Université de Montréal

STUDIES ON THE EXAGGERATED INFLAMMATORY RESPONSE CAUSED BY STREPTOCOCCUS SUIS AT SYSTEMIC AND CENTRAL NERVOUS SYSTEM LEVELS

par

MARÍA DE LA CRUZ DOMÍNGUEZ PUNARO

Département de pathologie et microbiologie

Faculté de médecine vétérinaire

Thèse présentée à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de
philosophiae doctor (Ph.D.)
en sciences vétérinaires
option microbiologie

Avril, 2010

Université de Montréal
Faculté de médecine vétérinaire

Cette thèse intitulée

STUDIES ON THE EXAGGERATED INFLAMMATORY RESPONSE CAUSED BY STREPTOCOCCUS SUIS AT SYSTEMIC AND CENTRAL NERVOUS SYSTEM LEVELS

présentée par

MARIA DE LA CRUZ DOMÍNGUEZ PUNARO

a été évaluée par un jury composé des personnes suivantes

John Fairbrother, président-rapporteur

Marcelo Gottschalk, directeur de recherche

Mariela A. Segura, codirectrice

Serge Rivest, codirecteur

Monique Doré, membre du jury

Jack P. Antel, examinateur externe

Marie Archambault, représentante du doyen

SUMMARY

Streptococcus suis serotype 2 is an important swine pathogen responsible for diverse infections, meningitis being its most striking feature. In addition, it is an emerging agent of zoonosis, which has gained worldwide attention due to important outbreaks in Asia. Understanding the pathogenesis of *S. suis* infections still represents a challenge. Up to present, the pro-inflammatory response due to *S. suis* has only been studied *in vitro*, and there is still a great need of appropriate experimental models for both septic shock and meningitis. In the present study, we successfully developed an *in vivo* model of *S. suis* infection in adult mice infected by the intraperitoneal route. This model served to investigate the pro-inflammatory events that take place at both the systemic and Central Nervous System (CNS) levels associated with this important pathogen. In addition, this model was useful to determine if susceptibility to *S. suis* infection may be influenced by the genetic background of the host.

The mouse model of *S. suis* infection was standardized in CD1 mice. Results showed sustained bacteremia during the 3 days post-infection (p.i.), accompanied by a quick and substantial release of different pro-inflammatory cytokines (TNF-α, IL-6, IL-12p40/p70, IFN-ɣ) and chemokines (KC, MCP-1 and RANTES) that lead to septic shock and 20% mortality in mice. Once the hallmark of the septic phase of *S. suis* infection was established in CD1 mice, research continued with the objective to confirm the role of inflammation in mortality and to determine if the genetic background of the host may influence the inflammatory response toward this pathogen and the further outcome of the disease. For this, the mouse model of *S. suis* infection was used with two genetically different inbred mouse strains, this is, C57BL/6 (B6) and A/J mice, which are considered as the prototype of Th1-type and Th2-type mice, respectively. Results demonstrated a striking susceptibility to *S. suis* infection in A/J mice in comparison to B6 mice, with 100% mortality in the former mice strain at 20 h p.i., and 16 % mortality at 36 h p.i. for the latter. Very interestingly, and similarly to CD1 mice, bacteremia did not seem to be responsible for the death of mice, as both mice strains presented similar amounts of bacteria in blood and organs. Thus, it was postulated that the higher mortality in *S. suis*-infected A/J mice was due to uncontrolled septic shock. In fact, A/J mice presented very high levels of TNF-α, IL-12p40/p70, IL-1β and IFN-ɣ, that significantly exceeded those found in B6 mice. Remarkably, chemokine levels were similar between strains, suggesting their limited participation in the development of septic shock by *S. suis*. A greater survival of B6 mice was partially related to a better regulation of the pro-inflammatory cytokine cascade, as they showed a higher production

of the anti-inflammatory cytokine IL-10 than A/J mice. The potential beneficial role of the IL-10 in mice infected with *S. suis* was confirmed using two approaches: the first, by blockage of the cell receptor of IL-10 (IL-10R) with an anti-mouse IL-10R monoclonal antibody (Mab) in B6 mice and the second by administrating recombinant mouse (rm)IL-10 (rmIL-10) to A/J mice. B6 mice that received the IL-10R MAb treatment before challenge with *S. suis* developed a clinical acute disease similar to that observed with A/J mice, with a striking and rapid increase in mortality and higher levels of TNF-α in comparison to those of infected mice that did not receive the treatment. Controversially, treatment with rmIL-10 significantly delayed the onset of septic shock in A/J mice infected with *S. suis*. These results show that survival from *S. suis* septic shock requires a tight regulation of pro- and anti-inflammatory mechanisms, and that the latter should be activated at the same time or soon after the onset of the pro-inflammatory response. This part of the study may represent a first step in the identification of host genes associated with resistance against *S. suis*.

One of the most important achievements of the mouse model of infection described in this project is the development of distinct clinical signs of neurological disease in CD1 mice from 4 days p.i. Indeed, in CD1 mice that survived sepsis due to *S. suis* infection, clinical signs of neurological disease and vestibular syndrome, which are quite similar to those observed in clinical cases of *S. suis* meningitis in both pigs and humans, were observed. Studies of the brains of infected CD1 mice using *in situ* hybridization combined with immunocytochemistry, demonstrated that the CNS inflammatory response began with a significant increase in the transcription of Toll-like receptor (TLR)2 and CD14 initially in the brain microvasculature and choroid plexuses, suggesting that *S. suis* may use these structures as portals of entry to the brain. There also was activation of NF-κB (as indicated by transcriptional activation of IκBα as a reporter system) and TNF-α, IL-1β and MCP-1, mainly in cells identified as microglia and to a lesser extent in astrocytes. These signals reached different brain structures, mainly the brain cortex, corpus callosum, hippocampus, choroid plexuses, thalamus, hypothalamus and meninges. All of these pro-inflammatory events were associated with extensive areas of inflammation and necrosis, severe demyelination and presence of antigens of *S. suis* inside microglia.

In vitro studies were conducted in order to better understand the interactions of *S. suis* and microglia. For this, mouse microglia were infected with a virulent wild type (WT) strain of *S. suis*. Two isogenic mutants deficient in capsule (CPS) or hemolysin production (suilysin, SLY) respectively, were also included for comparative purposes. The CPS was important for *S. suis* resistance to phagocytosis, and it also modulated the inflammatory response by hiding pro-inflammatory components from the bacterial cell wall. On the other hand, the absence of SLY, a potential cytotoxic factor, did not have a major impact on *S. suis* interactions with microglia. Studies with microglia helped to confirm previous findings *in vivo* in mice, as the WT *S. suis* strain induced the up-regulation of TLR2 and the

production of several pro-inflammatory mediators, including TNF-α and MCP-1. As observed in mice, *S. suis* induced NF-kB translocation, which was more rapid for cells stimulated with the CPS-deficient mutant, suggesting that bacterial cell wall components are potent inducers of NF-kB. Moreover, WT *S. suis* promoted phosphotyrosine, PKC and different mitogen-activated protein kinase (MAPK) events. However, microglia infected with the CPS-deficient mutant showed overall stronger and more sustained phosphorylation profiles. Finally, the CPS also modulated *S. suis*-induced inducible nitrogen oxide synthase (iNOS) expression and further nitric oxide production in microglia, which could be related to neurotoxicity and vasodilatation *in vivo*.

We are confident that our results may help to more fully understand the mechanisms underlying *S. suis* induction of inflammation, leading to the design of more efficient anti-inflammatory strategies for sepsis and meningitis. Finally, we believe this experimental model of infection in mice could also be useful for studying the pathogenesis of infections of the CNS, due to other bacteria.

Key words: *Streptococcus suis*, mouse, sepsis, meningitis, inflammation, cytokine, chemokine, microglia,TLR2, NF-κB, MAPK, nitric oxide.

RÉSUMÉ

Streptococcus suis de type 2 est un microorganisme pathogène d'importance chez le porc. Il est la cause de différentes pathologies ayant comme caractéristique commune la méningite. C'est également un agent émergeant de zoonose : des cas cliniques humains ont récemment été rapportés en Asie. Cependant, la pathogénèse de *S. suis* n'est pas encore complètement élucidée. Jusqu'à présent, la réponse pro-inflammatoire initiée par *S. suis* n'a été étudiée qu'*in vitro*. L'étude du choc septique et de la méningite requiert toujours des modèles expérimentaux appropriés. Au cours de cette étude, nous avons développé un modèle *in vivo* d'infection chez la souris qui utilise la voie d'inoculation intra-péritonéale. Ce modèle a servi à l'étude de la réponse pro-inflammatoire associée à ce pathogène, tant au niveau systémique qu'au niveau du système nerveux central (SNC). Il nous a également permis de déterminer si la sensibilité aux infections à *S. suis* pouvait être influencée par des prédispositions génétiques de l'hôte.

Le modèle d'infection par *S. suis* a été mis au point sur des souris de lignée CD1. Les résultats ont démontré une bactériémie élevée pendant les trois jours suivant l'infection. Celle-ci était accompagnée d'une libération rapide et importante de différentes cytokines pro-inflammatoires (TNF-α, IL-6, IL-12p40/p70, IFN-ɣ) et de chémokines (KC, MCP-1 and RANTES), qui ont entraîné un choc septique et la mort de 20 % des animaux. Ensuite, pour confirmer le rôle de l'inflammation sur la mortalité et pour déterminer si les caractéristiques génétiques de l'hôte pouvaient influencer la réponse inflammatoire et l'issue de la maladie, le modèle d'infection a été étendu à deux lignées murines consanguines différentes considérées comme résistante : la lignée C57BL/6 (B6), et sensible : la lignée A/J.

Les résultats ont démontré une importante différence de sensibilité entre les souris A/J et les souris B6, avec un taux de mortalité atteignant 100 % à 20 h post-infection (p.i.) pour la première lignée et de seulement 16 % à 36 h p.i. pour la seconde. La quantité de bactéries dans le sang et dans les organes internes était similaire pour les deux lignées. Donc, tout comme dans la lignée CD1, la bactériémie ne semblait pas être liée à la mort des souris. La différence entre les taux de mortalité a été attribuée à un choc septique non contrôlé chez les souris A/J infectées par *S. suis*. Les souris A/J présentaient des taux exceptionnellement élevés de TNF-α, IL-12p40/p70, IL-1β and IFN- γ, significativement supérieurs à ceux retrouvés dans la lignée B6. Par contre, les niveaux de chémokines étaient similaires entre les lignées, ce qui suggère que leur influence est limitée dans le développement du choc septique dû à *S. suis*. Les souris B6 avaient une production plus

élevée d'IL-10, une cytokine anti-inflammatoire, ce qui suppose que la cascade cytokinaire pro-inflammatoire était mieux contrôlée, entraînant un meilleur taux de survie. Le rôle bénéfique potentiel de l'IL-10 chez les souris infectées par *S. suis* a été confirmé par deux approches : d'une part en bloquant chez les souris B6 le récepteur cellulaire à l'IL-10 (IL-10R) par un anticorps monoclonal anti-IL-10R de souris et d'autre part en complémentant les souris A/J avec de l'IL-10 de souris recombinante. Les souris B6 ayant reçu le anticorps monoclonal anti-IL-10R avant d'être infectées par *S. suis* ont développé des signes cliniques aigus similaires à ceux observés chez les souris A/J, avec une mortalité rapide et élevée et des taux de TNF-α plus élevés que les souris infectées non traitées. Chez les souris A/J infectées par *S. suis*, le traitement avec l'IL-10 de souris recombinante a significativement retardé l'apparition du choc septique. Ces résultats montrent que la survie au choc septique dû à *S. suis* implique un contrôle très précis des mécanismes pro- et anti-inflammatoires et que la réponse anti-inflammatoire doit être activée simultanément ou très rapidement après le début de la réponse pro-inflammatoire. Grâce à ces expériences, nous avons donc fait un premier pas dans l'identification de gènes associés à la résistance envers *S. suis* chez l'hôte.

Une des réussites les plus importantes du modèle d'infection de la souris décrit dans ce projet est le fait que les souris CD1 ayant survécu à la septicémie présentaient dès 4 jours p.i. des signes cliniques neurologiques clairs et un syndrome vestibulaire relativement similaires à ceux observés lors de méningite à *S. suis* chez le porc et chez l'homme. L'analyse par hybridation *in situ* combinée à de l'immunohistochimie des cerveaux des souris CD1 infectées a montré que la réponse inflammatoire du SNC débutait avec une augmentation significative de la transcription du Toll-like receptor (TLR)2 et du CD14 dans les microvaisseaux cérébraux et dans les plexus choroïdes, ce qui suggère que *S. suis* pourrait se servir de ces structures comme portes d'entrée vers le cerveau. Aussi, le NF-κB (suivi par le système rapporteur de l'activation transcriptionnelle de IκBα), le TNF-α, l'IL-1β et le MCP-1 ont été activés, principalement dans des cellules identifiées comme de la microglie et dans une moindre mesure comme des astrocytes. Cette activation a également été observée dans différentes structures du cerveau, principalement le cortex cérébral, le corps calleux, l'hippocampe, les plexus choroïdes, le thalamus, l'hypothalamus et les méninges. Partout, cette réaction pro-inflammatoire était accompagnée de zones extensives d'inflammation et de nécrose, de démyélinisation sévère et de la présence d'antigènes de *S. suis* dans la microglie.

Nous avons mené ensuite des études *in vitro* pour mieux comprendre l'interaction entre *S. suis* et la microglie. Pour cela, nous avons infecté des cellules microgliales de souris avec la souche sauvage virulente (WT) de *S. suis*, ainsi qu'avec deux mutants isogéniques, un pour la capsule (CPS) et un autre pour la production d'hémolysine (suilysine). Nos résultats ont montré que la capsule était un important mécanisme de résistance à la phagocytose pour *S. suis* et qu'elle modulait la réponse inflammatoire, en dissimulant les

composants pro-inflammatoires de la paroi bactérienne. Par contre, l'absence d'hémolysine, qui est un facteur cytotoxique potentiel, n'a pas eu d'impact majeur sur l'interaction de *S. suis* avec la microglie. Ces études sur les cellules microgliales ont permis de confirmer les résultats obtenus précédemment *in vivo*. La souche WT a induit une régulation à la hausse du TLR2 ainsi que la production de plusieurs médiateurs pro-inflammatoires, dont le TNF-α et le MCP-1. *S. suis* a induit la translocation du NF-kB. Cet effet était plus rapide dans les cellules stimulées par le mutant déficient en CPS, ce qui suggère que les composants de la paroi cellulaire représentent de puissants inducteurs du NF-kB. De plus, la souche *S. suis* WT a stimulé l'expression de la phosphotyrosine, de la PKC et de différentes cascades liées à l'enzyme mitogen-activated protein kinase (MAPK). Cependant, les cellules microgliales infectées par le mutant déficient en CPS ont montré des profils de phosphorylation plus forts et plus soutenus que celles infectées par le WT. Finalement, la capsule a aussi modulé l'expression de l'oxyde nitrique synthétase inductible (iNOS) induite par *S. suis* et par la production subséquente d'oxyde nitrique par la microglie. Ceci pourrait être lié *in vivo* à la neurotoxicité et à la vasodilatation.

Nous pensons que ces résultats contribueront à une meilleure compréhension des mécanismes sous-tendant l'induction de l'inflammation par *S. suis*, ce qui devrait permettre, d'établir éventuellement des stratégies plus efficaces de lutte contre la septicémie et la méningite. Enfin, nous pensons que ce modèle expérimental d'infection chez la souris pourra être utilisé dans l'étude de la pathogénèse d'autres bactéries ayant le SNC pour cible.

Mots-clefs : *Streptococcus suis*, souris, septicémie, méningite, inflammation, cytokine, chémokine, cellules microgliales, TLR2, NF-κB, MAPK, oxyde nitrique.

TABLE OF CONTENTS

LIST OF TABLES

Annex 3. Article VI: Significant contribution of the *pgdA* gene to the virulence of *Streptococcus suis*.

Annex 5. Article VIII: New putative virulence factors of *Streptococcus suis* involved in invasion of porcine brain microvascular endothelial cells.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADS	Arginine deiminase system
AP-1	Activator protein-1
APCs	Antigen-presenting cells
ATP	Adenosin triphosphate
B6	C57BL/6
BBB	Blood-brain barrier
BMEC	Brain microvascular endothelial cells
BCSFB	Blood-cerebrospinal fluid barrier
CNS	Central Nervous System
CpG	Cytosine-phosphate-Guanosine
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
COX-2	Cyclo-oxygenase-2
CP	Choroid Plexus
CPe	Choroid Plexus epithelium
CS	Corticosterone
D-Ala	D-Alanyl
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DP	Dipeptidyl peptidase
dsRNA	Double stranded RNA
EF	Extracellular protein factor
EM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FBPS	Fibronectin/fibrinogen-binding protein
FB	Fibrinogen binding protein
FBPS	Fibronectin-fibrinogen binding protein
FN	Fibronectin
Gal	Galactose
GAPDH	Glyceraldehyde 3-phosphatase dehydrogenase
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GbO_3	Trihexosylceramide
GDH	Glutamate dehydrogenase
Glc	Glucose
GlcNac	N-acetylglucosamine
GlnA	Glutamine synthetase
h	Human
HA	Hyaluronic acid
HBMEC	Human brain microvascular endothelial cells
ICAM	Intracellular adhesion molecule

IFN-ɣ	Interferon gamma
Ig	Immunoglobulin
IIN	Innate Immune System
IIR	Innate Immune Response
IL	Interleukin
IL-1Ra	IL-1 receptor agonist
IN	Intranasal
iNOS	Inducible nitric oxide synthase
IRAK kinase	Interleukin-1 receptor
IV	Intravenous
JNK	c-Jun N-terminal kinase
KC	Keratinocyte-derived Chemokine
KO	Knockout
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic acid
PAMPs	Pathogen-associated molecular patterns
PLXTG	Leu-Pro-X-Thr-Gly
PRRS	Pathogen recognition receptors
LTA	Lipoteichoic acid
m	Mouse
mAb	Monoclonal antibody
MAPK kinase	Mitogen activated protein
MCP-1	Monocyte Chemotactic Protein-1
MEK kinases	Mitogen-activated/erk
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
MLST	Multi Locus Sequence Typing
MMP-9	Matrix metalloproteinase-9
MRF	Muramidase-released protein
mRNA	Messager ribonucleic acid
MSCRAM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MyD88	Myeloid Differentiation Primary Response Protein-88
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NeuNacN	N-acetyl neuraminic (sialic) acid
NF-kB kappaB	Nuclear transcription factor
NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OFS	Serum opacity factor
PBMEC	Porcine brain microvascular endothelial cells

PCPEC	Porcine choroid plexus epithelial cells
p.i.	post-infection
PG	Peptidoglycan
Rha	Rhamnose
PAF	Platelet –activating factor
PDGF	Platelet-derived growth factor
PRRS	Porcine Reproductive and Respiratory Syndrome
PCV	Porcine circovirus
PCR	Polymerase Chain Reaction
PECAM-1	Platelet –endothelial cell adhesion-1 molecule
PG	Peptidoglycan
PGE2	Prostaglandin E2
PLP	Proteolipid protein
RANTES	Regulated upon Activation, Normal T cell Expressed and presumably Secreted
RK	Regulated kinases
rm	Recombinant mouse
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative PCR
Sao	Surface antigene one
SAPK	JNK/stress activated protein kinases
sICAM	Soluble intracellular adhesion molecule
sIL-1Ra	Soluble IL-1 receptor agonist
SOD	Superoxide dismutase
SP-ase	Poliprotein signal peptidase
SLY	Suilysin
SPF	Specific-pathogen-free
SrtA	Sortase A
SsEno	*S. suis* Enolase
ssRNA	Single stranded RNA
ST	Sequence Typing
sTNF-αR	Soluble TNF-α receptor
STSS	Streptococcal toxic shock-like syndrome
TGF-β	Transforming growth factor beta
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL-1 receptor adapter protein
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha
TSLS	Toxic shock-like syndrome
US	United States of America
VCAM	Vascular cell adhesion molecule
WB	Western Blot
WT	Wild type
6PGD	6-Phosphogluconate dehydrogenase

A mi familia y amigos en México

A Alain

ACKNOWLEDGEMENTS

My gratitude to my advisor, Dr. Marcelo Gottschalk. I would like to thank you for all the time you consecrated to my research project, for your guidance, for always asking me to do my best and for finding the way to transform my weaknesses into my strongest points. I am also grateful to you for letting me attend several interesting scientific conferences and meetings and for funding me during my post-graduate studies. Lo que ha hecho por mi lo tendré presente.

Special thanks to my co-advisor, Dr. Mariela Segura. It has been a pleasure working with you. I am indebted for all your input to my PhD studies, for all the scientific discussions and the time you dedicated to me. Eres un excelente ejemplo de lo que debe ser una mujer en ciencia y soy afortunada de haber sido guiada tan acertadamente.

I would like to thank my co-advisor, Dr. Serge Rivest. I appreciate all your interest in my studies, your availability every time I needed it, your valuable advice and for sharing your knowledge with me. The help I received from the personnel of your laboratory, in particular of Natalie Laflamme and Marie-Michèle Plante, was very important to achieve the objectives of this thesis.

I am grateful to Dr. Martin Oliver, for your willingness to participate in my project, for allowing me to perform a part of my experiments in your laboratory and for the helpful discussions. I would like to thank all the members of your lab, for making me feel like if I were in my own lab. Special thanks to Irazú Contreras, for all her interest, time and explanations.

I appreciate also the invaluable collaboration and interest of Dr Danuta Radzioch to the present research project and for all her important suggestions.

I am grateful to the members of the different Committees and Juries: Dr. Monique Doré, Dr. Richard Drolet, Dr. Marie Archambault, Dr. Lucie Lamontagne, Dr. Jack Antel and Dr. John Fairbrother. Thank you for all your time and priceless advice.

Special thanks to Sonia Lacouture, research assistant at Dr Gottschalk's laboratory. Thank you very much Sonia, for your friendship, your help every time I needed it and for all the scientific discussions.

I would like to express my gratitude to the past and present students and post-doctoral fellows at Dr. Gottschalk's and Dr. Segura's laboratories, with whom I had the pleasure to work during my post-graduate studies: Geneviève Chabot-Roy, Richard Graveline, Bénédicte Bouchet, Ghyslaine Vanier, Miriam Esgleas Izquierdo, Nahuel Fittipaldi, Marie-Pier Lecours, Paul Lemire, Cynthia Calzas, Claude Lachance and Mathieu Houde. Thank you guys for your support, friendship and encouragement.

Thanks to all the professors from GREMIP for all the knowledge they shared with me, their advice and their encouragement.

This thesis has partly been funded by the Natural Sciences and Engineering Council of Canada (NSERC) and by the Centre de Recherche en Infectiologie Porcine (CRIP-FQRNT). Throughout my studies, I received the invaluable support of different scholarships and awards from the NSERC, the Université de Montréal (Faculté des études supérieures and Faculté de médicine vétérinaire), the CRIP-FQRNT, the J. A. DeSève Foundation and the Lucie Besner Award in Zoonosis. All the economic support I received gave me the tranquility to fully concentrate on my post-graduate studies. I thank the selection committees that allowed me to receive such recognitions.

I am grateful to Diane Rodier, Micheline St-Germain, Manon Coutellier and Hélène Boucher Rhéaume, for their assistance with the administrative matters, but also for the words of encouragement that I received from them.

I am indebted with the personnel of the Animal Care facilities of the FMV, in particular with Normand Lacasse and Marina Poulin, who always did their best to meet my needs for animal experimentation.

I would like to thank my family and friends in Mexico: my mom, my sister Silvia and her family (my brother in law Moisés, and my nephews Max and Ricky), my brother José María, and my friends Norma, Angie, Paty, Alex, Miguel, Rubén, Josefina and Fausto. Thanks for all your love, for believing in me and for your unconditional support so that I can pursue even my craziest goals. And to you daddy, as I know you still are by my side (†). A todos ustedes los quiero y espero estén contentos de verme terminar mis estudios de doctorado.

I was lucky to make new friendships here in Quebec and France, whose encouragement and company are well appreciated: Victoria, Adriana, Stephan, Karla and Brite. I hope these new friendships get stronger with time.

And…thank you Alain, for all your love, support, time and patience. I am blessed to have you in my life. Thanks also to Laura, for reminding me that there is a child inside me, and thanks to all the Guimont family, for their support and for making me feel part of a *famille quebecoise*.

Finally, I consider that animal experimentation is not a right, but a privilege. I was privileged to perform most of the experiments contained in the present thesis using the mouse as a model. I am indebted to these little fellows.

I. INTRODUCTION

Streptococcus suis is one of the most important pathogens affecting the swine industry worldwide, and it also is a relevant agent of zoonosis. In pigs, the most frequently reported pathologies include septicemia and central nervous system (CNS) infection, leading to meningitis, endocarditis, pneumonia and arthritis. In humans, *S. suis* infection has been considered as a work-related infection, as most of the cases described have occurred in people working in close relationship with pigs or raw pork products. In 2005, an important *S. suis* human outbreak in China, attracted worldwide attention to this pathogen, highlighting the menace it represents for human health. In this episode of *S. suis* infection, more than two hundred people were affected, including thirty nine deaths. Furthermore, in several Asian countries, cases of zoonotic infections by *S. suis* have escalated, and it is currently considered as the most important agent of adult meningitis in Vietnam. To date, thirty five *S. suis* serotypes have been reported, however, serotype 2 is still by far the most frequently isolated from both diseased animals and humans (at least in Europe and Asia), and it is also considered as the most virulent.

The mechanisms that *S. suis* exploits to invade and colonize the host and cause disease and the response of the host to such aggression have only been partially clarified. The capsular polysaccharide (CPS) of *S. suis* is known to play a crucial role in the progression of disease, as it hampers phagocytosis and helps bacteria to persist in the blood, allowing them to multiply and disseminate throughout the body, leading to septicemia and/or CNS disease. However, although all virulent *S. suis* strains are well capsulated, the presence alone of capsule does not warrant disease as most avirulent strains are also well encapsulated, indicating that other virulence factors are also essential. Many virulent strains of *S. suis* also possess a hemolysin (suilysin -SLY-, a member of thiol-activated cytolysin family of hemolysins) that may also participate in the development of the disease, although it does not seem to be a critical trait. Another proposed virulence factor is the serum opacity factor (OFS) which proved to be a relevant trait for the virulence of *S. suis*, as an isogenic mutant was greatly attenuated in virulence but not in colonization. However, the OFS is not present in all virulent strains. Recently, many putative virulence factors have

been described, amongst which, the D-alanylation of lipoteichoic acid (LTA) and N-acetylation of peptidoglycan (PG) have proved to be determinant for the virulence of this pathogen, as their respective isogenic mutants are not only more susceptible to the killing effect of different components from the innate immune sytem, but are also less virulent in pig and mouse infection models. Other potential *S. suis* proteins associated with virulence have been described, although they are not always present in all virulent strains. These include the extracellular protein factor (EF), the muramidase-released protein (MRP), a fibronectin/fibrinogen-binding protein (FBPS), a hialuronidase, different adhesins, and several proteases.

Despite much effort, elucidation of the pathogenesis of both systemic and CNS infections caused by *S. suis* remains a challenge. In the natural host, *S. suis* enters through the respiratory route and colonizes the tonsils, whereas in humans, the entrance is mainly through skin abrasions. Bacteria subsequently reach the bloodstream where the CPS will contribute to resistance to phagocytosis. Dissemination occurs as bacteria multiply and spread either freely or as cell-bound bacteria attached to monocytes/macrophages (the "modified Trojan horse" theory). This bacteremia often results in fatal septicemia and toxic shock-like syndrome. Several studies have demonstrated that *S. suis* induces the up-regulation of different adhesion molecules, as well as the production of diverse cytokines and chemokines in monocytes, which may trigger the inflammatory response and enhance the recruitment of leukocytes at sites of infection. However, all of these studies have been done *in vitro*.

If the host is able to resist septicemia, it may still develop meningitis, which is the most striking clinical feature of *S. suis* infection. In order to gain access to the CNS and provoke meningitis, *S. suis* should trespass the blood brain barrier (BBB). It has been demonstrated that *S. suis* is able to adhere to and to invade porcine brain microvascular endothelial cells (PBMEC), and that SLY is cytotoxic for these cells. There is also evidence of the relevance of the blood-cerebrospinal fluid barrier (CSF) as a possible gate for *S. suis* entry into the central nervous system. Indeed, *S. suis* is capable of invading porcine choroid plexus epithelial cells (PCPEC, the single-cell layer comprising the blood–CSF barrier), with

the activation of different intracellular signaling pathways involved in cellular uptake. Once in the brain, *S. suis* must face the local defense system, represented principally by microglia, which are the brain's macrophages. These cells are designed to respond quickly to pathogens and injury and to produce a wide variety of pro-inflammatory molecules. However, if stimulation is exaggerated, there will be an exacerbated local inflammatory response with negative consequences for the host. Studying the interactions between *S. suis* and microglia is necessary to have a better understanding of the development of the inflammatory response against this pathogen in the CNS and the possible pathological consequences.

As mentioned previously, certain of the pro-inflammatory events associated to *S. suis* have been studied *in vitro* only. Hence, and there is no *in vivo* confirmation of the machinery triggered by *S. suis* for the development of the inflammatory response at a systemic level or in the CNS, upon infection. Furthermore, to date, there is no available data on potential immune factors associated with genetic susceptibility to *S. suis* infection. The use of the mouse or murine cells as experimental models of *S. suis* infection is justified, since previous studies in the mouse showed that this animal species is susceptible to infection by this pathogen. However, these previous efforts were used to assess mainly disease outcomes and compare them to those observed for experimentally infected pigs.

In addition, *in vivo* research on *S. suis* infection permit the evaluation of the importance of different putative virulence factors. Increased knowledge on the mechanisms underlying *S. suis* induction of inflammation in the brain would be useful for the design of more efficient anti-inflammatory strategies in cases of meningitis that may be used in both swine and humans.

The main overall objective of this research project is to strengthen the current knowledge on the pathogenesis of *S. suis* infection by focusing on the development of the inflammatory response triggered by this important pathogen at both systemic and CNS levels.

The specific objectives of this research are:

1. To study the *in vivo* progression of *S. suis* infection at both the systemic and CNS levels using a standardized model. This objective focuses on the characterization of the inflammatory hallmark of systemic infection, including the development of clinical signs and measurement of a panel of pro-inflammatory markers at the systemic level.
2. To dissect in vitro some of the pro-inflammatory machinery activated by *S. suis* in murine microglia. This objective focuses on TLRs recognition, ability of microglia to phagocyte *S. suis*, as well as different intracellular signalling pathways that may lead to the production of different pro-inflammatory mediators and messenger molecules involved in inflammation.
3. To identify possible genetic immune traits associated with host susceptibility to *S. suis* infection. For this objective, the mouse model of infection will be used to compare the severity of infection between two genetically different inbred mouse strains. Different sepsis-clinical parameters and inflammatory mediators are evaluated to dissect possible immune factors associated with genetic susceptibility.

II. REVIEW OF THE LITERATURE

1. *Streptococcus suis*

1.1. General aspects of *S. suis*

S. suis is currently considered as one of the most important pathogens of the swine industry worldwide, responsible for substantial economical losses. The most frequent pathologies include meningitis, septicemia, arthritis, pneumonia and endocarditis [6]. It is also a zoonotic agent responsible for meningitis, septicemia, toxic shock-like syndrome and endocarditis [7, 8]. *S. suis* is a Gram-positive, facultatively anaerobic coccus, possessing cell wall antigenic determinants related to Lancefield group D, although it is genetically unrelated to other members of this group [6, 9]. *S. suis* occurs singly, in pairs and, rarely, in short chains, forming small, grey, colonies. Although some strains are β-hemolytic and produce a hemolysin on agar plates containing horse blood agar, all strains of this species are α -hemolytic when grown on blood agar plates containing sheep blood [10]. *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 [11, 12]. The NaCl (6.5%) test clearly differentiates between S. *suis* and *Streptococcus bovis* from the genus *Enterococcus.* The Voges-Proskauer test is critical and the most reliable for differentiating S. *suis* from S. *bovis* [12].

S. suis was first reported by veterinarians in the early 1950's from piglets and adult swine, from which α-hemolytic streptococci were isolated [13, 14]. Early classification of these bacteria determined that they belonged to groups R, S, R, S and T, according to Lancefield classification of streptococci [15]. However, this classification was inaccurate, as Elliot (1966) demonstrated that they belonged to group D. Misclassification was due to the fact that the LTA, the antigen from the cell wall that allows classification of streptococci into group D, was masked by antigens from the CPS used by de Moor [16]. *S. suis* was officially recognized as a species in 1987, when Kilpper-Bälz and Scheleifer used DNA hybridization and cell wall analysis to demonstrate that *S. suis* is not closely related to group D streptococci and that it constitutes a genetically homogeneus species [17].

Current classification of *S. suis* is based on its CPS antigens, and 35 serotypes, namely 1, 1/2, 2 - 34 have been described (Fig. 1) [6]. However, recent evidence suggested that serotypes 32 and 34 should be excluded from the *S. suis* species and re-designated as *Streptococcus*

orisratti [18]. Moreover, the 16S rRNA gene sequence analysis of the 35 reference strains of *S. suis* demonstrated that serotypes 32, 33 and 34 might belong to a different species [19]. In *S. suis* serotype 2, the antigen epitope portion corresponds to the N-Acetylneuraminic acid [20]. Some of the reference strains of these serotypes originated from diseased pigs whereas others were isolated from the nasal cavities of clinically healthy pigs [6]. Reference strains of serotypes 20, 31 were recovered from diseased calves, whereas serotype 33 was isolated from a lamb and reference strain of serotype 14 from a case of human meningitis [12, 21, 22]. The number of untypeable isolates is, in general, relatively low. These isolates are mostly recovered from sporadic cases of disease and it seems that there is no justification at the present time fot the characterization of new capsular types [23]. Some serotypes cross-react, indicating that some capsular antigenic determinants are shared. This is the case for serotype 1/2 cross-reaction with serotype 1 and serotype 2 antisera [24].

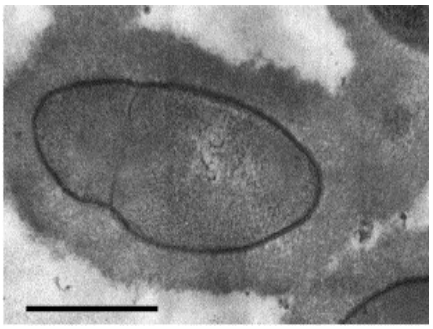


Fig. 1. Transmission electron microphotograph of thin sections of *S. suis* serotype 2 reference strain S735, which shows a thick capsule characteristic of this serotype. Original magnification 37,500×. Taken from [25].

Among all capsular types, *S. suis* serotype 2 is the most frequently isolated and is considered as the most virulent in several European and Asian countries [26-30]. In Japan, the prevalence of *S. suis* serotype 2 is around 28% [31], whereas in Spain and France it is of 51% and 69%, respectively [26, 27]. In North America, serotype distribution greatly differs from that reported in several European countries. In fact, recent data show that the incidence of *S. suis* serotype 2 strains recovered from diseased animals in Canada is relatively low (less than 25%) [32], whereas in the United States (US), most of *S. suis* field strains belong to serotype 3 (20% of the isolates) and serotype 2 (17%). The similarities between the results from Canada and US suggest a common status in North America [33].

1.2. *S. suis* infection and transmission

1.2.1. In swine

S. suis can affect pigs of all ages. However, the disease is most common following weaning and mixing, and the majority of cases occur between 3 and 12 weeks of age [34]. The incidence of the disease varies from herd to herd and also within a herd over a period of time. No seasonal incidence has been noted in pigs [35, 36]. Different management practices and/or the presence of other pathogens have been suggested as predisposing factors [37]. In fact, *S. suis* infection is associated with stressful management practices, such as excessive temperature fluctuation, high relative humidity, poor ventilation, crowding, an age spread of > 2 weeks between pigs in the same room, weaning and/or moving animals [37].

The natural habitat of *S. suis* is the upper respiratory tract (particularly the tonsils and nasal cavities) and the genital and alimentary tracts of pigs [6], although it can also be isolated from the lungs, the vagina, and the prepuce of pigs [38, 39]. In this species, *S. suis* is responsible for meningitis, meningoencephalitis, septicemia, arthritis and endocarditis [6]. Onset of infection is characterized by anorexia, depression and high fever, up to 42°C, glazed eyes, and reddening of mucous membranes. [6, 40-42]. After several days, diseased animals develop neurological signs, including opisthotonus, lateral recumbency, paddling, convulsions, and ataxia (Fig. 2) [40-43]. Some animals may also present lameness in one or more legs as arthritis develops [41, 42].

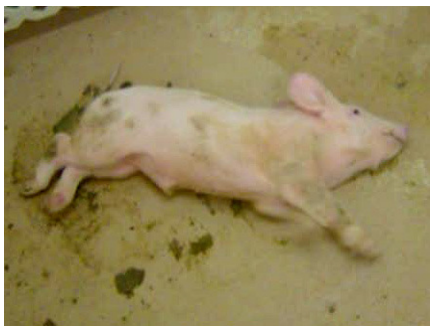


Fig. 2. Experimental infection with *S. suis* serotype 2 in a piglet. Typical clinical signs of *S. suis* meningitis: lateral recumbency and front limb-paddling.

In animals that have died due to septicemia, gross findings include bronchopneumonia, arthritis (Fig 3A) and diffuse inflammation of the heart (Fig. 3B), spleen, liver and serosae.

Histopathological examination of these organs usually reveals suppurative or fibrinopurulent inflammation [6, 43, 44]. In some cases, even though meningoencephalitis is evident, isolation of the pathogen from brain and meninges is negative, indicating that isolation of *S. suis* from other organs should be attempted to confirm the etiology [43].

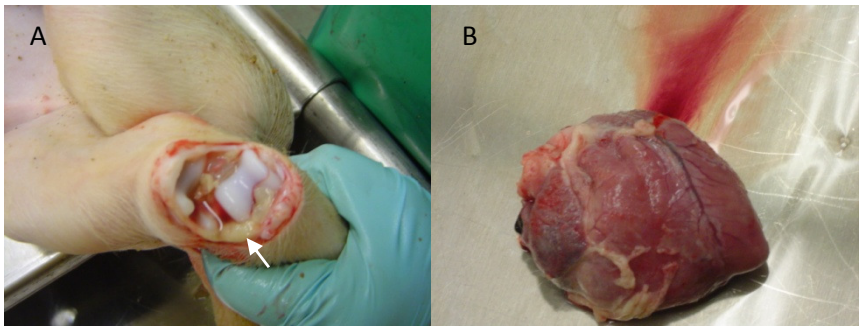


Fig. 3. Experimental infection with *S. suis* serotype 2 in a piglet. (A) Rear limb articulation, demonstrating that the synovial membrane is thickened (arrow), and that there is a fibrino-haemorragic exudate. (B) Heart, fibrinous exudate on the epicardium.

In the CNS, findings include meningeal haemorrhages, congestion and thickening of the dura mater, with flattening of gyri as a result of compression of the swollen brain by the dura mater and inner surface of the skull, shallowing of cerebullar sulci and increase in the volume of yellowish cerebrospinal fluid [42, 43]. Microscopically, the meninges are hemorrhagic, infiltrated by neutrophils and macrophages (Fig. 4A). Encephalitis is associated with marked cerebral edema characterized by broadening of perivascular spaces, massive infiltration of neutrophils, focal proliferation of microglial cells and gliosis (Fig. 4B). The choroid plexuses of the lateral, third, and fourth ventricles may show marked congestion associated with scant cellular exudates [42].

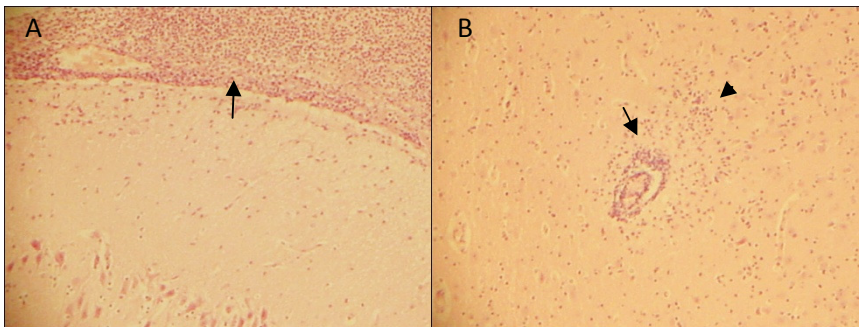


Figure 4. Clinical case of *S. suis* infection in a piglet. (A) Brain, showing severe thickening of the meninges, due to the massive infiltration of leukocytes (arrow). (B) Edema and infiltration of leukocytes in the perivascular space (arrow) and gliosis (arrow head).

Infected pigs generally have clinical signs and lesions referable to either the respiratory system or to the CNS, but not both [43]. *S. suis* is commonly isolated from the respiratory tract of pigs with respiratory disease. However, it is unclear whether the organism is a primary agent of pneumonia. Indeed, S. *suis* is often isolated in combination with other respiratory pathogens, such as *Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Bordetella bronchiseptica* and others [45, 46], suggesting that, in these cases, *S. suis* may act as an opportunistic pulmonary pathogen. This hypothesis is supported by the difficulty to reproduce respiratory clinical signs in experimentally infected animals. In fact pre-infection with *B. bronchiseptica* is needed for the induction of *S*. *suis* pneumonia during experimental trials [47]. In contrast, in pigs with meningitis or meningoencephalitis, S. *suis* is considered a primary pathogen since it is the only bacterial species isolated from the brain of these pigs [46]. Viral infections such as Aujeszky's disease virus, porcine reproductive and respiratory syndrome (PRRS) virus and porcine circovirus (PCV) may predispose pigs to infection with *S. suis* [48-50].

S.suis can be both vertically and horizontally transmitted. Piglets born from sows with uterine and/or vaginal infections are either infected or become infected at birth [51]. They can also acquire the bacterium after birth due to close contact with the sow, her feces and contact with piglets from other litters and the surrounding environment [6, 52]. Strategies for the elimination of *S. suis* based on off site production or early weaning have not been successful because many piglets are carriers of *S. suis* within the first days of life. Entrance of *S. suis* to naïve pig production facilities is through asymptomatic infected animals carrying pathogenic strains [6, 34]. It has been proposed that once a pig is infected with S. *suis* serotype 2, it may

remain a carrier for life [53]. Pigs may harbor different S. *suis* strains (even of different serotypes) in their nasal cavities and tonsils with no relationship with a specific pathological condition [54]. Multiple *S. suis* serotypes have also been isolated from diseased animals within the same herd [6, 46].

1.2.2. In humans

Human *S. suis* infections are most often reported from countries where pig-rearing is common. It has traditionally been considered as an occupational disease, as most of the cases are reported in people working with pigs or pig-derived products [55, 56]. Since the first report of human *S. suis* infection in Denmark in 1968 [57], many cases have been reported in several European countries (The Netherlands, Italy, Spain, United Kingdom, Belgium, Croatia, Austria, Sweden, Germany, Ireland, Hungary, France, Greece, Italy, Hungary, Portugal) and Asian countries (Japan, China, Hong Kong, Taiwan, Thailand, Singapore) as well as in Canada, the US, Australia, New Zealand, and Argentina [7, 58-61]. Most cases of *S. suis* infection have been attributed to serotype 2 strains [7]. However, cases due to serotype 4 [62], serotype 14 [63-65], and serotype 16 [66] have also been reported. Two human cases have been attributed to serotype 1, although the isolates from these cases were not confirmed with a serologic reaction using specific antisera [67].

However, the classical picture of *S. suis* infection as an occupational disease has drastically changed, as large outbreaks took place in China in 1998 and 2005 (see description below 1.2.2.1) [7, 68]**.** Currently, *S. suis* infection has become an important public health concern in several Asian countries but rate of *S. suis* human infection may not be precise, as in developing countries with intense pig farming, such as those in Southeast Asia, disease due to *S. suis* infection is not notifiable and under diagnosis is common [69]. In fact, *S. suis* is currently the primary cause of adult meningitis in Vietnam as well as the third most common cause of community-acquired bacterial meningitis in Hong Kong [69-72] .

S. suis causes a systemic infection in humans that affects several systems; meningitis is the most common clinical manifestation. The presenting features of *S. suis* meningitis are generally similar to those of other bacterial pyogenic meningitis and include frontal headache, fever, diaphoresis, vomiting, a stiff neck, confusion and generalized tonic clonic seizures. Sick people are often admitted to the hospital 2–5 days after the onset of clinical signs [56]. Besides

meningitis, patients may also present endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis and endophthalmitis [69, 73-75]. In some cases pulmonary embolism, lumbar epidural abscesses, aortic aneurisms and brain stem ophthalmoplegia leading to persistent dipoplia have also been reported [61, 76-78]. An association between *S. suis* infection and colon carcinoma has also been suggested [79].

Deafness and/or vestibular dysfunction, which may be permanent and disabling, are the most common sequela of *S. suis* meningitis, recorded in up to one-half of patients at presentation or a few days later. This hearing loss may be the result of damage to the eight craneal nerve or secondary to cochlear sepsis, resulting from passage of the organism from the sub-arachnoid space to the perilymph via the cochlear aqueduct [78, 80]. For unknown reasons, the recorded incidence of deafness following infection caused by *S. suis* is higher than that reported for other meningitis-causing bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*, and can reach 50% and 65% in Europe and Asia, respectively [75]. Deafness, however, has never been reported in non-meningitis cases of *S. suis* human infection [7]. The overall case fatality rate from *S. suis* infection reaches near 13% in Europe and 20% in Asia [74]. Interestingly, only five human cases of meningitis have been reported in North America (three in Canada and two in the US). However, recent information suggest that human infection with *S. suis* is more common in the US than currently thought, as swine-exposed persons had higher titers of antibodies to *S. suis* than did non–swine-exposed persons [81]. As *S. suis* can be mistaken for enterococci, *Listeria* spp., viridans streptococcus, or *S. pneumoniae*, it should be considered in the differential diagnosis of septicemia, especially when complicated by meningitis in adults with a recent history of contact with pigs or unprocessed pork [82].

The route of infection differs from the natural host, as in humans, entry of the organism occurs through small cuts in the skin, mucosa of the mouth and nasal cavity [7, 83]. Additionally, infection through ingestion by contaminated food has been proposed [84]. In general, high exposure to *S. suis* may lead to a colonization of the upper respiratory tract without producing any health consequences. In certain cases clinical disease will follow [7]. The bacterium may colonize the gastrointestinal tract, as suggested by the presence of diarrhea as a prodromal symptom [85]. The incubation period ranges from a few hours to two days [85]. Almost all cases of human infection can be assigned to close contact with pigs or to a high

degree of exposure to unprocessed pork meat, which in certain markets, has been found to be contaminated with *S. suis* [71, 86]. With few exceptions, most cases (in general adult males) are pig farmers, abattoir workers, people transporting pork, meat inspectors, butchers and veterinary practitioners [74, 75, 87]. Splenectomy, alcoholism and diabetes mellitus have been suggested as important predisposing factors for the development of serious *S. suis* disease [74, 88, 89]. Most studies showed that strains isolated from humans are phenotypically and genotypically similar to those recovered from swine within the same geographical region [90-95]. In addition, the virulence properties of strains isolated from pigs or humans appear to be similar [7].

1.2.2.1. Human outbreak in China

The first important outbreak of human *S. suis* serotype 2 infection took place in 1998, in the province of Jiangsu, China, resulting in fourteen deaths [96]. In the summer of 2005, another mores substantial episode of human infection was recorded in the province of Sichuan, China, with two hundred-fifteen cases of acute disease and a total of thirty-nine deaths. In the Sichuan episode, all infections occurred in backyard farmers who were directly exposed to infection during the handling of pigs that had died of unknown causes or been killed for comsumption, even if they were ill. In addition, the oral route of infection can not be ruled out [95]. These Chinese outbreaks did not follow the classical clinical presentation of *S. suis* infection, as they were characterized by a high incidence of systemic disease with elevated rates of mortality and a proportionally low number of cases of meningitis [82]. In all cases, there was no evidence of human to human infection. At first, Chinese cases were misdiagnosed as streptococcal toxic shock syndrome (STSS) as patients presented a sudden onset of disease, with high fever, diarrhea, hypotension, blood spots and petechia, clear erythematous blanching rash and dysfunction of multiple organs, such as acute respiratory distress syndrome, liver and heart failure, disseminated intravascular coagulation and acute renal failure [87, 95]. However, the presence of superantigens could not be demonstrated in the strains isolated during the Sichuan outbreak [28, 87, 95]. *S. suis* serotype 2 sequence type 7 strains emerged in 1996 and caused a STSS in the 1998 and 2005 Chinese outbreaks. The comparison between sequence type (ST) 7 strain SC84, isolated from a patient with STSS during the 2005 outbreak, and the sequence type 1 strain 31533, demonstrated some of the differences in their mechanisms for cytokine production. It was found that *S. suis* ST 7 strain SC84 possesses a stronger capacity to

stimulate T cells, naive T cells and peripheral blood mononuclear cell proliferation than does *S. suis* sequence type 1 strain 31533. The T cell response to both strains is dependent upon the presence of antigen-presenting cells and on their mitogenic effect over T cells [97].

Post-mortem examination of chinese patients revealed features of disseminated intravascular coagulation. Evidence of multiple organ damage was observed, involving several organs. Histologic findings included microthrombosis (hyaline thrombus) in organ capillaries; necrosis of parenchymal cells; and congestion, exudate, and hemorrhage of interstitial vessels of kidneys, lungs, and other organs [98]. Biochemical and Polymerase Chain Reaction (PCR) tests confirmed that all isolates from the 2005 outbreak were *S. suis* serotype 2 [28, 87, 95]. Phylogenetic analysis by Multilocus Sequence Typing (MLST) of *S. suis* strains isolated from both sick humans and pigs, showed that all strains belonged to the same clone responsible for the 1998 outbreak in the Jiangsu province [28, 87, 95]. MLST studies also showed that all but two strains from the 2005 outbreak belonged to the ST 7 (ST 1 complex) [28].

1.2.3. In other animal species

S. *suis* has been isolated from different animal species, including ruminants (calves and lambs), horses, cats, dogs, birds and hamsters [6, 99-106]. This suggests that *S. suis* may be pathogenic for more than one animal species. Similarly to the situation in pigs, it seems that *S. suis* colonizes the tonsils of these species [100, 107, 108]. Moreover, it is believed that hunters and poachers are at risk of infection as wild boars are a possible reservoir for *S. suis* [7, 109, 110]. Indeed, the prevalence of *S. suis* serotype 2 among wild boars (11%) is similar to that among domestic pigs (14%) in Germany [111].

1.3. Treatment and prevention of the infection

1.3.1. In swine

Control of *S. suis* infections in swine facilities is based on efficient management practices, such as use of chemoprophylactic agents, early weaning and vaccination [112]. The economic and zoonotic significance of this organism highlights the importance to monitor its drug resistance trends [113]. Sensibility to antimicrobial agents depends not only on the geographic origin of the strain, but also on the serotype and year of isolation [114]. In pigs, the most efficient antimicrobial drugs against *S. suis* infection include amoxicillin, ampicillin,

gentamicin, ceftiofur and enrofloxacin [37]. Antimicrobial resistance is observed against tetracyclines, sulphonamides, macrolides and clindamycin, and many isolates can develop a multi-resistance pattern [114]. However, continuous surveillance of the susceptibility pattern of clinical isolates and prudent use of antimicrobial agents is highly advised, as recent studies indicate that there is increasing resistance to penicillin, ampicillin and ceftiofur. Moreover, the resistance of *S. suis* to ceftiofur also poses potential health risks in humans since third-generation cephalosporins have been primary drugs used for the treatment of human meningitis in recent years [115]. As clinical signs of meningitis are associated to inflammation of the CNS, administration of anti-inflammatory drugs may be useful [6].

Different strategies of vaccination have been developed or are presently under investigation. First attempts of immunization included the use of autogenous bacterins, preparations from purified CPS or poorly encapsulated strains. Unfortunately, these approaches delivered, in general, unsatisfactory results [116]. In addition, autogenous vaccines may include extra disadvantages, such as lack of vaccine safety, efficacy of data and severe adverse reactions to oil adjuvants which are often added to this kind of vaccines. A further difficulty is that multiple strains belonging to different serotypes are often present in a single herd [117]. More recent vaccination strategies have included the use of *S. suis* isogenic mutants lacking putative virulence factors [118] and a purified SLY vaccine which induced good protection in swine experimentally infected with a serotype 2 strain [119]. This SLY-based vaccine may not be useful against North American *S. suis* strains that do not produce SLY [120]. Other possible vaccine candidates are purified bacterial cell wall components with putative immunogenic characteristics, such as the recently described HP0197 and 6-Phosphogluconate dehydrogenase (6PGD) proteins which confer protection in pigs and mice [121, 122]. Another potential vaccine candidate is Sao, a protein present on the surface of *S. suis*. The immunization of piglets with recombinant Sao resulted in development of a significant humoral antibody response. Nevertheless, this antibody response was not reflected in protection of pigs that were intratracheally challenged with a virulent *S. suis* serotype 2 strain [123]. However, administration of recombinant Sao together with a Quil A, provides cross-protection against *S. suis* serotype 2 disease in mouse and pig vaccination models [124]. The use of some other bacterial components as vaccine candidates, such as *S. suis* enolase (SsEno), a recently described *S. suis* adhesin with fibronectin-binding activity, has been controversial. In fact, one

study demonstrated that although subcutaneous immunization of mice with recombinant SsEno elicited strong immunoglobulin G (IgG) antibody responses, SsEno-vaccinated and nonvaccinated control groups showed similar mortality rates after challenge with highly virulent *S. suis* [125] (See annex, Article IV). In contrast to these results, other researchers showed that SsEno can confer complete protection against *S. suis* infection to mice, suggesting that enolase has potential as a vaccine candidate [126].

1.3.2. In humans

In humans suffering from *S. suis* infection, treatment is based on the use of antimicrobial agents. Penicillin G administrated by intravenous route is generally the first choice, as most *S. suis* isolates are susceptible to this antimicrobial [127-129]. Antimicrobial therapy also includes ampicillin, chloramphenicol, aminoglicosides and third generation cephalosporins, such as ceftriaxone [69, 70, 129]. Similarly to the pig, most human *S. suis* isolates are resistant to tetracycline and erythromycin [128]. Antimicrobial agents are administrated for up to four weeks [69]. Early administration of antimicrobials does not appear to have any influence on the development of post-meningitis hearing-loss [127].

Use of anti-inflammatory drugs such as dexamethasone as an adjunct treatment to reduce mortality and improve the outcome of meningitis remains controversial [130]. Based on results of individual cases, dexamethasone therapy may or may not be associated with protection against severe hearing loss [59, 131]. However, in a recent controlled trial (with treated and placebo groups), it was clearly shown that dexamethasone treatment during the first four days of hospitalization can significantly reduce the rate of severe hearing loss due to *S. suis*. Nevertheless, there was no difference in clearance of bacterial DNA between patients treated with dexamethasone and patients treated with placebo [128].

Currently, a human vaccine is not available, but simple preventive measures, such as wearing gloves during processing of pig meat or slaughtering, hand washing after handling raw pig meat, and thorough cooking of pork, should prevent the majority of cases. Travelers should be aware that dietary habits in some countries may pose a risk for infectious diseases, including *S. suis* infection [56].

1.4. Virulence factors of *S. suis*

One of the problems in identification of virulence factors of *S. suis* is the lack of agreement on virulence for this pathogen, as different parameters are used to define whether a strain is virulent or avirulent [6]. Different *S. suis* components have been proposed as virulence factors. However, most of them are either not critical for virulence or are present in virulent and avirulent strains. Besides, virulence of a certain number of these factors can not be completely ascertained, as there are no "knock-out" (KO) strains available for some factors. Moreover, most studies on *S. suis* virulence factors have been performed using serotype 2 strains, and there is a lack of information concerning possible virulence factors of other serotypes.

To decide if a field strain is virulent or avirulent, different research groups have proposed different criteria, such as the clinical condition of the animal from which the strain was isolated (clinically diseased or healthy animals); the presence of virulence-related proteins; and the virulence in pigs from high-health-status herds, specific-pathogen-free (SPF) pigs, and colostrum-deprived pigs or piglets of different ages from either conventional or SPF herds [132-135]. In fact, results of experimental infections of pigs with *S. suis* may depend, among other considerations, on the immunological status of the animals, the route of infection, the size of the inoculum and the presence of the organism as normal inhabitant of the upper respiratory tract of animals prior to the experimental infection [6, 136]. In addition, large discrepancies exist in the literature regarding even the virulence of the same strain of *S. suis* [137, 138]. To date, the CPS remains as the only factor that is considered to be decisive for *S. suis* virulence, although recent research has highlighted the importance of several components from the cell wall.

1.4.1. The capsular polysaccharide (CPS)

S. suis is a well-encapsulated bacterium [139], and it has been proposed that the CPS confers antiphagocytic properties [140-142]. The CPS of *S. suis* serotype 2 is composed of five different sugars: galactose (Gal), glucose (Glc), N-acetyl glucosamine (GlcNAc), rhamnose (Rha) and N-acetyl neuraminic (sialic) acid (NeuNAc) [20, 143]. The *cps* locus coding for the CPS of *S.*

suis serotype 2 has been cloned and characterized. This locus contains several genes that encode potential glucosyl-, galactosyl-, N-acetylglucosaminyl- and rhamnosyltransferase activities, as well as genes involved in the synthesis of sialic acid [144, 145].

The CPS is still considered as the only proven critical virulence factor, based on the studies with nonencapsulated isogenic mutants. The absence of CPS correlates with increased hydrophobicity and phagocytosis by murine and porcine phagocytes [140, 144, 146]. Comparable results were obtained with porcine neutrophils [147]. Indeed, it is now known that the CPS modulates the activation of relevant intracellular signalization pathways implicated in phagocytosis in murine macrophages [148]. Moreover, surface adhesion, without phagocytosis, of *S. suis* to murine macrophages is mediated, at least in part by the sialic acid component of the CPS [149]. The relevance of the CPS has also been evaluated in vivo, as nonencapsulated mutants were shown to be avirulent and cleared from circulation rapidly in both mouse and pig models of infection [140, 150].

Some research also proposes that thickness of the capsule has a positive correlation with virulence. In fact, isolates of *S. suis* serotype 2 recovered from diseased animals have been shown to possess a thicker capsule than those isolated from clinically healthy animals [151]. An increase of capsular thickness following *in vivo* growth has been noted for virulent but not for avirulent strains, and this increase in capsular thickness of virulent strains is also associated with resistance to killing by pig polymorphonuclear leukocytes [152]. However, other reports did not demonstrate any correlation between the thickness of capsular material and virulence [6]. Nevertheless, cells of serotype 2 reference strain are not covered by a thicker layer of CPS, compared to other serotypes [6]. It has been suggested that the invasive ability of strains of this serotype may depend on the composition of the capsular material which contains sialic acid [139], as this component has been associated with virulence of other bacterial agents of meningitis [153]. However, it has been shown that virulent and avirulent strains possess a capsule of similar size with similar concentrations of sialic acid [154]. Finally, factors other than the CPS would also be critical for virulence of *S. suis*, as most of non virulent strains are capsulated [6].

1.4.2. D-Alanylation of Lipoteichoic Acid (LTA)

LTA is an amphilic molecule found in most Gram-positive bacteria, and is considered as the equivalent of the lipopolysaccharide (LPS) in Gram-negative bacteria [155]. LTA is associated with development of shock by Gram-positive bacteria, as it activates leukocytes, stimulates an exacerbated generation of proinflammatory cytokines and cytotoxic molecules, and hence, causes a systemic inflammatory response syndrome that may lead to multiple organ failure and death [155]. This molecule is a polymer of polyphosphoglycerol substituted with a D-alanyl (D-Ala) ester or a glycosyl residue and anchored in the membrane by a terminal glycolipid moiety [156]. The D-alanylation of LTA allows Gram-positive bacteria to modulate their surface charge, regulate ligand binding and control the electromechanical properties of the cell wall [157]. Genetic studies of the biosynthesis of LTA in various Gram-positive bacteria, such as *Lactobacillus caseii* and *Bacillus subtilis* have shown that the incorporation of D-Ala residues requires the activity of four gene products, which are encoded by the *dlt* operon [158, 159]. However, there are few research data available dealing with the importance of D-alanylation of LTA and virulence of streptococci. Studies using isogenic mutants lacking a functional *dlta* operon (D-dltA-), have shown that in GBS, this operon is needed to resist the killing by defensins and phagocytic cells [157], whereas in Group A Streptococcus (GAS), *S. gordonii* and *S. pneumoniae*, it confers resistance to the killing effect of lysozyme and antimicrobial peptides (AMPs) [160-162].

The importance of D-alanylation of LTA in the virulence of *S. suis* serotype 2 has been recently evaluated. Use of a *S. suis* D-dltA- strain, demonstrated that, similarly to other streptococci, lack of LTA D-alanylation resulted in increased susceptibility to the action of cationic antimicrobial peptides. In addition, the D-dltA- mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Moreover, virulence of the D-dltA- mutant, in comparison to the parental strain, was attenuated in mouse and porcine models of infection, an effect that may be associated with a decrease in the ability to escape immune clearance mechanisms, a lower capacity to activate the inflammatory cascade and an impaired competence to move across host barriers [163] (See annex, Article V).

1.4.3. N-deacetylation of Peptidoglycan (PG)

The main cell wall component of Gram-positive bacteria is peptidoglycan (PG), which provides stress resistance and shape-determining properties to bacterial cell walls [155]. This polymer contains long sugar chains of two alternating sugar derivatives, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), which are highly cross-linked by peptide subunits and bridges [155, 164]. Among different bacterial species, the structure of the sugar chains is highly preserved, while the composition of the peptide subunits varies. PG synergizes with LTA to trigger the inflammatory response in the host and cause shock [165]. However, the host takes advantage of this rather invariant structure to recognize bacteria, through the nucleotide-binding oligomerization domain (NOD)1 and NOD2 proteins, which recognize muropeptides released during cell wall turnover, and also to kill the microorganisms through the hydrolytic activity of lysozyme [166, 167]. In order to circumvent the host's defenses, bacteria have developed specific mechanisms to modify the structure of their PG. For example, the *pgdA* gene from *S. pneumoniae* and *L. monocytogenes* N-deacetylate the NAG residues into glucosamine or muramic acid. This N-deacetylation of PG will help bacteria to avoid NOD1 and NOD2 recognition and will also increase the resistance to lysozyme [168, 169].

Similarly to the aforementioned bacteria, *S. suis* is able to N-deacetylate its PG by means of the *pgdA* gene. Studies demonstrated that expression of the *pgdA* gene was increased upon interaction of the bacterium with neutrophils *in vitro* as well as *in vivo* in experimentally inoculated mice, suggesting that *S. suis* may enhance PG N-deacetylation under these conditions. Virulence of an isogenic *pgdA* mutant was highly attenuated in comparison to its parental strain in mouse and pig models of infection, confirming that relevance of N-deacetylation of PG in the pathogenesis of *S. suis* infection. In addition, the *pgdA*- mutant was not able to persist in blood or to escape immune clearance mechanisms mediated by neutrophils [40] (See annex, Article VI).

1.4.4. Opacity Factor (OFS)

The presence of an Opacity Factor (OF) associated with virulence of *S. suis* (OFS) has been described [170]. This OFS, encoded by the *ofs* gene, shares structural homologies with members of the microbial surface components recognizing adhesive matrix molecules (MSCRAM) family and a C-terminal LPXTG sorting signal [171]. The N-terminal region of OFS is homologous to the respective regions of fibronectin-binding protein A of *Streptococcus dysgalactiae* and the serum opacity factor of GAS. Similar to these two proteins, the N-terminal domain of OFS was able to opacify horse serum [171]. The role of OFS in the pathogenesis of *S. suis* was evaluated in pigs experimentally infected with an isogenic mutant lacking the *ofs* gene. The mutant was severely attenuated in virulence but not in colonization [171]. These results encouraged further research on this mutant as a possible candidate for vaccination. However, experimental trials in pigs demonstrated that although pigs inoculated with the *ofs* mutant elicited humoral immune responses against different putative virulence factor of *S. suis*, the mutant strain failed to induce sufficient opsonizing activity and significant systemic protection against the parental strain [118]. The prevalence and variations of the *ofs* gene among different *S. suis* isolates from diseased and healthy pigs and human patients was further investigated. Regardless of their origin, only approximately 30 % of the isolates possessed a functional OFS [172].

1.4.5. Muramidase-released protein (MRP) and extracellular protein factor (EF)

These two proteins, known as muramidase-released protein (MRP) and extracellular factor (EF) protein, are among the first putative virulence factors described for *S. suis* serotype 2 [173]. MRP is a 136-kDa protein, present in the cell wall fraction and also released into the culture supernatant during bacterial growth. MRP is encoded by the *mrp* gene, is an LPXTG protein, and is therefore expected to be anchored to the cell wall PG by sortase A [173, 174]. On the other hand, EF is a 110-kDa protein only present in culture supernatants [173]. Serotype 2 strains with the phenotype $MRP^{+}EF^{+}$ induce severe clinical signs of disease in pigs, but strains with the phenotype $MRP^{-}EF^{-}$ do not [47, 175]. These proteins possesses different variants. Higher and lower molecular weight variants of MRP, respectively called MRP* (> 136-kDa) and MRP^{S} (< 136-kDa), and higher molecular weight EF proteins, called EF* (> 150-kDa) can be found in phenotypes such as $MRP^{*}EF^{-}$, $MRP^{S}EF^{-}$, $MRP^{S}EF^{+}$, $MRP^{-}EF^{*}$, and $MRP^{+}EF^{*}$ [27, 120,

176]. Also, it has been suggested that European *S. suis* serotype 2 strains that present MRP, EF and SLY (another putative virulence factor, see description below), that is, with a $MRP^{+}EF^{+}SLY^{+}$ phenotype, are more virulent than North American strains which do not possess such proteins [9]. However, when isogenic mutants were used to experimentally inoculate newborn germfree piglets, they proved to be as pathogenic as their parental strain [177]. Recent data have shown that the most common phenotypes in North American strains are $MRP^{+}EF^{-}SLY^{-}$ (40%) and $MRP^{-}EF^{-}SLY^{+}$ (35%), but that the presence of the gene did not always correlate with actual expression of the respective protein [33]. 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.

