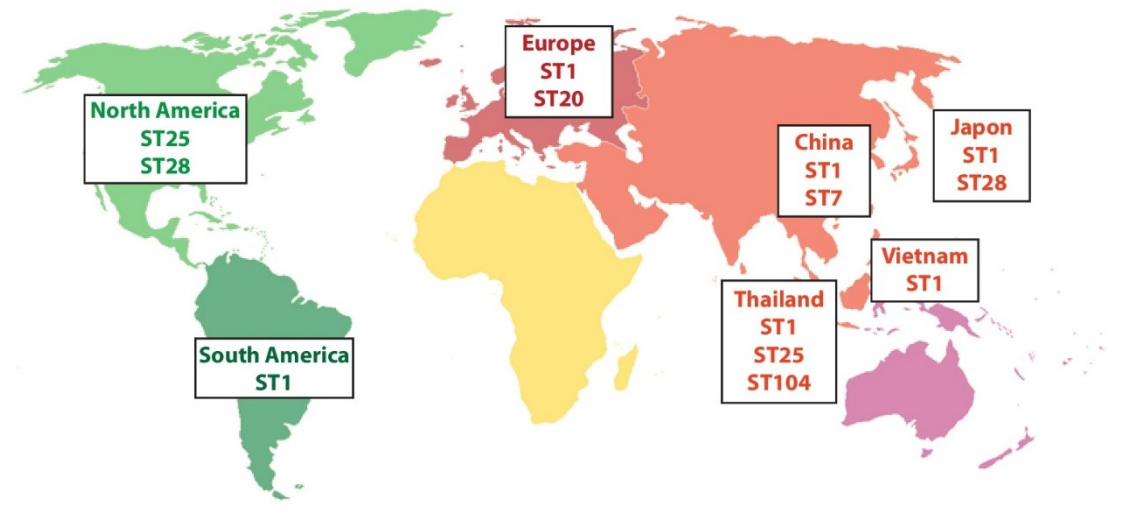

Figure A. Worldwide distribution of the most important S. *suis* serotype 2 sequence types isolated from both clinical pig and human cases of infection. Adapted from (10).

Different studies have tried to associate differences in genomic regions found in MLST to the virulence of the strain. Using a well-standardized mice model of systemic infection, Lachance *et al.* studied the difference in virulence among prevalent STs: the intermediately pathogenic ST25 North American strain (89-1591), a highly pathogenic ST1 European strain (P1/7), and the epidemic ST7 Chinese (SC84) strain (16). Mice infected with the North American 89-1591 strain showed no difference in survival with mock-infected mice, meanwhile survival curves of mice infected with the European P1/7 strain or Chinese SC84 strain differed greatly from mock-infected. Survival rates at the end of the experiment (60 h) were 90%, 40%, and 20% for 89-1591, P1/7 and, SC84, respectively. These results suggested that ST7 is more virulent than a ST1 European strain, but that they are both more than the ST25 North American strain. Interestingly, this study also showed that cytokines levels, but not bacterial burden, correlated with the degrees of virulence of ST7, ST1 and ST25 strains, suggesting a difference in these strain to activate the innate immune system (61). In the same line of studies, Auger *et al.* evaluated the virulence of three different STs (ST1, ST25, and ST28) from different geographical (Americas and Eurasia) and host (porcine and human) origins. However, results showed that prediction of virulence based on ST and geographical origin is difficult since strains

belonging to the same ST presented significant differences in virulence, which did not always correlate with a given geographical origin (62).

1.4 Virulence factors

While virulence between *S. suis* strains varies substantially, several bacterial components have been proposed as potential virulence factors, and many of these, have been proposed to be crucial. However, the identification of these factors has suffered from the lack of a clear definition of the term 'virulence' (29). Over the years, different criteria have been used to define a strain as virulent, from the clinical condition of the animal from which the strain was isolated, to diverse *in vitro* and *in vivo* studies. The differences in the model chosen, in addition to the variation in the experimental design, make it challenging to determine what is truly a virulence factor. Moreover, it is known that the presence of a certain virulence factor cannot define a strain as being virulent, while its absence cannot define it as avirulent (63). Additionally, it is important to remark that almost all of the studies on virulence and pathogenesis have used certain STs within the serotype 2, mainly ST1 and ST7 strains (15). Consequently, there is a lack of information concerning possible virulence factors of other serotypes.

1.4.1 The capsular polysaccharide

The *S. suis* serotype 2 CPS is a large extracellular structure made up of five different sugars: galactose (Gal), glucose (Glc), *N*-acetyl glucosamine (GlcNAc), rhamnose (Rha) and *N*-acetyl neuraminic (sialic) acid (NeuNAc) (64), and is still considered the only proven critical virulence factor. Several steps in the pathogenesis can be influenced by the presence of a thick CPS. For example, the adhesion of *S. suis* to host cells, which is mediated by cell wall components, seems to be reduced in the presence of the CPS, suggesting that the adhesin(s) involved in the process are partially masked by the capsule. Moreover, survival and dissemination after reaching the bloodstream were also shown to depend on the production of CPS since it confers protection against immune recognition and clearance (63). In fact, several studies with non-encapsulated mutants showed that the CPS protects *S. suis* from neutrophil and monocyte/macrophage–mediated phagocytosis and killing, and helps rapid clearance of the bacterium from the circulation (65-68). However, resistance to phagocytosis and clearance

should be considered multifactorial since encapsulated avirulent strains can be eliminated from blood within 48 h, suggesting that there are others factors involved in this process (69, 70).

In addition to its antiphagocytic properties, the *S. suis* CPS also plays a role in the modulation of the immune response. Several studies showed that activation of immune cells such as macrophages and DCs, by non-encapsulated mutants leads to an increased pro-inflammatory cytokines and chemokines production in comparison with the wild-type strain (67, 71-74). This suggests, that the CPS reduces the immune response by hiding cell wall immunostimulating components.

1.4.2 Muramidase-released protein and Extracellular factor

Two of the first virulence factors described for *S. suis* are the muramidase-released protein (MRP) and the extracellular factor (EF) (75). MRP is a 136-kDa protein, anchored to the cell wall peptidoglycan (PG) by sortase A and also released into the culture supernatant during bacterial growth (76, 77) On the other hand, EF is a 110-kDa protein only present in the culture supernatants (76).

Association of MRP and EF with virulence was observed in serotype 2 strains of *S. suis* and it seemed to be associated with certain countries. While strains with the phenotype MRP+EF+ have been isolated from acute cases of septicemia and/or meningitis (from either pig or human origin) in Europe and Asia, strains isolated from North America were phenotype MRP-EF- (78, 79). However, studies using isogenic mutants lacking both of these proteins appeared to be as virulent as the wild-type strain after experimental infection of new-born germfree pigs and similar results were also obtained with isogenic MRP-EF- mutants of *S. suis* serotype 1(75). Unfortunately, as the specific roles of MRP and EF in the pathogenesis of *S. suis* have not been fully clarified, they should be considered as virulence-associated markers.

1.4.3 Suilysin

Hemolysins have often been implicated as virulence factors in infections caused by various bacterial species. In fact, the pneumolysin, listeriolysin O, and streptolysin O, produced by *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Streptococcus pyogenes*, respectively, have been shown to contribute to the pathogenesis of disease caused by each

pathogen (80, 81). *S. suis* produces a 54-kDa thiol-activated hemolysin termed suilysin (SLY), that has the capacity to form transmembrane pores (82). SLY shares several characteristics with the previously mentioned toxins, including loss of activity upon oxidation, reactivation upon reduction, inhibition by small amounts of cholesterol, formation of transmembrane pores, and a multi-hit mechanism of action (83).

Interestingly, the gene coding this protein (*sly*) is highly conserved among *S. suis*. A study showed a 99.5% of homology between North America SC332 and the European 31533 (84). The high homology makes SLY a candidate for a vaccine against all SLY+ strains. Moreover, SLY has been showed to be very immunogenic since mouse immunization with the protein protects against a lethal dose (85). However, the role of this toxin as a virulence factor has not yet been confirmed.

In vivo studies using isogenic mutants lacking suilysin expression, showed that this protein plays a critical role in an intra-peritoneal mouse model of infection (50, 84). On the other hand, an intra-nasal mouse model using sublethal doses showed no differences in upper respiratory tract colonization between the wild-type strain and the suilysin knock-out mutant (86, 87). Concerning pig experimental models of infection, at either high or low dose of incubation, and independently of the infection route the mutant strain induced disease similarly to the WT strain, revealing that suilysin was not required for virulence. Still, SLY could play a role in the evolution of the disease by activating the host innate immune system and leading the induction of cytokines (88, 89).

In vitro studies have shown that suilysin plays important roles in the interactions with different host cells, in the induction of cell death, and in the inflammatory response. In fact, SLY positive strains were shown to be cytotoxic to endothelial and epithelial, as well as monocytes and macrophages (14, 65, 90-92). In addition, native purified SLY has been shown to induce the release of several pro-inflammatory cytokines by human and porcine BMEC (93-96), porcine peripheral blood cells (17), and porcine alveolar macrophages (88).

Finally, the presence of the gene coding SLY, similarly to MRP and EF, is correlated with high virulence of serotype 2 strains: ST1 strains are frequently MRP+EF+SLY+. Moreover, whereas SLY is present in most Asian and European *S. suis* serotype 2 strains, it is only present in a limited number of North American serotype 2 strains (15).

1.4.4 Cell wall modifications: *N*-deacetylation of peptidoglycan and D-alanylation of Lipoteichoic Acid

PG is the main component of the cell wall and it provides stress resistance and shape-determining properties (97). Since this structure can be recognized by the host, bacteria have developed different mechanisms to avoid immune recognition and killing. As it was demonstrated for *Streptococcus pneumoniae*, one of these mechanisms is the modification of the PG by means of *N*-deacetylation. (98). In the case of. *S. suis*, this modification is carried out by a deacetylase encoded by the *pgdA* gene. A study showed that *S. suis* enhance PG *N*-deacetylation in the context of an infection since expression of the *pgdA* gene was increased upon interaction of the bacteria with neutrophils. Moreover, in the same study, using an isogenic *pgdA* mutant, an attenuation in virulence in mouse and pig models of infection was demonstrated, suggesting again, an importance of this modification for *S. suis* infection.

Another strategy used by *S. suis* is the D-alanylation of its lipoteichoic acid (LTA), since it allows to modulate the surface charge and offers cationic antimicrobial peptides resistance. Similarly to *N*-acetylation, D-alanylation also plays a role in survival of this pathogen as demonstrated in mouse and pig models (99), where bacteria unable to produce this changes had a decreased ability to escape immune clearance a lower capacity to activate the inflammatory cascade and an impaired competence to across host barriers.

1.4.5 Others

There are many more virulence factors proposed, all summarized in Fittipaldi, *et al* (63) though little is known about which one in particular, can lead to the inflammatory activation and consequently to septic shock or meningitis. Among these:

- Bacterial adhesins are necessary for the attachment to the host cells. Examples are the enolase, the fibronectin-fibrinogen binding protein (FBP), and the glyceraldehyde-3-phosphatase dehydrogenase (GAPDH).
- Bacterial proteases play a critical role in the colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, and tissue damage during infection. Several proteases have been described for *S. suis*, including an arg-

aminopeptidase, a dipeptidyl peptidase (DPP) IV, a chymotrypsin-like, a caseinase, a phospholipase C, and a hyaluronate lyase.

- Sortases are membrane-associated transpeptidase responsible for the anchoring of surface proteins to the cell wall.
- Proteins such as Sao and glutamate dehydrogenase (GDH) have also been described to play a role in the pathogenesis of *S. suis* infection.

1.5 Pathogenesis

The study of the mechanisms involved in *S. suis* pathogenesis is complex since the development of the disease is influenced by environmental factors, immunity status of the host, and bacterial virulence factors. In fact, despite increasing research in the last years, the pathogenesis of infections due to *S. suis* is still not completely understood. In addition, most of the studies on this subject have been carried out using only strains from serotype 2 and concern only the development of meningitis (14, 100).

As represented in **Figure B**, *S. suis* pathogenesis can be simplified to 3 steps. Firstly, bacteria adhere and invade the epithelial cell layer of the upper respiratory tract (colonization), penetrate mucosal barriers, and gain access to blood circulation. Once in the bloodstream, *S. suis* can travel as free bacteria or associated to monocytes (bound and/or intracellular) and disseminate to organs such as spleen, liver, kidney, lung, and heart. If bacteremia is not controlled, it can lead to septicemia and/or septic shock. Finally, if host death by sepsis does not take place and bacteremia remains high, *S. suis* can also reach the central nervous system (CNS) and cross the barrier made of the brain microvascular endothelial cells and/or epithelial cells of the choroid plexus, causing meningitis (14). These 3 steps will be further explained in the next sections.

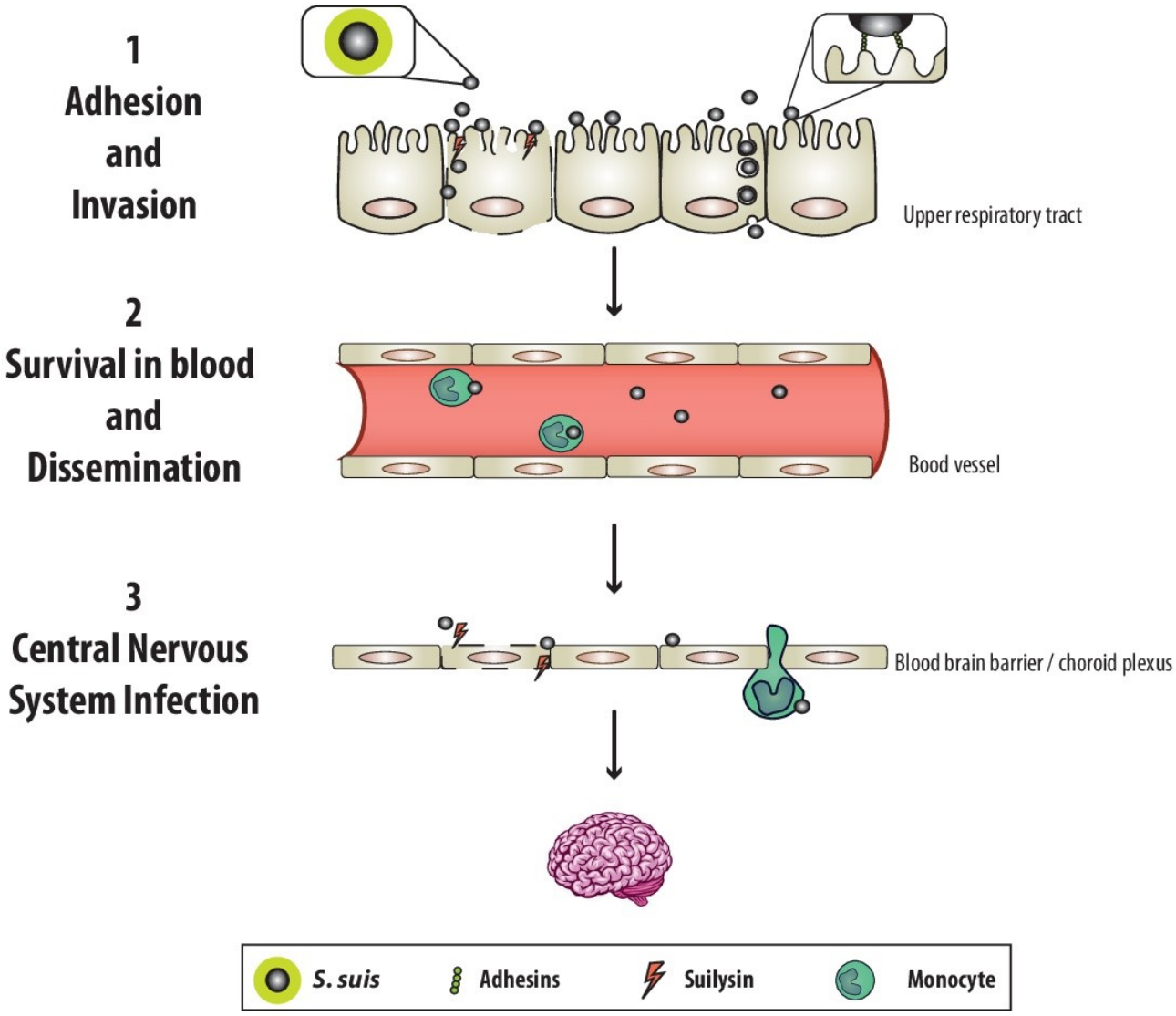

Figure B. Pathogenesis of *S. suis* infection involves 3 main steps. Adapted from (14).

1.5.1 STEP 1: Adhesion and invasion

As mentioned before, *S. suis* enters the natural host (pigs) via the upper respiratory tract and resides within the palatine or pharyngeal tonsils. Once colonized, some animals may remain healthy carriers, whereas others will develop bacteremia, septicemia, and/or meningitis. For these events to happen, the bacteria need to cross the epithelial barrier in order to reach the bloodstream (14). Regarding human infections, the bacterium enters mainly through skin wounds, thus having direct access to blood circulation. However, in cases of oral route of infection bacteria may interact with the mucosal epithelial cells in the intestine (100).

Despite many studies, it is still not completely understood how *S. suis* is able to breach the mucosal epithelia in the upper respiratory tract. *S. suis* possess multiple adhesins that allow interaction with the host. Generally, adhesion to host cells is mediated via hydrophobic interactions, cation-bridging, and receptor–ligand binding. Moreover, interaction with components of the extracellular matrix such as fibronectin, laminin and collagen can be used by bacteria to favorize interactions with cells (100). However, few studies have investigated the interactions between *S. suis* and epithelial cells and results suggest very low levels of adhesion and invasion (91, 101).

Norton *et al.* suggested that suilysin-positive *S. suis* strains can use invasion and cell lysis as a mechanism to breach the mucosal epithelium (92). Nevertheless, strains not producing this hemolytic toxin are also able to reach the circulation and disseminate (102), suggesting that other mechanisms could also be in play.

1.5.2 STEP 2: Survival in blood and dissemination

To disseminate throughout the host and cause bacteremia and septicemia, bacteria must be able to survive in blood. As mentioned before, the CPS is a key factor for the bacteria since its presence protects bacterium against the actions of the host immune innate system.

There are several hypotheses about how bacteria travel in the bloodstream. The one proposed by Williams and Blakemore in 1990 was named the “Trojan horse theory” (103). This model describes the uptake of bacteria by monocytes (in the absence of specific antibodies) followed by intracellular survival and invasion of organs or the CNS. Though the capsule might protect against phagocytosis, it could still allow an association of *S. suis* with these cells. Indeed,

a relatively high level of adhesion (without phagocytosis) of *S. suis* to phagocytic cells has recently been observed (104). Thus, traveling and dissemination to target organs by exploitation of host cells as "vehicles" was postulated, the so called "modified Trojan horse theory". Yet, is it also possible that extracellular *S. suis* bacteria travel free in circulation (14).

The interaction of *S. suis* with phagocytic cells in the bloodstream, and/or in organs is crucial for the development of the inflammatory response. It has been demonstrated that *S. suis* is capable of up-regulating important adhesion molecules and also several pro-inflammatory cytokines and chemokines (e.g. tumor necrosis factor alpha [TNF], IL-6, IL-1, IL-8, and monocyte chemotactic protein-1 [MCP-1]) *in vivo* and *in vitro* (71-73, 105, 106). This may result in multiple systemic effects, including contributing to the recruitment of leukocytes to the site of infection, increasing hematopoiesis and inducing fever. However, although activation of the immune system and synthesis of cytokines and chemokines contribute to the anti-infective process, their uncontrolled production can have adverse consequences for the host, leading to multi-organ failure and septic shock like syndrome.

1.5.3 STEP 3: Central nervous system invasion: Meningitis

To develop meningitis, *S. suis* needs to reach the CNS, and most importantly, cross the blood-brain barrier (BBB). The BBB is an anatomical and functional barrier that separates the brain from the intravascular compartment and maintains the homeostasis of the CNS. If the theory of the Trojan horse or the modified Trojan horse are correct, *S. suis* would arrive to the BBB inside or associated with monocytes. As the CNS is considered to be an immune privileged organ, normal circulation of monocytes into the CNS is still controversial. However the permeability to some immune cells could be modified as an adaption to the specific local microenvironment (14).

Because of the particular characteristics of the BBB, it is generally accepted that bacterial interactions with brain microvascular endothelial cells (BMEC) are mainly characterized by specific bacterial attachment with consequent invasion, toxicity and/or increase permeability. Therefore, in the case that the bacteria reach the CNS freely, it must adhere to the brain epithelium and invade it. Studies with human BMEC demonstrated that *S. suis* could adhere to but not invade this type of cells (90). It is possible that after the adherence of *S. suis*

to BMEC, the bacteria secrete toxic factors which would affect the endothelial cells. Such factors would increase BBB permeability, which could lead to the development of cerebral edema, increased intracranial pressure, and cerebral blood flow blockage characteristic of bacterial meningitis. Suilysin has been reported to be toxic to the BMEC, which could contribute to increased BBB permeability (90, 95). In fact, purified SLY has been shown to induce the release of several pro-inflammatory cytokines by human and porcine BMEC (93, 95). On the other hand, is it possible that suilysin-negative strains adhere to BMEC and result in cytokine production that would also lead to alteration in its permeability (93, 94). It has been shown that *S. suis* induces the release of pro-inflammatory cytokines and chemokines by human and porcine BMEC, murine microglia, and astrocytes (94, 106-108). However, the specific bacterial components responsible for exaggerated inflammatory reactions are not accurately known. In addition to cell wall components, bacterial CPS induces human macrophages to secrete prostaglandin E2 (PGE2) and matrix metalloproteinase 9, which may also be involved in disruption of the BBB (109).

Another model of entry of *S. suis* to the CNS has been suggested to be through the blood-cerebrospinal fluid barrier. In this model, *S. suis* invades the choroid plexus epithelial cells (CPEC), then transported within membrane-bound endocytic vacuoles to the apical side and finally exit by exocytosis onto the apical membrane of the blood–CSF barrier (110). In fact, a study with CPECs showed that *S. suis* induces necrosis and apoptosis (110). In addition, the translocation across the blood–CSF barrier activates neutrophils and affects the barrier´s function and integrity, further facilitating trafficking of bacteria and leukocytes (111).

2 Innate immune response

The vertebrate immune system comprises the innate and the adaptive immune system. While adaptive immunity is involved in the elimination of pathogens in the later phase of infection, and in the generation of immunological memory, the innate immunity is rapidly activated and represents the first line of defense against microorganisms. Therefore, it plays a crucial role in the early recognition of microbes and subsequent triggering of a pro-inflammatory response (112, 113). This system is composed by physical and chemical barriers, including the epidermis, the ciliated respiratory epithelium, the vascular endothelium, and mucosal surfaces with antimicrobial secretions. Additionally, among the cellular components, the innate system

includes macrophages, granulocytes, cytotoxic natural killer cells (NK), dendritic cells (DCs) and γδ T lymphocytes (114).

Conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) are recognized by specialized innate immune receptors, also known as pathogen recognition receptors (PRRs). The activation of the PRRs by pathogens leads to activation of the complement and coagulation cascades, opsonization and phagocytosis. Additionally, it will also activate diverse signaling pathways such as nuclear factor–kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), which promote the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors, which together orchestrate the early antimicrobial host response (112-117).

2.1 Pattern recognition receptors

Currently, five different families of PRRs have been identified. These families include transmembrane proteins such as the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and the receptor kinases, as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Among these, the TLRs and the NLRs are the most studied in the context of bacterial infections and will be described below.

2.1.1 Toll-like Receptors

The TLRs are a family of type I transmembrane proteins conserved from insects to humans (118). The cytosolic domain is termed the Toll/IL-1 receptor (TIR) domain and is the defining motif of the superfamily. Additionally, the extracellular portion formed by leucine-rich repeats (LRRs) is responsible for the recognition of PAMPs. Several TLRs have been reported in humans and mice (TLRs 1-13)**.** These receptors are not only expressed in immune cells, but also in a variety of other types, including vascular endothelial cells, adipocytes, cardiac myocytes, and intestinal epithelial cells. Moreover, their expression can be modulated in response to a variety of stimuli (119).

Based on their cellular location and their ligands, TLRs are classically defined into two subgroups. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 compose the first group. They are

expressed on the cellular surface and recognize main components of the microbial membrane like lipids, lipoproteins, and proteins. The other group is formed by TLR3, TLR7, TLR8 and TLR9 which are expressed in intracellular vesicles, like the endoplasmic reticulum, endosomes, and lysosomes, and recognize mainly nucleic acid from pathogens (17).

As mentioned before, after PAMP recognition, TLRs activate different intracellular signalling pathways that initiate and orchestrate the immune responses. These pathways begin with the recruitment of different adaptor molecules inside the cell. If the myeloid differentiation primary response 88 (MyD88)-dependent pathway is engaged, the cytosolic TIR domains of TLR1, TLR2, and TLR6 assemble with two proteins: MyD88 and the interleukin-1 receptor-associated kinase 4 (IRAK4). Consequently, IRAK4 undergoes (auto) phosphorylation and activates kinases IRAK1 and IRAK2. This step is followed by the recruitment of TNF receptor-associated factor 6 (TRAF 6) and the consequently activation of one of two possible signaling pathways: NF-κB activation or mitogen-activated protein (MAP) kinases activation (120) (121) which include the p38, c-Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinase (ERK) pathways.

On the other side, TLR3 and TLR4, use the TIR-domain-containing adapter-inducing interferon-β (TRIF) and the TIR domain-containing adaptor protein (TIRAP) as adaptor molecule instead of MyD88. This would lead to an alternative activation of NF-κB leading to the induction of inflammatory cytokines. TLR2 and TLR4 can also use TIRAP as an adaptor protein supplementary for the recruitment of MyD88 (120, 122).

2.1.2 NOD-like Receptors

The nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a specialized group of intracellular receptors that, like the TLRs, represent a key component of the host innate immune system. This family of proteins is defined by a tripartite structure consisting of: (*a*) a variable N-terminal protein-protein interaction domain, defined by the caspase recruitment domain (CARD), pyrin domain (PYD), acidic transactivating domain, or baculovirus inhibitor repeat (BIR); (*b*) a central NOD domain, which mediates self-oligomerization during activation (4); and (*c*) a C-terminal LRR that detects PAMPs (123). Similarly to TLRs, NLRs are expressed in immune cells, including both lymphocytes and

antigen-presenting cells (APCs) such as macrophages and dendritic cells, but also in non-immune cells, including epithelial and mesothelial cells (123).

Likewise, stimulation of the NLRs activates signaling pathways leading to production of pro-inflammatory mediators. The MAPK and NF-κB signaling pathways are two of the main targets of the NLRs, although not through the same adaptor proteins. Additionally, there is a third target that was not seen with the TLRs, which is activation of caspase-1 leading to IL-1β and IL-18 maturation. The complexes in charge of this activation are called inflammasomes and will be explained in detail in section 3: Interleukin 1 (123). In summary, they have the ability to regulate NF-κB and MAPKs signaling indicating that they can have an important role in the pathogenesis of a variety of inflammatory human diseases (123).

NOD1 and NOD2 are the best characterized intracellular receptors in the NLRs family. NOD1 is ubiquitously expressed, while NOD2 expression is restricted to monocytes, macrophages, dendritic cells, and intestinal Paneth cells (124). They recognize PG moieties found in bacteria. NOD1 recognizes d-γ-glutamyl-meso-diaminopimelic acid (iE-DAP) (125, 126) found in the structures of all Gram-negative and some Gram-positive bacteria like *B. subtilis* and *L. monocytogenes*. On the other side, NOD2 recognizes muramyl dipeptide (MDP) a component of the PG present in all the Gram-positive and Gram-negative bacteria (127, 128).

2.1.3 Importance of the PRRs in *S. suis* infection

Studies involving PRR activation after *S. suis* infection have demonstrated that mainly TLRs, particularly TLR2, TLR4, and TLR9, are implicated in its recognition. TLR2 is a versatile receptor, as it recognizes a variety of components, including lipoproteins, PG and LTA from Gram-positive bacteria. Using TLR2 deficient mice, Lachance *et al.* demonstrated an increased survival rate and a significant decrease of pro-inflammatory mediators after *S. suis* high virulent ST1 infection that could not be associated with a lower bacterial burden. These results suggested that there might be other receptors involved. Contrarily, when TLR2 deficient mice were challenged with epidemic high virulent ST7 strain no significant difference in survival and production of mediators in comparison with wild-type mice was observed. Together these results showed that infection of mice by highly pathogenic strains of *S. suis* may follow TLR2-dependent or independent pathways depending on the strain (129).

In vitro studies with murine macrophages and dendritic cells deficient in TLR2 showed a reduction in the secretion of pro-inflammatory products in response to encapsulated *S. suis* (105, 130). In this case, the response was totally abrogated in MyD88-deficient cells suggesting that a second pathway including other TLRs may play a role and might also participate directly or indirectly in the activation of the inflammatory cascade (105, 130). In addition, recent studies on peripheral blood mononuclear cells (PBMCs) showed an implication of the TLR2, TLR6, and TLR9 but not of TLR1 in response to *S. suis* ST1 and ST7 (131). The interactions with these receptors resulted in the release of several pro-inflammatory mediators, such as IL-6 and TNF, with the use of blocking antibodies for the TLRs reducing their concentrations (131). Moreover, a recent study with *S. suis* strains ST1, ST7 and ST25 showed the implication of TLR7 and TLR9 in the production of type-I interferon after recognition of genetic material (132).

Previous studies have demonstrated that TLR4 recognizes not only LPS from Gram-negative bacteria, but also certain toxins such as pneumolysin (133), listeriolysin O (134), and more recently *S. suis*-produced SLY (135). However, the capacity of *S. suis* and/or SLY to activate TLR4 remains controversial. In a study with whole live bacteria, the role of TLR4 in cytokine production was minimal since the production of only CXCL1 by DCs deficient in TLR4 was affected. On the other hand, a study with purified native SLY observed TLR4-dependent TNF production by peritoneal macrophages (135), suggesting that conditions tested, including concentration of toxin, could influence the results.

In the case of NOD receptors, their importance in the immunopathogenesis of infection was demonstrated for streptococci such as *S. pneumoniae* and *S. pyogenes* (136, 137). Unfortunately, there are not many studies about their role in the infection with *S. suis*. Lecours *et al.* studied dendritic cells deficient in NOD2 and demonstrated that only the release of IL-23 and CXCL1 was partially dependent on this receptor (105). In summary, the host response to *S. suis* is not limited to a specific receptor but requires a multimodal recognition system.

2.2 Inflammatory mediators

The secretion of cytokines and chemokines is one of the first steps necessary for the initiation of the innate immune response. They bind to specific receptors located in target

immune cells and have a specific effect on the interactions and communications between those cells (138).

Cytokines are soluble, low molecular weight glycoproteins which regulate both innate and specific immune responses and mainly act as inflammatory mediators. They are produced by a variety of cells, including monocytes macrophages, NK cells, endothelial cells, lymphocytes, and fibroblasts. Their action is pleiotropic, targeting multiple cells in diverse ways depending on timing and concentration. Some of these cytokines can appear early in the infection and initiate host responses. Others can appear late in the infection and help regulate or suppress the defense response. In addition some of them at low concentrations have a paracrine effect, whereas, at higher concentrations, such as in sepsis, cytokines have endocrine effects and act systemically (139, 140). The main pro-inflammatory cytokines produced in humans, mice and pigs are IL-1, IL-6, IL-18, IL-12, and TNF (138).

Chemokines are low molecular weight peptides, powerful activators, and chemoattractants for leukocyte subpopulations and some non-hemopoietic cells. They are secondary pro-inflammatory mediators that are induced by primary pro-inflammatory mediators such as IL-1 and TNF (141). Their receptors are transmembrane G-proteins and are expressed on different cell types and their binding and response to specific chemokines are highly variable. Their activation responses include chemotaxis, degranulation, release of superoxide anions, and changes in the avidity of integrins (142). There are two major subfamilies (CXC and CC) based on the position of the cysteine residue. All members of the CXC chemokine sub-family have an intervening amino acid between the first two cysteines and they are typically chemotactic for neutrophils, whereas members of the CC chemokine sub-family have two adjacent cysteines and attract and activate mononuclear cells (143).

2.2.1 Mediators involved in *S. suis* infection: Inflammation as a hallmark of the systemic infection

As mentioned above, several inflammatory and infectious diseases are associated with the production of cytokines and chemokines. It leads to the recruitment and activation of different leukocyte populations and to the alteration of the microvasculature. However, as seen

in cases of sepsis and septic shock, an uncontrolled production of this mediators can lead to a detrimental outcome (144, 145).

Recent studies showed that *S. suis* is not only able to interact with different types of immune cells, but also to induce the release of several pro-inflammatory cytokines and chemokines *in vitro*. Production and modulation of several mediators such as IL-1, IL-6, IL-8, IL-10, IL-12p-70, MCP-1, CXCL1, and TNF, amongst others, by different strains of *S. suis* was demonstrated in a whole-blood culture systems (146) in humans (147), murine DCs (72, 105), murine macrophages (71), monocytic THP-1 human cells (130, 148) and in PBMCs (131). These results demonstrate the full capacity of several strains of *S. suis* to induce inflammatory mediators.

On the other side, *in vivo* murine models of infection was developed to study septic shock and meningitis caused by *S. sui*s (106). These models are mainly used to study the pro-inflammatory response associated with *S. sui*s infection, both at systemic and CNS level. Results demonstrated the production of several pro-inflammatory cytokines (TNF, IL-6, IL 12p40/p70, IFN-γ) and chemokines (CXCL1, MCP-1, CCL5) leading to septic shock and death (106). The role of inflammation in mortality was confirmed by comparing two different mouse lines: C57BL/6 (considered resistant to infection with *S. suis*) and A/J (considered susceptible to infection) (149). The high mortality rate observed in the sensitive line was attributed to an uncontrolled septic shock. In fact, A/J mice showed unusually high levels of TNF, IL-12p40/p70, IL-1β, and IFN-γ, which were significantly higher than those found in the C57BL/6 mice. Interestingly, C57BL/6 mice had a higher production of IL-10, an anti-inflammatory cytokine, suggesting that the pro-inflammatory cascade is better controlled in these mice. Survival of septic shock seems to involve a precise control of pro- and anti-inflammatory mechanisms (149).

Further studies on the production of inflammatory mediators and its exacerbation, specially in the case of epidemic strain of *S. suis* (ST7) normally associated with STSLS, was performed by Lachance *et al.* using a mouse model (16). The host response was evaluated after *S. suis* infection with a North American intermediately pathogenic strain, a European highly pathogenic strain, and the Chinese epidemic strain. A whole-genome microarray approach demonstrated that pro-inflammatory genes were expressed at higher levels in mice infected with

the Chinese strain than those infected with either the European strain or the North American strain, which correlates with their level of virulence.

In summary, these studies showed that *S. suis* is able to interact with several immune cells and induce the synthesis of several inflammatory mediators. Generally, these cytokines and chemokines will orchestrate early host response, triggering the cascade of inflammation necessary to clear the infection. However, in the case of *S. suis*, an excessive inflammatory reaction can be observed, leading to septic shock and in the most severe cases, to host death. This is why excessive inflammation, no matter the host (mice, pigs or humans), is considered the hallmark of the *S. suis* infection.

3 Interleukin-1

IL-1 is the prototypic pro-inflammatory cytokine and is a central mediator of innate immunity and inflammation. It affects virtually all cells and organs and is a major pathogenic mediator of autoinflammatory, autoimmune, infectious, and degenerative diseases (21).

In fact, the term IL-1 refers to IL-1α and IL-1β, encoded by different genes. However, they bind the same receptor, and show similar biological activities in most studies (21). Importantly, differences between IL-1α and IL-1β are for the most part due to the cellular sources of each cytokine and the release mechanisms but not to differences in downstream events following receptor engagement (21)

3.1 Main functions of IL-1

In some instances, the production of IL-1 is a rapid and direct determinant of disease, whereas in others its production is an intermediate step (150). As an example, in autoimmune diseases, it acts by stimulation of other cells to produce cytokines or enzymes that then act on the target, whereas in septic shock, IL-1 can also act directly on the blood vessels to induce vasodilatation through the rapid production of activating factor, prostaglandins, and nitric oxide (151).

As mentioned before, IL-1 is one of the first and most potent pro-inflammatory cytokines released after a stimulus whether in sterile or septic inflammation (18). It is an inducer of several cytokines and chemokines, stimulates the synthesis of adhesion molecules, insulin, collagen,

collagenases, pro-coagulant proteins, proteoglycans, and several growth factors. Moreover, cells that themselves produce IL-1 will, in turn, respond to it more greatly. This positive feedback may be important for the augmentation or suppression of various biological properties during host responses to infection or inflammation (152).

IL-1 markedly prolongs the lifespan and stimulates the effector function of neutrophils and macrophages (153). In addition, IL-1 also causes degranulation of basophils and eosinophils, stimulates thromboxane synthesis in macrophages and neutrophils, and potentiates the activation of neutrophils by chemoattractant peptides (154). Furthermore, IL-1 has a role as co-stimulator of T cells (150), contributes to Th2 polarization (155) and is believed to also participate in antibody production. During the generation of the Th17 response, IL-1 appears essential given that T cells from mice deficient in IL-1R fail to induce IL-17 upon antigen challenge (156).

The effects of IL-1 on the CNS include fever (IL-1 is the classic endogenous pyrogen) and activation of the hypothalamus-pituitary-adrenal (HPA) axis (21). Consequently, leukocyte migration increases because of the high temperatures, while cortisol downstream of the HPA axis plays a regulatory function on immunity (21).

Lastly, metabolic effects have been also attributed to IL-1 including, among others, increased intestinal mucus production, decreased synthesis of adipocyte lipoprotein lipase, decreased hepatic albumin transcription, and increased hepatic metallothionein transcription (157).

Although IL-1 signaling plays essential roles in immunity and sterile inflammation, an uncontrolled production of IL-1 can be detrimental, as observed for autoinflammatory diseases such as rheumatoid arthritis and gout (22). For these, neutralization of IL-1β results in a rapid and sustained reduction in disease severity. Whereas treatment for autoimmune diseases often includes immunosuppressive drugs, neutralization of IL-1β is mostly anti-inflammatory (158).

3.2 Interleukin-1 Receptor

IL-1α and IL-1β independently bind to the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed (121). A third specific ligand, the IL-1 receptor antagonist (IL-1Ra), binds to the IL-1R1 with similar specificity and affinity but does not activate the receptor nor

trigger the downstream signaling (121). The IL-1 receptor accessory protein (IL-1RAcP) serves as a co-receptor that is required for signal transduction of IL-1/IL-1RI complexes. The type II IL-1R (IL-1R2) binds IL-1α and IL-1β but lacks a signaling-competent cytosolic part and thus serves as a decoy receptor (19). The pathway activated by IL-1 is similar to the one explained for TLR signaling in section 2.1.1. Briefly, IL-1 induces a conformational change in the first extracellular domain of the IL-1R1 that facilitates recruitment of IL-1RacP (159). Through cytosolic TIRs domains, the trimeric complex rapidly assembles with MyD88 and IRAK4. Upon binding, IRAK is phosphorylated, subsequently dissociated, and associates with TRAF6, which leads further downstream to the activation of the NF-κB and MAPKs pathways (121). IL-1 rapidly induces the expression of hundreds of genes such as the ones encoding IL-6, IL-8, MCP-1, and cyclooxygenase 2 (COX2) (121). Because of the ubiquitous presence of the receptor, they interact on different cell types, such as monocytes or macrophages (160, 161), epithelial (162), endothelial cells (163), chondrocytes (164), and fibroblasts (165). As mentioned before, IL-1α and IL-1β also induce expression of their own genes, which serves as a positive-feedback loop that amplifies the IL-1 response in an autocrine or paracrine manner (152, 162).

3.3 IL-1α

IL-1α is constitutively expressed in many cell types and in healthy tissues at steady state. Its expression can be rapidly increased in response to a variety of physiological stimuli, including oxidative stress, lipid overload, hormonal stimulation, exposure to cytokines (including IL-1β and IL-1α itself), and canonical proinflammatory mediators of microbial origin with TLR agonistic activities (166).

Unlike most secreted proteins, the synthesis of the precursor (proIL-1α), occurs in association with cytoskeletal structures (microtubules) and not in the rough endoplasmic reticulum (167). Importantly, the precursor and mature form of this cytokine are biologically active. After synthesis, proIL-1α can be localized in the cytosol, in the nucleus or displayed on the outer leaflet of the plasma membrane and more importantly such localization can change in response to specific stimuli (21). To be translocated to the nucleus, this cytokine possesses a nucleus localization signal (NLS). Once there, it binds transcription regulation factors and

activate the expression of proinflammatory cytokines and chemokines independently of IL-1R1 signaling. In the cytosol, glycosylation of proIL-1α may allow its translocation onto the outer surface of the cell. This membrane form accounts for no more than 5% of the total proIL-1α synthesized by the cell (19). Lastly, proIL-1α can be cleaved by activation of the calcium-dependent membrane-associated cysteine proteases called calpains (19, 167). However, after being released from dying cells proIL-1α can be also cleaved by extracellular proteases.

The main function attributed to proIL-1α is as a key 'alarmin' in the cell that alerts the host to injury or damage. (168, 169). In fact, the precursor can rapidly initiate a cascade of inflammatory cytokines and chemokines. This can also be conceptualized into an "inflammatory loop" where IL-1α expression is induced in response to oxidative, genotoxic and metabolic stressors, pro-inflammatory mediator or infection, and leads to the activation of IL-1R1 and the generation of more cytokines and the recruitment of myeloid cells to the site of the stress (166). The initial IL-1α–IL-1R1 signaling therefore initiates a loop of sustained and self-perpetuating inflammation that results in extensive tissue damage that occurs until IL-1R1 signaling is either exhausted or suppressed. In addition, proIL-1α has also been proposed as an autocrine growth factor. Some investigators have considered that intracellular proIL-1α regulates normal cellular differentiation, particularly in epithelial and ectodermal cells.

3.4 IL-1β

IL-1β is produced by hematopoietic cells such as blood monocytes, tissue macrophages, skin dendritic cells, and brain microglia (150). Although nearly all microbial products induce IL-1β via TLR ligands, IL-1 induces itself both *in vivo* and in monocytes *in vitro* (152). Following LPS stimulation, IL-1β mRNA levels rise rapidly within 14 minutes but begin to decline after 4 h owing to mRNA half-life or the action of microRNA (170). However, by using IL-1α as a stimulant or raising intracellular cAMP levels, mRNA levels are sustained for over 24 h compared with microbial stimulants (171). Moreover, there is a dissociation between transcription and translation that is characteristic of IL-1β since besides vigorous signal for transcription most of the IL-1β mRNA is degraded, and no significant translation into IL-1β takes place (19, 172). Even though IL-1β mRNA assembles into large polyribosomes, there is little significant elongation of the peptide (173). However, adding bacterial endotoxin or IL-1

itself to cells with high levels of steady state IL-1β mRNA results in augmented translation (19). One possible explanation is stabilization of the mRNA by microbial products (174). Another explanation is that IL-1β stabilized its own mRNA by preventing deadenylation (170). Studies on bacterial meningitis, have shown that brain cells such as astrocytes, microglial cells, endothelial cells, ependymal cells, and resident macrophages, react to invading pathogens by releasing early response inflammatory cytokines, among them IL-1β (175). High CSF levels of IL-1β significantly correlates with adverse outcome and severity of bacterial meningitis (176).

Following synthesis, proIL-1β remains primarily cytosolic and can be myristoylated on lysine residues as IL-1α (177) but has no known membrane form. Some IL-1β is found in lysosomes or associated with microtubules and either localization may play a role in secretion of the cytokine. Unlike the IL-1α precursor, the proIL-1β is not active, but after cleavage by caspase-1, the biologically active form is released into the extracellular space (21). The specific mechanisms of IL-1β activation by the inflammasomes will be discussed in the following section.

IL-1β is considered critical for local and systemic inflammation. In fact, mice deficient in IL-1β showed an impaired response to local inflammation by a subcutaneous injection of turpentine. Within the first 24 h, IL-1β deficient mice injected did not manifest an acute phase response, did not develop anorexia, had no circulation IL-6, and did not developed fever (178, 179), demonstrating the role of this particular cytokine. Moreover, these findings are consistent with those reported using anti-IL-1R type I antibodies in wild-type mice (178).

3.4.1 Inflammasome IL-1β-dependent and -independent maturation

As mention before, proIL-1β needs to be processed in order to be active. One of the enzymes in charge of this cleavage is caspase-1. Similar to proIL-1β, caspase-1 also requires activation via proteolytic processing. This step is mediated by multi-protein complexes, termed inflammasomes. They are organized in a very simple manner: a) a sensor molecule, which is particularly to each inflammasome and b) an adaptor protein, common to all the inflammasomes, that links the sensor molecule to pro-caspase-1 (180). To date, most inflammasomes described contain a nucleotide oligomerization domain (NOD)-like receptor (NLR) family as the sensor molecule (180). This includes the three best studied complexes: the NLR family pyrin domain-

containing 3 (NLRP3), the NLRP1, and the NLR family CARD domain-containing protein 4 (NLRC4) (181). Another well studied inflammasome, absent in melanoma 2 (AIM2), possesses a HIN (hematopoietic expression, interferon inducible nature, nuclear localization) domain (182). Several stimuli have been reported to trigger inflammasome formation and subsequent caspase-1 activation, ranging from particulate matter to toxins (180). A more detail explanation on the inflammasomes is described in the next sections.

On the other side, other mechanisms independently from the inflammasomes have been proposed for proIL-1β maturation. Proteolytic cleavage by extracellular enzymes might have an important role in regulating the activity of IL-1β. Indeed, during sterile inflammation, where the myeloid response into the affected tissues is dominated by neutrophils, the role of extracellular enzymes is more important than the one of the caspase-1 (158). Neutrophils contain a battery of proteases that release into the extracellular space such as elastase, cathepsin G, and protease 3, and they have been shown to cleave immature form of IL-1β, although less efficiently than caspase-1 (183). In addition, mast cell-derived protease and chymotrypsin-like serine protease chymase have been shown to convert proIL-1β into a biologically active form (184).

Another caspase-1-independent mechanism is carried out by caspase-8. Activation of TRIF signaling pathway by the TLR3 and TLR4 has been shown to drive efficient IL-1β processing even in caspase-1 knockout macrophages (185). Similar results were observed after other cellular stress stimuli such as inhibition of protein translation, chemotherapeutics or endoplasmic reticulum stress (186, 187).

3.4.1.1 NLRP3 inflammasome

The NLRP3 inflammasome, also known as cryopyrin, is the best characterized and has been linked with various human autoinflammatory and autoimmune diseases. Moreover, this complex is unique since its activation is regulated at both transcriptional and post-translational levels. Similar to IL-1β, a first signal induced by the TLR/NF-κB pathway upregulates the expression of NLRP3, the level of which is otherwise relatively low in numerous cell types (188). Signal 2 is transduced by PAMPs and damage-associated molecular patterns (DAMPs) to activate the functional NLRP3 inflammasome and to initiate assembly of the complex (180). Several molecules have been suggested to activate NLRP3 and to induce caspase-1 activation

and IL-1β maturation. These include urate crystals, ATP, bacterial pore-forming toxins, and particulate matter, including asbestos and silica (123).

Since the activators are quite different, it is possible that they are not directly recognized by the inflammasome. Instead, several mechanisms have been proposed for activation (189). A well-established mechanism of NLRP3 inflammasome activation is a decrease in the intracellular K^+ concentration. It was previously shown that the common NLRP3 activators ATP and nigericin cause a non-selective conductance of K^+ across the cell membrane and the alteration of intracellular ionic contents (190). Furthermore, a reduction in intracellular K^+ levels was found to be essential for other pore-forming toxins such as *S. aureus* and GBS hemolysins (191, 192). Moreover, this mechanism dependent on K^+ was also described for the other inflammasomes NLRP1, NLRC4, and AIM2 (189, 193, 194). Other mechanisms proposed are the lysosomal destabilization and rupture (195, 196), and the mitochondrial reactive oxygen species (ROS) generation (197).

3.4.1.2 NLRP1 inflammasome

The most know activator of the NLRP1 inflammasome, also known as NALP1, is the Anthrax lethal toxin (LeTx) an important virulence factor deployed by *Bacillus anthracis*, causative agent of anthrax disease. This toxin is composed of a protective antigen (PA) and a lethal factor (LF). While the PA forms a membrane pore through which LF is delivered, the LF is a zinc metalloprotease that cleaves and inactivates all mitogen-activated protein kinase kinases (198).

Another activator is the bacterial peptidoglycan muramyl dipeptide (MDP). Indeed, studies using a cell-free system with purified NLRP1 demonstrated that MDP was able to induce ATP binding and oligomerization of NLRP1 (199). Moreover, *Toxoplasma gondii* and host intracellular ATP depletion have also been shown to activate this inflammasome (198).

3.4.1.3 NLRC4 inflammasome

The NLRC4 inflammasome preciously known as IPAF was first discovered to be activated by flagellin delivery into the cytosol by *Legionella pneumophila* and *Salmonella Typhimurium*. However, studies using high doses of *S. Typhimurium* induced flagellin-

independent NLRC4 activation. This eventually led to the identification of the components of type III and IV secretion system (T3SS, T4SS) as additional activators of NLRC4 (200-202). NLRC4 thus recognizes T3SS/T4SS in two ways: indirectly by detecting flagellin, and directly by detecting some of the protein components of the secretion system (rod protein). Both flagellin and rod protein are unintentionally delivered to the mammalian cytosol by the bacterium through the secretion system. However, the direct binding of those PAMPs to NLRC4 was not demonstrated (203).

3.4.1.4 AIM2 inflammasome

The non-NLR AIM2 inflammasome can directly recognize and bind cytoplasmic double-stranded DNA (dsDNA) which may be encountered in the cytosol during pathogenic infection. This leads to the oligomerization of the inflammasome complex that recruits procaspase-1(204). A recent study demonstrated the importance of AIM2 in sensing both bacterial and viral pathogens and in triggering innate immunity using *Francisella tularensis* and *Listeria monocytogenes* (205).

4 Role of IL-1 in the infection caused by streptococci

The role of IL-1 in the infection caused by streptococci is somewhat described. Although the models of infection used are different and virulence between streptococci also differs, most of the studies observed a protective role of this cytokine during infection. This result is not surprising since IL-1 has a key role in the initiation of the cascade of inflammation and activation of the immune host response. However, a recent study with *S. pyogenes* showed the importance of having controlled levels of this cytokine since an excessive production is also detrimental for the host. **Table A** shows a summary of those studies, which are also explained in detail in the following section.

Table A. Role of IL-1 in the infection caused by streptococci

Pathogen	Route of infection	Method	Role	Reference
Streptococcus agalactiae (GBS)	Intraperitoneal	$ASC^{-/-}$; $NLRP3^{-/-}$; $Caspase\text{-}1^{-/-}$	Protective	(192)
	Intraperitoneal	$IL\text{-}1\beta^{-/-}$	Protective	(26)
	Intraperitoneal	$IL\text{-}1R^{-/-}$	Protective	(25)
Streptococcus pyogenes (GAS)	Intraveinal	Anakinra	Protective	(206)
	Subcutaneous	$IL\text{-}1R^{-/-}$; $Caspase\text{-}1^{-/-}$; Caspase-1 inhibitors	Protective	(27)
	Subcutaneous	$IFNAR^{-/-}$	Detrimental	(27)
Streptococcus pneumoniae	Intranasal	$IL\text{-}1R^{-/-}$	Protective	(23, 207)
	Intranasal	$IL\text{-}1\beta^{-/-}$; $IL\text{-}1\alpha^{-/-}$; $IL\text{-}1\alpha/\beta^{-/-}$	Protective	(24)

*Anakinra: IL-1R antagonist

4.1 *Streptococcus agalactiae*

Streptococcus agalactiae or Group B *Streptococcus* (GBS) is a frequent agent of life-threatening sepsis and meningitis in neonates and adults with predisposing conditions (26). The pathogenic potential of this bacterium dependent on the expression of a large variety of surface-exposed and secreted virulence factors (25). Innate immune recognition of GBS is accomplished

through selected TLRs, each of which activates an intracellular signaling pathway requiring MyD88. The crucial importance of TLR2 and MyD88-dependent pathways for anti-GBS defenses was shown by Biondo *et al.* (25). Additionally, Henneke *et al*. demonstrated that TLR2 and TLR6 are implicated in the activation by GBS "factors" (208) and that activation of NF-κB and p38 depends on MyD88 but not TLR2 and/or TLR4 (209).

Little is known about the role of the interleukin system in GBS disease. IL-1α was found to be markedly elevated in a rat model of i.p. GBS infection, and these levels correlated with those of other inflammatory mediators, such as TNF and IL-6, and with the bacterial burdens in infected organs (210). Moreover, IL-1α was not influenced by injection of anti-TNF monoclonal antibodies, suggesting that IL-1 cytokine production is not controlled by TNF.

Performing *in vitro* studies, Costa *et al.* (192) showed that murine bone-marrow derived dendritic cells (bmDCs) and macrophages (bmMs) responded to GBS by secreting IL-1β. Such production depended on MyD88, but not on TLR2. Moreover, DCs lacking NLPR3, ASC or caspase-1 were unable to release IL-1β, and mice lacking these mediators were considerably more susceptible to infection than wild-type mice suggesting, indirectly, a protective role of IL-1β in the GBS infection. Furthermore, studies with IL-1R-deficient mice infected i.p. with GBS showed that these mice displayed clinical signs of sepsis and neurological disease and died after challenge with a bacterial dose that did not cause illness or death in any of the wild-type animals (25). However, the ability of macrophages to kill GBS *in vitro* was not affected by a lack of IL-1R. Peritoneal macrophages showed a decreased production of the chemokines KC and MIP-1β in the absence of IL-1R, suggesting that IL-1 plays a role in attracting another immune cells (25). Additionally, another study demonstrated that IL-1β-deficient mice were markedly more sensitive to GBS infection, with most of them dying under challenge conditions that caused no deaths in wild-type control mice. Lethality was due to an inability to control local GBS and dissemination to target organs, such as the brain and kidneys. What is more, IL-1β-deficient mice had impaired production of the neutrophils chemokines CXCL1 and CXCL2 and neutrophil recruitment to the peritoneal cavity (26). Thus IL-1 could play a crucial role in host defenses against GBS by inducing high level production of chemokines, recruiting neutrophils to the infection site and controlling the dissemination of bacteria.

4.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a leading cause of bacterial infectious diseases, ranging from otitis media to the invasive infectious diseases pneumonia, sepsis, and meningitis (211). This pathogen produces a pore-forming toxin called pneumolysin (PLY) that is similar to SLY from *S. suis* (212). *In vitro* studies showed that PLY accesses the cytosolic compartment, causing cell death and the release of IL-1β. Shoma *et al.* (12) studied the involvement of PLY in the production of inflammatory cytokines. They showed that macrophages infected by *S. pneumoniae* produce IL-1α and IL-1β, but that production is impaired when using a PLY-negative mutant strain. Moreover, the infection with recombinant PLY (rPLY) not only produced a high level of IL-1 secretion in a TLR4 dependent manner but also activated caspase-1, needed for the cleavage of IL -1β. Fang *et al*. (10) showed that ASC inflammasomes, including AIM2 and NLRP3, are critical for caspase-1 activation and consequently IL-1β production. During *in vivo* experiments, they observed that ASC deficient mice were more susceptible than wild-type mice to *S. pneumoniae*, with impaired secretion of IL-1β into the bronchoalveolar lavage after intranasal infection, suggesting that ASC inflammasomes and IL-1β indirectly contribute to the protection of the host from infection with PLY-producing *S. pneumoniae*.

In addition, two studies using mice deficient in IL-1R showed the protective role of IL-1 in the infection by this pathogen. Using a murine model of pneumococcal nasopharyngeal colonization, Lemon *et al.* showed that mice deficient in the IL-1R had reduced numbers of neutrophils early after infection, fewer macrophages later in carriage, and prolonged bacterial colonization than wild-type counterpart (207). Likewise, Zwijnenburg *et al.* (23), using a model of bacterial meningitis, demonstrated that the lack of IL-1 impaired bacterial clearance and mortality was enhanced. Together these results suggest that IL-1 signaling is necessary to clear *S. pneumoniae* infection.

Moreover, Kafka *et al.* studied the role of IL-1 as well. To differentiate between the role of IL-1α and IL-1β they used IL-1α-, IL-1β-, and IL-1α/β-deficient mice that they inoculated intranasally with *S. pneumoniae*. IL-1β and IL-1α/β deficient mice displayed significantly lower survival rates and higher nasopharyngeal and lungs bacteria. Interestingly, treatment with recombinant IL-1β following infection improved survival. Moreover, local infiltrates of

neutrophils and relatively high preserved organ architecture were observed in the lungs of IL-1α deficient mice, while IL-1β and IL-1α/β deficient mice showed diffuse pneumonia and tissue damage. These results indicate a differential role of interleukin-1α from -1β and suggest that IL-1β has a major role in resistance to primary pneumococcal infection (24).

4.3 *Streptococcus pyogenes*

Streptococcus pyogenes or Group A *Streptococcus* (GAS) is a leading human pathogen responsible for a broad spectrum of clinical manifestations, including infections of the skin and upper respiratory tract, bacteremia, and occasionally sepsis and septic shock (213). Several studies showed the importance of DCs and macrophages in controlling the infection and in the development of protective immune response against GAS (214-216). Several studies with this pathogen showed that activation of signaling pathways such as p38, MAPK, and NF-κB, and cytokine production including IL-1β, IL-12, IL-6, and TNF by these cells following infection with this pathogen were dependent on MyD88. Particularly, production of IL-1β was showed to be dependent on caspase-1, NLRP3, and ASC. However, even though these results suggest that the signaling is through TLRs, TLR1, TLR2, TLR4, TLR9 were not involved in such production. These data further strengthen the notion that GAS is sensed by a yet unidentified receptor upstream of MyD88 (217-219).

During invasive GAS disease, blockade of IL-1β signaling *in vivo* by Anakinra, a nonglycosylated recombinant human IL-1β receptor antagonist, resulted in accelerated mortality demonstrating a protective role of this cytokine in this model of infection (206).

A recent study demonstrated the relationship between IL-1β and IFN-I, and the importance of the balance of both cytokines in the outcome of the infection by GAS. Previous *in vitro* studies showed that IFN-β expression is induced by bmMs and bmDCs infected with *S. pyogenes* (220, 221) and that IFN-I inhibits IL-1β production and inflammasome activation (222). Castiglia and collaborators worked with IFNAR-1-deficient mice and observed that lethality of these mice was caused by systemically exacerbated levels of IL-1β. High levels of the cytokine and histopathological changes were found in the site of the infection (subcutaneous injection), liver and spleen. Moreover, when the mice were treated with a caspase inhibitor or an IL-1R antagonist (Anakinra) they showed a full protection or an improved resistance to *S.*

pyogenes infection respectively. In addition, IL-1R- and caspase-1-deficient mice were more susceptible to the same model of infection, proving that IL-1R signaling is indispensable for bacterial clearance and survival. Thus, these results showed that IL-1β signaling must operate within a narrow range, because both excessive and defective IL-1β responses lead to lethal disease and that IFN-I signaling helps maintain the level of IL-1β activity within the desired range (27).

4.4 What do we know about *S. suis* and IL-1?

Little is known about the production of IL-1 in infections by *S. suis*. Most of the studies measure it as part of a group and do not evaluate the implication of this main pro-inflammatory cytokine alone. Thus, its production, regulation, and role following *S. suis* infection remain unknown.

Studies with human monocytic THP-1 cells infected by *S. suis* serotype 2 showed the release of IL-1β among other cytokines, such as TNF, IL-6, IL-8, and MCP-1 (148). Stimulation of these cells with high virulence heat-killed *S. suis* produced low levels of IL-1β, with production gradually decreasing proportionally to bacterial concentrations (148). Moreover, after stimulation with non-encapsulated mutant release was significantly higher, suggesting a role of cell wall components (130). Indeed, purified components of the cell wall showed to be partially responsible for this increase. On the contrary stimulation with purified CPS failed to induce significant levels of IL-1β.

A study using whole-blood culture system carried out by Segura *et al.*, showed the expression and secretion of IL-1β and other cytokines from porcine blood cells stimulated by live and heat-killed *S. suis* high virulence strain (146). Both heat-killed and live bacteria stimulate the expression of mRNA, whereas only live bacteria induced the secretion of high levels of the cytokine. When comparing the IL-1β production between the wild-type strain and the non-encapsulated mutant, no significant differences in the ability to induce the expression of IL-1β mRNA and the protein were found. Furthermore, a non-hemolytic mutant induced similar mRNA and protein levels of the pro-inflammatory cytokine as the parental strain, while purified hemolysin induced relatively high mRNA levels of IL-1β. However, purified hemolysin stimulated very low protein (100 pg/mL) levels of this cytokine.

Lecours *et al*. evaluated the ability of *S. suis* to interact with bone marrow-derived swine dendritic cells (pBMDCs). After stimulation with high virulence *S. suis* strain, DCs released several cytokines including IL-1β. Controversially, the non-encapsulated mutant did not induce higher levels (72). Additionally, a study with dendritic cells derived from monocytes purified from peripheral blood stimulated by the *S. suis* serotype 2 strain, triggered a strong release of several pro-inflammatory cytokines, including IL-1β, IL-8, TNF, IFN-γ, and IL-12 (223). Moreover, interaction of *S. suis* with primary porcine alveolar macrophages was studied by de Greeff *et al.* They showed that IL-1β, MIP-2α, and TNF genes among others, were expressed after 60 min of interaction between porcine macrophages and a non-encapsulated mutant of *S. suis*, whereas the parental strain induced lower levels of expression. RT-qPCR was used to confirm the microarray results (224).

Murine bmDCs have been shown to be a valid and interesting model to study the host immune response during *S. suis* infection (73). Lecours *et al.* (73, 105) showed the secretion of IL-1β among other cytokines like IL-6, TNF, IL-12p70, and IL-23, after infection for 16 h with different strains of *S. suis* serotype 2. Moreover, production of IL-1β was dramatically impaired in MyD88-deficient DCs for all strains tested and significantly reduced in TLR2 deficient bmDCs (105).

In vivo studies with a systemic model of *S. suis* infection showed relatively low levels of IL-1β in the plasma of A/J mice, and even lower levels in that of C57BL/6 mice. Since IL-1β is a potent cytokine, the low levels of this cytokines could be sufficient to exert biological effects as explained by the authors (149).

Regarding meningitis caused by *S. suis*, several studies have shown the ability of the bacteria to induce the release of pro-inflammatory cytokines and chemokines by human and porcine BMEC, murine microglia and astrocytes (94, 106, 107), but the specific bacterial components responsible for exaggerated inflammatory reactions are not known. Studies in microglia by Dominguez-Punaro *et al.* showed low levels of IL-1β after the stimulation with a high virulence strain of *S. suis* and even lower levels for the non-encapsulated and suilysin negative mutant strains (108). *In vivo* studies showed that *S. suis* infection induces production of inflammatory mediators in a time-dependent manner (106). Also, in immunohistochemistry studies, IL-1β was detected in the choroid plexus and cortex as early as 24 h p.i. and gradually

increased by day 5 p.i. in mice that exhibited clinical signs. At this time point, the IL-1β signal was clear in the choroid plexus, cortex, corpus callosum, and meninges (106).

III- Materials, Methods, and Results

Interleukin-1 signaling induced by *Streptococcus suis* serotype 2 is strain-dependent and contributes to bacterial clearance and inflammation during systemic infection

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Abstract

Streptococcus suis serotype 2 is an important porcine pathogen causing sudden death, septic shock, and meningitis, with exacerbated inflammation being a hallmark of the infection. In addition, it is also an emerging zoonotic agent. A rapid, effective, and balanced innate immune response against *S. suis* is critical to control bacterial growth and limit the spread of the pathogen without causing excessive inflammation. Even though interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced, its role in the *S. suis* pathogenesis has not been studied. We demonstrated that a classical virulent European sequence type (ST) 1 strain and the highly virulent ST7 strain induce important levels of IL-1 in systemic organs. Moreover, dendritic cells and macrophages, which are involved in the *S. suis* pathogenesis, are important sources of this cytokine, with the ST7 strain inducing higher levels. To better understand the underlying mechanisms involved in this production, different cellular pathways were studied. Independently of the strain, IL-1β production required MyD88 and involved recognition via TLR2 and possibly TLR7 and TLR9. This suggests that the bacterial components recognized are similar and conserved between *S. suis* strains. However, very high levels of the pore-forming toxin suilysin, only produced by the ST7 strain, are required for efficient maturation of pro-IL-1β via activation of the NLRP3, NLRP1, AIM2, and NLRC4 inflammasomes resulting from pore formation and ion efflux. Using IL-1R$^{-/-}$ mice, we demonstrated that IL-1 signaling may play a beneficial role during *S. suis* systemic infection by modulating the inflammation required to control and clear bacterial burden, thus promoting host survival. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role. A better understanding of the underlying mechanisms involved in the control of inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

Keywords: *Streptococcus suis* serotype 2; interleukin-1; dendritic cells; inflammation; systemic infection; suilysin

1. Introduction

Streptococcus suis causes sudden death and meningitis in pigs and is responsible for important economic losses to the swine industry. Additionally, *S. suis* is also a zoonotic agent causing meningitis and septic shock in humans and has become a public health concern, particularly in South-East Asia (Gottschalk, Xu *et al*. 2010, Gottschalk M 2010). Of the thirty-five described serotypes, serotype 2 is considered the most virulent and the most frequently isolated from both pigs and humans worldwide (Goyette-Desjardins, Auger *et al*. 2014). Using multilocus sequence typing, at least four predominant sequence types (STs) have been identified within serotype 2 strains: the virulent ST1 in Europe and Asia, the highly virulent ST7, responsible for two human outbreaks in China, and the intermediate and low virulent ST25 and ST28, respectively, in North America (Fittipaldi, Xu *et al*. 2011). Isolates with variable virulence belonging to the latter two STs have also been reported in Asia (Goyette-Desjardins, Auger *et al*. 2014, Kerdsin, Akeda *et al*. 2018).

Mechanisms used by *S. suis* to infect the host and induce disease remain poorly known. A variety of virulence factors have been proposed, including the capsular polysaccharide, which confers anti-phagocytic properties helping *S. suis* to persist in blood, multiply, and disseminate throughout the host, leading to sepsis and/or meningitis (Segura, Fittipaldi *et al*. 2017). Furthermore, many virulent strains of *S. suis*, including ST1 and ST7 strains, also produce an hemolysin similar to the pneumolysin of *Streptococcus pneumoniae*, called suilysin (SLY). This toxin may play an important role in bacterial dissemination and host inflammation as it is responsible for cell toxicity and inducing pro-inflammatory cytokines in many cell types (Lecours, Gottschalk *et al*. 2011, Tenenbaum, Seitz *et al*. 2016). Moreover, bacterial components such as lipoproteins (LPs) and lipoteichoic acid (LTA) modifications have also been suggested to be involved in the *S. suis* pathogenesis (Segura, Fittipaldi *et al*. 2017).

A rapid and effective innate immune response against *S. suis* is critical to control bacterial growth and limit the spread of the pathogen (Lachance, Gottschalk *et al*. 2013). Initial recognition by specialized membrane-associated or cytoplasmic receptors (pattern recognition receptors [PRRs]) mediates host immune responses by inducing the synthesis of diverse cytokines and chemokines through activation of nuclear factor-kappa B (NF-κB) and mitogen-

activated protein kinases (MAPKs) (Kawai and Akira 2010). Previous studies have shown that *S. suis* activates dendritic cells and macrophages through the Toll-like receptor (TLR) pathway (Graveline, Segura *et al*. 2007, Lecours, Segura *et al*. 2012). In fact, absence of the adaptor protein myeloid differentiation primary response 88 (MyD88), central to this pathway, results in abrogation of pro-inflammatory mediator production *in vitro* (Lecours, Segura *et al*. 2012). Moreover, being mostly an extracellular bacterium, recognition of *S. suis* occurs via surface-associated TLR2 and, possibly, TLR4 (Bi, Pian *et al*. 2015), although the latter remains to be confirmed. In case of internalization, however, *S. suis* may also activate the endosomal TLR7 and TLR9 (Auger, Santinon *et al*. 2017).