1.4.6. Hemolysin (suilysin, SLY)

Hemolysins have often been implicated as virulence factors in infections caused by various bacterial species. In 1994, a 54-kDa hemolysin, designated as suilysin (SLY), was identified and purified from culture supernatants of *S. suis* serotype 2 [178]. Subsequently, a 65-kDa hemolysin from the same serotype was described. In fact, these two proteins are the same toxin and the molecular mass difference are due to purification methods [179]. SLY is found among different serotypes of *S. suis*, including 1, 1/2, 3, 4, 5, 7, 8, 9, 14, 15, 17, 18, 19, 23 and 28, and it belongs to the formely named group of thiol-activated toxins that are now known as cholesterol-binding cytolytic toxins [180-182]. The hemolysin from *S. pneumoniae* (pneumolysin) is the most closely related orthologous gene known [182]. Other members of the same group include the streptolysin O (Group A Streptococcus -GAS-), listeriolysin (*Listeria monocytogenes*) and perfringolysin (*Clostridium perfringens*) [183]. SLY shares several characteristics with these toxins, such as 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 [179]. Cholesterol in the membrane of eukaryotic cells is thought to be the toxin-binding site [183, 184].

As with other putative virulence factors, the role of SLY in the pathogenesis of *S. suis* has not been fully elucidated. It has been demonstrated that it is cytotoxic to endothelial and epithelial cells, as well as neutrophils, monocytes and macrophages [147, 185-188]. In addition,

purified SLY induces the production of several pro-inflammatory cytokines by human and porcine BMEC [189-191], by porcine peripheral blood cells [192] and by pig alveolar macrophages [193]. SLY also enhances the upregulation of adhesion molecules in human monocytes [194] and it is implicated in the secretion of arachidonic acid, a precursor of prostaglandin, in human endothelial cells [195]. Together, this data provid evidence that indeed SLY plays an important role in bacterial dissemination, host inflammation and invasion of different tissues by *S. suis*. Nevertheless, some data are controversial, such as the failure to cause death following systemic administration to mice of a culture supernatant from a SLY-positive *S. suis* strain [196]. On the other hand, an allelic-replacement mutant of the *sly* gene was non-toxic for murine macrophage and also avirulent in a mouse infection model, whereas its virulence was only slightly attenuated in a porcine model of systemic infection [197]. Conversely, other researches showed that both the parental strain and a *sly* knockout mutant were resistant to bactericidal factors present in whole pig blood. Furthermore, in a pig challenge model, the mutant strain induced disease similarly to the wild type strain, with presence of clinical signs of disease and isolation of bacteria from different tissues [193]. Finally, similarly to MRP and EF, whereas SLY is present in most of Asian and European *S. suis* serotype 2 strains, it is only present in a limited number of North American serotype 2 strains [7, 26, 33, 47, 108, 120, 181].

1.4.7. Adhesins

1.4.7.1. Fibronectin-fibrinogen binding protein (FBPS)

Different strains of *S. suis* are able to bind to extracellular matrix (ECM) proteins, such as fibronectin [198]. *S. suis* serotype 2 possesses a 64 kDa protein with fibronectin (FN) and fibrinogen (FB)-binding activities (FBPS). This protein is the product of the *fbps* gene and is expressed by strains of serotype 2. However, the expression of the 64 kDa protein by all the serotypes of *S. suis* has not been studied. Therefore, it is possible that not all serotypes express the FBPS [199]. As this FBPS can bind to proteins from the extracellular matrix, it is possible that it may participate in tissue colonization by *S. suis.* Experimental infection of piglets with a *fbps* mutant showed that FBPS protein was not required for colonization of the tonsils but that it played a role in the colonization of the specific organs commonly involved in *S. suis* infection. Therefore, the *fbps* mutant was considered partly attenuated. The induction of specific

antibodies in piglets was observed following infection, suggesting that this protein might be an interesting candidate for a subunit vaccine [199].

1.4.7.2. Glyceraldehyde-3-phosphatase dehydrogenase (GAPDH)

S. suis possesses a 39 kDa glyceraldehide-3-phosphatase dehydrogenase (GAPDH) that can bind to albumin and results in increased adhesion of the pathogen to epithelial cells [200, 201]. Furthermore, the presence of albumin enhances the virulence of *S. suis* in mice [200]. It has been also demonstrated that GAPDH is implicated in binding to plasminogen of pig and human origin [202]. Moreover, adhesion and activation of plasminogen may be implicated in invasion and damage of tissues by *S. suis* [202]. The gene encoding the GAPDH of *S. suis* has been cloned and sequenced [203]. The *gapdh* gene has been found in isolates of serotypes 1, 9 and 7, in addition to serotype 2 [201]. However, the role of GAPDH in the pathogenesis of *S. suis* is not completely understood, as GAPDH is present in both virulent and non-virulent strains [200]. Interestingly, it has been reported that *S. suis* highly upregulates the expression of this gene *in vivo* in different porcine organs [204] and GAPDH is highly immunogenic in pigs [205].

1.4.7.3. Alpha-enolase

A 52 kDa protein with capacity to bind to the Fc fraction of IgG has been found in *S. suis* serotype 2 strains. This protein can interact with IgG from different species of mammals, including the human, pig, cow, rabbit and mouse, as well as with human IgA and chicken IgY [206, 207]. Also, a 60-kDa heat-shock protein with IgG binding protein has been characterized from *S. suis* serotype 2 [208]. Subsequent studies confirmed that these proteins were in fact identical [209]. This IgG-binding protein represents a common antigen found in all the *S. suis* serotypes including both virulent and avirulent strains of *S. suis* serotype 2, and it is found either associated with the cell surface or released in a soluble form during bacterial growth. It was found that this protein is indeed a *S. suis* α-enolase, designated as SsEno, that is expressed in all known serotypes of this pathogen. This enolase has fibronectin- and fibrinogen-binding activities and has proved to be relevant for the adhesion to and invasion of PBMEC, suggesting that it may contribute to the pathogenesis of *S. suis* meningitis [210]. In fact, Gram positive α-

enolases are cytoplasmic enzymes critical for sugar metabolism. However, they have been shown to be also surface located. Among other contributions to virulence, α-enolases are able to drive binding of pathogens to plasminogen [211, 212]. Hence, it was an exceptional finding that recombinant *S. suis* α-enolase expressed in *E. coli* was also able to bind fibronectin [207, 210]. Moreover, surface plasmon resonance demonstrated that *S. suis* α-enolase binds fibronectin with high (low nanomolar) affinity [210]. Since abolition of anchoring to the *S. suis* cell wall of LPXTG proteins does not result in full impairment of fibronectin binding by this pathogen [213], it might be hypothesized that α-enolase is one of the most important fibronectin-binding proteins. Interestingly, *S. suis* α-enolase has been shown to be expressed *in vivo* in pigs and is highly immunogenic, as demonstrated by the use of an immunoproteomic approach [214].

The potential of SsEno as a vaccine candidate has been studied. A research group demonstrated that SsEno is highly immunogenic in mice and elicits a strong immune response mediated by different subclasses of IgG. However, SsEno-vaccinated and nonvaccinated control groups showed similar mortality rates after challenge with a highly virulent *S. suis* strain. Similar results were obtained upon passive immunization of mice with hyperimmunized rabbit IgG anti-SsEno antibodies, not increasing the ability of mouse phagocytes to kill *S. suis* in vitro. It was concluded that although recombinant SsEno triggers a strong antibody response, it does not confer effective protection against infection with *S. suis* serotype 2 in mice [125] (see annex, Article IV). Alternatively, another research group identified the enolase gene from *S. suis* serotype 2, showing it to be an important trait for bacterial adherence. Moreover, they demonstrated that SsEno confers complete protection in mice and therefore it has potential as a vaccine candidate [126]. Further studies are needed to determine if indeed SsEno is a good vaccine candidate.

1.4.7.4. Pili

Pili have been shown to contribute to the virulence of different Gram-positive pathogenic species. These structures participate in adherence to host cells, colonization and systemic virulence [215]. Electron microscopy studies demonstrated that *S. suis* serotypes 1 to 8 and 1/2 present peritrichous, thin, and flexible pilus-like structures on their surface, that

measured up to 250 nm with a diameter of around 2 nm [139]. Morphologically similar structures were observed on hemagglutinating as well as on nonhemagglutinating strains of *S. suis* [216]. *S. suis* possesses four gene clusters encoding putative pili [217, 218]. Recent research evaluated the functionality of one of these clusters, known as the *S. suis* pilus *srtF* cluster. It was demonstrated that pili expressed by genes of this cluster are composed of the major pilin subunit but lack the ancillary protein (a putative adhesin). This phenotype is common among field isolates and results from nonsense mutations that prevent expression of the gene encoding the ancillary protein. Besides, as in other Gram-positive pathogens, the *srtF* cluster pilus is polymerized by the action of a dedicated sortase and anchored to the cell wall by the housekeeping sortase. However, *S. suis* pili are atypical compared to other Gram-positive pili, as it seems that their contribution to virulence of *S. suis* may be limited. In fact, abolishment of the expression of *srtF* cluster encoded pili does not impair interactions of *S. suis* with PBMEC. Furthermore, non-piliated mutants are as virulent as their WT strain in a murine model of *S. suis* infection [219].

1.4.8. Proteases

Bacterial proteases play a critical role in colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection [220]. Several proteases have been described for *S. suis*, including an Arg-aminopeptidase, a dipeptidyl peptidase (DP) IV, a chymotrypsin-like, a caseinase, a phospholipase C and a hyaluronate lyase [195, 221, 222].

The Arg-aminopeptidase of *S. suis* (55 kDa) can be found both extracellularly and cell-associated. It was suggested that its presence may be associated with production of ATP and other essential metabolic precursors via the arginine deiminase pathway [223].

The DPP IV protease (70 kDa) was found in the culture supernatant and on the cell surface of *S. suis* [223]. It is a specific protease that may cleave proteins after the X-Pro and X-Ala residues at the N-terminus of polypeptide chains [224]. Many cytokines have a proline in the second position in their polypeptide chains [225]. The *S. suis* DPP IV may result in local changes to host responses during the course of certain *S. suis*-associated infections. On the other hand, the removal of dipeptides from the N-termini of peptides can generate new

biologically active peptides. DPP IV may thus contribute significantly to the deregulation of inflammatory processes during meningitis. The *S. suis* DPP IV may also contribute to tissue destruction and systemic bacterial dissemination. [224]. It was recently demonstrated that DPP IV can interact with human fibronectin [226]. Moreover, inactivation of DPP IV appears to attenuate greatly the virulence of highly pathogen *S. suis* serotype 2 strains [226].

Caseinase (36 kDa), which putatively belongs to the class of metalloproteases, may have a nutritional function or participate in the maturation of different protein precursors. In fact, it may help in maturation of SLY [223]. The chymotrypsin-like protease, which belongs to the class of serine proteases, was detected in European, but not in North American, strains of serotype 2 tested [223].

A phospholipase C was detected in the culture supernatant and on the cell surface of *S. suis* serotype 2. This protease hydrolyses phosphatidylcholine into phosphate choline and diacylglycerol, a messenger molecule that activates protein kinase C, the latter participating in the production of arachidonic acid in target cells [224].

A hyaluronate lyase is also found in *S. suis* [221]. This enzyme catalyses the degradation of hyaluronic acid (HA), which is a glycosaminoglycan commonly found in different tissues, such as connective, epithelial and nervous tissues. *S. suis* may take advantage of this hyaluronate lyase to obtain growth factors or to facilitate tissue colonization [221]. This enzyme has been detected in *S. suis* serotypes 1, 1/2, 2, 3, 7, 9, 14 and 15 [227].

More recently, a novel serine protease that degrades porcine IL-8 was identified in *S. suis* serotype 2 [228]. *S. suis* can induce an exacerbated release of inflammatory mediators by swine endothelial cells that could cause a massive recruitment of leukocytes and subsequent BBB breakdown facilitating the pathogenesis of *S. suis*-induced meningitis [229]. Therefore, *S. suis* may secrete this IL-8 protease in order to delay recruitment of killer-neutrophils to the site of inflammation, allowing this pathogen to survive upon its arrival at CNS central nervous system [228].

In addition, *S. suis* produces an IgA1 protease. The purified recombinant protein shows good cleaving activity in IgA1 and is highly immunoreactive with convalescent sera. As the respective gene was found in most pathogenic strains but rarely in non-invasive isolates, the

IgA1 protease may play a crucial role in the pathogenesis of *S. suis* [230]. However, further studies are needed to prove this hypothesis.

S. suis also possesses a cell-surface subtilisine-like proteinase that plays a role in virulence. Mutants devoid of subtilisin-like proteinase activity have longer generation times, are more susceptible to killing by whole blood and are also less virulent in a mouse model of infection in comparison to their WT parental strain [231]. Moreover, it was found that the surface-associated subtilisin-like serine protease of *S. suis*, designated as SspA is also necessary for the development of disease in pigs, as an SspA isogenic mutant (*sspA*-) is less virulent in comparison to its WT parental strain [232].

1.4.9. Sortases

Many surface proteins which are covalently linked to the PG of Gram-positive bacteria have a consensus C-terminal motif Leu-Pro-X-Thr-Gly (LPXTG). In all Gram-positive bacteria examined so far, this sequence is cleaved, and the processed protein is attached to an amino group of a cross-bridge in the PG by enzymes known as housekeeping sortases [174, 233]. Moreover, many of these cell surface proteins possessing LPXTG motif have been reported to mediate bacterial interaction with host cells, namely the extracellular matrix, and thus play an distinguished role in virulence [234].

At first, five sortases were described for *S. suis* serotype 2, named as SrtA, SrtB, SrtC, SrtD and SrtE [233]. Complementary studies showed that SrtA belongs to the subtype A, whereas SrtB to D belong to group C [235]. Another sortase, also belonging to subtype C has been described [236]. SrtA is a transpeptidase associated to the membrane that clives LPXTG proteins between their T and G motifs. The relevance of SrtA in the pathogenesis of *S. suis* was examined using a *srtA* mutant. Interactions with host cells and extracellular matrix proteins, as well as virulence in a mouse infection model were investigated. It was found that the mutant was impaired in its interactions with porcine BMEC compared to the wild-type strain. In addition, it showed lower levels of adherence to plasma fibronectin, cellular fibronectin and collagen type I. However, disruption of *srtA* had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection [213] (See annex, Article VII). These results suggest that surface proteins anchored by SrtA are required for a normal level of bacterial binding but

are not essential for *S. suis* virulence. However, different results were obtained in a parallel study carried out using an isogenic *srtA* mutant of a highly virulent Chinese *S. suis* serotype 2 strain. In this study, deletion of *srtA* attenuated the full virulence of the virulent strain and impaired its colonizing potential in specific organs in challenged pigs. Furthermore, the *srtA* mutant displayed significant reduction in adherence to human cells (Hep-2 and human umbilical vein endothelial cells) [217].

1.4.10. Other proteins associated with virulence

Several other putative virulence factors have been ascribed to *S. suis*. Among them, we found the:

1) Surface antigen one (Sao) protein, located at the surface and exhibiting typical features of membrane-anchored surface proteins of Gram-positive bacteria, such as a signal sequence and an LPVTG membrane anchor motif [123]. Sao is a potential vaccine candidate as it elicits strong IgG and protective responses in mice and pigs [124].
2) Glutamate deshydrogenase (GDH), which is a 45 kDa protein exposed at the bacterial surface. The gene coding for GDH is conserved in all strains of *S. suis* serotype 2 tested [237]. As highly virulent strains produce electrophoretic types and sequence types of GDH distinct from those of moderately virulent and nonvirulent strains, it has been recently propose this may serve as useful markers in predicting the pathogenic behavior of strains of *S. suis* serotype 2 [238].
3) Lipoprotein signal peptidase (SP-ase), named as Lsp. SP-ase II is involved in the removal of the signal peptide from glyceride-modified prolipoproteins [239]. An isogenic mutant of *S. suis* serotype 2 unable to produce Lsp was constructed and shown to process lipoproteins incorrectly, including an *S. suis* homologue of the pneumococcal PsaA (pneumococcal surface protein A) lipoprotein [239]. In *S. pneumoniae* the PsaA lipoprotein interferes with complement-dependent host defense mechanisms [240]. However, in vivo trials carried out in pigs demonstrated that the *lsp* mutant is as virulent as its parental strain.
4) Putative phosphoglycerate mutase (38 kDa protein). This protein is found at the cell surface of *S. suis*. It is present in all known serotypes, except for serotypes 20, 26, 32

and 33 and shares a high degree of homology with sequences of unknown function from *S. pneumoniae*, *S. mutans*, *L. monocytogenes* and *Clostridium perfringens*. [241]. The recombinant protein was reactive with serum from pigs experimentally infected with virulent strains of *S. suis* serotype 2, suggesting that the protein is immunogenic and may serve as an antigen of diagnostic importance for the detection of most *S. suis* infections. Pigs immunized with the recombinant 38 kDa protein mounted antibody responses to the protein and were completely protected against challenge with a wild type strain of a homologous serotype, suggesting that it may be a good candidate for the development of a *S. suis* vaccine [241].

5) Superoxide dismutase (SOD). Superoxide ions (O_2^-) are a major antibacterial free radical in macrophage phagolysosomes. Thus, the capacity to secrete superoxide dismutases (SodA), which convert superoxide ions to molecular oxygen and H_2O_2, may be relevant in virulence. Indeed, these enzymes constitute one of the major defense mechanisms of cells against oxidative stress [242, 243]. The presence of a gene (*sodA*), encoding SodA as well as SOD activity was reported in *S. suis* serotype 2 strains [244].

6) Ssna nuclease. This is a 108 kDa protein located at the cell wall of *S. suis* serotypes 1 to 9. Its substrate specificity is for single- and double-stranded linear DNA. Screening of porcine clinical isolates from surface (nasal mucosa or palatine tonsil) or internal (joint, brain or other internal organ) locations revealed a significant relationship between expression of Ssna and isolation from an internal site [245].

7) Transmembranal 44 kDa protein. This protein of unknown function has been proposed as a virulence factor as well as an important immunogen of *S. suis* serotype 2 after two avirulent mutants were produced from a highly virulent *S. suis* strain [246]. It was demonstrated that the 44-kDa native protein, present in the parent strain, was absent in both mutants. Besides, complementary studies demonstrated that the protein was strongly immunogenic. However, the real contribution of this protein has not been further verified. [246].

8) Arginine deiminase system (ADS). In *S. suis* two cell wall-associated proteins with homologies to proteins from the ADS system of GAS were identified [247]. One of these proteins, of 47 kDa, is encoded by the *octS* gene and shares homologies with a orthine carbamoyl transferase, whereas the other protein, of 53 kDa, is encoded by

the *adiS* gene and is homologous to the streptococcal acid glycoprotein from *S. pyogenes* [247]. The ADS system catalyzes the conversion of arginine to ornithine, ammonia, and carbon dioxide. ADS are widely distributed among prokaryotic organisms and, in oral streptococci and GAS, they seem to provide protection against acidic stress by the production of ammonia to increase pH [248, 249]. Furthermore, it has been shown that the ADS of GAS is involved in adhesion to and invasion of epithelial cells [249]. Stgudies using an *adiS* mutant (called *arcA* mutant in the study), in which expression of the arcABC operon (from the ADS system) was abolished, demonstrated that this operon contributes to survival under acidic conditions [250].

9) Proteins binding to the extracellular matrix (ECM) and to plasmatic proteins. The ECM is a complex, structural network that gives support to epithelial and endothelial cells and surrounds connective tissue. The ECM is composed of different biomolecules, the most important being several glycoproteins, including collagen, fibronectin, elastin and laminin and glycosaminoglycans, such as heparin and heparan sulfate [251]. Following an injury or trauma, the ECM of the host is exposed and its components may serve as potential receptors for pathogen bacteria. In addition, bacteria may bind to different plasmatic and tissular, hiding their own immunologic epitopes with the aim to avoid recognition by the immune system. Recently, adherence of *S. suis* serotype 2 to different proteins from the ECM and from plasma was demonstrated [198]. In fact, all tested strains of *S. suis* bound to plasma and cellular fibronectin and collagen types I, III, and V, and some strains bound to fibrin, vitronectin, and laminin. An unencapsulated isogenic mutant bound to ECM proteins better than its parental encapsulated strain, suggesting that the CPS interferes with binding. On the other hand, pre-incubation with plasma fibronectin increased binding to collagen IV, suggesting that *S. suis* might use fibronectin as a bridging molecule. The results of heat treatment and proteolytic digestion suggest that adhesins for these ECM proteins are proteinaceous in nature [198]. However, the specific mechanisms implicated in these interactions remain to be studied.

10) Very recently, using a comparative proteomic approach with *S. suis* serotype 9 isolates from diseased and healthy pigs, several novel proteins that may be considered as putative virulence-associated factors were identified. These proteins are a DNA

nuclease (which seems to be different from Ssna nuclease), *o*-acetylserine lyase, PG-binding LysM, phosphoglycerate mutase, and putative 5'-nucleotidase [252]. More studies are needed in order to evaluate their respective role in the pathogenesis of *S. suis*.

1.5. Pathogenesis of *S. suis* infection

Most of the studies concerning the pathogenesis of *S. suis* have been carried out using strains from serotype 2 [6], and although exhaustive reseach has been performed, the knowledge on the mechanisms implicated in the development of the infection is still limited.

1.5.1. Portal of entry and colonization of tonsils

Both vertical and horizontal transmission of *S. suis* have been demonstrated in pigs [6]. In the case of horizontal transmission, most researchers believe that *S. suis* probably enters the host via the upper respiratory tract and resides within the palatine [253] or pharyngeal tonsils [254, 255]. Palatine tonsils in pigs are penetrated by numerous crypts which extend into, and branch extensively within, the lymphoid tissue. Stratified squamous non-keratinised epithelium covering the oropharyngeal surface is continuous with that lining the neck of crypts. Lymphoepithelium covers the tonsillar lymphoid tissue within the crypts. It consists of non-keratinised epithelial cells, M cells, goblet cells and many intraepithelial lymphoid cells [256]. The tonsils have been described as one of the primary sites of invasion by bacteria, with subsequent spread to the superpharyngeal lymph nodes [257]. In fact, *S. suis* antigens were identified by a specific fluorescent marker, and whole bacteria were frequently observed in the crypt lumen, but were also present in the epithelium and immediate sub-epithelial zone. In the inter-follicular tissue, the *S. suis* antigen was sometimes closely associated with cell nuclei and small amounts of particulate fluorescence were seen within a minority of tonsillar germinal centres [253]. Bacteria were found in association with cells of the myeloid lineage. Expression of CD16 and CD163 on these leukocytes suggested an association with mature macrophages in tonsils, which may lead to clearance or control of the micro-organism [253]. Other studies have confirmed that the presence of *S. suis* at the tonsillar crypts leads to an increase in the CD4 and

CD8 lymphocytes, suggesting the initiation of both humoral and cellular responses against *S. suis* might start within the crypt epithelium of the tonsils [258].

Once colonized by *S. suis*, some animals may remain as clinically healthy carriers, whereas others may develop bacteremia, septicemia and/or meningitis. For these events to happen, *S. suis* must first be able to disseminate from tonsils and/or other mucosal surfaces and reach the circulation (both blood and lymph). As mentioned above, in humans, the portal of entry is mainly through skin wounds [56] with access to blood circulation.

1.5.2. Trespassing of mucous membranes

S. suis is present at low levels on mucosal surfaces, and the mechanisms that this pathogen uses to traverse the first mucosal barriers to develop disease are still not completely understood. In fact, bacteria would need to breach mucosal epithelia in the upper respiratory tract to reach blood circulation [9]. It has been reported that virulent *S. suis* strains can adhere to epithelial cell lines of pig and human origin [186, 187]. The adhesin(s) involved seem(s) to be partially masked by the CPS and are a part of the cell wall [186]. In addition, *S. suis* isolates recovered from diseased animals adhere to frozen sections of the pig lung, whereas isolates from clinically healthy animals show a weak adherence. The thickness of the capsular material seems to influence the adherence activity, but is probably not the only mechanism involved [259]. As examples of new putative mechanisms involved in epithelial adhesion, we found the orphan transcriptional regulator, designated RevSC21 and the gene coding for glutamine synthetase (GlnA) [260, 261]. In the former case, deletion of the revSC21 gene of *S. suis* serotype 2 yielded a mutant strain which exhibited a significant decrease in adherence to human laryngeal epithelial cells, and lacked hemolytic activity. Complementary studies revealed that RevSC21 influences the expression of MRP, EF, SLY and CPS, among other virulence-associated factors [260]. In the latter example, and similarly to the revSC21 mutant, an isogenic mutant exhibited a significant decrease in adherence to the human laryngeal epithelial cells. Furthermore, it seems that GlnA plays a role in the colonization of the specific organs involved in *S. suis* infection [261].

In addition, *S. suis* may damage epithelial barriers to advance invasion of multiple tissues. In this regard, SLY positive strains may use cell disruption to reach the bloodstream as it

has been reported that suilysin is toxic for epithelial cells [186, 187, 262]. However, strains not producing this hemolytic toxin are also able to reach the circulation and disseminate [6]. The possibility that *S. suis* may be capable of traversing epithelial barriers without cytotoxicity is still under debate, as invasion of epithelial cells by receptor-mediated endocytosis has only been demonstrated for non-capsulated and non-typeable *S. suis* strains [263].

1.5.3. Blood dissemination: bacterial uptake and importance of phagocytic cells

In order to disseminate throughout the body and cause bacteremia, and septicemia, *S. suis* must be able to survive in blood. Development of septicemia is a critic step prior to the development of meningitis [9]. To survive in blood and resist the army of phagocytic cells, *S. suis* is equipped with a thick CPS that serves as a shield and hampers neutrophil and monocyte/macrophage-mediated killing [6, 264]. In fact, many studies support this theory, as contrary to capsulated strains, isogenic non-capsulated strains are highly sensitive to phagocytosis by murine and porcine macrophages [140, 141] and porcine neutrophils [264].

Still, resisting phagocytosis is not the same as dissemination. In order to cause a generalized infection and meningitis, *S. suis* must facilitate its travel throughout the bloodstream. Early findings suggested a “Trojan Horse” strategy, in which bacteria are taken up by monocytes (in the absence of specific antibodies), surviving within phagocytic cells, thus facilitating invasion of the CNS [265]. Studies carried out with flow cytometry also indicated uptake of *S. suis* by swine and human phagocytes [266]. In fact, this bacterial uptake could take place directly at the tonsils by macrophages or once the bacteria are in the bloodstream [9]. Nevertheless, most of the studies in the last decade regarding this subject demonstrate that *S. suis* may use different strategies to circulate, more precisely, either free or adhered to the surface of monocytes, a theory that is known as the “Modified Trojan Horse” [9]. Indeed, instead of being phagocytised, virulent capsulated *S. suis* remains attached to phagocytic cells [142, 149]. This phenomenon appears to be mediated by the presence of *S. suis* CPS that down-regulates central signaling pathways involved in macrophage phagocytosis [148].

In addition, *S. suis* possesses other weapons that can damage phagocytic cells. More precisely, SLY appears to affect complement-dependent killing by decreasing the opsonization

of *S. suis* and the bactericidal capacity of neutrophils and macrophages [149, 264] and thus, affecting the efficiency of the innate immune system.

1.5.4. *S. suis* and the development of the inflammatory response: *In vitro* studies

Interactions between *S. suis* and phagocytic cells are crucial for the development of the inflammatory response. In fact, *S. suis* is capable of up-regulating important adhesion molecules involved in inflammation, such as intercellular adhesion molecule-1 (ICAM-1, CD54), CD11a/CD18 and CD11c/CD18 in human monocytes. This may result in an increased leucocyte recruitment into sites of infection, thus providing a possible mechanism for some of the inflammatory features of meningitis caused by this pathogen [267]. Once in contact with murine and human macrophages, *S. suis* induces the secretion of different pro-inflammatory cytokines and chemokines, including tumor necrosis factor-alpha (TNF-α), Interleukin (IL)-6, IL-1β, IL-8 and monocyte chemotactic protein (MCP-1) [268, 269]. Porcine whole blood stimulated by live *S. suis* also releases high levels of TNF-α, IL-1β and IL-6 and intermediate levels of IL-8 and MCP-1. The bacterial cell wall is the major cytokine-inducing component [192]. Other studies support these findings, as porcine macrophages stimulated with a *S. suis* CPS mutant express a higher activation of pro-inflammatory genes, including TNF-α, IL-1β and MIP-2, in comparison to the well capsulated parental strain [270].

Moreover, stimulation of human monocytes by whole encapsulated *S. suis* or purified cell wall components triggers the release of a similar profile of cytokines and chemokines [271]. In experimentally infected pigs, *S. suis* induces the systemic production of IL-6 and IL-8 [272] (See annex, Article VIII). This production of pro-inflammatory mediators will have many systemic effects, contributing to the recruitment of leucocytes to the site of infection, enhancing haematopoiesis and inducing fever [273]. However, although inflammatory cytokines and chemokines contribute to the anti-infectious process, their production has severe side-effects, which will be discussed below.

1.5.5. Reaching the Central Nervous System (CNS)

1.5.5.1. Blood-brain barrier (BBB) disruption

To date, we know that in order to cause meningitis, *S. suis* colonizes the mucosa of the upper respiratory tract, with further invasion of the intravascular space, where it survives/multiplies to cause bacteremia and induces the inflammatory response. *S. suis* must then reach and invade the CNS. But how does *S. suis* induce meningitis? It is generally accepted that once in circulation (either free or in association with monocytes), meningitis-causing bacteria traverse the blood-brain barrier (BBB) and survive and multiply in the subarachnoid space to elicit inflammatory responses from the host, inciting pathophysiological alterations such as pleocytosis and BBB disruption [274]. However, in order to completely understand the development of the infection, we need to focus on the specific mechanisms that *S. suis* elicits to arrive and traverse the BBB. It is necessary to elucidate the strategy elicited by *S. suis* to get access to the CNS and study the interactions with endothelial cells from the BBB. It is also necessary to identify possible alternative portals of entry, such as the blood-cerebrospinal fluid (CSF) barrier (BCSFB) and compare the mechanisms of invasion elicited by *S. suis* with those used by other Gram-positive meningitis bacteria.

The BBB is a structural and functional barrier formed by brain microvascular endothelial cells (BMEC). BMECs possess distinct features such as tight junctions with a high electrical resistance between them, and low rates of pinocytosis [274-276]. These microvessels are covered by pericytes and outgrowths of astrocytes, which are referred to as astrocytic end-feet [277, 278] (Fig. 5). Pericytes are a very important cellular constituent of the BBB. They play a regulatory role in brain angiogenesis, endothelial cell tight junction formation, BBB differentiation, and contribute to the microvascular vasodynamic capacity and structural stability [279]. On the other hand, astrocytes provide the cellular link to the neurons and support the integrity of tight junctions [280]. The BBB functions as a high-resistance barrier to circulating macromolecules, protecting the brain from any microbes and toxins circulating in the blood. Disruption of the blood-brain barrier is a hallmark event in the pathophysiology of bacterial meningitis [274, 275].

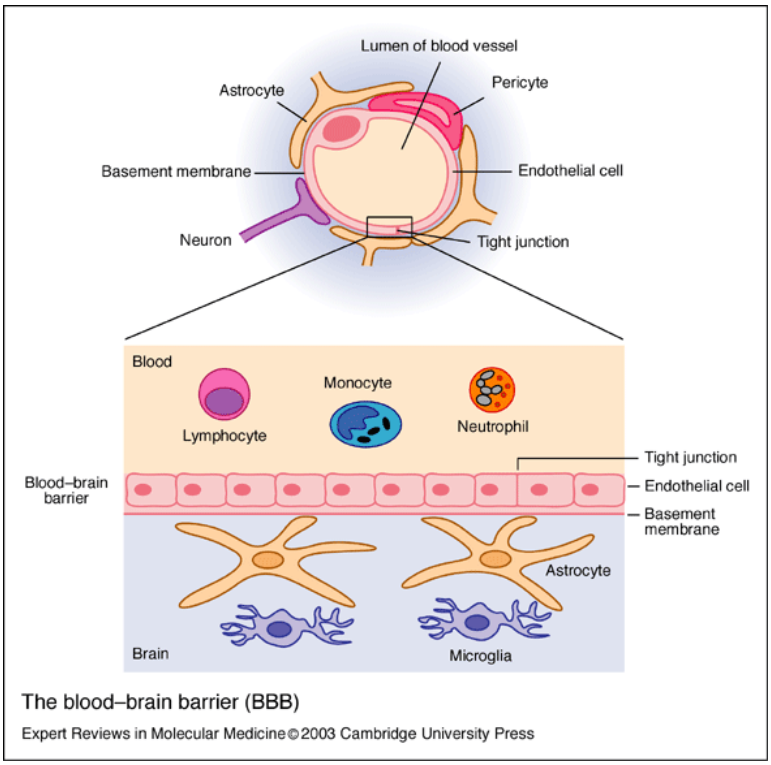


Fig. 5: The blood brain barrier. Taken from [278].

Several studies have addressed the interactions between *S. suis* and brain microvascular endothelial cells (BMEC) of either human (HBMEC) or porcine origin (PBMEC). Early studies demonstrated that this pathogen is capable of adhering to HBMEC, although cell invasion could not be confirmed. Moreover, as SLY was cytotoxic for HBMEC, it was suggested that SLY-positive *S. suis* strains use adherence and SLY-induced HBMEC injury, as opposed to direct cellular invasion, to proceed from the circulation to the central nervous system [281]. Interestingly and contrary to observations in epithelial cells, *S. suis* attachment to HBMEC seems to be independent of the CPS, as a noncapsulated mutant adhered similarly compared to its wild-type strain [281]. Once attached to HBMEC, bacterial cell wall components stimulate the production of several pro-inflammatory cytokines from HBMEC, including IL-6, IL-8 and MCP-1 [282], and stimulate the expression of adhesion molecules, such as soluble intercellular adhesion molecule-1 (sICAM-1) [283], whereas SLY induces the secretion of arachidonic acid [195]. Moreover, this bacterial attachment to BMEC seems to be specific, as *S. suis* does not adhere nor stimulate the production of pro-inflammatory cytokines from other types of endothelial cells, such as human umbilical vein endothelial cells [281]. Furthermore, bacterial

challenge stimulates production of different pro-inflammatory cytokines and chemokines, as well as that of pro-inflammatory mediators, including prostaglandin E(2) (PGE(2)), and matrix metalloproteinase 9 (MMP-9) in co-cultures of HBMEC and macrophages infected with *S. suis* [284]. The pro-inflammatory cytokine production along with the release of other pro-inflammatory mediators and expression of adhesion molecules may play a main role in initiating changes in permeability or adhesion properties of the same BMEC. This may provoke local hemodynamic changes, such as edema accumulation and hemorrhages and may allow the immune cells to infiltrate the CNS, and possibly influencing the activity of nearby cells, such as astrocytes and glial cells. [195, 282, 285].

Further studies revealed the ability of *S. suis* to adhere, and most importantly, to invade porcine BMEC (PBMEC) (Fig 6). In fact, viable bacteria were recuperated from PBMEC after several hours of incubation. In addition, the CPS of *S. suis* seemed to partially interfere with the adhesion and invasion abilities of the bacterium. This study also showed that invasion of *S. suis* in PBMEC requires actin microfilaments but not microtubular cytoskeletal elements. SLY proved to be a key element, as SLY-positive strains are toxic for PBMEC. Other researchers claim that *S. suis* adhesion to PBMEC is greatly influenced by the presence of CPS and by serum components. Surprisingly, and in disagreement with the previous results, they found that none of the *S. suis* tested was able to invade the PBMEC [286]

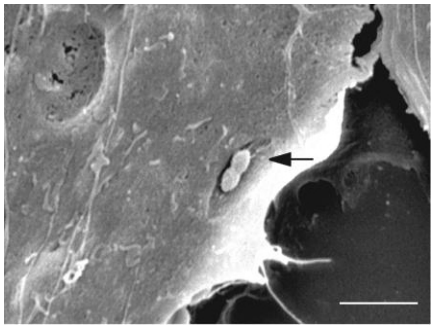


Fig. 6. *S. suis* invades PBMEC. The pathogen is observed forming small chains within invagination structures (arrow). Bars = 1.5 µm. Adapted from [4].

Other research examing molecular means by which *S. suis* interacts with PBMEC revealed that once in contact with these cells, *S. suis* will induce the expression of several putative virulence genes, including *dltA*, *pgdA* and *srtA* [236]. As mentioned in their respective sections (1.4.2 and 1.4.3), different in vitro and in vivo studies have confirmed the importance of *dltA* and *pgdA* genes in the pathogenesis of *S. suis* infection. In fact, their respective isogenic *S. suis* mutants are more susceptible to the killing effect of leukocytes and clinical presentation

of meningitis is significantly reduced in comparison to their parental strain An isogenic mutant lacking the *srtA* gene has a reduced capacity to adhere and invade PBMEC in comparison to its wild type strain (section 1.4.9) [287].

1.5.5.2. Possible entry through the blood-cerebrospinal fluid barrier (BCSFB)

The choroid plexus (CP) epithelium (CPe) is a villous organ that produces and secretes the bulk of the CSF into the ventricles of the brain. The CP consists of a single-layered cuboidal epithelium, which surrounds a highly vascular connective tissue core, and its surface is greatly increased by many villous projections. BBB barrier properties are present at the CPe/CSF interface, as the CPe presents apical tight junctions between cells, inhibiting paracellular diffusion of water-soluble molecules across this barrier. This barrier is known as the blood-cerebrospinal fluid (CSF) barrier (BCSFB). Besides its barrier function, CPe has a secretory function and produces the CSF. The barrier and secretory function of the CPe is maintained by the expression of numerous transport systems allowing the directed transport of ions and nutrients into the CSF and the removal of toxic agents out of the CSF [276, 288].

Transwell models of *in vitro* infection have demonstrated that the CPe is capable of restricting growth of *S. suis* upon activation with proinflammatory cytokines by creating an unfavorable microenvironment for the bacteria [289]. However, in contrast, *S. suis* seemed to affect the porcine CPe barrier function and integrity, as hemolytic strains of *S. suis* were cytotoxic for the CPe, and other death-cell mechanisms, such as apoptosis seemed to be involved [290, 291]. However, the development of an “inverted” Transwell filter system of porcine choroid plexus epithelial cells (PCPEC) from the same research group, permitted the investigation of possible bacterial invasion and translocation from the “blood side” (basolateral side) to the apical (CSF) side of the BCSFB, which better reproduces the normal physiological *in vivo* conditions. The basolateral cell membrane of the PCPEC is a significant obstacle that the bacteria encounter before they penetrate the BCSFB, the endothelium of the CP being fenestrated and having only minor barrier functions. In contrast, the PCPEC are firmly attached to each other by tight junctions and represent the structural basis of the BCSFB [292]. In this study, there was specific invasion and translocation of *S. suis* across the PCPEC exclusively from the basolateral side. This process seems to be influenced by bacterial viability, the presence of

the CPS and cytoskeletal regulation of PCPEC. Interestingly, and in striking contrast to previous research, no cell injury nor loss of barrier function was observed [293].

Anatomapathological findings at the CP of pigs infected by *S. suis* show disruption of the plexus brush border, a decrease in the number of Kolmer cells, and exudation of fibrin and inflammatory cells into the ventricles, reaching the arachnoid villi and olfactory nerve sheaths [265, 294]. Once in the CSF, *S. suis* may be free to survive and gain acccess to the brain parenchyma. Bacterial multiplication may be favored as the CSF contains almost no immunoglobulins, only a few leukocytes are present and no components of the Complement System are normally found [295].

1.6. Animal models for experimental infection with *S. suis*

Animal experimentation is an essential tool for the study of infectious diseases, including meningitis. These animal models may be useful to clarify mechanisms of disease pathogenesis, test novel drugs and vaccine candidates and characterize the role of bacterial and host factors and genetic susceptibility to infection [296]. Different animal species, including the pig, its natural host, have been used as animal models of *S. suis* infection. Results from these studies are very valuable and have set the reference for future research. However, to date, most of these studies have been performed with *S. suis* serotype 2. They have focused mainly on clinical aspects of the disease, comparison of the virulence of different *S. suis* strains, testing the relevance of different bacterial components, and evaluation of efficacy of vaccine candidates and of antimicrobials. Currently, *S. suis* is considered to be not only an important swine etiologic agent, but also a relevant emerging human pathogen. Therefore, there is an urgent need for suitable animal models that mimic clinical features found in infected swine and humans and that allow more comprehensive research on the activation and development of host immune responses to this important pathogen.

1.6.1. Swine

As the pig is the natural host of *S. suis*, early animal experimentation took place in this animal species. Experiments have been carried out using pigs of different ages and with diverse genetic background, testing different inoculation routes or amount of inoculum, and considering different parameters to determine virulence. In addition, availability of many of the techniques and reagents necessary to identify host factors associated with the development of the infection are not standardized for pig samples. Quality of animal facilities is also crucial to obtain trustworthy results. It is advisable that animals are housed in separate units into which air is forced through absolute filters (0.3 μm pore size) to prevent any contact of these animals with infectious agents [133].

The intranasal (IN) route of infection in pigs has been used to demonstrate that natural horizontal transmission of *S. suis* occurs by this means, and to draw attention to the tonsils as first site of colonization [255]. This route has also served to underline the possible association between *S. suis* and viruses. In fact, pigs infected experimentally with PRRSV and *S. suis* are more susceptible to develop pneumonia and bacteremia due to *S. suis* in comparison to pigs infected only with the bacterium. PRRSV-induced suppression of pulmonary intravascular macrophage function may in part explain PRRSV-associated increased susceptibility to *S. suis* infection [297]. Similar data were obtained in pregnant sows intranasally infected with PRRSV, whose piglets were more susceptible to *S. suis* infection than those born to non-infected sows [48]. IN infected pigs sometimes only develop subclinical infection, although this is transmissible among litters [298]. Treatment of these animals with acetic acid (also by the IN route) favors inducing the disease [299]. Infection of Göttingen minipigs with aerosolized *S. suis*, following exposure to acetic acid also by aerosol has also been proposed as an experimental model of infection. Since this breed is defined as free of *S. suis* and a range of other endemic porcine pathogens, this experimental model might be useful in the study of this infection[300].

On the other hand, the intravenous (IV) route of infection has been used in most of the experimental research with *S. suis* in pigs. This route is more advantageous as there is better control of the amount of bacteria inoculated, and it better reflects the septicemic and CNS

phases of disease. This latter advantage has facilitated the elucidation of virulence of several *S. suis* strains. In fact, the use this route in SPF piglets has been proposed as a standard model for *S. suis* infection. This standardized method proposes the use of 7-week old SPF piglets kept in isolation rooms with controlled air flow [133]. It allows the differentiaton of virulence of *S. suis* strains, based on clinical signs, zootechnical performance, lesions, and bacteriological findings [133, 301]. Some studies report that no clinical signs nor lesions are found in animals inoculated by the IV route [299]; however, this may be due to the amount of inoculum and size of experimental group. In general, IV infected animals develop several clinical signs, including depression, fever, lameness, seizures and paddling [40, 42, 133, 163, 272, 301]. Moreover, lesions at the CNS reflect for the most part those found in naturally infected pigs [42]. The IV route of *S. suis* infection has also been used for other purposes:

1) To identify immunogenic bacterial proteins that are induced or upregulated in vivo, and differences of their expression among *S. suis* strains [302].
2) To evaluate the importance of several putative virulence factors, such as the CPS, SLY, D-alanylation of LTA, N-acetylation of PG, FBPS and sortase A in the pathogenesis of infection [40, 119, 120, 122, 163, 199, 217, 272, 303].
3) To test the protective capacity of live vaccines or possible immunogenic molecules that may serve as vaccine candidates [116, 122, 205, 303-305].

In swine, the intracisternal (IC) model of infection has also been used, in particular for comparison with the IV route, obtaining similar results [294]. However, as this route bypasses several steps of the natural infection and handling of animals is more difficult, it has not been used for *S. suis* research in recent years.

1.6.2. Mouse

Experimental *S. suis* disease has also been investigated by using the mouse as model of infection. However, as encountered for the pig, it has been difficult to establish a standard model of mouse infection due to variability. Experiments have been performed with either outbred or inbred mouse strains infected with different *S. suis* strains and with dissimilar inoculum size. Moreover, as criteria for experimental *S. suis* infections in mice have not been established, it is difficult to compare the results among studies.

Nevertheless, *S. suis* experimentation in mice be more advantageous than the pig model of infection as manipulation of animals is easier and there is a wide selection of laboratory reagents, equipment and techniques available for use with mouse samples. To date, these studies have been performed with outbred mouse strains, such as CD1, BALB/c and CF-1, inoculated in most of the cases by the intraperitoneal (IP) route. The use of outbred mice strains offer phenotypic diversity that may be important to imitate the natural variation in response to infection. However, the inclusion of mouse inbred strains as models of *S. suis* infection should be considered. Inbred mice offer more uniform responses to experimental treatments due to their uniformity in the immune system. In addition, there is now a wide array of inbred "knock-out"(KO) mice available, with inactivation of specific genes that could facilitate the identification of host's factors participating in infection and may help understand the mechanisms of the host's susceptibility or resistance to infection.

S. suis research in mice has been performed to determine if the behavior of *S. suis* in this animal species resembles that in pigs [306, 307]. Moreover, as in the pig, mouse models have served to test the immunogenic potential of *S. suis* proteins or the use of *S. suis* strains as vaccine candidates [305, 308]. The mouse has also been used to assess the virulence of different *S. suis* strains or the importance of certain bacterial components for the development of the disease (CPS, SLY) [141, 197]. Kunming mice, which are the most popular outbred colony in China, have been used to test the efficacy of adjuvants to boost resistance against infection. Interestingly, in this case, infection with *S. suis* was performed by the oral route [309].

1.6.3. Others

Other animal species have been used in the research for *S. suis* with different purposes. For example, the hamster was used to determine the possible anatomical origin of hearing loss, a common sequela in humans that have suffered *S. suis* meningitis. In this case, the IP, intracranial and intrabullar routes of infection were examined, with 47%, 100% and 80% of successful reproduction of clinical meningitis, respectively (criteria of meningitis are not specified). *In vivo* studies of audiometry suggested there was hearing loss, and further histological examination of affected tissues allowed establishing that hearing loss is possibly associated to cochlear sepsis [310].

2. The innate immune response (IIR)

2.1. Components of the IIR

Immune systems in vertebrates are divided into two basic categories: innate and adaptive immunity. Adaptive immunity relies on antigen-specific receptors expressed on clonally expanded T and B lymphocytes that are generated by gene rearrangements and hyper mutations [311]. The innate immune system (IIS) is an evolutionarily ancient part of the host defense mechanism [312]. It constitutes the first line of host defense against pathogens and therefore plays a crucial role in the early recognition and subsequent triggering of a pro-inflammatory response [311, 313]. The IIS consists of physical and chemical barriers to infection, as well as of different cell types recognizing invading pathogens and activating antimicrobial immune responses. Physical and chemical defense mechanisms are represented by the epidermis, ciliated respiratory epithelium, vascular endothelium, and mucosal surfaces with antimicrobial secretions. Similarly, the cellular components of innate immunity include macrophages and granulocytes, cytotoxic natural killer cells (NK), antigen-presenting cells (APCs), that is, dendritic cells (DCs) and γδ T lymphocytes [314, 315]. In fact, one of the key components of the IIS lies behind the inflammatory response triggered by these cells [312].

The innate immune response (IIR) is not completely nonspecific, as was originally believed, as it is able to discriminate between self and a variety of pathogens. The IIR is activated through a limited number of germline-encoded pattern recognition receptors (PRRs) that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Table 1). The principal functions of PRRs include opsonization, activation of complement and coagulation cascades, phagocytosis, induction of apoptosis and activation of proinflammatory signalling pathways [312-317]. These PRRs are designed to recognize specific arrangements of key molecules called pathogen-associated molecular patterns (PAMPs). The PAMPs are vital structures of the microbial cell that have altered little over evolutionary time-spans and include LPS, lipoproteins, LTA, PG, lipoarabinomannan and oligosaccharides, among others [312, 317]. PAMPs are generated by microbes and not by the host, suggesting that PAMPs are good targets for innate immunity to discriminate between self- and nonself [312]. Among PRRs, TLRs have been highlighted as the key recognition structures of the IIS [312, 317].

2.2. Pattern recognition receptors: Family of Toll-like receptors (TLRs)

The family of TLRs, which are localized either to the cell surface or within endosomes, is the major and most extensively studied class of PRRs (Fig 7) [315, 318]. The Toll gene was identified as a gene essential for the dorsal-ventral development during embryogenesis as well as in the antifungal response in *Drosophila melanogaster* [315]. Structurally, TLRs are integral glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain. Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction [313, 315, 317, 319]. The TLR family now consists of thirteen mammalian members [313]. To date, ten TLRs have been identified in humans (hTLRs) while in the mouse twelve TLRs (mTLRs) have been recognized [319]. The genes corresponding to all ten TLRs genes identified in humans have been fully cloned in pigs [320]. Individual TLRs are differentially distributed within the cell. TLR1, TLR2, TLR4 and TLR6 are expressed on the cell surface (Fig 7), as demonstrated by positive staining of the cell surface by specific antibodies. In contrast, TLR3, TLR7, TLR8 and TLR9 have been shown to be expressed in intracellular compartments such as endosomes (Fig. 7) [5, 313]. Each TLR recognize distinct PAMPs derived from various microbial pathogens, including viruses, bacteria, fungi, and protozoa (Table 2 and Fig. 7) [5, 315, 321]. In addition to the recognition of PAMPs, TLRs may also be able to recognize endogenous signals such as heat shock proteins and products of necrotic cells and thus may be involved in auto-immune phenomena or activation of tissue repair [317].

TLRs are preferentially –but not exclusively- expressed in APCs, including macrophages and B lymphocytes [315, 317]. TLR signaling causes DCs to become APCs by the induction of co-stimulatory molecules (such as CD80 and CD86), the up-regulation of major histocompatibility complex (MHC) molecules and the secretion of cytokines and chemokines [319]. In fact, TLRs have been identified in most cell types, expressed either constitutively or in an inducible manner in the course of infection [315].

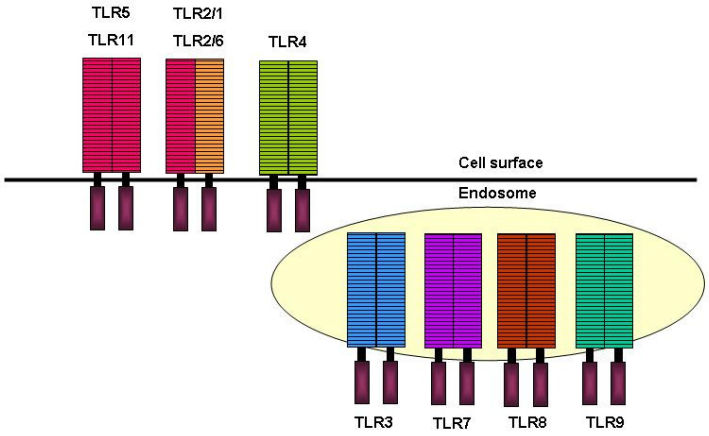


Fig. 7. TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. TLR11 participates in recognition of uropathogenic bacteria (ligand still unknown). Thus, the TLR family members recognize specific patterns of microbial components. Adapted from [5].

As aforementioned, TLRs play a central role in the recognition of a wide array of microbial components. Actually, most Gram-positive and -negative bacteria can activate additional TLRs via alternative PAMPs present in the cell membrane, cell wall, or intracellularly [315]. As general rule, it is considered that the lipid A portion of LPS from Gram-negative bacteria is recognized through the TLR4/MD2/CD14 complex [313, 322]. When LPS is present in the blood stream, it is immediately captured by LBP, which converts oligomeric micelles of LPS to a monomer for delivery to CD14. Then, CD14 concentrates LPS for binding to the TLR4/MD2 complex (Fig. 7) [313, 323].

2.3. Importance of TLR2

TLR2 is a versatile receptor, as it recognizes a variety of microbial components, including lipoproteins/lipopeptides from Gram positive/negative bacteria and PG, LTA from Gram-positive bacteria. These two last components can trigger a toxic shock syndrome similar to that induced by LPS [313, 314, 317]. MD2 enhances TLR2-mediated recognition, as it is physically associated with this receptor, but this association is weaker than with TLR4 [323]. Two characteristics have been proposed as mechanisms that could explain why TLR2 recognizes a wide spectrum of microbial components. The first explanation is that TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively (Table 2, Fig. 7) [5]. The second explanation involves the ability of TLR2 to recognize fungal-derived components. Indeed, TLR2 collaborates with distinct types of receptors such as dectin-1, that is a lectin family receptor for the fungal cell wall component β-glucan [5, 324].

In the case of Gram-positive cocci responsible for meningitis, it is now known that TLR2 is involved in the recognition of *S. pneumoniae* and in the expression of the pro-inflammatory response. In this case, TLR2 is the receptor for pneumococcal cell wall, including PG [325]. However, pneumolysin, a crucial virulence factor of *S. pneumoniae* virulence, triggers the inflammatory response via TLR4 [326], denoting that the host can synergistically activate different TLRs for *S. pneumoniae* recognition. As for GBS, researchers have found that this pathogen elicits cell recognition through TLR2 and a MyD88-dependent pathway, events that lead to cytokine and nitric oxide (NO) production, molecules that have a direct negative impact on neurons, as there is induction of apoptosis in these cells via a caspase-8 pathway [327, 328]. Moreover, GBS cell-recognition also requires CD14 and TLR6, that may act synergistically with TLR2 and function as co-receptors for secreted microbial products. It also seems that neither TLR1 nor TLR4 participate in GBS recognition [329]. Further downstream signaling pathways are by both MyD88 –dependent and –independent pathways. Whereas the former is necessary for NF-κB expression, bacterial uptake depends on the later mechanism [330]. In addition, Gram-positive bacteria can also trigger cytosolic PRRs, including NOD1/2 and the NALP1 inflammasome, both activated by PG [315, 331].

2.4. Pro-inflammatory signaling pathway

The net result of TLR engagement of a relevant PAMP is the triggering of downstream signaling pathways, ultimately resulting in the generation of an antimicrobial proinflammatory response [315]. This includes the production of inflammatory cytokines and chemokines, antimicrobial peptides, costimulatory molecules, MHC molecules, and other effectors necessary to arm the host cell against the invading pathogen [312]. Importantly, TLRs activate a common signaling pathway that will provoke the production of pro-inflammatory cytokines as well as alternative pathways that will induce appropriate and effector responses against different types of pathogens. In particular, TLR3, TLR4, TLR7, TLR8 and TLR9 induce antiviral responses by inducing type I IFN [311]. Interestingly, septic patients present an increased expression of CD14, TLR2 and TLR4 on monocytes, and death is associated with downregulation of TLR2 and CD14 expression on monocytes [332].

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. This allows them to use the same signaling components as IL-1R. Four TIR domain-containing adaptors (MyD88, TIRAP/MAL, TRIF, and TRAM) play a relevant role in TLR signaling pathways. Each TLR mediates distinctive responses in association with a different combination of these adapters [313]. To date, evidence indicates that TLR signaling pathways consist of a minimum of a MyD88-dependent pathway that is common to all TLRs, except for TLR3, and a MyD88-independent pathway (also known as TRIF-dependent pathway) that is restricted to TLR3 and TLR4 [5, 311, 315, 333].

2.4.1. MyD88 dependent pathway

In the MyD88 dependent pathway (Fig. 8), upon ligand binding TLRs/IL-1Rs dimerize (a), thereby undergoing conformational changes required for the recruitment of the adaptor molecule MyD88 (b). **T**he TIR domain-containing adapter MyD88 possesses a TIR domain in its C terminus and a death domain in its N terminus. Upon stimulation, MyD88 recruits IL-1 receptor kinase (IRAK) to TLRs through the interaction of both molecules' death domains. Two members of the IRAK family, IL-1 receptor-associated kinase (IRAK-1) and IRAK-4 (c) are activated by phosphorylation, dissociate from MyD88 and associate with TRAF6 (d). This last adaptor forms a complex with ubiquitin-conjugating enzymes (e) to activate TAK1 (f). TAK1 forms a complex with TAB1, TAB2 and TAB3. TAK1 leads to the activation of NF-κB (IκB) and activator protein-1 (AP-1) through the canonical IκB kinase (IKK) (g) complex and the MAPK respectively (h). AP-1 activation in TLR signaling is mostly mediated by MAP kinases such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK). Many TLR ligands activate these MAPK in similar kinetics.

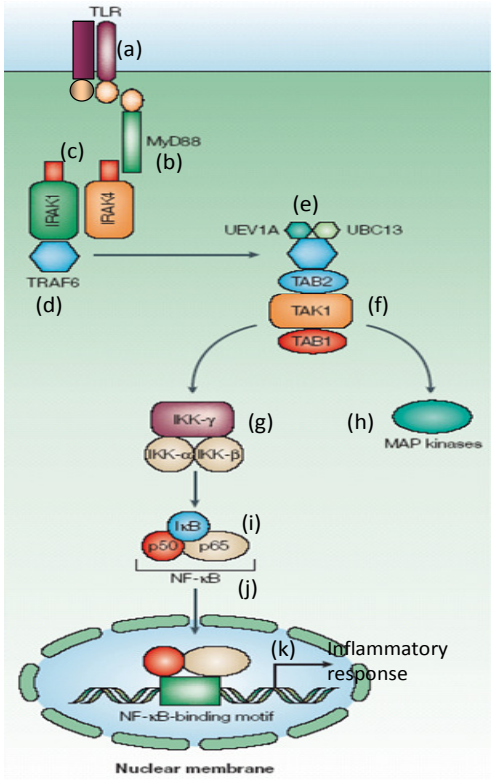


Fig. 8. MyD88 signalisation pathway. For details, see text. Adapted from [3].

The IKK complex phosphorylates the inhibitor of IκB (i), which becomes ubiquitinated and destroyed by the proteosome. IκB itself is a NF-κB target gene, and its de novo synthesis is a crucial auto-regulatory loop. Once released from its inhibitor, the NF-κB (j) moves to the nucleus, binds to specific DNA regions and up-regulates a number of pro-inflammatory genes involved in host defense (k). This signaling pathway is essential, for the expression of

inflammatory cytokine genes, including TNF-α, IL-6, IL-12, and IL-1β, free radicals, stressors and costimulatory molecules. In the case of the TLR2 and TLR4 signaling pathways, additional adapters TIRAP/MAL adaptors are also required for the activation of the MyD88- dependent pathway [3, 311-313, 317, 321].

2.4.2. MyD88 independent pathway

Triggering of IL-1R or TLR recruits IRAK to the receptor complex via adaptor MyD88, followed by TRAF6 activation, which finally leads to the activation of NF-κB and MAPK (Fig. 7). MyD88 KO macrophages are unresponsive to LPS and other bacterial components including PG, lipoproteins and CpG DNA, which proves the essential role of MyD88 in the response to all pathogen-derived immunostimulatory molecules [321]. However, although LPS-induced production of pro-inflammatory cytokines is not observed; activation of MAPK and NF-κB is still present but with delayed kinetics [5, 321].This indicates that the LPS response may be mediated by both MyD88-dependent and -independent pathways, each of which leads to the activation of MAPK and NF-κB [321]. The MyD88-independent pathway involves activation of the transcription factor IRF-3, and induction of IFN-β, which in turn, activates Stat1, leading to the induction of several IFN-inducible genes [321]. Viral infection or dsRNA, which are TLR3 ligands, also activate IRF-3 and thereby induce IFN-β in a MyD88-independent manner. Hence, TLR3 and TLR4 utilize the MyD88-independent component to induce IFN-β [5]. Nevertheless, the MyD88-dependent pathway is essential for the inflammatory response mediated by LPS [321].

2.4.3. Pro-inflammatory gene expression

Many proteins have NF-κB binding sites in their promoters. Indeed, once transcription factors such as NF-κB or AP-1 are upregulated, the cell responds in some way: either by phagocytosis, up-regulation of co-stimulatory molecules, maturation of APCs and transcription of genes producing pro- or anti-inflammatory cytokines. Adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adhesion molecule

(VCAM), are also up-regulated on the endothelium. Other enzymes that are part of the global response are cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthetase (iNOS), all of which contribute to the IIR against the invading pathogen. [317]. In addition, NF-κB has a crucial role in regulating the expression of anti-apoptotic proteins and affecting the susceptibility of cells to apoptosis [315, 317, 334, 335]. Moreover, the up-regulation of co-stimulatory molecules that enhances maturation of APC provides the link between the innate and acquired immune system [317].

2.5. MAPK signaling pathway

As mentioned, MAPK along with NF-κB are involved in signal transduction of genes associated with the control of many immune responses. Indeed MAPK are a family of kinases involved in numerous cellular processes which are activated by a dual phosphorylation mechanism involving phosphorylation of both Tyrosine- and Serine/Threonine residues [336]. MAPK pathway is activated not only through TLRs, but also through G-protein-coupled receptors, growth-factor receptors, cytokine receptors and receptors associated with environmental and genotoxic stress [337]. To date, four subfamilies of MAPK have been recognized: ERK1/ERK2, which is phosphorylated by the mitogen-activated/erk kinases (MEK or MKK) 1 and 2; the p38/regulated kinases (RK) subfamily, which is activated by MEK 3 and 4; the family of c-jun N-terminal kinases (JNK)/stress activated protein kinases (SAPK) that is activated by MKK4 and the recently discovered MEK/MKK6 [336]. These kinases stimulate AP-1 activity, a pivotal transcription factor that regulates T-cell activation, cytokine production, and the production of matrix metalloproteinases (MMP) [337]. Thus, MAPK are responsible for most cellular responses to cytokines and external stress signals and crucial for regulation of the production of inflammation mediators, such as COX2 and iNOS expression and NO production [338-341]. Moreover, MAPK signaling cascades regulate important cellular processes including cell proliferation, cell survival and death [342]. Numerous immunogenic molecules can trigger the MAPK signaling pathway, including LPS, double-stranded RNA, lipoproteins and cell wall components from Gram-positive bacteria, in particular LTA [336, 340, 341, 343-345].

3. Septic shock

3.1. Definition

The normal reaction to infection involves a series of complex immunologic processes. Activation of the immune system during microbial invasion is generally protective. A deficient immunologic defense may allow infection to become established. However, in certain circumstances, this response may be excessive because of a poorly regulated immune response to the invading pathogen. Consequently, the patient may experiment a wide spectrum of immunologic events, ranging from sepsis and severe sepsis to septic shock and death (Fig. 9) [2, 346].

Sepsis is defined as infection with evidence of systemic inflammation, consisting of two or more of the following: increased or decreased temperature or leucocyte count, tachycardia, and rapid breathing (Fig. 9) [2, 347-349]. Severe sepsis is sepsis complicated by organ dysfunction, tissue/organs hypoperfusion or hypotension, while septic shock is the syndrome characterized by a persistent arterial hypotension in a septic patient that persists after resuscitation with intravenous fluid (Fig. 9) [1, 2, 347-350]. This process is characterized by a massive release of pro-inflammatory mediators such as TNF-α, IL-6 and IL-1β. In addition, neutrophil apoptosis is significantly delayed by these inflammatory mediators. This allows them to function more effectively at sites of inflammation, thus leading to enhanced surveillance against infectious agents and a prolonged recruitment of other pro-inflammatory cells by the release of cytokines and chemokines [1]. Organ dysfunction in septic shock usually includes acute lung injury; coagulation abnormalities; thrombocytopenia; altered mental status; renal, liver, or cardiac failure; or hypoperfusion with lactic acidosis [348].

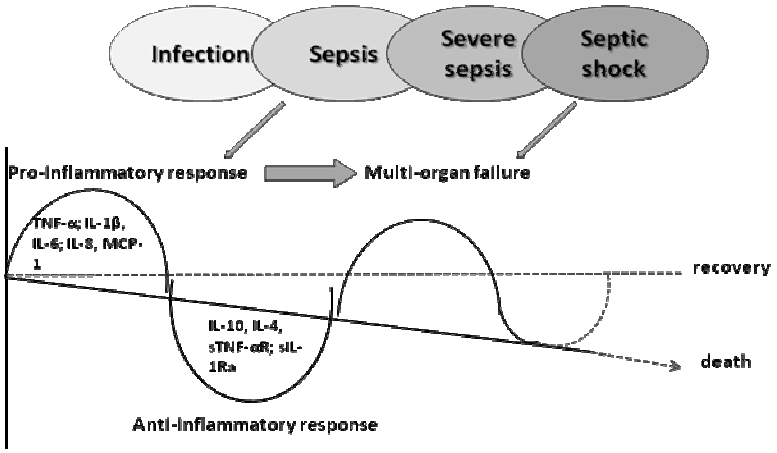


Fig. 9. Development of sepsis: The excessive inflammatory response to infectious agents can lead to septic shock. Following the release of pro-inflammatory mediators, compensatory anti-inflammatory mechanisms will appear. However, if pro- and anti-inflammatory mechanisms do not reach a balance, the disproportionate inflammatory response to microbial products finally may lead to tissue damage, haemodynamic changes, multiple organ failure and ultimately death. Adapted from [1, 2].