Amongst the mediators induced during inflammation, interleukin (IL)-1 is regarded as one of the most potent and earliest pro-inflammatory mediators produced (Giuliani, Sarti *et al*. 2017). It is involved in the recruitment of inflammatory cells and their activation, induction of other inflammatory factors such as lipid mediators and other cytokines, and participates in adaptive immunity and metabolism (Dinarello 1998, Gabay, Lamacchia *et al*. 2010, Biondo, Mancuso *et al*. 2014). In fact, there are two forms of IL-1, IL-1α and IL-1β, which are encoded by separate genes and synthesized as precursor peptides (pro-IL-1α and pro-IL-1β). While pro-IL-1α is biologically active and can exert intracellular or extracellular functions, a two steps mechanism is required for the complete maturation of IL-1β (Gabay, Lamacchia *et al*. 2010, Garlanda, Dinarello *et al*. 2013). Firstly, activation of PRRs leads to transcription and translation of pro-IL-1β. Next, the precursor is cleaved and activated mainly by caspase-1-dependent mechanisms (Afonina, Muller *et al*. 2015). Similar to proIL-1β, caspase-1 requires proteolytic processing, which is mediated by inflammasomes in response to a wide variety of stimuli. Though several inflammasomes have been described, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3), the NLRP1, the NLR family CARD domain-containing protein 4 (NLRC4), and the absent in melanoma 2 (AIM2) are the best characterized (Schroder, Muruve *et al*. 2009, Latz, Xiao *et al*. 2013). Once secreted, IL-1α and IL-1β bind to their shared receptor, IL-1 receptor (IL-1R), which is ubiquitously expressed, resulting in the synthesis of cytokines, chemokines, adhesion molecules, and acute phase proteins.

Although IL-1 signaling plays an essential role in immunity by participating in inflammatory response initiation, an uncontrolled production of IL-1 can lead to tissue damage and disease. In fact, IL-1 plays a protective role during both pneumococcal and Group B *Streptococcus* (GBS) infections, wherein a lack of IL-1 signaling contributes to a weak inflammatory response and higher bacterial burden (Zwijnenburg, van der Poll *et al*. 2003, Kafka, Ling *et al*. 2008, Biondo, Mancuso *et al*. 2014, Biondo, Mancuso *et al*. 2014). However, a recent study showed that lack of control of IL-1β production results in a lethal outcome in a mouse model of Group A *Streptococcus* (GAS) infection (Castiglia, Piersigilli *et al*. 2016, Valderrama and Nizet 2018)

During *S. suis* infection, the host response depends on the ability of innate immune mechanisms to control initial bacterial growth and limit the spread of the pathogen without causing excessive inflammation, a hallmark of the disease. However, regardless of the numerous studies on the *S. suis* pathogenesis, none have focused on the production and role of IL-1. Consequently, we assessed its implication during *S. suis* serotype 2 pathogenesis. Herein, we demonstrated that a classical virulent European ST1 strain and the highly virulent ST7 strain induce important levels of IL-1 in systemic organs. In fact, dendritic cells and macrophages, which are centrally involved in the *S. suis* pathogenesis, are important sources of this cytokine. Production of IL-1 by both strains involved recognition via TLR2 and, possibly, TLR7 and TLR9, suggesting that cell activation is not influenced by the virulence level of the strain. By contrast, pro-IL-1β maturation mechanisms were strain-dependent, with the elevated levels of the pore-forming toxin SLY, only produced by the ST7 strain, participating in this maturation via activation of the NLRP3, NLRP1, AIM2, and NLRC4 inflammasomes. Globally, we demonstrated that IL-1 plays a beneficial role during *S. suis* systemic infection caused by classical strains (ST1) by modulating the inflammation required to control and clear bacterial burden. Beyond a certain threshold, however, such as in the case of the highly virulent ST7, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role.

2. Materials and methods

2.1. Ethics statement

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

2.2. Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in **Table 1**. The classical virulent European reference ST1 P1/7 strain and the highly virulent Chinese ST7 SC84 strain were used throughout this study, including for construction of isogenic *sly*-deficient mutants. All *S. suis* strains were grown in Todd Hewitt broth (THB; Becton Dickinson, Mississauga, ON, Canada) as previously described (Segura, Gottschalk *et al*. 2004), diluted in culture medium before experiments with cells, and the final concentration (colony-forming units [CFU]/mL) determined by plating on THB agar (THA). For experimental mouse infections, bacteria were resuspended in THB. *Escherichia coli* was grown in Luria-Bertani (LB) broth or agar (Becton Dickinson). When needed, antibiotics (Sigma-Aldrich, Oakville, ON, Canada) were added to culture media at the following concentrations: for *E. coli*, ampicillin at 100 mg/mL, kanamycin and spectinomycin at 50 μg/mL; for *S. suis*, spectinomycin at 100 μg/mL.

2.3. Construction of the isogenic *sly*-deficient mutants

Precise in-frame deletion of the *sly* gene from *S. suis* strains P1/7 and SC84 was achieved using splicing-by-overlap-extension polymerase chain reaction (PCR) (Warrens, Jones *et al*. 1997). Oligonucleotide primers (**Table S1 in Supplementary Material**) were obtained from Integrated DNA Technologies (Coralville, IA, USA) and PCRs were carried out with the iProof proofreading DNA polymerase (BioRad Laboratories, Mississauga, ON, Canada) or with the Taq DNA polymerase (Qiagen, Valencia, CA, USA). Overlapping PCR-products were cloned into the plasmid pCR2.1 (Invitrogen, Burlington, ON, Canada), extracted using EcoRI, and cloned into the thermosensitive *E. coli*-*S. suis* shuttle vector pSET4s (Takamatsu, Osaki *et al*. 2001). Final constructions of pSET4s vectors were electroporated into competent *S. suis* cells

as previously described (Takamatsu, Osaki *et al*. 2001). Deletion of the *sly* gene was confirmed by PCR and sequencing.

2.4. Cloning, expression, and purification of recombinant suilysin (rSLY)

The region corresponding to the *sly* gene, excluding the signal peptide, was amplified by PCR. PCR amplicons were digested with NdeI and BamHI and cloned into the pIVEX2.4d vector (Roche, Mississauga, ON, Canada), which possesses a N-terminal His-tag, previously digested with the same enzyme. Primers used are listed in **Table S1 in Supplementary Material**. Protein synthesis was induced in the *E. coli* BL21 (DE3) strain using 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h, after which cells were lysed by sonication. The resulting recombinant His-tag suilysin, henceforth called rSLY, was purified by affinity chromatography using the HisPur Ni-NTA Spin Column Kit (Thermo Scientific, Rochelle, IL, USA) according to manufacturer's instructions. rSLY kept its hemolytic activity as evaluated using red blood cells (see below). Protein quantification was measured using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific).

2.5. Titration of hemolytic activity

Hemolytic titration was performed by preparing two-fold serial dilutions of bacterial culture supernatant in a solution of 0.145 M NaCl and 7 mM Na_2HPO, pH 7.2, using horse red blood cells as previously described (Gottschalk, Lacouture *et al*. 1995). The titer was defined as the reciprocal of the highest dilution with observed hemolysis. Results were expressed as the mean of at least three independent experiments.

2.6. Lipoteichoic acid preparation

Extraction and purification of LTA was performed as recently described (Hess, Waldow *et al*. 2017, Gisch, Auger *et al*. 2018). Yields of LTA preparations from 6 L of bacterial culture were 21.2 mg for strain P1/7, 20.9 mg for strain P1/7Δ*lgt*, 20.2 mg for strain SC84, and 30.9 mg for strain SC84Δ*lgt*.

2.7. Mice

MyD88$^{-/-}$ [B6.129P2(SJL)-MyD88$^{tm1.Defr}$/J], TRIF$^{-/-}$ [C57BL/6J-Ticam1^{Lps2}/J], TLR2$^{-/-}$ [B6.129-Tlr2^{tmKir}/J], TLR4$^{-/-}$ [B6.B10ScN-Tlr4$^{lps-del}$/JthJ], caspase-1$^{-/-}$ [B6N.129S2-*Casp1*^{tm1Flv}/J], NLRP3$^{-/-}$ [B6.129S6-*Nlrp3*^{tm1Bhk}/J], NLRP1$^{-/-}$ [B6.129S6-*Nlrp1b*^{tm1Bhk}/J], AIM2$^{-/-}$ [B6.129P2-*Aim2*$^{Gt(CSG445)Byg}$/J], NLRC4$^{-/-}$ (Franchi, Amer *et al*. 2006), and IL-1R$^{-/-}$ [B6.129S7-*Il1r1*^{tm1Imx}/J] mice on C57BL/6 background were housed under specific pathogen-free conditions alongside their wild-type counterparts. Mice were purchased from Jackson Research Laboratories (Bar Harbor, ME, USA), with the exception of NLRC4$^{-/-}$ mice, which were originally produced by Dr. G. Núñez (University of Michigan, USA) (Franchi, Kamada *et al*. 2012).

2.8. Generation of bone marrow-derived dendritic cells and macrophages

Hematopoietic stem cells from femurs and tibias of wild-type and knockout mice were used to generate bone marrow-derived dendritic cells (DCs) as previously described (Segura, Su *et al*. 2007, Lecours, Segura *et al*. 2012, Auger, Santinon *et al*. 2017) in complete culture medium, which was composed of RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (all from Gibco, Burlington, ON, Canada) and complemented with 10% granulocyte-macrophages colony-stimulating factor. For macrophages (MΦ), cells (5 x 10^5 cells/mL) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and complemented with 30% L929 cell-derived macrophage colony-stimulating factor supernatant (Weischenfeldt and Porse 2008). Cells were cultured for 8 days at 37 °C with 5% CO_2 and trypsinized using 0.05% trypsin-0.03% EDTA (Gibco) prior to infection. Cell purity, evaluated as previously described (Segura, Su *et al*. 2007, Lecours, Segura *et al*. 2012), was at least 85% CD11c$^+$ and F4/80$^+$ for DCs and MΦ, respectively.

2.9. *Streptococcus suis* infection of dendritic cells and macrophages

All activation studies were done in the absence of endotoxin contamination and under non-toxic conditions, the latter being evaluated by the lactate dehydrogenase (LDH) release with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Cells were resuspended at 1 x 10^6 cells/mL in complete medium and stimulated with the different *S. suis*

serotype 2 strains listed in **Table 1** (1 x 10^6 CFU/mL; initial multiplicity of infection=1). Conditions used were based on those previously published (Lecours, Gottschalk *et al*. 2011, Lecours, Segura *et al*. 2012). At indicated times intervals, supernatants were collected for cytokine measurements. For mRNA expression, cells were harvested in TRIzol (Invitrogen) 6 h following infection. Mock-infected cells served as negative controls. Activation of cells with LTA was performed using 30 µg/mL and supernatants collected 24 h later for IL-1β quantification. For signaling pathway studies, cells were pretreated for 30 min with 10 µM NF-κB inhibitor JSH-23, 10 µM p38 inhibitor SB0203580, 25 µM MEK1/2 inhibitor U0126 or 10 µM JNK inhibitor SP600125 (all from Calbiochem/EMD Millipore, San Diego, CA, USA). For assays with rSLY, a non-toxic concentration of 5 µg/mL was used. For cholesterol inhibition assays, 40 µg/mL of cholesterol (Sigma-Aldrich) was added to wells. When needed 2.5% of 10 mg/mL alhydrogel (Brenntag, Mülheim, Germany) was added as a NLRP3 activator. Finally, for experiments involving extracellular K^+, a stock solution of KCl (Laboratoire Mat. Inc., Quebec City, QC, Canada) was prepared and appropriately diluted.

2.10. *Streptococcus suis* DNA and RNA preparation and transfection of cells

For bacterial DNA and RNA isolation, bacteria were grown to mid-log phase. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions, including treatment with DNase I. For DNA preparation, bacteria were harvested in 10 mM Tris, 1 mM EDTA, pH 8.0, and treated with 10% SDS and 20 mg/mL proteinase K (Sigma-Aldrich) for 1 h at 37 °C. DNA was isolated using phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) (Gratz, Hartweger *et al*. 2011). After isolation, bacterial DNA was treated with 10 mg/mL RNase A (Roche) for 30 min at 37 °C. Cells were transfected with 1 µg of RNA or DNA complexed with DOTAP liposomal transfection agent (Sigma-Aldrich) as previously described (Mancuso, Gambuzza *et al*. 2009, Gratz, Hartweger *et al*. 2011, Auger, Santinon *et al*. 2017).

2.11. Cytokine and chemokine quantification in cell culture supernatants

Levels of IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF) in cell culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using pair-matched

antibodies from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's recommendations.

2.12. Determination of cell mRNA expression by RT-qPCR

Cell mRNA was extracted according to the manufacturer's instructions (TRIzol) and cDNA generated using the Quantitect cDNA Synthesis Kit (Qiagen, Mississauga, ON, Canada). Real-time qPCR was performed on the CFX-96 Touch Rapid Thermal Cycler System (Bio-Rad) using 250 nM of primers (Integrated DNA technologies) and the SsoFast Evagreen Supermix Kit (Bio-Rad). The cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 s and 57°C for 5 s. Melting curves were generated after each run to confirm the presence of a single PCR product. The sequences of primers used in this study are shown in **Table S1 in Supplementary Material** and were verified to have reaction efficiencies between 90% and 110%. The reference genes *Atp5b* and *Gapdh*, determined to be the most stably expressed using the algorithm geNorm, were used to normalize data. Fold changes in gene expression were calculated using the quantification cycle threshold (Cq) method using the CFX software man-ager v.3.0 (Bio-Rad). Samples from mock-infected cells served as calibrators.

2.13. *S. suis* serotype 2 mouse model of infection

Six-week-old male and female wild-type C57BL/6 and IL-1R$^{-/-}$ mice were used. Animals were acclimatized to standard laboratory conditions with unlimited access to water and rodent chow (Dominguez-Punaro Mde, Segura *et al*. 2008). These studies were carried out in strict accordance with the recommendations of and approved by the University of Montreal Animal Welfare Committee guidelines and policies, including euthanasia to minimize animal suffering, applied throughout this study when animals were seriously affected since mortality was not an end point measurement. The different *S. suis* serotype 2 strains, or the vehicle solution (sterile THB), were administered at a dose of 1 x 10^7 CFU by intraperitoneal inoculation. Survival was evaluated and mice were monitored at least three times daily until 7 days post-infection (p.i).

2.14. Measurement of plasma, liver, and spleen pro-inflammatory mediators

For kinetics of IL-1α and IL-1β production, wild-type mice were infected with each strain as described above. At various times p.i., blood was collected by intracardiac puncture following

euthanasia and anti-coagulated with EDTA (Sigma-Aldrich) as previously described (Claude Lachance 2013, Auger, Fittipaldi *et al*. 2016). Plasma supernatants were collected following centrifugation at 10 000 x *g* for 10 min, 4 °C. For liver and spleen, extraction buffer was prepared using complete Mini, EDTA-free, protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and organs homogenized using a POLYTRON PT 1200E system bundle (Kinematica, Lucerne, Switzerland). Homogenate supernatants were collected following centrifugation at 10 000 x *g* for 10 min, 4 °C, and stored at −80 °C. Levels of IL-1α and IL-1β were determined by ELISA as described, while IL-6, IL-12p70, interferon (IFN)-γ, C-C motif chemokine ligand (CCL) 2, CCL3, and C-X-C motif chemokine ligand (CXCL) 9 were measured using a custom-made cytokine Bio-Plex Pro™ assay (Bio-Rad) according to the manufacturer's instructions. Acquisition was performed on the MAGPIX platform (Luminex®) and data analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

2.15. Measurement of blood, spleen, and liver bacterial burden

Wild-type and IL-1R$^{-/-}$ were inoculated with *S. suis* as described above and blood bacterial burden was assessed 12 h and 48 h p.i. by collecting 5 µL of blood from the caudal vein. For liver and spleen, organs were collected and homogenized as described above. Bacterial burden was determined by plating appropriate dilutions on THA.

2.16. Statistical analyses

Normality of data was verified using the Shapiro–Wilk test. Accordingly, parametric (unpaired t-test) or non-parametric tests (Mann–Whitney rank sum test), where appropriate, were performed to evaluate statistical differences between groups. Log-rank (Mantel–Cox) tests were used to compare survival between wild-type and IL-1R$^{-/-}$ mice. Each test was repeated in at least three independent experiments. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *Streptococcus suis* serotype 2 induces elevated levels of IL-1 in systemic organs but not in plasma

Since IL-1 plays a key role in initiating the inflammatory cascade, systemic levels of both IL-1α and IL-1β were measured in plasma, liver, and spleen following infection with the classical European ST1 strain P1/7 and the highly virulent ST7 strain SC84. Levels of IL-1α and IL-1β were barely detectable in mock-infected animals and did not change between 6 h to 48 h. Similarly, IL-1β levels were scarcely detectable in plasma throughout the course of infection, including upon presentation of severe clinical signs of systemic disease, with no significant differences between strains (**Fig. 1A-B**). By contrast, levels of IL-1β in liver and spleen were high and reached maximum values during the first 12 h p.i., rapidly decreasing thereafter, with similar production patterns in both organs and between strains (**Fig. 1C-F**). IL-1α production patterns in plasma, liver, and spleen were similar to those of IL-1β (**Fig. S1 in Supplementary Material**). Collectively, these data demonstrate that strains P1/7 and SC84 induce elevated levels of IL-1 in organs and that this production remains locally.

3.2. *Streptococcus suis* induces IL-1 release from dendritic cells and macrophages in a strain-dependent manner

Given the elevated levels of IL-1 produced in liver and spleen and the fact that numerous cell types can produce IL-1, of which DCs and MΦ are highly important during *S. suis* infection (Lecours, Segura *et al*. 2012, Auger, Santinon *et al*. 2017), the capacity of these cells to produce IL-1 following *S. suis* infection was evaluated. Strain P1/7 induced modest levels of IL-1β in a time-dependent manner, with production by DCs being significantly greater than that by MΦ at 12 and 16 h ($p < 0.001$) (**Fig. 2A-B**). On the other hand, the highly virulent ST7 strain SC84 induced significantly higher levels of IL-1β compared to strain P1/7 (**Fig. 2C-D**) at 8 h, 12 h, and 16 h p.i. ($p < 0.05$) in both cell types. In fact, levels induced by strain SC84 16 h p.i. were approximately 40 times greater than those induced by strain P1/7. Interestingly, a slight delay in IL-1β production was observed by SC84-stimulated MΦ in comparison to DCs. Moreover, IL-1α kinetics by DCs and MΦ were similar to those obtained for IL-1β (**Fig. S2 in Supplementary Material**), suggesting that *S. suis* induces comparable production of these two cytokines from DCs and MΦ. Importantly, production was not the result of cell death, since

toxicity levels remained low (data not shown). Given that IL-1α and IL-1β production kinetics were similar and that both cell types responded similarly, subsequent experiments were only performed for IL-1β using DCs following 16 h of infection.

3.3. Role of Toll-like receptors and associated signaling pathways in *Streptococcus suis*-induced IL-1β production

To better comprehend the differential IL-1β production induced by strains P1/7 and SC84 from DCs, the role of different receptors and signaling pathways involved in this production was evaluated. The TLR pathway has been previously described to be activated by *S. suis* and involved in pro-inflammatory mediator production (Lecours, Segura *et al*. 2012, Auger, Santinon *et al*. 2017). As shown in **Fig. 3A**, production of IL-1β was almost completely abrogated in the absence of MyD88 following infection with both *S. suis* strains ($p < 0.01$). By contrast, production of IL-1β by both strains was independent of the TIR-domain-containing adapter-inducing IFN-β (TRIF) (**Fig. 3A**). Since *S. suis* is mostly considered an extracellular pathogen, its recognition by surface-associated receptors is crucial. While IL-1β production was significantly (but not totally) reduced in TLR2$^{-/-}$ DCs stimulated with P1/7 or SC84 ($p < 0.01$), no difference was observed with TLR4$^{-/-}$ DCs (**Fig. 3A**).

Given the implication of TLR2 in *S. suis*-induced IL-1β production, potential activators were investigated. Though somewhat controversial, LTA and LPs have been suggested to be activators of TLR2 in Gram-positive bacteria (Hashimoto, Tawaratsumida *et al*. 2006, Hashimoto, Tawaratsumida *et al*. 2006, Wichgers Schreur, Rebel *et al*. 2011). Consequently, LTA was extracted from both strains and used to activate DCs. As shown in **Fig. 3B**, LTA from strains P1/7 and SC84 induced high levels of IL-1β, with no differences between strains.

As previously described, however, current methods are unable to completely eliminate co-purified LPs from LTA preparations (Hashimoto, Tawaratsumida *et al*. 2006). As such, LTA was also extracted from *lgt*-deficient mutants (Δ*lgt*), in which absence of the lipoprotein diacylglyceryl transferase, a key enzyme in LP synthesis, renders LPs biological inactive and unrecognizable by TLR2 (Stoll, Dengjel *et al*. 2005, Gisch, Kohler *et al*. 2013). In accordance, not only LTA preparations from *lgt*-deficient mutants induce significantly less IL-1β than those

from wild-type strains ($p < 0.01$), but levels were in fact undetectable (**Fig. 3B**). In addition, IL-1β production was completely abolished in TLR2$^{-/-}$ DCs following activation with LTA preparations from wild-type strains ($p < 0.01$) (**Fig. 3B**). Taken together, these results indicate that co-purified LPs, but not *S. suis* LTA, are important inducers of IL-1β by DCs via recognition by TLR2.

Though considered mainly an extracellular bacterium, dependence of *S. suis*-induced IL-1β production on MyD88, but only partially on TLR2 (and not at all on TLR4), suggested a potential participation of endosomal TLRs. In fact, it was previously demonstrated that in the case of internalization, *S. suis* nucleic acids can induce DC activation (Auger, Santinon *et al*. 2017). As such, DNA and RNA were extracted from strains P1/7 and SC84 and complexed or not with DOTAP liposomal transfection reagent, which allows phagosomal delivery. *S. suis* DNA and RNA from both strains induced significant and similar IL-1β production from DCs only when complexed with DOTAP ($p < 0.05$) (**Fig. 3C**). At the same concentration, *S. suis* DNA was significantly more stimulating with regards to IL-1β production than RNA ($p < 0.01$). When Alum was added, a known activator of the NLRP3 inflammasome, a significantly higher production was observed for both genetic materials (**Fig. 3C**) ($p < 0.05$), probably due to additional processing of proIL-1β into its mature form (Li, Nookala *et al*. 2007). This recognition of RNA and DNA may suggest a certain involvement of TLR7 and TLR9, respectively, in *S. suis*-induced IL-1β production.

TLR activation triggers a variety of intracellular signalling pathways of which the NF-kB pathway and MAPK p38, Jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK) are amongst the most important. These pathways are implicated in transcriptional control of many pro-inflammatory genes including cytokines and chemokines (Johnson and Lapadat 2002, Lawrence 2009). To determine which of these pathways were involved in *S. suis*-induced IL-1β production, DCs were pre-treated with different inhibitors (NF-κB inhibitor [i] JSH-23, p38i SB203580, MEK1/2i U0126 or JNKi SP600125) or their vehicle as a negative control. As shown in **Fig. 3D**, treatment with NF-κBi significantly reduced secretion of IL-1β induced by both strains by approximately 50% ($p < 0.01$). Interestingly, differential inhibition by strains P1/7 and SC84 was observed for p38: while p38i had no effect on P1/7-induced IL-1β

production, it significantly reduced SC84-induced IL-1β ($p < 0.01$) (**Fig. 3D**). Finally, while treatment with MEK1/2i reduced IL-1β production by 90% for both strains ($p < 0.01$), JNKi had no effect on *S. suis*-induced IL-1β production from DCs (**Fig. 3D**). Consequently, these results indicate that, with the exception of p38 activation, *S. suis* strains P1/7 and SC84 use similar receptors and signaling pathways in the induction of IL-1β.

3.4. Inflammasome activation required for IL-1β maturation induced by *Streptococcus suis* is strain-dependent

Processing of proIL-1β into its mature form requires cleavage by proteolytic enzymes, the most important of which is caspase-1 (Garlanda, Dinarello *et al*. 2013, Dinarello 2018). To investigate whether maturation of *S. suis*-induced IL-1β requires this enzyme, caspase-1-deficient DCs were used. As shown in **Fig. 4A**, IL-1β production was reduced by more than 75% in caspase-$1^{-/-}$ DCs ($p < 0.01$), indicating that it is required for maturation of IL-1β following infection by both strains P1/7 and SC84. To determine the mechanisms by which *S. suis* might activate caspase-1, the role of different inflammasomes in IL-1β production was investigated. Presently, several inflammasomes have been described, with an even greater range of identified molecules that can trigger their activation (Latz, Xiao *et al*. 2013). Of these, NLRP1, NLRP3, AIM2, and NLRC4 are the best characterized inflammasomes (Latz, Xiao *et al*. 2013). Interestingly, a distinct pattern of inflammasome activation was observed for the two *S. suis* strains tested (**Fig. 4A**). While NLRP3- or AIM2-deficiency resulted in a partial decrease of IL-1β release following stimulation with P1/7 ($p < 0.05$), there was no significant involvement of NLRP1 or NLRC4 (**Fig. 4A**). Unexpectedly, IL-1β maturation induced by strain SC84 involved all four inflammasomes tested ($p < 0.05$), with a major implication of NLRP3 (**Fig. 4A**). IL-1β-specific activation by these inflammasomes was confirmed by lack of activity on the secretion of TNF, which is inflammasome-independent (**Fig. S3 in Supplementary Material**).

Since inflammasome activation was different between the two strains, we hypothesized that maturation, but not induction, of IL-1β could be responsible for the different levels of IL-1 β induced by both strains *in vitro*. To confirm this hypothesis, IL-1β mRNA levels induced by both strains were evaluated. In accordance, levels of IL-1β expression induced by strains P1/7 and SC84 were similar (**Fig. 4B**). This confirms that while both strains similarly activate DCs

to induce proIL-1β, the differential levels of IL-1β produced are the consequence of differences in maturation capacity.

3.5. *Streptococcus suis* induced-IL-1β production is blocked by additional extracellular potassium

Potassium (K^+) efflux has been described as a common denominator in the assembly and activation of inflammasomes (Latz, Xiao *et al*. 2013, Greaney, Leppla *et al*. 2015). Consequently, we evaluated production of IL-1β by *S. suis*-infected DCs exposed to increased concentrations of extracellular K^+ to inhibit its efflux. Interestingly, only 10 mM of extracellular K^+ was required to significantly inhibit IL-1β induced by both strains ($p < 0.05$) (**Fig. 5**). However, the effect was significantly greater for strain SC84 than for strain P1/7 ($p < 0.01$) (**Fig. 5**). Moreover, inhibition appeared to be dependent on K^+ concentration since increasing extracellular levels lead to a greater inhibition, regardless of strain. Importantly though, at concentrations equal to or greater than 40 mM, the decreased levels of IL-1β observed were non-specific due to cell death as determined by LDH release (data not shown). Unspecific inhibition of K^+ efflux at concentrations between 10 mM and 30 mM was discarded by measuring TNF and IL-6 production, which were not inhibited (**Fig. S4 in Supplementary Material**).

3.6. The elevated suilysin production by the ST7 strain is required for efficient proIL-1β maturation by dendritic cells

Given that pore-forming toxins have the ability to induce K^+ efflux (Latz, Xiao *et al*. 2013, Greaney, Leppla *et al*. 2015) and that *S. suis* produces SLY, a secreted cytolysin similar to the pneumolysin of *S. pneumoniae*, its role in IL-1β production was evaluated. It was previously suggested that the highly virulent *S. suis* ST7 strain responsible for the human outbreaks in China has an increased SLY production compared to other serotype 2 strains (He, Pian *et al*. 2014). We confirmed this by measuring the hemolytic activity in P1/7 and SC84 supernatants, with strain SC84 displaying a two-log greater titer than that of strain P1/7 (results not shown). To evaluate if SLY is implicated in *S. suis*-induced IL-1β release, *sly*-deficient isogenic mutants were constructed and used in parallel with their respective wild-type strains. As shown in **Fig. 6A**, no difference in IL-1β production was observed in the absence of SLY from strain P1/7,

suggesting that components other than the SLY (such as LPs) could be responsible for IL-1β production. By contrast, absence of SLY from strain SC84 resulted in a significant decrease of secreted IL-1β ($p < 0.001$), suggesting that the higher levels of SLY produced by this strain are implicated in IL-1β production (**Fig. 6A**). Moreover, IL-1β levels produced by SC84Δ*sly* were similar to those obtained with both wild-type P1/7 and P1/7Δ*sly*, suggesting that common bacterial components are probably responsible for the basal production of IL-1β by both strains (**Fig. 6A**). In addition, the lack of differences in IL-1β mRNA expression between the wild-type and *sly*-deficient mutants confirms that SLY participates in proIL-1β maturation rather than in IL-1β induction (**Fig. 6B**).

Since SLY is a cholesterol-dependent cytolysin, cholesterol inhibits its effects (Gottschalk, Lacouture *et al*. 1995). To evaluate the effect of cholesterol on *S. suis*-produced SLY and its consequences on IL-1β production, cholesterol was added to DCs infected with the different *S. suis* wild-type and *sly*-deficient strains. Importantly, cholesterol itself did not induce IL-1β production (**Fig. 7A**). While addition of cholesterol had no effect on IL-1β production by strains P1/7, P1/7Δ*sly* or SC84Δ*sly*, it significantly decreased production induced by the wild-type strain SC84 ($p < 0.001$), with detected levels similar to those measured for P1/7, P1/7Δ*sly*, and SC84Δ*sly*, confirming the role of SLY in the hyper-producing strain SC84 (**Fig. 7A**).

Further activation experiments using rSLY alone showed the production of moderate levels of IL-1β from DCs (**Fig. 7B**). Since SLY has been previously described to produce TNF following recognition by TLR4 (Bi, Pian *et al*. 2015), we activated TLR4$^{-/-}$ DCs with rSLY. However, IL-1β production was TLR4-independent **(Fig. S5 in Supplementary Material**), confirming results obtained with live bacteria. Meanwhile, addition of a non-toxic concentration of 5 µg/mL of rSLY resulted in a synergistic and similar increase of IL-1β for strains P1/7, P1/7Δ*sly*, and SC84Δ*sly* ($p < 0.01$) (**Fig. 7B**). Importantly, this effect was abolished following treatment with cholesterol, confirming an exclusive effect of rSLY (**Fig. 7B**).

3.7. Strain-dependent role of IL-1 signaling in host survival during *Streptococcus suis* systemic infection

Given the capacity of *S. suis* to induce IL-1 *in vitro* and *in vivo*, its implication in the balance and/or exacerbation of systemic inflammation induced by this pathogen, and subsequently, host survival was evaluated. Survival of IL-1R$^{-/-}$ mice was significantly decreased in comparison to wild-type counterparts following infection with strain P1/7 ($p < 0.01$), suggesting a beneficial role of IL-1 (**Fig. 8A**). Following infection with strain SC84, however, no statistical difference was observed between survival of wild-type and IL-1R$^{-/-}$ mice (**Fig. 8B**) indicating, that in the case of infection with a highly virulent strain no role can be distinguished.

To better understand this difference between strains, and since IL-1 plays a crucial role in both the initiation and amplification of inflammation, production of other pro-inflammatory mediators in plasma, spleen, and liver was evaluated 12 h p.i. Interestingly, IL-1α and IL-1β liver and spleen levels in WT and IL-1R$^{-/-}$ mice did not show any significant differences, indicating that IL-1 does not autoregulate after 12 h of infection with *S. suis* (**Fig. S6 in Supplementary Material**). As shown in **Fig. 9** and **Fig. 10**, significantly lower levels of IL-6, IL-12p70, IFN-γ, CCL2, CCL3, and CXCL9 were measured in the plasma, spleen, and liver of IL-1R$^{-/-}$ mice compared with wild-type mice following infection with P1/7 ($p < 0.05$), confirming that IL-1 is necessary for pro-inflammatory mediator modulation. However, following infection with strain SC84, levels of mediators where exacerbated in both wild-type and IL-1R$^{-/-}$ mice, indicating that IL-1 signaling cannot counterbalance the exacerbated inflammation induced by this highly virulent strain (**Fig. 9-10**).

During *S. suis* systemic infection, inflammation is required for clearance of bacteria, which, if uncontrolled, can lead to host death. Consequently, we evaluated bacterial load in blood, spleen and liver, 12 h and 48 h following infection with strains P1/7 and SC84. No differences were observed in bacterial burden of wild-type and IL-1R$^{-/-}$ mice 12 h p.i. in blood or organs, this regardless of strain (**Fig. 11**). Interestingly, 48 h p.i. following infection with P1/7 strain, bacterial burden was significantly higher in plasma, liver, and spleen of IL-1R$^{-/-}$ mice, in comparison to wild-type counterparts ($p < 0.01$) (**Fig. 11**). By contrast, no differences were observed following infection with strain SC84, and this for all organs (**Fig. 11**). Notably, bacterial load of wild-type mice infected with strain P1/7 or SC84 were similar 12 h and 48 h

p.i, indicating that the higher virulence of the latter is responsible for the elevated inflammation and detrimental host outcome.

4. Discussion

Previous studies showed low levels of IL-1 in plasma after experimental infection with *S. suis*, in comparison with other important pro-inflammatory cytokines such as TNF or IL-6, somehow underestimating the role of this cytokine (Dominguez-Punaro, Segura *et al*. 2007, Dominguez-Punaro Mde, Segura *et al*. 2008, Ye, Zheng *et al*. 2009, Lachance, Gottschalk *et al*. 2013). As such, the low levels of IL-1 observed in plasma after infection with both strains were not surprising and not related to the virulence levels of the strain. Several factors could explain this near lack of IL-1, including its short half-life in plasma (Kudo, Mizuno *et al*. 1990) and its association with other plasmatic proteins (Dinarello 1996, Lopez-Castejon and Brough 2011). To our knowledge, however, there has been no other study regarding plasmatic levels of IL-1 in similar models of infection (systemic infection). On the other hand, high levels of IL-1α and IL-1β were found in liver and spleen, which are two important filter organs. Previous studies with GBS also reported elevated levels of IL-1β in kidneys (Biondo, Mancuso *et al*. 2014). Therefore, although IL-1 cannot be found in plasma, activation of immune cells by infiltrated bacteria in liver and spleen could be responsible for the induction of IL-1, which remains locally.

To further analyze differences in IL-1 signaling between strains, *in vitro* studies with DCs and MΦs were performed. Interestingly, production by MΦ was somewhat delayed compared to that by DCs following infection with both *S. suis* strains. This seems to be a characteristic of extracellular pathogens, as it was previously reported for GBS, GAS, and *S. pneumoniae* (Harder, Franchi *et al*. 2009, McNeela, Burke *et al*. 2010, Costa, Gupta *et al*. 2012), and might be due to a less efficient capacity of macrophages to process proIL-1β into its mature form.

Unlike with most other cytokines, IL-1β production is controlled by a two-step signaling process. Firstly, activation of PRRs such as TLRs leads to the transcription of proIL-1β. Subsequently, a second signal induces cleavage of the precursor into active IL-1β through caspase-1- and inflammasome-dependent maturation. The higher levels of IL-1 induced by strain SC84, in comparison to strain P1/7, suggested differential cell activation or processing

mechanisms. However, the cellular activation leading to IL-1β production was similar for strains P1/7 and SC84, indicating that the recognized components are relatively well-conserved between these two strains, which might be the case for most *S. suis* serotype 2 strains. Indeed, production of IL-1β was MyD88-dependent and partially involved recognition of surface lipoproteins by TLR2. Comparable results were reported for GBS and *S. pneumoniae*, suggesting that recognized bacterial motifs might even be conserved amongst streptococci (Lee, Scanga *et al*. 2007, Costa, Gupta *et al*. 2012). Meanwhile, early studies suggested that certain toxins such as pneumolysin (Malley, Henneke *et al*. 2003, Shoma, Tsuchiya *et al*. 2008), listeriolysin O (Ito, Kawamura *et al*. 2005) and, more recently, SLY (Bi, Pian *et al*. 2015), may activate cells trough TLR4, an extracellular receptor which can signal via MyD88. However, the capacity of these toxins to activate TLR4 remains controversial. More recent studies with *S. pneumoniae* (including recombinant pneumolysin) showed that production of IL-1 was TLR4-independent (McNeela, Burke *et al*. 2010, Fang, Tsuchiya *et al*. 2011). In our study, IL-1β production by DCs induced by SLY-positive P1/7 and SC84 strains as well as rSLY was TLR4-independent, confirming preliminary results (Lecours, Segura *et al*. 2012). Moreover, IL-1β production was TRIF-independent, confirming the lack of TLR4 implication, since this adaptor protein is engaged by the latter and TLR3. To our knowledge, this is the first study evaluating the role of TRIF during *S. suis* infection. Finally, although considered a classical extracellular pathogen, *S. suis* strains P1/7 and SC84 can be internalized, albeit at low rates. When internalized, however, TLR7 and TLR9, which detect nucleic acids, were recently shown to recognize *S. suis*, resulting in IFN-β production (Auger, Santinon *et al*. 2017). In this study, we demonstrated that RNA and DNA from both strains also have the capacity to induce IL-1β, and equally so for both strains. Importantly, production was only observed when DNA and RNA were complexed with DOTAP, suggesting that recognition occurs in a process similar to that of IFN-β, following internalization and degradation (Auger, Santinon *et al*. 2017).

Following engagement of TLRs, activation of the MAPK and NF-κB signaling pathways results in the initiation of an inflammatory response leading to cytokine production. IL-1β production by DCs induced by both strains P1/7 and SC84 was dependent on the NF-κB and ERK pathways, but independent of JNK, similar to what has previously described for other cytokines induced by *S. suis* (Dominguez-Punaro Mde, Segura *et al*. 2010) and for other streptococci (Chung and

Dale 2004, N'Guessan, Hippenstiel *et al*. 2006, Bebien, Hensler *et al*. 2012). Meanwhile, IL-1β production induced by strain SC84, but not strain P1/7, was also p38-dependent, suggesting differential mechanisms, possibly due to differences in bacterial components or virulence. Indeed, pore-forming toxin secretion and its induced osmotic stress were observed to modulate MAPK phosphorylation for listeriolysin O and streptolysin O (Tang, Rosenshine *et al*. 1996, Bebien, Hensler *et al*. 2012). As such, the higher production of SLY by strain SC84 could be involved in p38 activation, which remains to be confirmed.

Receptors and pathways engaged by this pathogen could not explain the differences observed in IL-1β production between strains. Moreover, IL-1β gene induction confirmed that the first step involved in this production is the same. Consequently, the steps involved in its maturation were evaluated. Though IL-1β production induced by both strains depended on caspase-1, inflammasome activation was different between strains: while maturation of proIL-1β induced by strain P1/7 was only partially dependent on NLRP3 and AIM2, strain SC84 activated NLRP3 and, to a lesser extent AIM2, NLRP1 and, surprisingly, NLRC4. These differences in inflammasome activation between strains, both in the different inflammasomes activated and their implication levels, could explain the differential IL-1β levels produced by DCs. Although NLRP3 and AIM2 participate in IL-1β release by DCs and MΦ following infection by GBS and *S. pneumoniae* (Fang, Tsuchiya *et al*. 2011, Costa, Gupta *et al*. 2012), the implication of NLRP1 and NLRC4 have not yet been described following streptococcal infection (LaRock and Nizet 2015). The specific factors responsible for *S. suis*-dependent inflammasome activation are difficult to determine since all four inflammasomes can be activated by a wide range of molecules. Previous studies showed that NLRP1 could directly sense the protease activity of the *Bacillus anthracis* lethal toxin (Moayeri, Sastalla *et al*. 2012). Although activation of NLRP1 by streptococcal pore-forming toxins has not yet been evaluated, elevated levels of the *S. suis* SLY might be involved in a similar process. Moreover, strain SC84, unlike strain P1/7, also possesses a type IV secretion system encoded by its 89 K pathogenicity island (Li, Shen *et al*. 2011), which might be responsible for NLRC4 activation (Miao and Warren 2010), although this remains only an hypothesis. Regarding the AIM2 inflammasome, it has been previously shown to be activated by DNA (Rathinam, Jiang *et al*. 2010). In accordance, we observed that levels of IL-1β induced by DNA were higher than those induced by RNA.

Given the activation of a wide range of inflammasomes by strain SC84, it was hypothesized that ion fluxes (K^+) might be involved in the assembly of these four inflammasomes (Latz, Xiao *et al*. 2013, Greaney, Leppla *et al*. 2015). Indeed, bacterial pore-forming toxins can play a key role in IL-1β processing by generating K^+ efflux as previously described for pneumolysin and the β-hemolysin of GBS (Shoma, Tsuchiya *et al*. 2008, Biondo, Mancuso *et al*. 2014). In this study, we showed that SLY plays an important role for the SC84 strain, but not for P1/7, and that this role is associated only with IL-1β maturation. The reduction of IL-1β observed after addition of cholesterol, which is an inhibitor of SLY, further supports these results. The fact that levels of SLY produced by strain SC84 are much higher than those produced by strain P1/7 could explain these differences. Indeed, a role of SLY was observed for strain SC84, but not P1/7, when using the SLY-deficient mutant, confirming that a minimal level of SLY (threshold) is required. In other words, although cell activation by strains P1/7 and SC84 leads to similar levels of proIL-1β, the high levels of SLY produced by strain SC84 result in a more efficient maturation of IL-1β via pore formation, depletion of K^+ efflux, and a wider inflammasome activation. In accordance, the addition of rSLY to strain P1/7 or the SLY-deficient mutants synergistically increased IL-1β production by DCs. Interestingly, though rSLY itself induced some IL-1β secretion from DCs, levels were similar to those observed when cells were stimulated with Alum alone. Since Alum is a known activator of NLRP3, but not an inducer of IL-1β mRNA, it has been suggested to cause maturation and release of the IL-1β naturally synthetized by the cell in the absence of prior stimulation (Li, Nookala *et al*. 2007). In fact, addition of Alum to DNA and RNA increased the production of IL-1β, probably through activation of NLRP3 inflammasome. Noteworthy, the *sly*-deficient SC84 mutant produced similar IL-1β levels as those by strain P1/7, suggesting that both strains possess mechanisms other than SLY that participate in IL-1β maturation, which should be dissected in future studies using SLY-negative *S. suis* strains. Finally, the addition of extracellular K^+ inhibited *S. suis*-induced IL-1β production. Importantly, although only 10 mM of K^+ was sufficient to reduce IL-1β production, the effect was accentuated for strain SC84, which goes along with results showing a broad inflammasome activation by this strain. For strain P1/7, the K^+ efflux generated could be due to other yet unknown bacterial mechanisms that probably shared by other classical *S. suis* strains and responsible for "normal" inflammasome activation by this pathogen. Moreover, though

inhibition was concentration-dependent, addition of more than 40 mM had a cytotoxic effect on DCs, with 100% cell death when using 130 mM.

Following secretion, IL-1α and IL-1β bind their shared receptor, IL-1R, leading to cell activation, stimulation and secretion of diverse pro-inflammatory cytokines (positive feedback loop), recruitment of neutrophils and macrophages, and activation of killing mechanisms, amongst other effects (Weber, Wasiliew *et al*. 2010). Previous studies with GBS and *S. pneumoniae* showed a protective role of this cytokine during infection: absence of IL-1 signaling alters bacterial clearance and survival (Zwijnenburg, van der Poll *et al*. 2003, Kafka, Ling *et al*. 2008, Biondo, Mancuso *et al*. 2014, Biondo, Mancuso *et al*. 2014). In the case of *S. suis*, IL-1 signaling also plays a central and beneficial role following infection with the ST1 strain P1/7, which represents classical strains. Indeed, IL-1 signaling induced by strain P1/7 modulates the host innate immune response by increasing production of other pro-inflammatory cytokines and chemokines required for control of bacterial burden in blood and organs, which if unrestricted, causes host death (i.e. IL-1R$^{-/-}$ mice). However, and similarly to what has been described for type I IFN, the "protective effect" of IL-1 was not observed following infection with the highly virulent ST7 strain SC84 (Auger, Santinon *et al*. 2017). In fact, the levels of IL-1 induced by this strain were unable to modulate overall inflammation and host outcome since levels of inflammatory mediators were exacerbated. However, the similar bacterial load in P1/7- and SC84-infected wild-type mice indicates that the difference in the role of IL-1 signaling observed is not due to excessive bacterial burden by SC84, but rather by the exacerbated inflammation due to the virulence level of the strain. These results suggest that during *S. suis* systemic infection, the levels of induced inflammation play a critical role, and this regardless of the strain. In accordance, while IL-1 signaling initiates the cascade of inflammation, generating a positive loop, and stimulating the synthesis of more mediators necessary to fight the pathogen, IL-1 signaling itself cannot counterbalance exacerbated inflammation, resulting in host death. The latter is exemplified by the highly virulent ST7 strain SC84, which, possessing additional virulence factors such as the 89 K pathogenicity island (Zhao, Liu *et al*. 2011), is responsible for a greater innate immune system activation, resulting in a cytokine storm. In fact, this cytokine storm is associated with the streptococcal toxic shock-like syndrome caused by this strain (Tang, Wang *et al*. 2006). It should be noted, however, that even in the presence of IL-1

(i.e. wild-type mice), mice still succumb to *S. suis* infection, though to a significantly lower degree and rate, demonstrating the need in a balanced and controlled inflammation. Interestingly, *S. suis*-induced IL-1 did not autoregulate itself, suggesting that levels induced in the first hours of infection are sufficient to activate the immune system. This is in accordance with results obtained during systemic infection with GBS, during which levels of IL-1β are similar between wild-type and IL-1R$^{-/-}$ mice in kidneys, peritoneal lavage, and brain (Biondo, Mancuso *et al*. 2014).

In conclusion, this study demonstrates that a classical (P1/7) and highly virulent (SC84) *S. suis* strain induce IL-1 *in vivo*, but only in internal organs. While both strains similarly activate innate immune cells due to conserved bacterial components such as LPs, the pore-forming toxin SLY, which is highly produced by strain SC84 only, plays an important role in IL-1β maturation via activation of the NLRP1, NLRP3, AIM2, and NLRC4 inflammasomes. Based on these results, a model of the mechanisms involved in *S. suis*-induced IL-1β production by DCs is proposed (**Fig. 12**). Globally, *S. suis*-induced IL-1 plays a beneficial role during systemic infection by initiating the inflammatory cascade. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role. A better understanding of the underlying mechanisms involved in inflammation and subsequent bacterial burden could help to develop control measures for this important porcine and zoonotic agent.

5. Conflict of interest

The authors declare no conflict of interest. All authors have read and approved the manuscript.

6. Author contribution

Conceived and designed the experiments: AL, JPA, MS, MG

Performed the experiments: AL, JPA, AD, DR

Analyzed the data: AL, JPA, MS, MG

Provided research tools: SG, NG

Contributed to the writing of the manuscript: AL, JPA, MS, MG

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8. Abbreviations

AIM2, absent in melanoma 2; CCL, C-C motif chemokine ligand; CFU, colony-forming unit; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; GAS, Group A *Streptococcus*; GBS, Group B *Streptococcus*; IFN, interferon; IL, interleukin; IL-1R interleukin-1 receptor; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LP, lipoprotein; LTA, lipoteichoic acid; MΦ, macrophage; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK 1/2; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; NLR, NOD-like receptor; NLRC, NLR family CARD domain-containing protein; NLRP, NLR family pyrin domain-containing; NOD, nucleotide oligomerization domain; PCR, polymerase chain reaction; p.i., post-infection; PRR, pattern recognition receptor; p38, p38 mitogen-activated protein kinase; rSLY, recombinant SLY; SLY, suilysin; ST, sequence type; THA, Todd Hewitt broth agar; THB, Todd Hewitt broth; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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Tables and Figures

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

Strain/plasmid	General characteristics	Source/reference
Streptococcus suis		
P1/7	Classical virulent serotype 2 ST1 strain isolated from a pig with meningitis in the UK	(Slater, Allen *et al*. 2003)
P1/7Δ*sly*	Isogenic mutant strain derived from P1/7; in frame deletion of *sly* gene	This work
P1/7Δ*lgt*	Isogenic mutant strain derived from P1/7; in frame deletion of *lgt* gene	(1)
SC84	Highly virulent serotype 2 ST7 strain isolated from a human case of streptococcal toxic shock-like syndrome during the 2005 human outbreak in China	(Ye, Zheng *et al*. 2009)
SC84Δ*sly*	Isogenic mutant strain derived from SC84; in frame deletion of *sly* gene	This work
SC84Δ*lgt*	Isogenic mutant strain derived from SC84; in frame deletion of *lgt* gene	(1)
Escherichia coli		
TOP 10	F^- mrcA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen
BL21	F^-ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm rne131 (DE3)	Invitrogen
Plasmids		
pIVEX2.4d	Ap^r, pUC ori, T7 promoter, His tag-coding sequence	Roche Bioscience
pSET4Δ*sly*	pSET4s carrying the construct of *sly* gene for allelic replacement	This work
pIVEX*sly*	pET101 carrying *sly* gene for protein production	This work

Figure 1. ***Streptococcus suis*** **serotype 2 induces elevated levels of IL-1β in liver and spleen but not in plasma.**C57BL/6 mice were intraperitoneally inoculated with the *S. suis* strain P1/7 (white bars) or SC84 (black bars). Plasma (**A** & **B**), spleen (**C** & **D**), and liver (**E** & **F**) were collected at different times post-infection and levels of IL-1β were quantified by ELISA. Values for mock-infected controls did not change between 6 h and 48 h. As such, 0 h represents results for mock-infected mice throughout the experiment. Data are expressed as mean ± SEM of at least 3 independent experiments.

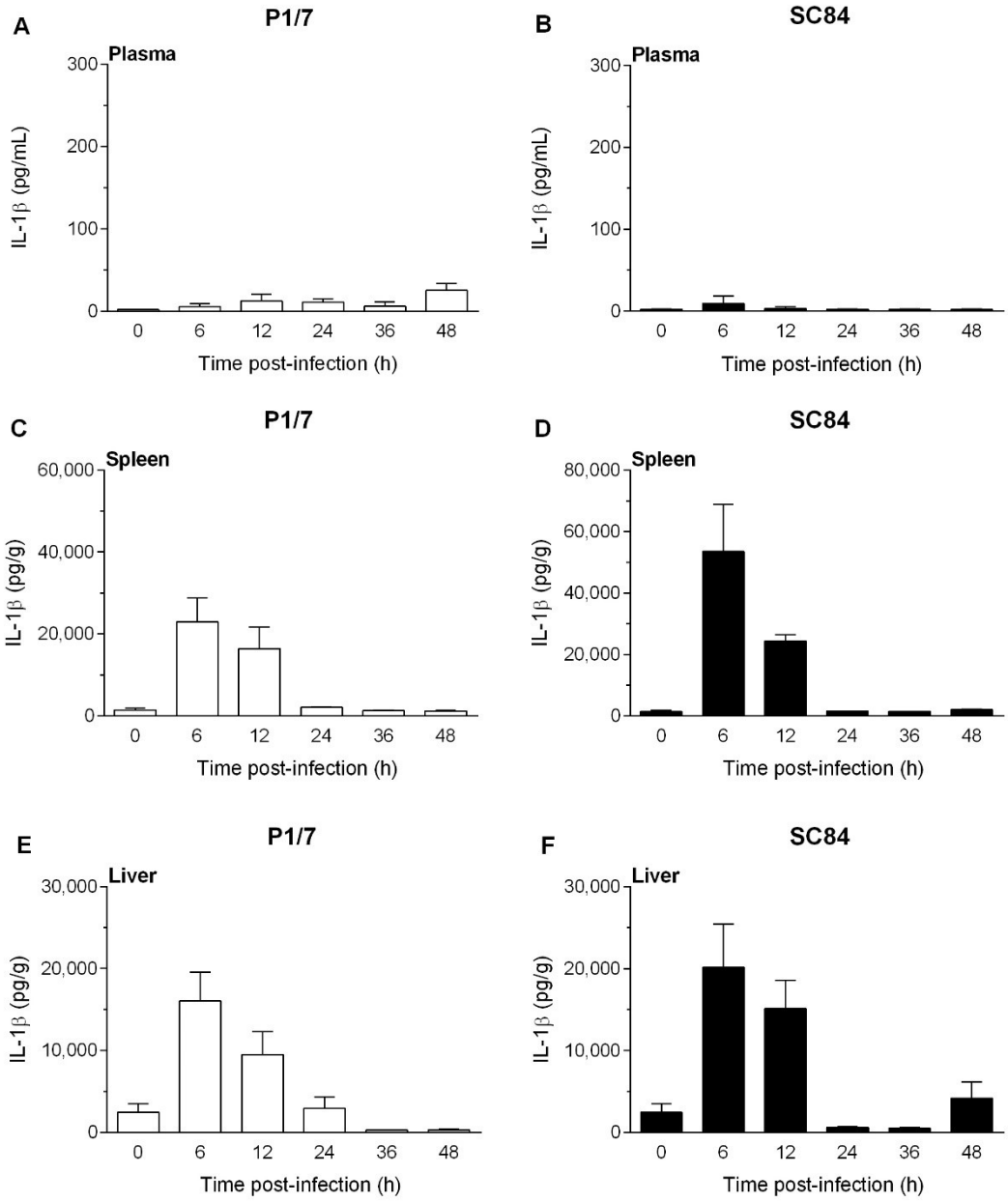


Figure 2. IL-1β released from dendritic cells (DC) and macrophages (MΦ) stimulated with *Streptococcus suis* is strain-dependent. IL-1β kinetics as measured by ELISA following infection of DCs (**A** & **C**) or MΦ (**B** & **D**) with strain P1/7 (white bars) or SC84 (black bars). Non-stimulated cells served as negative control (C-). Data represent the mean ± SEM of at least 3 independent experiments.

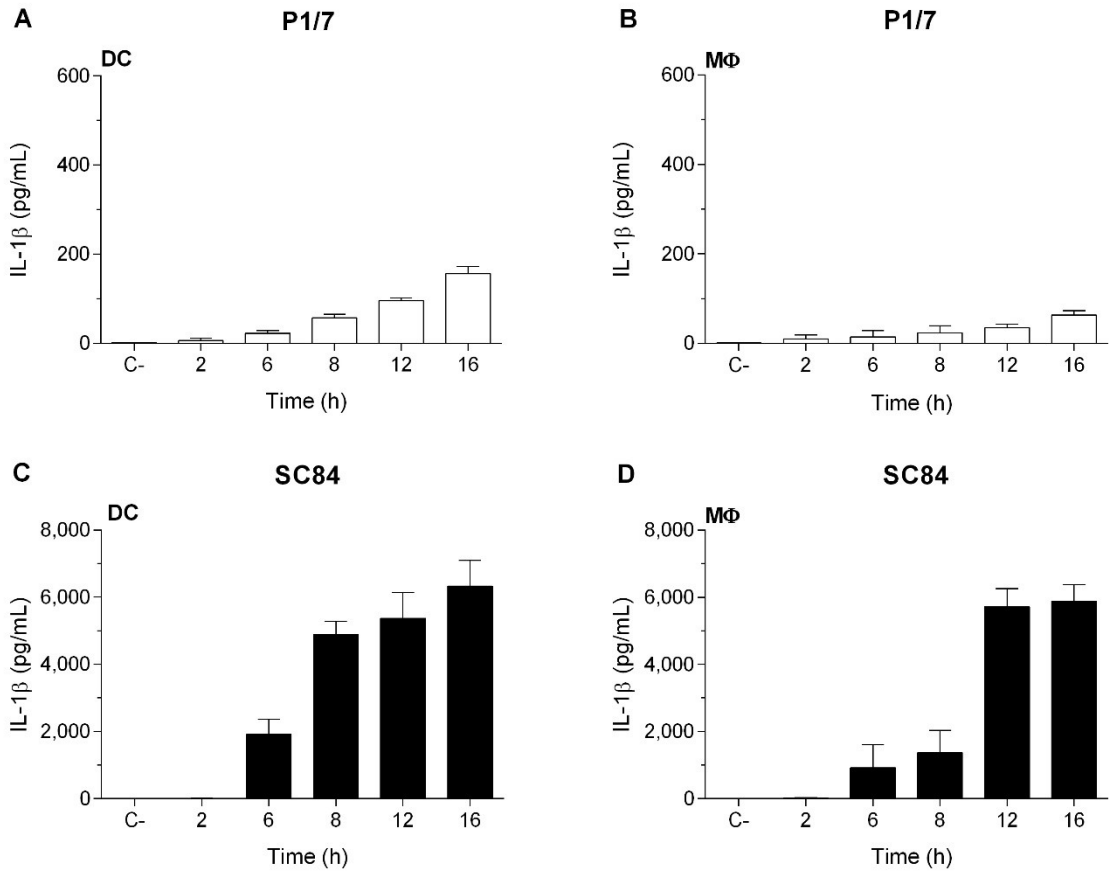


Figure 3. Role of Toll-like receptors (TLRs) and associated signaling pathways in *S. suis*-induced IL-1β production from dendritic cells (DCs). (**A**) Percentage of IL-1β production induced by *S. suis* strain P1/7 (white bars) or SC84 (black bars) 16 h following infection of DCs deficient for MyD88, TRIF, TLR2 or TLR4, with regards to wild-type (WT; normalized to 100%) counterparts (gray bar); (**B**) IL-1β production following activation of wild-type (WT) or TLR2$^{-/-}$ DCs with 30 µg/mL of LTA extracts from strains P1/7 or SC84 or their *lgt*-deficient mutants (Δ*lgt*); (**C**) IL-1β production by DCs following phagosomal delivery of 1 ug of *S. suis* RNA or DNA in the presence or absence of Alum; (**D**) Percentage of IL-1β production from DCs following pre-treatment with either NF-kB inhibitor (i), p38i, MEKi or JNKi and infection with *S. suis*, with regards to non-treated DCs. Data represent the mean ± SEM of at least 3 independent experiments. # ($p < 0.05$) indicates a significant difference with wild-type DCs; $ ($p < 0.05$) indicates a significant difference with negative control (elution buffer); * ($p < 0.05$) indicates a significant difference with non-treated DCs.

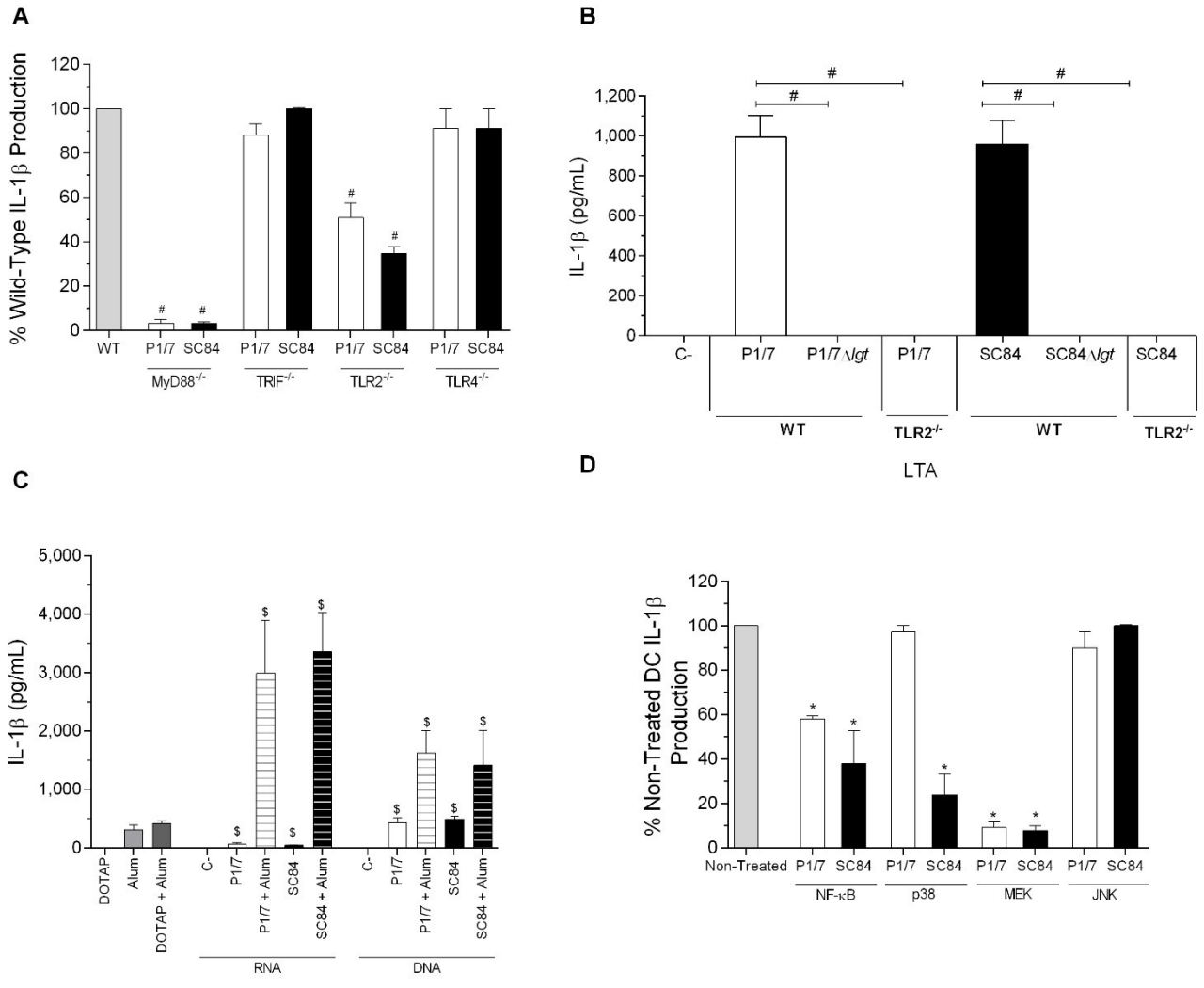


Figure 4. Inflammasome implication in *Streptococcus suis*-induced IL-1β dendritic cell (DC) production is strain-dependent. Percentage of IL-1β secretion by caspase-1 (CASP-1), NLRP3, AIM2, NLRP1 or NLRC4-deficient DCs induced by P1/7 (white bars) or SC84 (black bars) after 16 h of incubation, in comparison to wild-type counterparts; (**B**) DCs were infected with P1/7 and SC84 strains for 6 h and IL-1β mRNA expression was measured by RT-qPCR. Data are presented as 'fold' increase in mRNA expression relative to non-infected cells. Data represent the mean ± SEM of at least 3 independent experiments. * ($p < 0.05$) indicates a significantly difference with wild-type DCs.

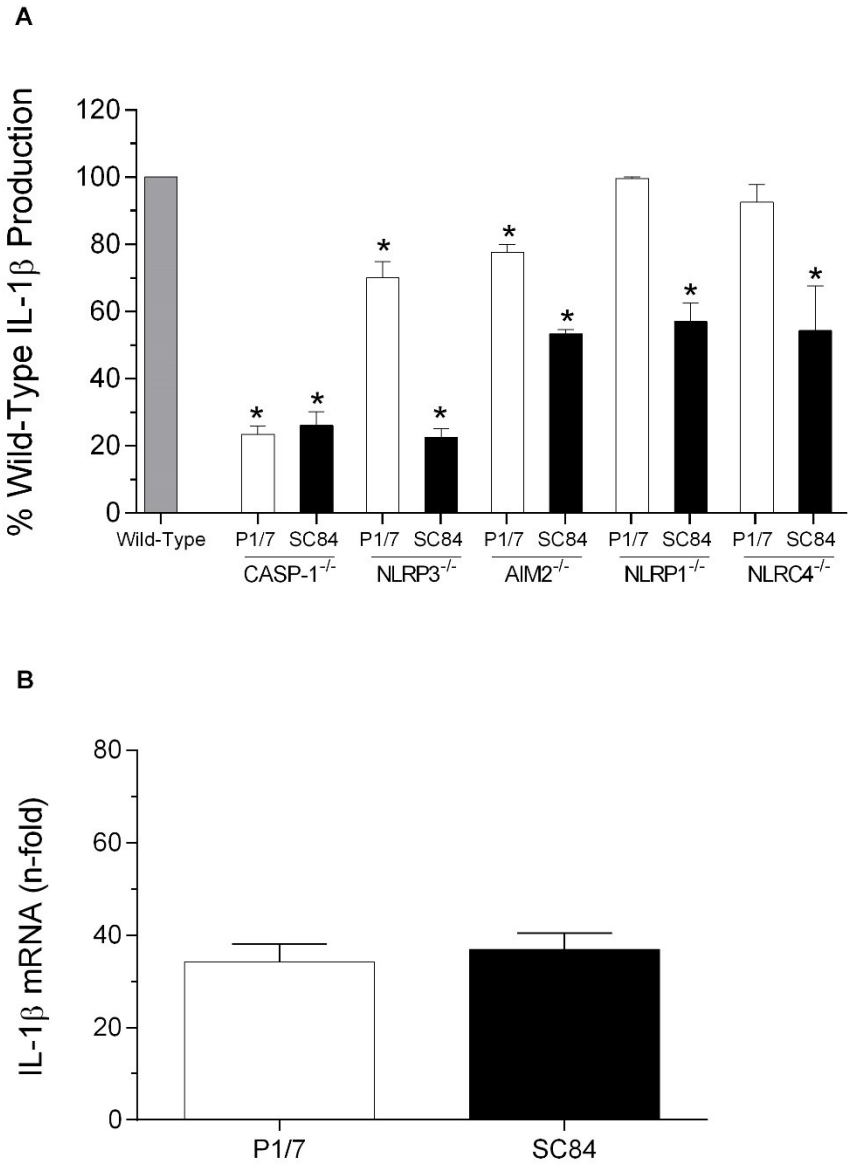


Figure 5. IL-1β secretion by dendritic cells (DCs) activated by *S. suis* is blocked by additional extracellular potassium (K^+). DCs were infected with either strain P1/7 or SC84 in the presence of different concentrations of KCl and IL-1β production was measured after 16 h by ELISA. Data represent the mean ± SEM of at least 3 independent experiments. # ($p < 0.05$) indicates a significant difference with non-treated DCs and * ($p < 0.05$) indicates a significant difference between P1/7 and SC84.

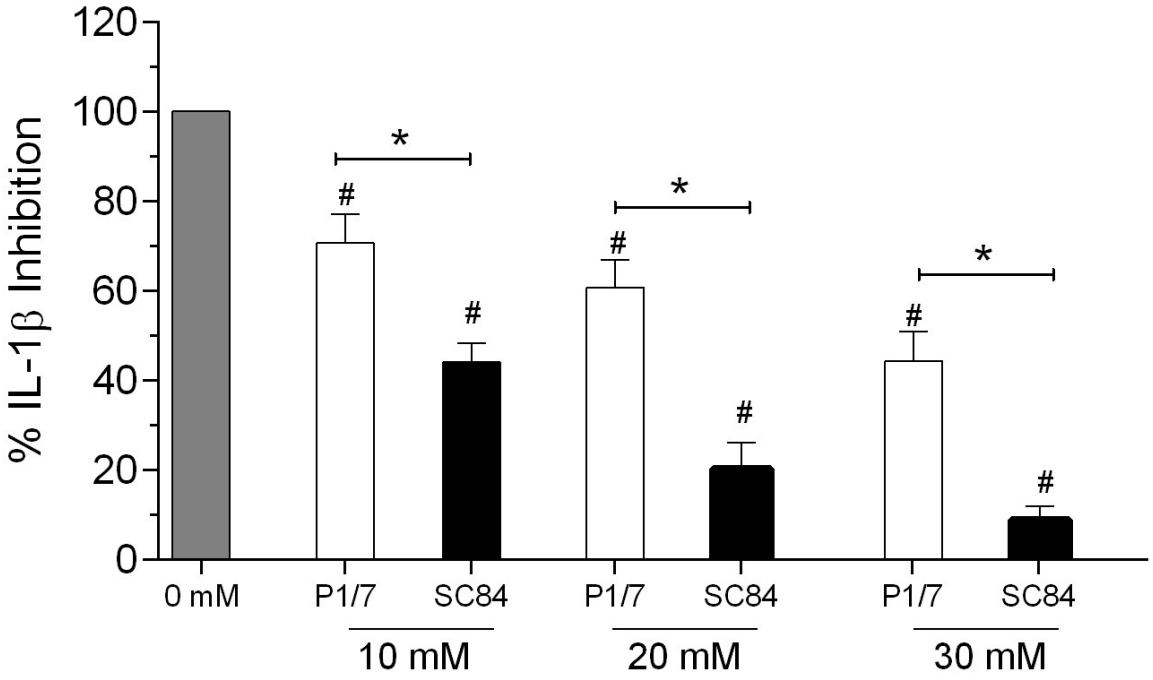


Figure 6. Suilysin (SLY) is involved in the maturation of *Streptococcus suis*-induced IL-1β by dendritic cells (DCs). (**A**) DCs were infected with the *S. suis* wild-type strains P1/7 and SC84 or their SLY-deficient mutants (Δ*sly*) for 16 h and IL-1β release was measured by ELISA; (**B**) DCs were infected with the different wild-type and mutant strains for 6 h and IL-1β mRNA expression was measured by RT-qPCR. Data are presented as 'fold' increase in mRNA expression relative to non-infected cells. Data represent the mean ± SEM of at least 3 independent experiments. * ($p < 0.05$) indicates a significant difference between SC84 and SC84Δ*sly*.