3.2. Development of septic shock

3.2.1. Cellular phase of the acute inflammatory response

A series of systemic pathogenic events are behind the transition from sepsis to severe sepsis/septic shock. This process begins with a neurohumoral, generalized pro- and anti-inflammatory response. The first set of events involves the cellular phase of the acute inflammatory response, in which leukocytes are delivered into exudates at the site of injury [351]. This phase is characterized by recruitment, rolling, firm adhesion to the endothelial surface, activation, aggregation, and subsequent transmigration of leukocytes to sites of inflammation through the endothelial cell junctions into the interstitium toward a chemoattracting gradient [1, 2, 348]. Leukocyte rolling is mediated through the expression of cell-adhesion molecules, such L-selectin and β_2 integrins (LFA-1 [CD11a/CD18]; Mac-1 [CD11b/CD18]; p150,95 [CD11c/CD18]; $\alpha d\beta_2$ [CD11d/CD18]) on leukocytes and E- and P-selectin, ICAM-1 and VCAM-1 on endothelium. Once leukocytes are attached to the vascular endothelium, they adhere to platelet-endothelial cell adhesion (PECAM-1) molecules present at

the endothelial cell junction and transmigrate through the junction into the perivascular tissue. Pseudopodia from neutrophils and other leukocytes extend between endothelial cells and come into contact with and bind to the basement membrane, where they express β_1 integrins (VL-1 to -6) that promote adhesion to extracellular matrix proteins such as laminin, fibronectin, vitronectin and collagen. This process is modulated by complement fragments (C5a), vasoactive amines, cytokines (TNF-α, IL-1β, IL-6), chemokines and membrane-derived mediators such as platelet-activating factor(PAF), platelet-derived growth factor (PDGF) and leukotrienes [1, 2, 348, 351]. This transendothelial migration of leukocytes leads to the accumulation of inflammatory cells in the connective tissue stroma and is accompanied by vascular leakage and injury [351]. These changes result in occlusion of post-capillary venules causing tissue ischemia. Neutrophil-derived proteases and toxic oxygen products can act synergistically on the endothelial surface, leading to endothelial injury, intercellular gap formation, and increased vascular permeability [2]. In addition, the level of endogenous activated Protein C, which modulates coagulation, controls inflammation and supports fibrinolysis is also decreased. The hallmarks of sepsis are excessive inflammation, excessive coagulation and suppression of fibrinolysis, leading to tissue hypoxia and ischemia with resultant organ dysfunction and death [350].

3.2.2. Disordered inflammation: Participation of the cytokine and chemokine pro-inflammatory network

As aforementioned, sepsis is characterized by a massive release of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-8, and macrophage chemotactic protein-1 (MCP-1) [1, 352-355] In addition, neutrophil apoptosis is significantly delayed by these inflammatory mediators. This allows them to function more effectively at sites of inflammation, thus leading to enhanced surveillance against infectious agents and a prolonged recruitment of other pro-inflammatory cells by the release of cytokines and chemokines [1].

3.2.2.1. Participation of cytokines

Cytokines are soluble, low molecular weight glycoproteins which act to regulate both innate and specific immune responses and act as inflammatory mediators. Individual cytokines

can be produced by multiple cells and are pleiotropic acting on multiple target cells in different ways depending on timing and concentration. At low concentrations cytokines have a paracrine effect, whereas at higher concentrations, such as in sepsis, cytokines have endocrine effects and act systemically [349]. During sepsis, a wide spectrum of pro-inflammatory cytokines are released, the following being the most relevant:

a) TNF- α

TNF- α (25 kDa) is a pleiotropic cytokine that plays a pivotal role in the pathogenesis of inflammation, cachexia, toxic shock and tissue injury [356]. It is released by cells of the monocyte/macrophage lineage, glial cells in brain, Kupffer cells in the liver, keratinocytes in the skin, mast cells, NK cells, T cells and B cells. TNF-α stimulating factors include LPS, enterotoxins, toxic shock syndrome toxins, bacterial cell wall, and products of the complement activation [357]. Circulating TNF-α interacts with at least two membrane-associated receptors named TNF-R1 and TNF-R2. Both TNF-Rs are widely expressed, as they are found in virtually all types of cells, excluding erythrocytes and they also exist in soluble forms in the serum [357]. TNF-R1 is mainly responsible for the induction of pro-inflammatory responses via NF-kB but can also induce apoptosis. The outcome of TNF-R1 engagement seems to depend on the association with certain membrane microdomains rich in cholesterol and sphingolipids, the so-called lipid rafts. The translocation of TNF-R1 after triggering lipid rafts seems to be essential for NF-kB activation, whereas interference with lipid raft organization leads to apoptosis. In contrast, TNF-R2 is less well defined and seems to be involved in supporting TNF-R1-mediated functions [1]. Pathologic manifestations of TNF-α production include hypotension, acute renal tubular necrosis, pulmonary edema, disseminated intravascular coagulopathy, and a cascade of cytokine and stress hormone release [356].

b) IL-1

IL-1 (17kDa) is a major inflammatory mediator produced primarily by monocytes and activated macrophages. The activity of IL-1 is mediated by two proteins, known as IL-1α and IL-1β, which are the products of separate genes. A high-affinity activating receptor (IL-1RI) for IL-1 is expressed by lymphocytes and fibroblasts, whereas a low-affinity decoy receptor (IL-1RII) is expressed on several other cell types. An unique feature of IL-1 is the existence of a naturally

occurring antagonist, IL-1Ra, which is expressed in neutrophils and monocytes [1]. Because IL-1α and IL-1β bind to the same receptor, there are no differences between the biological actions of IL-1α and IL-1β. However, IL-1α is a cell-associated cytokine that can also act as a transcription factor, whereas IL-1 β is secreted and, therefore, more likely to play a role in systemic inflammation [358]. In septic shock, IL-1 acts directly on the blood vessels to induce vasolilatation through the rapid production of small mediator molecules such as PAF, prostaglandins, and NO, which are potent vasodilatators. Other systemic effects of IL-1 are the ability to induce fever, sleep, anorexia and hypotension [359].

c) IL-6

Many cells, in particular fibroblasts, cells of the monocyte/macrophage lineage, and endothelial cells can secrete IL-6, a 26 kDa protein. This is a multi-functional cytokine that plays a central role in host defense mechanisms, regulation of immune responses, B cell differentiation, and is also a mediator for immunoglobulin class switching and hematopoiesis [360]. The biological activities of IL-6 partially overlap those of IL-1**.** Some of the pleiotropic effects of IL-1 are actually caused by IL-6 or by synergistic actions of IL-1 and IL-6. This cytokine stimulates hepatic protein synthesis during acute-phase responses and acts as an endogenous pyrogen. Because of these functions, IL-6 has been described as an alarm hormone: it is released by damaged tissues and induces the synthesis of acute-phase proteins in the liver that protect the host against inflammatory reactions [361, 362].

3.2.2.2. Participation of chemokines

The chemokines are small 8-to-10 kDa proteins. Two major subfamilies, CXC and CC chemokines, have been extensively investigated in sepsis and in endotoxemia. In the CXC chemokines, the first two cysteine residues are separated by a single amino acid and they are typically chemotactic for neutrophils, whereas in the CC chemokines, the first two cysteine residues are adjacent to each other, and they attract and activate mononuclear cells [363].

a) IL-8

IL-8, a CXC chemokine, is produced by a wide array of cells, including endothelial cells, monocytes/macrophages and neutrophils. IL-8 plays a role in the pathophysiology of sepsis because this cytokine is able to activate and degranulate neutrophils. In fact, IL-8 is released into the circulation in animal models for septic shock and upon intravenous administration of endotoxin. Moreover, plasma levels of IL-8 are increased in up to 89% of in patients with sepsis, with no significant differences between those with Gram-positive or Gram-negative infections. IL-8 is able to induce shape change, release of lysosomal enzymes, and degranulation of neutrophils. It also elicits respiratory burst, and activates arachidonate-5-lipoxygenase in neutrophils, processes that enhance inflammation. IL-8 may promote adherence of neutrophils to the endothelium by increasing, β_2-integrin expression and may regulate transendothelial migration of these cells. Moreover, when produced at local sites, IL-8 elicits edema formation due to neutrophil-mediated endothelial damage and subsequent plasma leakage [364, 365].

b) MCP-1(CCL2)

MCP-1, a prototype of CC chemokines, is a potent chemoattractant and a regulatory mediator involved in a variety of inflammatory diseases. This chemokine is secreted mainly by monocytes, although MCP-1 expression is also found in different types of cells, including fibroblasts, endothelial and epithelial cells. Similarly to IL-1 and IL-6, MCP-1 stimulates monocytes/macrophages to elicit respiratory burst, expression of β_2-integrins, release of lysosomal enzymes, induction of production of different cytokines and attraction of T cells and NK cells [352, 365]. More precisely, MCP-1 has been implicated as a remarkable mediator of monocyte and lymphocyte infiltration of tissues in a wide variety of inflammatory diseases, such as glomerulonephritis, rheumatoid arthritis, and bacterial meningitis. It has been shown that inhibition of MCP-1 expression results in reduced transmigration of monocytes through blood vessels and in diminished recruitment of T lymphocytes, suggesting a critical role in acute inflammation [363].

3.2.3. Anti-inflammatory response

During sepsis, the anti-infectious agemt response is closely linked to an overwhelming pro-inflammatory process. All mediators contribute in synergy to tissue injury, organ dysfunction, and possibly to lethality. The early hyper pro-inflammatory phase of sepsis is followed by counterregulatory mechanisms, and the hyper- and anti-inflammatory states alternate during the following course of sepsis or the two may co-exist at the same time. The anti-inflammatory response relies on a few cytokines, mainly IL-10, IL-4 and transforming growth factor-beta (TGF-β) and some soluble factors, including soluble TNF-receptor (sTNF-αR) and IL-1 receptor antagonist (IL-1Ra). There is also participation of glucocorticoids, neuromediators (adrenalin, acetylcholine), and some other factors (heat shock proteins, adenosine). These mediators modify the immune status of circulating leukocytes or they reduce pro-inflammatory cytokine production in response to Toll-like receptor agonists [1, 366].

a) IL-10

Plasma IL-10 concentrations in patients with septic shock are significantly higher than those with only sepsis. It is believed that the magnitude of the IL-10 response appears to correlate both with the severity of the inflammatory insult and the plasma concentration of pro-inflammatory cytokines. In fact, it is known that IL-10 production in sepsis is a direct consequence of pro-inflammatory cytokine production, predominantly TNF-α. IL-10 (18 kDa) is produced by T_H2 lymphocytes, monocytes and epithelial cells. Being a T_H2 cytokine, IL-10 mainly inhibits gene expression and synthesis of pro-inflammatory cytokines and chemokines from macrophages. More precisely, it suppresses the synthesis of IL-1α, IL-1β, TNF-α, IL-6, IL-8, IL-12, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein (MIP)-1α, regulated upon activation—normal T cell expressed and secreted (RANTES), leukemia-inhibiting factor (LIF), and IL-10 itself. In addition, IL-10 induces neutrophil apoptosis during the resolution of inflammation. Cell signaling after the engagement of IL-10 to its receptor includes phosphorylation of JAK1 and TYK2 and phosphorylation of

STAT-3, very similar to that of interferon-α/β. IL-10 also inhibits the synthesis of NO and the synthesis of gelatinase and collagenase [1, 355, 367].

b) IL-4

IL-4 is a 15 kD protein produced by activated T cells, mast cells, and basophils. IL-4 stimulates the T_H2 cell differentiation and proliferation, and enhances B cell secretion of IgE. Moreover, it suppresses production of IL-1β and other monocyte-derived cytokines, including TNF-α, IL-6 and IL-8, although it enhances IL-1Ra synthesis. IL-4 also induces fibroblast proliferation and production of extracellular matrix proteins [368, 369].

c) TGF-β

TGF-β is a pleotrophic cytokine expressed in the vessel wall that promotes several anti-inflammatory effects, such as the production of anti-inflammatory cytokines and the inhibition of leukocyte adhesion to the endothelium. In addition, TGF-β also inhibits the migration of neutrophils through endothelial monolayers activated by TNF-α. This cytokine achieves its anti-inflammatory properties by inhibiting the expression of at least two genes, E-selectin and IL-8, which are essential in the inflammatory pathway [1, 370].

d) sIL-1Ra

IL-1Ra receptor is one of the three members of the IL-1 gene family, and it functions as a specific receptor agonist. Processing of IL-1α or IL-1β, to “mature” forms require specific cellular proteases. In contrast, IL-1Ra evolved with a signal peptide and is readily transported out of the cells and termed secreted IL- 1Ra (sIL-1Ra). The major function of sIL-1Ra is to regulate the pleiotropic effects of IL-1 by competitively blocking its binding to cell surface receptors. Thus, sIL-1Ra functions as a major naturally occurring anti-inflammatory protein; when the balance between IL-1 and IL-1Ra is upset, inflammatory disease and tissue damage may ensue. Maintenance of a balance between IL-1 and IL1-Ra is important in preventing the development or progression of inflammatory disease in certain organs. Recently, three

intracellular isoforms for IL-1Ra have been described, and identified as icIL-1Ra1, 2, and 3, although their role is still poorly defined [371, 372].

There are two IL-1 receptors (IL-1R), the type I receptor (IL-1RI) transducing a signal, whereas the type II receptor (IL-1RII) binds IL-1 but does not transduce a signal. In fact, IL- IRII acts as a sink for IL-18 and has been termed a “decoy” receptor. When IL-1 binds to IL-1R1, a complex is formed that then binds to the IL-1R accessory protein (IL-1R-AcP), resulting in high-affinity binding. The extracellular or ‘‘soluble’ ’ portions of the IL-1RI (IL-1sRI) and IL-1RII (IL-1sRII) circulate in health and disease functioning as natural “buffers” binding IL-1α, IL-1β, or IL-IRa. In addition, several cytokines exert a negative influence on both the production and activity of IL-1 [371, 372].

e) sTNF-αR

TNF-α has two receptors, TNF-α receptor I (TNF-αRI) (55kDa) and TNF-αRII (75 kDa). The TNF-αRI is the main mediator of the TNF-α signaling to cells, whereas the TNF-αRII, which has a secondary role in signaling, serves also for “ligand passing,” this is, for channeling TNF-α to the TNF-αRI, thus facilitating its binding to it. Both receptors for TNF-α exist also in soluble forms, probably derived by proteolytic cleavage from the cell surface forms. Once released, soluble receptors (sTNF-αRI and sTNF-αRII) inhibit TNF-α activity by binding to TNF-α and preventing binding of the ligand to TNF-α cell receptors. This regulatory process may modulate TNF-α activity in response to inflammation. Interestingly, these soluble receptors are found in the circulation of patients with sepsis and in response to endotoxemia and their rise indicates worsening of sepsis. In addition, renal insufficiency may contribute to the persistently high levels by inhibiting the elimination of soluble receptors [373, 374].

f) Glucocorticoids and neuromediators

One of the more intriguing subjects of investigation is the connection between the central nervous system, the adrenal cortex and the resolution of inflammation. Glucocorticoids (GCs), which are produced by the adrenal cortex, are main players in this concept. GCs are the

most powerful endogenous inhibitors of the innate immune reaction throughout the organism. The secretion of cortisol in humans, or corticosterone in animals, occurs as the result of the hypothalamic-pituitary-adrenal (HPA) axis activation. Inflammatory and stress-related sensory information triggers corticotropin-releasing factor (CRF)-secreting neurons in the paraventricular nucleus (PVN) of the hypothalamus. This leads to the release of ACTH from the pituitary, which then stimulates the release of GCs from the zona fasciculata of the adrenal cortex. Numerous studies have suggested that IL-1β, IL-6, TNF-α and other pro-inflammatory cytokines, lead to an increased pituitary release of adrenocorticotrophic hormone into the serum, which mediates increased cortisol/corticosterone production in the adrenal cortex, ultimately decreasing the inflammatory response. In the 'cholinergic anti-inflammatory pathway', vagus nerve stimulation with a subsequent release of acetylcholine attenuates systemic inflammatory responses by the inhibition of the release of TNF-α and other cytokines from macrophages. It was recently demonstrated that the nicotinic acetylcholine receptor α7 subunit is essential for inhibiting cytokine synthesis by the cholinergic anti-inflammatory pathway. In addition, the adenosine A2a receptor also seems to be necessary for down-regulation of the pro-inflammatory immune response [1, 375].

3.3. Toxic shock syndrome (TSS) and toxic shock like syndrome(TSLS)

Infections of the chest, abdomen, genitourinary system, and primary bloodstream (including meningitis) result in more than 80% of cases of sepsis. Interestingly, it has been observed that the occurrence of sepsis due to Gram-negative bacterial infections has diminished over the years to 25–30%, while sepsis caused by Gram-positive infections accounts for 30–50% of cases [347]. However, several Gram-positive species are also capable of producing disease through toxin production in addition to classic sepsis syndromes. Toxic shock syndrome (TSS) is an acute, multi-system, toxin-mediated illness, typically resulting in shock and multi-organ failure early in its clinical course. It represents the most fulminant expression of a spectrum of diseases caused by toxin-producing strains of *S. aureus* and *S. pyogenes* (GAS). In addition to these pathogens, some Gram-negative bacteria, *Mycoplasma* spp, and certain viruses are known to produce toxins known as superantigens (SAgs). These SAgs bypass conventional mechanisms of MHC-limited antigen processing, whereby antigens are processed

into peptide fragments within APCs. In fact, SAgs share the ability to trigger excessive and non-conventional T-cell activation, this is, on $CD4^+$ and $CD8^+$ T cells, which bear the T cell receptor (TcR) αβ and sometimes on those bearing the γδ TcR. The staphylococcal and streptococcal SAgs identified to date are single-chain proteins expressed as precursor molecules, which are then cleaved to release the functional extracellular toxin. Unlike conventional peptide antigens, SAgs bind most often to invariant regions of the MHC class II molecules at the surface of APCs outside the classical antigen-binding groove. They are presented as unprocessed proteins to T cells that express appropriate motifs on the variable domain of the β chain (Vβ) of the TcR. This process results in the formation of ternary MHC class II molecule-SAg-TcR complexes leading to the proliferation of the targeted T cells in a Vβ-restricted fashion. Streptococcal and staphylococcal SAgs exhibit a remarkable spectrum of biological and pharmacological activities. These activities include the pyrogenic effects elicited by these toxins as a consequence of the release of IL-1 and TNF-α, and their action on the hypothalamus; blockade of the reticuloendothelial system (monocytes/macrophages located in the reticular connective tissue) and enhancement of host susceptibility to lethal shock by endotoxin; and immunosuppression of humoral and cell-mediated responses, deletion of T cell repertoire, anergy and apoptosis of lymphocytes. Moreover, T cell activation leads to recruitment of further T and B cells to the site of infection. Clonal T cell expansion continues, as well as activation of APCs, further amplifying the release of pro-inflammatory mediators. A complex interplay exists between the cytokines released during this pro-inflammatory cascade, with IFN-γ rapidly inducing TNF-α and IL-6 expression. [346, 376].

Toxic shock-like syndrome (TSLS), caused by GAS is a syndrome with a rapid and fulminant course closely resembling the TSS caused by *S. aureus* and GAS SAgs. The streptococcal TSLS has a rapid course and frequently fatal outcome. GAS produces a variety of extracellular substances that have different antigenic and biologic activities. These exotoxins are often associated with severe suppurative soft-tissue infection, such as skin or muscle and other soft tissue, and may cause a syndrome of fever, shock, a rash that later desquamates, hypotension and multi-organ-system dysfunction. Although streptococcal TSLS is most often associated with exotoxin A-producing infections of soft tissues, the syndrome has also been reported in association with exotoxin types B and C, and with strains producing combinations of the exotoxins. Streptococcal pyrogenic exotoxins types A, B, and C, are responsible for the

fever and rash of scarlet fever, and each has potent toxin properties that alter host defense by increasing susceptibility to endotoxin shock, decreasing antibody response, inhibiting macrophage function, and activating lymphocytes. TSLS has been associated with the reappearance of highly mucoid exotoxin-producing strains of streptococci that have a greater tendency to produce potent exotoxins than previous strains. In fact, the observed increase in the incidence and severity of invasive group A streptococcal infections coincides with the resurgence of genetically related, highly virulent strains of GAS that express an invasive phenotype and carry a specific gene for pyrogenic exotoxin A [377, 378].

It is thought that *S. suis* ST7 has recently acquired putative virulence factors responsible for TSLS. Indeed, as mentioned in section 1.3.2.1, *S. suis* ST 7 strains possesses a stronger capacity to stimulate T cells, naive T cells and peripheral blood mononuclear cell proliferation than *S. suis* ST1. As explained, *S. suis* ST7 infection in humans is characterized by acute onset of fever, chills, headaches, dizziness, malaise, abdominal pain, diarrhea, organ function impairment, and death [97, 98].

3.4. Genetic influence of the host on the development of sepsis

Variation in responses to pathogens is influenced by exposure history, environment and the host's genetic status. There is now evidence that specific genetic factors influence a host's susceptibility or immunity to many infectious agents, and modulate severity and outcome of infectious diseases, which may have important clinical implications. Thus, variation in key innate immunity genes may explain variation in individuals' responses to infection. If so, it would be useful to evaluate genotypes of critically ill patients to understand individual susceptibility and response to infection, which could lead ultimately to specific patient-tailored therapy based on genotype [379]. Investigation of single nucleotide polymorphisms (SNPs) in candidate genes associated with sepsis and septic shock may provide valuable clues to understand the origin of such variability.

In humans, TNF-α polymorphism (-308A allele) is associated with increased secretion of TNF-α from macrophages *in vitro* and elevated TNF-α blood concentration *in vivo.* There is

evidence that the TNF-α-308A allele is associated with adverse outcome in a variety of infectious and inflammatory diseases including cerebral malaria and meningococcal disease. The TNF-α promoter gene is in linkage disequilibrium with several alleles that may be involved with the control of TNF-α secretion, or that may be independent risk factors for the development of meningococcal disease or other forms of sepsis [379]. On the other hand, SNPs in CD14, mannose-binding lectin (MBL) and hTLR2 are associated with increased prevalence of sepsis associated with positive bacterial cultures, but not with altered prevalence of septic shock or decreased survival. Furthermore, CD14 SNPs are associated with Gram-negative bacterial infections, and TLR2 with Gram-positive bacterial infections, MBL not being associated with a particular organism class [380]. These results reinforce the theory that SNPs in innate immune receptors may alter recognition and clearance of bacteria without changing outcomes in critically ill adults with systemic inflammatory response syndrome. Morevoer, hTLR4 mutations are associated with an increased incidence of Gram-negative infections and development of sepsis [381]. However, results may be contradictory, as other research has provided evidence that there is no association between SNPs of hTLR4 and outcome of Gram negative sepsis. This would also be the case for SNPS of TNF-α, IL-1β, plasminogen activator-1 (PAI-1), urokinase plasminogen activator (uPA) and CD14 [382]. Allelic variation in HLA class II molecules plays a major role in modulating severity of GAS sepsis, primarily because they serve as binding and signaling receptors for superantigens, which are the primary trigger of the severe inflammatory responses that can lead to organ failure and shock [383].

In the case of meningitis-associated bacteria, some patients may develop meningitis, whereas others may present with sepsis or even septic shock. This variability of clinical presentations of the same disease among affected individuals may be associated with host genetic factors. For example, in the case of infection by *N. meningitidis*, complement deficiencies and defects in sensing or opsonophagocytic pathways, such as SNPs of TLR4 and combinations of inefficient variants of Fcγ-receptors, seem to have the most important role in genetically susceptibility [384]. In the case of *S. pneumoniae* infections, homozygotes for MBL codon variants, could be at substantially increased risk of invasive pneumococcal disease in humans [385]. Moreover, and similarly to *N. meningitidis* infection, genetic variability in the TLR4, CD14 and Fcγ-receptors genes is associated with an increased risk of developing invasive disease in patients who are infected with *S. pneumoniae* [386]. To date, there are no studies

regarding the possible influence of host's genetic factors on the development of *S. suis* infection.

In order to systematically identify additional gene variants that modulate susceptibility to severe sepsis or other infections, and to elucidate how they influence disease outcome, researches take advantage of animal experimental models of the disease with attributes that incorporate roughly the same level of genetic variation as that of human populations [383]. Different inbred mouse lines have been used to study differential susceptibility to several infections; unfortunately, these models are limited in their genetic variability and cannot be used to map modifier loci. However, the generation of genetically diverse mouse reference population of recombinant inbred strains, which can be linked to an immortal population of human monozygotic twins, is considered as the ideal model for many studies. Recombinant inbred strains are generated by the crossing of two inbred strains followed by at least twenty consecutive generations mating among siblings. These types of strains are frequently used for studying genetic variation and mapping quantitative trait loci (QTL) influencing disease [383].

4. The innate immune response in the brain

For a long time, the CNS has been considered as an immune privileged organ, as, unlike the peripheral organs, it exhibits a relative absence of lymphatic drainage of the parenchyma, has a limited number of APCs, and the BBB meticulously filtrates circulating elements. Although this is partly true, the CNS has its own innate immune system, which is activated in response to both cerebral and systemic immune challenges. On one hand, activation of the innate immune system and cytokine production controls infections, remove debris and promote repair in the CNS, but on the other hand, they may promote various CNS pathologies, ranging from acute injuries to chronic degeneration. As in peripheral tissues, the brain contains a resident set of bone marrow derived mononuclear phagocytes that include the microglia in the parenchyma, perivascular macrophages and macrophages in the meninges and choroid plexus. However, unlike most other tissues, the CNS contains very few mast cells or DCs. In certain immune-mediated lesions within the CNS, DCs can be found in the brain parenchyma and may

contribute to the chronicity of the disease. However, the absence of DCs in the normal brain is likely to be a major contributor to the particular immunological status within the brain parenchyma [387, 388].

4.1. Triggering of the inflammatory response in the brain

Most of the research on the development of the inflammatory response in the brain has been developed using LPS, a TLR4 ligand widely known for its rapid and potent induction of cytokine production. The use of LPS in animal models of brain inflammation has been of great help for depicting the spatio-temporal expression of genes involved in this response. Indeed, the intraperitoneal injection of LPS in mice leads to a robust induction of IkBα (index of NF-κB activation) in cells of endothelial type along the blood vessels of the entire brain. The same effect is observed when animals are injected with either IL-1β or TNF-α, but not IL-6. Moreover, the IkBα transcript is also found in scattered small cells, which are positive for microglia markers (using ant-OX-42 antibodies). These results indicate that the brain responds to systemic inflammatory insults and that specific cells of the CNS are involved in such a response [389]. Interestingly, complementary studies suggested that the inflammatory response in the brain starts in the circumventricular organs (structures devoid of BBB), and notably, that TLR4 may be the recognizing molecule for Gram-negative bacterial components only in response to systemic infection, whereas CD14 may have a more complex role in the pro-inflammatory signal transduction events in the brain parenchyma. These conclusions were made as the systemic inoculation of LPS in mice lead to an up-regulation of TLR4 mainly in the leptomeninges, choroid plexus, and circumventricular organs (subfornical organ, organum vasculosum of the lamina terminalis, median eminence, and area postrema), but only scattered small cells displayed a convincing positive signal within the brain parenchyma. These brain structures also exhibited constitutive expression of CD14, which was up-regulated in different cells of myeloid origin throughout the entire brain parenchyma. However, and in contrast to the strong up-regulation of the gene encoding CD14, neither LPS nor the challenge with IL-1β caused a convincing increase in the TLR4 mRNA levels throughout the CNS [390]. Nevertheless, IL-1β is not the only cytokine that has a direct effect on the inflammatory response in the brain. In fact, TNF-α has a similar effect, as rats that are intracerebroventricularly challenged with recombinant rat TNF-α, show a strong up-regulation of IkBα, TNF-α, and CD14 mRNA in

parenchymal microglial cells localized in different regions of the brain. In the case of CD14 mRNA, the signal is mainly detected at the right caudoputamen, cerebral cortex, hippocampal and septal formation, and parenchymal elements around the third ventricle, beneath the leptomeninges, and near the central canal and the fourth ventricle, whereas IkBα and TNF-α signals are observed in regions near the ventricles. These findings provide the evidence that microglial-derived TNF-α is responsible for the production of the CD14 during endotoxemia. This autocrine/paracrine stimulatory loop may be of great importance in controlling the inflammatory events that take place in the CNS during innate immune response as well as under pathological conditions [391].

Mice that receive a systemic challenge with LPS, show a robust up-regulation of TLR2 within cerebral tissue, which is first detected in regions devoid of BBB and few blood vessels and microcapillaries and then in brain parenchyma in cells identified as microglia. Moreover, LPS-challenge also induces a rapid induction of IκBα and up-regulation of MyD88, suggesting that LPS-induced TLR2 transcription may be dependent on the NF-κB pathway. However, mice challenged with bacterial components that are considered as TLR2 ligands (LTA, PG or a combination of both), failed to induce the up-regulation of the receptor [392].

Similar studies more recently confirmed the relevance of chemokine up-regulation in the brain. The systemic challenge with LPS provokes up-regulation of MCP-1 mRNA particularly in endothelial cells of the microvasculature and in brain areas devoid of BBB, indicating that MCP-1 may therefore play a critical role in the cerebral innate immune response and contribute to the early chemotaxic events during cerebral inflammation [393].

4.2. Central role of microglia

The term microglia refers to cells that reside within the parenchyma of the CNS, that share many if not all the properties of macrophages in other tissues. Moreover, although microglia are "brain macrophages," they are distinguished due to their parenchymal location and certain functional differences, from other types of brain macrophages such as meningeal and perivascular macrophages and perivascular cells or pericytes, which are enclosed by a perivascular basement membrane within blood vessels and are not part of the CNS

parenchyma. Besides, they are ontogenetically related to cells of the mononuclear phagocyte lineage, unlike all other cell types in the CNS [388, 394-397].

Microglia account for up to 10% of the overall number of brain cells. They are present within all brain regions including both grey and white matter [398]. One of the characteristics of microglia is their activation at a very early stage in response to injury. Microglia activation often precedes reactions of any other cell type in the brain. In their non-activated or resting state, they have a characteristic "ramified" morphology not seen in resident macrophages of other organ systems, adapted to the specialized microenvironment of the CNS. Certain cell surface markers of importance in immune regulation, such as MHC class II molecules and complement type-3 receptor (CR3; CD11b/CD18 complex) are constitutively expressed on ramified microglia in the normal adult brain. Microglia are able to respond selectively to molecules involved in neurotransmission, as they have receptors for CNS signaling molecules such as adenosine triphosphate (ATP) and calcitonin gene-related peptide (CGRP), and can react both with changes in their extracellular ionic milieu and by activation of transcriptional mechanisms. This allows them in their ‘resting’ state to continuously monitor the physiological integrity of their micro-environment and to react rapidly in the event of pathological events [388, 394-398].

Ramified microglia have the capacity to dramatically change their morphology in response to a variety of CNS insults such as microbial invasion; they retract processes and round cell body, acquiring a reactive “amoeboid” form. In addition, they quickly up-regulate a large number of receptor types and secrete innumerable products that are thought to contribute to the defense and, potentially, damage of the infected brain. Signs of microglial activation include increased expression of CR-3, up-regulation of MHC class I and II antigens, which enable microglia to interact directly with immunocompetent cells such as T cells and presence of phagolysosomes, which denotes transition into phagocytic cells. There also is synthesis of a variety of potentially harmful soluble factors, including reactive oxygen and nitrogen intermediates, proteolytic enzymes, arachidonic acid metabolites and pro-inflammatory, potentially cytotoxic cytokines such as TNF-α and IL-1 [388, 394-397].

Microglia can unequivocally be distinguished from other resident, non-neuronal cells in the CNS by immunocytochemistry or immunofluorescence or confocal microscopy by using anti-serum against the CR3 (OX-42 antibody). In addition, these cells constitutively express

galactose-containing glycoconjugates that bind isolectin B4 from *Griffonia simplicifolia* (Fig 10) [395].

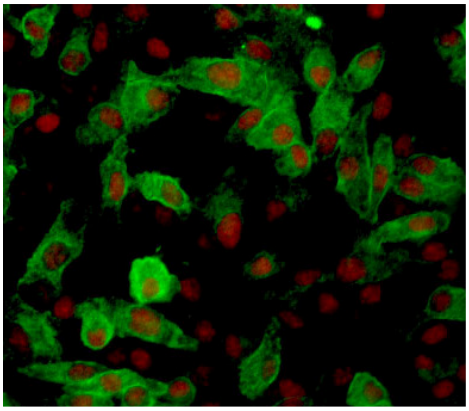


Fig. 10. Primary microglia from newborn C57BL/6 mice. Immunofluorescence microscopy. Microglia were identified by fluorescein isothiocyanate (FITC)-labeled *G. simplicifolia* isolectin B4 (Vector Laboratories). Nuclei were labeled with Propidium Iodide (SIGMA). (Domínguez-Punaro, *et al*; unpublished data).

Bsibsi *et al* [399], performed cultures of primary microglia from human postmortem brain tissues obtained from healthy donors with the aim of investigating the expression of TLRs by these cells. They found that microglia express mRNA encoding a wide range of different TLR family members, with a marked expression of both TLR2 and TLR3. In addition, microglia also express TLR1, TLR4, TLR5, TLR6, TLR7, and TLR8 and lower but detectable levels of TLR9. Immunocytochemical studies demonstrated that both TLR3 and TLR4 are exclusively localized in vesicular structures inside microglia and not on the surface of the cells. In addition, as only a minority of cultured microglia cells express sufficient levels of TLRs to be detectable by immunocytochemical staining, microglial TLR expression may be regulated and dependent on a certain level of activation that is reached only in a minority of cells in culture [399]. It was later confirmed that, similarly to human microglia, mouse microglia express mRNA for TLR1-9 [400]. Furthermore, cell activation was investigated after stimulation of inactive microglia with various TLR agonists, including LPS (TLR4), PG (TLR2), polyinosinic-polycytidylic acid (TLR3), CpG DNA (TLR9), and infection with viable Theiler's murine encephalomyelitis virus (which interestingly, activated TLRs 2, 3, 5, and 9). It was also observed that microglia stimulated with the different TLR agonists expressed pro-inflammatory cytokines, including IL-12, IL-18, and TNF-α, denoting a Th1-type pro-inflammatory response. In addition, there also was expression of chemokines (MIP-1α, MCP-1, and RANTES), which in vivo may be required for attracting peripheral macrophages and T cells to the CNS. Most importantly, microglia activation via TLR agonists up-regulated expression of co-stimulatory molecules (B7-1, B7-2, CD40, and ICAM-1) and MHC class II, which may render the cells functionally capable to present Ag to $CD4^+$ Th1

cells [400]. Moreover, microglia stimulated with different TLRs agonists (LPS, CpG, oligodesoxynucleotide, and Tripalmitoyl-S-glyceryl-cysteine), release substantial amounts of NO [401].

The accessibility to microglia obtained from KO mice lacking selected genes involved in the innate immune response, has allowed the dissection of some of the mechanisms used by microglia to mount an inflammatory response against meningitis-causing bacteria, which at the same time may have negative consequences for the host. For example, experiments using co-cultures of microglia and neurons or neurons alone infected with GBS, demonstrated that GBS induces neuronal injury only in the presence of microglia. Moreover, experiments using TLR2 $^{-/-}$ and MyD88$^{-/-}$ microglia indicate that this microglial activation and neuronal apoptosis induced by GBS require these signal transduction molecules. Furthermore, NO, a molecule that is largely responsible for GBS-induced neuronal injury, was produced only in the wild type, but not in TLR2$^{-/-}$ or MyD88$^{-/-}$ cells [327]. Complementary studies revealed that both TLR2 and MyD88 are involved in GBS-induced apoptosis in microglia via caspase-8 activation [328].

Purified components from *S. pneumoniae* are strong inducers of the inflammatory response in microglia. Indeed, when microglia are stimulated with purified cell walls from *S. pneumoniae*, another important meningitis agent, there is release of different pro-inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-12, MIP-1α and MIP-2. In addition, the use of a ERK1/2 inhibitor (tyrphostin AG126) blocks this cytokine release, revealing the participation of the MAPK pathway in the production of pro-inflammatory molecules in microglia [344, 402]. In addition, pneumolysin is mainly responsible for the release of NO and TNF-α from mouse microglia and it also induces apoptosis of these cells. In this case, apoptosis is attributed to damage to mitochondria, followed by the release of apoptosis-inducing factor (AIF) from the mitochondria [401, 403, 404]. In additional studies, mouse microglial cultures were pre-stimulated with different TLRS agonists, such as Pam3CSK4 (TLR1/2), LPS and CpG and then exposed to either encapsulated or nonencapsulated strains of *S. pneumoniae*. Results revealed that those cells pre-stimulated with TLR1/2, 4 and 9 agonists, ingested and killed more bacteria than non-stimulated cells, suggesting the participation of several TLRs in the inflammatory response against this pathogen [405]. Both bactericidal activity and secretory response from microglia are significantly influenced by a Pneumococcal surface protein C (PspC) molecule, present on the bacterial surface. Lack of PsPC renders *S.*

pneumoniae more susceptible to the phagocytic and killing activities of microglia and also stimulates the release of TNF-α, MIP-2, IL-10 and NO [406].

S. aureus is the main causal agent of brain abscesses in humans. Mouse microglia exhibit bactericidal activity against this pathogen. In addition, microglia show an increase in the up-regulation of TLR1, TLR2, TLR6, and CD14 following stimulation with heat-inactivated *S. aureus* or PGN. The increase in the expression of these TLRs and in CD14 following bacterial stimulation might serve as a mechanism to potentiate the antibacterial immune response in the brain prior to leukocyte recruitment in vivo. Moreover, up-regulation of the mentioned receptors is accompanied by enhanced of MHC Class II, CD40, CD80, and CD86 expression. These later findings suggest that the ability of microglia to present antigens may be enhanced following stimulation with *S. aureus* and this could serve as a means to amplify the expansion of antigen-specific T cells to quell CNS infections initiated by this pathogen. Finally, microglia respond to heat-inactivated *S. suis* or its PG by secreting substantial quantities of IL-1α, IL-1β, TNF-α, IL-6, IL-1Ra, MIP-1α, MIP-2, and MCP-1 [407]. The importance of TLR2 in the recognition of *S. aureus* was further evaluated by comparing the inflammatory response from $TLR2^{-/-}$ and wild type microglia. TLR2 was found to play a pivotal role in PG recognition and subsequent activation in primary microglia, as demonstrated by the attenuated expression of pro-inflammatory cytokines and chimokines in PG-treated $TLR2^{-/-}$ microglia compared with wild type cells. In contrast, the responses of $TLR2^{-/-}$ and wild type microglia to *S. aureus* were qualitatively similar, indicating that alternative receptors are responsible for recognizing intact bacteria [408]. Moreover, the pro-inflammatory response against *S. aureus* is partially attenuated when using specific agonist of PPAR-γ, a Peroxisome proliferator-activated receptor (PPARs), member of the nuclear receptor superfamily of proteins that regulate the expression of genes involved in reproduction, metabolism, development, and immune responses. More precisely, the use of the prostaglandin metabolite 15-deoxy-Δ12,14- prostaglandin J2 (15d-PGJ2), which is a natural agonist for PPAR-γ, inhibits cytokine and CC chemokine production by microglia stimulated with heat-killed *S. aureus*. Moreover, 15d-PGJ2 also selectively inhibited the *S. aureus*-dependent increase in microglial TLR2, CD14, MHC class II, and CD40 expression. These findings suggest that 15d-PGJ2 may exert beneficial effects during the chronic stage of brain abscess by minimizing damage to normal brain parenchyma that is a common sequelae of disease [409].

The remarkable participation of microglia in mounting the inflammatory response against Gram-negative bacteria responsible for meningitis has also been studied with *Borrelia burgdorferi*, the causative agent of Lyme disease. In fact, microglia react rapidly when infected with this pathogen, producing different pro-inflammatory mediators, such as IL-6, TNF-α, and PGE2. This reaction is associated with up-regulation of TLR2 and CD14 and further induction of NF-κB [410]. However, *B. burgdorferi*, as well as *N. meningitidis* induce a delayed production of IL-10 from microglia, which in vivo, may be associated with the suppression of inflammation seen within the brain parenchyma during chronic bacterial infections. Moreover, it would also indicate a decrease in the bactericidal capacity of these cells and may serve to demonstrate the ability of microglia to limit potentially damaging inflammation within the CNS in response to invading pathogens [411].

Microglia express functional NOD2 proteins, which may represent a potentially distinguished mechanism by which this cell type can respond to bacterial infections of the CNS. In fact, NOD2 protein expression is elevated following exposure to specific bacterial ligands for TLRs. These findings are supported by the observation that muramyl dipeptide (MDP), which elicits cellular responses via NOD2, can also elevate levels of this receptor, and by the demonstration that microglia express Rip2 kinase and GRIM-19, essential downstream effector molecules for NOD2-mediated cell responses. Finally, NOD2 is also up-regulated after exposure to *B. burgdorferi*, as well as *N. meningitidis* [412]. Moreover, recent data demonstrate that NOD2 is required, at least in part, for the astrogliosis, demyelination, behavioral changes, and elevated inflammatory cytokine levels observed following in vivo infection with these two pathogens [413]. To date, there are no published reports on the participation of these important intracellular receptors in the development of meningitis by *S. suis*.

4.3. Participation of other glial cells

Astrocytes are the major glial cell type in the CNS. In the human brain, astrocyte bodies and processes cover around 11% of the cortex area, 0.80% of this value being represented by astrocyte processes, which form perivascular sheaths [414]. The principal method for identifying astrocytes is immunohistochemistry or immunofluorescence and confocal microscopy with antisera raised against glial fibrillary acidic protein (GFAP) an intermediate filament present in the cell body and processes of all astrocytes [414-416] (Fig. 11).

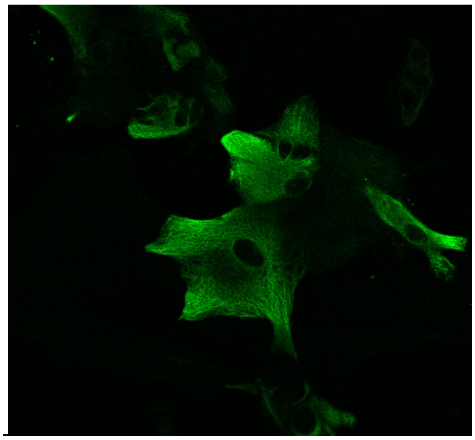


Fig. 11. Primary astrocytes from newborn C57BL/6 mice. Confocal microscopy. Astrocytes were identified with rabbit anti-GFAP antibody (Abcam) and Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen). The staining demonstrates the network of intermediate filaments in the cytoplasm (Zheng and Domínguez-Punaro, *et al*, unpublished data).

Traditionally, astrocytes have been considered to have a supportive or structural role in the brain. However, they are also a remarkable source for neuroactive substances, such as growth factors, eicosanoids, and neurosteroids, which may subsequently influence neuronal development, survival and neurosecretion [415]. In addition, in recent years, astrocytes have been recognized as potentially important contributors to inflammatory immune responses within the brain. In fact, production of pro-inflammatory cytokines in the brain, such as IL-1β and TNF-α increases dramatically in neuropathological states, as they contribute to the breakdown of the BBB and recruitment of leukocytes into the CNS. Furthermore, expression of adhesion molecules (VCAM-1 and ICAM-1) make a major contribution to the extravasation of leukocytes across the BBB. Interestingly, IL-1β induces sustained expression of IL-8 and VCAM-1 and ICAM-1 in astrocytes. This suggests that astrocytes may participate in the development of chronic inflammatory lesions in the brain. Moreover, IL-1β is able to induce a sustained activation of NF-κB in astrocytes, which may also lead to a chronic inflammatory state [417, 418].

Primary murine astrocytes express mRNA encoding for different members of the TLR family. In fact, these cells have a low basal expression of mRNA for TLR2-5 and 9. These receptors are functional, as binding to their specific ligands leads to the translocation of NF-κB to the nucleus and up-regulation of the expression of mRNA-encoding IL-6 [419]. In addition, these cells express only basal levels for TLR2-3 [327, 399]. As opposed to microglia, which mainly express TLR3 in intracellular compartments, astrocytes appear to have both a high intracellular and a high surface expression of TLR3. This possibly reflects the fact that astrocytes

are not professional phagocytes and thus would require a high surface expression of TLR3 for an efficient detection of extracellular pathogens [420]. Moreover, astrocyte response to LTA includes activation of PI3K/Akt and ERK1/2 MAPK pathways and activation of the IKK/NF-κB cascade. Once activated and translocated to the nucleus, NF-κB binds to κB-binding site of MMP-9 promoter, turning on transcription of MMP-9 [421].

The participation of astrocytes to the development of meningitis associated with Gram-positive bacteria also has been studied. In the case of *S. pneumoniae*, purified pneumococcal cell walls are able to induce tyrosine phosphorylation and activation of ERK1/2 and p38 MAPK pathways in a dose-dependent fashion. Inhibitors of tyrosine phosphorylation are able to alleviate this effect and block cytokine production of astrocytes. Moreover, these responses of astrocytes require the presence of sCD14 present in serum [336]. It seems that astrocytes may play a key role in the initial antibacterial immune response against S*. aureus* or its purified cell walls, an event that involves TLR2, with further production of pro-inflammatory cytokines and chemokine production [422]. In addition, up-regulation of GFAP expression in the brain is noteworthy to contain the development of *S. aureus* abscesses. $GFAP^{-/-}$ mice develop, in comparison to wild type mice, larger and more poorly demarcated inflammatory lesions, as well as a significantly increased intracerebral bacterial load, a diffuse leukocytic infiltration of the contralateral hemisphere, purulent ventriculitis, vasculitis, and severe brain edema. Wild type mice also present activated astrocytes showing a strong up-regulation of their GFAP expression in the abscesses [423]. Finally, *S. aureus* or its PG are able to modify the structure of gap junctions, that are formed by the hexameric organization of protein subunits called connexins (Cx). These gap junctions establish communication among astrocytes, and they may influence neuron homeostasis at sites distant from the primary focus of infection. This event is partially dependent on the p38 MAPK pathway [424].

Astrocytes also possess functional NOD receptors. It has been found, that similarly to microglia, NOD2, in association with its downstream effector molecule, Rip2 kinase, participate in the inflammatory response *N. meningitidis* and *B. burgdorferi*, that lead to the production of different pro-inflammatory cytokines [412, 413].

Some of the mechanisms involved in regulation of the inflammatory response in astrocytes are being elucidated. It was recently found that in rat astrocytes, down-regulation mechanisms of TLR2-4 include the participation of monomeric GTPases of the Rho family. Rho

proteins are activated by signals emanating from different surface receptors, including TLRs and act as molecular turn-off switches to control a variety of cellular processes, e.g., membrane trafficking, endocytosis, cytoskeletal remodeling, cell growth and differentiation, transcription, and apoptosis [425]. As these results demonstrated the importance of astrocytes in mounting the inflammatory response in the CNS, it will be necessary to evaluate their role in the meningitis caused by *S. suis*.

Studies evaluating the participation of oligodendrocytes in the development of the CNS inflammatory response are scarce, but it is known that oligodendrocytes can at least express mRNA encoding for TLR2-3. Oligodendrocytes seem to be very sensitive to pro-inflammatory events in their microenvironment. For example, up-regulation of TLR4 from microglia stimulated with LPS provokes death of oligodendrocytes, an effect that may be associated with the release of pro-inflammatory molecules from microglia. This finding may be associated with the demyelination observed in chronic lesions of meningitis [327, 399, 426].

4.4. Animal models of meningitis

There is an urgent need for animal models in the field of infectious diseases, particularly in the research on meningitis and septic shock. The usefulness of an animal model of meningitis/septic shock will vary depending on: 1) if the objective of the study is to focus on bacterial factors of virulence that may facilitate colonization and invasion of tissues, 2) if the main goal is to reproduce the development of pathogenic events leading to inflammation (either generalized or at the CNS), 3) if we want to analyze the host immune response and the progress of possible negative consequences or 4) if we want to test new therapeutic drugs or vaccines. There is no substitute for using animal studies if we want to understand in depth the dynamics of host–pathogen interactions or the complex interactions between the different cell types and organs that are involved in the host response to a pathogen. Numerous animal models have been developed; including monkeys, rats, mice and rabbit models [427-429]. Nevertheless, mice are an attractive organism in which to understand human or zoonotic infectious diseases. Despite some differences, the immune systems of mice and humans are similar and they can often be challenged with the same, or similar, pathogens [427-429].

One of the most important concerns in developing a model of infection (meningitis/septic shock) is the site of bacterial inoculation. On one hand, the pathogen is inoculated into animals intranasally, intraorally, subcutaneously, intravenously, or intraperitoneally. This methodology allows, depending on the chosen route, study of nasopharyngeal colonization, mucosal invasion, intravascular survival, bacterial multiplication and tissue invasion, including the CNS. Therefore, these types of models allow researchers to focus on bacterial and host factors that determine the development of septicemia and the potential of a pathogen to invade the CNS. Because it is considered that adult animals will not reliably develop meningitis after intranasal or intraperitoneal challenge with live organisms, infant animals must be used for these models. On the other hand, adult animals are infected by direct inoculation of the pathogen into the CSF (intracisternal route), and in the case of mice, also by the intracranial route (IC) or by direct intracerebral instillation (through the right orbital surface of the zygomatic bone, at the posterior corner of the right eye). These models can reliably produce lethal infections with a predictable time course. However, these types of experimental infections bypass the natural dissemination of bacteria from the intravascular compartment to the CNS. Nevertheless, these models are considered to be useful for studying pathogen and host factors leading to brain and meningeal inflammation, for studying complications resulting from CNS inflammation, and for evaluating potentially useful agents for therapy of bacterial meningitis [427-429].

1) The adult rabbit model

This model has been developed for the most part using New Zealand white rabbits (2 - 3 kg weight). Animals are anesthetized, and a helmet formed with dental acrylic is attached to the skull by screws. This helmet secures the anesthetized animal in a stereotactic frame that holds a geared electrode introducer with a spinal needle and facilitates puncture of the cisterna magna. Animals are infected by direct injection of a bacterial suspension into the cisterna magna [430-432]. The size of the inoculum that induces lethal infection depends mainly on the infecting microorganism, and it varies depending whether the objective of the study is to develop fatal disease or meningitis. For example, with *S. pneumoniae*, a dose of 1×10^6 CFU/ml produces meningitis in all animals, although as much as 1×10^9 CFU/ml is needed in order to produce a fatal disease. As a rule, only encapsulated bacteria are capable of establishing a productive infection in the subarachnoid space, whereas unencapsulated pathogens induce a

transient meningeal inflammation but are ultimately cleared from the CSF. In addition to live organisms, heat-killed bacteria, purified bacterial products, or host factors (cytokines) can be instilled into the CSF with the object of evaluating their inflammatory activity [432].

2) The adult rat model

This model uses adult Wistar rats and requires anesthesia of animals (usually a combination of ketamine/xylazine). Then there is removal of 75 µl of CSF via intracisternal puncture using a micromanipulator fitted to a 25-gauge needle, followed by inoculation of 50 µl of the challenge organism (1×10^6 CFU/ml). This model has been tested with *S. pneumoniae*, *E. coli* and *H. influenza*. After inoculation, meningitis is allowed to develop for defined durations. This model has been useful to determine bacterial and leukocyte counts in CSF, as well as bacterial counts in the blood, although for short periods, not longer than 8 hours after inoculation, as well as to assess possible differences in pathogenicity between capsulated and non capsulated strains. Moreover, brains from sick animals have been used to analyze possible changes at the cerebral capillary endothelium and integrity of the BBB [433]. This same model, with some modifications, has been useful in the study of the pathogenesis of meningitis by *S. pneumoniae*, providing the following advantages: (1) it allows the continuous measurement of multiple pathophysiologic parameters, such as temperature, cerebral blood flow and intracranial pressure (measured by Laser-Doppler flowmetry), (2) it is performed under standardized, controlled conditions, such as monitoring of mean arterial blood pressure and 3) animals can be equipped with a cranial window for the visualization of pial vessels, to determine their diameter and integrity by light microscope and video equipment. The advantages of the adult rat model is that it allows taking repetitive samples of blood and CSF, although quantities are smaller than these in the rabbit model [428, 429, 434].

3) The adult mouse model

In adult mice, meningitis is reproduced using mainly the intracerebral, the intracisternal, and the intracranial routes of infection. The intraperitoneal route has been used to a lesser extent. However, as mentioned above, the three mentioned approaches bypass several steps of the disease, and development of clinical signs is very rapid, making the design of long-term studies difficult, and it may be necessary to provide antimicrobial treatment so

that animals can survive longer. Moreover, manipulation of animals is complicated, as anesthesia is necessary, and precise handling is required to locate the best site of inoculation.

The intracerebral route has been used in particular for the study of the pathophysiology of *L. monocytogenes* meningoencephalitis. It has also proved useful for cytokine-induced meningitis. In this case, mice are anesthetized, and a superficial dorsal skin incision is made on the lower back to visualize the underlying lumbar vertebrae. Pro-inflammatory cytokines (such as IL-1β) are then injected via lumbar puncture at vertebrate level L-1 or L-2. Mice injected with pro-inflammatory cytokines develop significant CSF pleocytosis and disruption of the BBB within 4 hours after injection [435, 436].

The intracisternal route is an approach widely used for the research in *S. pneumoniae* meningitis. For this, mice should be under short-term anesthesia with halothane and then, they receive a 15 µl intracisternal injection containing 1×10^7 CFU/ml of *S. pneumoniae*. All animals surviving for more than 24 h after infection receive antimicrobial therapy with ceftriaxone. At specific time points, mice are anaesthetized for a second time (ketamine/xylazine) and a catheter is placed into the cisterna magna to determine leukocyte counts in the CSF and to measure the intracranial pressure. On necropsy, brain samples are taken to determine bacterial counts, to evaluate histopathological lesions and to measure the presence of cytokines. In this approach, all infected animals develop meningitis. Under antimicrobial therapy, about 80–90% of infected mice recover from the disease. However, motor residues and hearing impairment can still be observed in recovered mice. Histopathological alterations such as leukocyte infiltration, vasculitis and abscess formation may be observed from 24 h after inoculation [437]. Moreover, this route is also used to infect rats/mice with *S. pneumoniae* and study the mechanisms underlying meningitis-associated hearing loss by means of auditory brainstem response audiometry. Infected animals present deafness, which may be mild and partially reversible during the acute stage or severe with permanent hearing loss. Suppurative labyrinthitis is accompanied by BBB disruption, which correlates closely with hearing loss during the acute stage but not after recovery. In chronic lesions, there is hair cell damage, and fibrocytic occlusion of the cochlea; spiral ganglion neuronal density is markedly decreased and correlates with the severity of permanent hearing loss [438, 439].

The intracranial route is considered as an alternative method to the intracisternal route, in particular in research concerning *S. pneumoniae*. Bacteria are usually inoculated into the right forebrain of adult mice. Observed parameters (blood and CSF bacterial titers, CSF leukocyte counts and meningeal inflammation) increase gradually and all animals die within 45 h. Therefore, antimicrobial treatment with ceftriaxone must be started at around 21 h after infection, because treatment at later time points is associated with high mortality (44% in case of treatment at 24 h). Examination of brain sections of intracranially infected animals revealed a purulent infiltrate surrounded by reactive astrocytes and microglia that developed within 36 h at the site of infection, corresponding to early stage of cerebritis. However, an advantage of the model is its practicability with secure injection of the whole inoculum into the brain [440, 441].

Recently, the intracranial subarachnoidal route of infection was proposed as a variation for the intracranial route. Adult mice are placed under anesthesia (ketamine/xylazine) and infected with *S. pneumoniae* into the subarachnoid space through a soft point located 3.5 mm rostral from the bregma. The bregma is the intersection of the coronal and sagittal sutures of the skull and can be recognised in mice by visual examination of the exposed skull. The stereotaxic coordinates of such inoculation point are 0 mm (× plane), 3.5 mm rostral (y plane) and 2 mm ventral (z plane) from the bregma. In moribund mice, systemic dissemination of pneumococci to blood and spleen was observed. Histological analysis of the brain of animals infected with *S. pneumoniae* confirmed the induction of meningitis closely resembling the disease in humans [296, 442].

The intraperitoneal route has been used to study the pathogenesis of meningitis caused by *H. influenzae*, although two in-vivo passages to enhance virulence of bacteria are required, and a minimum dose of 1×10^4 CFU/ml is necessary to cause disease. Many of the animals display hind leg paralysis, become moribund and/or convulse at around 12 h post-infection [443]. Moreover, adult mice infected by the intraperitoneal route with *N. meningitidis*, produce a rapid but transient bacteremia and symptoms of infection (inactivity, lachrymation) that last only during 9 h post-infection. Bacteria were not recovered from the blood at 18 h post-infection [444]. Mice infected with *S. pneumoniae* by this route, show bacteremia that lasts up to 24 h, and cerebral deposition of *S. pneumoniae* can be found from 6 h of infection. However all animals die from septic shock thirty-six hours after infection, before

the development of meningitis. Antimicrobial treatment of animals with cefazolin (an antimicrobial agent without CNS penetration) is required to stabilize peripheral bacterial titers, resulting in animal survival for at least 5 days [445].

4) The infant rat model

This model has been extensively used for the study of the pathogenesis of meningitis caused by *H. influenza* and *E. coli*. The experimental model uses albino Sprague-Dawley COBS/CD suckling rats (around 5 days old), caged with their mothers. Bacteria are atraumatically instilled into the nose. The inoculum per animal varies between 1×10^3 and 10^9 CFU/ml. This approach results in development of bacteremia after 2 days (73% of animals), depending on the dose. When the inoculum size is decreased, the incidence of bacteremia decreases [446]. This model has demonstrated that intranasal inoculation can result in bacteremia and meningitis, and that the magnitude of bacteremia is directly proportional to the inoculum size. However, this model has several limitations, including the size of the animals, which only results in small sample volumes, variability in the time course, modest brain damage and low case-fatality rate [429].

5) The infant mouse model

This model consists of 11 day-old mice infected by the intracranial route with *S. pneumoniae*. All mice develop meningitis sixteen hours after infection, as documented by positive bacterial cultures of the CSF, although no clear signs of CNS disease are observed. Development of brain lesions depends not only on the bacterial strain used, but also on mouse strain selected, as at 40 h post-infection, there is 60% of survival in infected C57BL/6, 50% in CD1 mice, and 0% in BALB/c. Histological evaluations of brain sections reveal apoptosis in the dentate gyrus of the hippocampus in 27% of infected C57BL/6 and in 5% of infected CD1 mice [447]. The infant mouse model has also been used in *N. meningitidis* research. In this case, bacteria are inoculated into the nostrils of infant mice and the numbers of organisms in the nasal passages, lungs, blood and brains are determined. Intranasal instillation results in consistent nasal colonisation, which usually develops into a lung infection. In many cases, the lung infection precedes bacteremia, which occasionally results in death of the mice. The severity of the infection and the transition to bacteremia is enhanced by intraperitoneal

treatment of the mice with iron dextran or human transferrin. The requirement for lung colonization to precede bacteremia and the need for injection of iron compounds limit the use of this model of meningitis [448].

4.5. Studying meningitis with KO mice: The example of *Streptococcus pneumoniae*

Despite the use of antimicrobial agents, the prognosis of bacterial meningitis is still poor due to central CNS complications. Experimental studies with animal models have given new insights into its pathophysiology during the acute phase of the disease. Targeted gene KO in mice have been used to characterize the mechanism of gene action during infectious processes, including meningitis, representing a gold tool for this research. Most mouse mutants with a defective immune system have a broad vulnerability to infection and show signs of immunodeficiency. By using KO mice, new knowledge of the roles of the different receptors, adaptor molecules, cytokines, proteases, and oxidants involved in the inflammatory cascade has emerged. It is believed that in the future, temporal and cell type-specific control of gene expression will provide even more information on the impact of a particular gene on meningitis-induced brain damage [449].

The generation of a KO mice requires a targeted insertion of DNA into embryonic stem cells by homologous recombination [450]. Typically, a targeting vector is designed in a way that a positive selectable marker gene (for example, neomycin resistance gene, *neo*^{r}) is flanked by large sequences of cloned genomic DNA homologous to the endogenous target gene. The homologous sequences enable insertion of the vector into the target gene by homologous recombination, while the *neo*^{r} gene disrupts the wild type DNA sequence, replacing the original allele by a non-functional one ("null" allele, *gene*$^{+/-}$). Mating of heterozygous mice with one "null" allele will produce a strain of KO mice homozygous for the non-functional gene (*gene*$^{-/-}$). To date, hundreds of KO mice have been genetically engineered and numerous animals have been designed with specific mutations of genes related to the immune or CNS, offering new tools for research on bacterial meningitis [427].

S. pneumoniae is a leading cause of bacterial pneumonia, meningitis, and sepsis in children worldwide. In pneumococcal meningitis, the most common form of bacterial meningitis in adults, death occurs in 25–30% of all patients and neurological and neuropsychological sequelae are reported to affect up to 50% of survivors. Clinical and neuropathological studies have shown that the fatal outcome of the disease is mainly a result of complications secondary to bacterial meningitis. These complications include hearing loss, intracranial hypertension due to brain edema or hydrocephalus, arterial and venous cerebrovascular alterations (cerebral ischemia, venous thrombosis), intracranial hemorrhage, and systemic spread of bacteria leading to septic shock and multi-organ failure [451].

The use of KO mice in *S. pneumoniae* research was used firstly to confirm that TLR2 is involved in the host immune response, initiating the recognition of the pathogen and triggering mechanisms involved in bacterial killing and production of pro-inflammatory mediators. Interestingly, the use of $TLR2^{-/-}$ mice allowed establishing that the host reaction against *S. pneumoniae* does not depend solely on this receptor. More precisely, infected $TLR2^{-/-}$ mice show an increase in disease severity, higher brain and blood bacterial titers and aggravated intracranial complications in comparison to wild type mice. However, expression of different pro-inflammatory mediators is similar between $TLR2^{-/-}$ mice and wild type mice, indicating that there should be receptors other than TLR2 that still sense infection with *S. pneumoniae* and trigger the inflammatory response [325, 452]. In this regard, it is now accepted that pneumolysin recognition is TLR4-dependent, and once the receptor is bound to its ligand, there is release of TNF-α and IL-6, and may induce apoptosis of cells of the myeloid/monocytic lineage [453]. Furthermore, it was found that TLR9 plays a protective role in the lungs at an early stage of infection prior to the entry of circulating inflammatory cells, as $TLR9^{-/-}$ mice are more susceptible to the intranasal challenge with *S. pneumoniae*, showing impaired pneumococcal uptake and in pneumococcal killing [454].

The use of KO mice has also been useful to determine the importance of MyD88 as an adaptor molecule to induce the inflammatory response against *S. pneumoniae*. MyD88-deficient mice display a markedly diminished inflammatory host response in the CNS, as evidenced by reduced CSF pleocytosis and expression of cytokines, chemokines and complement factors, accompanied by a decreased of intracranial complications. Nevertheless,

MyD88 deficiency is associated with a worsening of disease that seems to be attributable to severe bacteremia. Surprisingly, the role of MyD88 or even other adaptor molecules or receptors may depend on the site of the infection, as, although MyD88-deficient mice showed a reduced inflammatory response in the CNS, they were able to mount an exacerbated inflammatory response in the lung, in comparison to wild-type animals [455]. The relevance of the transcription factor NF-κB was also evaluated by a similar approach, comparing the susceptibility to pneumococcus infection between wild type mice and KO mice deficient in the subunit p50 of NF-κB. It was observed that p50 KO mice have an impaired bacterial clearing, along with an enhanced inflammatory host response and increased mortality, thus confirming the importance of NF-κB activation in the pro-inflammatory response of the host [456].

Many in vitro and in vivo studies have highlighted the relevance of different cytokines (IL-1β, TNF-α and IL-6) in the pro-inflammatory response towards *S. pneumoniae*. Studies with mice deficient in one of these cytokines or their receptors have provided a deeper insight into their role during bacterial meningitis. In a murine model of pneumococcal CNS inflammation, the lack of TNF-α was associated with worse clinical outcome and shorter survival time compared with wild type mice, but meningeal inflammation was not distinguishable between the groups. However, bacterial titers were significantly higher in the blood and spleen of TNF-$\alpha^{-/-}$ mice, indicating that the clearance of *S. pneumoniae* from the bloodstream was severely affected. By contrast, mice with targeted disruption of the two TNF receptors p55 and p75 showed decreased meningeal inflammation with reduced leukocyte infiltration. These results indicate that other TNF receptor ligands than TNF-α may contribute to the inflammatory reaction in pneumococcal meningitis [457]. In IL-6-deficient mice, infection with pneumococci caused a marked increase in CSF leukocyte counts compared with wild type mice. This was associated with a higher expression of IL-1β, TNF-α and MIP-2 in IL-$6^{-/-}$ mice, indicating that in wild type animals these pro-inflammatory mediators are down-regulated by IL-6 [458].

Finally, the importance of different reactive molecules, including nitrogen intermediates, such as NO, in the pathogenesis of *S. pneumoniae* have also been studied using KO mice. NO is produced by three different NO synthases (NOS): two constitutive isoforms, which are present in endothelial cells (eNOS) and neuronal cells (nNOS), and one inducible isoform iNOS, which is expressed in many cells, including neutrophils, macrophages, microglia

and astrocytes, in response to stimulation with cytokines or bacterial cell-wall components. The significance of pharmocological studies using NOS inhibitors is limited by three major problems: 1) NOS inhibitors are not completely selective for one isoform, 2) their bioavailability is questionable, and 3) they may exert additional pharmacological effects unrelated to the NOS system. Therefore, mice deficient in one distinct isoform have offered a powerful tool to elucidate the role of eNOS and iNOS in experimental bacterial meningitis [449]. In pneumococcal meningitis, $iNOS^{-/-}$ mice showed significantly lower concentrations of pro-inflammatory cytokines and also a reduction in the BBB disruption in comparison to wild type mice. However, iNOS-deficient mice presented increased CSF leukocyte counts in reponse to pneumococcal challenge. There is not a clear explanation for this effect, but it provides evidence that NO is able to modulate the adhesiveness of leukocytes to the endothelium [459].