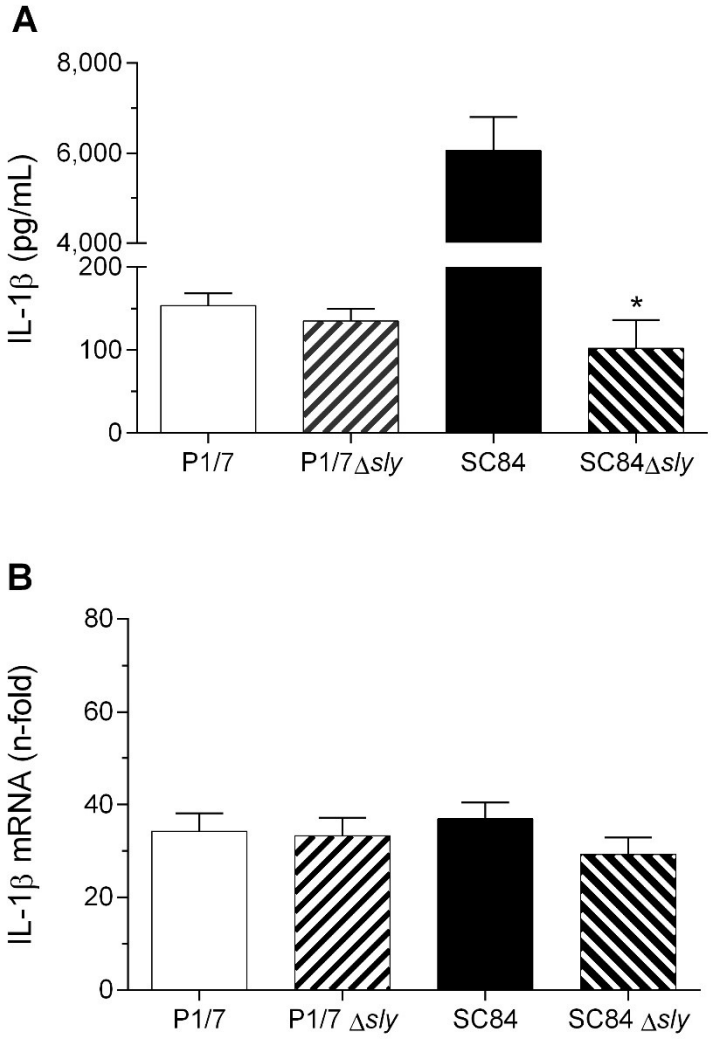


Figure 7. Co-stimulation with recombinant suilysin (rSLY) enhances *Streptococcus suis*-induced IL-1β production by dendritic cells (DCs), which is inhibited by cholesterol (CHOL). (**A**) DCs were stimulated with the different strains of *S. suis* in the presence or absence of CHOL for 16 h and IL-1β production was measured by ELISA; (**B**) DCs were infected with the different strains of *S. suis* alone, in combination with 5 µg/mL of rSLY or with 5 µg/mL of rSLY and CHOL. Data represent the mean ± SEM of at least 3 independent experiments. * ($p < 0.05$) indicates a significant difference with bacteria alone.

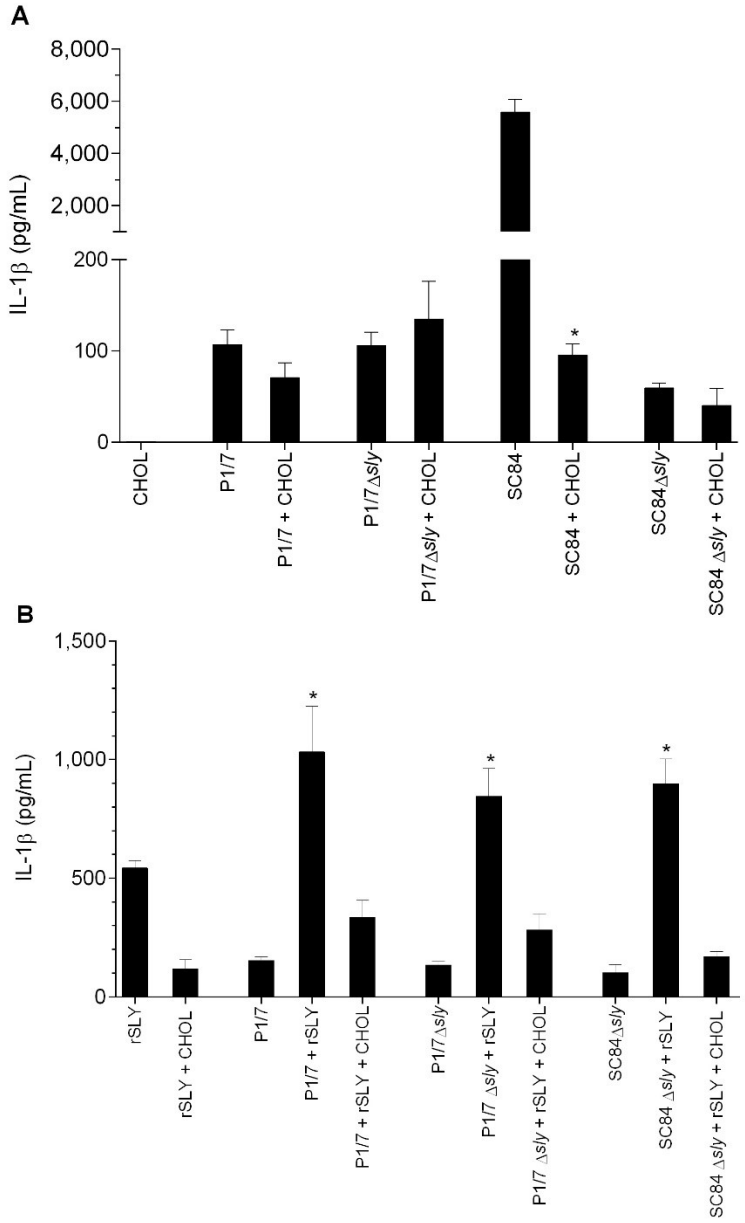


Figure 8. Survival of wild-type (WT) and IL-1 receptor-deficient (IL-1R$^{-/-}$) mice following *Streptococcus suis* systemic infection. WT and IL-1R$^{-/-}$ mice were inoculated with strain P1/7 (A) or SC84 (B) and survival was monitored. Data represent survival curves (n=15). * ($p < 0.05$) indicates a significant difference between survival of WT and IL-1R$^{-/-}$ mice.

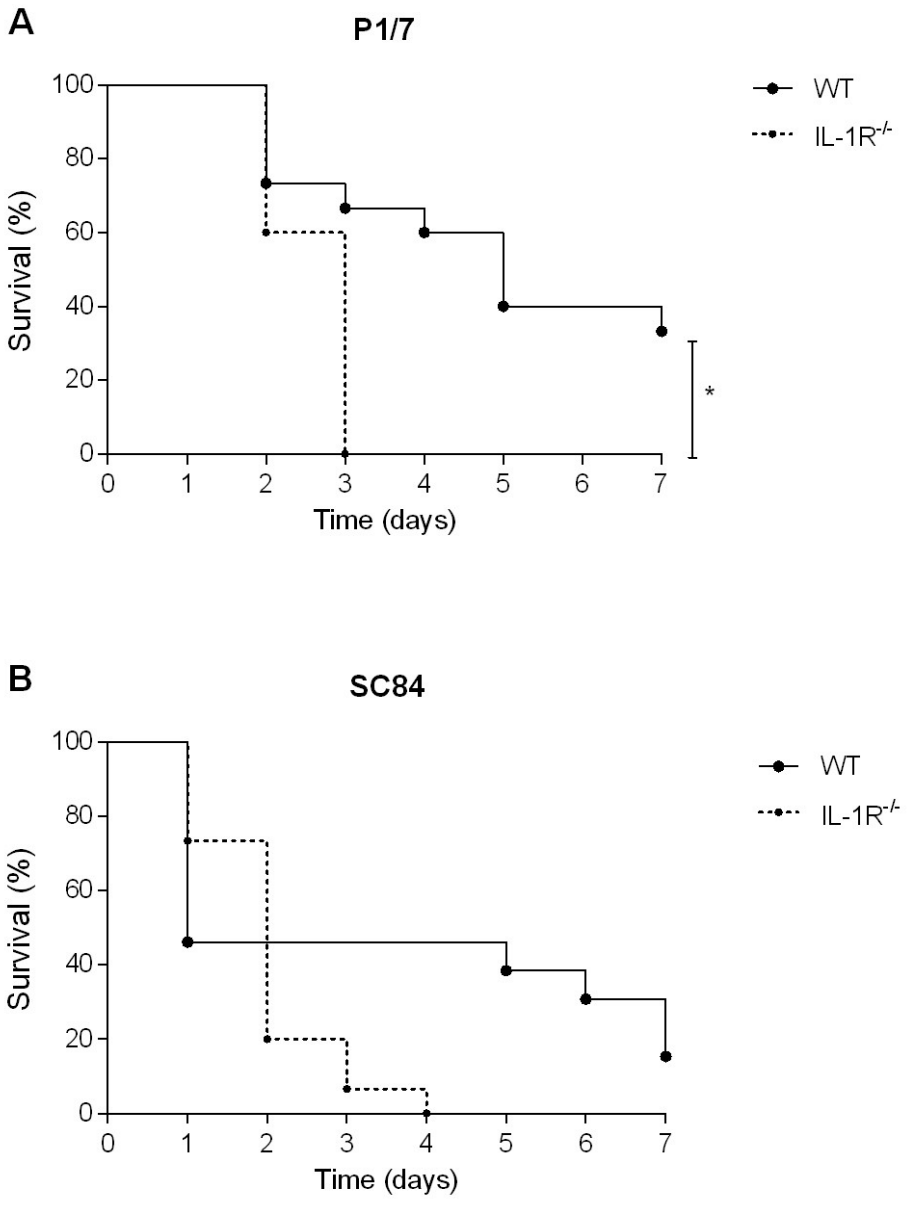


Figure 9. Plasma pro-inflammatory mediator production during *Streptococcus suis* systemic infection. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following infection with strain P1/7 or SC84. Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

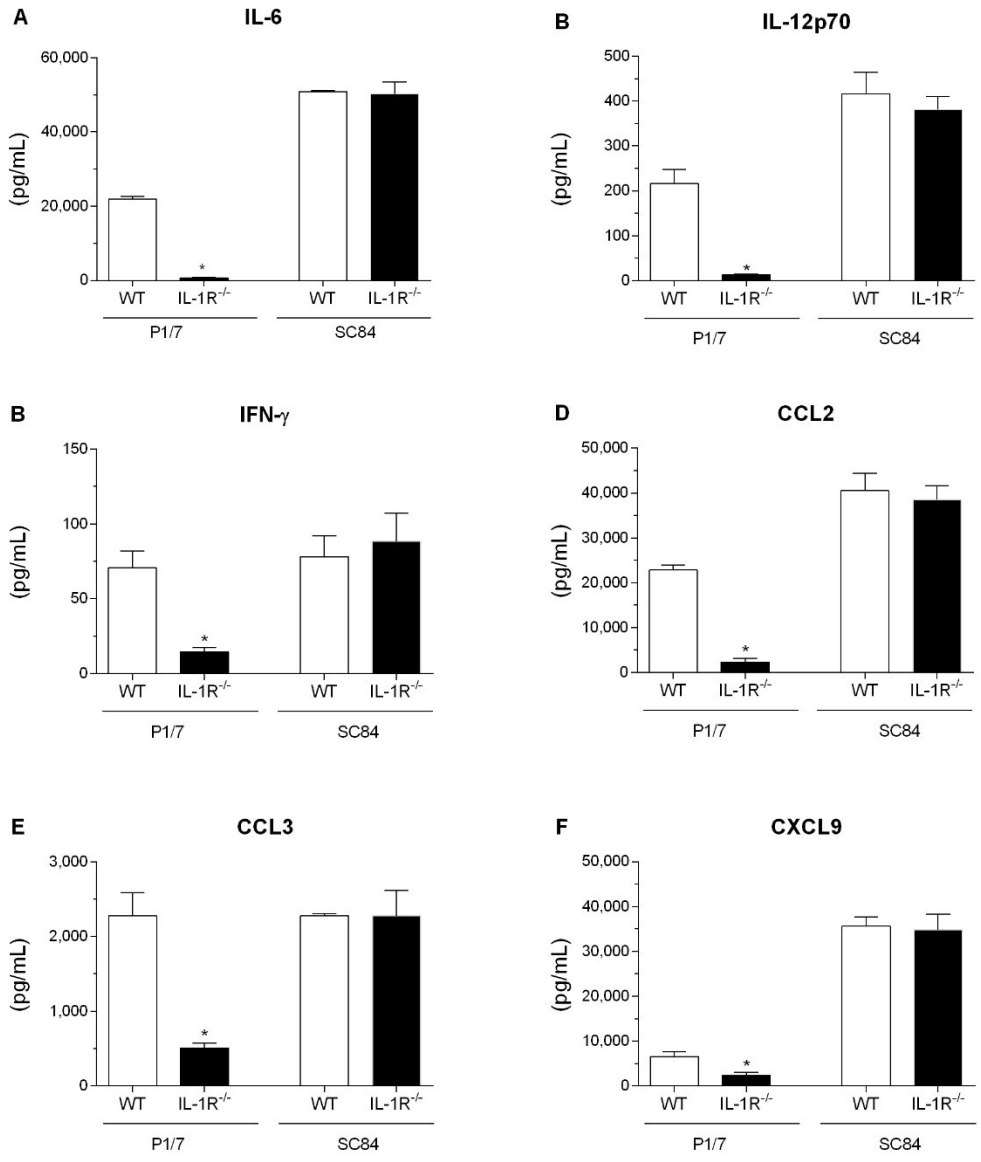


Figure 10. Pro-inflammatory mediator production in spleen and liver during *Streptococcus suis* systemic infection Spleen and liver levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following infection with strain P1/7 or SC84 strain. Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

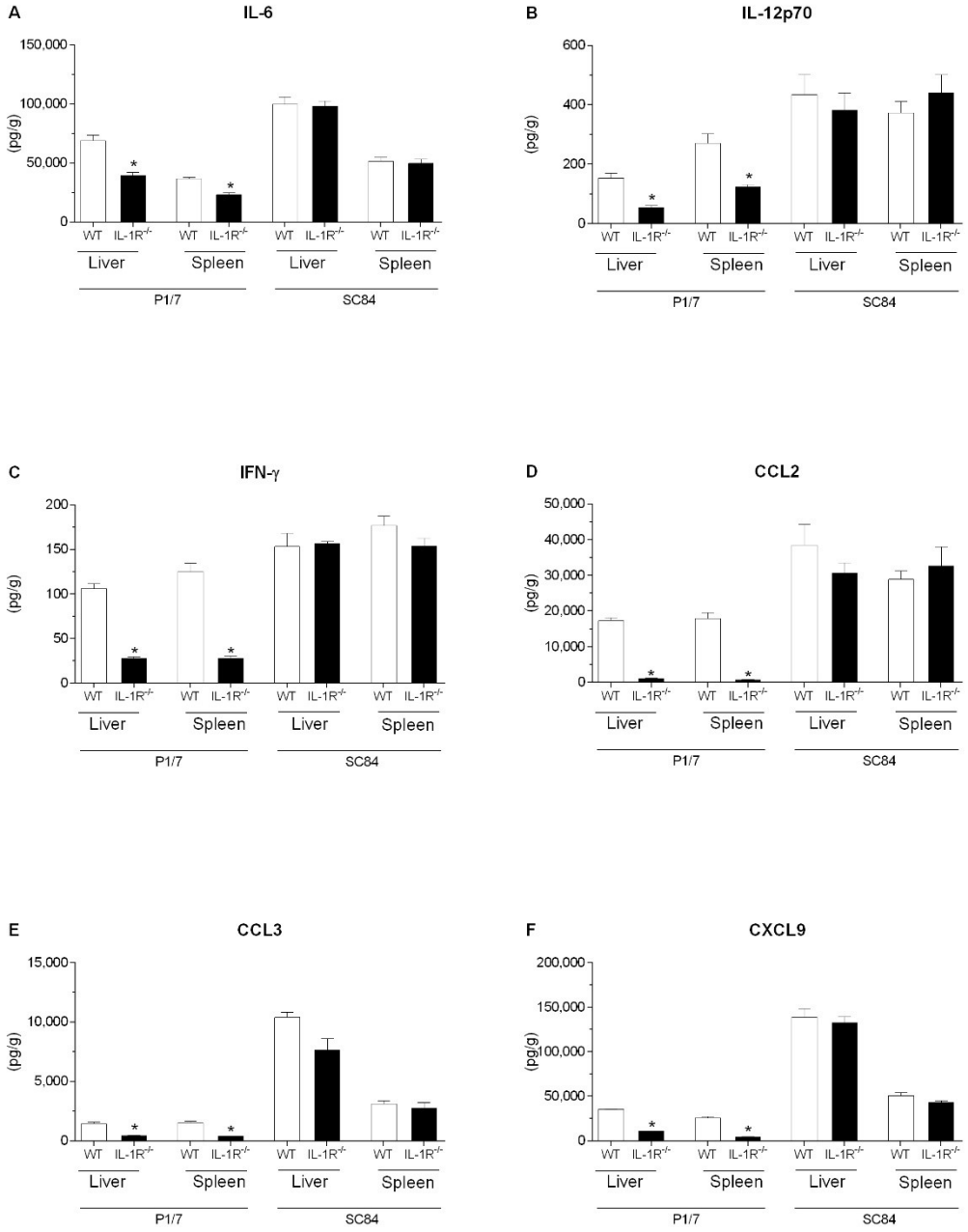


Figure 11. IL-1 signaling is required for control of bacterial burden in blood, liver, and spleen. Bacterial burden in blood (**A** & **B**), liver **(C** & **D),** and spleen (**E** & **F**) of wild-type (WT) and IL-1R$^{-/-}$ mice infected with strain P1/7 or SC84 12 h (left panel) or 48 h (right panel) post-infection. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. Data represent the geometric mean of at least six individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R $^{-/-}$ mice.

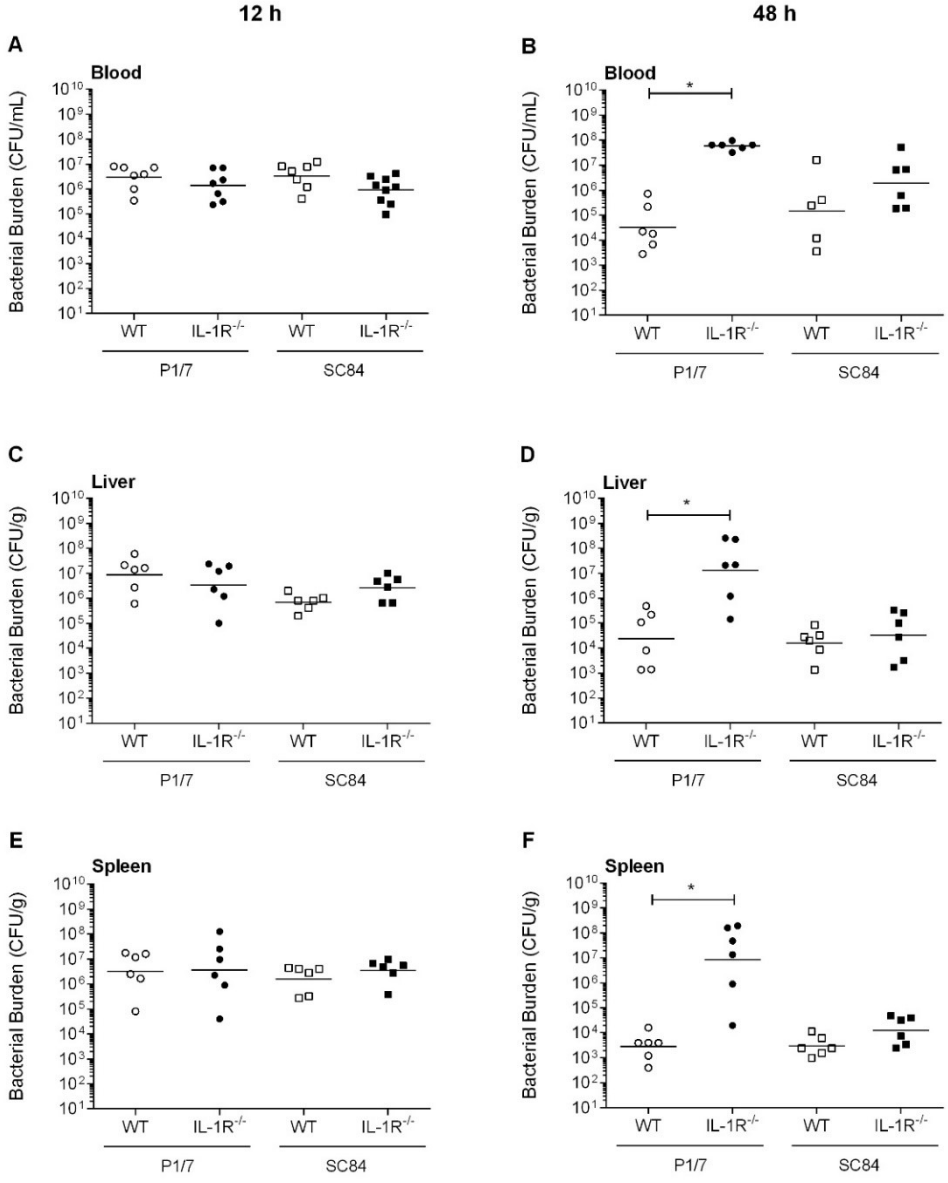


Figure 12. Model of the mechanisms involved in *Streptococcus suis*-induced IL-1β production by dendritic cells (DCs). **1A:** Strain-independent recognition of *S. suis* by DCs requires MyD88-dependent signaling and partially involves TLR2 activation via recognition of surface lipoproteins (LPs); **1B:** If internalized, *S. suis* DNA and RNA can induce the production of IL-1β, possibly via recognition by endosomal receptors TLR7 and TLR9; **1C:** Recognition of *S. suis* leads to activation of the NF-κB and MEK pathways for both strains, alongside p38 for SC84; **1D:** Strains P1/7 and SC84 induce comparable transcription of IL-1β mRNA; **2A:** For strain P1/7, low levels of suilysin (SLY) and other not yet identified bacterial components lead to partial NLRP3 and AIM2 inflammasome activation; **2B:** Caspase-1 cleavage leads to maturation of moderate levels of IL-1β that are then secreted; **3A & 3B:** For strain SC84, secretion of high levels of SLY induces an important K^+ efflux that results in a activation of multiple inflammasomes, including NLRP3, NLRP1, AIM2, and NLRC4; however, other bacterial components could also influence this activation. **3C:** Increased caspase-1 cleavage leads to a more efficient maturation of the proIL-1β, resulting in the secretion of high levels of IL-1β.

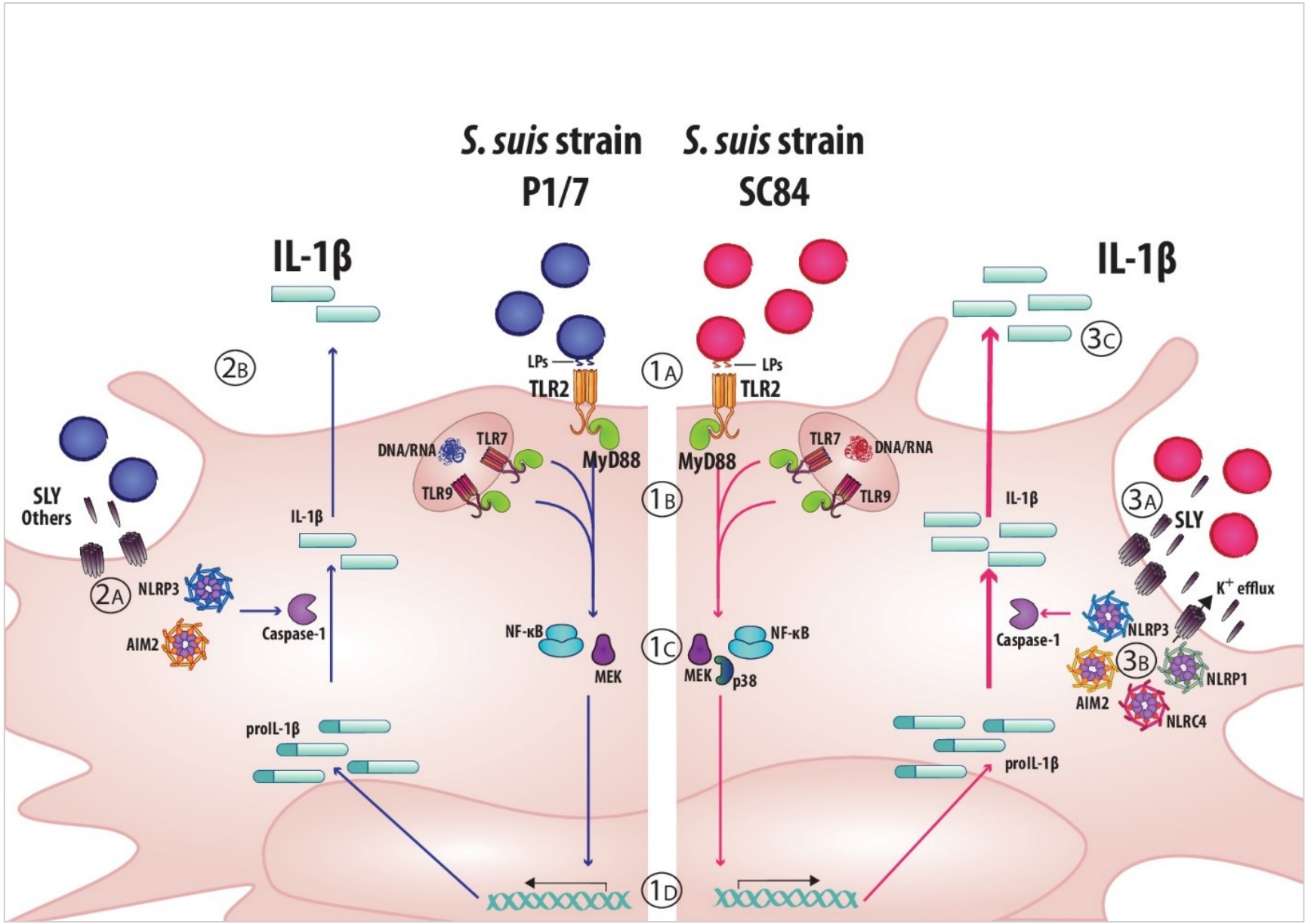

S. suis strain P1/7
S. suis strain SC84
IL-1β
IL-1β
LPs
TLR2
LPs
TLR2
DNA/RNA
TLR7
TLR9
MyD88
MyD88
TLR7
DNA/RNA
TLR9
SLY
Others
IL-1β
IL-1β
NLRP3
AIM2
Caspase-1
NF-κB
MEK
NF-κB
MEK
p38
Caspase-1
SLY
K+ efflux
NLRP3
NLRP1
AIM2
NLRC4
proIL-1β
proIL-1β
1A
1B
1C
1D
2A
2B
3A
3B
3C

Table S1. Oligonucleotide primers used in this study

Primer name	Sequence (5' – 3')
sly -ID1	GAAGTGACTGCTGACAAGATGC
sly –ID2	GCGCAATACTGATAAGCGTTGG
sly -ID3	CAATCCAGGTGTTCCGATTTCG
sly -ID4	TGCAGGAGATCTGCGACTAAG
sly -ID5	CGCAGATATGCGGATGAAG
sly -ID6	CACCTCATCCGCATATGCCAAACTGACTAT
sly -ID7	ATAGTCAGTTTGGCATATGCGGATGAGGTG
sly –ID8	CCAGTAAGAGACCAGCAACAGG
PT101 *sly*	F: GCGC**CATATG**CATATGGATTCCAAACAAGATATTAAT[1]
	R: CGCG**GGATCC**TTACTCTATCACCTCATCCGC[2]
Atp5b	F: ACC AGC CCA CCC TAG CCA CC
	R: TGC AGG GGC AGG GTC AGT CA
Gapdh	F: CCC GTA GAC AAA ATG GTG AAG
	R: GAC TGT GCC GTT GAA TTT G
Il1a	F: TCG GGA GGA GAC GAC TCT AA
	R: TGA GTT TTG GTG TTT CTG GC
Il1b	F: AGG TCA AAG GTT TGG AAG CA
	R: TGA AGC TAT GGC AAC TG

[1] NdeI site in bold

[2] BamHI site in bold

Figure S1. ***Streptococcus suis*** **serotype 2 induces elevated levels of IL-1α in liver and spleen, but not in plasma.** C57BL/6 mice were intraperitoneally inoculated with *S. suis* strain P1/7 (white bars) or SC84 (black bars). Plasma (**A** & **B**), spleen (**C** & **D**), and liver (**E** & **F**) were collected at different times post-infection and levels of IL-1α were quantified by ELISA. Values for mock-infected controls did not change between 6 h and 48 h. As such, 0 h represents results for mock-infected mice throughout the experiment. Data are expressed as mean ± SEM of at least 3 independent experiments.

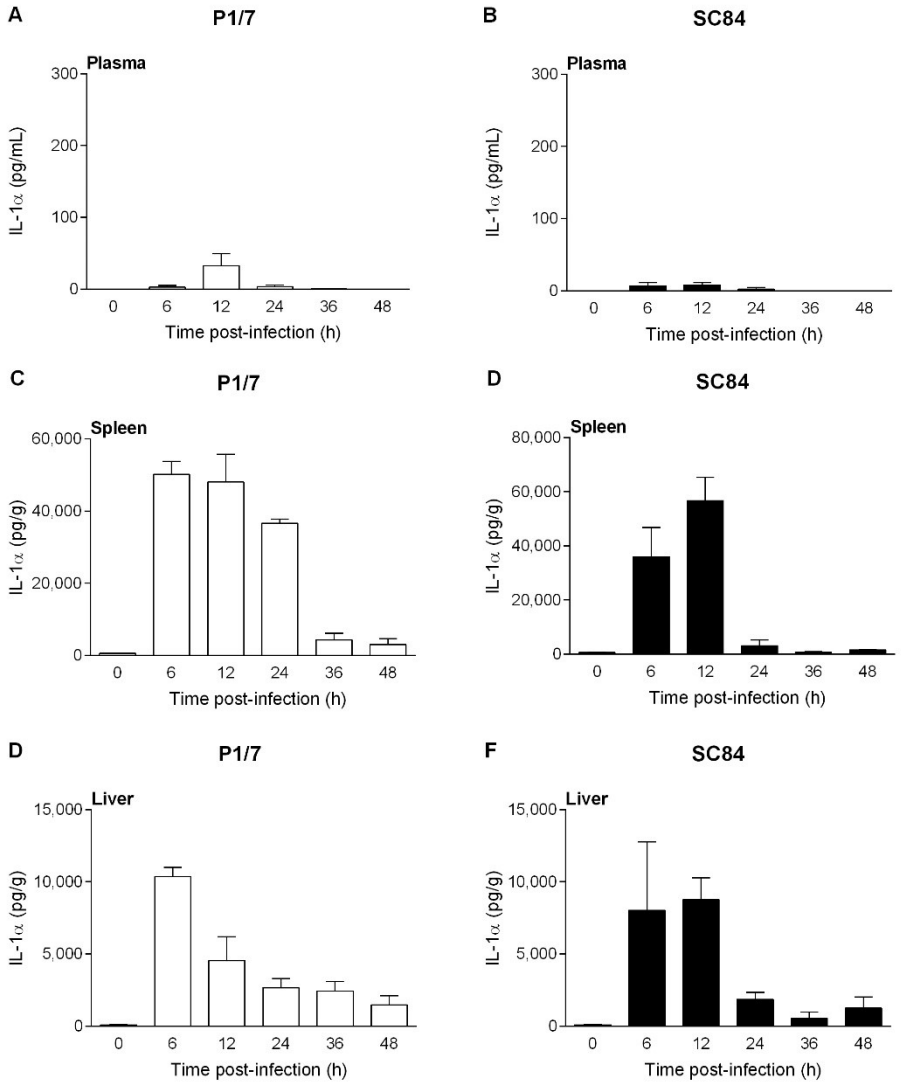


Figure S2. IL-1α release from dendritic cells (DCs) and macrophages (MΦ) stimulated with *Streptococcus suis* is strain-dependent. IL-1α kinetics as measured by ELISA following infection of DCs (**A** & **C**) or MΦ (**B** & **D**) with strain P1/7 (white bars) or SC84 (black bars). Non-stimulated cells served as negative control (C-). Data represent the mean ± SEM of at least 3 independent experiments.

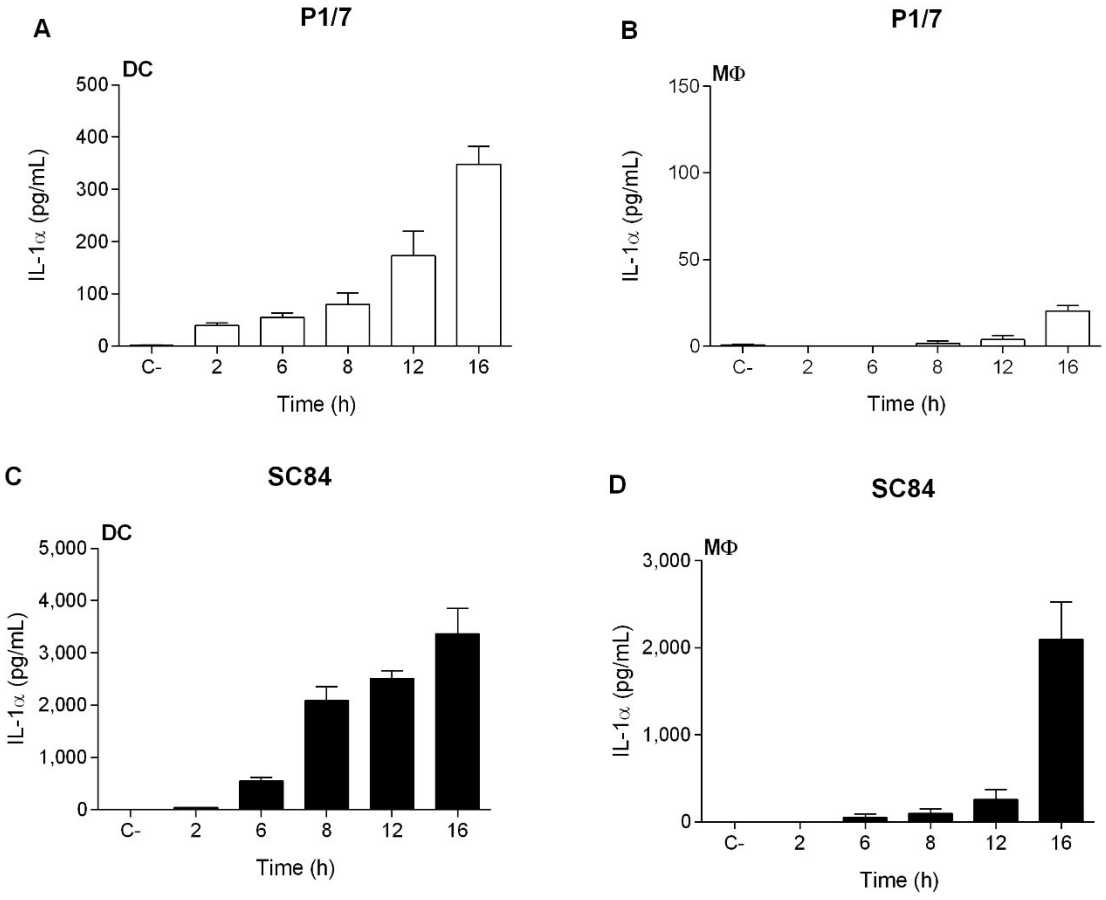


Figure S3. ***Streptococcus suis*-induced TNF production is inflammasome-independent.** Percentage of TNF secretion by caspase-1 (CASP-1), NLRP3, AIM2, NLRP1 or NLRC4-deficient dendritic cells induced by strain P1/7 (white bars) or SC84 (black bars) after 16 h, in comparison to wild-type counterparts. Data represent the mean ± SEM of at least 3 independent experiments.

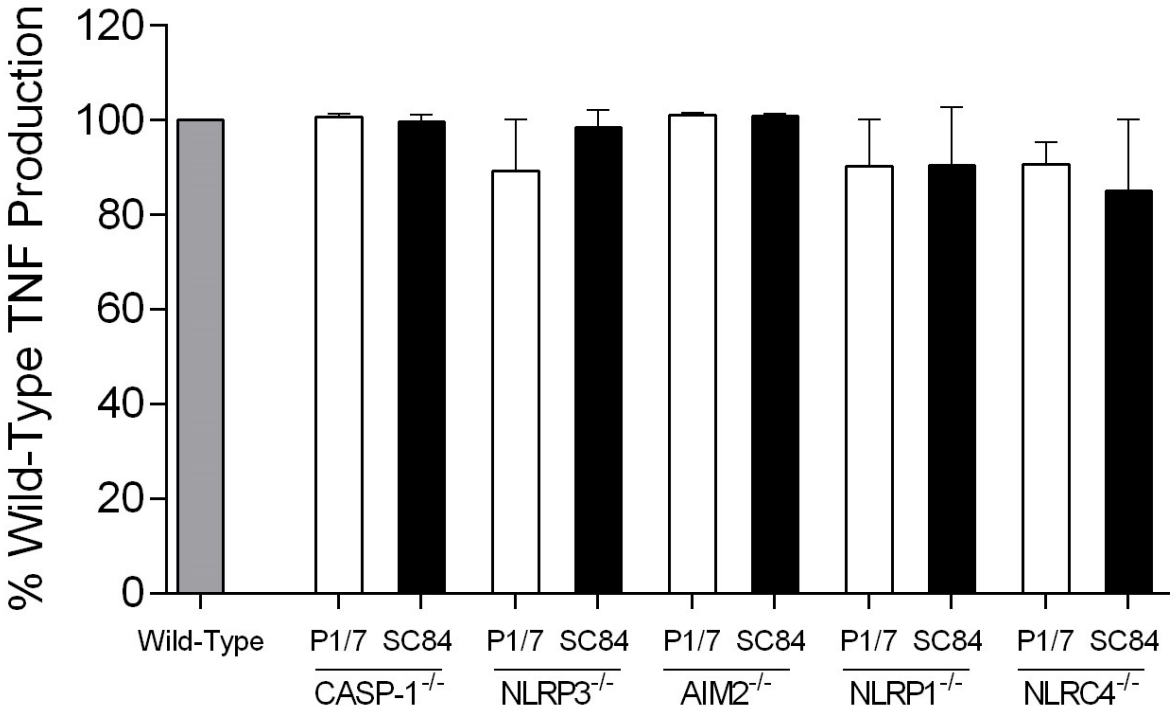


Figure S4. ***Streptococcus suis*-induced IL-6 and TNF secretion by dendritic cells (DCs) is independent of additional extracellular potassium (K^+) concentrations.** DCs were infected with either strain P1/7 or SC84 in the presence of different concentrations of KCl and IL-6 (**A**) or TNF (**B**) production was measured after 16 h by ELISA. Data represent the mean ± SEM of at least 3 independent experiments.

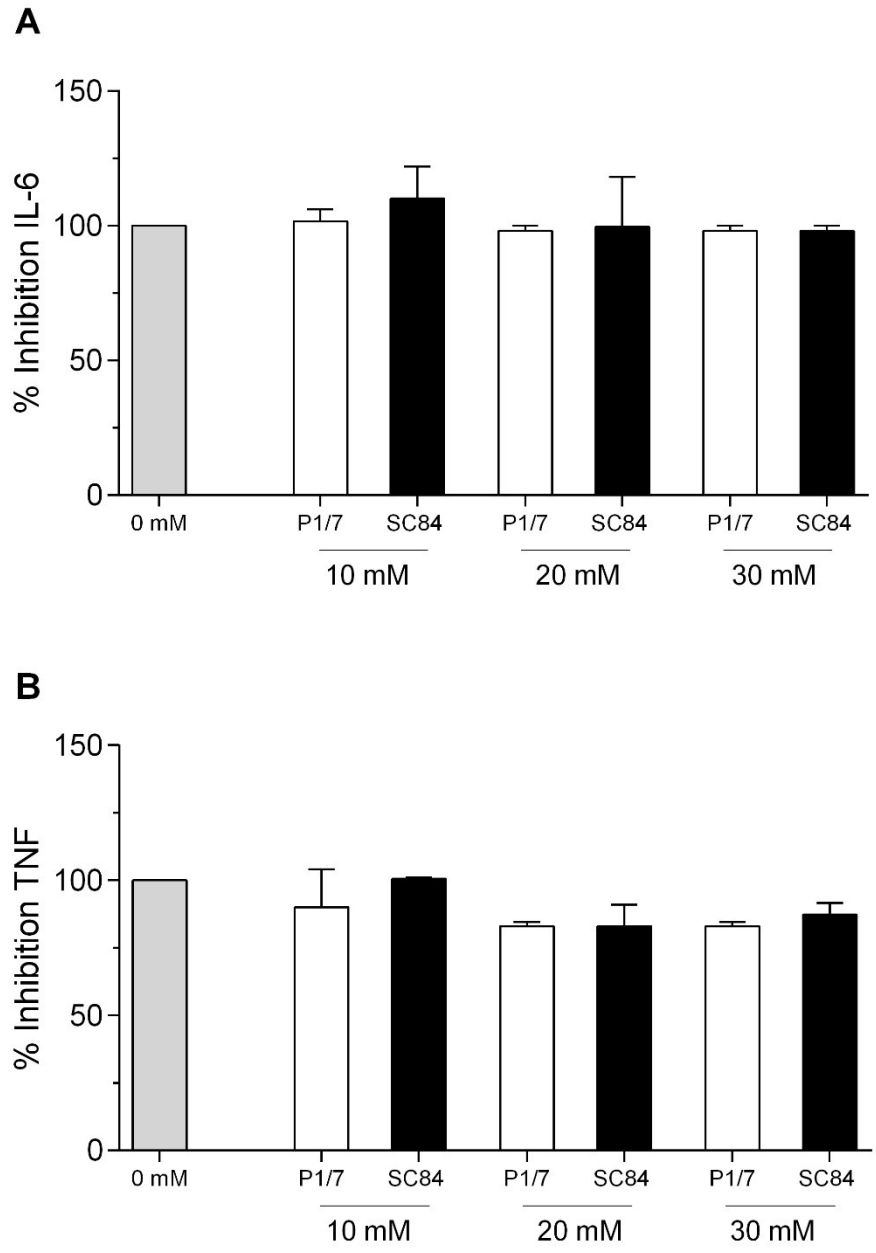


Figure S5. IL-1β production by recombinant suilysin (rSLY) is Toll-like receptor (TLR) 4-independent. IL-1β secretion by wild-type (WT) and TLR4$^{-/-}$ mice stimulated for 16 h with rSLY (5 μg/mL). Data represent the mean ± SEM of at least 3 independent experiments.

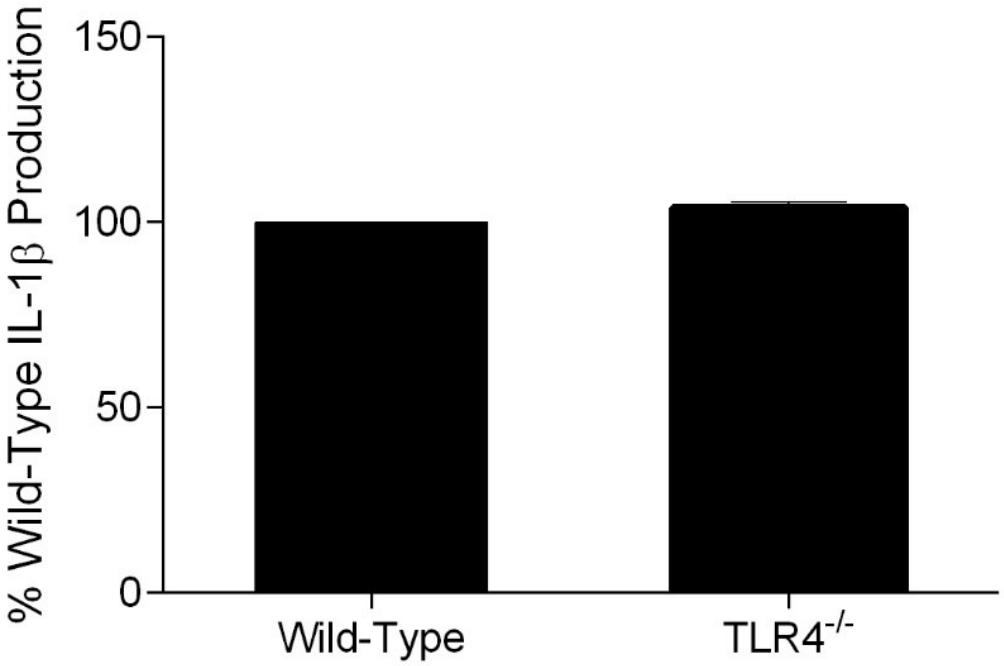
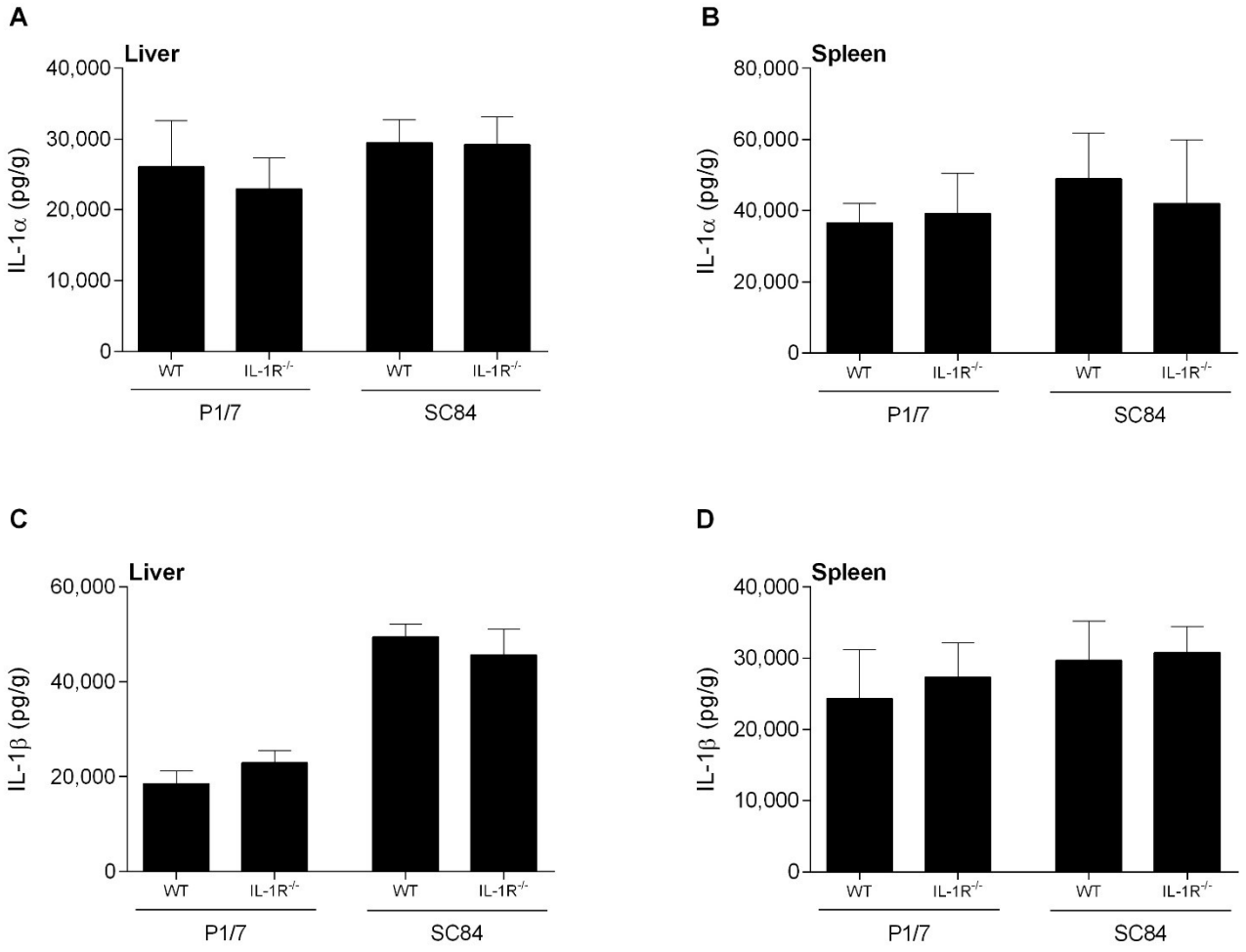


Figure S6. IL-1 does not modulate its own production following *Streptococcus suis* infection. Spleen and liver levels of IL-1α (A & B) and IL-1β (C & D) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following infection with strain P1/7 or SC84. Data represent the mean ± SEM of at least 4 individuals.



IV- Discussion

Innate immunity plays a central role in the pathogenesis of *S. suis* since the host's ability to control bacterial growth and dissemination relies mainly on its activation. However, a massive release of pro-inflammatory mediators by immune cells can lead to an excessive and uncontrolled inflammation, which is a hallmark of *S. suis* infection. Among these mediators, IL-1 is regarded as one of the earliest and most potent cytokines produced in sterile and bacterial inflammation. However, there are no studies focusing on *S. suis*-induced IL-1 production. Consequently, the role of IL-1 signaling as well as the mechanisms involved in its production in the context of systemic *S. suis* infection were evaluated.

Of the 35 serotypes of *S. suis* described for the moment, serotype 2 is the most commonly isolated from diseased animals and humans in most countries. Within serotype 2, strains are genotypically and phenotypically heterogeneous and consequently virulence is also diverse. In this study, two *S. suis* serotype 2 strains were evaluated: the classical virulent European ST1 strain (P1/7) and the highly virulent ST7 strain (SC84), responsible for the 2005 human outbreak in China. In addition, for comparison purposes, a representative typical intermediately pathogenic ST25 strain (89-1591) was also used (see Annex II). This North American strain, unlike P1/7 and SC84 strains, does not produce pore-forming toxin SLY (12). Comparison of the different strains provided insight regarding the differential mechanisms involved in IL-1 production and, consequently a better knowledge of the *S. suis* serotype 2 pathogenesis.

1- *S. suis* induced high levels of IL-1 in systemic organs but not in plasma.

Although IL-1 plays an important role during bacterial inflammation, previous studies on *S. suis* showed relatively low levels of IL-1β in plasma. In fact, when comparing with other important pro-inflammatory cytokines such as TNF and IL-6 (106, 149), the low values of IL-1 found lead to believe that this cytokine does not play a major role during *S. suis* systemic infection. Consequently, the low levels of IL-1β in plasma observed in this work following infection with strain P1/7 were expected. Moreover, similar results were obtained following infection with strains 89-1591. Being of a lower virulence than strain P1/7, results were not

surprising either (**see Annex II – Fig. 1**). Curiously, however, following infection with strain SC84, a strain which causes septic shock and induces an excessive inflammatory reaction, low values of IL-1 were obtained as well. Importantly, the low levels were found throughout the infection, even when mice presented symptoms of infection. These results suggested that the levels of this cytokine remain low in bloodstream independently of strain virulence. Importantly, levels of IL-1α, which were never assessed before in the context of *S. suis* infection, were also low, regardless of the strain or the time post-infection. Significant elevation in plasma IL-1 have been detected in animal and humans injected with LPS, but circulation IL-1 levels were still relatively low compared with levels of IL-6 and TNF (225). It is important to remark that IL-1 has an important effect on the vascular wall, so a high level of this cytokine could rapidly induce severe hypotension and death, which suggests the need for a controlled level. Furthermore, the near lack of IL-1 in plasma could be also due to the fact that unlike other cytokines, significant amounts of IL-1 remain inside cells (225). Moreover, IL-1 has a short half-life in plasma and also can associate with other plasmatic proteins such as α-2-macroglobulin and the complement, facts that could make its measure more difficult (225, 226).

After gaining access to the bloodstream, *S. suis* can disseminate and reach important organs such as liver and spleen. In fact, the high bacterial loads in these organs, which were previously described (16, 149) could lead to the activation of resident immune cells. In fact, Lachance *et al.* demonstrated that a great number of genes, particularly pro-inflammatory mediators, were upregulated following infection with several strains of *S. suis* using an Illumina whole-genome microarray assay on spleen samples, and that the number and level of those genes correlated with *S. suis* virulence degree (16). In this study, the evaluation of IL-1 levels in liver and spleen showed significantly high levels of IL-1α and IL-1β following infection with the three evaluated strains. The pattern was similar between strains with the highest values during the first 24 h, which then returned to basal (**see Annex II – Fig. 1**).

Together, these results suggest that although mice present symptoms of systemic infection, high levels of IL-1 cannot be observed in plasma, no matter the virulence of the strain, and probably due to intrinsic characteristics of the cytokine. However, a strong activation of resident immune cells by infiltrating bacteria in liver and spleen leads to a high IL-1 production, which remains locally in these organs no matter the virulence degree. The elevated levels of

IL-1 might be important for immune cell recruitment and later bacterial clearance. However, the elevated levels of IL-1 might also participate in the development of septic shock and organ failure frequently observed in cases of *S. suis* infection.

2- IL-1 production induced by *Streptococcus suis* serotype 2 is strain-dependent

IL-1 being an important cytokine, and given the elevated levels found in liver and spleen, it was of interest to the study the mechanisms involved in IL-1 production. Moreover, understanding the differences between strains could help clarify the pathogenesis of the infection by this bacterium.

Several studies previously demonstrated interactions between *S. suis* and different cell types. Murine dendritic cells (DCs) and macrophages (MΦ) were widely used and shown to be a good model for the study of bacterial internalization, interactions between *S. suis* and immune receptors, and cytokine-induced pathways. Moreover, DCs and MΦ are phagocytic cells, residents in filter organs such as liver and spleen, and probably some of the cell types involved in the high IL-1 production observed *in vivo*. In our study, we observed a modest production of IL-1 after stimulation of DCs with strain P1/7. By contrast, very high levels of IL-1α and IL-1β were induced by strain SC84. These results, in accordance with the virulence degree of the strain, suggested that extra virulence factors are probably involved in an enhanced cell activation. Surprisingly, however, DCs stimulated with the intermediate virulence strain 89-1591 produced higher levels than P1/7 (**see Annex II – Fig. 2 and 3**) further indicating that cell activation leading to IL-1 production differs between strains. Interestingly, overall production of IL-1 by MΦ was somewhat delayed when compared to DCs following infection with strains P1/7 and SC84. This characteristic was already described for other extracellular pathogens including GBS, GAS, and *S. pneumoniae* (192, 218, 227), and might be due to a lower capacity of these cells to process the cytokine into the mature form.

Studies have shown that recognition of *S. suis* generally requires implication of the TLR pathway, which results in production of several pro-inflammatory mediators, including IL-1

(105). The use of the adaptor protein MyD88 after TLR engagement was demonstrated to be an important step in cytokine production during *S. suis* infection since most of the mediators tested were critically reduced in the absence of this adaptor protein ($MyD88^{-/-}$ cells) (105). Importantly unlike with most other cytokines, IL-1β production is controlled by a two-step signaling system. Firstly, activation of TLRs leads to the transcription of proIL-1β. Subsequently, a second signal induces cleavage of the precursor into active IL-1β through caspase-1- and inflammasome-dependent maturation. In this study, we identified that *S. suis* is able to activate both signals. However, while the cellular activation leading to IL-1β production was similar for strains 89-1591, P1/7, and SC84, maturation of the cytokine was strain-dependent, which resulted in differential levels of IL-1β secretion.

More precisely, IL-1 production induced by *S. suis* strains 89-1591, P1/7, and SC84 depended on the adaptor protein MyD88, with a partial involvement of TLR2 resulting from recognition of surface lipoproteins (**see Annex II - Fig. 4 and 5A**). This demonstrates that components involved in activation of DCs are relatively well-conserved between the strains, fact that might be the case for all *S. suis* serotype 2 strains. Comparable results were also reported for GBS and *S. pneumoniae* suggesting that the recognized bacterial motifs are probably conserved even between streptococci (192, 228). Involvement of TLR4 was also evaluated since it can recognize not only LPS from Gram-negative bacteria, but also certain toxins such as pneumolysin (133, 229), listeriolysin O (134), and more recently *S. suis*-produced SLY (135). In our study, IL-1β production by DCs induced by strain 89-1591 was TLR4-independent, which is not surprising since it does not produce SLY. However, production induced by P1/7 and SC84, which both produce SLY, was also TLR4-independent. These results are in accordance with previous studies from our lab (105). In fact, TLR4 was not involved in rSLY-induced IL-1β production by DCs, demonstrating that unlike with other toxins, TLR4 does not play a role in IL-1β production. Moreover, IL-1β production was TRIF-independent. To our knowledge, this is the first study evaluating the role of TRIF during *S. suis* infection. This further confirms the lack of TLR4 implication in *S. suis*-induced IL-1β production since this adaptor protein is engaged by TLR4 (and TLR3).

Since production was almost abrogated in the absence of MyD88, yet implication of TLR2 was only partial, the involvement of endosomal TLR7 and TLR9, which detect nucleic

acids, was hypothesized. Although considered a classical extracellular pathogen, *S. suis* strains P1/7 and SC84 can be internalized, albeit at low rates. In contrast, strain 89-1591 is significantly more internalized (132). In fact, when internalized, TLR7 and TLR9 were shown to recognize *S. suis*, resulting in IFN-β production (132). In this study, we demonstrated that RNA and DNA from the different strains induced IL-1β production. Importantly, such production was only observed when DNA and RNA were complexed with DOTAP, suggesting that recognition occurs in a process similar to that of IFN-β, following internalization and degradation of bacterium (**see Annex II - Fig. 5B**).

Following engagement of TLRs, activation of the MAPK and NF-κB signaling pathways results in the initiation of an inflammatory response leading to cytokine production. In fact, previous studies with *S. suis* and other pathogens such as GBS, *S. pneumoniae*, and GAS have demonstrated an implication of these pathways in the production of several cytokines by various cell types (108, 230-232). In accordance, IL-1β production by DCs induced by strains P1/7 and SC84 was dependent on the NF-κB and ERK pathways. Meanwhile, IL-1β production induced by strain SC84, but not strain P1/7, was also p38-dependent, suggesting a differential mechanism in its activation, possibly due to differences in bacterial components or virulence. For example, pore-forming toxin secretion and its induced osmotic stress were described to modulate MAPK phosphorylation for listeriolysin O and streptolysin O (230, 233). As such, the higher production of SLY by strain SC84 could be involved in p38 activation, which remains to be confirmed. Moreover, pathways used by strain 89-1591 are under evaluation.

So far, the receptors involved in IL-1β production, as well as the pathways engaged by this pathogen, could not explain the differences observed between strains. Moreover, in accordance with these results, the study of IL-1β gene induction indicated that the three strains evaluated induced the same levels of IL-1β mRNA (**see Annex II - Fig. 6**), As mentioned before, the production of mature IL-1β involves cleavage of proIL-1β, mainly by caspase-1. As expected, IL-1β production by DCs activated by strains 89-1591, P1/7 or SC84 depended on this protease (**see Annex II - Fig. 7**). In fact, unlike with mast cells and neutrophils, which also produce numerous proteases, maturation of IL-1β in DCs has only been reported to be caspase-1-dependent (183, 192). Similar to proIL-1β, caspase-1 also requires activation via proteolytic processing. This step is mediated by multi-protein complexes, termed inflammasomes,

consisting of a sensor protein and an adaptor molecule that links it to pro-caspase-1 (181). To date, most inflammasomes described contain a NOD-like receptor (NLR) family as the sensor molecule (180). This includes the three best-studied complexes: the NLRP3, the NLRP1, and the NLRC4 (181). In addition, the AIM2 inflammasome has a DNA-binding HIN domain instead (182). In our study, we observed a differential pattern in inflammasome activation between strains. While maturation of proIL-1β induced by strain P1/7 was only partially dependent on the NLRP3 and AIM2 inflammasomes, and weakly so, strain 89-1591 activated these inflammasomes more importantly (**see Annex II - Fig. 7**). Interestingly, strain SC84 activated NLRP3 and, to a lesser extent the AIM2, NLRP1 and, surprisingly, NLRC4 inflammasomes. These differences in inflammasome activation between strains, both in the different inflammasomes activated and their implication levels, could explain the differential IL-1β levels produced by DCs. Although NLRP3 and AIM2 participate in IL-1β release by DCs and MΦ following infection by GBS and *S. pneumoniae* (192, 234), the implication of NLRP1 and NLRC4 have not yet been described following streptococcal infection (235).

Several stimuli have been reported to trigger inflammasome formation, subsequent caspase-1 activation, and IL-1β maturation. Therefore, it is difficult to determine the specific factors responsible for *S. suis*-dependent inflammasome activation. Previous studies showed that NLRP1 could directly sense the protease activity of the *Bacillus anthracis* lethal toxin (236). Although activation of NLRP1 by streptococcal pore-forming toxins has not yet been evaluated, elevated levels of the *S. suis* SLY might be involved in a similar process. Moreover, strain SC84, unlike strain P1/7, also possesses a type IV secretion system encoded by its 89 K pathogenicity island (49), which might be responsible for NLRC4 activation (201) although this remains only an hypothesis. Regarding the AIM2 inflammasome, it has been previously shown to be activated by DNA (205). In accordance, we observed that levels of IL-1β induced by DNA were higher than those induced by RNA. Furthermore, a greater internalization of strain 89-1591 could be responsible for the stronger activation of AIM2 via DNA. Indeed, production of IL-1β depended greatly on internalization for this strain, as shown by inhibition of actin polymerization with cytochalasin D (**see Annex II – Fig. 8**). What its more, activation by DNA was reduced in AIM2$^{-/-}$ cells, further confirming this hypothesis (**see Annex II – Fig. 9**).

In addition, another theory based on ion effluxes might explain the broad inflammasome activation observed for strain SC84 since potassium efflux was demonstrated to be a common denominator in the assembly and activation of the four evaluated inflammasomes (180, 237). In fact, previous studies demonstrated that low intracellular K^+ concentrations are required to activate inflammasomes (189, 193, 194). In accordance, the addition of extracellular K^+ blocked its efflux, and consequently assembly of these inflammasomes and IL-1β maturation induced by strains P1/7 and SC84. Importantly, although only 10 mM of K^+ was sufficient to reduce IL-1β production by both *S. suis* strains, the effect was accentuated for SC84, a fact that goes along with previous results showing a broader inflammasome activation by this strain. Moreover, even though inhibition was concentration-dependent, addition of more than 40 mM had a cytotoxic effect on DCs, with 100% cell death when using 130 mM.

Bacterial pore-forming toxins could play a key role in this theory since they can generate K^+ efflux through the membrane, thus reducing the intracellular concentration of this ion, causing inflammasome assembly, and consequently IL-1β maturation (237). In fact, previous studies showed that these types of toxins, which include pneumolysin and the β-hemolysin of GBS, were required for IL-1β production by MΦ and DCs stimulated with live bacteria *in vitro* (25, 229). In this study, we showed that SLY plays an important role for the SC84 strain, but not for P1/7, and that this role is associated only with IL-1β maturation. The reduction of IL-1β observed after addition of cholesterol, which is an inhibitor of SLY, further supports these results. The fact that levels of SLY produced by strain SC84 are much higher than those produced by strain P1/7 could explain these differences. Indeed, the fact that a role of SLY was observed for strain SC84, but not P1/7, when using the SLY-deficient mutant confirms that a minimal level of SLY (threshold) is required. In other words, although cell activation by strains P1/7 and SC84 leads to similar levels of proIL-1β, the high levels of SLY produced by strain SC84 result in a more efficient maturation of IL-1β via pore formation, depletion of K^+ efflux, and a wider inflammasome activation.

To further evaluate the role of SLY, the recombinant protein was produced and purified. Though rSLY itself induced some IL-1β secretion from DCs, levels were similar to those observed when cells were stimulated with Alum alone. Since Alum is a known activator of NLRP3, but not an inducer of IL-1β mRNA, it has been suggested to cause maturation and

release of the IL-1β naturally synthesized by the cell in the absence of prior stimulation (238). In fact, addition of Alum to DNA and RNA increased the production of IL-1β, probably through activation of NLRP3 inflammasome. As such, rSLY may very well have the same effect. Addition of rSLY to strain P1/7 or SLY-deficient mutants synergistically increased IL-1β production by DCs, supporting this hypothesis. Noteworthy, the *sly*-deficient SC84 mutant was able to produce similar IL-1β levels to those produced by strain P1/7, suggesting that classical *S. suis* strains possess other mechanisms that participate in IL-1β maturation. Moreover, strain 89-1591-induced IL-1β was also able to activate the inflammasomes in a different way, further indicating that other components are in play.

Together, these data demonstrate that DCs and MΦ are an important source of IL-1 following *S. suis* infection. Moreover, we showed that the intermediate virulent strain 89-1591, the virulent strain P1/7, and the highly virulent strain SC84, similarly activate innate immune cells due to conserved bacterial components such as LPs. However, the pore-forming toxin SLY, which is highly produced by strain SC84 only, plays an important role in IL-1β maturation via activation of the NLRP1, NLRP3, AIM2, and NLRC4 inflammasomes.

3- The role of IL-1 in *S. suis* systemic infection depends on inflammation

As previously mentioned, IL-1 is extremely important for the initiation and amplification of inflammation. In fact, after being secreted, IL-1α and IL-1β bind their shared receptor, IL-1R, which is ubiquitously expressed, resulting in cell activation, stimulation of the secretion of other pro-inflammatory cytokines (positive feedback loop), recruitment of neutrophils and macrophages, and activation of killing mechanisms, amongst other effects (121). Since high levels of this cytokine were found in liver and spleen, its role was further evaluated using IL-1R knock-out mice (IL-1R$^{-/-}$). It is important to note that although these mice have the ability to produce IL-1, the cytokine has no effect since there is no other receptor than IL-1R to transduce its signal.

Previous studies with GBS and *S. pneumoniae* showed a protective role of this cytokine during infection since absence of IL-1 signaling (whether via lack of IL-1R, the cytokine itself or any mediator involved in its production) altered bacterial clearance and survival (23-26). However, a recent study with GAS demonstrated that levels of IL-1β must be within a certain range since both excessive and defective IL-1β responses led to lethal disease (27). In this study, we observed a beneficial role of IL-1 following infection with *S. suis* strains 89-1591 and P1/7 since survival of IL-1R$^{-/-}$ mice was significantly reduced compared to that of their wild-type counterparts (**see Annex II – Fig. 11**). Moreover, IL-1 signaling induced by these two strains was necessary to modulate the host innate immune response by increasing production of other pro-inflammatory cytokines and chemokines (IL-6, IL-12p70, IFN-γ, CCL2, CCL3, and CXCL9) (**see Annex II – Fig. 12-14**). In addition, the lack of IL-1 signaling, and by consequence the lack of inflammatory mediators, impeded control of bacterial growth in blood and in internal organs, resulting in host death. Interestingly, due to its lower virulence and reduced immunomodulation, differences in bacteremia and bacterial load in liver and spleen following infection with strain 89-1591 were only observed later in the infection (48 h and 72 h p.i.) (**see Annex II – Fig. 15**). Nevertheless, even in the presence of IL-1 (i.e wild-type mice), a certain number of mice still succumbed to infection due to an exacerbated inflammatory response induced by the pathogen, demonstrating the need in a balanced and controlled inflammation during *S. suis* infection.

It is interesting to further discuss the case of the highly virulent strain SC84. Similar to what has been described for type I IFN (132), the "protective effect" of IL-1 was not observed following infection with this strain: survival following infection with a standard dose showed no significant differences between wild-type and IL-1R$^{-/-}$ mice 7 days p.i. In accordance, there were no differences in bacterial load in blood, liver or spleen between wild-type and IL-1R$^{-/-}$ mice 12 h and 48 h p.i. In fact, the similar bacterial load in 89-1591-, P1/7-, and SC84-infected wild-type mice indicates that the difference in the role of IL-1 signaling observed is not due to excessive bacterial burden by SC84 but rather to strain virulence. These results are in accordance with Lachance *et al*., who also observed that strain virulence and induced mortality do not depend on bacterial load (16). Indeed, independently of the presence or absence of IL-1 signaling, levels of pro-inflammatory mediators were extremely high following infection with

strain SC84, demonstrating an uncontrolled inflammatory reaction. In fact, the levels of IL-1 induced by this strain are unable to modulate overall inflammation and host outcome. We believe that the highly virulent ST7 strain SC84, which possesses additional virulence factors such as an 89 K pathogenicity island (239) and higher levels of SLY, could be responsible for a greater innate immune system activation, resulting in a cytokine storm. In fact, this cytokine storm is associated with the streptococcal toxic shock-like syndrome caused by this strain (46).

These results suggest that the levels of induced inflammation play a critical role during *S. suis* systemic infection, and this regardless of the strain. In accordance, using a non-lethal dose of strain SC84, during which inflammation may be controlled, IL-1 plays a beneficial role as well (**see Annex I – Fig. 1**). Moreover, similar to what was observed with strains P1/7 and 89-1591, IL-1 signaling stimulated the synthesis of more inflammatory mediators required for bacterial clearance (**see Annex I – Fig. 2, 3, and 4**). In addition, using a higher dose of P1/7, the role of IL-1 could not be distinguished due to an inability of the host to clear bacteria (**see Annex I – Fig. 5 and 6**), similarly to during infection with a standard dose of the highly virulent strain SC84.