Table 1. Pattern recognition receptors (PRRs): Soluble receptors

Soluble receptors:	Members	Role	Ref.
Collectins	Surfactant proteins A & D (SP-A, SP-D) and the mannose-binding lectin (MBL) and MBL-associated proteins (MASPs)	SP-A and SP-D act as opsonins, increasing the clearance of the organism. The bacterial targets of both molecules are LTA and PG. MBL and MASPs bind poly-mannose components of microbial cell walls. They also activate the complement cascade.	[460]
Complement cascade (CC)	CC is activated by three pathways: the classical, alternative, and lectin pathways.	CC eliminates bacteria by a direct attack, forming a membrane attack complex and producing opsonins C3b and iC3b for opsonophagocytosis.	[317, 461]
Pentraxins	It includes the short pentraxins such as C-reactive protein and serum amyloid P (SAP) and the long pentraxins. C-reactive protein and SAP are acute phase proteins synthesised by the liver as part of the acute inflammatory response.	C-reactive protein binds phosphorylcholine and mannose residues found on bacterial or fungal cell walls and both CRP and SAP act as opsonins. They can activate the classical pathway of CC. The short pentraxins are also taken up by Fcγ receptors on phagocytic cells.	[317]
Others	Lipopolysaccharide-binding protein (LBP) and soluble/membrane associated CD14	LBP is an acute phase protein produced in the liver and bounds LPS present in the serum. It increases the transfer of LPS to a receptor, CD14, a glycosylphosphatidyl inositol anchored cell surface glycoprotein found on macrophages that functions as a binding receptor for LPS. In CD14-negative cells such as endothelial cells and fibroblasts, the soluble form of CD14 present in serum can functionally replace membrane-bound CD14. This LBP/CD14 complex then links TLR4, which ultimately leads to intra-cellular signalling and cytokine secretion by the macrophage.	[321, 462]

Table 1. Pattern recognition receptors (PRRs). Continuation: Cell-associated and intracellular receptors.

Cell-associated receptors:	Members	Role	Ref.
Membrane-bound receptors	TLRs	TLRs are explained in detail in the text	
	CD14	CD14 role is explained in the text	
	MD2	160-amino acid protein that is associated with TLR2/4 on the cell surface.	[323, 463]
Scavenger receptors	SR-AI, SR-AII and MARCO	They bind LPS, LTA as well as whole bacteria. They clear tissue of invading organisms by acting as receptors for phagocytosis.	[317, 464]
C-type lectin family	Mannose receptors (MR)	Mannose-binding lectin (MBL) binds repeating arrays of sugar groups on microbial surfaces with its lectin domains. MBL activates the "alternative," or lectin, complement pathway in an antibody-independent manner. MBL also is an opsonin that enhances phagocytosis. MBL binds yeasts, viruses, and Gram-negative bacteria and Gram-positive bacteria with low affinity. MR are present on macrophages. Their main function is phagocytosis of microbial pathogens, and their delivery into the lysosomal compartment where they are destroyed by lysosomal enzymes.	[465]
Complement receptors		Organisms coated with complement components are bound to complement receptors and also ingested by phagocytosis.	[317, 461]
Formyl-peptide receptors		Found on neutrophils and monocytes. They are in part responsible for chemotaxis of neutrophils to sites of infection.	[466]
Intra-cellular receptors	**Members**	**Role**	**Ref.**
Nucleotide binding and oligomerization domain (NOD) receptors	NOD1 and NOD2	Sense the cytosolic presence of the PG fragments meso-DAP and muramyl dipeptide, respectively and activate the inflammatory response.	[312]
Others	Protein kinase R (PKR)	Activated by double stranded RNA. PKR also upregulates NF-κB and MAP kinases, leading to the production of interferons.	[312, 317]

Table 2. TLRs recognize molecular patterns associated with a broad range of pathogens including bacteria, fungi, protozoa, and viruses. Adapted from [311, 313, 318, 321, 467].

TLR	Ligand
TLR1	Triacyl lipopeptides (bacteria)
TLR2	PG, LTA (from Gram positive bacteria), lipoprotein, lipopeptides, atypical LPS, lipoarabinomannan (mycobacteria)
	Zymosan, phospholipomannan (fungi)
	GPI anchor (protozoa)
	Envelope protein (virus)
TLR3	Poly (I:C), dsRNA (virus)
TLR4	LPS from Gram negative bacteria (bacteria)
	RSV fusion protein (virus)
	Glycoinositolphospholipids (protozoa)
	Mannan, glucuronoxylomannan (fungi)
TLR5	Flagellin (bacteria)
TLR6	Diacyl lipopeptides (bacteria)
TLR7/TLR8	Synthetic imidazoquinoline-like molecules, ssRNA (virus) Immune function of TLR8 remains unknown in mice
TLR9	CpG DNA (bacteria, protozoa, virus)
	Hemozoin (protozoa)
TLR10 (pseudogene in mouse)	Unknown, however, it heterodimerizes with TLR1and TLR2
TLR11 (pseudogene in human)	Unknown components of uropathogenic bacteria
	Profilin like molecule (protozoa)
TLR12 (mouse)	Unknown
TLR13 (mouse)	Unknown

III. MATERIALS, METHODS AND RESULTS

ARTICLE I:

"Streptococcus suis serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection"

María C. Domínguez-Punaro[*], Mariela Segura[§], Marie-Michèle Plante[†], Sonia Lacouture[*], Serge Rivest[†], and Marcelo Gottschalk[2*]

[*]Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada. [†]Laboratory of Molecular Endocrinology, Centre Hospitalier de l'Université Laval, St-Foy, Québec, Canada. [§]Centre for the Study of Host Resistance, The Research Institute of the McGill University Health Centre, Montréal, Québec, Canada.

Published in: *J. Immunol.* 2007 Aug 1;179(3):1842-54

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the experimental design; I substantially performed research, analyzed data and wrote the paper.

Abstract

Streptococcus suis, an important swine and human pathogen, causes septic shock and meningitis. The pathogenesis of both systemic and central nervous system (CNS) infections caused by *S. suis* is poorly understood. A hematogenous model of infection in CD1 mice was developed to study the systemic release of cytokines during the septic shock phase and the pro-inflammatory events in the CNS associated with this pathogen. Using a liquid array system, high levels of systemic TNF-α, IL-6, IL-12, IFN-γ, CCL2, CXCL1 and CCL5 were observed 24 h after infection and might be responsible for the sudden death of 20% of animals. Infected mice that survived the early sepsis later developed clinical signs of meningitis and exhibited lesions in the meninges and in numerous regions of the brain, such as the cortex, hippocampus, thalamus, hypothalamus and corpus callosum. Bacterial Ags were found in association with microglia residing only in the affected zones. *In situ* hybridization combined with immunocytochemistry showed transcriptional activation of TLR2 and TLR3 as well as CD14, NF-κB, IL-1β, CCL2 and TNF-α, mainly in myeloid cells located in affected cerebral structures. Early transcriptional activation of TLR2, CD14 and inflammatory cytokines in the choroid plexus and cells lining the brain endothelium suggests that these structures are potential entry sites for the bacteria into the CNS. Our data indicate an important role of the inflammatory response in the pathogenesis of *S. suis* infection in mice. This experimental model may be useful for studying the mechanisms underlying sepsis and meningitis during bacterial infection.

Introduction

Infections caused by *Streptococcus suis* serotype 2 are a problem worldwide in the swine industry. In swine, *S. suis* infection is associated with meningitis, septicemia, endocarditis and arthritis (1). Moreover, *S. suis* is an agent of zoonosis, afflicting people in close contact with infected pigs or pork-derived products (2). The clinical presentation of *S. suis* infection may vary from asymptomatic bacteremia to fulminant systemic disorder. Meningitis is the most striking feature; the presence of fibrin, oedema and cellular infiltrates in the meninges and choroid plexus along with adjacent encephalitis are the most frequently observed histopathological characteristics (3). People who survive the infection may develop important sequelae such as permanent deafness (2, 4). Recently, a serious *S. suis* outbreak in China resulted in more than 200 human cases with a fatality rate of nearly 20%. Symptoms reported in this outbreak include high fever, malaise, nausea and vomiting, followed by nervous symptoms, subcutaneous hemorrhage, septic shock, and coma in severe cases (5). Virulence factors associated with *S. suis* are poorly defined. To date, the antiphagocytic capsular polysaccharide (CPS)[3] as well as other putative virulence factors, such as the hemolysin (suilysin), cell-wall associated and extracellular proteins, fibronectin-binding proteins and a serum opacity factor, have been described (6, 7).

Elucidation of the pathogenesis of both systemic and CNS infections caused by *S. suis* remains a challenge. It is believed that pigs are infected with *S. suis* via airborne transmission and that bacteria, once in the blood, interact with different leukocyte populations as well as endothelial cells and then migrate to different organs and cause tissue damage (8). In the event, for unknown reasons, that bacteria fail to cause acute fatal septicemia, *S. suis* is able to reach the CNS through mechanisms that are only partially understood (9). It has been demonstrated that *S. suis* adheres to human brain microvascular endothelial cells (BMEC) and exerts a cytotoxic effect that involves suilysin (9). In addition, interactions between *S. suis* and epithelial cells from the choroid plexus have been observed using an in vitro model in which this pathogen induces a loss of blood-cerebrospinal fluid barrier function (10). Both mechanisms

might lead to a breakdown of the blood brain barrier (BBB), as described for other pathogens (11, 12).

Clinical and neuropathological studies with meningitis-associated bacteria have shown that a fatal disease outcome can be attributed to the host inflammatory response which leads to intracranial complications, including brain edema, increased intracranial pressure and cerebrovascular insults. Indeed, up-regulation of pro-inflammatory mediators and increased leukocyte trafficking may contribute to the breakdown of the BBB (13). *S. suis* induces BMEC to release arachidonic acid and different pro-inflammatory cytokines and chemokines, which might help bacteria migrate through the BBB and modulate local inflammation (14, 15). Additionally, *S. suis* induces phagocytes to secrete cytokines, chemokines, PGE_2 and matrix metalloproteinase-9, mediators which are also implicated in the disruption of the BBB (16-19). Lastly, *S. suis* up-regulates the expression of adhesion molecules by monocytes, consequently increasing adherence to endothelial cells (20). Although the exact mechanisms underlying the inflammatory response induced by *S. suis* are unknown, in vitro experiments showed that cytokine and chemokine production by *S. suis*-activated phagocytes is mediated through CD14-dependent and independent pathways (18). More recently, we demonstrated in vitro that TLR2 plays an important role in the recognition of *S. suis* through a MyD88-dependent mechanism (21).

The CNS, although commonly considered an immunologically privileged site, is capable of mounting a coordinated innate immune response along with the expression of different immunomodulatory mediators (22). Different types of cells may play an inflammatory role. Microglial cells, the macrophage-like population within the CNS, represent the first line of defense against invading pathogens and have pro-inflammatory effector functions (23). Astrocytes, the major glial cell type in the CNS, also contribute to the inflammatory response in the brain. Both types of cells express constitutively low levels of mRNA encoding different TLRs which can be notably increased following the binding of specific ligands (23, 24). To date, the inflammatory

activities in the brain as well as the pro-inflammatory role of both microglia and astrocytes during CNS infections associated with *S. suis* have not been studied.

The recently documented increase in severity of *S. suis* infection in humans underscores the critical need to better understand factors associated with pathogenesis of *S. suis* infection (5). In the present study, we developed an adult mouse model of streptococcal meningitis and encephalitis after i.p. infection with a virulent *S. suis* serotype 2 strain. Using this model, we studied the systemic release of different cytokines during the septic shock phase observed within hours after infection. In addition, the cerebral innate immune response in mice that survived the septic phase, but developed clinical signs of meningitis at later stages of the infection, was studied using mRNA in situ hybridization. To our knowledge, this is the first report of a streptococcal meningitis/encephalitis model using an i.p. infection route in adult mice.

Materials and methods

Bacterial strain, growth conditions and production of antiserum

S. suis serotype 2 strain 31533 was used for the experimental infection. This is an encapsulated, suilysin-positive virulent strain that has been widely used in several cell stimulation studies (15, 17-19, 25). Bacteria were grown overnight onto sheep blood agar plates at 37°C and isolated colonies used as inocula for 5 ml of Todd-Hewitt broth (THB) (Difco Laboratories), which was incubated for 8 h at 37°C with agitation. Working cultures were prepared by transferring 10 µl of 1/1000 dilutions of 8 h-cultures into 30 ml of THB which was incubated for 16 h at 37°C with agitation. Stationary-phase bacteria were washed twice in PBS (pH 7.3). Bacterial pellet was then resuspended in THB until an OD_{600nm} value of 0.4 was achieved, which corresponded to 5×10^8 CFU/ml. The inoculum for experimental infection was prepared with a 1:10 dilution of the resuspended bacteria in THB to obtain a final concentration of 5×10^7 CFU/ml. This final suspension was plated onto blood agar to accurately determine the CFU/ml. Specific polyclonal antiserum against *S. suis* serotype 2 was obtained as previously described (26).

Experimental infection

Female, 6-week-old CD1 mice (Charles River) were acclimated to standard laboratory conditions of 12-h light/12-h dark cycle with free access to rodent chow and water. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals by the Animal Welfare Committee of the Université de Montréal. A total of 145 animals were included in the study. On the day of the experiment, 1 ml volume of the bacterial suspension (5×10^7 CFU/ml) or the vehicle solution (sterile THB) was administrated by i.p. injection. Three series of

preliminary and independent trials were performed for both systemic and CNS infection studies to establish optimal bacterial dose and time points. Preliminary data obtained in these pre-studies showed results consistent with those obtained in the major experiments described bellow.

Study of the systemic innate immune response

A total of 36 infected and 12 non-infected mice were assigned to the bacteriological and histopathological studies as well as the systemic measurement of pro-inflammatory molecules. Samples were taken at 3, 6, 12, 24, 30 and 36 h and at 3, 6, 9 and 12 days (d) post infection (p.i.) and the following studies performed:

A. Clinical parameters of disease and mortality. A group of 10 mice were monitored three times daily for mortality and clinical signs of septic disease, such as depression, swollen eyes, rough hair coat, lethargy, and nervous signs of meningitis.

B. Determination of viable bacteria in organs. At each designated time, 3 infected and 1 non-infected mice were anaesthetized with CO_2. Blood was collected by cardiac puncture and the brain, liver and spleen were obtained aseptically. The organs (0.05g/organ) were trimmed, placed in 500 µl of PBS (pH 7.3) and homogenized with a vortex. Then, 50 µl of 10^{-2} and 10^{-4} dilutions of the homogenate in PBS was plated onto blood agar plates. Blood samples (50 µl) were also plated. All samples were plated using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech). Blood agar plates were incubated overnight at 37°C. Colonies were counted and expressed as CFU/0.05g for organ samples or CFU/ml for blood samples. In addition, a 5 ml THB tube was inoculated with 300 µl of the remaining supernatant from homogenates or with 10 µl of blood and cultured at 37°C overnight with agitation. These THB cultures were plated only when the cultures plated with the Spiral Plater were negative due to low numbers of viable bacteria in the organs. Although not quantitative, a positive THB culture indicates the presence of bacteria in the organ.

C. Histopathological studies. Samples from the brain, heart, liver and spleen were fixed in 10% buffered formalin. After paraffin embedding, 4-mm–wide tissue sections were stained with hematoxylin-eosin according to standard protocol and examined under light microscopy.

D. Plasma collection and measurement of cytokines and chemokines. Blood from CO_2-anaesthetized mice was collected by cardiac puncture into heparinized tubes and centrifuged at 10,000 x *g* for 10 min to obtain plasma. Samples were preserved at -80°C until analysis. Levels of IL-1β, IL-6, IL-10, IL-12 (p40/p70), TNF-α, IFN-γ, CCL2 (MCP-1), CXCL1 (KC) and CCL5 (RANTES) in plasma were determined using a liquid multi-array system (Luminex Co.). Commercial multiplex coated beads, biotinylated antibodies and Beadlyte microtiter 96 well filter plates were obtained from Upstate Group Inc. Each multiplex assay was performed in duplicate following the manufacturer's specifications. Data were collected using the Luminex-100 system Version IS 2.2 and analyzed by MasterPlex Quantitation Software (MiraiBio Inc.). Standard curves for each cytokine and chemokine were obtained using the reference concentrations supplied by the manufacturer. Results are the mean of two independent experiments.

Study of the cerebral innate immune response

A total of 55 infected and 22 non-infected mice were analyzed in this set of experiments. For all studies, samples were taken at 3, 6, 12, 24, and 36 h as well as at 2, 3, 5, 7, 9 and 14 d p.i. At each designated time, 5 infected and 2 non-infected mice were deeply anaesthetized with an i.p. injection of a mixture of ketamine hydrochloride (Bimeda-MTC) and xylazine (Bayer), and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M borax buffer (pH 9.5 at 4°C). Brains were rapidly removed from the skulls, post-fixed for 2-8 d, and placed in a solution containing 10% sucrose diluted in 4% paraformaldehyde-borax buffer overnight at 4°C. The frozen brains were mounted onto a microtome (Reicher-Jung, Instruments Company) and cut into 25 µm coronal sections from the olfactory bulb to

the end of the medulla. The sections were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at -20°C.

cRNA probes

Plasmids for the synthesis of the cRNA probes have described previously (27, 28). Plasmids were linearized, and the sense and antisense riboprobes were synthesized as reported (27, 28). Radioactive cRNA copies were obtained by incubating 250 ng of linearized plasmids in 6 mM $MgCl_2$, 40 mM Tris (pH 7.9), 2 mM spermidine, 10 mM NaCl, 10 mM ATP/GTP/CTP, 100 µCi of α-^{35}S-UTP (Dupont-NEN; #NEG 039H), 20 U RNAsin (Promega), and 10 U of either T7, SP6 or T3 RNA polymerase for 60 min at 37°C. Unincorporated nucleotides were removed using ammonium acetate precipitation method, and 100 µl of DNase solution (1 µl DNase, 5 µl of 5 mg/ml tRNA, 94 µl of 10 mM Tris/ 10 mM $MgCl_2$) was added 10 min before phenol-chloroform extraction. The cRNA was precipitated with 80 µl of 5 M ammonium acetate and 500 µl of 100% ethanol for 20 min on dry ice. The pellet was dried and resuspended in 50 µl of 10 mM Tris/1 mM EDTA. A probe containing 10^7 cpm was mixed into 1 ml of hybridization solution (500 µl formamine, 60 µl 5 M NaCl, 10 µl 1 M Tris (pH8.0), 2 µl 0.5 M EDTA (pH 8.0), 50 µl 20x Denhart's solution, 200 µl 50% dextran sulphate, 50 µl 10 mg/ml tRNA, 10 µl 1M DDT, (118 µl DEPC water – volume of probe used). This solution was mixed and heated for 10 min at 65°C before being spotted on slides (27, 28).

In situ hybridization histochemistry

Histochemical localization of TLR2, TLR3, TLR4, CD14, IκB (an index of NF-κB activation), IL-1β, IL-6, TNF-α, CCL2 (MCP-1) and mouse proteolipid protein (PLP) mRNA was performed on every twelfth section of the whole rostro-caudal extent of each brain using *in situ* hybridization with ^{35}S-labeled cRNA probes as described previously

(27-29). All solutions were treated with DEPC and sterilized to prevent RNA degradation. Briefly, tissue sections mounted onto poly-L-lysine-coated slides were desiccated overnight under vacuum, fixed in 4% PFA for 30 min, and digested with 10 µl/ml of proteinase K in 0.1 M Tris HCl (pH 8.0) and 50 mM EDTA (pH 8.0) at 37°C for 25 min. Brain sections were rinsed in sterile DEPC water, followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of ethanol (50, 70, 95, and 100%). After vacuum drying for a minimum of 2 h, 90 µl of hybridization mixture (10^7 cpm/ml) was spotted onto each slide, sealed under a coverslip, and incubated at 60°C overnight (~15-20 h) in a slide warmer. Coverslips were then removed and the slides rinsed in 4X standard saline citrate (SSC) at room temperature (SSC 1X is composed of 0.15 M NaCl and 15 mM trisodium citrate buffer at pH 7.0). Sections were digested with 20 µg/ml of RNase A at 37°C for 30 min, rinsed in descending concentrations of SSC (2X, 1X, 0.5X SSC), washed in 0.1X SSC for 30 min at 60°C, dehydrated with graded concentrations of ethanol, and vacuum-dried for 2 h. Sections were exposed at 4°C to X-ray films (Biomax, Kodak) for 1-3 days. Slides were defatted in xylene, dipped in NTB-2 nuclear emulsion (Kodak; diluted 1:1 with distilled water), exposed for 7 (IκBα and IL-1β transcripts), 8 (PLP transcript), 14 (CD14, TNF-α and CCL2 transcripts), 15 (TLR2 and TLR3 transcripts), 19 (TLR4) or 31 d (IL-6), then developed in a D19 developer (Kodak) for 3.5 min at 14-15°C, washed 15 s in water, and fixed in rapid fixer (Kodak) for 5 min. Tissues were rinsed in running distilled water for 1-2 h, counterstained with thionin (0.25%), dehydrated with graded concentrations of ethanol, cleared with xylene, and sealed with distrene plasticizer xylene mounting medium.

Qualitative analysis

The anatomical identification of brain structures was based on the Paxinos and Franklin's Atlas (30). The relative intensity of TLR2, TLR3, TLR4, CD14, IκBα, IL-1β, IL-6, TNF-α, CCL2 and PLP mRNA expression throughout the brain of each infected animal

was determined on x-ray film images and graded according to the scale of undetectable (-), low to undetectable (+/-), low (+), moderate (++), strong (+++), or very strong (++++) signal (31).

Combined immunohistochemistry and in situ hybridization

In situ hybridization was combined with immunohistochemistry to identify the cellular sources expressing TLR2, IκBα, and CCL2 (MCP-1) in the brain parenchyma of mice after systemic injection of *S. suis*. Every twelfth section of the brain was processed by the avidin–biotin method with peroxidase as a substrate. Microglial cells were labeled with an Ab against the anti-ionized calcium binding adapter molecule 1 (*iba*1, kindly provided by Dr. Y. Imai, Nationale Institute of Neuroscience, Kodaira, Japan), as previously described (32). Astrocytes were labeled with an Ab against glial fibrillary acidic protein (GFAP) (DAKO). Briefly, sections were rinsed with Potassium-PBS (KPBS; 2.2 mM $K_2H_2PO_4$, 1.8 mM KH_2PO_4 and 138 mM NaCl in milliQ water at pH 7.4), incubated with primary Ab, washed once with KPBS before incubation with biotinylated secondary Abs (Vector Laboratories), and then rinsed again with KPBS before a final incubation with an avidin-biotin-peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories). After several washes in KPBS, brain sections were reacted in 0.05% 3,3'-diaminobenzidine (DAB) and 0.003% hydrogen peroxide. Thereafter, sections were rinsed in KPBS, mounted, desiccated, fixed in 4% PFA, and digested by proteinase K. Pre-hybridization, hybridization, and post-hybridization steps were performed as described above, but shorter dehydration times (ethanol 50, 70, 95, 100%) were used to prevent discoloration of immunoreactive cells. The slides were dried and then exposed and developed as described above.

Immunohistochemistry for S. suis

Immunohistochemical localization of *S. suis* was also performed on every twelfth section of the whole rostro-caudal extent of each brain. Brain sections were washed 3 times for 15 min each in KPBS, followed by 20 min incubation at room temperature with solution A (0.4% Triton X, 4% goat serum and 1% BSA in KPBS). The sections were incubated with a primary polyclonal Ab against *S. suis* (1:6000 in solution A) for 18 h at 4°C, followed by three 5-min washes with solution B (0.02% Triton X and 0.05% BSA in KPBS), incubation with the secondary Ab diluted 1:500 in solution B of a commercial biotinylated anti-rabbit IgG (Vector Laboratories) for 90 min, and three 15-min washes with solution B. Immunoreaction was displayed by the addition of ABC labeled polymer and the substrate DAB (Vector Laboratories) as per the manufacturer's instructions. The sections were washed 3 times for 15 min each in KPBS and then counterstained with Nissl stain (0.25% thionine solution) to obtain a general index of cellular morphology. The tissues were mounted onto slides and observed under light microscopy.

Determination of acute demyelination

The myelin content was determined via Sudan black B staining (SBB) as described before (29). Briefly, brain sections mounted onto poly-L-lysine-coated slides were dehydrated with graded concentrations of ethanol (50 and 70%; 2 min each) and incubated for 1 h in SBB solution (Sigma, 0.3% saturated solution in 70% ethanol). The sections were rinsed in 70% ethanol until the desired contrast was reached and then washed with water. Tissues were dehydrated with graded concentrations of ethanol (50 and 70%; 2 min each), cleared with xylene for 1 min (two times), and cover slipped. In addition, the level of demyelination was correlated to the level of PLP mRNA expression in specific areas of the brain using in situ hybridization as described above.

Results

Systemic and CNS symptoms appear at different times after i.p. infection

During the course of infection, clinical signs of sepsis were depression, rough hair coat, swollen eyes, weakness, and death. Mortality of 20% due to septicemia was observed before 48 h p.i. The appearance of clinical signs of meningitis was observed in 40% of remaining infected mice between days 4 and 9 p.i. These mice exhibited normal characteristics before the sudden appearance of nervous signs, which consisted of hyperexcitation, episthotonus, opisthotonus (video1.mov), bending of the head towards one side, walking in circles (video2.mov), and strong locomotive problems of mainly paresis of the forelimbs. Some mice displayed sudden spinning while in lateral recumbence (video3.mov). In addition, mice developed unilateral or bilateral exophthalmia, with corneal opacity, probably in association with increased intracranial pressure.

Bacteria survive several days in mice after i.p. infection

After i.p. infection with *S. suis*, mice developed bacteremia which lasted for several days. *S. suis* was isolated from all infected mice that were tested. Bacteria were found in the blood, liver, spleen, and brain. From 3 h to day 3 p.i., bacterial counts in the blood exceeded 1 x 10^8 CFU/ml, then decreased between days 6 and 9 p.i. to a median of 2 x 10^2 CFU/ml, and finally disappeared at day 12 p.i. (Fig. 1*A*). Viable counts in the liver and spleen were comparable and presented similar kinetics. From 3 h to day 3 p.i., bacterial counts from these organs were higher than 1 x 10^7 CFU/organ in all sampled animals. Counts diminished drastically at day 6 p.i., and by day 12 p.i. were negative in the spleen or qualitatively positive in the liver (growth after broth enrichment) (Fig. 1*A*). In contrast, bacterial counts in the brain showed a different pattern. While in other organs viable counts were very similar and showed well-defined kinetics among

sampled mice, bacterial loads in the brain were highly variable between individual mice and between sampling times (Fig. 1*B*). No clear kinetic pattern of bacterial counts could be identified in the brain. Nevertheless, bacterial counts were relatively high as early as 3 h p.i., ranging from 10^4 to 10^8 CFU/0.05 g of brain. This range of bacterial load was observed throughout the different p.i. time points up to day 3 day p.i. Although bacterial loads decreased by day 6 p.i., the presence of *S. suis* in the brain could be detected qualitatively at day 12 p.i. (Fig. 1*B*).

S. suis- associated lesions were correlated with CNS symptoms

Although it was possible to isolate bacteria from various tissues at different p.i. time points, histopathological lesions associated with the infection were found in the brain of infected mice that developed clinical signs of meningitis. These mice presented bacterial loads ranging from 10^3 to 10^4 CFU/0.05 g of tissue. No significant histopathological changes in the brain, heart, liver or spleen were observed in mice that succumbed during the early phase of sepsis, which occurred during the first 48 h p.i. From day 6 p.i., the most important changes in the brain of mice with clinical nervous symptoms comprised the presence of neutrophils across blood vessel walls, hemorrhagic foci and malacia mainly at the somatosensory cortex, striatum, hippocampus, thalamus and hypothalamus, together with gliosis and the presence of inflammatory foci composed primarily of neutrophils. At these time points, it was possible to observe great numbers of bacteria inside blood vessels (bacterial emboli), surrounded by a rim of neutrophils (Fig. 2*A*). As the infection progressed, by day 9 p.i., there was complete destruction of the corpus callosum, including the external capsule, where numerous neutrophils and cellular debris were observed (Fig. 2*B*). In several mice, the meninges were thickened and severely infiltrated by a mixture of neutrophils, macrophages and lymphocytes (Fig. 2*C*). Occasionally, in severely affected mice, a moderate dilation of the aqueduct was apparent. Outside the brain, histopathological lesions were observed only in the heart of some mice that developed clinical nervous

symptoms. These lesions, consisting of dense infiltrates of neutrophils in the myocardium of the interventricular septum (suppurative myocarditis), and thrombi composed of fibrin, great numbers of neutrophils and bacteria, were found on the wall of the right atrio-ventricular valves (valvular endocarditis).

Intraperitoneal infection with S. suis induces a rapid pro-inflammatory systemic response in mice

Results showed that the systemic cytokine and chemokine production in mice after infection with *S. suis* varied over time and among different cytokines. Production of pro-inflammatory TNF-α, one of the most important host mediators in the pathogenesis of septic shock, showed a high but transitory peak at 6 h p.i. and a drastic return to basal levels at 12 h p.i, regardless of the infection outcome (Fig. 3*A*). This rapid TNF-α production in plasma may indicate an important early participation of phagocytic cells during the onset of the inflammatory response against *S. suis* and the initiation of an amplification loop leading to the production of several other pro-inflammatory cytokines. Along with TNF-α, IL-6 and IL-12(p40/p70) were also rapidly induced. IL-6, an important inducer of acute phase proteins (33, 34), was expected to be elevated in the later phase of infection (35, 36) but, similar to TNF-α, its systemic levels were significantly high only during the first 12 h p.i., peaking at 97,000 pg/ml at 6 h p.i. (Fig. 3*B*). Th1-promoting IL-12, which mediates the transition to adaptive immunity, was also induced early after *S. suis* systemic infection. Levels of IL-12p40 in plasma increased abruptly and also returned rapidly to basal levels of non-infected controls as early as 6 h p.i. (Fig. 3*C*). Interestingly, IL-12p40 production preceded the appearance of biological IL-12p70 as suggested previously (37). Indeed, the pattern of IL-12p70 expression was similar to the aforementioned pro-inflammatory cytokines, peaking at 6 h p.i and gradually returning to baseline by 30 h p.i. (Fig. 1*D*). In contrast, production of IFN-γ, a critical Th1 cytokine, was rapidly induced at 6 h p.i., remained at high levels, and reached a plateau at 24 to 36 h p.i. (Fig. 3*E*). Surprisingly, IL-1β, known

to play an important role in inflammation (38), was induced only slightly after systemic *S. suis* infection (Fig. 3*F*). It should be noted that these cytokines were no longer detected at day 6 p.i. (data not shown).

We also measured the production of the chemokines CXCL1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) in mice infected with *S. suis*. While CXCL8 (IL-8) has not been identified in the mouse, CXCL1 is one of the CXCL8 homologs believed to be important in the trafficking and activation of neutrophils in mice (39, 40). Levels of this key chemotactic factor were extremely high and rapidly induced, reaching peak production between 6 and 12 h p.i. and returning to baseline at day 3 p.i. (Fig 3*G*). The expression of CCL2 (MCP-1), a prototypic inflammatory chemokine which mainly targets monocytes and T lymphocytes (40), was increased at 3 h p.i., peaking between 3 h and 24 h p.i, and gradually decreasing thereafter (Fig. 3*H*). Since very little is known regarding the role of CCL5 (RANTES), a highly potent chemoattractant for both monocytes and T cells (41), in Gram-positive systemic infections, we measured its production during the septicemic phase of *S. suis* infection in mice. Results showed that CCL5 was also induced as early as 3 h p.i., reaching maximal expression at 6 h p.i. and slowly diminishing to levels of non-infected mice at day 3 p.i. (Fig. 3*I*). No significant levels of any chemokines were observed after day 6 p.i. (data not shown).

We also assessed the expression of anti-inflammatory IL-10. In infected mice, IL-10 in plasma was apparent at 6 h p.i. and gradually increased to peak levels between 24 and 30 h p.i., with values greater than 6000 pg/ml (Fig. 3*J*). Thereafter, it disappeared rapidly and was undetectable after day 6 p.i. (data not shown).

S. suis up-regulates the expression of genes encoding for several pro-inflammatory mediators at different structures of the brain

In situ hybridization was used to examine the spatiotemporal expression of different pro-inflammatory genes after i.p. infection of mice with *S. suis* at different p.i. time

points (Table I). This technique was combined with immunohistochemistry to study the ability of microglia/macrophages and astrocytes to express diverse pro-inflammatory genes as described below.

Expression of TLR2, TLR3 and TLR4

A modest constitutive TLR2 hybridization signal (+/-) was observed at the choroid plexus of the lateral ventricles in all vehicle-treated mice from 3 to 12 h p.i. In infected mice, transcriptional activation of TLR2 gene was first detected at 24 h p.i. in the meninges as well as in different areas of the somatosensory cortex, striatum, the choroid plexus, hippocampus, thalamus and hypothalamus. The spatiotemporal intensity of the hybridization signal varied from weak but positive (-/+ or +) at 24 h p.i. to moderate (++) at 3-4 days p.i. TLR2 mRNA expression was bilateral and in most cases symmetrical (Table I and data not shown). As the infection progressed, the signal became stronger, reaching peak expression at day 5 p.i. (+ to ++++, Fig. 4*A*). As depicted in Fig. 5*A*, TLR2 gene expression was particularly intense in the corpus callosum, hippocampus, and thalamus, coinciding with findings from histopathological studies also showing that similar structures were the most affected. Such a robust signal in small-scattered cells indicated that microglia/macrophages were probably the type of cells expressing this innate immune receptor (Fig. 6*A*). TLR2 hybridization signal was also present in cells lining the endothelium of the brain capillaries (Fig. 6*A*). Participation of microglia/macrophages was confirmed by dual labeling (TLR2 mRNA in *iba*1-immunoreactive cells). These data provide evidence that systemic *S. suis* infection activates transcription of TLR2 of either resident or blood-derived microglia (Fig. 7*A*). TLR2 signal started to diminish after day 9 p.i., returning to basal levels of control mice at day 14 p.i. (Table I).

It is notable that *S. suis* was able to induce the transcriptional activation of TLR3, which was not expressed constitutively in non-infected mice. Although TLR3 signal was generally weaker in comparison with the expression of other genes studied here, it followed a pattern of expression similar to TLR2 (Table I). In infected animals, a weak positive (-/+) TLR3 hybridization signal was detected at 24 h p.i., mainly in the choroid plexus of the lateral ventricles as well as the thalamus and hypothalamus. Peak TLR3 hybridization signal (+ to ++) in mice with clinical signs of cerebral inflammation was observed between days 5 and 7 p.i. The brains of these animals showed a weak to moderate scattered pattern of TLR3 at the cortex, thalamus, hypothalamus, choroid plexus of lateral ventricles, hippocampus and corpus callosum. In contrast to TLR2, TLR3 expression was not observed in the meninges. From day 9 p.i. the expression of TLR3 decreased to normal levels (Table I). Finally, TLR4 hybridization signal was not detected in any group at any time during the experiment (data not shown). Previous studies showed that the TLR4 probe accurately recognizes its target gene (42) and is therefore not induced in the brain of *S. suis*-infected mice.

Expression of CD14

In contrast to constitutive expression that was nearly undetectable, *S. suis*-infected mice showed spatiotemporal expression of CD14 at levels similar to some degree as other pro-inflammatory transcripts (Table I). A modest positive bilateral signal was observed as early as 3 h p.i. (-/+) and peaked at day 5 p.i. (+ to ++). At this time point, CD14 expression was detected in the corpus callosum, thalamus, hypothalamus and meninges (Fig. 4*B*). In the corpus callosum, CD14 signal was lower compared to that of TLR2 (Fig 5*B*), although it could be detected in the cells of blood vessels (Fig. 5*B* and 6*B*). In contrast to TLR2 and TLR3, expression of this receptor was weaker in the choroid plexus, cortex and hippocampus.

Expression of IκBα

Basal expression (+/-) of IκBα, an indirect marker of NF-κB activity, was found mainly in the choroid plexus. In *S. suis*-infected mice, however, the signal increased in this structure at 3 h p.i. and reached maximal expression levels at day 5 p.i. (+ to +++, Fig. 4C and Table I). IκBα distribution resembled that of TLR2, especially in the corpus callosum, hippocampus and hypothalamus (Fig. 5*C*). The message was also found in the cortex, thalamus and meninges (Fig. 4*C* and Table 1), as well as in blood vessels (Fig. 5*C* and 6*C*). IκBα mRNA was detected in both myeloid cells (data not shown) and astrocytes (Fig. 7*B*). Following peak expression, the signal gradually decreased to basal levels by 14 d p.i. (data not shown).

Expression of pro-inflammatory cytokines and chemokines in the brain

There was no constitutive expression of TNF-α, IL-1β, IL-6 and CCL2 (MCP-1) in brains of control mice. However, the expression levels of these immune mediators were up-regulated to different extents in the brain of *S. suis*-infected mice (Table I). 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, IL-1β signal was clear in the choroid plexus, cortex, corpus callosum, and meninges (++) (Table I). On the other hand, IL-6 gene expression was found in the CNS of only a few mice and it was not considered significant (Table I). It should be noted that we previously demonstrated that this IL-6 probe hybridizes IL-6 in the brain of LPS-treated mice (43). For TNF-α, a weak (+) mRNA expression appeared at 24 h p.i. in the corpus callosum. At day 5 p.i., TNF-α signal increased and was confined to the entire corpus callosum and the choroid plexus, while other brain structures and blood vessels failed to express TNF-α (Table I).

A weak positive (-/+) signal for CCL2 (MCP-1) was observed in the choroid plexus at 3 h p.i. One day following inoculation, the signal intensified (+ to ++) in the cortex,

choroid plexus, hippocampus, thalamus, corpus callosum and meninges (Table I). Similar to other transcripts, CCL2 expression levels peaked by day 5 p.i. (Fig. 4*D* and 5*D*). Given the particularly widespread and strong levels of expression of this chemokine, we also evaluated the participation of microglia and astrocytes in its production. Dual labeling provided anatomical evidence that cells expressing CCL2 transcript were mainly of the microglia/macrophage subset (Fig. 7*C*), although a modest participation of astrocytes was also observed (Fig. 7*D*). Strong expression of CCL2 mRNA was also observed in cells lining the endothelium of the brain capillaries (Fig. 6*D*). CCL2 expression decreased considerably after day 5 p.i. and was similar to control mice at day 14 p.i.

Presence of S. suis in different anatomical sites as detected by immunohistochemistry

S. suis Ags were detected by immunohistochemistry in different structures of the brain in mice showing nervous clinical signs. No antigenic labeling was observed in control mice (Fig. 8*A* and 8*B*). Although *S. suis* Ags were observed in different areas of the brain, their presence was more striking at the corpus callosum and cerebral cortex, where histopathological lesions and expression of inflammatory mediators were observed (Fig. 8*C* and 8*D*). Interestingly, both Nissl and hematoxylin-eosin staining indicated degeneration of the corpus collosum of infected mice (Fig. 2*B* and 8*D*). The presence of *S. suis* Ags was largely associated with large, elongated cells with oval nuclei that had morphological characteristics compatible with macrophages (Fig. 8*E*).

The inflammatory response to S. suis provokes demyelination

PLP, synthesized by oligodendrocytes, is the most abundant protein of CNS myelin (44). As the infection progressed, the inflammatory response to *S. suis* correlated with a decrease in the expression of PLP mRNA in specific areas of the brain, indicating demyelination. At day 5 p.i., this loss in myelin content was observed in the corpus

callosum (Fig 9*D*-*F*), whereas vehicle-treated mice did not exhibit any significant changes in PLP mRNA expression (Fig 9*A*-*C*). To confirm the loss of myelin, brain sections were stained with SBB. While control mice showed no alterations (Fig. 9*G*-*I*), histological examination of infected mice revealed an acute loss of myelin staining (Fig. 9*J*-*K*). As observed with the PLP mRNA probe, this loss of myelin was restricted to the corpus callosum (Fig. 9*L*).

Discussion

To successfully induce meningitis, a pathogen must be able to invade and survive in the intravascular space, cross the BBB, survive and multiply in the cerebrospinal fluid (CSF) and then cause damage to the CNS (45). Different animal models have been used to study infections caused by meningeal bacterial pathogens, specifically the pathogenesis of meningitis, the host immune response induced after infection, and the efficacy of novel drugs and vaccines. Most models use infant animals because adult animals do not reliably develop brain pathology after intranasal, i.v. or i.p. challenge with live microorganisms (45). In addition, models using i.v. or i.p. challenges in adult animals typically result in bacteremia and septicemia, leading sometimes to rapid death of infected animals with modest, if any, brain pathology. Experimental infections leading to meningitis are usually performed by direct inoculation of the pathogen into the CSF or by direct intracerebral instillation (46, 47). However, this model bypasses several steps, such as bacterial dissemination and increased permeability of the BBB, which is the natural portal of entry into the CNS for most meningeal pathogens. In the present study, we used a CD1 mouse model of *S. suis* infection to study the two phases of disease: an early septic shock-like syndrome leading to death and a second late phase that induces evident brain damage. Using this model we consistently reproduced clinical signs of affected nervous system and damage to the CNS in adult animals by means of a hematogenous infection with a Gram positive bacterial pathogen.

Interestingly, CD1 mice appear to be specifically and consistently susceptible to CNS infections by *S*. *suis*, although this pathogen does not induce clinical signs of meningitis in other mouse strains (BALB/c, C57BL/6 and A/J) tested (unpublished observations). The i.p. route may confer certain advantages compared with direct inoculation into the CNS. First, the technique causes minimal trauma and anesthesia is not required. Second, it better represents the natural route of infection since most pathogenic bacteria cause bacteremia before reaching the CNS via mechanisms that are only partially understood.

In the model used in the present study, a virulent *S. suis* strain caused 20% mortality within 48 h p.i. The number of CFU in the blood, liver and spleen was routinely high in all infected mice until day 3 p.i, although the presence of high levels of bacteremia was not always fatal. Variable numbers of bacteria were found in the brain, indicating a pattern of *S. suis* colonization that is unique to this organ. Surprisingly, more than 100 CFU/sample were found in the brain of some mice after 6-9 days p.i. in the absence of clinical signs; however, animals were sacrificed at specified time points in our kinetics study and therefore it is not known whether these mice would have developed nervous disease at a later time.

The use of a multiplex system in this study allowed us to quantitatively measure the kinetics of several cytokines simultaneously in a small volume of plasma. High levels of TNF-α, IL-6, IL-12, IFN-γ, CCL2 (MCP-1), CXCL1 (KC) and CCL5 (RANTES) were observed in vivo within 24 h p.i. and might be responsible in part for the sudden death of 20% of animals. In fact, the presence of high number of bacteria and the lack of specific lesions in mice that died during the first 24-48 h suggest that the exacerbated inflammatory response might be the cause of death. It is not clear why some animals succumbed while others survived this acute phase. A general analysis of cytokine patterns suggests that infection with *S. suis* serotype 2 induces a Th1-type immune response. TNF-α, the first cytokine observed in the systemic inflammatory cascade following *S. suis* infection, has been previously implicated in the pathogenesis of Gram-positive septic shock-like syndrome (36, 48). It is important to note the remarkably high

levels of IL-6 production in *S. suis*-infected mice. Indeed, high levels of both TNF-α and IL-6 correlate inversely with survival time in patients with sepsis (49). Previous studies of mice infected with *S. pneumoniae,* Group A (GAS) or Group B streptococci (GBS) have reported elevated IL-6 in plasma, albeit at lower levels than those found here (48, 50). Likewise, IFN-ɣ contributes to the immune control of invading pathogens but also may cause pathology leading to death when its production is excessive or uncontrolled (50). It has been shown that high levels of IL-12 produced early during GAS infection, as those observed in this study with *S. suis*, may influence NK cells and others to produce IFN-ɣ, suggesting a positive feedback regulation in the pro-inflammatory cytokine cascade (50, 51). Levels of IL-1β were not as high as those observed for other type 1 cytokines; however, this finding does not necessary preclude a causative role for IL-1 in *S. suis* disease, as already suggested for other pathogens (52). As for the chemokines, high levels of CXCL1 (KC) and CCL2 (MCP-1) were detected during the first 36 h p.i. in the sepsis phase of infection, and their presence in plasma lasted longer than most of the pro-inflammatory cytokines tested. Although there are very few studies on the activity of CCL5 (RANTES) in sepsis (39), it has been shown that its levels in plasma during meningococcal disease show a different pattern than those of other chemokines (53). However, in our study, CCL5 followed a pattern similar to other chemokines. It has been shown that different meningitis-causing bacteria induce distinct inflammatory responses, including either high or low levels of CCL5 folowing interaction with cells of the human meninges (54). Finally, the relatively delayed IL-10 up-regulation might indicate a negative feedback mechanism to control the extent of the inflammatory response, as described in experimental pneumococcal meningitis (55). Given that numbers of viable bacteria remained constant during the first 3 d p.i., IL-10 production did not appear to influence bacterial clearance from the blood or various organs and instead likely contributed to mouse survival by preventing immunopathology associated with excessive pro-inflammatory cytokines.

We showed that most *S. suis*-infected mice that survive septicemia later developed CNS clinical signs such as locomotion problems, episthotonus, opisthotonus,

bending of the head laterally and walking in circles, which could be considered characteristic of brain inflammation. *S. suis* infection clearly induced inflammation and suppurative and necrotizing lesions in specific anatomical sites of the brain parenchyma, such as the meninges, cortex, hippocampus, thalamus, hypothalamus, and corpus callosum. In addition, a strong activation of TLR2 in the same regions of the brain parenchyma was observed several days after the presence of *S. suis* bacteremia. Previous studies showed that mice systemically inoculated with peptidoglycan (PGN), lipoteichoic acid (LTA) or a combination of both fail to modulate TLR2 expression in the CNS (27). However, intracisternal injection of live or killed *S. pneumoniae*, PGN and LTA triggers an immune reaction via TLR2 (56). These conflicting results may reflect different routes of inoculation. We hypothesize that local multiplication of *S. suis*, which might expose or release high amounts of PGN, LTA and other surface components, might be responsible for TLR2 up-regulation. The role of this receptor in protective immunity and immunopathology in Gram positive meningitis is controversial (57). Indeed, it has been demonstrated that TLR2 plays a dual role in GBS infection depending on the bacterial dose, that is, it has a protective role at low doses but exerts detrimental effects, such as causing septic shock-like syndrome, at high doses (48).

In our model, results show that TLR2 activation coincided with up-regulated expression of CD14 receptor and activation of IκBα (index of NF-κB activation), IL-1β, TNF-α and MCP-1 in similar anatomical regions among mice with CNS symptoms. However, it is not possible to ascertain that the increased mRNA expression of inflammatory mediators was directly related to TLR2 signaling pathway. In fact, there was also a moderate induction of TLR3 mRNA expression which followed a pattern similar to TLR2. Thus, we cannot rule out the possibility that *S. suis* can activate other TLRs besides TLR2 and possibly other components of the innate immune system, as previously reported by our in vitro studies (18, 21). In agreement with these findings, mice with *S. pneumoniae*-induced meningitis have increased TLR2, TLR4 and TLR9 mRNA expression while hippocampal cultures exposed to the same bacteria also trigger up-regulated TLR2 and TLR3 mRNA expression. In addition, TLR2-deficient mice

infected intracraneally with *S. pneumoniae* express different pro-inflammatory cytokines in the CNS, indicating TLR2-independent recognition pathways (56, 58, 59). Finally, we confirmed previous data that support no involvement of TLR4 in this response (21). The pneumolysin of *S. pneumoniae* was reported to activate TLR4 (60). Although *S. suis* produces a toxin, named suilysin, with high homology to pneumolysin (6), it appears that these two toxins do not engage the same activation pathways. It should be noted that no correlation could be established between positive in situ hybridization signals and bacterial loads in the brain. In fact, the technique requires perfusion of mice with paraformaldehyde and such a treatment does not allow further sampling for other types of studies, such as bacteriology. However, results from immunohistochemistry showed the presence of high loads of bacterial Ags in association with cells which morphologically resembled microglia residing only in the affected zones, indicating a high concentration of bacteria in these regions.

Transcriptional activation of cytokine mRNA in the CNS at a time coincident with the presence of clinical signs may indicate an important role of inflammation in the development of *S. suis*-related meningitis. Based largely on in vitro data, it has been suggested that CSF cytokines are secreted predominantly by microglia or astrocytes within the brain parenchyma (61). In this study, we showed that, in general, microglia/macrophage (or infiltrating monocyte) cells and, to a lesser extent, astrocytes were activated and these cell types were probably the cellular sources of cytokine induction in the brain parenchyma. This hypothesis is supported by the strong transcriptional activation of NF-κB and MCP-1 mRNA in these cells. Infiltrating monocytes would contribute not only to in situ inflammation but also to bacterial infiltration into the CNS given that monocytes carry externally associated bacteria, as suggested by the “modified Trojan Horse” theory for *S. suis* invasion of the CNS (6). Finally, endothelial and ependymal cells might also play a supporting role in inducing inflammation in the CNS (61).

S. suis does not appear to induce the expression of pro-inflammatory mediators in leaky areas of the brain (regions excluding the BBB) and does not affect the so-called

circumventricular organs, as previously reported for LPS (22, 27, 62, 63). Indeed, microscopic analysis of brain sections showed that cells expressing transcripts for TLR2, CD14, IκBα and CCL2 (MCP-1) were associated with microvascular vessels early after infection, suggesting that endothelial cells play an important role in the pathogenesis of *S. suis*-related meningitis. Furthermore, previous in vitro reports from our laboratory show that the BBB may be the portal of entry for the bacteria into the brain (9, 25) and that BMEC might also contribute to the initiation of inflammatory process by producing, among others, IL-6, CXCL8 (IL-8) and CCL2 after contact with *S. suis*. (15). Interestingly, these in vitro studies showed an absence of TNF-α and IL-1β activation in *S. suis*-infected BMEC (15), a finding that is consistent with the lack of a positive transcription signal for these cytokines in microvascular vessels as demonstrated here and in other studies (64). In this study, shortly after infection, TLR2 and CD14 mRNA up-regulation was observed in cells lining the brain microvasculature. Although data are limited, TLR2 expression in brain endothelial cells has been reported (64) while endothelial cell expression of CD14 is controversial (65-67). On the other hand, both TLR2 and CD14 may be induced in perivascular microglia and monocytes, which participate in many inflammatory events in the CNS (68, 69). Finally, it has also been suggested that *S. suis*, which can affect the viability of porcine choroid plexus epithelial cells, may also invade the CNS through the choroid plexus (10). In agreement with this hypothesis, the present study showed robust and rapid expression of TLR2, TLR3, CD14, IκBα, IL-1β, TNF-α, and CCL2 (MCP-1) in the choroid plexus early after *S. suis* infection.

In conclusion, results obtained in the present study provide important insights into the mechanisms underlying the innate immune response against the infection caused by *S. suis* at both systemic and CNS-specific levels. This is the first report of an hematogenous infection model that consistently reproduces clinical signs of an affected nervous system and damage to the CNS in adult animals with a bacterial pathogen. The adult model of CNS infection developed in this study may be useful to further study mechanisms involved in bacterial invasion of the CNS and brain inflammation caused by Gram positive bacteria.

Acknowledgments:

We are grateful to Natalie Laflamme (Centre Hospitalier de l'Université Laval, St-Foy, QC, Canada) and Claude Lachance (McGill University Health Centre Research Institute, Montréal, QC, Canada) for technical support.

Footnotes:

[1]This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant # 0680154280, CIHR (SR) and by the Centre de recherche en infectiologie porcine (CRIP-FQRNT).

[2]Address correspondence and reprint request to Dr. M. Gottschalk, GREMIP, Faculté de médicine vétérinaire, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, Québec, J2S 2M2, Canada. Tel.:1-450-773-8521, ext. 1-8374. Fax: 1-450-778-8108.

[3]Abbreviations used in this paper: CPS, capsule polysaccharide; BBB, blood brain barrier; BMEC, brain microvascular endothelial cells; THB, Todd-Hewitt broth; d, days; p.i., post infection; PFA; paraformaldehyde; DDT, dithiothreitol; DEPC, diethylpyrocarbonate; PLP, proteolipid protein; TEA, triethanolamine; SCC; standard saline citrate; *iba*1, ionized calcium binding adapter molecule 1; GFAP, glial fibrillary acidic protein; KPBS, Potassium-PBS; ABC, avidin-biotin-peroxidase complex; DAB, 3,3'-diaminobenzidine; SBB, Sudan black B staining; CSF, cerebro-spinal fluid; GAS, Group A Streptococci; GBS, Group B Streptococci; PGN, peptidoglycan; LTA, lipoteichoic acid.

Table I. ***mRNA expression of different receptors and pro-inflammatory mediators in the brain of mice intraperitoneally infected with S. suis***[a]

Expression at 3 h p.i.

Structure	TLR2	TLR3	CD14	IκBα	IL-1β	IL-6	TNF-α	CCL2
Meninges	-	-	-	-	-	-	-	-
Choroid plexus	+/-	-	+/-	+	-	+/-	-	+/-
Blood vessels	-	-	-	-	-	-	-	-
Cortex	-	-	-	-	-	-	-	-
Hippocampus	-	-	-	-	-	-	-	-
Corpus callosum	-	-	-	-	-	-	-	-
Thalamus	-	-	-	-	-	-	-	-
Hypothalamus	-	-	-	-	-	-	-	-

Expression at 24 h p.i.

Structure	TLR2	TLR3	CD14	IκBα	IL-1β	IL-6	TNF-α	CCL2
Meninges	+	-	+/-	+	-	-	-	+
Choroid plexus	+	+/-	+	+	+	+/-	-	+
Blood vessels	+/-	-	++	++	-	+/-	-	+
Cortex	+	-	+	+	+	+/-	-	++
Hippocampus	+	-	+/-	+	-	-	-	+
Corpus callosum	+/-	-	++	+	-	-	+	++
Thalamus	+	+/-	+	+	-	-	-	+
Hypothalamus	+	+/-	+	+	-	-	-	+/-

Expression at 5 d p.i.

Structure	TLR2	TLR3	CD14	IκBα	IL-1β	IL-6	TNF-α	CCL2
Meninges	+	-	++	++	++	v	-	+
Choroid plexus	++	++	+	++	++	v	++	++
Blood vessels	++	-	++	+++	-	v	-	++
Cortex	++	++	+	+	++	v	-	+++
Hippocampus	+++	++	+/-	+++	+	v	-	+
Corpus callosum	++++	++	++	+++	++	v	++	++++
Thalamus	+++	+	++	++	+/-	v	-	++
Hypothalamus	+	+	++	+++	+/-	v	-	+/-

Expression at 9 d p.i.

Structure	TLR2	TLR3	CD14	IκBα	IL-1β	IL-6	TNF-α	CCL2
Meninges	+/-	-	+/-	+/-	+/-	+/-	-	-
Choroid plexus	+/-	+/-	+/-	+	+/-	+/-	+/-	+/-
Blood vessels	+/-	-	+/-	+	-	+/-	-	+/-
Cortex	+/-	-	+/-	+/-	+/-	+/-	-	+/-
Hippocampus	+	-	+/-	+/-	+/-	+/-	-	+/-
Corpus callosum	+	-	+/-	+	+/-	+/-	+/-	+
Thalamus	+	-	+/-	+/-	+/-	+/-	-	+/-
Hypothalamus	+/-	-	+/-	+/-	+/-	+/-	-	+/-

[a] ++++, very strong signal; +++, strong signal, ++, moderate signal; +, low but positive signal; +/-, low to undetectable; -, undetectable; for an average of 3 mice per group at each post-infection time (p.i.). v: variable results in different mice. CCL2: MCP-1

ARTICLE I. FIGURES.

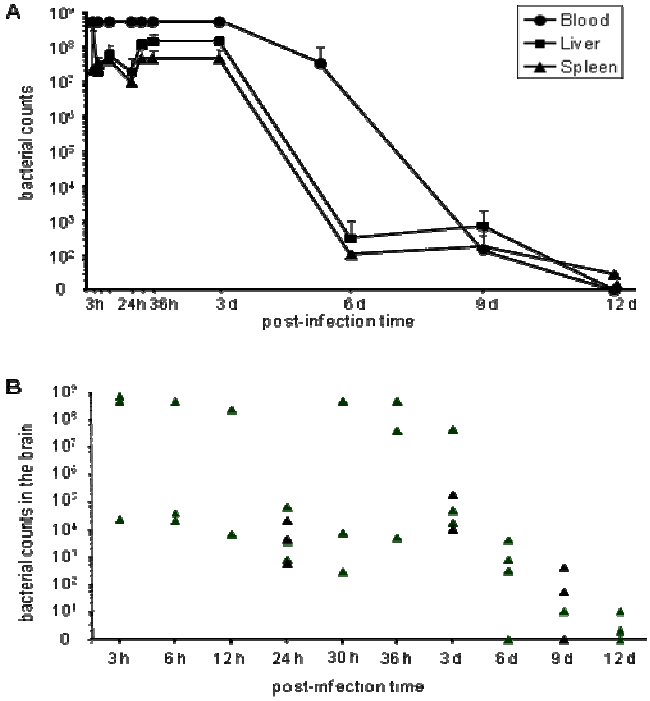


Fig.1 1. Bacterial distribution in different organs from mice infected i.p. with *S. suis*. (*A*) Bacterial loads in the liver and spleen are expressed as CFU/0.05 g of tissue and in the blood as CFU/ml. Results are expressed as mean ± SEM of at least 3 infected mice per time point. (*B*) Bacterial counts in the brain. Results are expressed as CFU/0.05 g of tissue. Each ▲ represents one mouse.

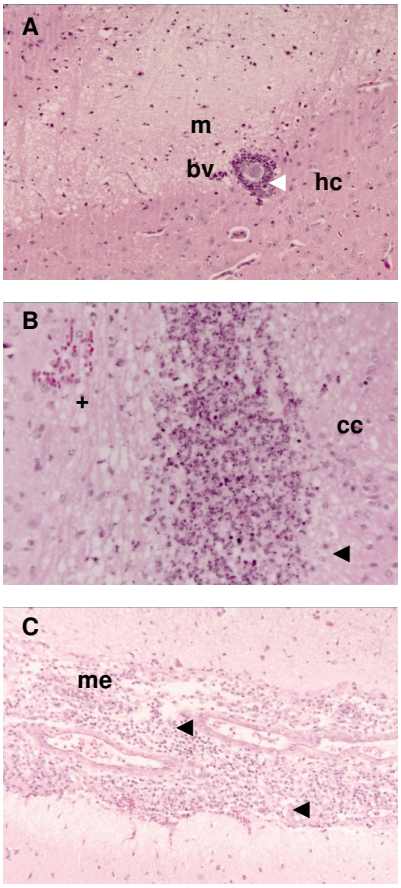


Fig. 2. Histopathological findings in the brains of mice with clinical signs of meningitis. (A) Micrograph of a brain from an infected mouse at day 6 p.i. showing a blood vessel (bv) surrounded by a rim of neutrophils (n) with bacteria partially occluding the lumen (white arrow head). Areas of malacia (m) are observed nearby. Hippocampus (hc). **(B)** Micrograph of a brain from an infected mouse at day 9 p.i. showing complete destruction of the corpus callosum (cc). The normal tissue was replaced by numerous infiltrating neutrophils (black arrow head). Some hemorrhagic foci are also observed (+). **(C)** Micrograph of a brain from a mouse at day 9 p.i. showing the meninges (me) severely thickened and diffusely infiltrated by numerous macrophages and neutrophils (black arrow heads). Hematoxilin-eosin staining, 400X magnification. Bacterial loads in these mice ranged from 10^3 to 10^4 CFU/0.05 g of brain tissue.

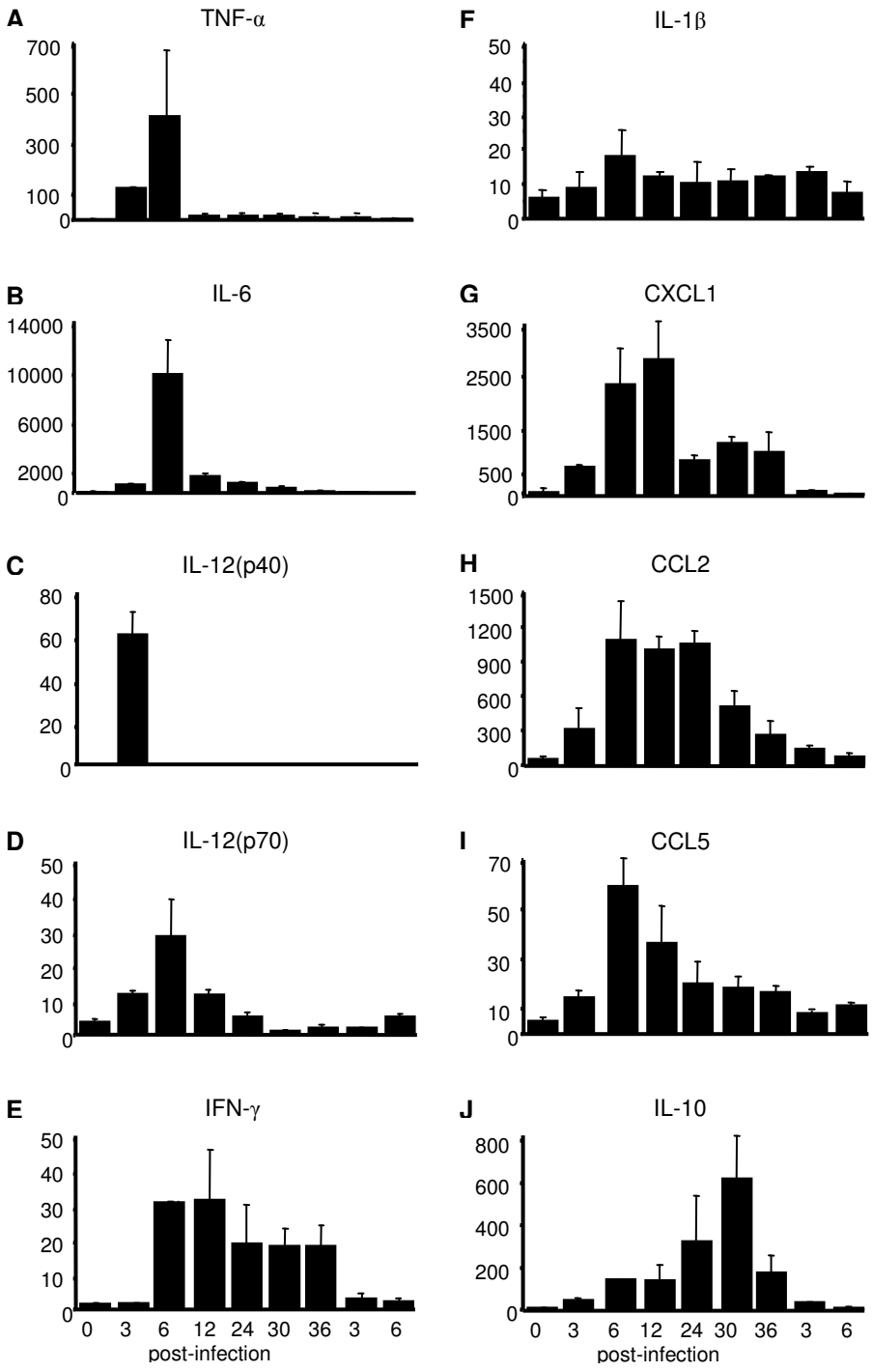


Fig. 3. *S. suis* infection induces production of inflammatory mediators in a time-dependent manner. (A) TNF-α, (B) IL-6, (C) IL-12p40, (D) IL-12p70, (E) IFN-γ, (F) IL-1β, (G) CXCL1/KC, (H) CCL2/MCP-1, (I) CCL5/RANTES and (J) IL-10. Cytokine concentrations were assayed by a liquid multi-array system (Luminex), as described in the Material and Methods. Data are expressed as mean ± SEM pg/ml and are representative of two independent experiments. Time 0 h

represents the mean (SEM) results from negative mice. Median bacterial loads in the blood of mice used for cytokine measurements are presented in Figure 1*A*.