Globally, as illustrated in **Figure I** below, we demonstrated that IL-1 signaling plays a beneficial role in *S. suis* serotype 2 systemic infection. Beyond a certain threshold, however, *S. suis*-induced inflammation cannot be counterbalanced by this signaling, making it difficult to discriminate its role. In other words, while IL-1 signaling initiates the cascade of inflammation, generating a positive loop, and stimulating the synthesis of more mediators necessary to fight the pathogen, IL-1 signaling itself cannot counterbalance exacerbated inflammation, resulting in host death.

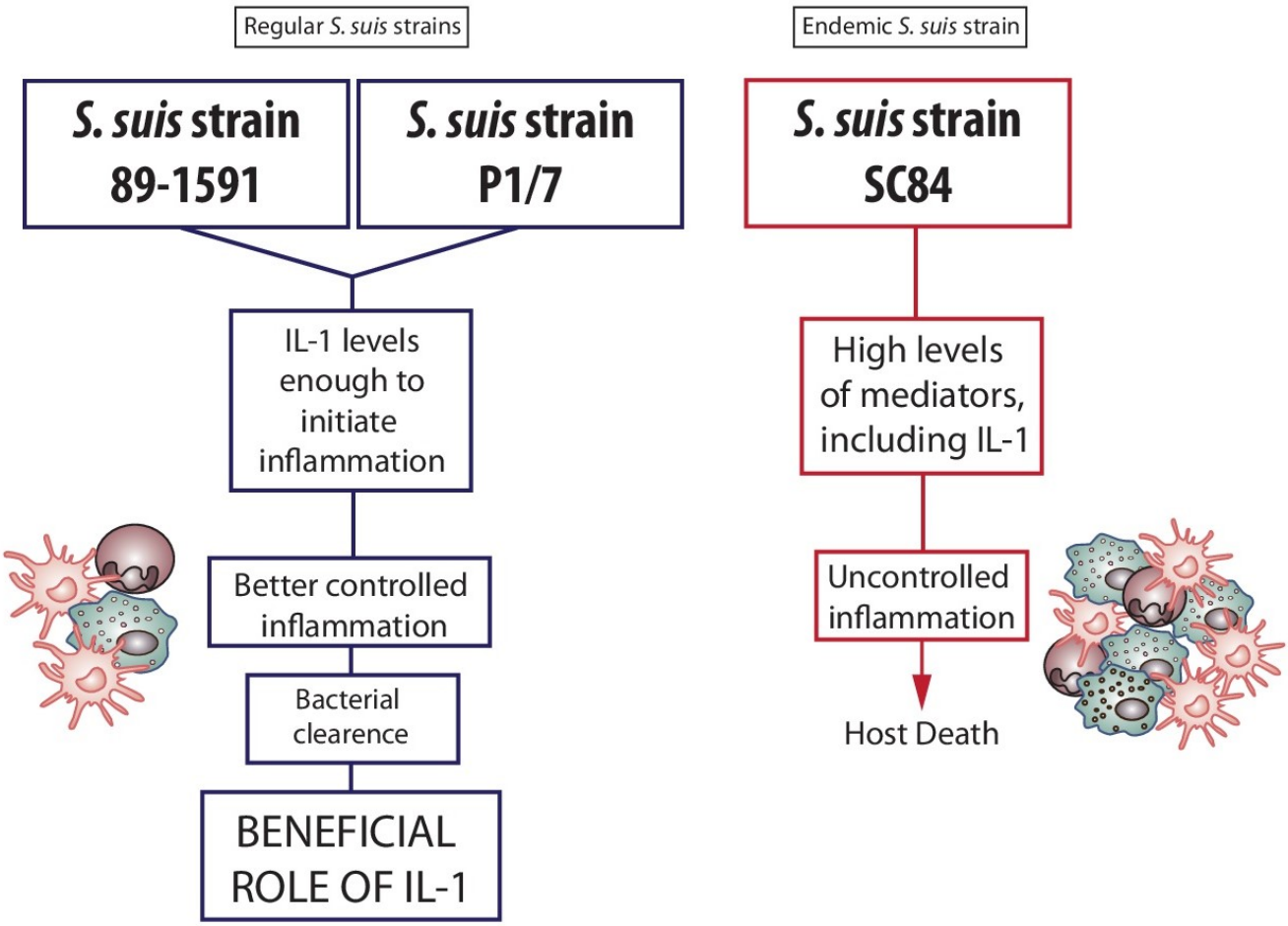


Figure I. Role of IL-1 in the pathogenesis of the infection caused by *S. suis* serotype 2.

V- General Conclusions and Perspectives

Conclusions

- ✓ The present work demonstrated that during *S. suis* serotype 2 infection, high levels of IL-1 are rapidly produced in both liver and spleen. This production remains locally within these two major filtering organs.

- ✓ Innate immune cells such as dendritic cells and macrophages were demonstrated to be a major source of IL-1. Additionally, levels of IL-1 induced by strain SC84 were higher than those induced by strains 89-1591 and P1/7.

- ✓ The first step required to produce mature and biologically active IL-1β (from TLR activation to gene transcription) was similar for the three strains evaluated, no matter their virulence. By contrast, the second step, responsible for proIL-1β maturation, was different between strains, accounting for the varying levels of IL-1β observed.

- ✓ High levels of pore-forming toxin SLY, produced mainly by strain SC84, play an important role in IL-1β maturation, leading to activation of not only NLRP3 and AIM2, but also of NLRP1 and NLRC4. Their role in streptococcal infections had not been described before. Additionally, such activation was due to pore formation and K^+ efflux.

- ✓ Finally, *S. suis*-induced IL-1 plays a beneficial role during systemic infection by initiating the inflammatory cascade required to clear the infection. However, beyond a certain threshold, *S. suis*-induced inflammation cannot be counterbalanced by this signaling pathway, making it difficult to precisely discriminate its role.

Perspectives

- ✓ Given the importance of *S. suis* as both a porcine and zoonotic pathogen, a better understanding of the mechanisms involved in the control of inflammation and bacterial burden can provide biomedical foundations for the development of effective control measures for this pathogen.
- ✓ Is the role of IL-1 found during *S. suis* systemic infection also the case during meningitis? The study on the production of IL-1 in brain after intracisternal infection will help us evaluate if IL-1 plays an important role or not during stages of meningitis.
- ✓ Experiments in pigs would be necessary to validate the results obtained in this study.
- ✓ As a long-term perspective, we propose a potential use of IL-1β in the prevention of infections caused by *S. suis* serotype 2 or as an immune booster.

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Annex I: Inflammation induced by *S. suis* plays a major role in host survival

Figure 1. Survival of wild-type (WT) and IL-1 receptor-deficient (IL-1R$^{-/-}$) mice after intraperitoneal infection with a non-lethal dose of *Streptococcus suis* strain SC84. Six-weeks old WT and IL-1R-/- mice were inoculated with a non-lethal dose (5 x 10^6 CFU) of strain SC84 and survival was monitored during the acute systemic infection until 10 days post-infection. Data represent survival curves (n=15). *** ($p < 0.001$) indicate a significant difference between survival of WT and IL-1R$^{-/-}$ mice.

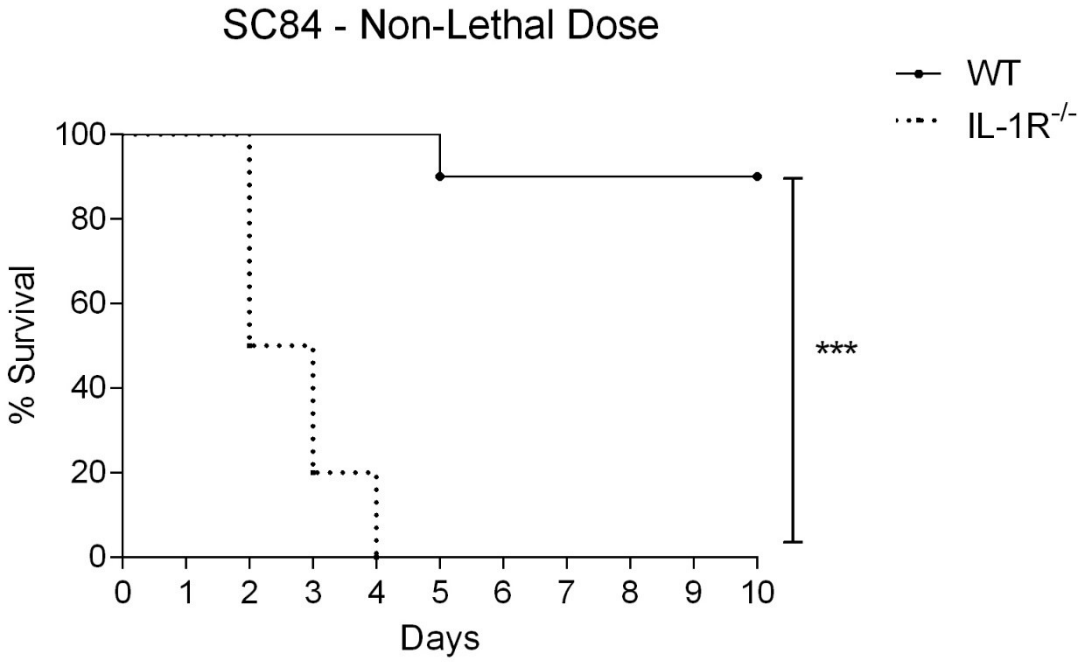


Figure 2. IL-1 modulates plasma pro-inflammatory mediators during *Streptococcus suis* systemic infection caused by a non-lethal dose of strain SC84. Plasma levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following the infection with a non-lethal dose of strain SC84 (5 x 10^6 CFU). Data represent the mean ± SEM (n = 4). * ($p < 0.05$) indicates a significant difference between wild-type and IL-1R$^{-/-}$ mice.

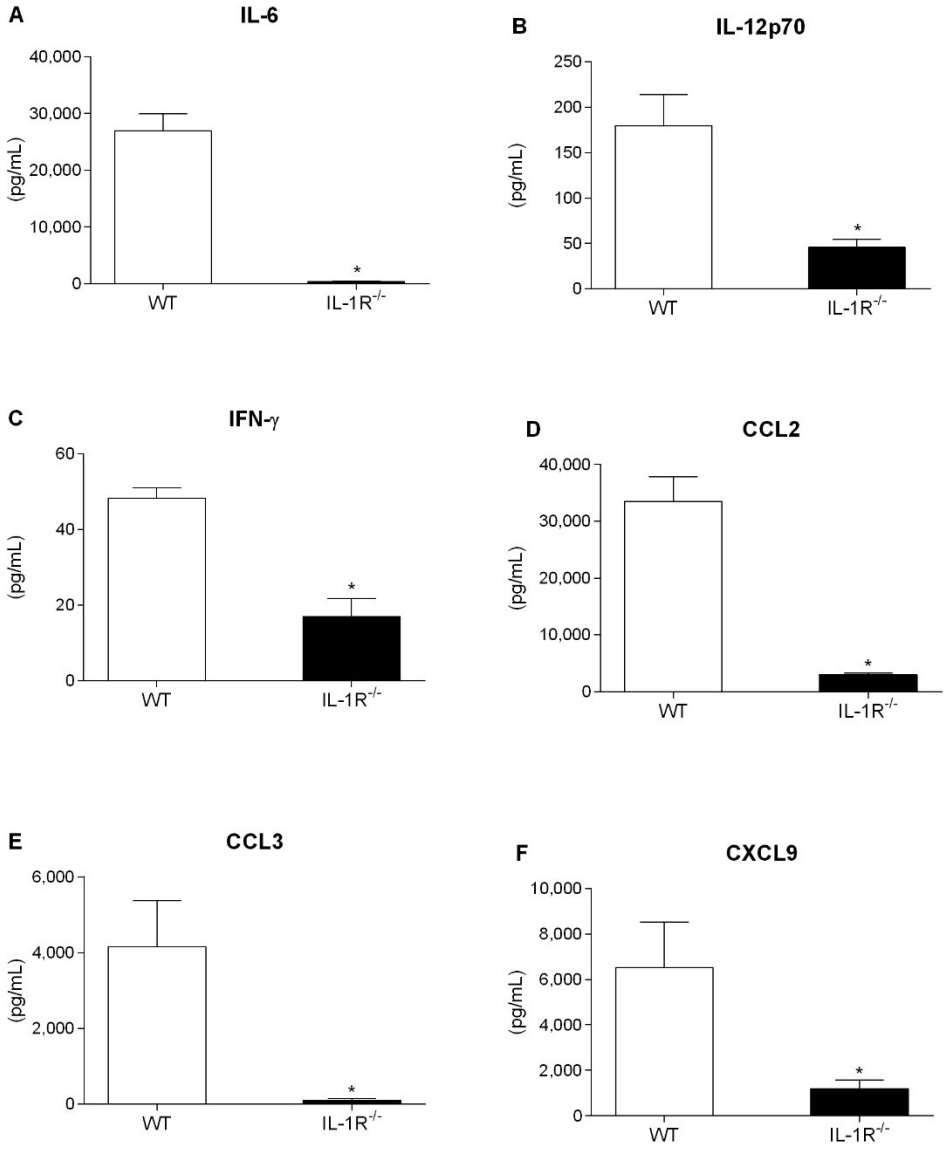
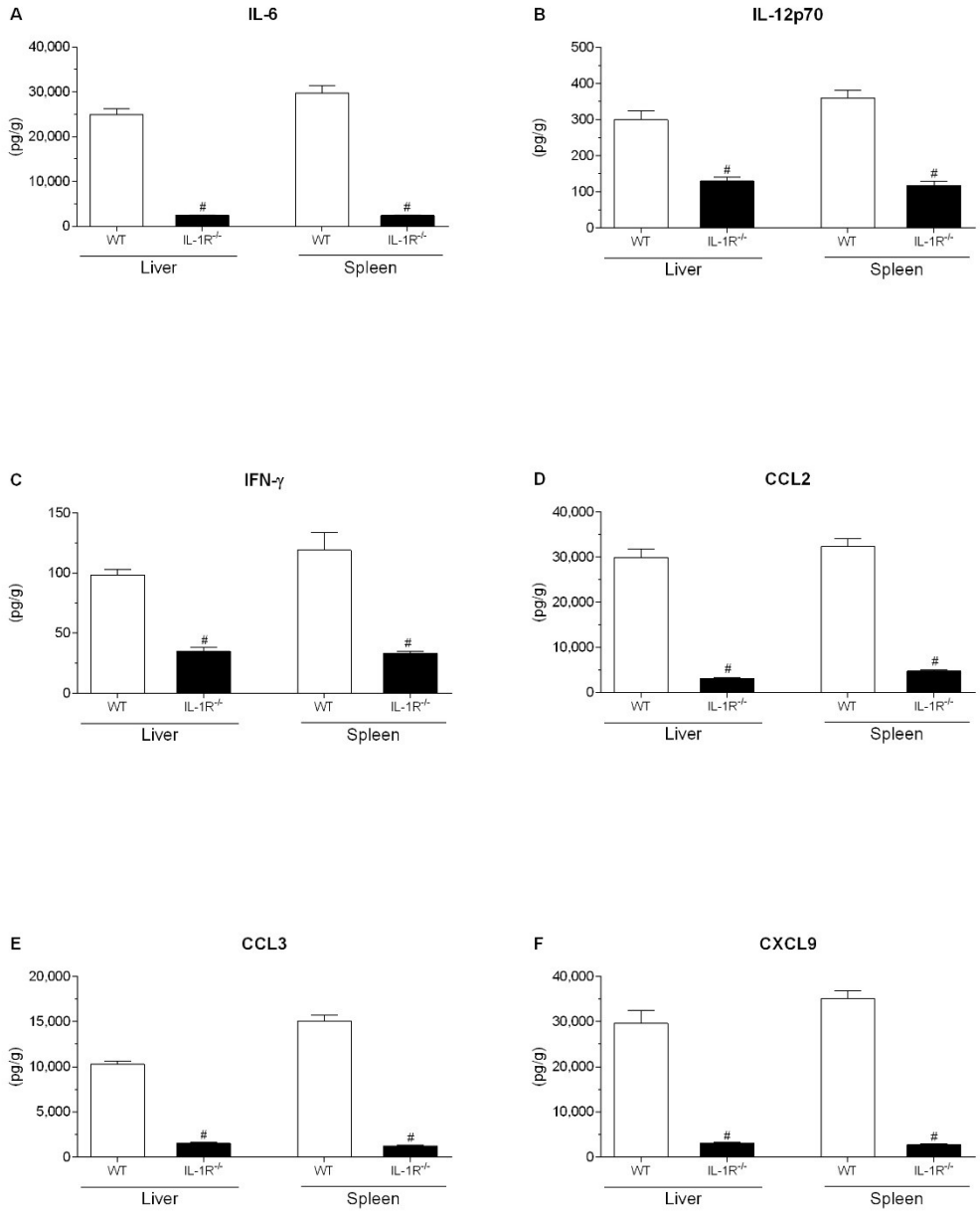


Figure 3. IL-1 modulates pro-inflammatory mediators in spleen and liver during



***Streptococcus suis* systemic infection caused by a non-lethal dose of strain SC84.** Spleen and liver levels of IL-6 (**A**), IL-12p70 (**B**), IFN-γ (**C**), CCL2 (**D**), CCL3 (**E**), and CXCL9 (**F**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h following the infection with a non-lethal dose of strain SC84 (5 x 10^6 CFU). Data represent the mean ± SEM (n = 4). $^{\#}$ ($p < 0.01$) indicate a significant difference between wild-type and IL-1R$^{-/-}$ mice.

Figure 4. IL-1 is required for control of bacterial burden in blood, liver, and spleen during *Streptococcus suis* systemic infection caused by a non-lethal dose of strain SC84. Bacterial burden in blood (A & B), liver (C & D), and spleen (E & F) of wild-type (WT) and IL-1R$^{-/-}$ mice infected with a non-lethal dose of strain SC84 (5 x 10^6 CFU) 12 h (left panel) or 48 h (right panel) post-infection. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. Data represent the geometric mean (n = 6 to 9). $^{\#}$ ($p < 0.01$) indicate a significant difference between WT and IL-1R $^{-/-}$ mice.

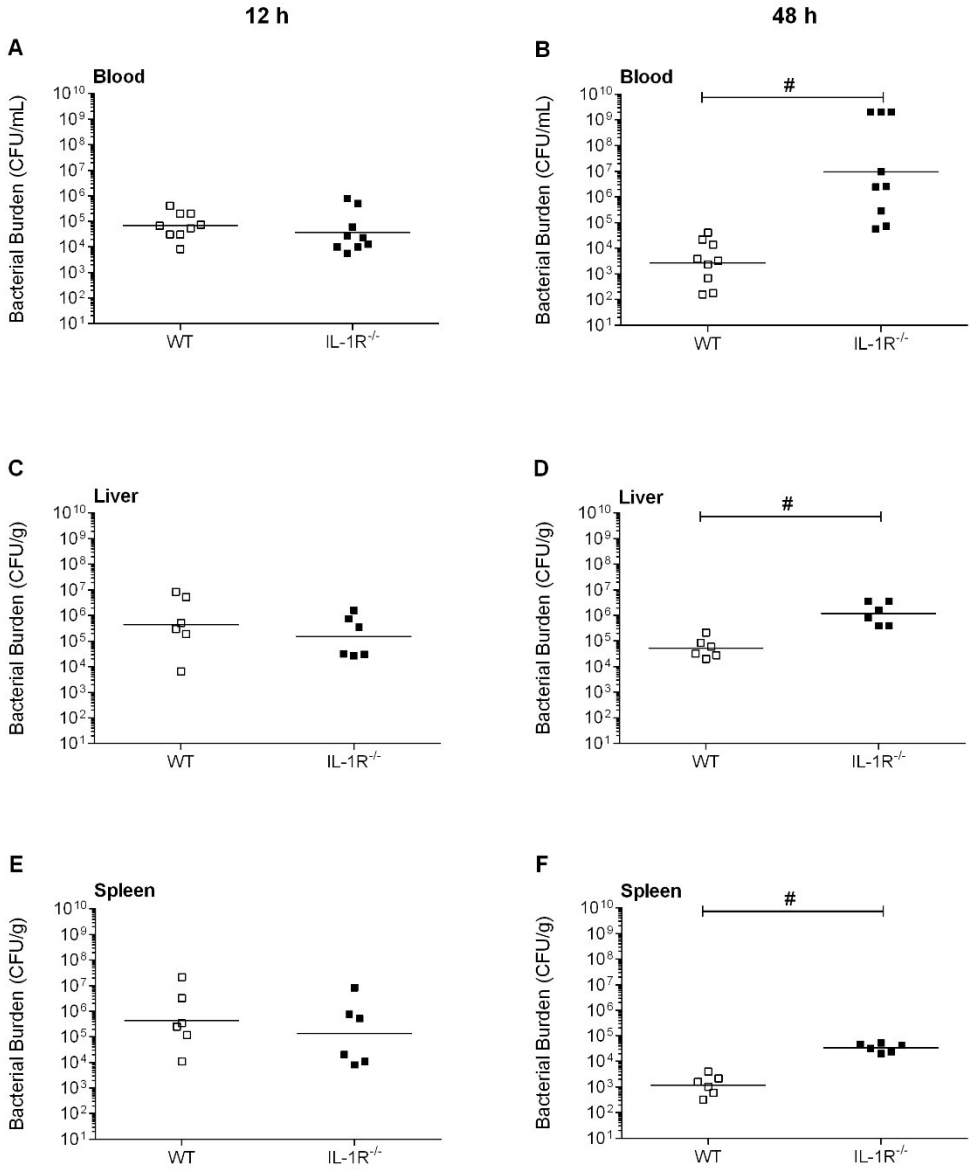


Figure 5. Survival of wild-type (WT) and IL-1 receptor-deficient (IL-1R$^{-/-}$) mice after intraperitoneal infection with a high dose of *Streptococcus suis* strain P/7. Six-weeks old WT and IL-1R$^{-/-}$ mice were inoculated with a high dose (5 x 10^7 CFU) of strain P1/7 and survival was monitored during the acute systemic infection until 10 days post-infection. Data represent survival curves (n=15).

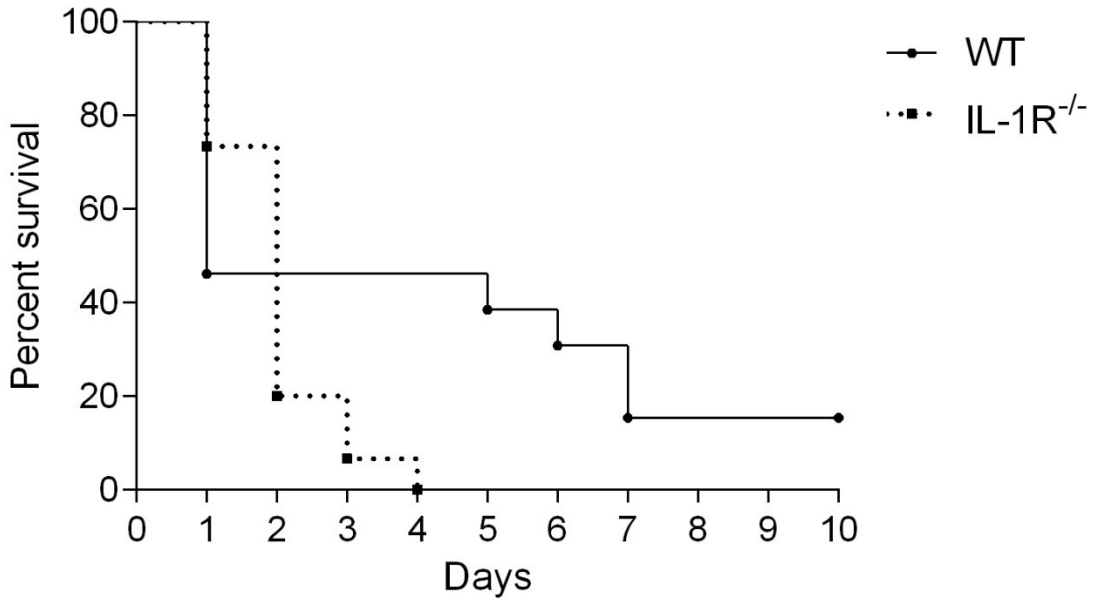
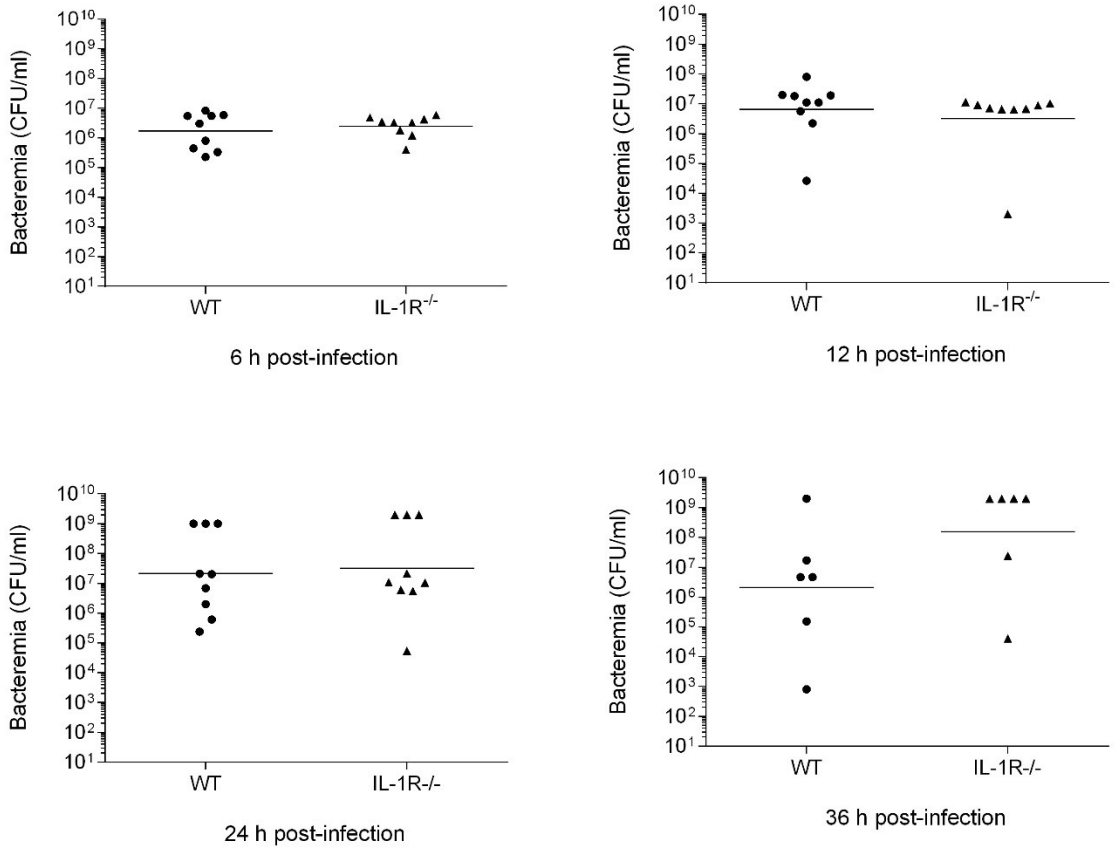


Figure 6. Bacterial burden in blood during *Streptococcus suis* systemic infection caused by a high dose of strain P1/7. Bacterial burden in blood of wild-type (WT) and IL-1R$^{-/-}$ mice infected with a high dose of strain P1/7 (5 x 10^7 CFU) 6 h, 12 h, 24 h or 48 h post-infection. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. Data represent the geometric mean (n = 6 to 9).



Annex II: *S. suis* serotype 2 strain 89-1591-induced IL-1

Figure 1. ***S. suis*** **strain 89-1591 induces elevated levels of IL-1 in liver and spleen, but not in plasma.**C57BL/6 mice were inoculated intraperitoneally with a standard dose (1 x 10^7 CFU) of *S. suis* strain 89-1591. Plasma (**A&B**), liver (**C&D**), and spleen (**E&F**) were collected at different times post-infection and the levels of IL-1α and IL-1β were quantified by ELISA. Values for uninfected controls did not show statistically significant changes from 6 h to 48 h. As such, 0 h represents results for non-infected mice throughout the experiment. Data are expressed as mean ± SEM (n = 3).

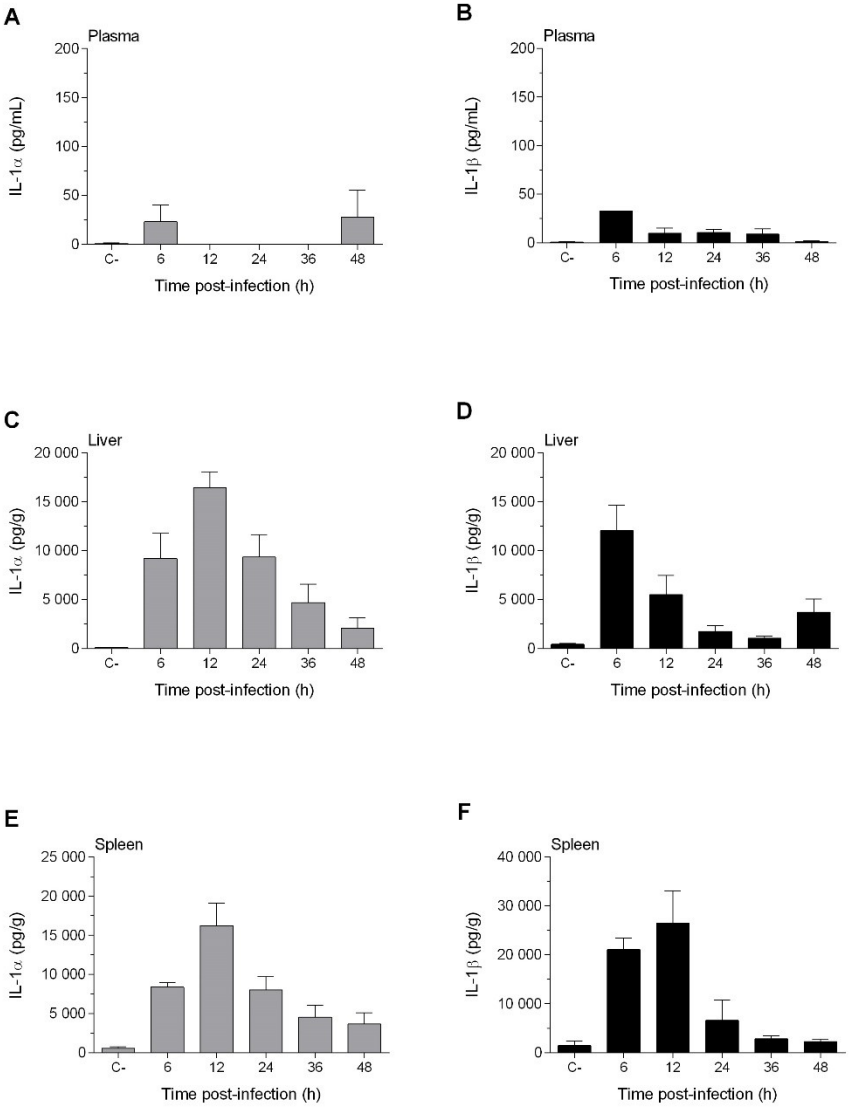


Figure 2. IL-1 released from dendritic cells (DCs) stimulated with *Streptococcus suis* strain 89-1591. IL-1α (**A**) and IL-1β (**B**) kinetics as measured by ELISA following infection of DCs with *S. suis* strain 89-1591. Non-stimulated cells served as negative control (C-). Data represent the mean ± SEM (n=5).

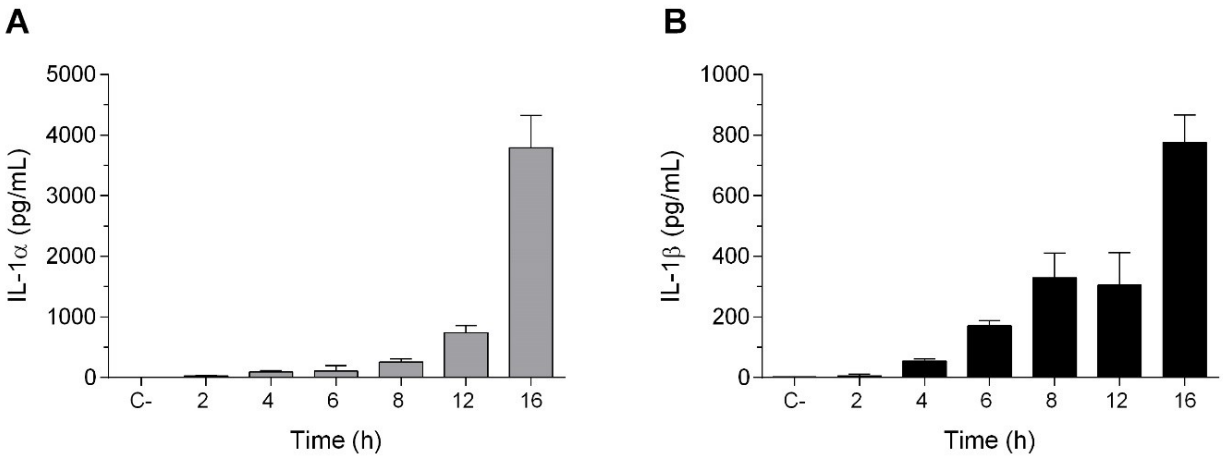


Figure 3. Comparison of the IL-1β production by dendritic cells stimulated with *S. suis* serotype 2 strains 89-1591, P1/7, and SC84. Levels of IL-1β produced by dendritic cells as measure by ELISAafter 16 h incubation time. Data represent the mean ± SEM (n=4). *** ($p < 0.01$) indicate a significant difference between 89-1591, P1/7 and SC84.