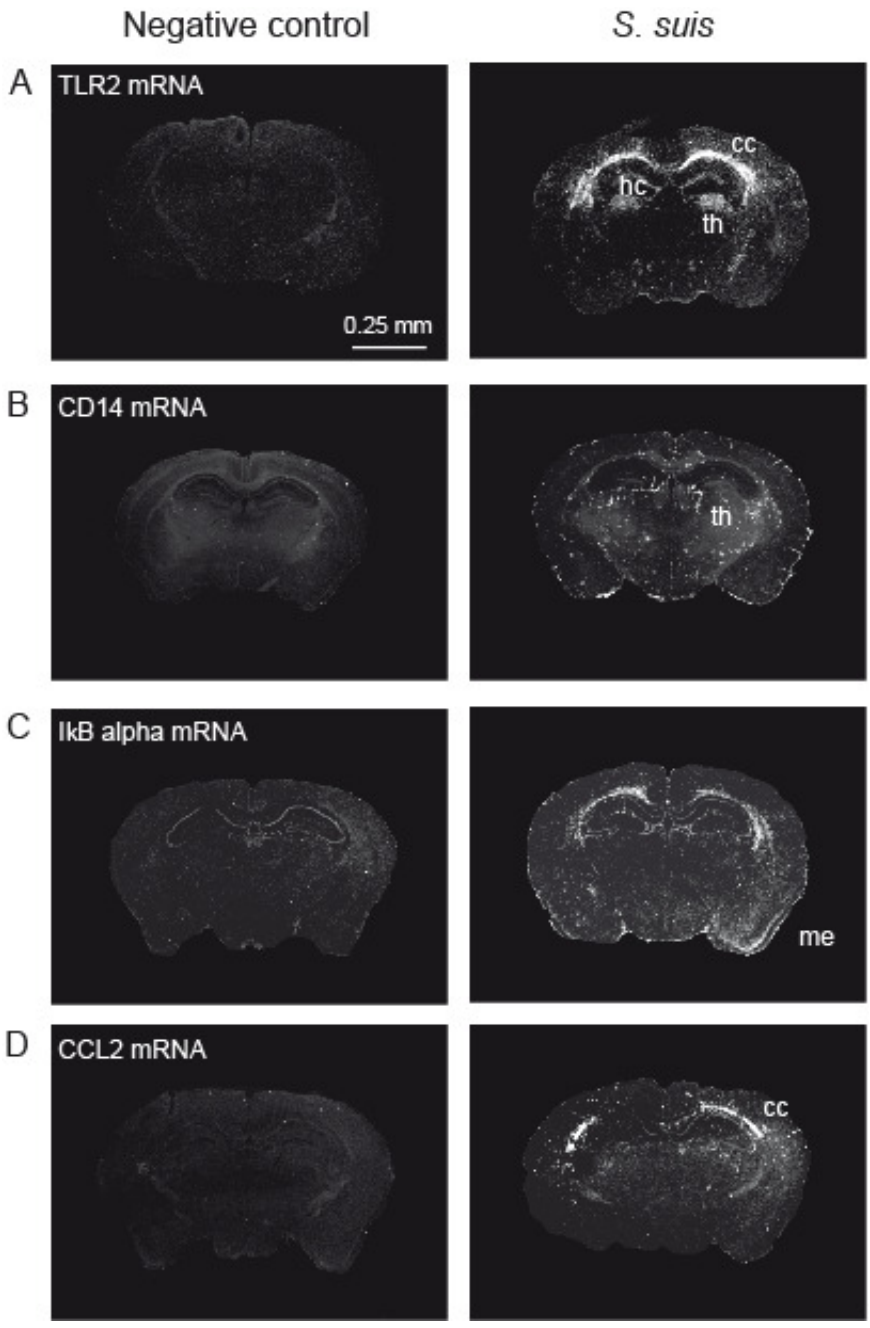


Fig. 4. Coronal sections showing the *in situ* hybridization results from *S. suis*-infected mice and non-infected (negative) controls. The dark-field microphotographs of nuclear emulsion-dipped coronal sections show the spatial expression of TLR2 (*A*), CD14 (*B*), IκBα (*C*), and CCL2/MCP-1 (*D*) mRNA at day 5 p.i. The positive hybridization signal appears as an

agglomeration of silver grains in specific structures of the brain, such as the hippocampus (hc), corpus callosum (cc), thalamus (th), and meninges (me).

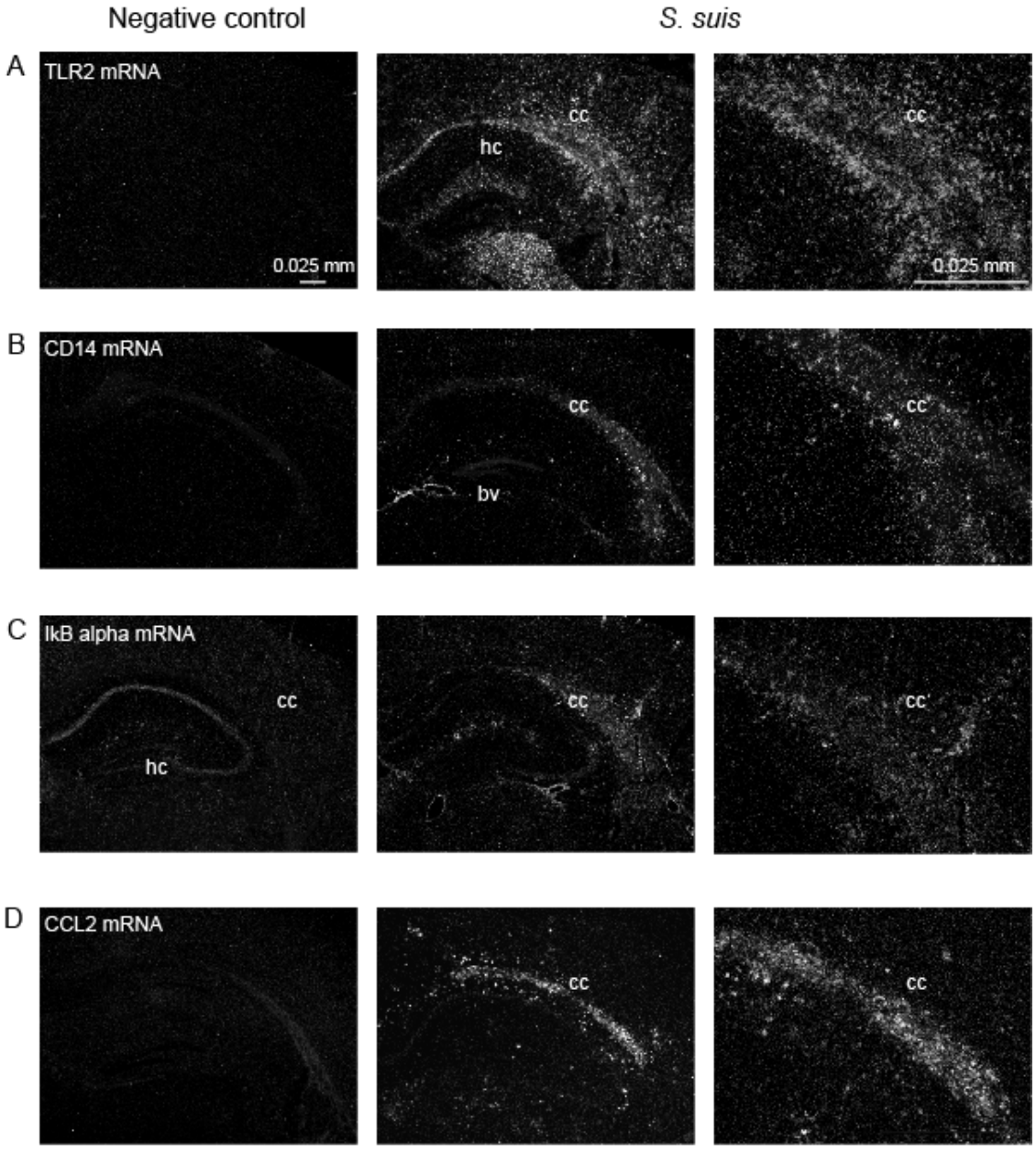


Fig. 5. *S. suis* is able to induce strong transcriptional activation of genes encoding TLR2 (*A*), CD14 (*B*), IκBα (*C*), and CCL2/MCP-1 (*D*) in various structures of the brain. These dark-field microphotographs are magnifications of coronal sections depicted by Figure 4 and are representative examples of the expression patterns of the different transcripts in the hippocampus (hc), corpus callosum (cc) and blood vessels (bv) at day 5 p.i.

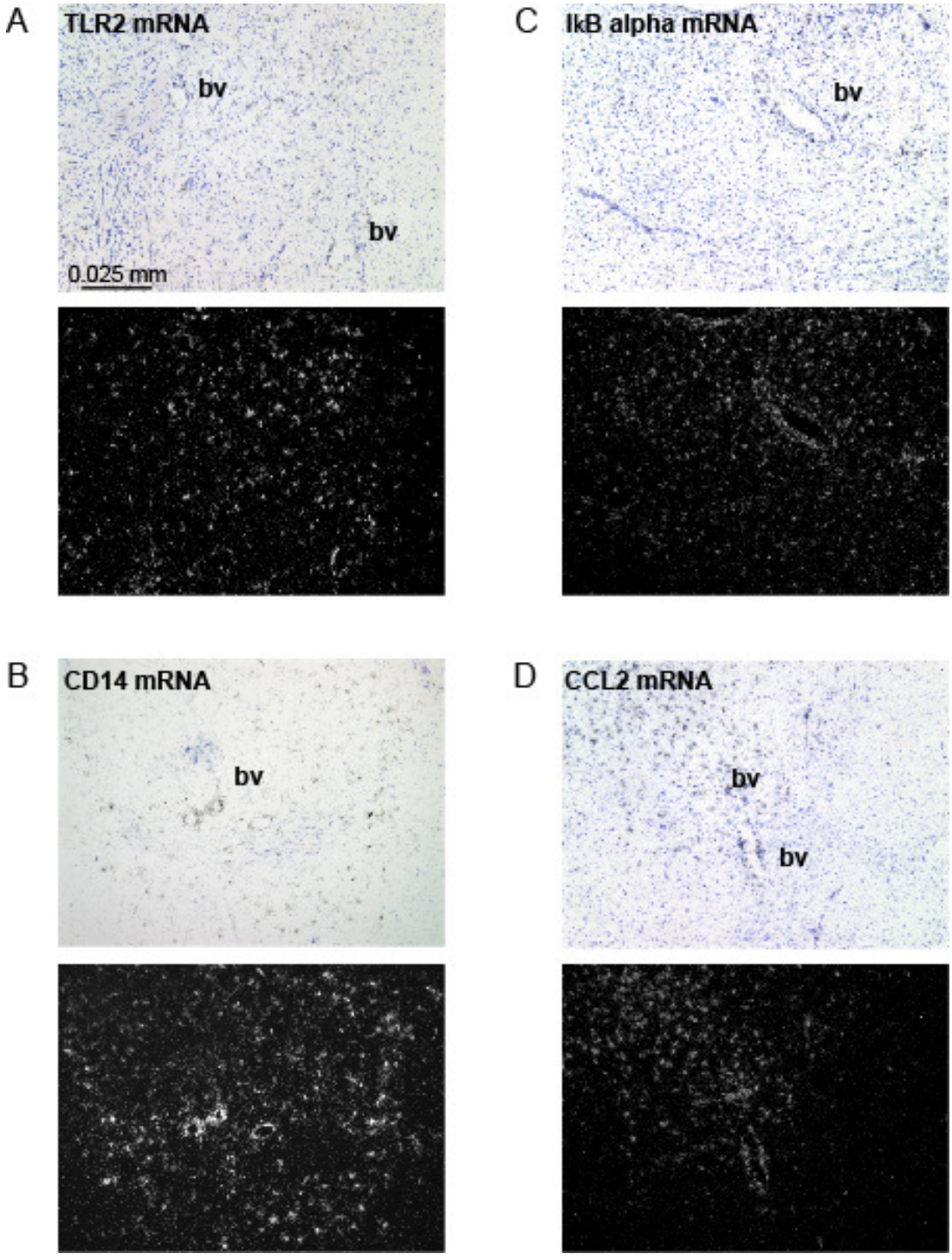


Fig. 6. *S. suis* infection induces transcriptional activation of genes encoding pro-inflammatory molecules in the brain and blood vessels (bv) of mice. The dark-field photomicrographs of nuclear emulsion-dipped coronal sections from infected mice at day 5 p.i. show the presence of positive hybridization signal in parenchymal cells and also in cells lining the endothelium of the brain capillaries, as clearly indicated by corresponding bright-field photomicrographs.

Hybridization signals are depicted for TLR2 (*A*), CD14 (*B*), IκBα (*C*), and CCL2/MCP-1 (*D*) transcripts.

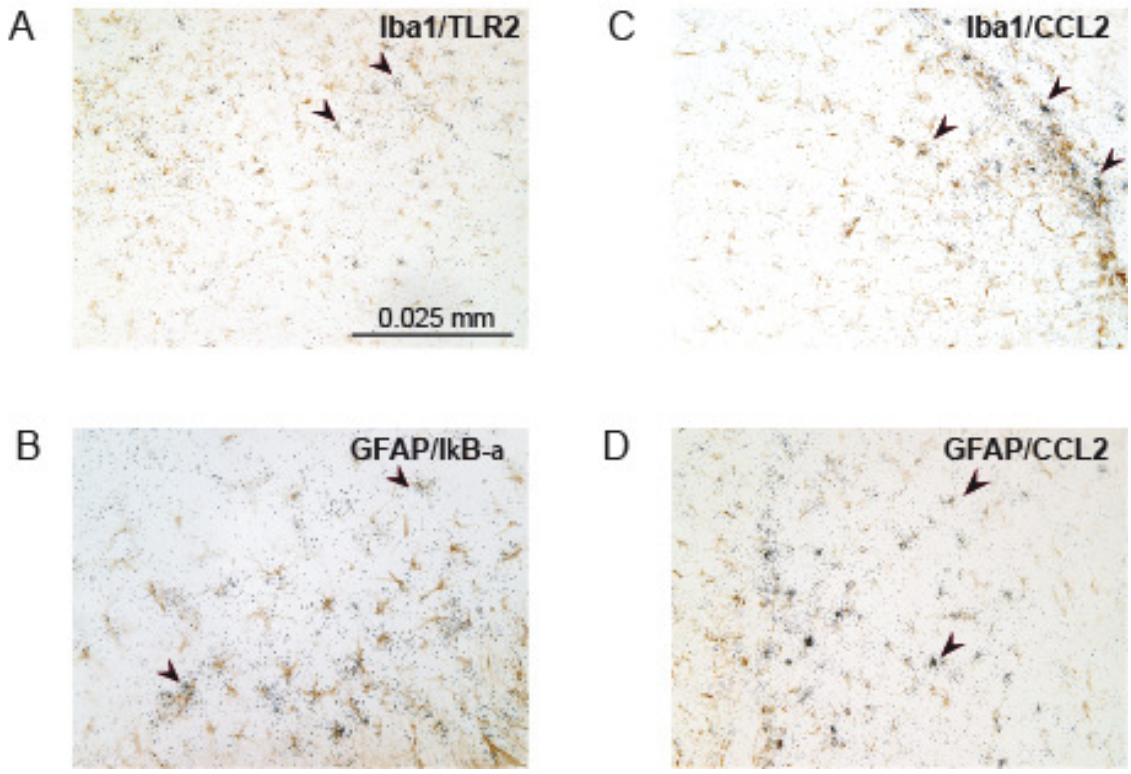


Fig. 7. Combined immunohistochemistry and *in situ* hybridization labeling suggests a key role of microglia/macrophages in the inflammatory response provoked by i.p. *S. suis* infection in mice. Microglial cells were labeled with an antibody against ionized binding adapter molecule 1 (*iba1*) and glial fibrillary acidic protein (GFAP) antisera was used to stain astrocytes (brown cell bodies and ramifications). In situ hybridization was thereafter performed for TLR2, IκBα or CCL2/MCP-1 mRNA (silver grains). All microphotographs are representative of infected mice at day 5 p.i. The presence of mRNA encoding either TLR2 (*A*) or CCL2/MCP-1 (*C*) within parenchymal microglia appears as an agglomeration of silver grains inside the cell cytoplasm (black arrow heads). Few positive astrocytes for IκB (*B*) and CCL2/MCP-1 (*D*) transcripts were also detected.

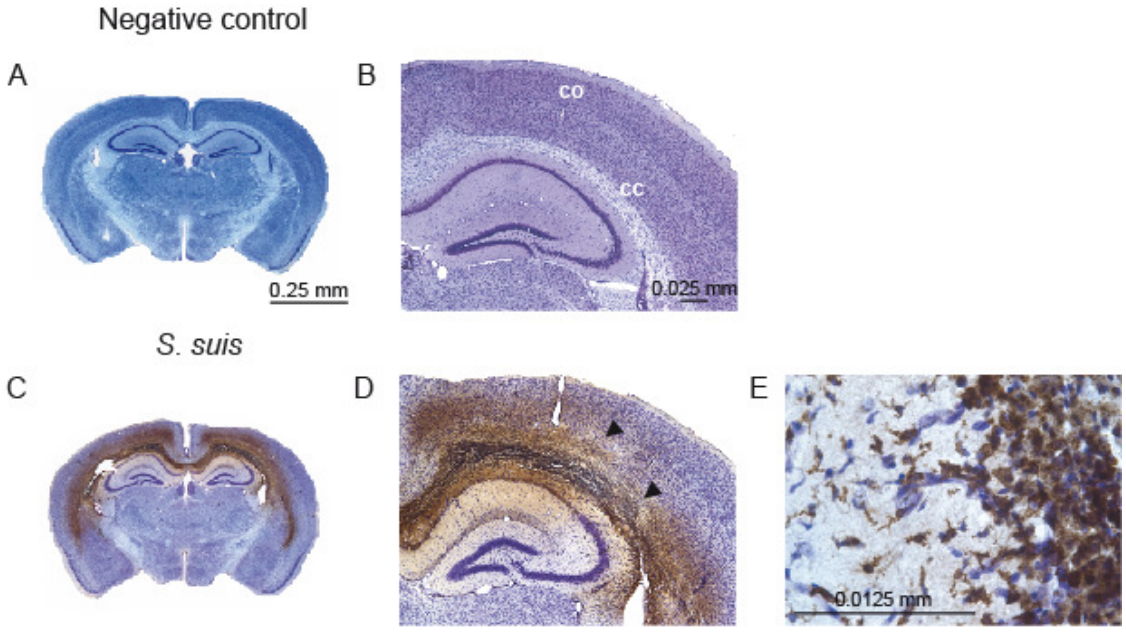
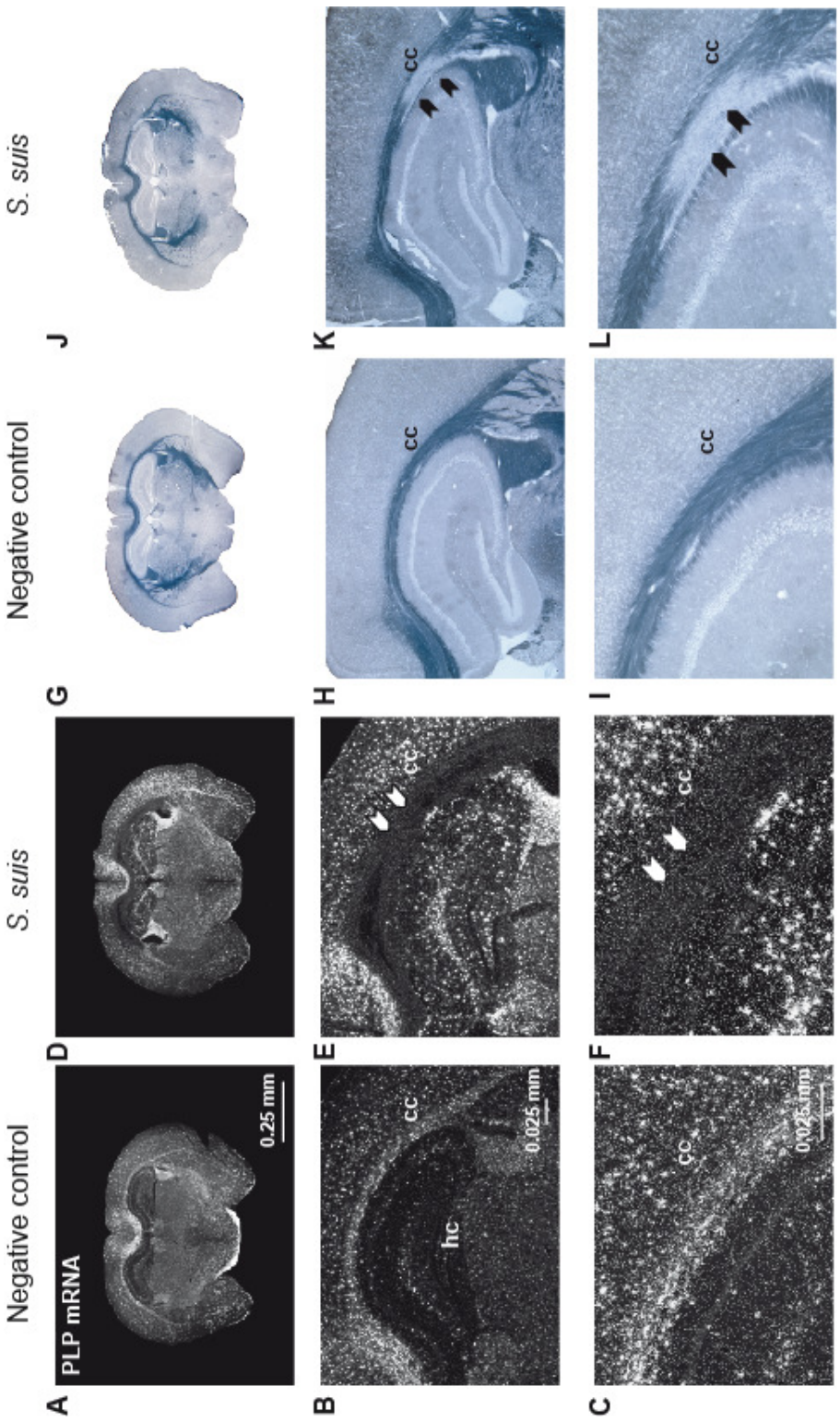


Fig. 8. *S. suis* Ags were labeled by immunoperoxidase using a polyclonal antiserum. (A and B) Nissl-stained coronal section from a control mouse showing the absence of bacterial Ags or tissue degeneration. **(C)** Representative section of an infected mouse at day 5 p.i. showing the bilateral localization of *S. suis* Ags in the brain. **(D)** Localization of *S. suis* Ags occurred in specific areas of the brain parenchyma, mainly the cortex (co) and corpus callosum (cc). **(E)** *S. suis* Ags were largely found in associated with cells with morphological resemblance to macrophages/microglia. Nissl staining revealed areas of corpus collosum degeneration **(C and D)** as indicated by black arrow heads.

Fig.9. PLP transcript expression was diminished in the brain of *S. suis*-infected mice. (A-C) Brain sections from control mice showing normal levels of PLP expression. **(D-F)** Brain sections of infected mice at day 5 p.i. showing the reduced expression of PLP mRNA, mainly in the corpus callosum (cc). In **(E)** and **(F),** the regions with a decreased level of PLP transcript in the corpus callosum are indicated by white arrow heads. Please note that PLP mRNA expression levels remain similar to control mice in other areas of the brain**. (G-I)** SBB-stained brain sections from control mice showing absence of histological changes. The presence of myelin in the (cc) is apparent. **(J-L)** SBB-stained brain sections of infected mice at day 5 p.i. showing a clear and localized loss of myelin at the level of the corpus callosum (cc). In **(K)** and **(L)** the localized demyelination in the (cc) is indicated by black arrow heads.


Negative control
S. suis
Negative control
S. suis
A
B
C
D
E
F
G
H
I
J
K
L
PLP mRNA
0.25 mm
0.025 mm
0.025 mm
cc
cc
cc
hc
cc
cc
cc

SUPPLEMENTAL DATA LEGENDS

Video 1.

A CD1 mouse presenting clinical signs of CNS inflammation (episthotonus and opisthotonus) which suddenly appeared at day 6 after i.p. infection with a virulent strain of *S. suis* serotype 2. Animals were immediately and humanely sacrificed after the video was recorded as approved and recommended by The Animal Welfare Committee of the Université de Montréal, Canada.

Video 2.

A CD1 mouse presenting clinical signs of CNS inflammation (locomotion problems, bending of the head laterally and walking in circles) which suddenly appeared at day 5 after i.p. infection with a virulent strain of *S. suis* serotype 2. Animals were immediately and humanely sacrificed after the video was recorded as approved and recommended by The Animal Welfare Committee of the Université de Montréal, Canada.

Video 3.

A CD1 mouse presenting serious clinical signs of CNS inflammation (locomotion problems, 360° rotation of the body) at day 9 after i.p. infection with a virulent strain of *S. suis* serotype 2. Animals were immediately and humanely sacrificed after the video was recorded as approved and recommended by The Animal Welfare Committee of the Université de Montréal, Canada.

References

1. Higgins, R., and M. Gottschalk. 2005. Diseases of Swine. In *Streptococcal diseases* 9th ed. B. Straw, J. J. Zimmerman, S. D'Allaire, and D. J. Taylor, eds. Iowa State University Press, Ames, IA. 769-783.

2. Dupas, D., M. Vignon, and C. Geraut. 1992. *Streptococcus suis* meningitis. A severe noncompensated occupational disease. *J. Occup. Med.* 34:1102-1105.

3. Madsen, L. W., B. Svensmark, K. Elvestad, B. Aalbaek, and H. E. Jensen. 2002. *Streptococcus suis* serotype 2 infection in pigs: new diagnostic and pathogenetic aspects. *J. Comp. Pathol.* 126:57-65.

4. Yen, M. Y., Y. C. Liu, J. H. Wang, Y. S. Chen, Y. H. Wang, and D. L. Cheng. 1994. *Streptococcus suis* meningitis complicated with permanent perceptive deafness: report of a case. *J. Formos. Med. Assoc.* 93:349-351.

5. Ye, C., X. Zhu, H. Jing, H. Du, M. Segura, H. Zheng, B. Kan, L. Wang, X. Bai, Y. Zhou, Z. Cui, S. Zhang, D. Jin, N. Sun, X. Luo, J. Zhang, Z. Gong, X. Wang, L. Wang, H. Sun, Z. Li, Q. Sun, H. Liu, B. Dong, C. Ke, H. Yuan, H. Wang, K. Tian, Y. Wang, M. Gottschalk, and J. Xu. 2006. *Streptococcus suis* sequence type 7 outbreak, Sichuan, China. *Emerg. Infect. Dis.* 12:1203-1208.

6. Segura, M., and M. Gottschalk. 2004. Extracellular virulence factors of streptococci associated with animal diseases. *Front. Biosci.* 9:1157-1188.

7. Baums, C. G., U. Kaim, M. Fulde, G. Ramachandran, R. Goethe, and P. Valentin-Weigand. 2006. Identification of a novel virulence determinant with serum opacification activity in *Streptococcus suis*. *Infect. Immun.* 74:6154-6162.

8. Madsen, L. W., H. Bak, B. Nielsen, H. E. Jensen, B. Aalbaek, and J. H. Riising. 2002. Bacterial colonization and invasion in pigs experimentally exposed to *Streptococcus suis* serotype 2 in aerosol. *J. Vet. Med. B* 49:211-215.

9. Gottschalk, M., and M. Segura. 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. *Vet. Microbiol.* 76:259-272.

10. Tenenbaum, T., R. Adam, I. Eggelnpohler, D. Malaton, A. Seibt, G. E. Novotny, H. J. Galla, and H. Schroten. 2005. Strain-dependent disruption of blood-cerebrospinal fluid barrier by *Streptoccocus suis* in vitro. *FEMS Immunol. Med. Microbiol.* 44:25-34.

11. Rubin, L. L., and J. M. Staddon. 1999. The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.* 22:11-28.

12. Huang, S. H., M. F. Stins, and K. S. Kim. 2000. Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. *Microbes Infect.* 2:1237-1244.

13. Koedel, U., W. M. Scheld, and H. W. Pfister. 2002. Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect. Dis.* 2:721-736.

14. Jobin, M. C., J. Fortin, P. J. Willson, M. Gottschalk, and D. Grenier. 2005. Acquisition of plasmin activity and induction of arachidonic acid release by *Streptococcus suis* in contact with human brain microvascular endothelial cells. *FEMS Microbiol. Lett.* 252:105-111.

15. Vadeboncoeur, N., M. Segura, M. Doré, and M. Gottschalk. 2003. Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. *FEMS Immunol. Med. Microbiol.* 35:49-58.

16. Jobin, M. C., M. Gottschalk, and D. Grenier. 2006. Upregulation of prostaglandin E2 and matrix metalloproteinase 9 production by human macrophage-like cells: synergistic effect of capsular material and cell wall from *Streptococcus suis*. *Microb. Pathog.* 40:29-34.

17. Segura, M., J. Stankova, and M. Gottschalk. 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. *Infect. Immun.* 67: 4646-4654.

18. Segura, M., N. Vadeboncoeur, and M. Gottschalk. 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. *Clin. Exp. Immunol.* 127:243-254.

19. Segura, M., G. Vanier, D. Al-Numani, S. Lacouture, M. Olivier, and M. Gottschalk. 2006. Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system. *FEMS Immunol. Med. Microbiol.* 47: 92-106.

20. Al-Numani, D., M. Segura, M. Doré, and M. Gottschalk. 2003. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by *Streptococcus suis* serotype 2. *Clin. Exp. Immunol.* 133:67-77.

21. Graveline, R., M. Segura, D. Radzioch, and M. Gottschalk. 2007. TLR2-dependent recognition of *Streptococcus suis* is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness *Int. Immunol.* 19:375-389.

22. Nguyen, M. D., J. P. Julien, and S. Rivest. 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat. Rev. Neurosci.* 3:216-227.

23. Olson, J. K., and S. D. Miller. 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J. Immunol.* 173:3916-3924.

24. Jack, C. S., N. Arbour, J. Manusow, V. Montgrain, M. Blai, E. McCrea, A. Shapiro, and J. P. Antel. 2005. TLR signaling tailors innate immune responses in human microglia and astrocytes. *J. Immunol.* 175:4320-4330.

25. Vanier, G., M. Segura, P. Friedl, S. Lacouture, and M. Gottschalk. 2004. Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. *Infect. Immun.* 72:1441-1449.

26. Higgins, R., and M. Gottschalk. 1990. An update on *Streptococcus suis* identification. *J. Vet. Diagn. Invest.* 2:249-252.

27. Laflamme, N., G. Soucy, and S. Rivest. 2001. Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding Toll-like receptor 2 in the CNS. *J. Neurochem.* 79: 648-657.

28. Laflamme, N., H. Echchannaoui, R. Landmann, and S. Rivest. 2003. Cooperation between toll-like receptor 2 and 4 in the brain of mice challenged with cell wall components derived from gram-negative and gram-positive bacteria. *Eur. J. Immunol.* 33:1127-1138.

29. Glezer, I., A. Lapointe, and S. Rivest. 2006. Innate immunity triggers oligodendrocyte progenitor reactivity and confines damages to brain injuries. *FASEB J.* 20:750-752.

30. Paxinos, G., and K. B. J. Franklin. 2001. *The mouse brain in stereotaxic coordinates*. Academic Press, San Diego, CA.

31. Vallieres, L., and S. Rivest. 1999. Interleukin-6 is a needed proinflammatory cytokine in the prolonged neural activity and transcriptional activation of corticotropin-releasing factor during endotoxemia. *Endocrinology* 140:3890-3903.

32. Babcock, A. A., W. A. Kuziel, S. Rivest, and T. Owens. 2003. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. *J. Neurosci.* 23:7922-7930.

33. Song, M., and J. A. Kellum. 2005. Interleukin-6. *Crit. Care Med.* 33(Suppl.):S463-S465.

34. Dofferhoff, A. S., E. Vellenga, L. P.C., A. v. Zanten, P. O. Mulder, and J. Weits. 1991. Tumour necrosis factor (cachectin) and other cytokines in septic shock: a review of the literature. *Neth. J. Med.* 39:45-62.

35. Ebong, S., D. Call, J. Nemzek, G. Bolgos, D. Newcomb, and D. Remick. 1999. Immunopathologic alterations in murine models of sepsis of increasing severity. *Infect. Immun.* 67:6603-6610.

36. Teti, G., G. Mancuso, and F. Tomasello. 1993. Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection. *Infect. Immun.* 61:227-235.

37. Abdi, K. 2002. IL-12: the role of p40 versus p75. *Scand. J. Immunol.* 56:1-11.

38. Allan, S. M., P. J. Tyrrell, and N. J. Rothwell. 2005. Interleukin-1 and neuronal injury. *Nat. Rev. Immunol.* 5:629-640.

39. Ley, C. 2003. Arrest cytokines. *Microcirculation* 10:289-295.

40. Bagglioni, M. 2001. Chemokines in pathology and medicine. *J. Intern. Med.* 250: 91-104.

41. Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 347:669-671.

42. Sergerie, Y., G. Boivin, D. Gosselin, and S. Rivest. 2007. Delayed but not early glucocorticoid treatment protects the host during experimental herpes simplex virus encephalitis in mice. *J. Infect. Dis.* 195:817-825.

43. Brochu, S., M. Olivier, and S. Rivest. 1999. Neuronal activity and transcription of proinflammatory cytokines, IkappaBalpha, and iNOS in the mouse brain during acute endotoxemia and chronic infection with Trypanosoma brucei brucei. *J Neurosci Res* 57:801-816.

44. Greer, J. M., and M. B. Less. 2002. Myelin proteolipid protein- the first 50 years. *Int. J. Biochem. Cell. Biol.* 34:211-215.

45. Koedel, U., and H. W. Pfister. 1999. Models of experimental bacterial meningitis. Role and limitations. *Infect. Dis. Clin. North. Am.* 13:549-577.

46. Gerber, J., G. Raivich, A. Wellmer, C. Noeske, T. Kunst, W. Bruck, and R. Nau. 2001. A mouse model of *Streptococcus pneumoniae* meningitis mimicking several features of human disease. *Acta Neuropathol. (Berl)* 101:499-508.

47. Chiavolini, D., S. Tripodi, R. Parigi, O. M.R., E. Blasi, M. Cintorino, G. Pozzi, and S. Ricci. 2004. Method for inducing experimental pneumococcal meningitis in outbred mice. *BMC Microbiol.* 4:36-45.

48. Mancuso, G., A. Midiri, C. Beninati, C. Biondo, R. Galbo, S. Akira, P. Henneke, D. Golenbock, and G. Teti. 2004. Dual role of TLR2 and myeloid differentiation

factor 88 in a mouse model of invasive group B streptococcal disease. *J. Immunol.* 172:6324-6329.

49. Norrby-Teglund, A., K. Pauksens, M. Norgren, and S. E. Holm. 1995. Correlation between serum TNF alpha and IL6 levels and severity of group A streptococcal infections. *Scand. J. Infect. Dis.* 27:125-130.

50. Goldmann, O., G. S. Chhatwal, and E. Medina. 2005. Contribution of natural killer cells to the pathogenesis of septic shock induced by *Streptococcus pyogenes* in mice. *J. Infect. Dis.* 191:1280-1286.

51. Raeder, R. H., L. Barker-Merrill, T. Lester, D. Boyle, and D. W. Metzger. 2000. A pivotal role for interferon-gamma in protection against group A streptococcal skin infection. *J. Infect. Dis.* 181:639-645.

52. Dinarello, C. A. 2005. Interleukin-1beta. *Crit. Care Med.* 33:S460-462.

53. Moller, A. S., A. Bjerre, B. Brusletto, G. B. Joo, P. Brandtzaeg, and P. Kierulf. 2005. Chemokine patterns in meningococcal disease. *J. Infect. Dis.* 191:768-775.

54. Fowler, M. I., R. O. Weller, J. E. Heckels, and M. Christodoulides. 2004. Different meningitis-causing bacteria induce distinct inflammatory responses on interaction with cells of the human meninges. *Cell Microbiol.* 6:555-567.

55. Koedel, U., A. Bernatowicz, K. Frei, A. Fontana, and H. W. Pfister. 1996. Systemically (but not intrathecally) administered IL-10 attenuates pathophysiologic alterations in experimental pneumococcal meningitis. *J. Immunol.* 157:5185-5191.

56. Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H. W. Pfister, and C. J. Kirschning. 2003. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J. Immunol.* 170:438-444.

57. Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli, and R. Landmann. 2002. Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammmation. *J. Infect. Dis.* 186:798-806.

58. Bottcher, T., M. v. Mering, S. Ebert, U. Mayding-Lamade, U. Kuhnt, J. Gerber, and R. Nau. 2003. Differential regulation of Toll-like receptor mRNAs in experimental murine central nervous system infections. *Neurosci. Lett.* 344:17-20.

59. Letiembre, M., H. Echchannaoui, P. Bachmann, F. Ferracin, C. Nieto, M. Espinosa, and R. Landmann. 2005. Toll-like receptor 2 deficiency delays

pneumococcal phagocytosis and impairs oxidative killing by granulocytes. *Infect. Immun.* 73:8397-8401.

60. Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paten, M. R. Wessels, and D. T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci.* 100:1966-1971.

61. Kim, Y. S., J. Honkaniemi, F. R. Sharp, M. G. Tauber, and 2004. Expression of proinflammatory cytokines tumor necrosis factor-alpha and interleukin-1beta in the brain during experimental group B streptococcal meningitis. *Mol. Brain Res.* 128:95-102.

62. Laflamme, N., and S. Rivest. 2001. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J.* 15:155-163.

63. Rivest, S. 2003. Molecular insights on the cerebral innate immune system. *Brain Behav. Immun.* 17:13-19.

64. Constantin, D., A. Cordenier, K. Robinson, D. A. Ala'Aldeen, and S. Murphy. 2004. *Neisseria meningitidis*-induced death of cerebrovascular endothelium: mechanisms triggering transcriptional activation of inducible nitric oxide synthase. *J. Neurochem.* 89:1166-1174.

65. Jermann, H., C. Hii, G. Hodge, and A. Ferrante. 2001. Synthesis and surface expression of CD14 by human endothelial cells. *Infect. Immun.* 69:479-485.

66. Beekhuizen, H., I. Blokland, A. J. Corsel van Tilburg, F. Koning, and R. van Furth. 1991. CD14 contributes to adherence of human monoctyes to cytokine-stimulated endothelial cells. *J. Immunol.* 147:3761-3767.

67. Singh, A. K., and Y. Jiang. 2004. How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology* 201:197-207.

68. Glod, J., D. Kobiler, M. Noel, R. Koneru, S. Lehrer, D. Medina, D. Maric, and H. A. Fine. 2006. Monocytes form a vascular barrier and participate in vessel repair after brain injury. *Blood* 107:940-946.

69. Guillemin, G. J., and B. J. Brew. 2004. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. *J. Leukoc. Biol.* 75:388-397

ARTICLE II:

"In vitro characterization of the microglial inflammatory response to *Streptococcus suis* Serotype 2, a relevant zoonotic emerging agent of meningitis"

María de la Cruz Domínguez-Punaro,[1] Mariela Segura,[1] Irazú Contreras,[2] Claude Lachance,[1] Mathieu Houde,[1] Marie-Pier Lecours,[1] Martin Olivier,[2] Marcelo Gottschalk[1]

Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, J2S 7C6, Canada[1]; Department of Microbiology and Immunology, McGill University, Montreal, QC, H3A 2B4, Canada[2]

* Corresponding author. Mailing address : Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, J2S 7C6, Canada. Phone: (450) 773-8521 × 1-8374. Fax: (450) 778-8108.

Accepted for publication in Infect. Immun. (article in press).

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the experimental design; I substantially performed research, analyzed data and wrote the paper.

Abstract

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

Introduction

Streptococcus suis is one of the most important swine pathogens worldwide as well as an important agent of zoonosis. Up today, 35 serotypes have been described, although serotype 2 is still the most frequently isolated from both, swine and humans. In swine, meningitis is the most striking feature of the infection, although other pathologies such as septicemia, endocarditis, pneumonia and arthritis have been described (3, 31, 66). The most common pathology associated to *S. suis* infection in humans is also meningitis, although cases of septicemia with septic shock, endocarditis, and several other clinical manifestations have been reported (65, 67, 69). As a zoonotic agent, *S. suis* infection has been traditionally considered as an occupational hazard, since most of the cases described in Western countries have occurred in people working in close relationship with pigs or raw pork products. The situation in Asian countries is completely different. In addition to an important human Chinese outbreak caused by *S. suis* in 2005 (69), this pathogen has recently been reported as the third most common culture-confirmed cause of community-acquired bacterial meningitis in Hong Kong (33) and the most frequent cause of bacterial meningitis in adults in Vietnam (44). People who survive *S. suis* infection may be handicapped as severe post-infection sequels, such as deafness, may develop (65, 66).

In recent years, an important number of studies describing the proposed virulence factors of *S. suis* serotype 2 have been published (5, 26). However, few candidates have been shown to be critical for virulence. Among them, the capsular

polysaccharide (CPS) has been considered as an important anti-phagocytic factor (13). Although not essential for virulence, a hemolysin (suilysin) produced by most virulent strains in Eurasia has also been shown to be toxic for cells of murine, human, and swine origins (12, 14, 54, 64).

The pathogenesis of infection of *S. suis* has been partially elucidated. In swine, infection occurs through the respiratory route with subsequent colonization of the tonsils while in humans, the access is mainly through skin cuts and/or the oral route (4, 25, 26). Once *S. suis* reaches the bloodstream, it travels either free or associated to monocytes (24), with invasion of different tissues and organs. High mortality observed at this stage of the disease may be associated to septic shock with an exacerbated release of pro-inflammatory cytokines (17, 18). However, if the host overcomes septicemia, *S. suis* may still invade the central nervous system (CNS) and cause meningitis and, in some cases, encephalitis. Mechanisms used by the pathogen to gain access to the brain and induce local inflammation are still under debate. It is likely that access is by transcytose and/or toxicity to microvascular endothelial cells (64) and/or the choroid plexus epithelial cells that are part of the blood-brain barrier (BBB) (63). Increase of BBB permeability due to inflammation cannot be ruled out (24).

Recently, our laboratory developed an in vivo mouse model of meningitis/encephalitis after *S. suis* infection via the intraperitoneal route (17). Using this model, an important inflammatory response in the CNS with the expression of different pro-inflammatory genes, including Toll-like receptor (TLR)-2, CD14, IκBα (an

index of NF-κB expression), IL-1β, TNF-α, and MCP-1 was observed. Interestingly, the expression of these genes and bacterial antigens were found by immunohistochemistry to be probably associated with microglia and astrocytes (17). Microglia, the macrophage-like population within the CNS, represents the first line of defense against invading pathogens and has proinflammatory effector functions (48). Although previous findings draw attention to the implication of these cells in the development of meningitis and encephalitis, it is critical to dissect how these cells initiate key pro-inflammatory mechanisms in order to respond to *S. suis* infection.

The goal of this study was to explore the murine microglial response to a virulent strain of *S. suis* as well as isogenic mutants defective in either capsule or suilysin production. The ability of microglia to phagocyte *S. suis*, to activate TLRs, to secrete different pro-inflammatory mediators as well as to activate important inflammatory intracellular signaling pathways was evaluated.

Materials and methods

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

Bacterial strains and growth conditions. Three strains of *S. suis* capsular serotype 2 were used, the virulent wild-type suilysin-positive strain 31533 (WT) originally isolated from a case of porcine meningitis and widely used in previous studies (18, 19, 42, 58, 59), as well as two isogenic mutants obtained by allelic exchange, the suilysin-negative mutant SX911 (SLY-) (45) and the non-capsulated mutant B218 (CPS-) (23). Bacteria were grown overnight on sheep blood agar plates at 37°C and isolated colonies were inoculated into 5 ml of Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI), which

was incubated for 8 h at 37°C with agitation. Working cultures were prepared by transferring 10 µl of 1/1000 dilutions of 8 h-cultures into 30 ml of THB, which was incubated for 16 h at 37°C with agitation. Stationary-phase bacteria were washed twice in phosphate-buffered saline pH 7.3 (PBS). For experiments, the bacterial pellet was resuspended and diluted in cell culture medium to a final concentration of 1×10^6 CFU/ml, unless specified. When necessary, the WT and CPS- *S. suis* strains were heat killed as previously described at 60°C for 45 min (minimal experimental conditions required for *S. suis* killing) (57).

Phagocytosis assays. Phagocytosis assays were performed as already described (60) with some modifications. Briefly, microglial cells were infected by removing culture medium and adding different *S. suis* strains resuspended in cell culture media at a MOI of 2:1. In selected experiments, bacterial opsonization was performed by incubating bacteria in the presence of 20% (v/v) of complete normal or heat inactivated mouse serum in PBS for 30 min at 37°C with shaking prior to cell infection. Mouse serum from C57BL/6 mice was inactivated by incubation for 30 min at 56°C. After 15, 30 and 60 min of infection, cell monolayers were washed twice with warm culture media, and reincubated for 1 h with medium containing penicillin G (5 µg/ml) and gentamicin (100 µg/ml) to kill extracellular bacteria (both antibiotics were from Sigma-Aldrich, Oakville, ON, Canada). Previous studies with *S. suis* showed that this concentration of antibiotics is able to kill any remaining extracellular bacteria (56, 60). In addition, supernatant

controls were taken in every test to confirm antibiotic efficacy. After antibiotic treatment, cell monolayers were washed three times and the medium was replaced with 1 ml of sterile distilled water to lyse microglial cells. After vigorous pipetting to ensure complete cell lysis, viable intracellular streptococci were determined by quantitative plating of serial dilutions of the lysates on THB agar. All samples were plated using an Autoplate 400 Automated Spiral Plater (SpiralBiotech Inc., Norwood, MA). Each test was repeated four times in independent experiments, and the number of CFU recovered per well (mean number ± SEM) was determined.

Phagocytosis was confirmed by confocal microscopy. Microglial cells were plated on coverslips and infected with *S. suis* WT and CPS- strains. After 2 h of bacteria-cell contact, coverslips were washed with culture media to remove non-associated bacteria, and cells fixed with methanol/acetone (80:20) for 20 min at -20°C, washed and blocked with PBS containing 2% fetal bovine serum for 10 min. Coverslips were incubated for 1 h with optimal dilutions of rabbit hyperimmune anti-*S. suis* serum, produced as described previously (36), and with rat anti-LAMP1 antibody (Developmental Studies Hybridoma Bank, Iowa, IA). After washing, coverslips were incubated with the secondary antibodies Alexa-Fluor 488 goat anti-rabbit IgG and Alexa-Fluor 568 goat anti-rat IgG (Invitrogen) for 30 min, washed and mounted on glass slides with mowiol containing DABCO and DAPI to stain the nuclei. Samples were observed with an IX-80 confocal microscope integrated into the FV-1000 imagery system and analysed using the fluoview software (Olympus, Markham, ON, Canada).

Cytokine and chemokine detection by ELISA. Microglial cells were infected with *S. suis* WT and mutant strains included in the study at a MOI of 2:1. Purified *Escherichia coli* lipopolysaccharide (LPS) at 1µg/ml (Sigma-Aldrich) was used as positive control. After 12 h incubation (optimal incubation time as observed in kinetic studies, data not shown), the supernatant was recovered to measure levels of IL-1β, IL-6, TNF-α, MCP-1 and the chemokine interferon-inducible protein of 10 kD/CXC chemokine ligand 10 (CXCL10/IP-10) by a sandwich ELISA, using commercially available antibody pairs (R&D Systems, Minneapolis, MN), as previously described (58). Standard curves were included in each ELISA plate (Nunc, Ville Mont Royal, QC, Canada) as two-fold dilutions of recombinant (R&D Systems) mouse IL-1β (1000 to 39 pg/ml), IL-6 (1250 to 39 pg/ml), TNF-α (1000 to 8 pg/ml), MCP-1 (500 to 8 pg/ml) or IP-10 (4000 to 31 pg/ml). Supernatant dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to determine the level of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate.

Analysis of TLRs gene expression by real time reverse transcriptase-quantitative PCR. Microglial cells were infected with WT and CPS- *S. suis* strains at a MOI of 2:1 for 0, 1, 2, 4 and 8 h. Following infection, medium was removed and cells were washed with cell culture medium. Total cellular RNA was prepared from cells using Trizol (Invitrogen) following standard procedures. Next, 1 µg of total RNA was reverse-

transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) and the resulting cDNA was amplified using the SsoFast^{TM} EvaGreen$^{®}$ Supermix kit (Bio-Rad, Hercules, CA). The PCR amplification program for all cDNA samples consisted of an enzyme activation step of 3 min at 98°C, followed by 40 cycles of a denaturing step for 3 s at 98 °C and an annealing/extension step for 5 s at 57 °C. Each 10 µl reaction contained 250 nM each of forward and reverse primers. The primers used for amplification of the different target cDNA are listed in Table 1 and were all tested to achieve an amplification efficiency between 90% and 110%. The primer sequences were all designed from NCBI GenBank mRNA sequence using web-based software primerquest from Integrated DNA technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/). The Bio-Rad CFX-96 sequence detector was used for amplification of target cDNA of various TLRs and quantitation of differences between the different groups was calculated using the $2^{-\Delta\Delta Ct}$ method. β-actin and β_2 microglobulin (β2m) were used as normalizing genes to compensate for potential differences in cDNA amounts between the various samples. These two genes were selected from candidate normalizing genes using the "Normfinder V19" algorithm (2) as their expression was found to be the most stable in these experimental conditions. The non-infected BV-2 microglia group was used as the calibrator reference in the analysis.

Measurement of nitric oxide production. Microglia cells were seeded and stimulated with killed bacteria as previously described (56), with some modifications. Briefly, heat-killed *S. suis* WT and CPS- strains (concentration equivalent to 1×10^9 CFU/ml) were

incubated with cells (5×10^5 cells/well) for 6, 12, 24, and 48 h. LPS (1µg/ml) was used as positive control. Nitric oxide (NO) production was determined by measuring nitrite, a stable end product of NO. Microglial culture supernatant was assayed by mixing with Griess reagent (Promega, Madison, WI). Sulfonamide (1%) and N-1 (1-naphthyl)-ethylenediamine dihydrochloride (0.1%) were added to the supernatant. After 30 min of incubation at room temperature, the absorbance was read at 540 nm. The nitrite concentration (expressed as µM/ml) was calculated using standard solutions of sodium nitrite prepared in culture medium.

Signaling pathway analysis by immunoblotting. Western blotting was performed to analyze the phosphorylation state of protein kinase C (PKC) pathway, several mitogen-activated protein kinases (MAPKs), including stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), extracellular signal-regulated kinase (ERK) and p38, as well as total tyrosine (Tyr) phosphorylation and inducible nitric oxide synthase (iNOS) expression, as previously described (38, 50). Briefly, cells were plated in a 6-well plate and stimulated with *S. suis* WT and CPS- strains at a MOI of 2:1. LPS (1µg/ml) was used as positive control. After different incubation times (see Results), cells were lysed in cold buffer containing 0.5 M Tris (pH 6.8), 0.5 M EDTA, 1% β-mercaptoethanol (v/v) (Biorad, Mississauga, ON, Canada), 10 mM EGTA, 10% IGEPAL (v/v), 1 mM sodium orthovanadate (Na_3VO_4) (all from Sigma-Aldrich) and protease inhibitors aprotinin (10 µg/ml) (Sigma-Aldrich) and leupeptin (5 µg/ml) (Roche, Mississauga, ON, Canada). The lysates (60µg/lane) were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were

blocked in Tris-buffered saline/0.1% Tween containing 1% bovine serum albumin (or 5% skim milk for anti-α-actin), for 1 h at room temperature. Membranes were then washed and incubated overnight at 4°C with one of the following antibodies: anti-phospho-ERK 1/2 (Thr^{202}/Tyr^{204}), anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-phospho-(Ser) PKC substrate antibody (all from Cell Signaling, Danvers, MA), anti-phospho SAPK/JNK 1/2 (Thr^{183}/Tyr^{185}) (Invitrogen), anti-phospho-Tyr (clone 4G10, Upstate, Lake Placid, NY) or anti-iNOS (BD Systems, San Jose, CA). After washing, membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated antibodies (Sigma-Aldrich) for 1 h, and proteins were visualized with ECL Plus Western Blotting reagent (Amersham, Arlington Heigths, IL). Membranes were then stripped using the Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), and blotting was repeated using anti-protein antibodies (per the manufacturer of the anti-phospho-protein). Blotting with anti-α-actin antibody (Sigma-Aldrich) was used as loading control for p-Tyr, p-PKC and iNOS blots.

MAPK inhibition assays. In selected experiments, microglial cells were pretreated with the ERK inhibitor Apigenin (50 μM), the SAPK-JNK inhibitor SP600125 (50 μM) or the p38 inhibitor SB203580 (75 μM) 1 h prior to infection (all from Biomol Research Laboratories, Plymouth Meeting, PA). Western blot analysis of the corresponding phosphorylated proteins was done as described above after 2 h or 4 h incubation in presence of bacteria. Cytokine measurement was performed as aforementioned after 12 h incubation. Inhibitors were used at maximal subcytotoxic concentrations as determined by the Cyto Tox 96 Non-Radioactive Cytotoxicity Assay. An inhibitor was considered cytotoxic if viability was < 90% of untreated control after 12 h.

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

Statistical analysis. Each test was done at least in triplicate biological repetitions. Differences were analyzed for significance by using the Student unpaired *t* test (two-

tailed *P* value). A *P* value of <0.05 was considered significant. All data are presented as mean ± SEM. All statistical analyses were performed using Sigma Plot software (v.11; Systat Software, San Jose, CA).

RESULTS

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

To confirm the intracellular location of bacteria, confocal microscopy was performed using hyperimmune serum against *S. suis* and an antibody specific for LAMP1, a protein enriched in phagolysosomes. Confocal analysis showed that only few

numbers of WT *S. suis* were internalized by microglia (Fig. 2). However, the CPS- mutant was found in higher numbers not only attached to the cell membrane, forming small chains, but also inside the cytoplasm and associated to numerous phagolysosomes of microglial cells (Fig. 2).

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

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

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

***S. suis* infection induces TLR2 gene expression.** TLRs are receptors that play a key role in the innate immune response. Having observed differences in expression of cytokine levels following infection of microglia by the WT strain and the CPS- mutant, we were interested in examining whether gene expression of different TLRs was modified. To this effect, microglia cells were infected with WT and CPS- strains of *S. suis* for different

times and TLR1, TLR2, TLR4, TLR6, and TLR9 gene expression analyzed by real-time PCR. Microglial cell infection by both *S. suis* strains failed to increase TLR1, TLR4, TLR6, and TLR9 gene expression over basal levels (data not shown). However, TLR2 gene expression was significantly increased in microglia between 4 and 8 h of incubation by either the WT strain or its CPS- mutant, when compared to non-infected cells ($P < 0.05$) (Fig. 4). Interestingly, the CPS- mutant was able to induce TLR2 gene expression in microglial cells at a higher level than the WT *S. suis* strain ($P < 0.001$). This finding is in agreement with the fact that CPS- mutant was also associated with a general higher cytokine production by these cells.

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

Microglial cell profile of tyrosine phosphorylation is modulated during *S. suis* infection. Studies with different pathogens have demonstrated that bacterial attachment can lead to activation of host signal transduction cascades, predominantly through Tyr phosphorylation of proteins that contribute to different signal transduction mechanisms, including internalization of pathogens into host cells (22). Thus, modulation of protein phosphotyrosine residues in response to *S. suis* infection and the effect of CPS in this process were assessed. As shown in Fig. 6A, the Tyr phosphorylation response towards *S. suis* is considerably down-modulated by the presence of the capsule. Indeed, the WT strain induces a modest Tyr phosphorylation of some proteins, which is more apparent at 2h post-infection, while the CPS- mutant leads to dramatic changes in the phosphorylation state of numerous proteins. These changes occur not only earlier in comparison to the WT strain (15 min), but also the level of phosphorylation is stronger and generally increases over time. It is interesting to note that during the course of infection, the phosphorylation level of some proteins of ~ 110, 70-75 and 25-30 kDa temporarily decreased, suggesting that either different bacterial components and/or different steps of the infection (adhesion vs internalization) may influence Tyr phosphorylation patterns (Fig. 6A).

Murine microglial cells display PKC activity after *S. suis* infection. PKC represents a family of serine/threonine kinases that play central roles in multiple signaling events, such as regulation of the immune response by MAPKs and gene transcription activation

(62). Using an antibody specific to PKC substrates containing phospho-serine, phosphorylation levels of several proteins in microglial cells was shown to be different after infection with the WT strain compared to the CPS- mutant (Fig. 6B). In fact, the WT strain showed a discrete phosphorylation of several proteins, ranging from ~ 25 to 60 kDa that occurred at different times post-infection depending on the protein. In contrast, the phosphorylation pattern of PKC substrates observed for the CPS- mutant was particularly stronger and more stable reaching maximal phosphorylation at 2 h post-infection for most of the proteins. Similarly to that observed for phosphotyrosine (Fig. 6A), PKC-dependent phosphorylation of some proteins showed a shift between de phosphorylated and the non-phosphorylated state over the infection period.

***S. suis* infection activates MAPK phosphorylation in murine microglia.** It is known that MAPK signal transduction pathways are involved in microglial activation leading to production of different pro-inflammatory mediators that play an essential role in host response against pathogens (6, 51). To this end, the time-course phosphorylation of the three MAPK signaling pathways, ERK 1/2, SAPK/JNK and p38, in microglial cells was investigated after stimulation with *S. suis* WT strain and CPS- mutant (Fig 7). A specific phosphorylation pattern was observed for each MAPK evaluated, that seemed to be influenced also by the strain tested. ERK 1/2 activation in response to the WT strain was observed as soon as 15 min; however, this phosphorylation was down-regulated between 30 min and 1 h post-incubation, though a second phase of phosphorylation

was found at 2 h. No activation of this MAPK was found when using longer incubation times (data not shown). A similar ERK 1/2 activation pattern was detected with the CPS- mutant. Similarly to the WT strain, there was dephosphorylation of ERK 1/2 at 1 h and a second and final pick of phosphorylation at 2 h. When cells were stimulated with WT *S. suis* the phosphorylated form of JNK was also found at 15 min post-stimulation, but similarly to ERK 1/2, phosphorylated JNK was down-regulated from 30 min to 1 h. A second phase of activation was found at 2 h that extended up to 4 h of bacteria-cell contact. Interestingly, when using the CPS- mutant, a prompt and lasting JNK phosphorylation was detected, from 15 min to 4 h of stimulation. This phosphorylation seemed to be slightly down-regulated from 30 min to 1 h. In the case of p38, a very slight activation was found in WT strain-infected migroglial cells between 30 min and 2 h post-infection. However, when microglial cells were stimulated with the CPS- mutant, p38 phosphorylation was more marked and extended from 1 h to 4 h post-infection. These results show that *S. suis* is not only capable of inducing activation of different MAPK signaling pathways, but also that cell wall components seem to be mainly implicated in this phenomenon.

The involvement of ERK 1/2, JNK and p38 in regulation of microglial production of pro-inflammatory cytokines and chemokines in response to *S. suis* infection was confirmed using specific inhibitors. For this purpose, microglial cells were treated with subcytotoxic doses ERK 1/2 (Apigenin), JNK (SP600125) or p38 (SB203580) inhibitors, and then infected with either *S. suis* WT strain or its CPS- mutant. Fig. 8A shows Western blot results of this selective MAPK inhibition, with an evident abrogation in

phosphorylation of all three MAPKs. In parallel, it was possible to confirm that MAPK activity was involved in cytokine and chemokine production, as microglia treated with MAPK inhibitors prior to *S. suis* infection showed a strong diminution of TNF-α and MCP-1 production (Fig. 8B and 8C), for either the WT strain or its CPS- mutant.

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

DISCUSSION

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

Microglial cells have recently been shown to be able to phagocytose and kill Gram-positive bacteria, including well encapsulated pathogenic *S. pneumoniae* (52). In

the case of *S. suis*, previous studies carried out with murine, porcine and human phagocytes indicated that *S. suis* capsule is critical for bacterial resistance to phagocytosis (12, 56, 61). Similarly, phagocytosis and confocal microscopy results from the present study show that microglial cells hardly ingest well encapsulated *S. suis*, whereas the CPS- mutant was significantly more ingested than the WT strain. The absence of the suilysin, a virulence factor present mainly in Eurasian strains (54), has already been associated to a partial susceptibility of encapsulated *S. suis* to killing by neutrophils and dendritic cells in the presence of complement (5)12) (M. P. Lecours, M. Gottschalk, M. Houde, P. Lemire, N. Fittipaldi, and M. Segura, submitted for publication). Since components from the complement cascade can be found in the brain (26), their possible effect on phagocytosis of *S. suis* by microglia was evaluated. Although the presence of serum significantly increased the phagocytosis rate of *S. suis*, complement components do not seem to be implicated in such a process. In addition, the capsulated *S. suis* SLY- mutant behaved similarly to the WT strain, indicating a particular behavior of the microglial cells, different from other phagocytes (5, 12). The observed increased rate of phagocytosis in the presence of serum might be due to other proteins, such as albumin and fibronectin (9).

Results of cell activation by *S. suis* provide support to the relevance of microglia in the development of the inflammatory response against this pathogen, as shown by the production of pro-inflammatory cytokines and chemokines. This confirms previous in vivo findings in the brain of mice where high mRNA expression levels of different pro-inflammatory mediators were observed in cells suspected to be microglia (18). Indeed,

high levels of TNF-α and MCP-1 but relatively low levels of IL-1β were observed after in vitro *S. suis* activation of microglial cells. Interestingly, the WT encapsulated strain did not induce IL-6 production. It has been previously shown that, although highly secreted in the bloodstream during the septicemic phase, IL-6 mRNA was not expressed in the brain of *S. suis* infected mice (18). Results obtained in this study confirm this observation. For so far unknown reasons, the lack of IL-6 production by microglia differs from what was observed with other phagocytic cells and *S. suis* (30, 57, 58) as well as with other streptococci and microglial cells (44).

The pneumolysin produced by *S. pneumoniae*, which presents a high homology with the suilysin, was shown to play an active role in inflammation (37). This does not seem to be the case for the suilysin, since the *S. suis* SLY- mutant induces similar cytokine levels than the WT strain. On the other hand, the capsule seems to be critical for modulating production of pro-inflammatory mediators, as the CPS- mutant induced the release of significant higher levels of all pro-inflammatory mediators. These findings support the assumption that several cell wall components, such as lipoteichoic acid (LTA), peptidoglycan (PG) and lipoproteins, partially masked by the capsule, are potent pro-inflammatory inducers, as recently suggested (24, 25, 70). Finally, as a humble production of IP-10 for capsulated *S. suis* strains in comparison to the CPS- mutant was also noted, we might hypothesize that the CPS might influence the onset of the adaptive inflammatory response, as recently shown for dendritic cells (M. P. Lecours, M. Gottschalk, M. Houde, P. Lemire, N. Fittipaldi and M. Segura, submitted for publication). As a consequence, less T lymphocytes would be attracted to the site of infection.

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

A sustained and therefore uncontrolled production of toxic products released from microglia may cause irreversible damage to neurons. NO plays a significant role in

macrophage bactericidal functions, however, it is also involved in a variety of brain insults, including neurotoxicity, increase of intracranial pressure and meningeal inflammation (41). It was observed that both *S. suis* WT and CPS- strains efficiently enhance the expression of iNOS in a time-dependent manner, that transformed in the release of NO from microglia, although levels were again higher with the CPS- mutant. Cell wall components of other Gram positive bacteria, such as LTA, have already been reported to be involved in NO production by microglial cells (15).

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

It was also examined whether *S. suis* activates the three classical MAPK intracellular signaling pathways and if their phosphorylation was involved in the production of pro-inflammatory mediators. As expected, the CPS- mutant proved to be particularly potent in MAPK activation, as phosphorylation patterns were stronger and more sustained than those obtained with the WT strain. MAPK phosphorylation levels, in particular p-ERK 1/2 and p-JNK, followed a biphasic pattern. This noticeable dephosphorylation of MAPK proteins highlights the intimate cross-talk between these signaling pathways and pathogen derived components and likely has an impact on microglia proliferation and/or activation (20). Interestingly, phosphorylation levels of p38, a MAPK that plays an important role in activation of inflammatory responses (53), were subtly increased by WT *S. suis*, but more noticeable with the CPS- mutant, emphasizing the relevance of CPS in regulation of pro-inflammatory events. It is likely that, again, hidden cell wall components are the principal mediators of MAPK pathway activation. In fact, recent studies revealed that a capsule deficient mutant of *S. suis* was able to induce higher levels of transcriptional expression of different putative genes from the MAPK pathway than the parental strain (17). Furthermore, purified cell wall preparations from *S. suis* and other meningitis-causing bacteria were shown to trigger the phosphorylation of MAPK signal transduction pathway (15, 63). EstA cell surface protein is a recently described *S. pneumoniae* virulence factor that induces MAPK phosphorylation and NF-κB translocation (39). The *estA* gene is also found in *S. suis* (40), so we may hypothesize that MAPK and other intracellular signaling pathway activation do not depend solely on a few *S. suis* constituents, but that many of them participate in

the activation of the pro-inflammatory machinery. The use of pharmacological MAPK inhibitors, revealed an almost complete abrogation in cytokine release from microglia infected with either *S. suis* WT or CPS- strains, confirming the importance of ERK 1/2, JNK and p38 in the inflammatory response against this pathogen. MAPK pathways are molecular targets for drug development, and their inhibitors will undoubtedly be one of the next group of drugs developed for the treatment of human diseases (53), so these results may open the door to future studies, using animal models of *S. suis* meningitis to evaluate the in vivo efficiency of such drugs.

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

Finally, it might also be argued that activation of microglial cells may be a direct consequence of phagocytosis. In fact, cytochalasin treatment significantly reduced cytokine release by *S. suis* infected cells (data not shown). However, levels of cytokines produced by treated cells infected with the non phagocytosed well encapsulated WT strain were also significantly reduced, suggesting that phagocytosis alone was not responsible for cell activation. Rearrangement of the actin cytoskeleton may be necessary to for the formation of a fully active receptor (16)complex, which may indeed be affected by cytochalasin treatment as reported in other systems (16, 33, 65). In fact, the regulation of inflammatory cytokine production is very complex and is controlled at transcriptional, post-transcriptional, and post-translational levels. Alteration of cell receptors and/or actin networks could conceivably affect most of these levels of regulation by altering cell surface-mediated events. However, it is also possible that additional distinct mechanisms exist that are not related to alterations in receptor complexes and are probably mediated by phagolysosome 'in-out' signals. Further studies are needed to address this issue.

In conclusion, results obtained in the present study demonstrate that *S. suis* phagocytosis by microglia and consequent activation of these cells is highly influenced by the presence of the capsule, and probably involves recognition of cell wall components that requires participation of TLR2-dependent pathway, activation of different signaling pathways, translocation of NF-κB and production of different pro-inflammatory mediators and neurotoxic metabolites. The results obtained may

contribute to understand the participation of microglia in the meningitis caused by *S. suis* and the genesis of brain injury associated to this pathogen.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to MG (#154280 and Discovery Accelerator Supplement #380299) and from the Canadian Institutes of Health Research to MO. M.C.D.P. and M-P. L. are recipients of postgraduate scholarships from NSERC and I.C. is the beneficiary of a postgraduate scholarship from CONACyT-Mexico.

REFERENCES

1. **Akira, S., K. Takeda, and T. Kaisho.** 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. **2:**675-680.

2. **Andersen, C. L., J. L. Jensen, and T. F. Ørntoft.** 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. **64:**5245-5250.

3. **Bahloul, H., A. Mofredj, A. Mrabet, G. Gineyt, and P. Rousselier.** 2008. *Streptococcus suis* meningitis after oral contamination? Med. Mal. Infect. **38:**281-282.

4. **Baums, C. G., and P. Valentin-Weigand.** 2009. Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development. Anim. Health Res. Rev. **10:**65-83.

5. **Benga, L., M. Fulde, C. Neis, R. Goethe, and P. Valentin-Weigand.** 2008. Polysaccharide capsule and suilysin contribute to extracellular survival of *Streptococcus suis* co-cultivated with primary porcine phagocytes. Vet. Microbiol. **132:**211-219.

6. **Bhat, N. R., and F. Fan.** 2002. Adenovirus infection induces microglial activation: involvement of mitogen-activated protein kinase pathways. Brain Res. **948:**93-101.

7. **Blasi, E., R. Barluzzi, V. Bocchini, R. Mazzolla, and F. Bistoni.** 1990. Immortalization of murine microglial cells by a *v-raf/v-myc* carrying retrovirus. J. Neuroimmunol. **27:**229-237.

8. **Bocchini, V., R. Mazzolla, R. Barluzzi, E. Blasi, P. Sick, and H. Kettenmann.** 1992. An immortalized cell line expresses properties of activated microglial cells. J. Neurosc. Res. **31:**616-621.

9. **Brazeau, B., M. Gottschalk, S. Vincelette, and B. Martineau-Doizé.** 1996. *In vitro* phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology **142:**1231-1237.

10. **Brown, G. C.** 2007. Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase. Biochem. Soc. Trans. **35:**1119-1121.

11. **Castrillo, A., D. J. Pennington, F. Otto, P. J. Parker, M. J. Owen, and L. Boscá.** 2001. Protein Kinase C-epsilon is required for macrophage activation and defense against bacterial infection. J. Exp. Med. **194:**1231-1242.

12. **Chabot-Roy, G., P. J. Willson, M. Segura, S. Lacouture, and M. Gottschalk.** 2006. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. Microb. Pathog. **41:**21-32.

13. **Charland, N., J. Harel, M. Kobisch, S. Lacasse, and M. Gottschalk.** 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology **144:**325-332.

14. **Charland, N., V. Nizet, C. E. Rubens, K. S. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect. Immun. **68:** 637-643.

15. **Chien, H., K. Y. Yeh, F. Jiang-Sjieh, I. W. Wei, C. Y. Chang, and C. H. Wu.** 2005. Signal transduction pathways of nitric oxide release in primary microglial culture challenged with gram-positive bacterial constituent, lipoteichoic acid. Neuroscience **133:**423-436.

16. **Dai, Q., and S. B. Pruett.** 2006. Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton and associated TNF-alpha production. Alcohol Clin. Exp. Res. **30:**1436-1444.

17. **De Greeff, A., L. Benga, P. J. Wichgers Schreur, P. Valentin-Weigand, J. M. J. Rebel, and H. E. Smith.** 2009. Involvement of NF-κB and MAP-kinases in the transcriptional response of alveolar macrophages to *Streptococcus suis*. Vet. Microbiol. **141:**59-67.

18. **Domínguez-Punaro, M. C., M. Segura, M. M. Plante, S. Lacouture, S. Rivest, and M. Gottschalk.** 2007. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J. Immunol. **179:**1842-1854.

19. **Domínguez-Punaro, M. C., M. Segura, D. Radzioch, S. Rivest, and M. Gottschalk.** 2008. Comparison of the susceptibilities of C57BL/6 and A/J mouse strains to *Streptococcus suis* serotype 2 infection. Infect. Immun. **76:**3901-3910.

20. **Fettucciari, K., I. Fetriconi, A. Bartoli, R. Rossi, and P. Marconi.** 2003. Involvement of mitogen-activated protein kinases in Group B *Streptococcus*-induced macrophage apoptosis. Pharmacol. Res. **47:**355-362.

21. **Fiebich, B. L., R. D. Butcher, and P. J. Gebicke-Haerter.** 1998. Protein kinase C-mediated inducible nitric oxide synthase expression in cultured microglial cells. J. Neuroimmunol. **92:**170-178.

22. **Finlay, B. B., and P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science **276:** 718-725.

23. **Fittipaldi, N., J. Harel, B. D'Amours, S. Lacouture, M. Kobisch, and M. Gottschalk.** 2007. Potential use of an unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant as a live attenuated vaccine in swine. Vaccine **25:**3524-3535.

24. **Fittipaldi, N., T. Sekizaki, D. Takamatsu, M. C. Domínguez-Punaro, J. Harel, N. K. Bui, W. Vollmer, and M. Gottschalk.** 2008. Significant contribution of the *pgdA* gene to the virulence of *Streptococcus suis*. Mol. Microbiol. **70:**1120-1135.

25. **Fittipaldi, N., T. Sekizaki, D. Takamatsu, J. Harel, M. C. Domínguez-Punaro, S. Von Aulock, C. Draing, C. Marois, M. Kobisch, and M. Gottschalk.** 2008. D-alanylation of lipoteichoic acid contributes to the virulence of *Streptococcus suis*. Infect. Immun. **76:**587-594.

26. **Francis, K., J. van Beek, C. Canova, J. W. Neal, and P. Gasque.** 2003. Innate immunity and brain inflammation: the key role of complement. Expert. Rev. Mol. Med. **23:**1-19.

27. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **76:**259-272.

28. **Gottschalk, M., M. Segura, and J. Xu.** 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim. Health Res. Rev. **8:**29-45.

29. **Gottschalk, M., J. Xu, C. Calzas, and M. Segura.** 2010. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. **5:**371-391.

30. **Graveline, R., M. Segura, D. Radzioch, and M. Gottschalk.** 2007. TLR2-dependent recognition of *Streptococcus suis* is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int. Immunol. **19:**375-389.

31. **Hanisch, U. K.** 2002. Microglia as a source and target of cytokines. Glia **40:**140-155.

32. **Hanisch, U. K., M. Prinz, K. Angstwurm, K. G. Häusler, O. Kann, H. Kettenmann, and J. R. Weber.** 2001. The protein tyrosine kinase inhibitor AG126 prevents the massive microglial cytokine induction by pneumococcal cell walls. Eur. J. Immunol. **31:**2104-2115.

33. **Hawkes, D. J., and J. Mak.** 2006. Lipid membrane: a novel target for viral and bacterial pathogens. Curr. Drug Targets **7:**1615-1621.

34. **Henn, A., S. Lund, M. Hedtjärn, A. Schrattenholz, P. Pörzgen, and M. Leist.** 2009. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX **26:**83-94.

35. **Higgins, R., and M. Gottschalk.** 2005. Streptococcal diseases, p. 769-783. *In* B. E. Straw, S. D'Allaire, W. L. Mengelin, and D. J. Taylor (ed.), Diseases of Swine. Iowa State University Press, Aimes, Iowa.

36. **Higgins, R., and M. Gottschalk.** 1990. An update on *Streptococcus suis* identification. J. Vet. Diagn. Invest. **2:**249-252.

37. **Jacobs, A. A., P. L. Loeffen, A. J. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742-1748.

38. **Jaramillo, M., D. Channe Gowda, D. Radzioch, and M. Olivier.** 2003. Hemozoin incrases IFN-γ-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase-and NF-κB-dependent pathways. J. Immunol. **171:**4243-4253.

39. **Kang, E. H., E. Gebru, M. H. Kim, H. Cheng, and S. C. Park.** 2009. EstA protein, a novel virulence factor of *Streptococcus pneumoniae*, induces nitric oxide and pro-inflammatory cytokine production in RAW 264.7 macrophages through NF-κB/MAPK. Microb. Pathog. **47:**196-201.

40. **Kim, M. H., B. S. Kang, S. Kim, K. J. Kim, C. H. Lee, B. C. Oh, S. C. Park, and T. K. Oh.** 2008. The crystal structure of the estA protein, a virulence factor from *Streptococcus pneumoniae*. Proteins **70:**578-583.

41. **Kim, Y. S., and M. G. Täuber.** 1996. Neurotoxicity of glia activated by Gram-positive bacterial products depends on nitric oxide production. Infect. Immun. **64:**3148-3153.

42. **Kobish, M., M. Gottschalk, P. Morvan, R. Cariolet, G. Bénévent, and J. P. Joloy.** 1995. Experimental infection of SPF piglets with *Streptococcus suis* serotype 2. J. Rech. Porcine France **27:**97-102.

43. **Lee, J., S. Shin, C. H. Teng, S. J. Hong, and K. S. Kim.** 2005. FimH adhesin of *Escherichia coli* K1 type 1 fimbriae activates BV-2 microglia. Biochem. Biophys. Res. Commun. **334:**917-923.

44. **Liu, X., V. S. Chauhan, A. B. Young, and I. Marriott.** 2010. NOD2 mediates inflammatory responses of primary murine glia to *Streptococcus pneumoniae*. Glia **58:**839-847.

45. **Lun, S., J. Perez-Casal, W. Connor, and P. Willson.** 2003. Role of the suilysin in pathogenesis of *Streptococcus suis* capsular type 2. Microb. Pathogen. **34:**27-37.

46. **Marques, C. P., M. C. Cheeran, J. M. Palmquist, S. Hu, and J. R. Lokensgard.** 2008. Microglia are the major cellular source of inducible nitric oxide synthase during experimental herpes encephalitis. J. Neurovirol. **14:**229-238.

47. **Neher, J. J., and G. C. Brown.** 2007. Neurodegeneration in models of Gram-positive bacterial infections of the central nervous system. Biochem. Soc. Trans. **35:**1166-1167.

48. **Newton, A. C.** 1995. Protein kinase C: structure, function, and regulation. J. Biol. Chem. **270:**28495-28498.

49. **Olson, J. K., and S. D. Miller.** 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J. Immunol. **173:**3916-3924.

50. **Pouliot, P., I. Plante, M.-A. Raquil, P. A. Tessier, and M. Olivier.** 2008. Myeloid-related proteins rapidly modulate macrophage nitric oxide production during innate immune response. J . Immunol. **181:**3539-3601.

51. **Pyo, H., I. Jou, S. Jung, S. Hong, and E. H. Joe.** 1988. Mitogen-activated protein kinases activated by lipopolysaccharide and beta-amyloid in cultured rat microglia. Neuroreport. **30:**871-874.

52. **Ribes, S., S. Ebert, T. Regen, A. Agarwal, S. C. Tauber, D. Czesnik, A. Spreer, S. Bunkowski, H. Eiffert, U. K. Hanisch, S. Hammerschmidt, and R. Nau.** 2009. Toll-like receptor stimulation enhances phagocytosis intracellular killing of nonencapsulated and encapsulated *Streptococcus pneumoniae* by murine microglia. Infect Immun **23:**ahead of printing.

53. **Schindler, J. F., J. B. Monahan, and W. G. Smith.** 2007. p38 Pathway Kinases as Anti-inflammatory Drug Targets. J. Dent. Res. **86:**800-811.

54. **Segers, R. P., T. Kenter, L. A. de Haan, and A. A. Jacobs.** 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiol. Lett. **167:**255-261.

55. **Segura, M., and M. Gottschalk.** 2002. *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infect. Immun. **70:** 4312-4322.

56. **Segura, M., M. Gottschalk, and M. Olivier.** 2004. Encapsulated *Streptococcus suis* inhibits activation of signaling pathways involved in phagocytosis. Infect. Immun. **72:**5322-5330.

57. **Segura, M., J. Stankova, and M. Gottschalk.** 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.

58. **Segura, M., N. Vadeboncoeur, and M. Gottschalk.** 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. **127:**243-254.

59. **Segura, M., G. Vanier, D. Al-Numani, S. Lacouture, M. Olivier, and M. Gottschalk.** 2006. Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system. FEMS Immunol. Med. Microbiol. **47:** 92-106.

60. **Segura, M. A., P. Cléroux, and M. Gottschalk.** 1998. *Streptococcus suis* and group B *Streptococcus* differ in their interactions with murine macrophages. FEMS Immunol. Med. Microbiol. **21:**189-195.

61. **Smith, H. E., M. Damman, J. van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750-1756.

62. **Tan, S. L., and P. J. Parker.** 2003. Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem. J. **376:** 545–552.

63. **Tanabe, S., M. Gottschalk, and D. Grenier.** 2008. Hemoglobin and *Streptococcus suis* cell wall in synergy to potentiate the inflammatory response of monocyte-derived macrophages. Innate Immun. **14:**357-363.

64. **Tenenbaum, T., R. Adam, I. Eggelnpohler, D. Malaton, A. Seibt, G. E. Novotny, H. J. Galla, and H. Schroten.** 2005. Strain-dependent disruption of blood-cerebrospinal fluid barrier by *Streptoccocus suis* in vitro. FEMS Immunol. Med. Microbiol. **44:**25-34.

65. **Triantafilou, M., K. Miyake, D. T. Golenbock, and K. Triantafilou.** 2002. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J. Cell Sci. **115:**2603-12611.

66. **Vanier, G., M. Segura, P. Friedl, S. Lacouture, and M. Gottschalk.** 2004. Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. Infect. Immun. **72:**1441-1449.

67. **Wangkaew, S., R. Chaiwarith, P. Tharavichitkul, and K. Supparatpinyo.** 2006. *Streptococcus suis* infection: a series of 41 cases from Chiang Mai University Hospital. J. Infect. **52:**455-460.

68. **Wangsomboonsiri, W., T. Luksananun, S. Saksornchai, K. Ketwong, and S. Sungkanuparph.** 2008. *Streptococcus suis* infection and risk factors for mortality. J. Infect. **57:**392-396.

69. **Wertheim, H. F. L., H. D. Trung Nghia, W. Taylor, and C. Schultsz.** 2009. *Streptococcus suis*: An Emerging Human Pathogen. Clin. Infect. Dis. **48:**617–625.

70. **Wichgers Schreur, P. J., J. M. Rebel, M. A. Smits, J. P. van Putten, and H. E. Smith.** 2009. Differential activation of the Toll-like receptor 2/6 complex by lipoproteins of *Streptococcus suis* serotypes 2 and 9. Vet. Microbiol. **In press**.

71. **Yu, H., H. Jing, Z. Chen, H. Zheng, X. Zhu, H. Wang, S. Wang, L. Liu, R. Zu, L. Luo, N. Xiang, H. Liu, X. Liu, Y. Shu, S. S. Lee, S. K. Chuang, Y. Wang, J. Xu, and W. Yang.** 2006. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg. Infect. Dis. **12:**914-920.

72. **Zhao, W., W. Xie, W. Le, D. R. Beers, Y. He, J. S. Henkel, E. P. Simpson, A. A. Yen, Q. Xiao, and S. H. Appel.** 2004. Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism. J. Neuropathol. Exp. Neurol. **63:**964-077.

Table 1: Primers used in real-time quantitative PCR for the amplification and detection of TLRs

Gene	Forward Primer	Reverse Primer	Amplicon size
TLR1	CACAGCTCCTTGGTTTTAATG	TGGGTATAGGACGTTTCTGTAG	102 bp
TLR2	TGGAGCATCCGAATTGCATCACCG	GAGCGGCCATCACACACCCC	193 bp
TLR3	CGGGGGTCCAACTGGAGAACCT	GGGCGTTGTTCAAGAGGAGGGC	198 bp
TLR4	GCCTCCCTGGCTCCTGGCTA	AGGGACTTTGCTGAGTTTCTGATCCA	139 bp
TLR6	CCGTCAGTGCTGGAAATAG	CGATGGGTTTTCTGTCTTGG	108 bp
TLR9	CAGTTTGTCAGAGGGAGC	ACTTCAGGAACAGCCAATTG	198 bp
β-Actin	CCAACCGTGAAAAGATGACC	AGCATAGCCCTCGTAGATG	170 bp
β2m	ATGGCTCGCTCGGTGACCCT	TTCTCCGGTGGGTGGCGTGA	110 bp

ARTICLE II. FIGURES.

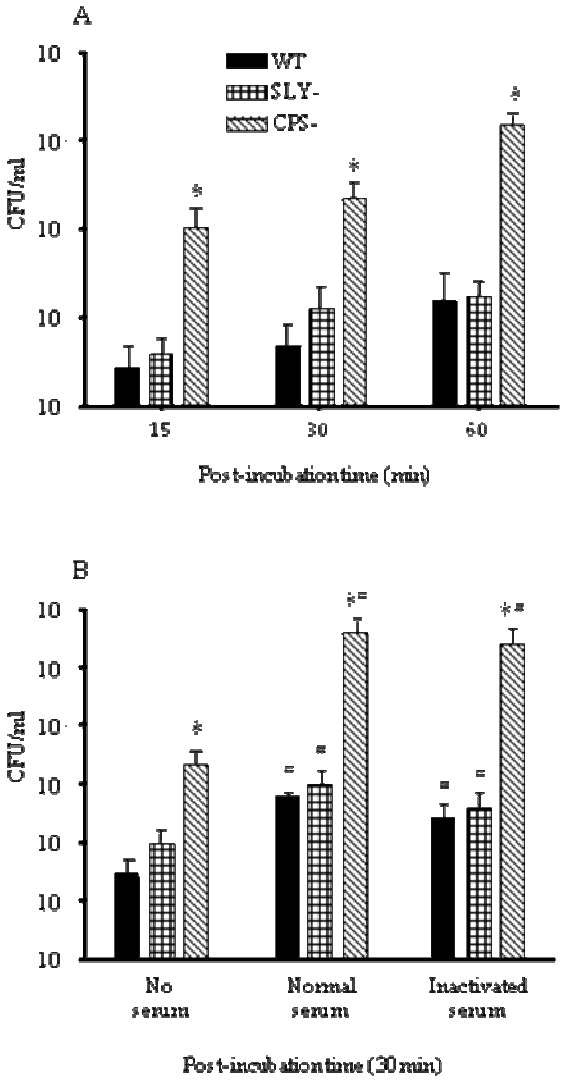


FIG. 1. Phagocytosis of *S. suis* by murine microglial cells. (A) Kinetics of phagocytosis of *S. suis* strains (1x 10^6) by murine microglia after 15, 30 and 60 min infection times. **P* <0.05 compared to phagocytosis levels obtained with the wild-type strain. (B) Effect of opsonization on phagocytosis at 30 min post-infection. Bacteria were non-opsonized (no serum) or pre-opsonized with 20% of either normal or inactivated mouse serum. **P* <0.05 compared to phagocytosis levels obtained with the wild-type strain and # *P* < 0.05, indicates statistically significant differences between non-opsonized strains and their respective normal serum- or inactivated serum-opsonized counterparts. Numbers of internalized bacteria were determined by quantitative plating after 1 h of antibiotic treatment, and results are expressed as CFU recovered bacteria per ml (means ± SEM obtained from three independent experiments). WT, wild-type strain. CPS-, non-encapsulated mutant. SLY-, suilysin-negative mutant.

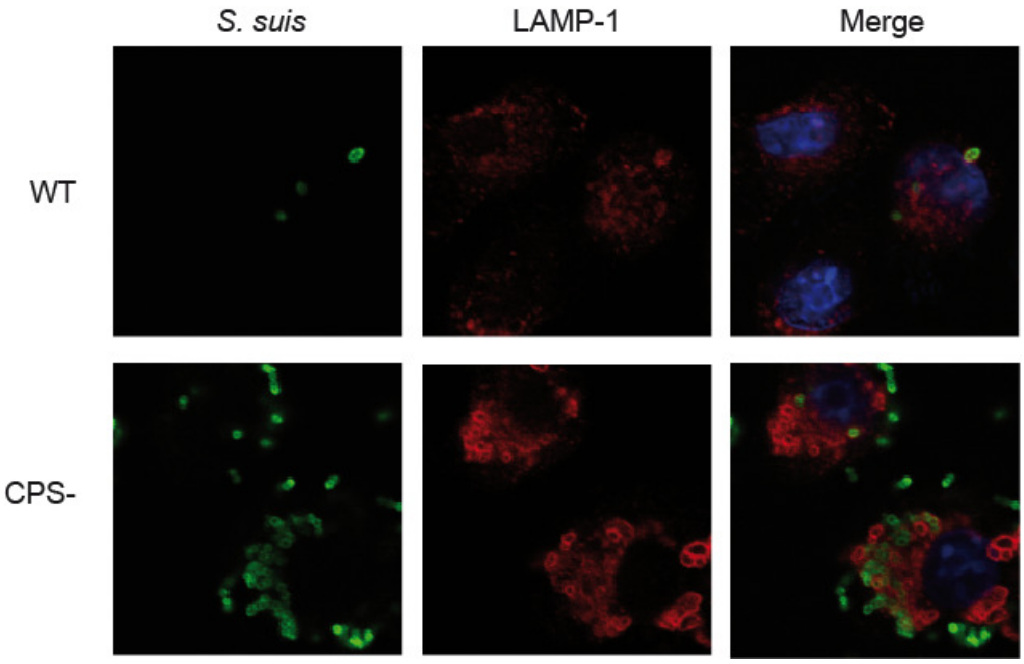


FIG. 2. Interaction of murine microglial cells with *S. suis*. Microglia were infected with either *S. suis* wild-type (WT) strain (A) or the non-encapsulated (CPS-) mutant (B) for a 2 h period. Cells were then washed, and bacteria were visualized with rabbit anti-*S. suis* serum and Alexa Fluor 488-conjugated goat anti-rabbit IgG (green), while phagolysosomes from BV-2 cells were evidenced with rat anti-LAMP1 antibody and Alexa Fluor 568-conjugated goat anti-rat IgG (red). The nuclei was stained with DAPI (blue). Images were examined with a confocal laser scanning microscope.

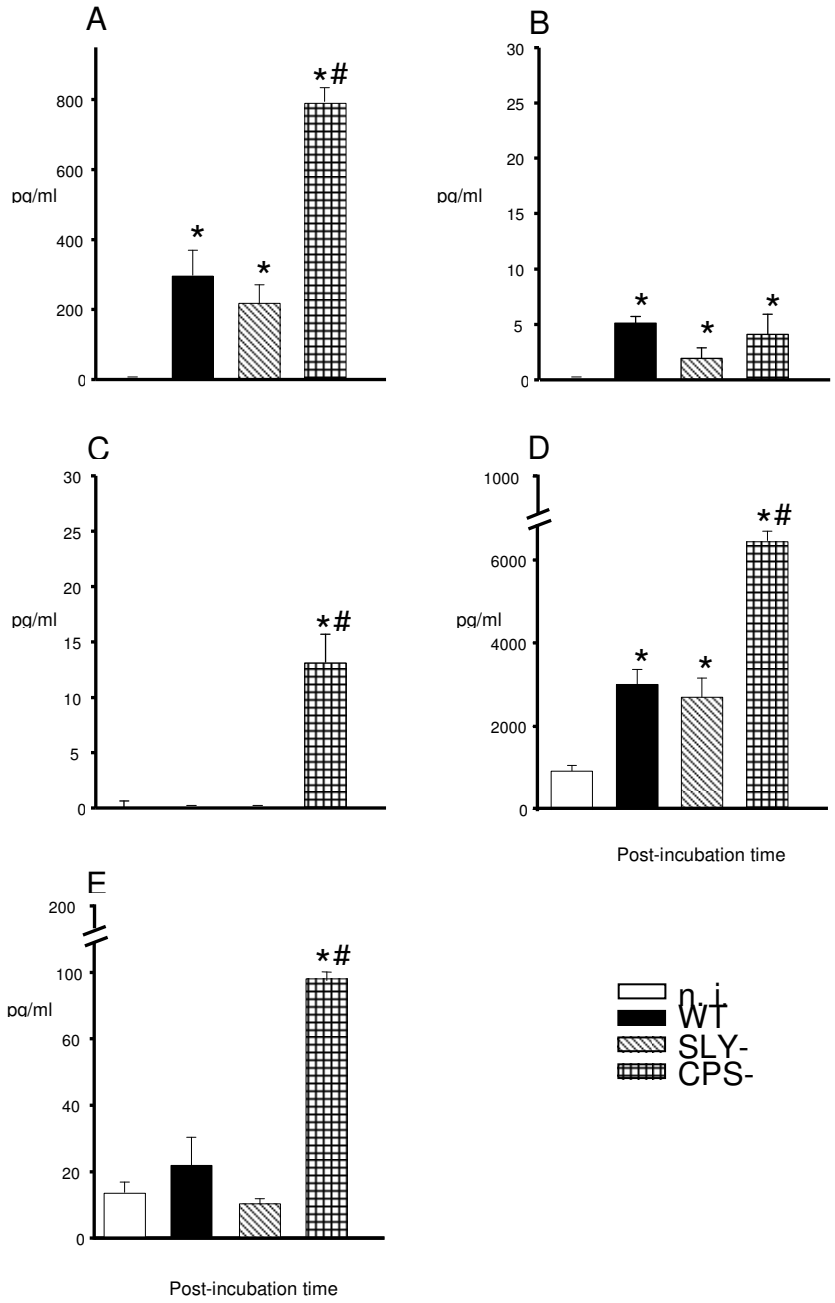


FIG. 3. Comparative study of cytokine production. (A) TNF- α, (B) IL-1β, (C) IL-6, (D) MCP-1, (E) IP-10. Murine microglial cells were incubated with the different *S. suis* strains (1 x 10^6). Culture supernatant were harvested at 12 h post-stimulation and analyzed for cytokine production by ELISA. Data are expressed as mean ± s.d. (in pg/ml) from at least three independent experiments. n.i., non-infected cells. WT, wild-type strain. CPS-, non-encapsulated mutant. SLY-, suilysin-negative mutant. * $P < 0.05$, denotes significant differences with n.i. cells; # $P < 0.05$, denotes significant differences with WT *S. suis* strain.

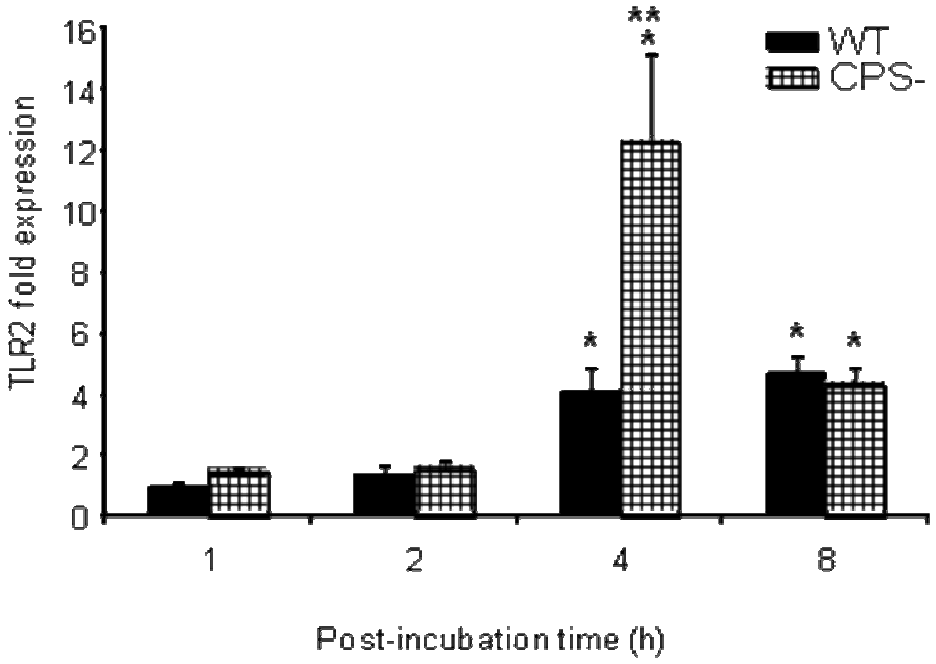


FIG. 4. Increase of TLR2 mRNA expression in the murine microglia cell line BV-2 following *S. suis* exposure. Microglia was stimulated for 1, 2, 4 and 8 h with 1×10^6 *S. suis*. Total RNA was isolated from microglia at the indicated time points and analyzed for TLR2 mRNA expression by real-time quantitative PCR as described in materials and methods. The levels of TLR2 gene expression following *S. suis* was calculated after normalizing cycle thresholds against the 'housekeeping' genes β-Actin and β_2 microglobulin (β2m) using the $2^{-\Delta\Delta Ct}$ method. The results are presented as fold-induction value relative to non-infected microglia (mean ± SEM). * $P < 0.05$ indicates significant differences between infected and non-infected cells. ** $P < 0.001$ indicates significant differences between microglia stimulated with *S. suis* wild-type (WT) versus cells infected with *S. suis* non-encapsulated (CPS-) mutant. Results are mean (± SEM) of three independent experiments.

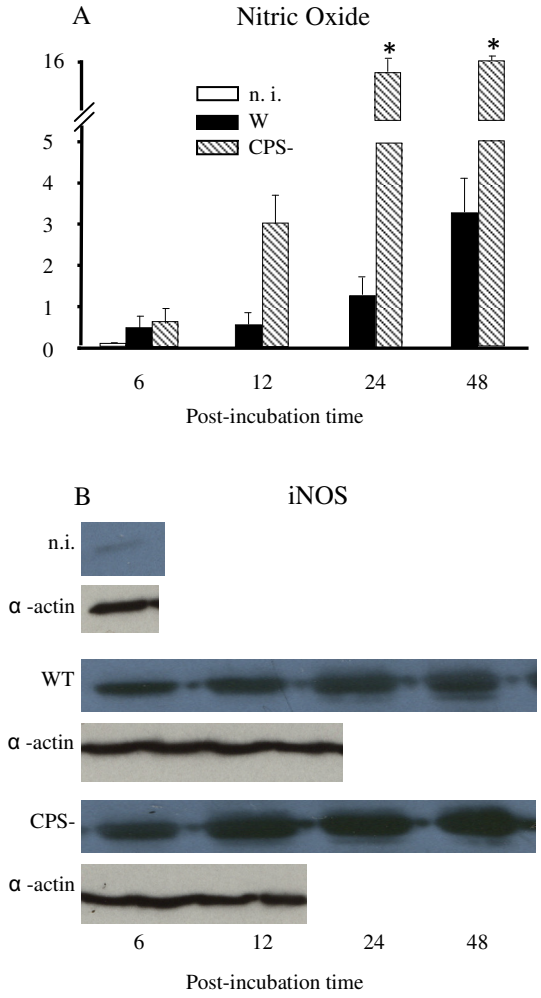


FIG. 5. Time-course increase of nitric oxide production (A) and nitric oxide synthase (iNOS) expression (B) by murine microglial cells treated with *S. suis*. Heat killed *S. suis* wild-type (WT) or non-encapsulated (CPS-) strains (1 ×10^9) were incubated with microglia for 6, 12, 24 and 48 h. (A) Microglia supernatants were collected to measure nitric oxide production by Griess reaction method. Data are expressed as the mean ± SEM (in µM/ml) of three independent experiments. **P* <0.05 denotes significant differences versus WT *S. sui*s. n. i., non-infected cells. (B) Representative western blot analysis of murine microglia extracts using an iNOS-specific antibody. Blotting with anti-α-actin antibody was used as loading control.

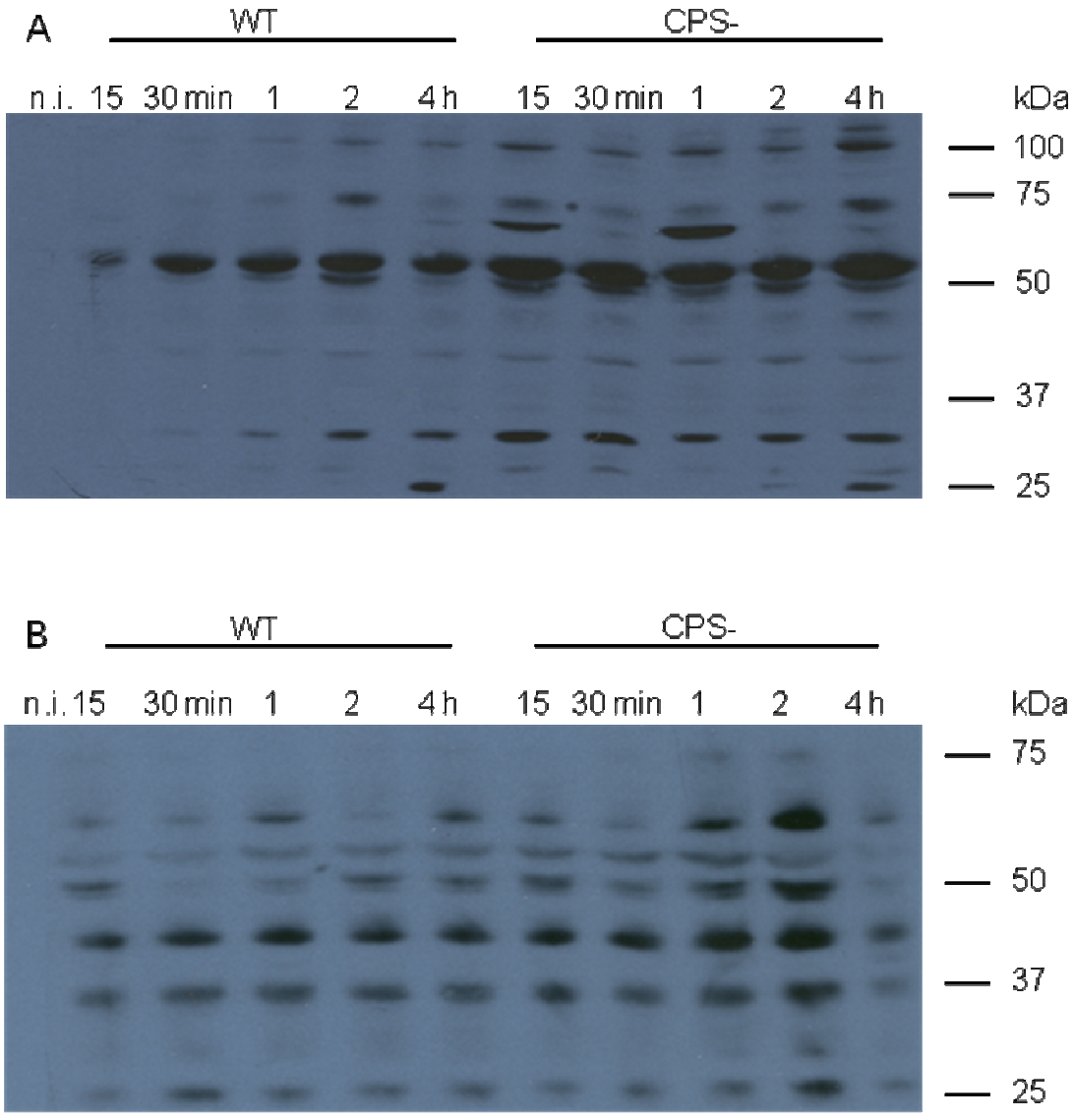


FIG. 6. *S. suis* induced levels of (A) tyrosine (p-Tyr) and (B) serine phosphorylation (p-PKC). Murine microglia BV-2 was infected for 15, 30 min, 1, 2 or 4 h with either the wild-type (WT) strain or its non-encapsulated (CPS-) mutant (1 x 10^6). Cell lysates (total proteins) from non-infected (n.i.) cells and infected cells were subjected to Western blot. p-Tyr and p-PKC protein levels were revealed by using anti-p-Tyr (clone 4G10) or anti-p-PKC monoclonal antibodies, respectively. The results are representative of three individual experiments.

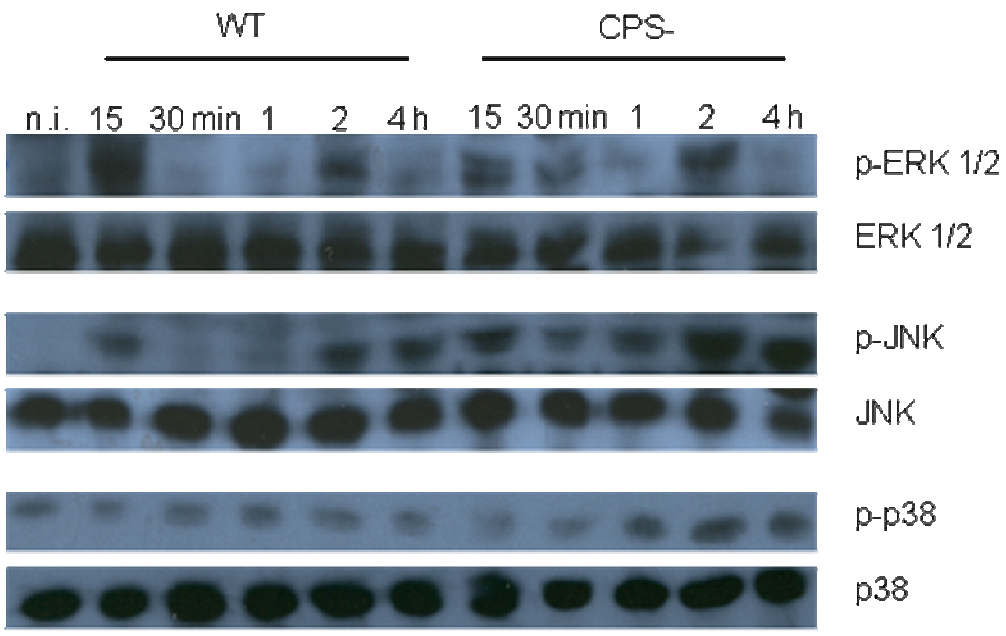


FIG. 7. Time course phosphorylation of MAPKs in BV-2 microglia. Cells were infected with either *S. suis* wild-type (WT) strain or its non-encapsulated (CPS-) mutant (1 x 10^6). Cell extracts were recovered at 15, 30 min, 1, 2 and 4 h post-incubation and were subjected to western blot analysis by using antibodies specific for phospho-MAPKs (p-ERK, p-JNK and p-p38). Following analysis, blots were stripped and reprobed with an antibody specific for ERK, JNK or p38 to verify uniformity in gel loading. Results are representative of three independent experiments. n. i., non-infected cells.

FIG. 8.

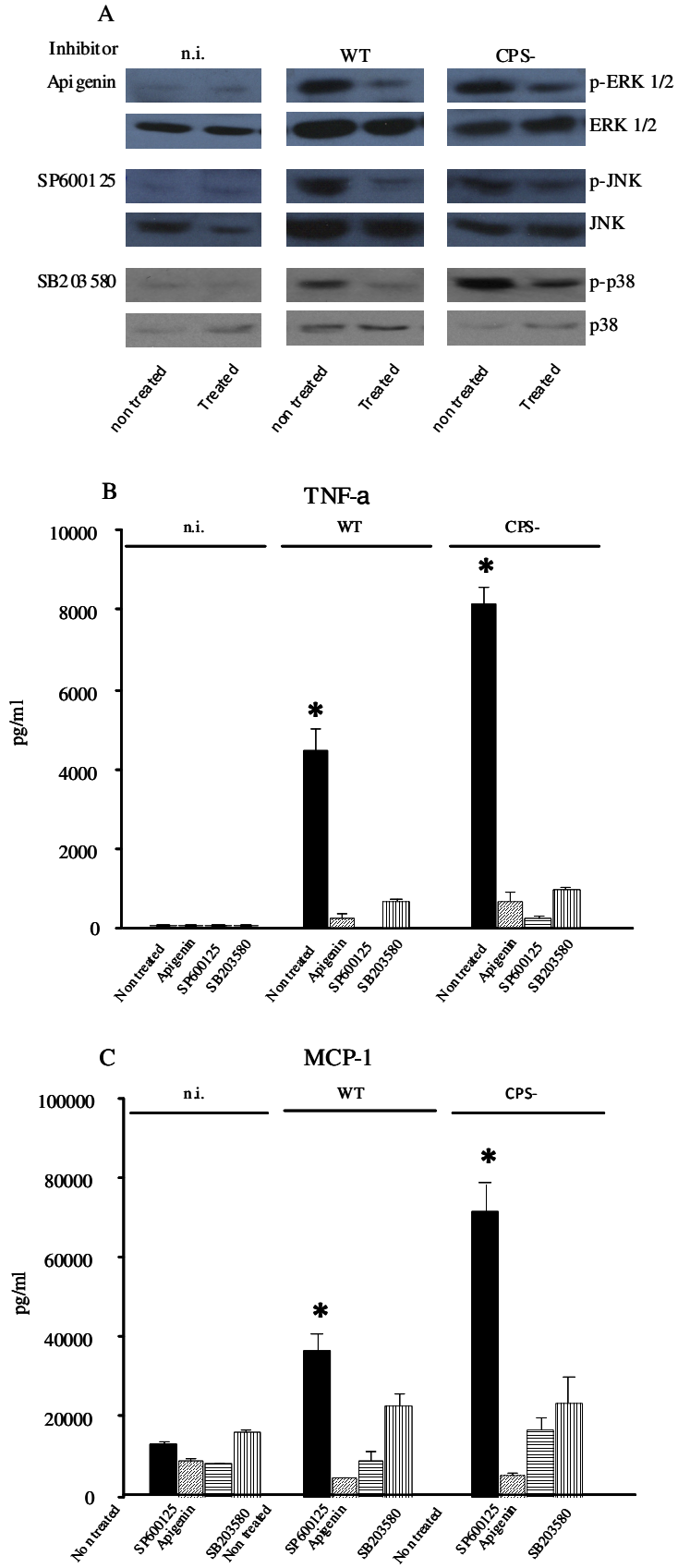

A
Inhibitor
n.i.
WT
CPS-
Apigenin
SP600125
SB203580
p-ERK 1/2
ERK 1/2
p-JNK
JNK
p-p38
p38
non treated
Treated
B
TNF-a
pg/ml
10000
8000
6000
4000
2000
0
Non treated
Apigenin
SP600125
SB203580
C
MCP-1
100000
80000
60000
40000
20000
0

FIG. 8. Pharmacologic inhibition of MAPKs. Murine microglial cells were treated with various inhibitors 1 h prior to infection with *S. suis* wild-type (WT) strain or its non-encapsulated (CPS-) mutant (1×10^6). Apigenin (50 µM), SP600125 (50 µM) and SB203580 (75 µM) inhibit ERK 1/2, JNK and p38, respectively. Inhibitors were all used at maximal subcytotoxic dosis for a total of 13 h. (A) To confirm inhibition of MAPK phosphorylation, cells extracts were recovered after 2 h (p-ERK, p-JNK) or 4 h (p-p38) of bacteria-cell contact, and then analyzed by western blot using specific antibodies for each one of the proteins tested. Results are representative of three independent experiments. To evidence inhibition in cytokine production, cells were infected for 12 h and supernatant recovered for detection of (B) TNF-α and (C) MCP-1 production by ELISA. Data are expressed as mean ± SEM (in pg/ml) from three independent experiments. n.i., non-infected cells. * P <0.05, denotes significant differences with cells treated with MAPK inhibitors.

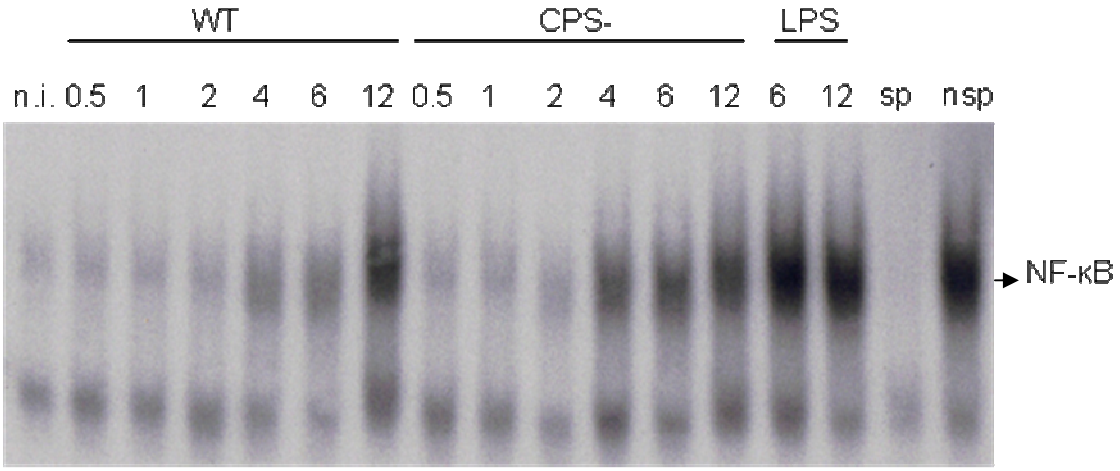


FIG. 9. *S. suis* activates nuclear factor NF-κB in BV-2 microglia cells. Cells were infected with either *S. suis* wild-type (WT) strain or its non-encapsulated (CPS-) mutant (1×10^6).for 0.5 to 12 h. Non-infected (n.i.) cells were used as negative controls. LPS (1 µg/ml) served as positive control. Cells were lysed and nuclear extracts were submitted to an electrophoretic mobility shift assay (EMSA). The presence of NF-κB-activated proteins in the cell nuclei was demonstrated by binding to oligonucleotide probes containing a single copy of the NF-κB-motif 5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3' end labelled with γ-^{32}P ATP. Binding reactions were electrophorezed on native 4% polyacrylamide gels to separate bound and unbound DNA probe. sp, specific probe. nsp, non-specific probe.

ARTICLE III:

"Comparison of the Susceptibility of C57BL/6 and A/J Mouse Strains to *Streptococcus suis* Serotype 2 Infection"

María de la Cruz Domínguez-Punaro,[1] Mariela Segura,[1] Danuta Radzioch,[2] Serge Rivest,[3] Marcelo Gottschalk [1]

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, J2S 7C6, Canada[1]; Centre for the Study of Host Resistance, McGill University Health Centre Research Institute, Montreal, QC, H3G 1A4, Canada[2]; Laboratory of Molecular Endocrinology, Centre Hospitalier de l'Université Laval, St-Foy, QC, G1V 4H2, Canada[3]

* Corresponding author. Mailing address: Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, J2S 7C6, Canada. Phone: (450) 773-8521 × 1-8374. Fax: (450) 778-8108.

Published in : *Infect. Immun.* 2008 Sep;76(9):3901-10

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the experimental design; I substantially performed research, analyzed data and wrote the paper.

ABSTRACT

Streptococcus suis is an important swine and human pathogen. Assessment of susceptibility to *S .suis* using animal models has been limited to monitoring mortality rates. We recently developed a hematogenous model of *S. suis* infection in adult CD1 outbred mice to study the in vivo development of an early septic shock-like syndrome that leads to death and a late phase that clearly induces CNS damage, including meningitis. In the present study, we compared the severity of septic shock-like syndrome caused by *S. suis* between adult C57BL/6 (B6) and A/J inbred mice. Clinical parameters, pro-inflammatory mediators and bacterial clearance were measured to dissect potential immune factors associated with genetic susceptibility to *S. suis* infection. Results showed that A/J mice were significantly more susceptible than B6 mice to *S. suis* infection, especially during the acute septic phase of infection (100% of A/J and 16% of B6 mice died before 24 h post-infection). The greater susceptibility of A/J mice was associated with an exaggerated inflammatory response, as indicated by their higher production of TNF-α, IL-12 p40/p70, IFN-γ and IL-1β, but not with different bacterial loads in the blood. In addition, IL-10 was shown to be responsible, at least in part, for the higher survival in B6 mice. Our findings demonstrate that A/J mice are very susceptible to *S. suis* infection and provide evidence that the balance between pro and anti-inflammatory mediators is crucial for host survival during the septic phase.

INTRODUCTION

Streptococcus suis is one of the most important pathogens affecting the swine industry. This pathogen not only causes septicemia but also affects the central nervous system (CNS) and other tissues, leading to meningitis, endocarditis, pneumonia and arthritis. Although 35 serotypes have been described so far, serotype 2 is still the most frequently isolated from diseased animals (20). The ability of *S. suis* to cause disease is not limited to pigs. In humans, the organism is responsible for cases of septicemia with septic shock, meningitis, endocarditis and other pathologies (2). Human *S. suis* infections are reported mainly in people with occupational exposure to infected pigs or raw pork products, and latest serious outbreaks in Asia have led to critical recognition of *S. suis* as an emerging agent of zoonosis. The most recent outbreak in China resulted in more than 200 human cases which were characterized by an unusual clinical presentation of toxic shock-like syndrome with an increased death rate as high as 20% (50). In Vietnam, *S. suis* is by far the most common cause of adult meningitis (31).

The pathogenesis of infection is poorly understood. To induce clinical disease in swine, it is believed that *S. suis* enters through the respiratory route to colonize the tonsils. In humans, however, the entrance is mainly through skin abrasions, although other routes of infections have also been proposed (3, 16). Thereafter, by mechanisms presently not well understood, it reaches the bloodstream, where it disseminates freely or as cell-bound bacteria attached to phagocytes (15) and causes bacteremia that can, in some cases, lead to fatal septicemia. If the host survives this critical phase of infection, *S. suis* may colonize the CNS and cause meningitis. Our knowledge of the

bacterial components involved in the pathogenesis of *S. suis* infection is also limited. These components include the antiphagocytic polysaccharide capsule, a hemolysin known as suilysin, different cell-wall associated and extracellular proteins, including enzymes (15, 20, 38). The wide distribution of these factors among strains isolated either from asymptomatic or clinically ill animals suggests that pathogenesis of *S. suis* is multi-factorial.

Clinical septicemia with septic shock and meningitis may be related to an exacerbated or uncontrolled inflammatory response that is also, in the case of meningtis, accompanied by an increase in the permeability of the blood-brain barrier (BBB) (15). For example, *S. suis* is able to up-regulate different adhesion molecules expressed by monocytes, thereby increasing leukocyte recruitment to sites of infection and providing a boost to the inflammatory response (1). In addition, it induces the production of different pro-inflammatory cytokines and chemokines by human, mouse and swine leukocytes and by human and swine brain microvascular endothelial cells (BMECs) (39-41, 46). It was reported only recently that human and murine monocytes/macrophages recognize whole *S. suis* or its purified cell wall components mainly through a Toll-like receptor (TLR)-2-dependent pathway, with the possible participation of CD14. This recognition was associated with a triggering of the inflammatory response via a MyD88-dependent downstream signaling pathway and subsequent production of pro-inflammatory cytokines and chemokines (17, 40).

Assessment of the susceptibility to *S. suis* infection in experimental animal models has been limited to the evaluation of overall mortality (4, 23, 49). Recently, our

laboratory developed a hematogenous model of *S. suis* infection in adult CD1 outbred mice to study the *in vivo* development of an early septic shock-like syndrome that causes high mortality and a late phase that clearly induces CNS damage, including meningitis (11). We observed that during the septic phase of infection, *S. suis* induces a Th1 response that is characterized by an up-regulation of key pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-12 (p40/p70), IL-6, IFN-γ, as well as chemokines including CCL2 (MCP-1), CXCL1 (KC) and CCL5 (RANTES). We postulate that these inflammatory mediators are responsible for exacerbating the inflammation and for high host mortality occuring during the septic phase of infection. In mice that develop clinical meningitis, *S. suis* is also able to trigger the expression of pro-inflammatory genes in the brain, including TLR2, TLR3, CD14, IκBα (an index of NFκB expression), IL-1β, TNF-α, and MCP-1 (11).

Development of a suitable animal model enabling researches not only to assess mortality caused by *S. suis* infection, but also to gain new insights into host immune responses to infection with Gram positive bacteria might allow to delineate factors modulating host susceptibility to disease. The aim of this study was to compare the severity of septic shock-like syndrome in adult C57BL/6 versus A/J inbred mice. We measured sepsis-related clinical parameters, pro and anti-inflammatory mediators and bacterial clearance to dissect potential immune factors associated with genetic susceptibility to *S. suis* infection.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. suis* serotype 2 strain 31533 was used for all experimental infections. This encapsulated, suilysin-positive virulent strain has been used largely in cell stimulation studies (39-41) as well as for experimental infections in mice and swine (11, 26). Bacteria were grown overnight on sheep blood agar plates at 37°C and isolated colonies were inoculated into 5 ml of Todd-Hewitt broth (THB) (Difco laboratories, Detroit, MI, USA), which was incubated for 8 h at 37°C with agitation. Working cultures were prepared by transferring 10 µl of 1/1000 dilutions of 8 h-cultures into 30 ml of THB which was incubated for 16 h at 37°C with agitation. Stationary-phase bacteria were washed twice in PBS (pH 7.3). The bacterial pellet was then resuspended and adjusted to a concentration of 5×10^8 CFU/ml. The inoculum for experimental infection was diluted in THB to obtain a final concentration of 1×10^7 CFU/ml. This final suspension was plated onto blood agar to accurately determine the CFU/ml.

Mice and experimental infections. Female 6 to 8-week-old C57BL/6J (B6) or A/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were acclimatized to standard laboratory conditions of 12-h light/12-h dark cycle with free access to rodent chow and water. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals, and approved by the Animal Welfare Committee of the Université de Montréal. On the day of the experiment, a 1 ml volume of either the bacterial suspension (1×10^7 CFU/ml) or the

vehicle solution (sterile THB) was administrated by intraperitoneal injection (i.p.). Two independent preliminary trials were performed to establish the optimal bacterial dose and time points (data not shown).

Study of mortality and clinical signs. A total of 19 mice per mouse strain were closely monitored daily to record mortality and clinical signs of disease, such as depression, rough appearance of hair coat and swollen eyes (11). Weight loss associated with infection was also recorded daily. Mice exhibiting extreme lethargy were considered moribund and were humanely euthanized.

Histopathology studies. For histopathological examination, samples of the brain, heart, liver and spleen were fixed in 10% buffered formalin. After paraffin embedding, 4 μm wide tissue sections were stained with hematoxylin-eosin according to standard protocol and examined under light microscopy.

Study of the systemic inflammatory response. A total of 38 B6 and 36 A/J mice were assessed for bacteriology and systemic levels of inflammatory mediators. Samples were taken at 3, 6, 12, 24, 36, 48 and 72 h post infection (p.i.) and analyzed as described below.

Determination of viable bacteria in organs. At each time point, at least 3 infected and 2 non-infected mice per mouse strain were euthanized with CO_2 and sampled. Blood was collected by cardiac puncture, and the brain, liver and spleen were obtained aseptically. The organs (0.05 g/organ) were trimmed, placed in 500 µl of PBS (pH 7.3), and homogenized with a vortex. Then, 50 µl of serial dilutions of the homogenate in PBS was plated onto blood agar plates. Blood samples (50 µl) were also processed. All samples were plated using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Inc., Norwood, MA). Blood agar plates were incubated overnight at 37°C. Colonies were counted and expressed as CFU/0.05g for organ samples or CFU/ml for blood samples.

Plasma collection and measurement of cytokines and chemokines. Blood from CO_2-anesthetized mice was collected by cardiac puncture into heparinized tubes and centrifuged at 10,000 × *g* for 10 min to obtain plasma. Samples were preserved at -80°C until analysis. Levels of IL-1β, IL-6, IL-10, IL-12 (p40/p70), TNF-α, IFN-γ, CCL2 (MCP-1), CXCL1 (KC) and CCL5 (RANTES) in plasma were determined using a liquid multiarray system (Luminex Co., Austin, TX, USA). Commercial Multiplex-coated beads, biotinylated antibodies and 96-well filter plates were obtained from Upstate Group, Inc. (Lake Placid, NY, USA). Each multiplex assay was performed in duplicate following the manufacturer's specifications. Data were collected using the Luminex-100 system Version IS 2.2 and analyzed by MasterPlex Quantitation Software (MiraiBio, Inc., Alamada, CA, USA). Standard curves for each cytokine and chemokine were obtained

using the reference cytokine and chemokine concentrations supplied by the manufacturer.

Treatment of mice with antibodies or recombinant proteins. The importance of IL-10 for the survival of mice to *S. suis* infection was determined using two separate approaches. In the first approach, neutralization of IL-10 in B6 mice was performed via a single i.p. injection of 1 mg/ml of rat anti-murine IL-10 receptor (IL-10R) monoclonal antibody (mAb) 1B1.3a. The anti-IL-10R mAb was administrated into 10 B6 mice 1 h before infection with *S. suis*. The control group, which consisted of 6 B6 mice, received 1 ml of pyrogen-free saline containing an equivalent quantity of normal rat serum IgG (Jackson ImmunoResearch, West Grove, PA, USA). In the second approach, the role of IL-10 in modulating the survival of A/J mice to *S. suis* infection was tested by i.p. administration of recombinant mouse (rm) IL-10 (R&D Systems, Minneapolis, MN) in 10 A/J mice. At different times relative to the infection, each mouse received a cumulative dose of 2.6 µg rmIL-10 diluted in 300 µl of pyrogen- free saline. The control group of 10 A/J mice received vehicle alone. Injection doses of rmIL-10 or mAb 1B1.3a were selected based on previous publications (5, 7, 8, 21, 25).

Statistical analyses. Overall survival for the different treatment groups were described using Kaplan-Meier plots. Survival curves were compared using the log-rank test. Values for bacterial loads in different tissues as well as serum cytokine levels between A/J and B6 mice were compared using the Mann-Whitney test. A $P < 0.05$ was considered as statistically significant. All analyses were performed using the Sigma Plot System (v.9; Systat Software, San Jose, CA).

RESULTS

C57BL/6 and A/J mouse strains show important differences in their clinical susceptibility to *S. suis* infection. The inbred mouse strains A/J and B6 showed significant differences in clinical behavior and mortality after i.p. infection with *S. suis*. In A/J mice, clinical signs related to infection were observed at 2 h p.i., which quickly worsened and resulted in a mortality rate of 84% at 12 h p.i. and then 100% at 20 h p.i. (Fig. 1). Based on the clinical profile and progression, all deaths were considered related to septicemia. This was confirmed by bacteriological isolation of *S. suis* in different tissues (see below). *S. suis* infection in B6 mice had a slower and different pattern of progression, with subtle clinical signs of acute disease appearing at 8 h p.i. B6 mice showed higher survival compared with A/J mice; at 24 h p.i., only 16% of B6 mice died due to septicemia (Fig. 1). Interestingly, from day 3 p.i. onward, 44% of surviving B6 mice developed sudden neurological signs, such as recumbence, difficult mobility and rear limbs weakness. Furthermore, 57% of B6 mice which showed neurological signs also presented unexpected clinical signs suggestive of cardiac distress. *S. suis* was isolated from different organs of all mice that died from the infection. Weight loss was another clinical characteristic observed in B6 mice infected with *S. suis*. At day 3 p.i., 69% of surviving B6 mice had suffered important weight loss of between 21 and 40% of their initial body weight. However, at day 15 p.i., the remainder of the surviving mice had recovered their initial body weight.

No histopathological lesions were observed in the different tissues isolated from A/J and B6 mice that died during the first 48 h p.i. However, in B6 mice which died at

day 3 p.i. or later, histopathological studies confirmed that the neurological signs were consistent with encephalitis and meningitis, while clinical signs of cardiac distress suggested endocarditis. The main histopathological findings in the brain of mice with clinical signs of neurological disease were the presence of foci of malacia, hemorrhages and gliosis (Fig. 2A). *S. suis* was observed as bacterial emboli contained inside blood vessels with thickened walls due to neutrophil infiltration (Fig. 2B). A severe infiltration of the meninges mainly with macrophages and lymphocytes, as well as fibrin, was also observed (Fig. 2C). Endocarditis was diagnosed on the basis of partial occlusion of the atrio-ventricular right valves by thrombi formed by fibrin, a mix of inflammatory cells, cellular debris and bacteria (Fig. 2D).

***S. suis* is present in different tissues.** Intraperitoneal infection with *S. suis* in both A/J and B6 induced sustained bacteremia, as demonstrated by the recovery of bacteria from infected mice at different p.i. time points. A/J mice had high and sustained bacterial loads in the blood, liver and spleen, with more than 1×10^8 CFU per ml or organ at 3 to 12 h p.i. (Fig. 3A-C). Interestingly, the number of *S. suis* recovered from the brain was lower compared with other organs although it increased with time from 5×10^4 CFU/organ at 3 h p.i. to 8×10^5 CFU/organ at 12 h p.i. (Fig. 3D). No data could be obtained at later times in this mouse strain because 100% of A/J mice died within 20 h p.i. Similar to A/J mice, B6 mice showed bacterial loads in the blood of above 1×10^8 CFU/ml and these loads remained constant up to 48 h p.i. Bacterial loads remained elevated at 72 h p.i. (~1×10^5 CFU/ml), demonstrating the ability of virulent *S.*

suis to survive successfully in the bloodstream (Fig. 3A). Likewise, bacterial loads were high in the liver and spleen of infected B6 mice, with more than 1×10^7 CFU/organ during the first 36 h p.i. (Fig. 3B and 3C). Similar to A/J mice, *S. suis* was found in lower numbers in the brain; however, in these mice, the presence of the pathogen was sustained throughout the experiment, with 1×10^4 to ~1×10^5 CFU/organ at 72 h p.i. (Fig. 3D). There were no statistical differences in site-specific bacterial loads between the A/J and B6 mouse strains at any of the time points tested throughout the experiment ($P > 0.05$).

Profile of the inflammatory response induced by *S. suis* differs remarkably between A/J and C57BL/6 mice. There were important differences in the kinetics of cytokine and chemokine production between the two mouse strains in response to infection with *S. suis*. The inflammatory response in A/J mice could be determined only during the first 12 h p.i. given that, as discussed earlier, all A/J mice died within 20 h after *S. suis* infection. Indeed, in A/J compared with B6 mice, *S. suis* infection induced a rapid systemic production of diverse pro-inflammatory cytokines. There were notable differences in the kinetics of cytokine production, namely for TNF-α, IL-12 (p40/p70), IFN-γ and IL-1β, between the two mouse strains. A significant difference in TNF-α secretion between the mouse strains were found at 6 h p.i. ($P = 0.018$). In A/J mice, TNF-α, one of the most important mediators of septic shock, increased rapidly, peaked at 6 h and was still elevated at 12 h p.i. (Fig. 4A). In B/6 mice, TNF-α increased more slowly, peaking at 12 h p.i. and then declining to basal levels at 24 h p.i. (Fig. 4A). This

rapid and important upregulation of TNF-α in plasma of *S. suis*-infected A/J mice may be related to the early development of clinical signs resembling septic shock and associated death in these mice. A/J mice also presented a rapid and high increase in IL-12p40 production compared to the modest induction of IL-12p40 observed in B6 mice (P = 0.024 at 6 h p.i.) (Fig. 4B). Levels of bioactive IL-12p70, a key cytokine bridging innate and adaptive immunity and IFN-γ, a critical marker of sepsis, were significantly higher in A/J mice than in B6 mice (Fig. 4C and 4D). Production of IL-1β was lower than that of other cytokines, although a significant and sustained expression was observed in A/J compared with B6 mice at 6 h and 12 h p.i. (P = 0.002 and 0.023, respectively) (Fig. 4E). Interestingly, the expression of IL-6, another classical indicator of sepsis, was elevated and sustained in both mouse strains, without any significant difference. (P > 0.05) (Fig. 4F).

Concerning the chemokines, expression of KC, an important neutrophil chemoattractant, was rapidly induced, appearing as early as 3 h p.i., similarly in both mouse strains (P > 0.05) (Fig. 5A). Very little or no KC expression was detected in A/J mice after 12 h p.i, whereas in B6 mice *S. suis*-induced KC production started to decrease only at 36 h p.i. (Fig. 5A). High levels of MCP-1, a molecule with monocyte chemoattractant properties, were observed in both infected mouse strains which showed no significant differences (P > 0.05) (Fig. 5B). Similar to KC and MCP-1, the kinetics of RANTES expression were comparable between the two mouse strains although the levels detected were relatively modest (P > 0.05).

IL-10 plays an important role in controlling the severity of *S. suis*-induced septic infection. As described above, A/J mice showed an exacerbated inflammatory systemic response to *S. suis* infection and, to some extent, were also unable to mount an appropriate anti-inflammatory response. As shown in Fig. 6A, A/J mice secreted relatively low amounts of IL-10, with a highest mean level of 620 pg/ml detected at 12 h p.i. In contrast, B6 mice were able to secrete significantly higher and more sustained levels of IL-10 from 12 h to 36 h p.i., with a mean production peak of 2,610 pg/ml at 12 h p.i. (P = 0.014 vs. A/J mice) (Fig. 6A). We used two different approaches to elucidate the potential role of IL-10 in mediating host protection against lethal *S. suis* infection. First, systemic IL-10R was blocked in B6 mice to assess whether interference with the IL-10 pathway would increase susceptibility to infection and exacerbate mortality. During the first 12 h p.i., B6 mice treated with an anti-IL-10R mAb or vehicle control both showed similar clinical signs such as rough hair coat, depression and recumbence. However, the severity of disease increased with time only in the group which received the anti-IL-10R mAb. Indeed, during the course of the septic phase of disease, B6 mice treated with the anti-IL-10 mAb had significantly higher mortality than the control mice (P = 0.012). Of the B6 mice treated with anti-IL-10 mAb, 50% died at 20 h p.i. and 90% at 24 h p.i. (Fig. 6B). In contrast, only 17% of control mice died at 30 h p.i. and 50% at 72 h p.i. (Fig. 6B). Overall, the survival curves during the first 24 h p.i. were comparable between A/J mice (Fig. 1) and B6 mice treated with anti- IL-10R mAb (Fig. 6B). Confirming the protective role of IL-10, Fig. 7 shows that anti-IL-10R mAb-treated B6 mice produced significantly higher levels of TNF-α 24 h p.i. compared to non-treated B6

mice. Interestingly, levels of TNF-α in treated B6 mice were similar to those observed in A/J mice (Fig. 4A).

In the second approach, we investigated whether administration of rmIL-10 to A/J mice would enhance their survival after *S. suis* infection. To this end, A/J mice received 1 μg of rmIL-10 1 h before and 3 h after i.p. infection with *S. suis* and a final dose of 0.6 μg at 24 h p.i. We observed striking differences in mortality between A/J mice treated with rmIL-10 and non-treated controls; at 20 h p.i., only 10% of the treated mice died compared with 80% of the non-treated controls ($P < 0.001$). At 36 h p.i., 100% of the non-treated controls succumbed to their infection (Fig. 6C), as reported above (Fig. 1), compared with 40% of the treated mice. Although the administration of rmIL-10 significantly prolonged the survival of infected A/J mice, it did not confer complete protection against *S. suis,* as 70% of rmIL-10-treated A/J mice died at 48 h p.i.