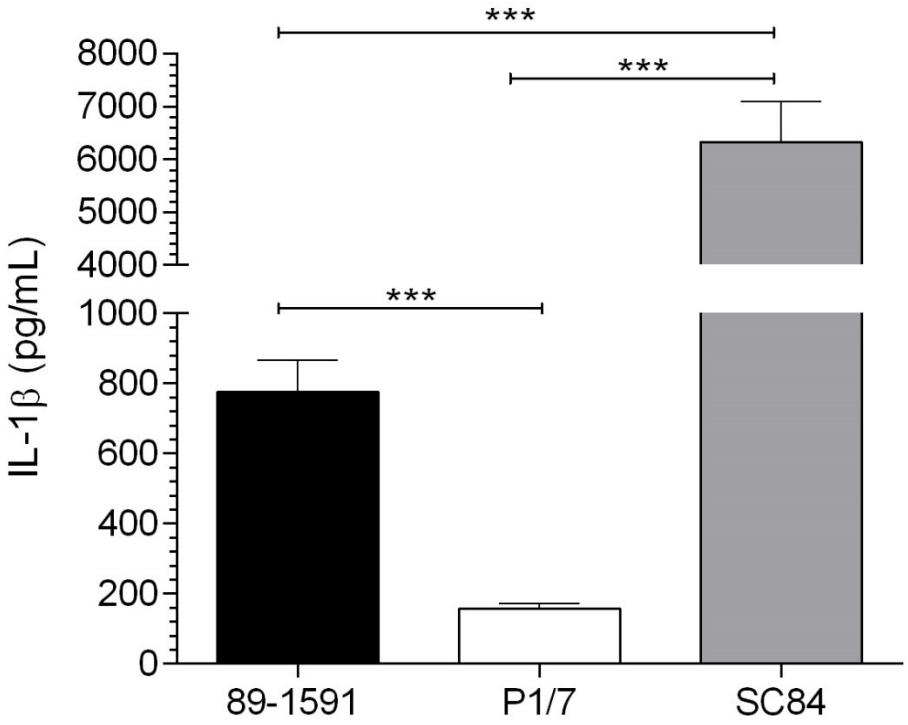


Figure 4. Role of Toll-like receptors (TLRs) and associated adaptor proteins in *S. suis*-induced IL-1β production from dendritic cells. Percentage of IL-1β production induced by *S. suis* strain 89-1591 16 h following infection of dendritic cells deficient for either MyD88, TRIF, TLR2, or TLR4, with regards to wild-type (WT; considered to be 100%) counterparts. Data represent the mean ± SEM (n=4); * ($p < 0.05$) indicate a significant difference with wild-type DCs.

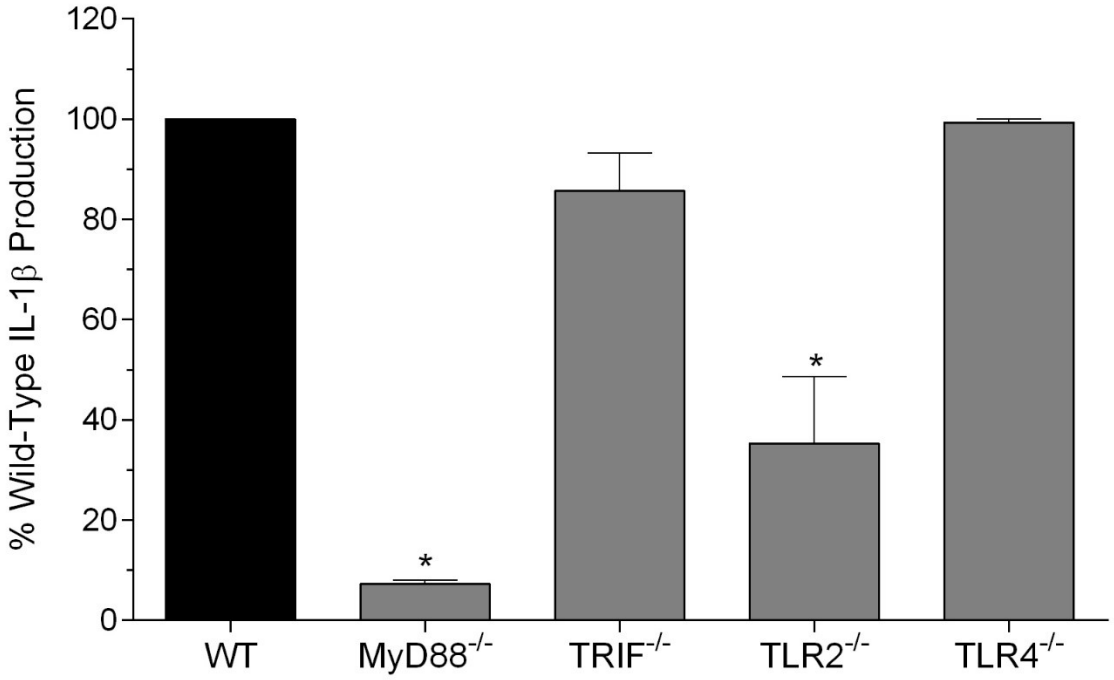


Figure 5. Strain 89-1591 bacterial components stimulate the production of IL-1β.(A) IL-1β production following activation of wild-type (WT) or TLR2$^{-/-}$ DCs with 30 µg/mL of LTA extracts from strain 89-1591 or their *lgt*-deficient mutant (Δ*lgt*). (**B**) IL-1β production by DCs following phagosomal delivery of 1 ug of *S. suis* RNA or DNA. Data represent the mean ± SEM (n = 4); * ($p < 0.05$) indicates a significant difference with LTA from wild-type strain 89-1591 (**A**) or with negative control (C-) (**B**).

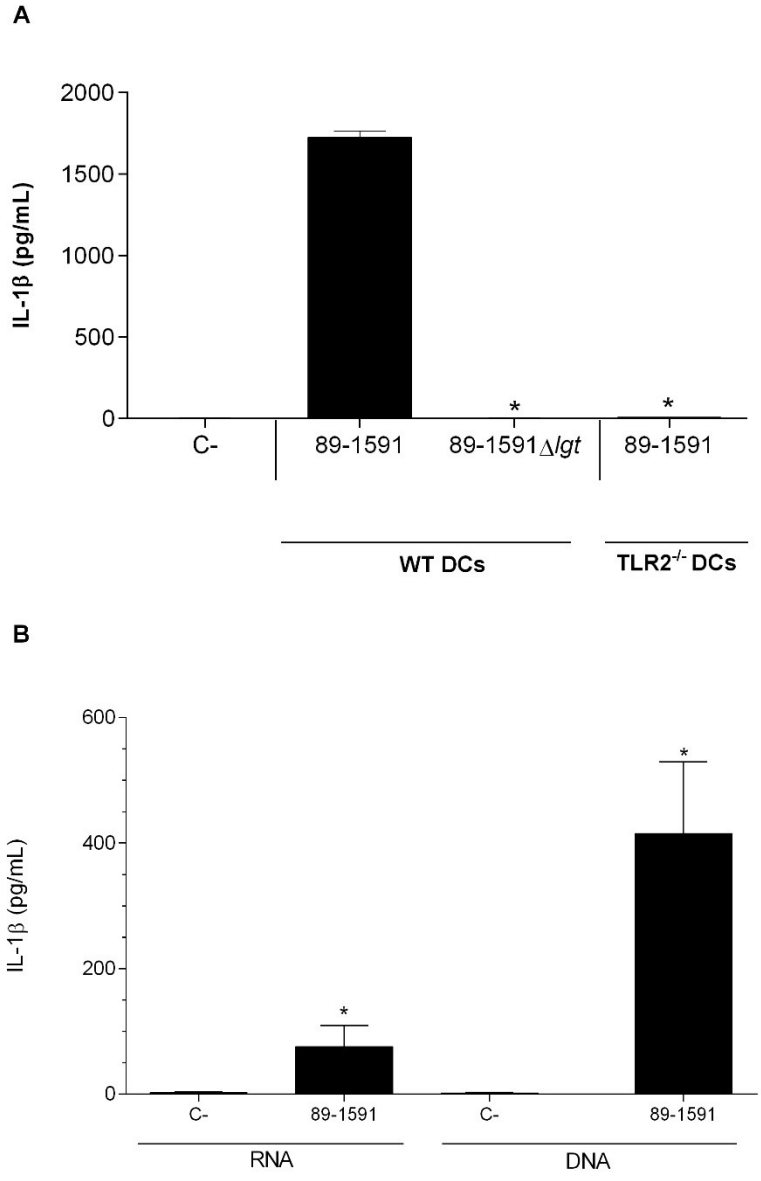


Figure 6. Comparison of the IL-1β mRNA production by dendritic cells stimulated with *S. suis* serotype 2 strains 89-1591, P1/7, and SC84.Levels of IL-1β mRNA induced by dendritic cells as measure by ELISA after 6 h incubation time. Data represent the mean ± SEM (n=3).

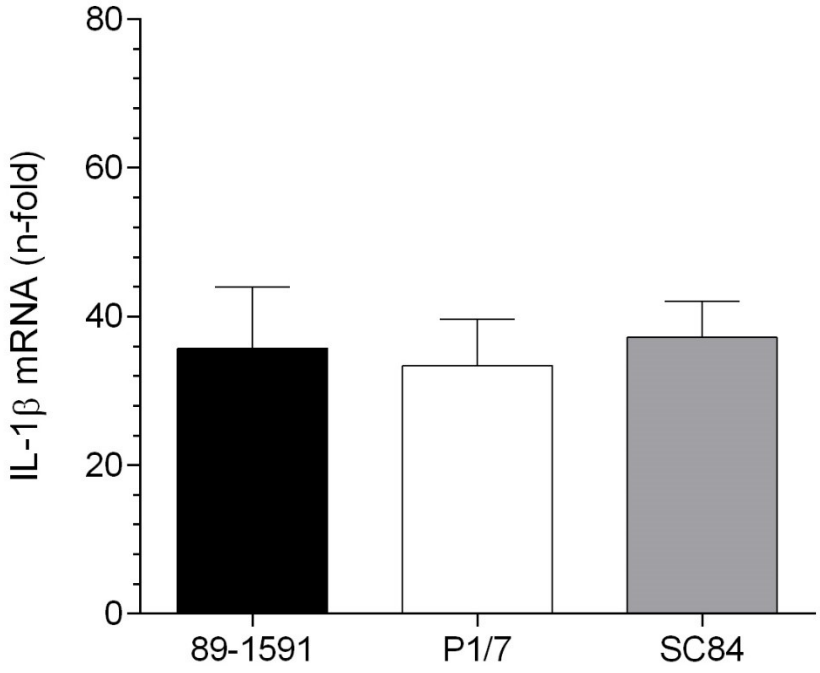


Figure 7. Strain 89-1591-induced IL-1β is caspase-1, NLRP3, and AIM2 dependent. Percentage of IL-1β secretion by caspase-1 (CASP-1), NLRP3, AIM2, NLRP1, and NLRC4-deficient DCs induced by 89-1591 after 16 h, in comparison to wild-type (WT; considered 100%) counterparts. Data represent the mean ± SEM (n = 4); * ($p < 0.05$) indicates a significantly difference obtained with WT DCs.

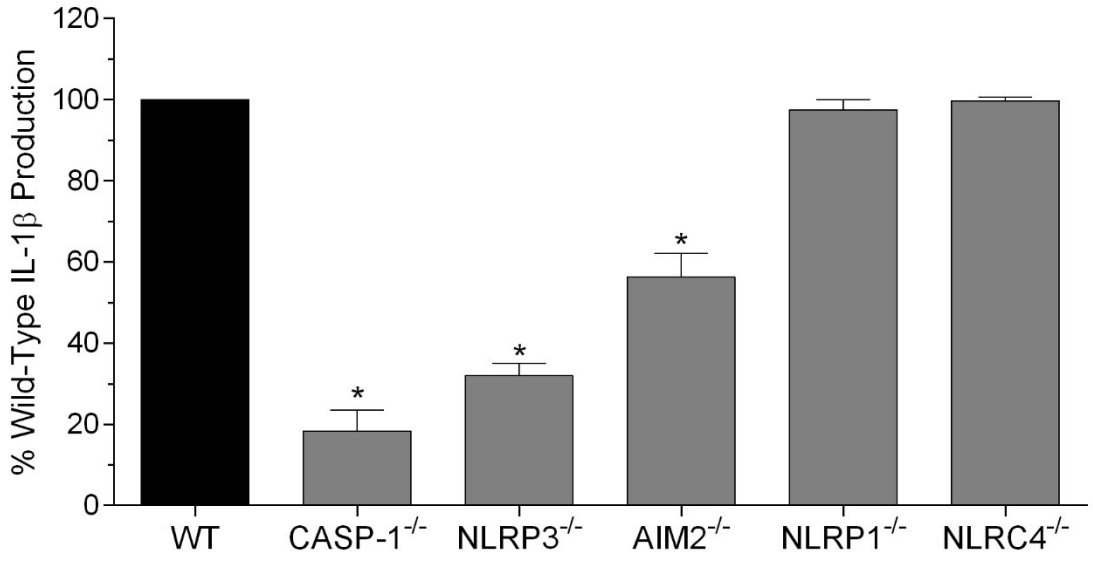


Figure 8. Strain 89-1591 induced IL-1β production depends on internalization. Implication of actin polymerization (5 μM cytochalasin D) on IL-1β production 16 h following infection with *S. suis* strain 89-1591. Data represent the mean ± SEM (n = 4); * ($p < 0.05$) indicates a significant difference with DCs treated with DMSO (vehicle) considered 100%.

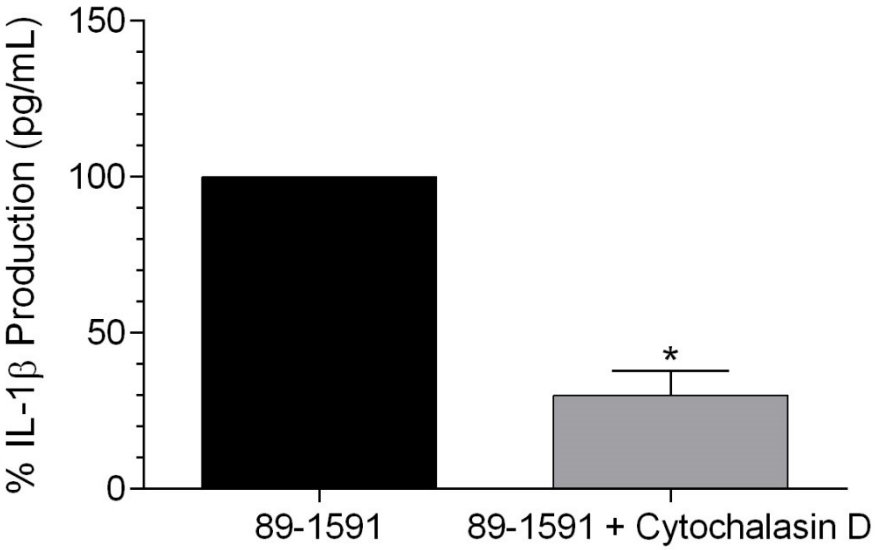


Figure 9. Production of IL-1β by dendritic cells stimulated with 89-1591 DNA depends on AIM2 inflammasome. Percentage of IL-1β secretion by AIM2-deficient DCs induced by 89-1591 DNA after 16 h, in comparison to wild-type (WT; considered 100%) counterpart. Data represent the mean ± SEM (n = 4); * ($p < 0.05$) indicates a significantly difference obtained with WT DCs.

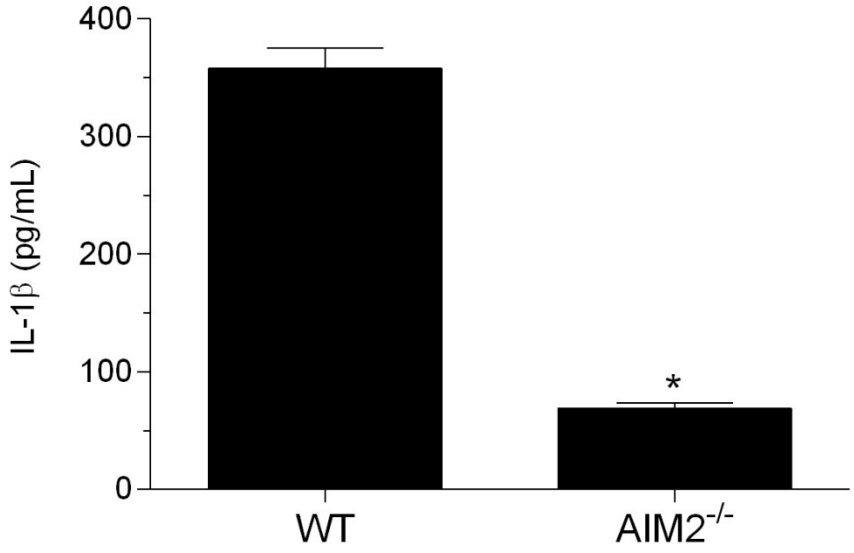


Figure 10. Mechanisms of IL-1β maturation by strain 89-1591. Bacteria recognition depends on MyD88, with a partial involvement of TLR2 resulting from recognition of surface lipoproteins (LPs). If internalized, bacterial DNA and RNA can induce the production of IL-1β, possibly through recognition on endosomal receptors TLR7 and TLR9. Activation of the NLRP3 and AIM2 inflammasomes by mechanisms not known yet lead to secretion of IL-1β. Internalization could play an important role since DNA products can activate AIM2 inflammasome.

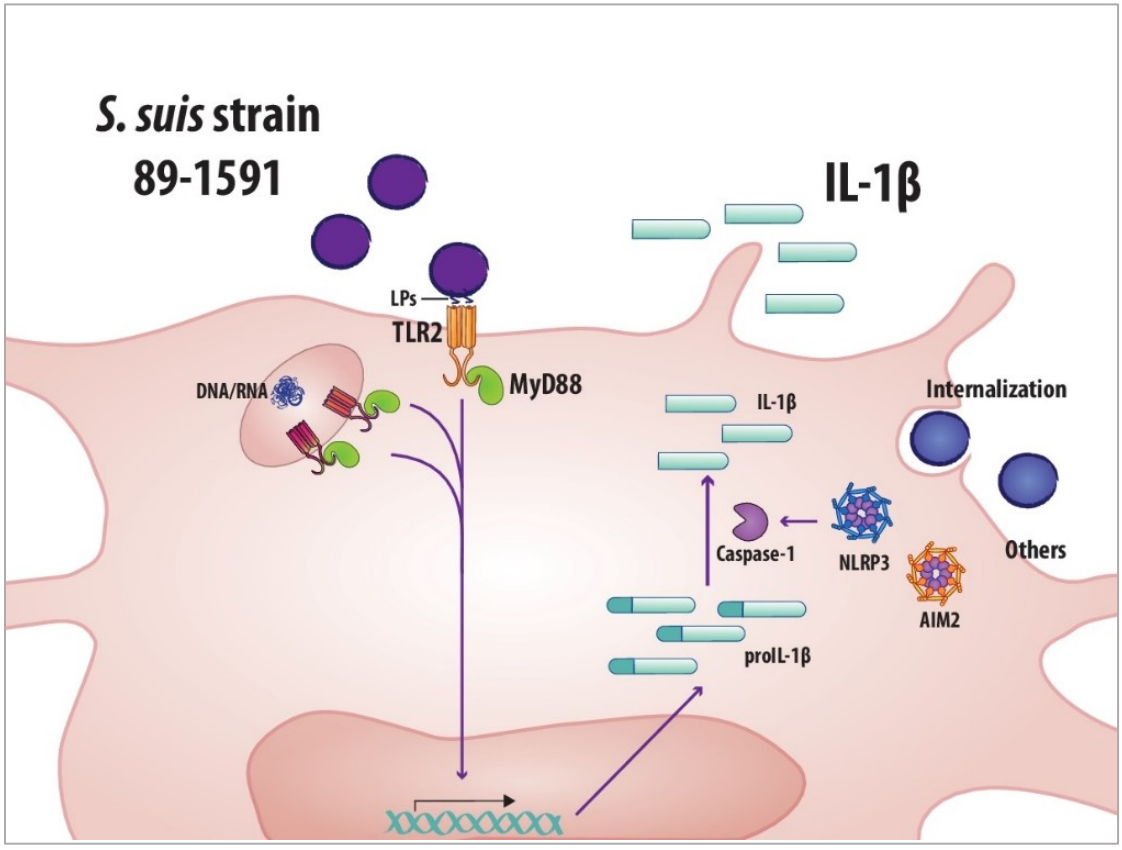


Figure 11. Survival of wild-type (WT) and IL-1 receptor-deficient (IL-1R$^{-/-}$) mice after intraperitoneal infection with *Streptococcus suis*. Six-weeks old WT and IL-1R$^{-/-}$ mice were inoculated intraperitoneally with a standard dose (1 x 10^7 CFU) of strain 89-1591 and survival was monitored for 10 days post-infection. Data represent survival curves (n=15). $^{\#}$ ($p < 0.01$) indicate a significant difference between survival of WT and IL-1R$^{-/-}$ mice.

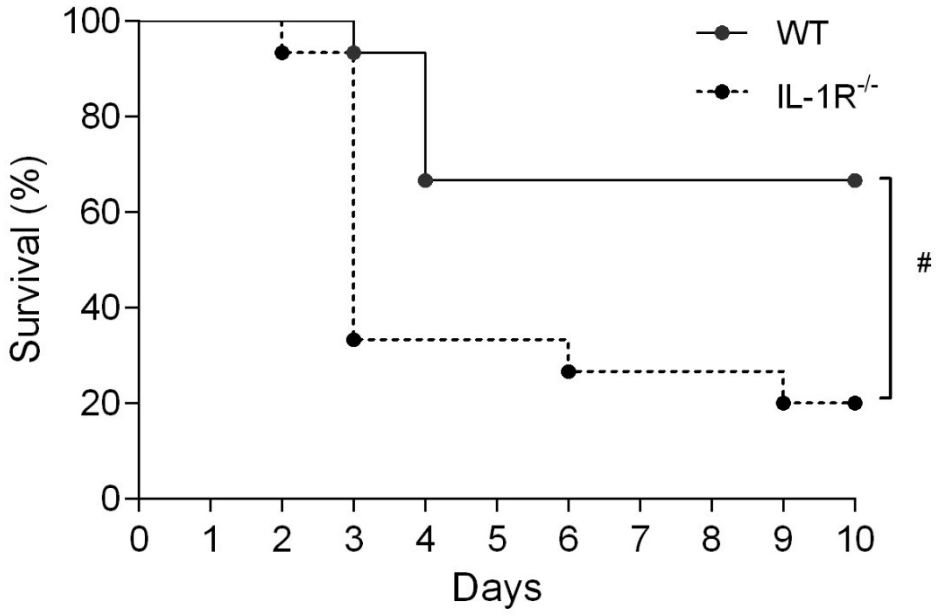


Figure 12. Pro-inflammatory mediator production in plasma during *Streptococcus suis* systemic infection. Plasma levels of IL-6 (**A**), IFN-γ (**B**), CCL3 (**C**), and CXCL9 (**D**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h, 48 h, and 72 h following infection with a standard dose of strain 89-1591 (1 x 10^7 CFU) Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

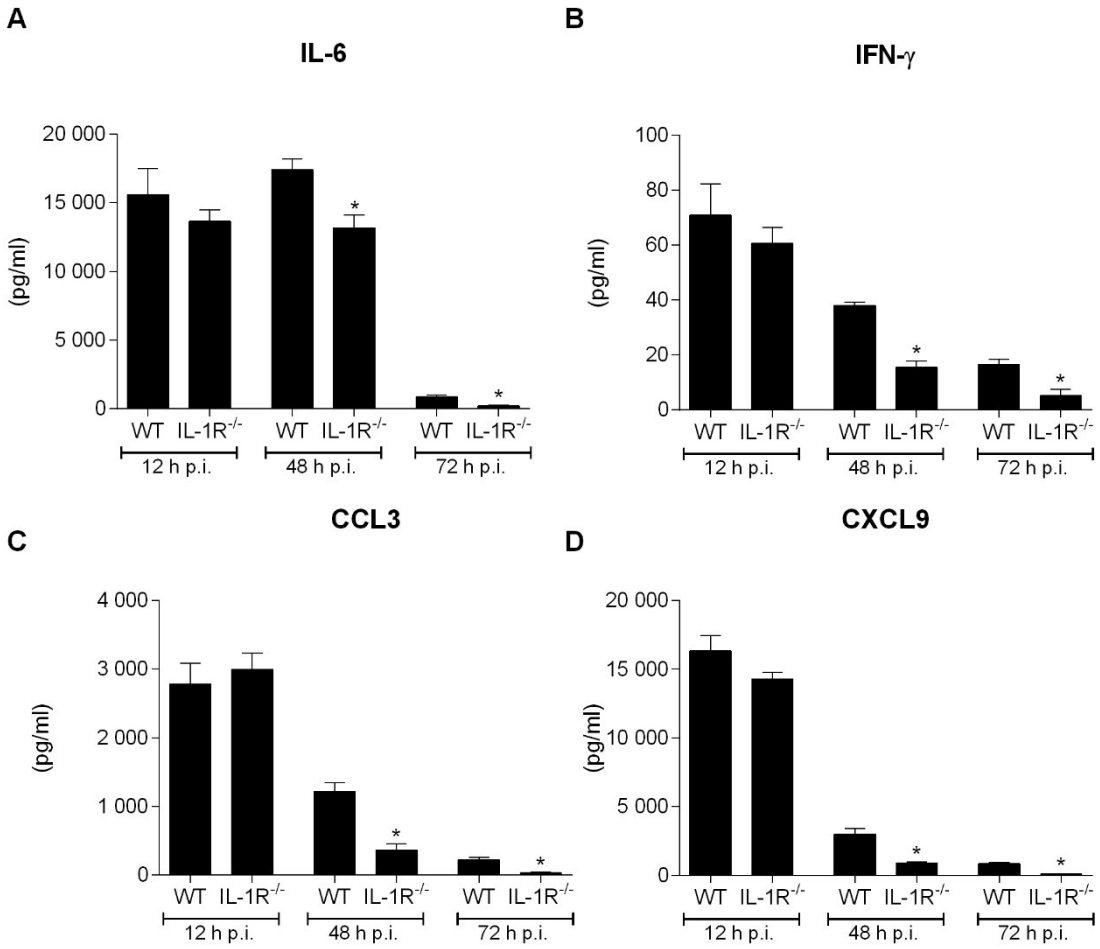


Figure 13. Pro-inflammatory mediator production in liver during *Streptococcus suis* systemic infection. Liver levels of IL-6 (**A**), IFN-γ (**B**), CCL3 (**C**), and CXCL9 (**D**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h, 48 h, and 72 h following infection with a standard dose of strain 89-1591 (1 x 10^7 CFU). Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

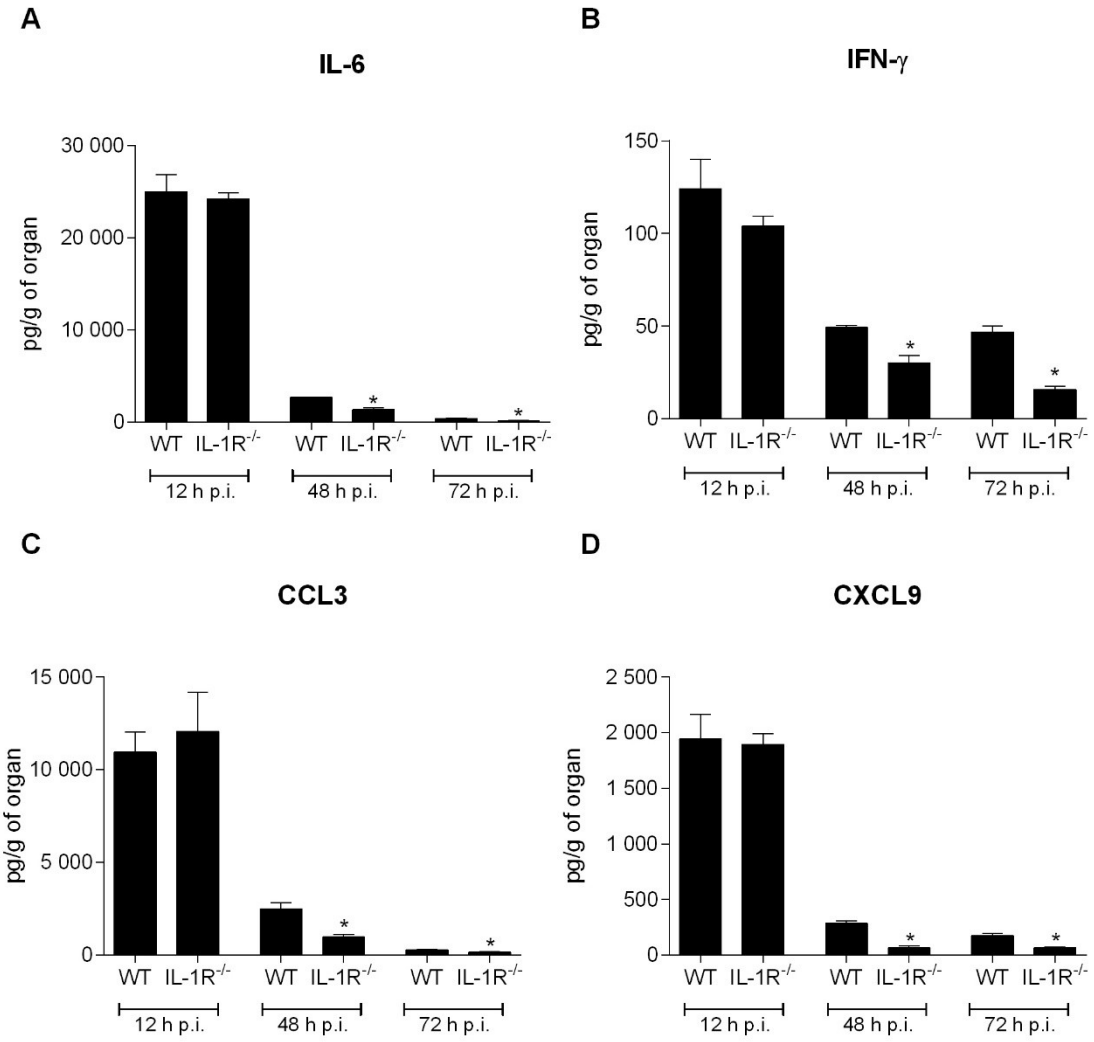


Figure 14. Pro-inflammatory mediator production in spleen during *Streptococcus suis* systemic infection. Spleen levels of IL-6 (**A**), IFN-γ (**B**), CCL3 (**C**), and CXCL9 (**D**) in wild-type (WT) and IL-1R$^{-/-}$ mice 12 h, 48 h, and 72 h following infection with a standard dose of strain 89-1591 (1 x 10^7 CFU). Data represent the mean ± SEM of at least four individuals. * ($p < 0.05$) indicates a significant difference between WT and IL-1R$^{-/-}$ mice.

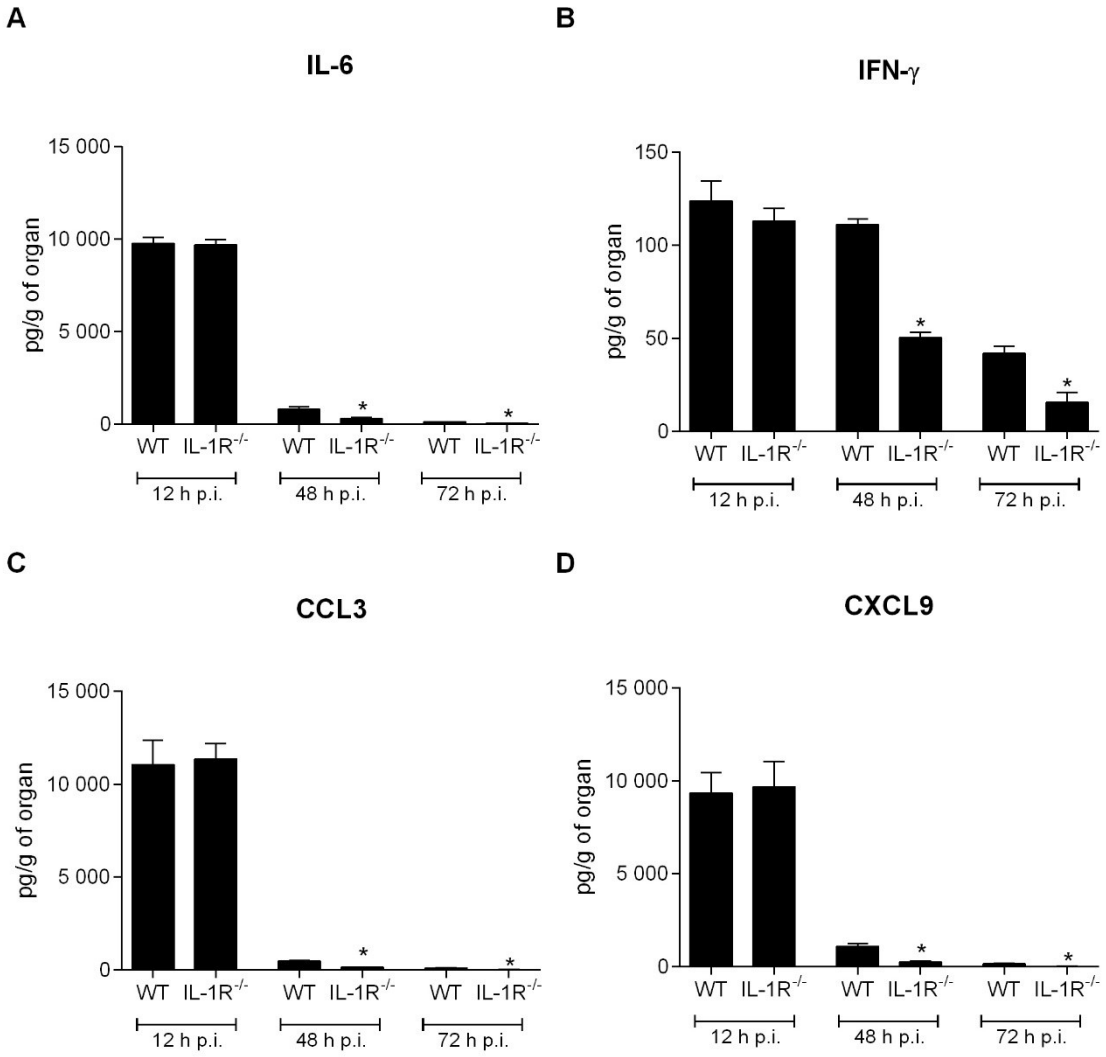


Figure 15. IL-1 is required for control of bacterial burden in blood. Bacterial burden in blood (A), liver (B), and spleen (C) of wild-type (WT) and IL-1R$^{-/-}$ mice infected with a standard dose of strain 89-1591 (1 x 10^7 CFU) at 12 h, 48 h and 72 h post-infection. A blood bacterial burden of 2 x 10^9 CFU/mL, corresponding to the average burden upon euthanasia, was attributed to euthanized mice. Data represent the geometric mean (n = 10). * ($p < 0.05$) indicate a significant difference between WT and IL-1R$^{-/-}$ mice.