DISCUSSION

S. suis is one of the most important causative agents of septicemia, septic shock and meningitis in pigs as well as in humans (20, 33). Most of past research on the activation of the innate immune response by *S. suis* has been conducted using in vitro models of infection. However, animal models are essential to achieve a better understanding of the activation and development of host immune responses to this important pathogen. Although in some diseases mouse models do not reflect the disease outcome in the natural host, we have recently shown for the first time that an adult mouse model for *S. suis* using i.p. route of infection in CD1 mice clearly reproduce clinical signs and lesions observed in swine (11). Kataoka et al, also showed that clinical signs, disease outcome and lesions in mouse models were similar to those in experimentally infected pigs using the same virulent and avirulent bacterial strains. In that study, authors observed differences in susceptibility among several mouse strains and suggested that differences in genetic background or immune responses might explain these discrepancies. However, no further analyses were carried out (23). In this study, we compared the susceptibility of two genetically different inbred mouse strains, A/J and B6, to *S. suis* infection. These mouse strains have been used frequently to investigate the genetic control of host immunity to infectious pathogens. Previous studies using these mouse strains have focused mainly on sepsis induced by endotoxin (LPS) challenge, cecal ligation and puncture (CLP) and systemic candidiasis (28, 30, 34, 42, 44, 45). Study of genetic factors underlying susceptibility to systemic streptococcal infection has been limited mainly to *Streptococcus pneumoniae* and Group A

Streptococci (GAS, *Streptococcus pyogenes*) (13, 14, 22, 24, 28), and most of these studies evaluated only mortality and bacterial load in different tissues. To our knowledge, no studies describing host susceptibility and inflammatory response to *S. suis* infection have been published to date.

In a previous study from our laboratory, *S. suis* showed intermediate virulence in outbred CD1 mice (11). Based on clinical behavior and mortality, the present study clearly demonstrated that A/J mice are significantly more susceptible to *S. suis* infection than B6 mice, especially during the acute septic phase of infection. Indeed, the susceptibility of B6 mice to *S. suis* infection shown in this study, is comparable to that previously observed in CD1 mice. These results are in agreement with studies of *C. albicans* or GAS infection (14, 30) to which A/J mice are highly susceptible while B6 mice show intermediate susceptibility. Nevertheless, overall mortality to GAS infection is similar between B6 and A/J mice, although it is delayed for 48 h in B6 mice (28). Interestingly, these results differ from studies of LPS and cecal ligation and single puncture (CLP) in which A/J mice were considerably more tolerant than B6 mice (42). B6 mice show either high or intermediate susceptibility to infection with *S pneumoniae*, depending on the serotype used (29). Therefore, the genetic susceptibility of the mouse strain may vary depending on the pathogen encountered or injury received.

Consistent with previous studies, mortality in both A/J and B6 mice during the first 48 h after *S. suis* infection can be attributed to a septic shock-like condition in which there is uncontrolled bacteremia and/or an exacerbated production of pro-inflammatory cytokines and chemokines (30, 45). In the present study, the number of

CFUs in the blood, liver, spleen and brain were not significantly different between the mouse strains, arguing against the possibility that the higher mortality in *S. suis*-infected A/J mice was due to uncontrolled bacteremia. Compared with B6 mice, A/J mice are known to carry a loss-of-function mutation in the structural gene of the C5 component of the complement pathway (32, 35). In a model of *C. albicans* infection, there was no conclusive evidence that this deficiency is responsible for the higher fungal replication in the kidney of A/J versus B6 mice (30, 45) and direct fungicidal activity by complement could not be demonstrated (30). Similarly, complement alone, in the absence of specific antibodies, does not contribute to phagocytosis and killing of *S. suis* by monocytes and neutrophils (6). If the complement were essential for bactericidal activity against *S. suis,* higher bacterial load would have been expected in A/J than B6 mice, whereas this study demonstrated no significant differences in the bacteria load between the two strains.

Although a deficiency of complement might not modulate bacterial clearance, some studies suggests that it may play a role in the dysregulated cytokine response observed in A/J mice (30). Accordingly, the high mortality of A/J mice after *S. suis* infection might be caused by an uncontrolled release of pro-inflammatory cytokines. This hypothesis is supported by the lack of specific histopathological findings in A/J (as well as B6) mice which had succumbed to infection within the first 48 h p.i. Different cytokines released early after infection are thought to play opposing roles: pro-inflammatory cytokines are necessary to control infection, although excessive or uncontrolled responses may disturb homeostasis and lead to increased disease severity

and mortality (47). In the present study, we observed that A/J mice had significantly higher production of TNF-α, IL-12p40, IL-12p70, IFN-γ and IL-1β compared with B6 mice, particularly during the first 6 to 12 h p.i. Production of TNF-α, one of the most important acute phase cytokines and a reliable indicator of septic shock in both Gram positive and negative bacterial infections (9, 43), was comparable between B6 (this study) and CD1 mice (11). On the other hand, A/J mice produced twice as much TNF-α as B6 or CD1 mice did. Likewise, A/J mice produced 3 to 19-fold higher levels of IL-12p40, IL-12p70 and IFN-γ than did B6 (this study) or CD1 mice (11). As previously observed in CD1 mice, the release of IL-12p40 preceded that of IL-12p70 following *S. suis* infection (11). In A/J mice, increased IL-12 expression may have been associated with induction of high levels of IFN-γ, a cytokine known for its potent ability to activate macrophages and enhance TNF-α synthesis, thereby exacerbating mortality as previously reported (18, 19). A/J mice had relatively low levels of IL-1 β, as previously reported in CD1 mice (11), but these levels were consistently higher than those in B6 mice and might be sufficient to exert a biological effect. Both A/J and B6 mice produced high levels of IL-6 but to a lesser extent than did CD1 mice (11). Interestingly, there were no significant differences in any of the three chemokines measured, KC, MCP-1 and RANTES, between both mouse strains at all time points. The levels of chemokines observed here were nearly identical to those reported in CD1 mice (11). Since both A/J an B6 mice infected with *S. suis* had secreted similar levels of these chemokines, the difference in mortality observed between the two strains makes these chemokines to

be unlikely candidates contributing to the difference in the development of septic shock-like syndrome after *S. suis* infection between the two mouse strains.

In previous studies of sepsis in mice, a massive secretion of inflammatory mediators was postulated as a likely cause of severe disease and mortality (37). Available data with *S. suis* also suggest that this may be the case in both, swine and humans. We have demonstrated that *S. suis* stimulated swine cells in a whole cell blood system to induce high levels of pro-inflammatory cytokines (41). In addition, we have recently shown that pigs experimentally infected with virulent *S. suis* show high levels of IL-6 and IL-8 (G. Vanier, et al, presented at the First Symposium of the Centre de recherché en infectiologie porcine, Saint-Hyacinthe, QC, Canada, 28-29 May 2007). In humans, *S. suis* is able to induce the release of pro-inflammatory cytokines from brain microvascular endothelial cells (46), and cytokine levels in humans with streptococcal toxic shock-like syndrome due to *S. suis* infection were extremely high and shown to be identical to those observed in C57BL/6 mice experimentally infected with the same strain that cause the Chinese outbreak (C. Ye, H. Zheng, J. Zhang, H. Jing, L. Wang, Y. Xiong, W. Wang, Q. Sun, X. Luo, H. Du , M. Gottschalk, and J. Xu, submitted for publication). Thus, levels of cytokines are a prognosis of human disease severity too.

Differential survival of genetically different mouse strains after infection with invading pathogens has been linked to the ability to control this exacerbated pro-inflammatory cytokine production. As such, Råberg et al. defined “resistance” as the manner in which a host can limit pathogen burden, whereas “tolerance” is defined by the manner in which the host can limit the damage done by a given pathogen burden

(36). The data presented here indicate that the two mice strains have similar loads throughout the first 12 hours after which all A/J mice succumb to infection. This indicates that at least over the first 12 hours, while both mice strains experience the same pathogen load, B6 mice are able to limit the induced damage and thus seem more tolerant. Indeed, the higher survival of B6 mice during the septic phase might be related to their ability to mount a competent anti-inflammatory response, as demonstrated by the high and sustained production of IL-10 from 12 h to 36 p.i. Similar results in CD1 mice were previously reported (11). Other studies have also demonstrated the importance of IL-10 for controlling cytokine production (10, 12) and for survival of mice to septic shock (48). Indeed, high mortality in IL-10 gene knock-out mice is associated with elevated circulating TNF-α levels, and more importantly, early treatment with rIL-10 delays the onset of irreversible shock (27). In the present study, we demonstrated that early systemic blocking of the IL-10R in B6 mice is detrimental for host survival to the septic phase of *S. suis* infection as anti-IL-10R mAb treatment before *S. suis* infection resulted in 90% mortality in B6 mice as early as 24 h p.i. with concomitant and dramatic increase in TNF-□ levels. S*. suis*-infected A/J mice, on the other hand, showed low and nearly undetectable IL-10 production. We hypothesized that the low IL-10 production might be partially responsible for the elevated levels of pro-inflammatory cytokines and the septic shock observed in these mice. In support of this concept, A/J mice treated with rmIL-10 showed 90% survival compared with 20% survival in non-treated mice.

Together these findings suggest that, in *S. suis*-infected mice, IL-10 plays an important role in the early control of sepsis-related inflammatory responses and consequently can improve host survival. Similar protection conferred by rIL-10 treatment has been reported in mice after challenge with CPL, LPS, or GBS infection (8, 21, 25). Interestingly, in agreement with these studies, rmIL-10 treatment delayed but did not abolish mortality due to *S. suis* in A/J mice, indicating that other immunoregulatory factors are also involved in controlling the inflammatory response and influencing long-term survival.

Although B6 mice were more tolerant than A/J mice to the acute phase of infection, surviving B6 mice remained susceptible to developing *S. suis*-induced meningitis and endocarditis. These pathologies are similar to those described in *S. suis*-infected swine, humans, as well as CD1 mice (2, 11, 20). The clinical neurological signs in *S. suis*-infected B6 mice were not as marked as those observed in CD1 mice (11). Nevertheless, our histopathological analysis confirmed neurological disease in the infected B6 mice. These findings raise the possibility that B6 mice are a potentially useful model for future studies of the pathogenesis of *S. suis* infection.

Our findings show a clear susceptibility of A/J mice to *S. suis* infection and provide evidence that the balance between pro and anti-inflammatory mediators is crucial for survival during the septic phase. The different responses elicited in A/J versus B6 mice following systemic infection with *S. suis* warrant future studies using a genome-wide scan with F_2 populations derived from both mouse strains.

ACKNOWLEDGEMENTS

IL-10R (1B1.3a) mAb was kindly provided by Dr. M. Stevenson, Centre for the Study of Host Resistance, Research Institute of the McGill University Heath Centre, Montreal, QC, Canada. We are grateful to Claude Lachance (Research Institute of the McGill University Health Centre, Montréal, QC, Canada) and Sonia Lacouture (Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada) for technical support. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Grant no. 0680154280 and Centre de recherche en infectiologie porcine (Fonds québécois de la recherche sur la nature et les technologies). M.C.D.P. is recipient of a NSERC postgraduate scholarship.

ARTICLE II. FIGURES

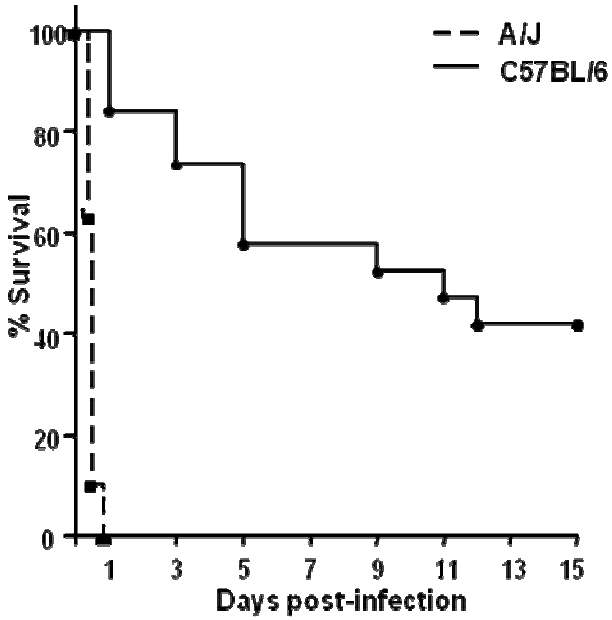


Fig. 1. Survival curves of A/J and C57BL/6 (B6) mice after *S. suis* infection. Mice were injected i.p. with *S. suis* serotype 2 (1×10^7 CFU/ml) and mortality recorded daily for 15 days. *S. suis*-infected A/J mice showed significantly greater mortality during the first 20 h p.i. compared with B6 mice ($P < 0.001$).

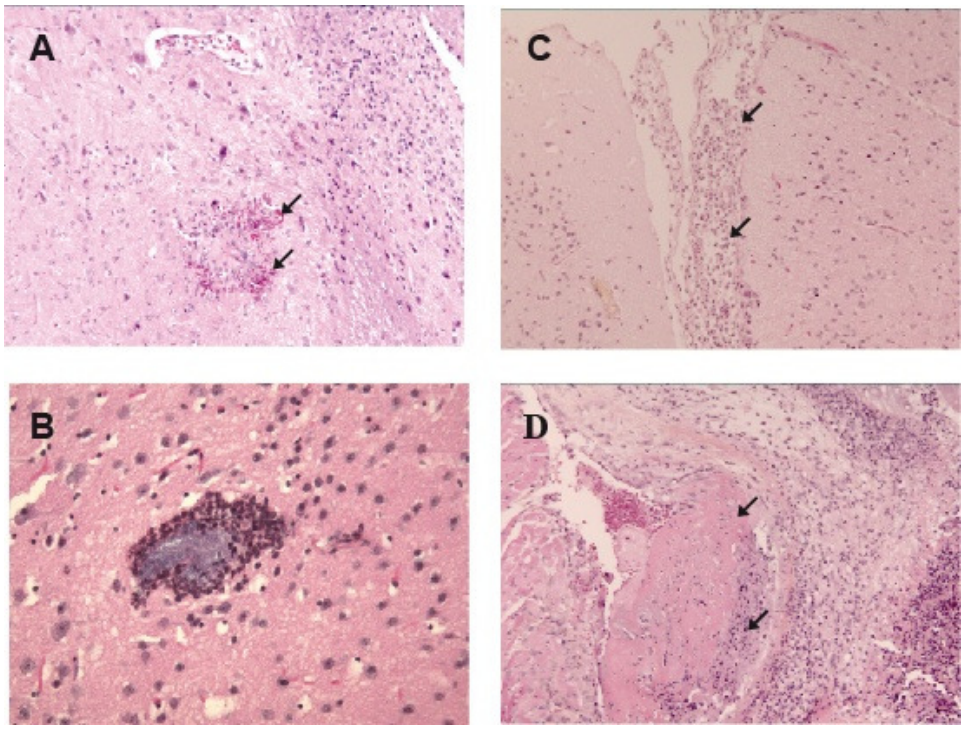


Fig.. 2. Relevant histopathological findings in C57BL/6 mice presenting clinical signs of neurological disease or sudden death (day 9 p.i.) after infection i.p. with *S. suis*. Hematoxilin-eosin staining of brain and heart tissue samples was performed. **(A)** Micrograph of the brain parenchyma showing an area of malacia and hemorrhages (black arrows); magnification, ×100.

(B) Micrograph of the brain cortex in which is evident the presence of a bacterial embolus (*) surrounded by neutrophils; magnification, ×400. **(C)** Micrograph of the meninges, which are severely and diffusely infiltrated by macrophages and other non-suppurative inflammatory cells (black arrows); magnification, ×100. **(D)** Micrograph of the heart at the atrioventricular valves with a thrombus occluding most of the valvular lumen (black arrows). The thrombus comprises fibrin, inflammatory cells and bacteria; magnification, ×200.

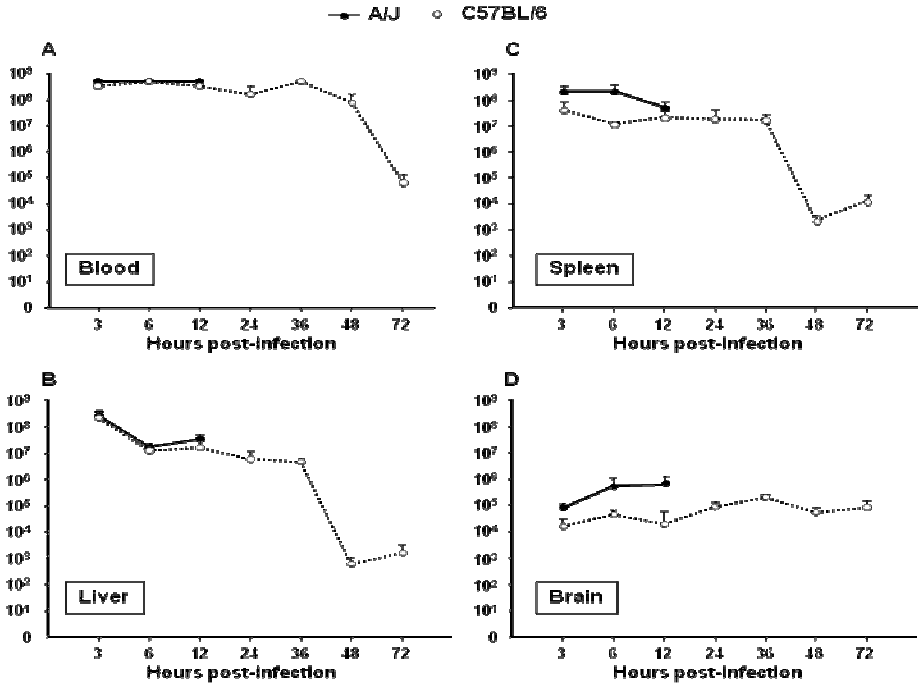


Fig. 3. Bacterial loads in different organs from A/J and C57BL/6 mice infected i.p. with *S. suis*. Bacterial loads are expressed as CFU/ml for blood (A), and as CFU/0.05 g of tissue for the liver (B), spleen (C) and brain (D). Results are expressed as mean ± SEM of at least 3 infected mice per p.i. time point. No significant differences were found between the two mouse strains throughout the experiment ($P > 0.05$).

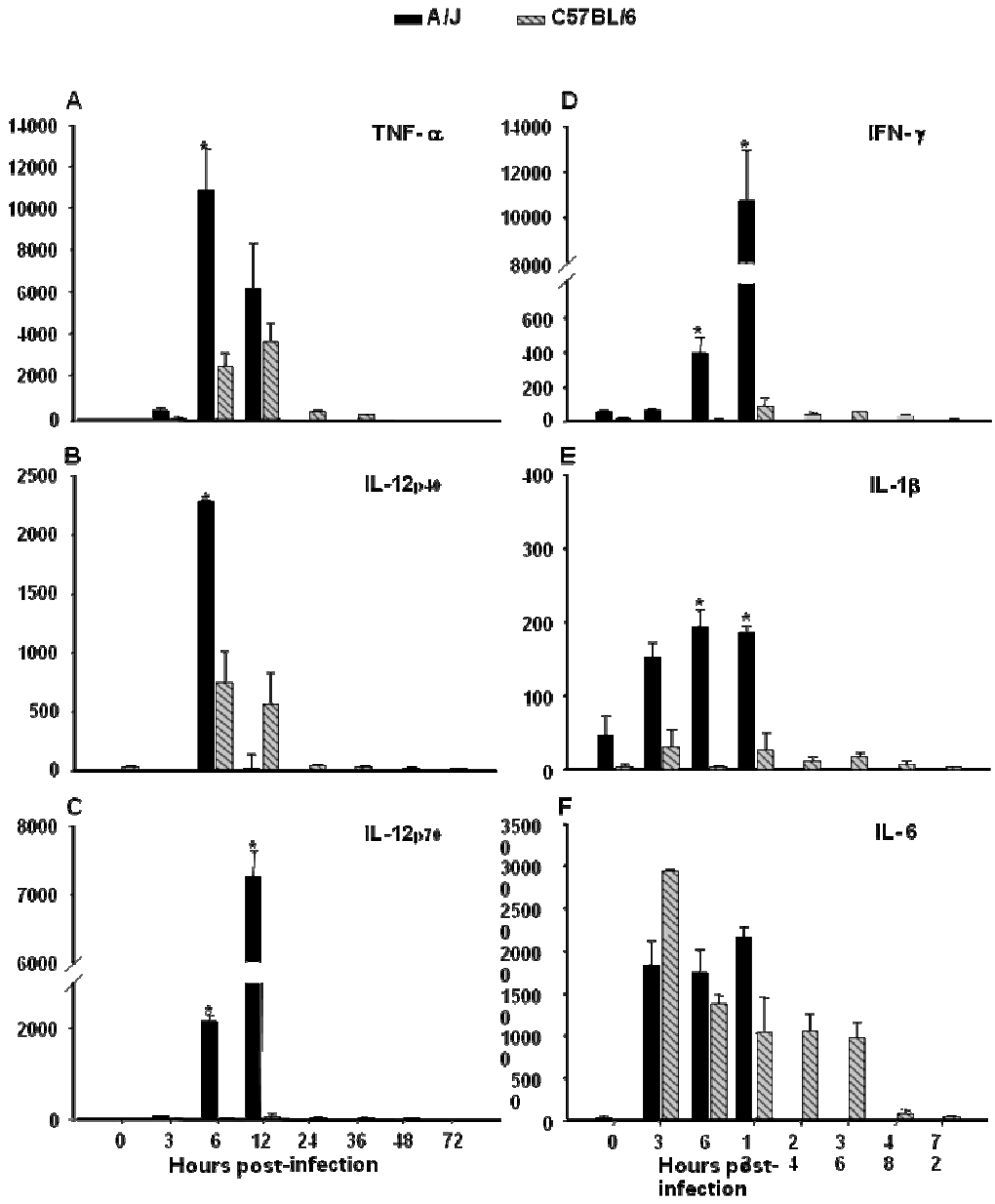


Fig. 4. Kinetics of the expression of inflammatory mediators in A/J and C57BL/6 mice infected i.p. with *S. suis*. (A) TNF-α, (B) IL-12p40, (C) IL-12p70, (D) IFN-γ, (E) IL-1β, and (F) IL-6. Cytokine concentration in sera was assayed by a liquid multiarray system (Luminex), as explained in the Materials and Methods. Data are expressed as mean ± SEM pg/ml. Values of uninfected controls did not show statistically significant changes from 3 h to 72 h. As such, time 0 h represents the mean (± SEM) results of non-infected mice through out the experiment. The asterisk (*) represents significant differences between mouse strains ($P < 0.05$).

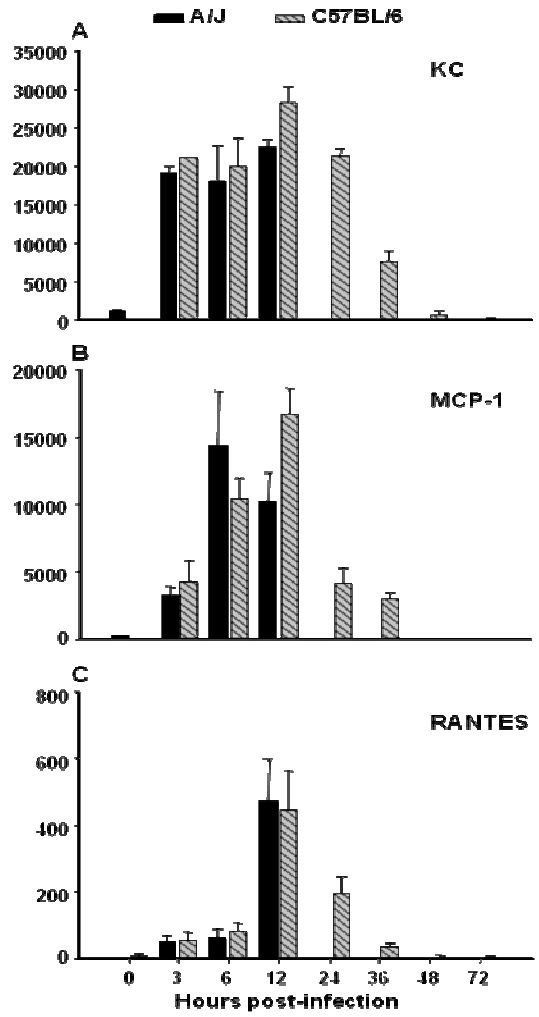


Fig. 5. Kinetics of serum chemokine expression in A/J and C57BL/6 mice infected i.p. with *S. suis*. (A) KC, (B) MCP-1, and (C) RANTES. Chemokine concentration was assayed by a liquid multiarray system (Luminex), as explained in the Materials and Methods. Data are expressed as mean ± SEM pg/ml. Time 0 h represents the mean (± SEM) results of non-infected mice through out the experiment.

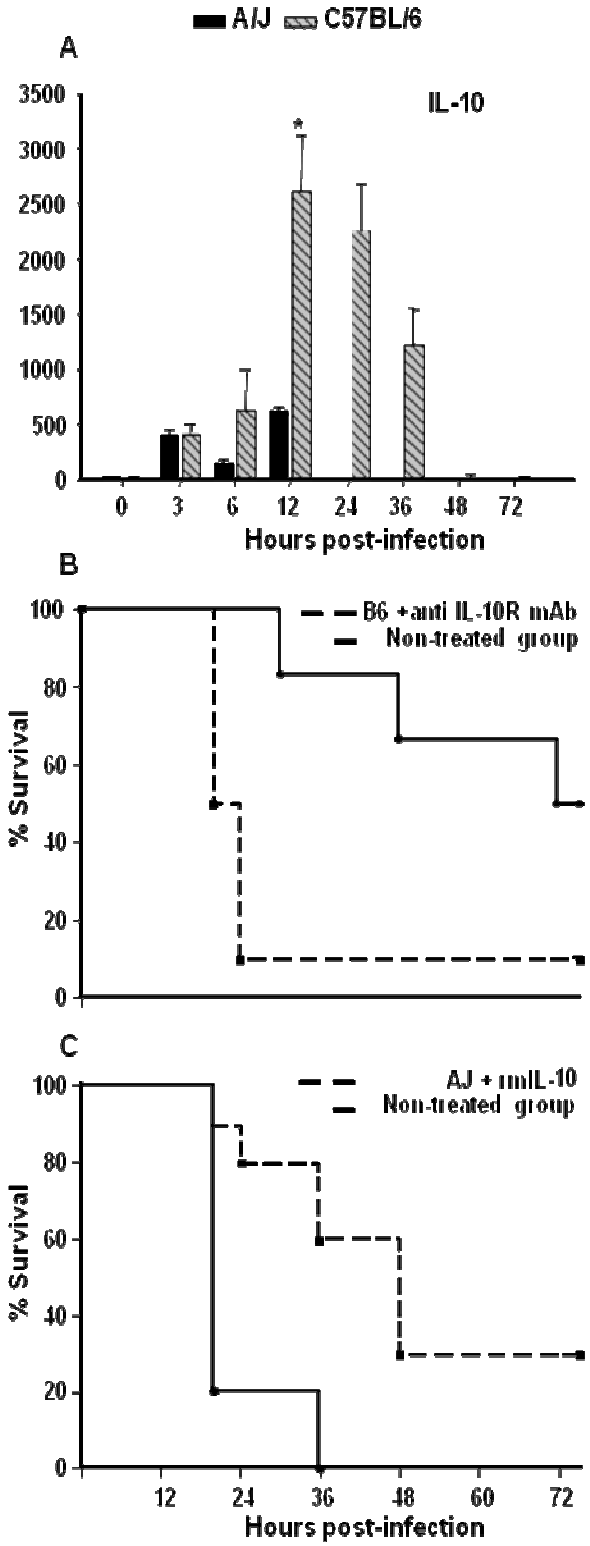


Fig. 6. Role of IL-10 in the survival of A/J and B6 mice after *S. suis* infection. A) Kinetics of serum IL-10 production in A/J and C57BL/6 mice infected i.p. with *S. suis*. IL-10 concentration was assayed by a liquid multiarray system (Luminex), as explained in the Materials and Methods. Data are expressed as mean ± SEM pg/ml. Time 0 h represents the mean (± SEM) results of non-infected mice through out the experiment. The asterisk (*) represents significant differences between mouse strains (P <0.05). **B) Survival of C57BL/6 mice treated with anti-IL-10R mAb (1 mg/ml) prior to challenge with *S. suis*.** Blockage of IL-10R impedes the protective role of IL-10 and thus renders animals significantly more susceptible to *S. suis* infection. This is evidenced by a more rapid course of mortality in treated C57BL/6 mice compared with non-treated controls (P = 0.012). **C) Survival of A/J mice treated with rmIL-10 prior to challenge with *S. suis*.** A/J mice were treated with rmIL-10 at different final concentrations and times relative to the infection. It is notable that rmIL-10 treatment has a protective effect, significantly delaying mortality compared with that observed in the non-treated group (P < 0.001).

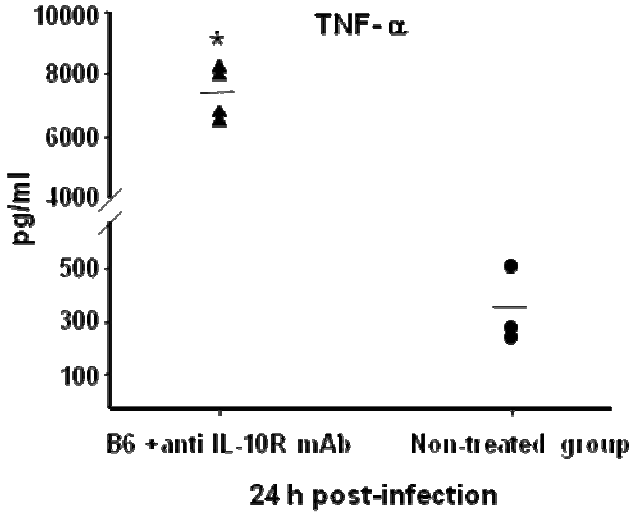


FIG. 7. Effect of blockage of IL-10R on cytokine production. C57BL/6 mice were treated with anti-IL-10R mAb (1 mg/ml) before i.p. infection with *S. suis* serotype 2 (1 × 10^7 CFU/ml). Serum samples were taken at 24 h p.i., and TNF-α levels measured by ELISA kit (R & D System). The asterisk (*) indicates significant differences between treated and non treated groups (*P* <0.001). Symbols represent values from individual mice while the horizontal lines indicate the median for each group.

REFERENCES

1. **Al-Numani, D., M. Segura, M. Doré, and M. Gottschalk.** 2003. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by *Streptococcus suis* serotype 2. Clin. Exp. Immunol. **133:**67-77.

2. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.

3. **Bahloul, H., A. Mofredj, A. Mrabet, G. Gineyt, and P. Rousselier.** 2008. *Streptococcus suis* meningitis after oral contamination? Med. Mal. Infect. **38:**281-282.

4. **Beaudoin, M., R. Higgins, J. Harel, and M. Gottschalk.** 1992. Studies on a murine model for evaluation of virulence of *Streptococcus suis* capsular type 2 isolates. FEMS Microbiol. Lett. **99:**111-116.

5. **Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern, and M. B. Oldstone.** 2006. Interleukin-10 determines viral clearance or persistence in vivo. Nat. Med. **12:**1301-1309.

6. **Chabot-Roy, G., P. J. Willson, M. Segura, S. Lacouture, and M. Gottschalk.** 2006. Phagocytosis and killing o f *Streptococcus suis* by porcine neutrophils. Microb. Pathog. **41:**21-32.

7. **Chiu, B.-C., V. R. Stolberg, M. Freeman, and S. W. Chensue.** 2007. Mononuclear Phagocyte-Derived Interleukin-10 Suppresses the Innate Pulmonary Granuloma Cytokine Response in Aged Mice. Am. J. Pathol. **171:**829-837.

8. **Cusumano, V., F. Genovese, G. Mancuso, M. Carbone, M. T. Fera, and G. Teti.** 1996. Interleukin-10 protects neonatal mice from lethal Group B Streptococcal infection. Infect. Immun. **64:**2850-2852.

9. **De Maio, A., M. L. Mooney, L. E. Matesic, C. N. Paidas, and R. H. Reeves.** 1998. Genetic component in the inflammatory response induced by bacterial lipopolisaccharide. Shock **10:**319-323.

10. **de Waal Malefyt, R., J. Abrams, B. Bennet, C. G. Figdor, and J. E. de Vries.** 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. **174:**1209-1220.

11. **Domínguez-Punaro, M. C., M. Segura, M. M. Plante, S. Lacouture, S. Rivest, and M. Gottschalk.** 2007. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J. Immunol. **179:**1842-1854.

12. **Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'garra.** 1991. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. **147:**3815-3822.

13. **Gingles, N. A., J. E. Alexander, A. Kadioglu, P. W. Andrew, A. Kerr, T. J. Mitchell, E. Hopes, D. P., S. Brown, H. B. Jones, S. Little, G. C. Booth, and W. L. McPheat.** 2001. Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. Infect. Immun. **69:**426-434.

14. **Goldmann, O., G. S. Chhatwal, and E. Medina.** 2004. Role of host genetic factors in susceptibility to group A streptococcal infections. Indian. J. Med. Res. **119 Suppl:**141-143.

15. **Gottschalk, M., and M. Segura.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **76:**259-272.

16. **Gottschalk, M., M. Segura, and J. Xu.** 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim. Health Res. Rev. **8:**29-45.

17. **Graveline, R., M. Segura, D. Radzioch, and M. Gottschalk.** 2007. TLR2-dependent recognition of *Streptococcus suis* is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int. Immunol. **19:**375-389.

18. **Heinzel, F. P.** 1990. The role of IFN-γ in the pathology of experimental endotoxemia. J. Immunol. **145:**2920-2924.

19. **Heinzel, F. P., R. M. Rerko, P. Ling, J. Hakimi, and D. S. Schoenhaut.** 1994. Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of Gamma Interferon. Infect. Immun. **62:**4244-4249.

20. **Higgins, R., and M. Gottschalk.** 2005. Streptococcal diseases, p. 769-783. *In* B. E. Straw, S. D'Allaire, W. L. Mengelin, and D. J. Taylor (ed.), Diseases of Swine. Iowa State University Press, Aimes, Iowa.

21. **Howard, M., T. Muchamuel, S. Andrade, and S. Menon.** 1993. Interleukin 10 protects mice from lethal endotoxemia. J. Exp. Res. **177:**1205-1208.

22. **Kadioglu, A., and P. W. Andrew.** 2005. Susceptibility and resistance to pneumococcal disease in mice. Brief Funct. Genomic Proteomic **4:**241-247.

23. **Kataoka, Y., M. Haritani, M. Mori, M. Kishima, C. Sugimoto, M. Nakazawa, and K. Yamamoto.** 1991. Experimental infections of mice and pigs with *Streptococcus suis* type 2. J. Vet. Med. Sci. **53:**1043-1049.

24. **Kerr, A. R., J. J. Irvine, J. J. Search, N. A. Gingles, A. Kadioglu, P. W. Andrew, W. L. McPheat, C. G. Booth, and T. J. Mitchell.** 2002. Role of

inflammatory mediators in resistance and susceptibility to pneumococcal infection. Infect. Immun. **30:**1547-1557.

25. **Latifi, S. Q., M. A. O'Riordan, and A. D. Levine.** 2002. Interleukin-10 controls the onset of irreversible septic shock. Infect. Immun. **70:**4441-4446.

26. **Lun, S., J. Perez-Casal, W. Connor, and P. Willson.** 2003. Role of the suilysin in pathogenesis of *Streptococcus suis* capsular type 2. Microb. Pathogen. **34:**27-37.

27. **Manley, M. O., M. A. O'Riordan, A. D. Levine, and S. Q. Latifi.** 2005. Interleukin 10 extends the effectiveness of standard therapy during late sepsis with serum interleukin 6 levels predicting autcome. Shock **23:**521-526.

28. **Medina, E., and A. Lengeling.** 2005. Genetic regulation of host responses to group A streptococcus in mice. Brief Funct. Genomic Proteomic **4:**248-257.

29. **Mizrachi-Nebelzahl, Y., S. Liftshitz, R. Teitelbaum, S. Novick, A. Levi, D. Benharroch, E. Ling, and R. Dagan.** 2003. Differential activation of the immune system by virulent *Streptococcus pneumoniae* strains determines recovery or death of the host. Clin. Exp. Immunol. **134:**23-31.

30. **Mullick, A., M. Elias, S. Picard, L. Bourget, O. Jovevski, S. Gauthier, A. Tuite, P. Harakidas, C. Bihum, B. Maisse, and P. Gros.** 2004. Dysregulated inflammatory response to *Candida albicans* in a C5-deficient mouse strain. Infect. Immun. **72:**5868-5876.

31. **Nguyen Thi, H. M., H. Ngo Thi, T. N. Tran Vu, L. Le Dieu, H. C. Tran Thi, X. S. Dinh, H. P. Nguyen, V. C. Ly, S. D. To, J. Campbell, T. N. Ho Dang, N. M. Tran, V. V. C. Nguyen, M. D. de Jong, T. C. Nguyen, T. H. Tran, J. Farrar, and C. Schultsz.** 2008. *Streptococcus suis* meningitis in adults in Vietnam. Emerg. Infect. Dis. **In press**.

32. **Nilsson, U. R., and H. J. Muller-Eberhard.** 1967. Deficiency of the fifth component of complement in mice with an inherited complement defect. J. Exp. Med. **125:**1-16.

33. **Normile, D.** 2005. Infectious diseases. WHO probes deadliness of China's pig-borne disease. Science **309:**1308-1309.

34. **O'Malley, J., L. E. Matesic, M. C. Zink, J. D. Strandberg, M. L. Mooney, A. De Maio, and R. H. Reeves.** 1998. Comparison of acute endotoxin-induced lesions in A/J and C57BL/6 mice. J. Hered. **89:**525-530.

35. **Ooi, Y. M., and H. R. Colten.** 1979. Genetic defect in secretion of complement in C5 in mice. Nature **282:**207-208.

36. **Råberg, L., D. Sim, and A. F. Read.** 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science **318:**812-814.

37. **Remick, D. G., D. R. Call, S. J. Ebong, D. E. Newcomb, P. Nybom, J. A. Nemzek, and G. E. Bolgos.** 2001. Combination immunotheraphy with soluble tumor necrosis factor receptors plus interleukin 1 receptor antagonist decreases sepsis mortality. Crit. Care Med. **29:**473-481.

38. **Segura, M., and M. Gottschalk.** 2004. Extracellular virulence factors of streptococci associated with animal diseases. Front. Biosci. **9:**1157-1188.

39. **Segura, M., J. Stankova, and M. Gottschalk.** 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect. Immun. **67:**4646-4654.

40. **Segura, M., N. Vadeboncoeur, and M. Gottschalk.** 2002. CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by *Streptococcus suis* capsular type 2. Clin. Exp. Immunol. **127:**243-254.

41. **Segura, M., G. Vanier, D. Al-Numani, S. Lacouture, M. Olivier, and M. Gottschalk.** 2006. Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system. FEMS Immunol. Med. Microbiol. **47:** 92-106.

42. **Stewart, D., W. B. Fulton, C. Wilson, C. L. Monitto, C. N. Paidas, R. H. Reeves, and A. De Maio.** 2002. Genetic contribution to the septic response in a mouse model. Shock **18:**342-347.

43. **Teti, G., G. Mancuso, and F. Tomasello.** 1993. Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection. Infect. Immun. **61:**227-235.

44. **Trentzsch, H., D. Stewart, C. N. Paidas, and A. De Maio.** 2003. The combination of polymicrobial sepsis and endotoxin results in an inflammatory process that could not be predicted from the independent insults. J Surg Res **111:**203-208.

45. **Tuite, A., S. Picard, A. Mullick, and P. Gros.** 2005. Genetic control of susceptibility to *Candida albicans* in susceptible A/J and resistant C57BL/6 mice. Genes Immun **6:**672-682.

46. **Vadeboncoeur, N., M. Segura, M. Doré, and M. Gottschalk.** 2003. Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by *Streptococcus suis* serotype 2. FEMS Immunol. Med. Microbiol. **35:**49-58.

47. **van Amersfoot, E. S., T. J. Berkel, and J. Kuiper.** 2003. Receptors, mediators and mechanisms involved in bacterial sepsis and septic shock. Clin. Microbiol. Rev. **8:**974-983.

48. **van der Poll, T., A. Marchant, W. A. Buurman, L. Berman, C. V. Keogh, D. D. Lazarus, L. Nguyen, M. Gold**1995. Endogenous IL-10 protects mice from death during septic peritonitis. J. Immunol. **155:**5397-5401.

49. **Williams, A. E., W. F. Blakemore, and T. J. L. Alexander.** 1988. A murine model of *Streptococcus suis* type 2 meningitis in the pig. Research Vet. Science **45:**394-399.

50. **Yu, H., H. Jing, Z. Chen, H. Zheng, X. Zhu, H. Wang, S. Wang, L. Liu, R. Zu, L. Luo, N. Xiang, H. Liu, X. Liu, Y. Shu, S. S. Lee, S. K. Chuang, Y. Wang, J. Xu, and W. Yang.** 2006. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg. Infect. Dis. **12:**914-920.

IV.DISCUSSION

1. **Validation of a mouse model of infection for the study of the pathogenesis of *S. suis* infection**

As previously mentioned, the pig is the natural host of *S. suis* infection, and logically, it should be the best animal model. However, there are many obstacles at this respect. First, there is a lack of uniformity in experimental animals, as research has been carried out using pigs of different ages and with diverse genetic backgrounds, testing different inoculation routes or dose of inoculum, and considering different parameters to determine virulence. Second, many of the techniques and reagents necessary to identify host factors associated with the development of the infection and subsequent inflammation are not standardized for pig samples. Third, the use of the pig as animal model is limited due to handling of the animals, and the availability of facilities to carry out such experiments. To date, there is only one standardized pig model of *S. suis* infection, which requires the use of specific-pathogen-free (SPF) piglets, inoculated by the intravenous route, and kept in separated units, with filtered air-flow [133]. This model has been useful to determine virulence of field *S. suis* strains, and to measure different clinical signs, zootechnical performances, lesions, and bacteriological findings [133].

Mice may be an excellent alternative to carry out research on the pathogenesis of *S. suis* infection and to understand the mechanisms this pathogen uses to cause disease in pigs and humans. On one hand, as previously mentioned, the pig should be the first choice to develop an animal model for *S. suis* research, but unfortunately the lack of standardized models and availability of reagents limit its use. However, the mouse has been used to study the pathogenesis of some pig pathogens, including *Pasteurella multocida* (Progressive atrophic rhinitis) [468], *Actinobacillus suis* [469] and Pseudorabies virus [470]. It is also widely accepted that mice are an excellent organism to understand human infectious diseases. Despite some differences, the immune systems of mice and humans are similar [427]. In addition, their physiological responses can be studied in an environment in which pathogen associated variables such as strain, virulence, dose, and route of infection can be carefully controlled. In addition, a wide variety of immunological and biochemical reagents in the form of assays, markers, and antibodies are available to characterize innate and adaptive immune responses in these infection models [471]. The

mouse has been widely used in the study of the pathogenesis of infections due to many meningitis- and sepsis-causing bacteria, including *S. pneumoniae* [432], *L. monocytogenes* [435], *H. influenzae* [443], *N. meningitidis* [444], and Group B *Streptococcus* (GBS) [472], among others.

In meningitis models of infection using adult mice, they are typically infected with live microorganisms by the intracisternal, intracerabral or intracranial routes of infection [296, 432, 440], which are traumatic, require anesthesia and special handling of the animal. In addition, these models bypass several steps, such as bacterial dissemination and increased permeability of the blood-brain barrier (BBB), which is the natural portal of entry into the CNS for most meningeal pathogens. In contrast, challenge of adult animals using the intravenous or intraperitoneal routes, usually results in bacteremia and septicemia, leading sometimes to rapid death of infected animals with modest, if any, brain pathology [432, 445]. In the case of infant animals are used, results are highly influenced by the route of infection chosen and mice strain selected [447, 448].

Although many researchers have used the mouse as model of infection to study *S. suis* infections, they have mainly evaluated overall mortality [473, 474]. In the present research, we successfully developed a mouse model of *S. suis* infection using the intraperitoneal route of infection, which better represents the natural course of infection (Articles I and III). With this model, we were able to study the two phases of *S. suis* infection, characterized by an early septic shock-like syndrome leading to death and a second late phase that induces evident CNS damage and clear clinical signs of neurologic disease. The use of this model allowed us to have a more detailed picture of the development of events leading to inflammation (systemic and the CNS), we were able to analyze the expression (at both mRNA and protein levels) of relevant pro-inflammatory mediators, and we studied the clinical progress of infection and development of sepsis and meningitis. It also served to study possible genetic host factors that may influence the progress of the disease (Article II). Furthermore, the CD1 model allowed us to compare the virulence of several strains deficient in putative *S. suis* virulence factors that may facilitate the development of inflammation and invasion of tissues (Annex, articles V to VIII) [40, 163, 272, 287]. In addition the capsule (CPS) and suilysin (SLY), components from the cell wall may help bacteria to develop a sustained bacteremia and colonize tissues. In this

regard, the CD1 model has been used to evaluate the role of some of these cell wall components. Indeed, the D-alanylation of lipoteichoic acid (LTA), has been postulated as a crucial virulence factor of *S. suis*. In vitro studies using a *S. suis* strain deficient in D-alanylation of LTA (*dltA*-), provided evidence that this virulence factor helps bacteria resist the action of cationic antimicrobial peptides and killing by neutrophils. Challenge studies in CD1 mice demonstrated that *in vivo*, the D-alanylation of LTA helps results in sustained bacteremia and therefore, facilitates invasion of tissues. When mice were infected with a high dose of either a wild type strain (WT)or its the *dltA*- mutant, severe clinical signs associated with septicemia, and similar bacterial concentrations in blood and different organs were observed. This phase of the disease culminated in the death of several mice from both groups during the first 72 h post-infection. However, when mice were infected with an intermediate bacterial dose, no deaths associated with septicemia in any group. The findings obtained in mice were validated in a pig *S. suis* model of infection, in which the absence of D-alanylation of LTA also impaired the virulence of the *dltA*- mutant (Annex, Article V) [163]. Other components of the cell wall possibly involved in the development of *S. suis* disease include the N-acetylation of peptidoglycan (PG), that may help bacteria to avoid recognition by phagocytic cells and to resist the effect of lysozyme. Indeed, the absence of N-acetylation of *S. suis* PG severely impaired the ability of the bacteria to cause sustained bacteremia and colonize different tissues in the mouse *S. suis* model. No major clinical signs associated with *S. suis* infection, no deaths were recorded and bacteria was cleared from circulation during the first three days of infection, mice inoculated with a *S. suis* mutant deficient in PG N-acetylation (*pgdA*-). In contrast, sustained levels of *S. suis* for several days in blood and different organs in mice inoculated with the WT strain. Moreover, sustained bacteremia in mice infected with the WT strain was accompanied by a strong production of pro-inflammatory cytokines. Similar findings were observed when virulence of the *pgdA*- mutant was assessed in a pig model of *S. suis* infection, validating the results obtained with mice (Annex, Article VI) [40].

The repeatability of the bacteremia and mortality results in CD1 mice infected with different WT strains of *S. suis*, allowed us to use this model of infection to also investigate the importance of many putative virulence factors in the development of septicemia.

Different *S. suis* cell surfaces proteins possess a Leu-Pro-X-Thr-Gly (LPXTG) motif that may serve to mediate bacterial interactions with host cells, namely those from the extracellular matrix. These LPXTG cell surface proteins are anchored to the *S. suis* cell wall by a Sortase A (srtA), whose role in disease was evaluated by constructing a *S. suis* strain deficient in sortase A (*srtA*-) and comparing its virulence against its WT parental strain. The *in vivo* trials in the CD1 *S. suis* model, revealed no difference in virulence between the WT strain and the *srtA*- mutant, as the two strains caused sustained bacteremia and similar mortality, indicating that anchorage of LPXTG proteins alone is not sufficient for virulence in mice (Annex, Article VII) [287]. Another example of the importance of bacteremia for the development of the disease and further mortality in the CD1 model is the evaluation of *S. suis* mutants deficient in cell surface subtilisin-like proteinase. Proteases are hydrolytic enzymes that catalyze the cleavage of peptide bonds and are critical virulence factors for numerous microbial pathogens. These enzymes hydrolyze a variety of host proteins, including serum and tissue components, thus helping to neutralize the host immune defense system and causing tissue destruction and invasion. The subtilisine-like protease of *S. suis* contains a cell wall sorting signal LPXTG that is responsible for covalently anchoring proteins to cell wall PG. CD1 mice infected with two *S. suis* mutants deficient in subtilisine-like proteinase presented a significantly higher survival rate in comparison to those mice infected with the parental WT strain. Survival was associated to the impairment of these two subtilisine-like protease deficient mutants in their ability to cause sustained bacteremia and septicemia [475].

In addition, this mouse model of infection was used to screen the virulence of five *S. suis* mutants that have a reduced invasion to porcine brain microvascular endothelial cells (PBMEC). The *in vivo* experiments confirmed that sustained bacteremia is crucial for the development of the disease, and that although some putative virulence factors may have a more active role when performing *in vitro* experiments, they are not critical for the development of the infection. Indeed, only two of the five mutants tested presented a significant diminution in virulence in comparison to their WT parental strain, causing less mortality and less severe clinical signs in mice. In fact, these two less aggressive mutants

rapidly disappeared from blood and were less invasive in different tissues (Annex, Article VIII) [272].

Finally, the CD1 model was used to evaluate potential vaccine candidates. *S. suis* possesses a alpha-enolase (SsEno) that binds specifically to fibronectin and plasminogen and that has a role in the bacterial adhesion and invasion of PBMEC [210]. It was postulated that similarly to other bacterial fibronectin-binding proteins, SsEno may contribute to the virulence of *S. suis* [210]. In addition, as purified SsEno is highly immunogenic in mice, it was hypothesized that it could be useful as a vaccine candidate. However, vaccination trials demonstrated that although vaccination with SsEno elicits a strong humoral response, it is not protective as, immunized and non immunized mice challenged with a virulent WT *S. suis* strain, presented similar clinical signs and mortality rates (Annex, Article IV) [125].

2. Development of the acute phase of *S. suis* infection

2.1 Septicemia with high mortality is a first phase of *S. suis* infection: the role of the exaggerated inflammatory response

In CD1 mice, virulent *S. suis* induced a high and constant bacteremia for several days, and number of CFU in blood, liver and spleen were routinely high in all mice. This sustained bacteremia is likely to be attributed to the resistance of *S. suis* to phagocytosis by neutrophils and macrophages in the absence of specific IgG [142, 264]. Bacteria-released components, such as SLY may enhance bacterial dissemination due to their cytotoxic effect on phagocytic, endothelial and epithelial cells [4, 149, 282, 476]. Moreover, SLY may prevent complement deposition on *S. suis*. The toxin might reduce the amount of complement available for the opsonization of the bacteria, as has been observed with swine neutrophils and, more recently, with mouse dendritic cells [264, 477]. However, bacteremia was not always fatal, as in CD1 mice it caused only 20% mortality within 48 h post-infection (p.i.). In contrast to our findings, in other streptococcal diseases, the ability

of the host to decrease the bacterial loads seems to be critical for survival. For example, in mouse models of septicemia with *S. pneumoniae*, blood bacterial titers are not sustained, as they decrease during the first 36 h p.i. with a new increase to the original inoculation dose at 48 h p.i. although no mortality is recorded during this second period [478].

Different studies have demonstrated that the outcome of the immune response to infection is determined, in part, by the degree of inflammation it generates. Inflammatory cytokines, produced by cells including macrophages and neutrophils, are fundamental in generating an effective primary immune response and in clearing infection. However, excessive production of pro-inflammatory cytokines not only enhances immune responses to fight the invading pathogen, but also has deleterious effects that perturb regular hemodynamic and metabolic balances. In humans with severe sepsis, the production of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, among others, causes septic shock which has a mortality of >50% even when appropriate antimicrobial therapy is administered [479, 480].

Few studies have measured the kinetics of production of pro-inflammatory cytokines at the protein level *in vivo* after an experimental infection with Gram-positive bacteria [481-484]; most of these studies measured a limited number of mediators. The use of a multiplex system in this study allowed us to quantitatively measure the kinetics of production of several cytokines simultaneously in a small volume of plasma *in vivo* [485]. We took advantage of this system to compare the levels of cytokine between infected and non infected mice and to ascertain which particular cytokines were involved in the development of septic shock. Although it is not possible to make a direct comparison between *in vitro* and *in vivo* studies, kinetics of systemic cytokine and chemokine production found in the CD1 mouse model share some similarities with previous *in vitro* reports using the same strain of *S. suis.* These reports showed that high levels of TNF-α, and significant levels of IL-1β, IL-6, IL-8 and MCP-1, were released by activated mouse and human monocytic cells as well as by total swine whole blood leukocytes [486-488]. Moreover, in a pig model of *S. suis* infection, a substantial production of IL-6 was observed, which correlated with a high fever index [272].

In the present *in vivo* studies, high levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-12 and IFN-γ and the chemokines MCP-1, KC and RANTES were observed during the first 24 h p.i. and were probably, at least in part, responsible for the sudden death of 20% of CD1. A general examination of the cytokine production suggests that in infected CD1 mice *S. suis* would develop a Th1 type immune response. We observed that the inflammatory systemic response began with the early secretion of TNF-α. This cytokine that is mainly secreted by monocytes in response to inflammatory stimuli has been associated in the pathogenesis of toxic shock-like syndrome (TSLS) caused by Gram-positive bacteria, especially when high bacterial doses are used. Rodents infected with GBS demonstrated a significant increase of TNF-α levels within 12 - 72 h after challenge, high levels of production correlating with mortality. TNF-α production is followed by high levels of IL-1 and IL-6 [489, 490]. In humans with sepsis, TNF-α is thought to be a major factor contributing to multiple organ failure related to a high mortality rate [373]. High levels of systemic TNF-α may also contribute to increased permeability of the BBB [491], facilitating invasion of *S. suis* to the brain. Infected mice also showed high levels of IL-6. However, the impact of some cytokines in the outcome of sepsis remains controversial, as is the case with IL-6. This cytokine is considered to have pro-inflammatory and anti-inflammatory effects, one evident feature being the ability to induce production of acute phase proteins in the liver [492]. It is known that IL-6 is greatly up-regulated in the serum of patients with bacterial infection of sepsis, [361], and many studies have reported a positive correlation between IL-6 serum levels and outcomes in septic patients [493]. Production levels of IL-1β were not as high as other cytokines, although they may be high enough to have biological consequences in our mouse model, and may synergize the effects of TNF-α and stimulate the secretion of other pro-inflammatory cytokines.

2.2 The development of septic shock due to *S. suis* in mice is influenced by genetic host factors

Once the hallmark of the septic phase of *S. suis* infection was established in CD1 mice, the objective of subsequent research was to confirm the role of inflammation in mortality observed during the septic phase and also to determine if the genetic

background of the host may influence the response towards this pathogen and further outcome of the disease. In fact, previous research had indicated that genetically determined variations in the immune system could result in greater susceptibility to infection, or development of different forms of disease in some individuals [494]. Understanding the genetic basis of differential susceptibility to bacterial infection in inbred mouse strains has proven extremely informative for understanding the interface of host:pathogen interaction and identifying normal defence mechanisms of phagocytes that may fail in permissive hosts [471]. In addition, understanding the mechanisms underlying disease resistance or susceptibility to *S. suis* may be useful for the development of effective drug therapy. For this, the mouse model of *S. suis* infection was used with two genetically different inbred mouse strains: A/J and C57BL/6 (B6) mice (Article III). Indeed, study of inbred strains of mice has resulted in the identification of several host resistance loci that regulate natural and acquired immunity in response to several pathogens. The genetically inbred mouse strains included in this study are considered as the prototype of resistant (B6) and susceptible (A/J) mice strains. They have been used frequently to investigate the genetic control of host immunity to infectious pathogens such as *L. monocytogenes*, *Candida albicans*, *S. aureus* and in models of sepsis with lipopolysaccharide (LPS) or cecal ligation [494-498]. Moreover, A/J and B6 mice strains have been successfully used for the study of the influence of the genetic control of immune responses in the outcome of streptococcal infections [484, 494, 499]. Nevertheless, most of these studies evaluated only mortality and bacterial loads in different tissues.

Results obtained demonstrated a stricking susceptibility to *S. suis* infection in A/J mice in comparison to B6 mice, as the former reached 100% of mortality at 20 h p.i., whereas mortality in the latter mouse strain reached up to 16 % at 36 h p.i. Very interestingly, and similarly to CD1 mice (Article I), bacteremia did not seem to be responsible for the death of mice. In fact, bacterial loads in different tissues were comparable between the two mouse strains; with sustained bacteremia during the first 3 days p. i. and kinetics resembling those observed in CD1 mice. We postulated then, that the higher mortality in *S. suis*-infected A/J mice was due to uncontrolled cytokine production leading to sepsis and deadly septic shock. A/J mice are known to carry a loss-of-function mutation in the structural gene of the C5 component of the complement pathway

[500]. C5 plays several critical roles in host response to infection, including target lysis and phagocyte recruitment. However, if complement was essential for bactericidal activity against *S. suis*, bacterial loads should be higher in A/J than B6 mice, whereas no significant differences in the bacterial loads between the strains were recorded. In fact, A/J mice presented outstanding levels of TNF-α, IL-12p40/p70, IL-1β and IFN-γ, levels of which significantly exceeded those found in B6 mice. A similar and potentially autodestructive cytokine pattern is found in susceptible mice strains infected with GAS [484, 501]. The remarkable cytokine production in A/J mice would lead to a series of pathogenic events that may lead to multiple organ dysfunction and death. Remarkably, chemokine levels (KC, MCP-1 and RANTES) were similar between strains, suggesting that they might have a limited participation in the development of septic shock due to *S. suis*.

Conversely, compared to A/J mice, better survival of B6 mice was partially related to a better regulation of the pro-inflammatory cytokine cascade. Indeed, B6 mice not only presented lower levels of several pro-inflammatory cytokines, but also showed a higher production of the anti-inflammatory cytokine IL-10 (discussed below). In addition, it seemed that following to *S. suis* infection, B6 mice present a Th1 type immune response. This would agree with previous findings stating that B6 mice portray a Th1-response in response to different pathogens, with a well controlled pro-inflammatory response that appears to be beneficial during sepsis [502, 503]. However, some other reports of mortality and severity of sepsis in B6 mice, concluded that a massive secretion of pro-inflammatory mediators was likely the cause of death [504]. This clearly exemplifies that the type of immune response is influenced by both pathogen and host factors. Very recently, independent research groups have also found that B6 mice inoculated with *S. suis* ST7 (involved in the Chinese human outbreak), develop a strong inflammatory response with high levels of TNF-α, IL-1β, IL-6, KC and MCP-1 [97]. The cytokine levels they observed are indeed higher than those obtained when mice were infected with an European virulent strain (the same strain that we used in our studies).

Interestingly, the 2005 Chinese *S. suis* human outbreak was characterized by a very high prevalence of patients with TSLS that presented increased serum levels of TNF-α IL-1β, IL-6, IL-12p70, IFN-γ and IL-8 [505]. The cytokine profile found in A/J mice resembles that

observed during the Chinese outbreak. It has been assumed that *S. suis* ST7 has acquired putative virulence factors responsible for the TSLS, although it will be interesting to study if the genetic background of the host plays a role, in addition to possible changes in the bacterial genome [97].

2.3 Role of anti-inflammatory mediators in the survival of mice following septic shock dute to *S. suis*

The better survival of B6 mice could be due to the fact that they better controlled the release of pro-inflammatory cytokines and mounted a higher anti-inflammatory response, in comparison to A/J mice. IL-10 is a crucial down regulation-mechanism of inflammation which efficiently blocks the production of different pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1β and IL-6 from human/mouse monocytes and human neutrophils stimulated with LPS or lipoproteins from pathogens [506-509]. The results obtained in this study concerning the systemic production of IL-10, suggested that survival of B6 mice during the septic phase may be related to the capacity of their innate immune response to mount a competent anti-inflammatory response, whereas in the case of A/J mice, the production of IL-10 remained low and almost undetectable (Article III). Moreover, in mouse models of sepsis, IL-10 induces inhibition of TNF-α secretion and decreases mortality [510-512]. In humans, IL-10 seems to have a protective function in the progress to organ failure in sepsis. IL-10 suppresses TNF-α production and inhibits the expression of adhesion molecules and TNF-α receptors on neutrophils [513].

We focused on IL-10 research using two approaches: the first, by blocking the cell receptor of IL-10 (IL-10R) with an anti-mouse IL-10R monoclonal antibody (Mab) in B6 mice and the second by providing recombinant mouse (rm)IL-10 (rmIL-10) to A/J mice. We expected in the first approach an increase in mortality in B6 mice caused by cytokine outburst whereas in the second we believed A/J mice would better control the development of sepsis. B6 mice that received the parental administration of IL-10R MAb before challenge with *S. suis* developed a clinical acute disease that resembled that observed in A/J mice, with an striking and fast increase in mortality and higher levels of TNF-α in comparison to infected mice that did not receive the treatment. Conversely,

treatment with rmIL-10 delayed the onset of septic shock in A/J mice infected with *S. suis*. These results show that survival to *S. suis* septic shock requires a tight regulation of pro- and anti-inflammatory mechanisms, and that the latter should be activated at the same time or soon after the onset of the pro-inflammatory response. The beneficial effect of parental administration of IL-10 has been documented in other streptococcal diseases. For example, when adult mice receive anti-IL-10 antibodies at the time of infection with GBS, they show an increase in mortality and in severity of lesions, accompanied by an early and sustained production of pro-inflammatory cytokines. The opposite is observed if animals receive a treatment with rmIL-10 [514]. The administration of rmIL-10 has similar effect in infant models of GBS infection and in mouse models of septic shock with LPS [515-517]. We cannot conclude that resistance/susceptibility to *S. suis* infection in mice relies solely on the systemic production of IL-10, as with the rmIL-10 treatment in A/J, we observed a delay in the onset of septic shock, although mice finally succumbed to infection. There would be other regulators of the inflammatory response involved, which we have not yet identified. Possible candidates to study are the secretion of the endogenous IL-1Ra, which blocks the biological action of IL-1, polymorphisms in the promoter for the TNF-α gene [518], secretion of sTNF-αR that antagonizes TNF-α activity and participation of endogenous glucocorticoids (i.e. adrenocortical corticosterone (CS)), which among other biological effects, block the production of TNF-α [519].

Currently, A/J mice are believed to be relatively deficient in the prophylactic activities of CS. In fact, B6 and A/J demonstrate relevant differences concerning the endogenous production of CS, as B6 adrenals exhibit abundant CS-producing cells, which have a cord-like array distribution, whereas A/J adrenals contain few, randomly arranged CS-producing cells [520]. Moreover, B6 mice have a significantly higher adrenal CS content than A/J mice [521]. Thus, we can argue that, once infected with *S. suis*, A/J mice will not have a sustained production of CS to help regulate the inflammatory response. The phenotype picture presented here is the first step in the identification of host genes associated with resistance against *S. suis*. Further steps should include studies with B6 IL-$10^{-/-}$ deficient mice, as well as generation of recombinant inbred strains (B6 × A/J) to study

the phenotypic and genetic variations among them, and cloning of possible host target genes.

3) Development of Central Nervous System disease by *S. suis*: the role of an exaggerated inflammatory response

3.1 A mouse model of *S. suis* infection with clear clinical signs and lesions of encephalitis/meningitis

One of the most remarkable achievements of the mouse model of infection described in this project is the development of clear clinical signs of neurological disease from 4 days p.i. (Article I). Interestingly, bacterial loads in the brains of the mice were lower in comparison to those in other sampled tissues, suggesting a particular mode of colonization for this organ that may be related to the degree of BBB dysruption. Moreover, *S. suis* may be present in the brain, without clinical signs of disease, as the bacteria were recovered from the brains of CD1 mice 6 days p.i. in the absence of clinical signs, indicating that a certain minimum number of invading bacteria might be needed to cause CNS disease. It is worth mentioning that assessment of bacterial concentrations from different organs (and determination of pro-inflammatory mediators in plasma) required sacrifice and sampling of animals at specific time points, and therefore it was not possible to determine if these mice with late positive cultures in brain would have developed nervous disease at later time.

The presence of clinical signs of CNS in mice infected by the intraperitoneal route with *S. suis*, represents a striking difference to mouse infection models of meningitis of Gram-positive cocci. As mentioned previously, in most of these experimental models, bacteria are inoculated directly into the CNS, inducing a rapid exacerbated inflammatory response in the brain and 100% of mortality of animals during the first 72 h p.i. unless antimicrobial treatment is included. In GBS infection of mice pups, clinical signs include apathy, anorexia, and pallor of the skin, whereas adult mice infected with *S. pneumoniae* are lethargic and unable to walk, opisthotonus and epileptic seizures being infrequently

observed [440, 522]. In the present model of *S. suis* meningitis, clear neurological signs were observed in CD1 mice who survived to the septic phase of infection. Neurological disease started with subtle changes in behaviour, and transformed quickly into disorientation, episthotonos, opisthotonus, walking in circles with the head towards one side, uncontrolled spinning (rolling) when in lateral recumbency, forelimb tilt, and in some cases, abnormal limb position at rest (crossed forelimbs), suggesting propioceptive deficits. All the aforementioned clinical signs closely resemble to those present in pigs infected with *S. suis* [6, 40, 133] (Annex, article V), and they represent CNS inflammation that is accompanied by vestibular syndrome.

CD1 animals with clinical signs of CNS disease showed inflammation with suppurative and necrotizing lesions in the meninges and in different anatomical sites of the brain parenchyma, such as the cortex, hippocampus, corpus callosum, thalamus and hypothalamus (Article I). It is known the hippocampus is an area of the brain specialized in spatial learning and memory [523], and learning deficiencies are important sequelae in people that recover from bacterial meningitis [524]. In fact, spatial learning and memory deficits have been associated with lesions in the hippocampal formation, and inactivating synaptic transmission in the dorsal hippocampal formation reversibly impairs encoding and retrieval of spatial memory [525, 526]. Moreover, mice with pneumococcal meningitis suffer neuronal damage at the hippocampus and develop long term spatial memory and learning deficits [527]. As we found severe damage to the hippocampus, it would be interesting to adapt this model to study if meningitis caused by this pathogen is also accompanied by long-term learning deficiencies and to evaluate the effect of treatment with anti-inflammatory drugs that seem to diminish the negative effect of these sequelae. We are not able to explain why *S. suis* seems to prefer these brain areas. Currently, bacterial thrombi inside blood vessels and bacterial antigens in different structures of the brain of CD1 mice, including the hippocampus and corpus callosum are identified by means of immunohistochemistry using a primary polyclonal antibody against S. *suis*. Data obtained in our studies studies, as well as those of independent research groups [293], support the theory that *S. suis* can get access to the brain by traversing the BBB and the blood-cerebrospinal fluid barrier (BCSFB) (see discussion below). As the corpus callosum is located on the roof of the lateral ventricles (which are full of cerebrospinal fluid (CSF)), it is

possible that the relevant presence of *S. suis* antigens in these areas is by passage of bacteria contained in the CSF to this brain structure. Moreover, as in mice experimentally infected with pneumoccoccus, bacteremia plays a significant role in development of apoptosis in the dentate gyrus of the hippocampus during meningitis [528]. We hypothesize that *S. suis* might also induce neuronal apoptosis in these structures. More research is needed to confirm this hypothesis.

A very interesting finding is the presence of bilateral areas of demyelination mostly restricted to the corpus callosum (Article I). Studies revealed that transcript expression of mouse proteolipid protein (PLP) - synthesized by oligodendrocytes - was diminished in these areas. The origin of such degenerative lesion may be associated to microglia [529], and will be discussed in the correspondent section.

The distribution pattern of these lesions shares some similarities with experimental *S. pneumoniae* infection in mice, in which there is inflammation of meninges, accompanied by inflammation and necrosis of the neocortex, dentate gyrus of the hippocampus and corpus striatum, although there also are brain abscesses due to the inoculation technique used [440]. In addition, some mice with *S. suis* meningitis develop a patent dilation of lateral ventricles that may cause an increase of intracranial pressure. This may be associated with the production TNF-α that can alter the permeability of the BCSFB [530]. Local production of nitric oxide by microglia (Article II) and astrocytes (unpublished publications) may also participate to increase the intracranial pressure and augmentation of meningeal inflammation.

3.2 *S. suis* infection in mice is also associated with vestibular syndrome and other pathologies

As aforementioned, some of the clinical signs in infected CD1 suggested vestibular syndrome. The vestibular system maintains balance and body posture; it detects motion of the head. It is located in the labyrinth, at the inner ear [531]. In animals, dysfunction of the vestibular system is commonly recognized by signs such as head tilt, recumbency, circling, and rolling. Nystagmus (either horizontal, rotary, vertical, or positional) can also be seen. It can result from lesions to the vestibular receptors in the membranous labyrinth or the vestibulocochlear nerve (VIII cranial nerve) by inflammation, trauma, neoplasia, etc. [532,

533]. In humans suffering from meningitis due to *S. suis*, as there is cochlear and vestibular damage accompanied by hearing loss [131, 534], it is possible that CD1 mice with these particular clinical signs demonstrate the same pathology as humans infected with *S. suis*. In fact, as a complement of my work during my PhD, we recently demonstrated that CD1 mice with clinical signs of meningitis and vestibular syndrome present damage in inner ear structures, with severe inflammatory changes at the cochlea and suppurative labyrinthitis that is aggravated in perilymphatic spaces, with presence of high numbers of streptococci (ongoing studies, unpublished observations) (Fig. 1). These inflammatory changes are very similar to those observed in pigs and guinea pigs experimentally infected with *S. suis*, which develop otitis interna characterized by a mixed inflammatory exudate comprising neutrophils, lymphocytes, and less often macrophages in the perilymphatic ducts of the cochlea and perineuritis along the vestibulocochlear nerve [310, 535]. Rats experimentally infected with *S. pneumoniae* develop a similar pathology in inner ear structures, as there is disruption of the blood-labyrinth barrier that leads to extravasation of inflammatory cells in different structures of the cochlea, including the labyrinth spiral ligament, the stria vascularis and the spiral limbus. By means of Auditory Brainstem Response measurements, it has been determined that rats with such pathological changes in the inner ear manifest severe hear impairment [438]. All the clinical and histopathological data of inner ear damage by *S. suis* in CD1 mice provide additonal evidence of the accuracy of this experimental model studying the pathogenesis of *S. suis* infection and reinforce its usefulness in future research with the objective to determine if indeed *S. suis* causes deafness in mice. Our experimental model of infection may serve to study the participation of different pathways and reactive molecules from the host implicated and possible use of novel drugs that may help alleviate the negative consequences of such important sequelae of meningitis.

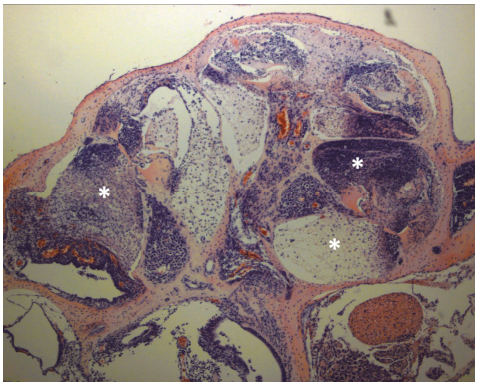


Fig. 1. Inner ear (cochlea) of CD1 mice with clinical signs of meningitis and vestibular syndrome. Presence of excessive inflammatory cells (white *) at the scala tympani, scala media and scala vestibuli (Domínguez-Punaro, *et al*, unpublished data).

Finally, around 2-3 days after infection some CD1 mice (with or without neurologic clinical signs) present a cloudy cornea ("white eye"), which is usually unilateral. Microscopic examination reveals severe acute endophthalmitis with presence of neutrophils and bacteria (data not shown). Interestingly, in humans, endophthalmitis may be associated with bacterial infections, including those due to *S. suis* [536, 537]. In addition, some infected mice may also manifest endocarditis, which is another pathology commonly found in pigs and humans affected by *S. suis* [538, 539].

3.3 Triggering of the inflammatory response at the Central Nervous System

The study of pro-inflammatory events at the CNS, similarly to the systemic studies, was carried out in mice sampled at specific time points and from those included in the mortality study that showed clear clinical signs of meningitis (Article I). We used the mouse model of *S. suis* infection to characterize the *in vivo* pro-inflammatory events that take place in the brain and identify the CNS cells that participate in the inflammatory response against this pathogen. For this, we used the *in situ* hybridization (ISH) technique. This unique technique localizes gene sequences *in situ* and visualizes the product of gene expression while preserving cell integrity within the heterogeneous tissue, permitting anatomically meaningful interpretations [540].

Our results showed an early activation of TLR2 and CD14 genes first in cells lining the brain microvasculature, the meninges and choroid plexuses, supporting the hypothesis that *S. suis* traverses the BBB and the BCSFB to gain access to the brain. In fact, previous *in vitro* studies had underlined the fact that *S. suis* can adhere and invade PBMEC, with the

release of pro-inflammatory cytokines [4]. Trespassing of the BBB may be enhanced by the strong systemic secretion of TNF-α presented in mice. Moreover, *S suis* has the ability to compromise barrier function of porcine choroid plexus epithelial cells [541] that may be enhanced by SLY production that is cytotoxic for endothelial cells [4].

These findings also support previous *in vitro* studies finding that recognition of this pathogen is through a TLR2-dependent pathway in collaboration with CD14. Indeed, studies with THP-1 human monocytes revealed that virulent encapsulated *S. suis* or its purified cell wall components activate the expression of TLR2 [271] in association with CD14 [269, 271], events that are followed by the release of high amounts of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-8 and MCP-1) [271]. In complementary studies, mouse macrophages deficient in TLR2 showed impaired (but still positive) cytokine responses to encapsulated bacteria. As for CD14, it has been traditionally recognized as a molecule participating in LPS recognition [542]. In rats, the systemic injection of LPS up-regulates the gene encoding CD14 early in brain areas devoid of BBB and later in the brain parenchyma [391]. However, CD14 also participates in the detection of bacterial components from Gram-positive bacteria, including brain pathogens. It has been shown that heat-killed *S. aureus* and *S. pneumoniae* induce activation of transcription of TLR2 in a CD14-dependent fashion [543]. Complementary studies demonstrated that the lipoteichoic acid from these two pathogens triggers innate immune recognition via LPS-binding protein-CD14-TLR2 [544].

In mice infected with *S. suis*, there is activation of IκBα (index of NF-κB activation). It has been postulated that IκBα expression in cells of the BBB is a general mechanism that takes place during systemic inflammation [388, 545]. Translocation of NF-κB leads to the expression of different pro-inflammatory genes. We could not determine if the increased mRNA expression of inflammatory mediators observed in mice is directly related to TLR2-NF-κB signaling pathway, although previous studies with mouse macrophages infected with *S. suis* demonstrate that blocking of TLR2 significantly diminishes cytokine and chemokine production [271].

The *in vivo* pro-inflammatory picture in the brain was completed by studying the expression of mRNA transcripts for several pro-inflammatory mediators, that is, IL-1β, TNF-α, IL-6 and MCP-1. Interestingly, the transcripts for these mediators were always observed at different degrees of intensity in the choroid plexuses, cortex, corpus callosum and different areas of the brain, coinciding with those areas where inflammation, necrosis, demyelination and *S. suis* antigens were found. However, whereas MCP-1 was identified also in brain microvasculature, IL-1β and TNF-α were not. This agrees with previous studies with *S. suis*, which show an absence of TNF-α and IL-1β activation in brain microvascular endothelial cells (BMEC) [282]. Various studies highlight the production of similar panels of cytokines and chemokines in the brain inflammatory response towards different brain pathogens, such as *H. influenzae*, *S. pneumoniae* [546] and *S. aureus* [547]. Very interestingly, in *S. suis* mouse infection, it seems that participation of IL-6 is more relevant at the systemic level, but not at the CNS. Indeed, there was a low and inconstant IL-6 gene expression in the brain of infected CD1 mice, mostly restricted to blood vessels, choroid plexus and cortex. This was further confirmed in microglial cells (see below).

It is important to note a striking difference between our model and previous studies describing the development of the inflammatory response in the brain with respect to bacterial antigens. In several studies, it was found that the parental injection of LPS in rodents leads to cytokine gene expression in the circumventricular organs, which are devoid of BBB and further activation of microglia in the brain parenchyma [388, 390, 392, 548]. Similarly, mice parentally infected with *Salmonella enterica* serovar Typhimurium, demonstrate increases in TLR2 mRNA levels in the circumventricular organs and brain parenchyma and infiltrating blood derived-myeloid cells [549]. In comparison, the intracisternal injection of live or killed *S. pneumoniae*, LTA or PG, triggers an immune reaction via TLR2 [452]. However, mice with *S. pneumoniae*-induced meningitis demonstrate increased TLR2, TLR4, and TLR9 mRNA expression, whereas TLR2-deficient mice infected by the intracranial route with *S. pneumoniae* express different pro-inflammatory cytokines in the CNS, indicating TLR2-independent recognition pathways [452, 550, 551].

Surprisingly, in the brains of *S. suis* infected mice, we observed a moderate induction of TLR3 mRNA expression that followed the same pattern of expression of TLR2 (Article I). This receptor is known to recognize viral double-stranded RNA. Thus, the hypothesis that *S. suis* can activate other TLRs in adittion to TLR2 and possibly other components of the innate immune system in a non-specific manner cannot be ruled out. In fact, *in vitro* results showed that *S. suis* activates THP-1 monocytes in a TLR2-dependent and, to a certain extent, -independent (but MyD88-dependent) manner, indicating that other TLRs are also involved in *S. suis* recognition. Other bacterial agents can also induce an increase in TLR3 expression, as hippocampal cultures exposed to *S. pneumoniae* showed up-regulation of both TLR2 and TLR3 mRNA expression [551]. Another example is the up-regulation of TLR3 in human epithelial airways cells by *H. influenzae* [552, 553]. It is possible that the TLR3 up-regulation observed in the brain of *S. suis*-infected mice might leads to activation of the MyD88-independent pathway. Indeed, it has been demonstrated that *in vitro*, CPS from *S. suis* contributes to MCP-1 production in a MyD88-independent manner [271]. The MyD88-independent pathway involves activation of the transcription factor IRF-3, and induction of IFN-β, which in turn, activates Stat1, leading to the induction of several IFN-inducible genes [321]. Viral infection or dsRNA, which are classical TLR3 ligands, also activate IRF-3 and thereby induce IFN-β in a MyD88-independent manner. Hence, TLR3 utilizes the MyD88-independent component to induce IFN-β [5]. *S. suis* might use similar mechanisms. It is worth noting that MCP-1, along with RANTES and IP-10 are IFN-β-inducible genes [554].

The pro-inflammatory signals in the mice brain increased quickly with time, reaching different areas of the brain and being much more evident in those animals with neurologic clinical signs. It is noteworthy to mention that the presence of these signals had a positive correlation with the anatomical distribution of lesions. ISH in combination with immunohistochemistry identified cells resembling microglia or blood-derived monocytes, and to a lesser extent astrocytes, as those responsible for the pro-inflammatory gene activation. Therefore, *in vitro* studies were conducted to better understand the role of mouse microglia in the inflammatory response towards *S. suis* (Article II). Microglia are the first line of the brain and the main role of activated microglia is believed to be in brain

defense. However, if the activation of these cells is exacerbated, they may play a harmful role, significantly affecting the development of the inflammatory response and leading to a pathological state. Indeed, microglia produce a wide range of pro-inflammatory mediators, and a variety of biologically active molecules including cytotoxic and neurotrophic molecules that may harm the brain tissue.

Studies with mouse microglia (Article II) initially evaluated their possible protective role in the development of *S. suis* meningitis. Activated microglia transform into phagocytes and may limit the multiplication of pathogens in the brain [398]. The use of mouse microglia cells allowed us to determine that in these cells, phagocytosis of *S. suis* is severely restricted by the presence of the CPS, similarly to findings in mouse and human macrophages [142, 148], pig neutrophils [264] and mouse dendritic cells [477]. These results were confirmed by confocal microscopy studies, which showed that only a few WT *S suis* were ingested by microglia, whereas the CPS- strain was found in high numbers inside phagolysosomes. The presence of serum significantly increased the rate of phagocytosis of capsulated bacteria, although again, phagocytosis of the CPS- mutant significantly exceeded those of its WT strain. The absence of the SLY has already been associated with a partial susceptibility of encapsulated *S. suis* to phagocytosis by neutrophils and dendritic cells in the presence of complement [264, 477]. Since fragments from the complement cascade can be found in the brain [278], their possible effect of on phagocytosis of *S. suis* by microglia was evaluated. Results from the present study show that complement components do not seem to affect phagocytosis of the capsulated *S. suis* SLY- mutant by microglial cells, indicating a different behavior of phagocytes from different locations. The observed increased rate of phagocytosis in the presence of serum might be due to proteins other than complement fractions, such as albumin [555]. As murine microglia were not able to phagocyte virulent *S. suis*, it is likely that *in vivo*, this would facilitate multiplication of the pathogen thus creating a destructive environment that would attract more inflammatory cells to the site of infection and enhance the inflammatory response. Therefore, we foccused on the pro-inflammatory activity of microglia in contact with *S. suis*, to try to understand how their activation may be necessary for the host, even though this may lead to negative consequences.

Microglia infected with *S. suis* are capable of producing different pro-inflammatory cytokines and chemokines, highlighting their relevance in the development of the inflammatory response towards this pathogen. The release of substantial quantities of TNF-α and MCP-1 by microglia following contact with virulent *S. suis* was a significant finding, supporting the previous observations in infected mice, in which there was a strong up-regulation of genes encoding TNF-α and MCP-1 in different areas of the brain and meninges (Article I). Microglia released low levels of IL-1β, still, which nevertheless may be enough to have a biological impact in the brain. It is likely that microglia are not the only glial cell implicated in the inflammatory response towards *S. suis*, as *in vivo* experiments in mice suggested that astrocytes also play a pro-inflammatory role. In fact, recent experiments from our laboratory, complementing those of the present thesis, demonstrate that astrocytes infected with *S. suis* can release high levels of TNF-α and MCP-1, with a more modest production of IL-1β and similarly to microglia, the bacterial CPS is implicated in modulating cytokine production (data not shown). In contrast to observations in other phagocytic cells and *S. suis* [268, 269, 271] and with other streptococci and microglial cells [556], microglial cells activated with *S. suis* do not produce significant levels of IL-6. Nevertheless, this result agrees with findings in the brain of infected mice where there was no expression of IL-6 (Article I). It seems that SLY does not have a major role in cytokine release from microglia, as *S. suis* deficient in this putative virulence factor induced similar cytokine levels to its parental strain. This is in contrast to pneumolysin of *S. pneumoniae*, which presents a high homology with SLY and is known to play an active role in inflammation [306].

The CPS seems to be critical for modulating the production of pro-inflammatory mediators, as the CPS- mutant induced, in most of the cases, the release of significant higher levels of pro-inflammatory mediators. This supports the assumption that several components of the cell wall (i. e. LTA, PG and lipoproteins), partially masked by the CPS, are potent pro-inflammatory activators. In fact, the importance of LTA and PG in the inflammatory response against *S. suis* has been recently assessed [477]. In addition, cell wall lipoproteins have recently been described as being responsible for cell activation [557]. Finally, as a low production of IP-10 for capsulated *S. suis* strains in comparison to

that for the CPS- mutant was also noted, we might hypothesize that the CPS might influence the onset of the adaptative inflammatory response, as recently shown for dendritic cells [477]. As a consequence, less lymphocytes T will be attracted to the site of infection.

Following demonstration that microglia can actively respond to the insult represented by *S. suis* by producing pro-inflammatory cytokines/chemokines, research was focused on studying mechanisms involved in pathogen recognition, as well intracellular pathways implicated in the development of the inflammatory response and production of cytotoxic effector molecules. As no differences were found between the SLY-deficient strain and its parental WT strain, signalization studies were conducted by comparing the WT strain and its CPS- mutant.

It has recently been demonstrated that TLRs, which are crucial pattern recognition receptors in innate immunity, are expressed in microglia [400]. TLR activation will set in motion a broad spectrum of intracellular events to initiate inflammatory response, including MAPK signaling pathways, activation of NF-κB and cytokine production [322]. Studies in mice had demonstrated up-regulation of TLR2 in different areas of the brain parenchyma following infection with virulent *S. suis*, and it was suggested that microglia or blood –derived macrophages were mainly responsible for recognition of the pathogen and further initiation of the inflammatory response (Article I). Interestingly, the present results demonstrate microglia in contact with *S. suis*, up-regulate the expression of TLR2 mRNA in a time-dependent manner. This is more pronounced in the case of a *S. suis* strain deficient in CPS, indicating an active role of cell wall components in TLR2-mediated recognition. Moreover, these findings agree with previous results obtained with human monocytes and *S. suis* [271]. It is likely that, during *in vivo* infections, local multiplication of *S. suis*, stimulates the release of high amounts of LTA and PG that will activate TLR2 up-regulation. It is worth noticing that under the *in vitro* conditions used, TLR1, TLR4, TLR6 and TLR9 gene expression was not modified. However, it is not possible to exclude their participation in *S. suis* recognition, as their basal expression may be high enough to trigger the inflammatory response. This differs from recent research, demonstrating that bacterial lipoproteins from

S. suis activate the TLR2/6 but not the TLR1/2 complex from epithelial cells [557]. It would be necessary to perform further studies with KO microglia ($TLR2^{-/-}$, $TLR1^{-/-}$, $TLR6^{-/-}$) to verify the participation of these receptors.

The *in vitro* studies with mouse microglia also confirm the important participation of NF-κB as a key transcription factor involved in triggering the inflammatory response against *S. suis*, as results obtained *in vivo* in mice suggested (Article I). We determined that *S. suis* induces the translocation of NF-κB to the nucleus in microglia (Article II). Both *S. suis* WT and its CPS- mutant were able to increase the binding activity of NF-κB, it in a time-dependent fashion. As the DNA binding activity of NF-κB was faster and more intense when cells were infected with non-capsulated *S. suis*, it is very probable that cell wall components, which are completely exposed in the CPS- mutant, influence the activation of NF-κB as observed by others [345, 558]. As aforementioned, in mice infected with *S. suis*, the IκBα transcript (index of NF-κB activation) was observed in cells identified as astrocytes as well as in microglia. Our recent research with mouse astrocytes confirms this finding. We have demonstrated translocation of NF-κBp65 to the nucleus of these cells following infection with *S. suis*. Interestingly and similarly to microglia, this p65 translocation is influenced by the presence of CPS (unpublished observations). Moreover, TLR2 participation and NF-κB translocation in astrocytes, with further induction of the inflammatory response has been recently documented for *S. aureus* [559].

TLRs downstream signaling involves activation of PKC-family of proteins, Tyr protein and the Mitogen Activated Protien Kinases (MAPK) system [3], in addition to NF-κB. MAPK activation has gained recent attention as a crucial pathway for glia activation [338]. However, despite the relevant role these proteins may play, very little is known about their participation in *S. suis* inflammatory response (Article II). The lower and biphasic patterns of phosphorylation for PKC substrates and Tyr protein observed in mouse microglia infected with *S. suis* WT strain in comparison to those with the CPS- mutant may indicate that capsulated *S. suis* may modulate microglia signaling events most likely to avoid phagocytosis as it was previously documented [148]. Our results obtained indicated that the CPS interferes with the MAPK-mediated response, as phosphorylation patterns (p-ERK, p-JNK, p-p38) found with the CPS- mutant were faster, stronger and more stable than

those obtained with the WT strain. Nevertheless, the partial exposition of cell wall components in the WT strain is enough to trigger MAPK signalization pathways. This supports recent studies, where purified cell wall preparations from *S. suis* triggered the activation of the MAPK pathway in human macrophages [560]. Similarly to the present research, following infection of pig macrophages with whole *S. suis* or a non-capsulated mutant, an increase in the transcriptional response involving different genes from the MAPK pathway was observed, in particular with a CPS deficient strain [270]. EstA protein, a recently described virulence factor of *S. pneumoniae*, present at the bacterial cell surface, induces MAPK phosphorylation and NF-κB translocation [561]. As the *estA* gene is also found in *S. suis* [562], it is probable that MAPK activation and other intracellular signaling pathways do not depend solely on a few *S. suis* constituents, but many of them participate at the same time to activate the pro-inflammatory machinery. The pharmacological inhibition of MAPK revealed an almost complete abrogation in the release of TNF-α and MCP-1 from microglia infected with either *S. suis* WT or CPS- strains, demonstrating the importance of MAPK in the inflammatory response against this pathogen. Similar findings have been observed when ERK, JNK or p38 are pharmacologically inhibited in microglia stimulated with LPS [396, 563, 564]. In the case of *S. pneumoniae*, LTA is capable of triggering the MAPK signalization pathway with cytokine production, iNOS induction and release of nitric oxide (NO). This latter is p-38 MAPK dependent [345]. It would be interesting to determine if in the case of *S. suis*, NO production by microglia is triggered by the same pathway or if it is mainly NF-κB-dependent. We cannot conclude that this MAPK induction is related to TLRs activation as it has been proved for other meningitis-causing bacteria, like *S. aureus* [565]. To do so, we should either block TLRs (i.e. TLR2) with specific antibodies or preferably, use TLR KO microglia. In both cases, we would expect abrogation of MAPK phosphorylation followed by a decrease in cytokine production. Moreover, as ERK, JNK, and p38 pathways are all molecular targets for drug development, it would be interesting to test the efficacy of some of these MAPK-inhibitor drugs in our mouse model of *S. suis* meningitis.

As previously stated, mice infected with *S. suis* presented severe demyelinization, restricted mainly to the corpus callosum (Article I), leading to postulate that microglia may be involved in this process. In fact, parenteral LPS treatment in rabbits induces

oligodendrocyte damage but only in the presence of microglial cells [529]. There are several mechanisms by which activated microglia may induce white matter injury. When activated, microglial cells have been shown to release reactive nitrogen and reactive oxygen species (like NO) into the surrounding areas, causing damage to oligodendrocytes. Activated microglia also release excitotoxic metabolites, such as glutamate and quinolinic acid, which may cause glutamate receptor– or N-methyl-D-aspartic acid receptor–mediated injury to oligodendrocytes [529]. *In vitro* studies with murine microglia (Article II) demonstrated that indeed these cells react to the insult represented by *S. suis* with the expression of iNOS and the release of NO in a time-dependent manner, events that are influenced by the presence of the capsule. We may theorize therefore that *in vivo*, NO release from microglia may damage oligodendrocytes and contribute to the loss of myelin or even cause direct neurotoxicity. Moreover, astrocytes may also enhance damage to oligodendrocytes as they also express iNOS following contact with *S. suis*, with kinetics similar to those of microglia (unpublished data).

3.4 Possible future research on the development of S. suis meningitis using C57BL/6 mice

Interestingly, inbred mice of strain C57BL/6 develop clinical signs of CNS disease (Article III), although not as evident as in CD1 mice, and these include recumbency, problems in locomotion and rear limb weakness, sometimes accompanied by head tilt and their brain lesions have a close resemblance to those observed in CD1 mice (Article I). Preliminary studies with the C57BL/6 mouse model also show increase in the expression of TLR2 with a spatiotemporal pattern that mimics that found in CD1 mice (unpublished observations) (Fig. 2).

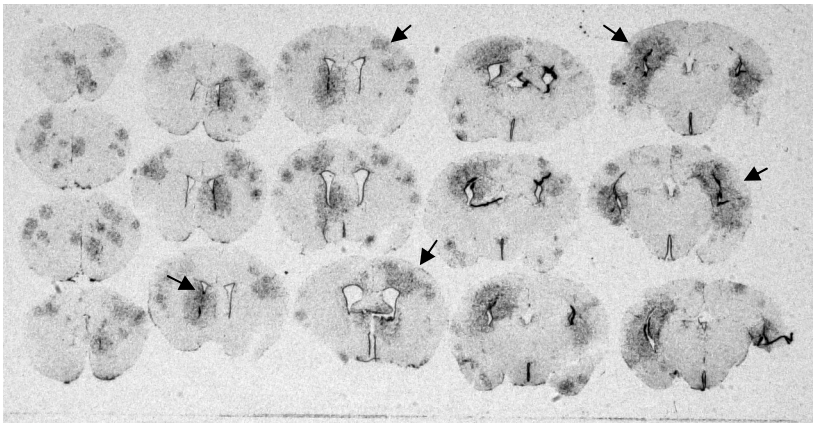


Fig. 2. TLR2 expression in brain of C57BL/6 mice infected with *S. suis*. *In situ* hybridization, brain coronal sections, from rostral (upper left) to caudal (down right). TLR2 expression is manifested as "black spots" (see black arrows) in different sections of the brain and in areas surrounding the choroid plexuses (Domínguez-Punaro, *et al*, unpublished observations).

These results encourage further research using different C57BL/6 knockout mice to pursue the study of signalization pathways involved sepsis, meningitis and inner ear damage by *S. suis*. KO mice strains that should be tested include TLR2$^{-/-}$, TLR1$^{-/-}$, TLR6 $^{-/-}$ and MyD88$^{-/-}$ mice.

In summary, this research project using a mouse model of infection enhanced the knowledge of the pathogenesis of *S. suis* infection. With this model, we studied the development of septic shock and evaluated a possible genetic role in the susceptibility of the disease. We also evaluatd the *in vivo* progress of the inflammatory response and associated lesions at the Central Nervous System. Moreover, the *in vivo* results obtained in the brain suggested the active participation of microglia in the local inflammatory response. Therefore, studies were conducted to understand some of the mechanisms implicated in the interactions of microglia and *S. suis*. The main observations found at the systemic level are summarized in Fig. 3, while those concerning the Central Nervous System are shown in Fig. 4.

Fig. 3.

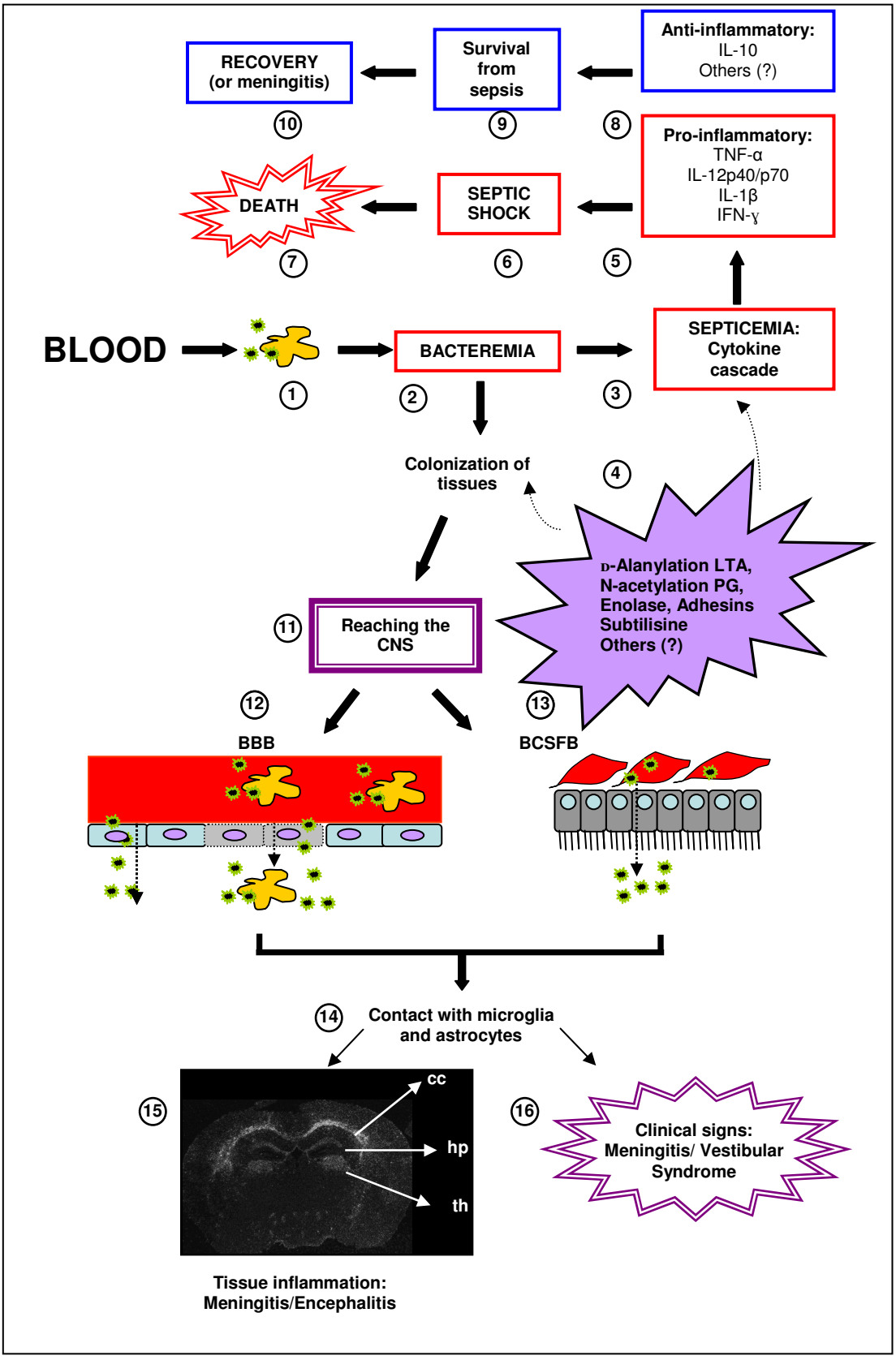

RECOVERY
(or meningitis)
Survival
from
sepsis
Anti-inflammatory:
IL-10
Others (?)
10
9
8
DEATH
SEPTIC
SHOCK
Pro-inflammatory:
TNF-α
IL-12p40/p70
IL-1β
IFN-γ
7
6
5
BLOOD
BACTEREMIA
SEPTICEMIA:
Cytokine
cascade
1
2
3
Colonization of
tissues
4
D-Alanylation LTA,
N-acetylation PG,
Enolase, Adhesins
Subtilisine
Others (?)
11
Reaching the
CNS
12
13
BBB
BCSFB
14
Contact with microglia
and astrocytes
15
cc
hp
th
16
Clinical signs:
Meningitis/ Vestibular
Syndrome
Tissue inflammation:
Meningitis/Encephalitis

Fig. 3. Proposed hypothesis for the development of *S. suis* infection. When bacteria reach the bloodstream, they will resist phagocytosis by phagocytic cells due to the presence of the CPS (1). Bacteria travel in the blood either attached to these cells or free, resulting in bacteremia (2), which may lead to septicemia (3). Different virulence factors play istrategic roles for tissue colonization by *S. suis* (4). During the septic phase of disease, there will be an exaggerated systemic production of pro-inflammatory cytokines and chemokines (5) that may cause septic shock (6) and ultimately death (7). Activation of compensatory anti-inflammatory mechanisms, such as production of IL-10 (8) are crucial for survival from sepsis in some cases (9) and recovery of disease (10). However, if the animal survives this acute phase of infection, *S. suis* may still get access to the CNS (11) through either the BBB (12) and/or the BCSFB (13). Traversing of the BBB may be enhanced by cytotoxic factors, such as SLY and production of pro-inflammatory mediators. Once in the CNS, the inflammatory response will be orchestrated mainly by microglia (see Figure 4), and to a lesser extent by astrocytes (14). This inflammatory response will include activation of receptors, such as CD14, TLR2, and TLR3 and production of pro-inflammatory cytokines and chemokines, such as IL-1β, TNF-α, MCP-1 (among others) in different areas of the brain (15), including the microvasculature, the corpus callosum (cc), the hippocampus (hp) and the thalamus (th). This will lead to the development high levels of inflammation with clinical signs of CNS and lesions of meningitis/encephalitis (16), accompanied by demyelination of specific brain structures.

Fig. 4.

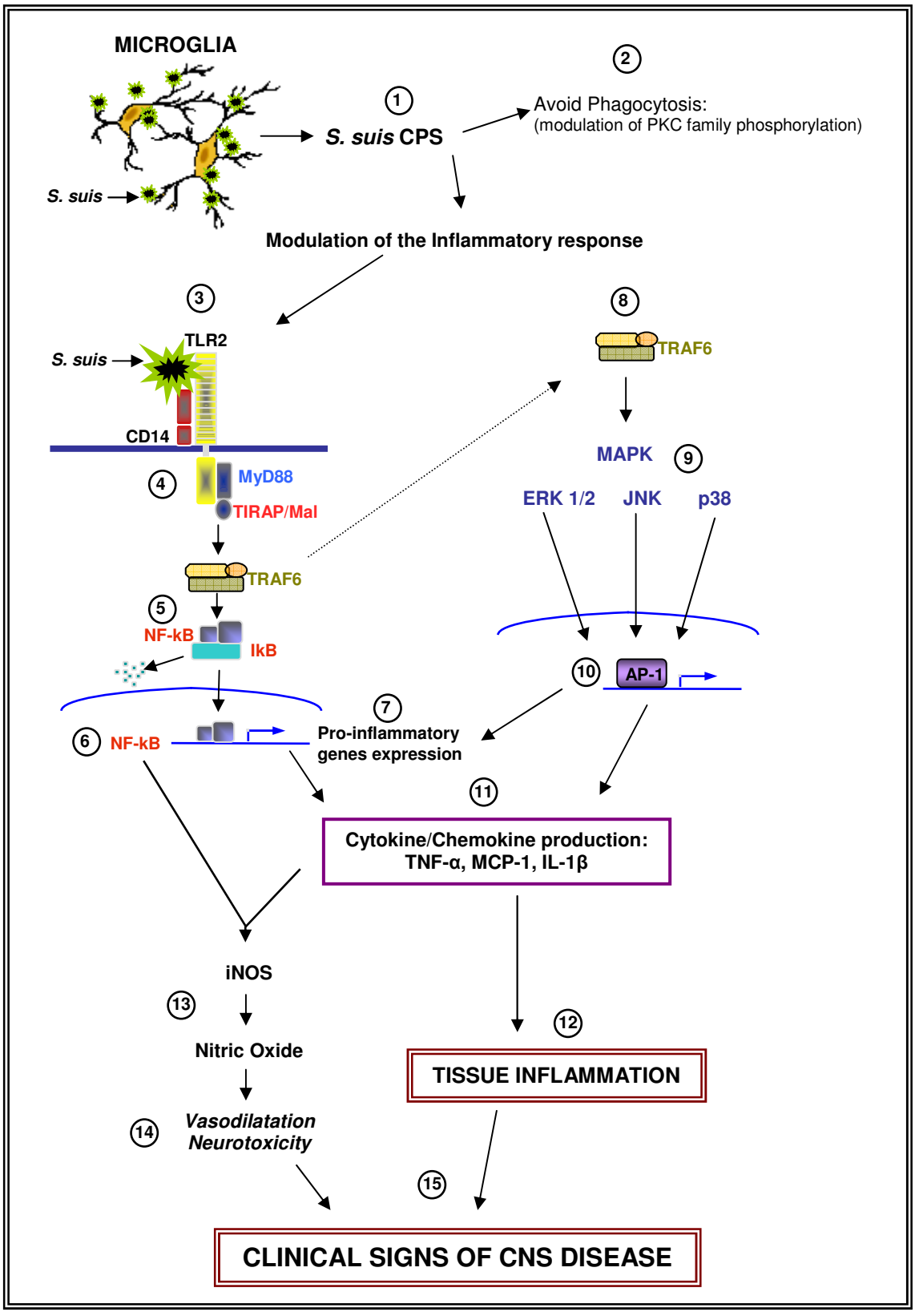

MICROGLIA
S. suis
1
S. suis CPS
2
Avoid Phagocytosis:
(modulation of PKC family phosphorylation)
Modulation of the Inflammatory response
3
S. suis
TLR2
CD14
4
MyD88
TIRAP/Mal
TRAF6
5
NF-kB
IkB
6
NF-kB
7
Pro-inflammatory
genes expression
8
TRAF6
MAPK
9
ERK 1/2
JNK
p38
10
AP-1
11
Cytokine/Chemokine production:
TNF-α, MCP-1, IL-1β
iNOS
13
Nitric Oxide
14
Vasodilatation
Neurotoxicity
12
TISSUE INFLAMMATION
15
CLINICAL SIGNS OF CNS DISEASE

Fig. 4. Proposed role of microglial cells in inflammation caused by *S. suis* at the CNS. Based on the results obtained in the present thesis, we can hypothesize that once in contact with microglia, the CPS of *S. suis* (1) will modulate different intracellular signalization pathways, including phagocytosis, in part by inhibition of the phosphorylation of PKC family of proteins (2). Nevertheless, once in contact with microglia, *S. suis* will be recognized through TLR2 in collaboration with CD14 (3) leading to the activation of the MyD88-downstream pathway (4), including up-regulation of IkBα (5) and translocation of NF-kB to the nucleus (6) to induce up-regulation of pro-inflammatory genes (7). Activation of MyD88 may also lead to TRAF6/MAPK/AP-1 signalization pathways (8-10), also with the up-regulation of pro-inflammatory genes (7). The signalization cascade (NF-kB and AP-1 pathways) will cause the production of pro-inflammatory cytokines and chemokines (11) that will increase tissue inflammation (12). Furthermore, there will also be activation of the iNOS pathway and further release of NO (13), which may also have negative effects, including vasodilatation and demyelination, which lead to neurotoxicity (14). All these inflammatory and degenerative events would lead to clinical signs of CNS disease (15).

V. GENERAL CONCLUSIONS AND PERSPECTIVES

General conclusions

Results from the present work demonstrate that:

- The standardized mouse model of *S. suis* infection is useful for studying the mechanisms underlying sepsis and meningitis during bacterial infection. This model accurately reproduces the septic and clinical phases of infection, as well as mimicry CNS lesions found in natural infections in pigs and humans.

- Death of mice during the acute phase of disease is associated with an exacerbated production of pro-inflammatory cytokines and weak participation of negative regulatory mechanisms.

- Susceptibility to *S. suis* infection may be caused by a genetic predisposition of the host that will manifest a dysregulated inflammatory response.

- Inflammation in the CNS of mice infected with *S. suis* starts at the brain microvessels, and extends quickly to different areas of the brain parenchyma and meninges. This inflammatory response initiates with the up-regulation of TLR2 with the participation of CD14 and NF-κB, followed by the expression of genes of several pro-inflammatory cytokines and chemokines in cells identified as microglia and to a lesser extent in astrocytes.

- Microglia are active participants in the inflammatory response in *S. suis* infections, as once in contact with *S. suis*, they react by the up-regulation of TLR2, following with translocation of NF-κB to the nucleus and production of different cytokines, chemokines and reactive oxygen metabolites. In addition, the MAPK-signalization pathway is also actively involved in the inflammatory response *to*wards *S. suis* in microglia.

Perspec*tives*

Possible future steps for this research include:

- Use of the mouse model of *S. suis* infection to:
  - Identify harmful and beneficial effects of the host immune response in different phases of the disease (septic shock/meningitis).
  - Test novel drugs (antimicrobial agents, anti-inflammatory drugs) that may help stop the progression of *S. suis* infection or the inflammatory consequences of the disease.
  - Study the molecular mechanisms underlying deafness present in cases of meningitis by *S. suis* and other Gram-positive pathogens (on going studies).
  - Study the virulence of *S. suis* strains lacking in putative virulence factors.
  - Develop novel effective vaccination strategies.

- Development of F_2 populations derived from resistant × susceptible mouse strains to perform genome-wide scan studies and delineate the genetic background associated to susceptibility to *S. suis* infection.

- Delineate the *in vivo* relevance of the pro-inflammatory intracellular signalization pathways (MAPKs, iNOS) identified in microglia challenged with *S. suis*.

- Determine the relevance of astrocytes in the inflammatory response against *S. suis* (manuscript in preparation).

- Establishment of co-cultures of primary microglia or astrocytes with neurons to study the possible damage caused to the latter cells once glial cells have started the inflammatory response against *S. suis*.

VI.REFERENCES

1. Weigand, M.A., et al., *The systemic inflammatory response syndrome.* Best Pract. Res. Clin. Anaesthesiol., 2004. **18**: p. 455-475.
2. Tsiotou, A.G., et al., *Septic shock; current pathogenetic concepts from a clinical perspective.* Med Sci Monit, 2005. **11**(3): p. RA76-85.
3. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**: p. 499-511.
4. Vanier, G., et al., *Invasion of porcine brain microvascular endothelial cells by Streptococcus suis serotype 2.* Infect. Immun., 2004. **72**: p. 1441-1449.
5. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity* Int. Immunol., 2005. **17**: p. 1-14.
6. Higgins, R. and M. Gottschalk, *Streptococcocal Diseases*, in *Diseases of swine*, B.E. Straw, et al., Editors. 2006, Blackwell Publishing. p. 769-783.
7. Gottschalk, M., M. Segura, and J. Xu, *Streptococcus suis infections in humans: the Chinese experience and the situation in North America.* Anim Health Res Rev, 2007. **8**(1): p. 29-45.
8. Tang, J., et al., *Streptococcal toxic shock syndrome caused by Streptococcus suis serotype 2.* PLoS Medicine, 2006. **3**: p. e151.
9. Gottschalk, M. and M. Segura, *The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions.* Vet. Microbiol., 2000. **76**: p. 259-272.
10. Hommez, J., et al., *Identification and characterization of Streptococcus suis.* Vet. Microbiol., 1986. **11**: p. 349-355.
11. Devriese, L.A., et al., *Characteristics of different Streptococcus suis ecovars and description of a simplified identification method.* Vet Microbiol, 1991. **26**(1-2): p. 141-50.
12. Higgins, R. and M. Gottschalk, *An update on Streptococcus suis identification.* J Vet Diagn Invest, 1990. **2**(3): p. 249-52.
13. Field, H.I., D. Buntain, and J.T. Done, *Studies on pig mortality. I. Streptococcal meningitis and arthritis. .* Vet. Rec., 1954. **66**: p. 453–455.
14. Jansen, E.J. and C.A. Van Dorssen, *Meningoenceohalitis bij varkens door streptococcen.* Tijdschr Diergeneeskd, 1951. **76**: p. 815-832.
15. de Moor, C.E., *Septicaemic infections in pigs, caused by haemolytic Streptococci of new Lancefield groupsdesignated R, S, and T.* Antonie Van Leeuwenhoek, 1963. **29**: p. 272-280.
16. Elliott, S.D., *Streptococcal infection in young pigs. I. An immunochemical study of the causative agent (PM Streptococcus).* J. Hyg. (Lond). , 1966. **64**: p. 205-212.
17. Kilpper-Balz, R. and K.H. Schleifer, *Streptococcus suis sp. nov., nom. rev.* International Journal of Systematic Bacteriology, 1987. **37**: p. 160-162.
18. Hill, J.E., et al., *Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that Streptococcus suis serotypes 32 and 34, isolated from pigs, are Streptococcus orisratti.* Vet Microbiol, 2005. **107**(1-2): p. 63-9.
19. Chatellier, S., et al., *Phylogenetic diversity of Streptococcus suis strains of various serotypes as revealed by 16S rRNA gene sequence comparison.* Int. J. Syst. Bacteriol. , 1998. **48**: p. 581-589.

20. Katsumi, M., et al., *Comparative preparation methods of sialylated capsule antigen from Streptococcus suis type 2 with type specific antigenicity.* J Vet Med Sci, 1996. **58**(10): p. 947-52.
21. Gottschalk, M., et al., *Isolation and characterization of Streptococcus suis capsular types 9-22.* J Vet Diagn Invest, 1991. **3**(1): p. 60-5.
22. Higgins, R., et al., *Description of six new capsular types (29-34) of Streptococcus suis.* J Vet Diagn Invest, 1995. **7**(3): p. 405-6.
23. Higgins, R. and M. Gottschalk, *Distribution of Streptococcus suis capsular types in 2000.* Can. Vet. J., 2001. **42**: p. 223.
24. Perch, B., K.B. Pedersen, and J. Henrichsen, *Serology of capsulated streptococci pathogenic for pigs: six new serotypes of Streptococcus suis.* J Clin Microbiol, 1983. **17**(6): p. 993-6.
25. Fittipaldi, N., et al., *Potential use of an unencapsulated and aromatic amino acid-auxotrophic Streptococcus suis mutant as a live attenuated vaccine in swine.* Vaccine, 2007. **25**(18): p. 3524-35.
26. Berthelot-Herault, F., et al., *Production of muraminidase-released protein (MRP), extracellular factor (EF) and suilysin by field isolates of Streptococcus suis capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France.* Vet Res, 2000. **31**(5): p. 473-9.
27. Wisselink, H.J., et al., *Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of Streptococcus suis strains isolated from diseased pigs in seven European countries.* Vet Microbiol, 2000. **74**(3): p. 237-48.
28. Ye, C., et al., *Streptococcus suis sequence type 7 outbreak, Sichuan, China.* Emerg Infect Dis, 2006. **12**(8): p. 1203-8.
29. Lun, Z.R., et al., *Streptococcus suis: an emerging zoonotic pathogen.* Lancet Infect Dis, 2007. **7**(3): p. 201-9.
30. Takamatsu, D., et al., *Streptococcus suis in humans, Thailand.* Emerg Infect Dis, 2008. **14**(1): p. 181-3.
31. Kataoka, Y., et al., *The epidemiological studies of Streptococcus suis infections in Japan farms from 1987 to 1991.* J. Vet. Med. Sci., 1993. **55**: p. 623-626.
32. Messier, S., S. Lacouture, and M. Gottschalk, *Distribution of Streptococcus suis capsular types from 2001 to 2007.* Can Vet J, 2008. **49**(5): p. 461-2.
33. Fittipaldi, N., et al., *Serotype distribution and production of muramidase-released protein, extracellular factor and suilysin by field strains of Streptococcus suis isolated in the United States.* vet. Microbiol., 2009. **18**: p. 310-317.
34. MacInnes, J.I. and R. Desrosiers, *Agents of the "suis-ide diseases" of swine: Actinobacillus suis, Haemophilus parasuis, and Streptococcus suis.* Can. J. Vet. Research, 1999. **63**: p. 83-89.
35. Clements, M.R., et al., *Streptococcus suis type II infection. A new industrial disease?* Practitioner, 1982. **226**(1364): p. 323-325.
36. Clifton-Hadley, F.A., *Streptococcus suis type 2 infections.* Br Vet J, 1983. **139**(1): p. 1-5.
37. Dee, S.A., et al., *Effect of management practices on the Streptococcus suis carrier rate in nursery swine.* J Am Vet Med Assoc, 1993. **203**(2): p. 295-299.
38. Clifton-Hadley, F.A., *Studies of Streptococcus suis type 2 infection in pigs.* Vet Res Commun, 1984. **8**(3): p. 217-227.
39. Tarradas, C., et al., *Identification of Streptococcus suis isolated from swine: proposal for biochemical parameters.* J Clin Microbiol, 1994. **32**(2): p. 578-580.

40. Fittipaldi, N., et al., *Significant contribution of the pgdA gene to the virulence of Streptococcus suis.* Mol. Microbiol., 2008. **70**: p. 1120-1135.
41. Madsen, L.W., et al., *Experimental Infection of Conventional Pigs with Streptococcus suis serotype 2 by Aerosolic Exposure.* Acta Vet. Scand., 2001. **42**: p. 303–306.
42. Zheng, P., et al., *Pathologic analysis of the brain from Streptococcus suis type 2 experimentally infected pigs.* Vet. Pathol., 2009. **46**: p. 531-535.
43. Reams, R.Y., et al., *Streptococcus suis infection in swine: a retrospective study of 256 cases. Part II. Clinical signs, gross and microscopic lesions, and coexisting microorganisms.* J Vet Diagn Invest, 1994. **6**(3): p. 326-34.
44. Galina, L., J.E. Collins, and C. Pijoan, *Porcine Streptococcus suis in Minnesota.* J. Vet. Diagn. Invest., 1992. **4**(2): p. 195-196.
45. Galina, L., J.E. Collins, and C. Pijoan, *Porcine Streptococcus suis in Minnesota.* J Vet Diagn Invest, 1992. **4**(2): p. 195-196.
46. Reams, R.Y., et al., *Multiple serotypes and strains of Streptococcus suis in naturally infected swine herds.* J Vet Diagn Invest, 1996. **8**(1): p. 119-21.
47. Vecht, U., et al., *Virulence of Streptococcus suis type 2 strains in newborn germfree pigs depends on phenotype.* Infect Immun, 1992. **60**(2): p. 550-556.
48. Feng, W., et al., *In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility of piglets to challenge by Streptococcus suis type II.* J Virol, 2001. **75**(10): p. 4889-4895.
49. Schmitt, C.S., et al., *Influence of ampicillin, ceftiofur, attenuated live PRRSV vaccine, and reduced dose Streptococcus suis exposure on disease associated with PRRSV and S. suis coinfection.* Vet Microbiol, 2001. **78**(1): p. 29-37.
50. Pallares, F.J., et al., *Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS).* J Vet Diagn Invest, 2002. **14**(6): p. 515-519.
51. Cloutier, G., et al., *Epidemiology of Streptococcus suis serotype 5 infection in a pig herd with and without clinical disease.* Vet Microbiol, 2003. **97**(1-2): p. 135-51.
52. Berthelot-Herault, F., et al., *Experimental airborne transmission of Streptococcus suis capsular type 2 in pigs.* Vet Microbiol, 2001. **82**(1): p. 69-80.
53. Robertson, I.D. and D.K. Blackmore, *Prevalence of Streptococcus suis types 1 and 2 in domestic pigs in Australia and New Zealand.* Vet Rec, 1989. **124**(15): p. 391-394.
54. Torremorell, M. and C. Pijoan, *Prolonged persistence of an epidemic Streptococcus suis strain in a closed pig population.* Vet Rec, 1998. **143**(14): p. 394-395.
55. Arends, J.P. and H.C. Zanen, *Meningitis caused by Streptococcus suis in humans.* Rev. Infect. Dis., 1988. **10**: p. 131-137.
56. Wertheim, H.F.L., et al., *Streptococcus suis: An Emerging Human Pathogen.* Clin. Infect. Dis., 2009. **48**: p. 617–625.
57. Perch, B., P. Kristjansen, and K. Skadhauge, *Group R streptococci pathogenic for man. Two cases of meningitis and one fatal case of sepsis.* Acta Pathol Microbiol Scand, 1968. **74**(1): p. 69-76.
58. Taipa, R., V. Lopes, and M. Magalhaes, *Streptococcus suis meningitis: first case report from Portugal.* J Infect, 2008. **56**(6): p. 482-483.
59. Fittipaldi, N., et al., *Streptococcus suis meningitis, Hawaii.* Emerg Infect Dis, 2009. **15**: p. 2067-2069.
60. Lee, G.T., et al., *Streptococcus suis meningitis, United States.* Emerg Infect Dis, 2008. **14**(1): p. 183-185.

61. Ibaraki, M., et al., *A Japanese case of Streptococcus suis meningitis associated with lumbar epidural abscess.* Rinsho Shinkeigaku, 2003. **4**: p. 176-179.
62. Arends, J.P. and H.C. Zanen, *Meningitis caused by Streptococcus suis in humans.* Rev Infect Dis, 1988. **10**(1): p. 131-137.
63. Gottschalk, M., et al., *Description of 14 new capsular types of Streptococcus suis.* J Clin Microbiol, 1989. **27**(12): p. 2633-2636.
64. Kerdsin, A., et al., *Clonal dissemination of human isolates of Streptococcus suis serotype 14 in Thailand.* J. Med. Microbiol., 2009. **58**: p. 1508-1513.
65. Haleis, A., et al., *Meningitis caused by Streptococcus suis serotype 14, North America.* Emerg. Infect. Dis. , 2009. **15**: p. 350-352.
66. Nghia, H.D., et al., *Human case of Streptococcus suis serotype 16 infection.* Emerg Infect Dis, 2008. **14**(1): p. 155-157.
67. Kopic, J., M.T. Paradzik, and N. Pandak, *Streptococcus suis infection as a cause of severe illness: 2 cases from Croatia.* Scand J Infect Dis, 2002. **34**(9): p. 683-684.
68. Segura, M., *Streptococcus suis: An Emerging Human Threat.* J. Infect. Dis., 2009. **199** (1):4-6.
69. Wertheim, H.F.L., et al., *Streptococcus suis, an important cause of adult bacterial meningitis in Northern Vietnam.* PLoS ONE, 2009. **4**: p. e5973.
70. Hui, A.C., et al., *Bacterial meningitis in Hong Kong: 10-years' experience.* Clin Neurol Neurosurg, 2005. **107**(5): p. 366-70.
71. Ip, M., et al., *Streptococcus suis in Hong Kong.* Diagn Microbiol Infect Dis, 2007. **57**(1): p. 15-20.
72. Mai, N.T., et al., *Streptococcus suis Meningitis in Adults in Vietnam.* Clin Infect Dis, 2008.
73. Arend, S.M., et al., *Septicaemia, meningitis and spondylodiscitis caused by Streptococcus suis type 2.* Infection, 1995. **23**(2): p. 128.
74. Huang, Y.T., et al., *Streptococcus suis infection.* J Microbiol Immunol Infect, 2005. **38**(5): p. 306-13.
75. Walsh, B., A. Williams, and J. Satsangi, *Streptococcus suis type 2: pathogenesis and clinical disease.* Reviews in Medical Microbiology, 1992. **3**: p. 65-71.
76. Ishigaki, K., et al., *A case of Streptococcus suis endocarditis, probably bovine-transmitted, complicated by pulmonary embolism and spondylitis.* Kansenshogaku Zasshi, 2009. **83**: p. 544-548.
77. Laohapensang, K., R.B. Rutherford, and S. Arworn, *Mycotic Abdominal Aortic Aneurysm Due to Streptococcus suis: A Case Report.* Surg. Infect. (Larchmt), 2010 **11** (2):179-181.
78. Meecham, J.S. and R.C. Worth, *Persistent diplopia following Streptococcus suis type 2 meningitis.* J. R. Soc. Med., 1992. **85**: p. 579-580.
79. Voutsadakis, I.A., *Streptococcus suis endocarditis and colon carcinoma: a case report.* Clin Colorectal Cancer, 2006. **6**(3): p. 226-228.
80. Huang, Y.T., et al., *Streptococcus suis infection.* J. Microbiol. Immunol. Infect., 2005. **38**: p. 306-313.
81. Smith, T.C., et al., *Exposure to Streptococcus suis among US swine workers.* Emerg Infect Dis, 2008. **14**(12): p. 1925-1927.
82. Gottschalk, M., M. Segura, and J. Xu, *Streptococcus suis infections in humans: the Chinese experience and the situation in North America.* Anim. Health Res. Rev. , 2007. **8**: p. 29-45.
83. Heath, P.J., et al., *Streptococcus suis serotype 14 as a cause of pig disease in the UK.* Vet Rec, 1996. **139**(18): p. 450-451.

84. Bahloul, H., et al., *[Streptococcus suis meningitis after oral contamination?].* Med Mal Infect, 2008. **38**(5): p. 281-282.
85. Fongcom, A., et al., *Streptococcus suis infection in northern Thailand.* J Med Assoc Thai, 2001. **84**(10): p. 1502-1508.
86. Cheung, P.Y., et al., *Streptococcus suis in retail markets: How prevalent is it in raw pork?* Int J Food Microbiol, 2008. **127**(3): p. 316-320.
87. Tang, J., et al., *Streptococcal toxic shock syndrome caused by Streptococcus suis serotype 2.* PLoS Med, 2006. **3**(5): p. e151.
88. Gallagher, F., *Streptococcus infection and splenectomy.* Lancet, 2001. **357**(9262): p. 1129-1130.
89. Watkins, E.J., et al., *Septicaemia in a pig-farm worker.* Lancet, 2001. **357**(9249): p. 38.
90. Berthelot-Herault, F., et al., *Genetic diversity of Streptococcus suis strains isolated from pigs and humans as revealed by pulsed-field gel electrophoresis.* J Clin Microbiol, 2002. **40**(2): p. 615-619.
91. Chatellier, S., et al., *Relatedness of Streptococcus suis serotype 2 isolates from different geographic origins as evaluated by molecular fingerprinting and phenotyping.* J Clin Microbiol, 1999. **37**(2): p. 362-366.
92. Marois, C., et al., *Molecular characterization of Streptococcus suis strains by 16S-23S intergenic spacer polymerase chain reaction and restriction fragment length polymorphism analysis.* Can J Vet Res, 2006. **70**(2): p. 94-104.
93. Pedroli, S., et al., *[Streptococcus suis bacteremia].* Presse Med, 2003. **32**(13 Pt 1): p. 599-601.
94. Rehm, T., et al., *Amplified fragment length polymorphism of Streptococcus suis strains correlates with their profile of virulence-associated genes and clinical background.* J Med Microbiol, 2007. **56**(Pt 1): p. 102-109.
95. Yu, H., et al., *Human Streptococcus suis outbreak, Sichuan, China.* Emerg Infect Dis, 2006. **12**(6): p. 914-920.
96. Zhu, F., et al., *[Homogeneity study on the Streptococcus suis isolated from human and swine].* Zhonghua Liu Xing Bing Xue Za Zhi, 2000. **21**(6): p. 427-429.
97. Zheng, H., et al., *Mitogenic effect contributes to increased virulence of Streptococcus suis sequence type 7 to cause streptococcal toxic shock-like syndrome.* Clin Exp Immunol, 2008. **153**(3): p. 385-391.
98. Yu, H., et al., *Human Streptococcus suis outbreak, Sichuan, China.* Emerg. Infect. Dis., 2006. **12**: p. 914-920.
99. Devriese, L.A., et al., *Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats.* J Appl Bacteriol, 1992. **73**(5): p. 421-425.
100. Devriese, L.A., et al., *Streptococcus suis infection in fallow deer.* Vet Rec, 1993. **132**(11): p. 283.
101. Devriese, L.A. and F. Haesebrouck, *Streptococcus suis infections in horses and cats.* Vet Rec, 1992. **130**(17): p. 380.
102. Devriese, L.A., et al., *Streptococcus suis infection in birds.* Avian Pathology, 1994. **23**: p. 721-724.
103. Devriese, L.A., et al., *Streptococcus suis meningitis in a horse.* Vet Rec, 1990. **127**(3): p. 68.
104. Hommez, J., et al., *Streptococcus suis and other streptococcal species as a cause of extramammary infection in ruminants.* Vet Rec, 1988. **123**(24): p. 626-627.

105. Salasia, S.I., C. Lammler, and L.A. Devriese, *Serotypes and putative virulence markers of Streptococcus suis isolates from cats and dogs.* Res Vet Sci, 1994. **57**(2): p. 2592-61.
106. Hojo, K., et al., *Predominant bacteria recovered from a periodontitis site in a hamster model raised by silk-ligature with Prophyromonas gingivalis infection.* Biosci Biotechnol Biochem, 2008. **72**(5): p. 1348-1351.
107. Cruz Colque, J.I., L.A. Devriese, and F. Haesebrouck, *Streptococci and enterococci associated with tonsils of cattle.* Lett Appl Microbiol, 1993. **16**(2): p. 72-74.
108. Segers, R.P., et al., *Characterisation of the gene encoding suilysin from Streptococcus suis and expression in field strains.* FEMS Microbiol Lett, 1998. **167**(2): p. 255-261.
109. Halaby, T., et al., *Streptococcus suis meningitis, a poacher's risk.* Eur J Clin Microbiol Infect Dis, 2000. **19**(12): p. 943-945.
110. Rosenkranz, M., et al., *Streptococcus suis meningitis and septicemia contracted from a wild boar in Germany.* J Neurol, 2003. **250**(7): p. 869-870.
111. Baums, C.G., et al., *Prevalence of Streptococcus suis genotypes in wild boars of Northwestern Germany.* Appl Environ Microbiol, 2007. **73**(3): p. 711-717.
112. Kataoka, Y., T. Yoshida, and T. Sawada, *A 10-year survey of antimicrobial susceptibility of Streptococcus suis isolates from swine in Japan.* J. Vet. Med. Sci. , 2000. **62**: p. 1053-1057.
113. Martel, A., et al., *Prevalence and mechanism of resistance against macrolides and lincosamides in Streptococcus suis isolates* vet. Microbiol., 2001. **83**: p. 287-297.
114. Aarestrup, F.M., S.E. Jorsal, and N.E. Jensen, *Serological characterization and antimicrobial susceptibility of Streptococcus suis isolates from diagnostic samples in Denmark and Sweden.* Vet. Microbiol., 1998. **63**: p. 71–80.
115. Zhang, C., et al., *In vitro antimicrobial susceptibility of Streptococcus suis strains isolated from clinically healthy sows in China.* Vet. Microbiol., 2008. **131**: p. 386-392.
116. Busque, P., et al., *Immunization of pigs against Streptococcus suis serotype 2 infection using a live avirulent strain.* Can. J. Vet. Research, 1997. **61**: p. 275–279.
117. Haesebrouck, F., et al., *Efficacy of vaccines against bacterial diseases in swine: what can we expect? .* Vet. Microbiol., 2004. **100**: p. 255-268
118. Kock, C., et al., *Intranasal immunization with a live Streptococcus suis isogenic ofs mutant elicited suilysin-neutralization titers but failed to induce opsonizing antibodies and protection.* Vet. Immunol. Immunopathol. , 2009. **15**: p. 135-145.
119. Jacobs, A.A.C., A.J.G. van den Berg, and P.L.W. Loeffen, *Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of Streptococcus suis.* Vet. Rec., 1996. **139**: p. 225–228.
120. Gottschalk, M., et al., *Production of virulence-related proteins by Canadian strains of Streptococcus suis capsular type 2.* Can J Vet Res, 1998. **62**(1): p. 75-79.
121. Zhang, A., et al., *Identification of a surface protective antigen, HP0197 of Streptococcus suis serotype 2.* Vaccine, 2009. **27**: p. 5209-5213.
122. Tan, C., et al., *Vaccination with Streptococcus suis serotype 2 recombinant 6PGD protein provides protection against S. suis infection in swine.* FEMS Microbiol. Lett. , 2009. **296**: p. 78-83.
123. Li, Y., et al., *Identification of a surface protein of Streptococcus suis and evaluation of its immunogenic and protective capacity in pigs.* Infect Immun, 2006. **74**(1): p. 305-312.
124. Li, Y., et al., *Immunization with recombinant Sao protein confers protection against Streptococcus suis infection.* Clin Vaccine Immunol, 2007. **14**(8): p. 937-43.
125. Esgleas, M., et al., *Immunization with SsEno fails to protect mice against challenge with Streptococcus suis serotype 2.* FEMS Microbiol. Lett., 2009. 294: p. 82-88.

126. Feng, Y., et al., *Streptococcus suis enolase functions as a protective antigen displayed on the bacterial cell surface.* J. Infect. Dis., 2009. **200**: p. 1583-1592.
127. Lutticken, R., et al., *Meningitis caused by Streptococcus suis: case report and review of the literature.* Infection, 1986. **14**(4): p. 181-5.
128. Mai, N.T., et al., *Streptococcus suis meningitis in adults in Vietnam.* Clin. Infect. Dis., 2008. **46**: p. 659-667.
129. Gottschalk, M., et al., *Streptococcus suis: a new emerging or an old neglected zoonotic pathogen?* Future Microbiol., 2010. **5**: p. 371-391.
130. Greenwood, B.M., *Corticosteroids for acute bacterial meningitis.* N. Engl. J. Med. , 2007. **357**: p. 2507-2509.
131. Navacharoen, N., et al., *Hearing and vestibular loss in Streptococcus suis infection from swine and traditional raw pork exposure in northern Thailand.* J. Laryngol. Otol., 2009. **123**: p. 857-862.
132. Beaudoin, M., et al., *Studies on a murine model for evaluation of virulence of Streptococcus suis capsular type 2 isolates.* FEMS Microbiol Lett, 1992. **78**(2-3): p. 111-116.
133. Berthelot-Herault, F., et al., *Experimental infection of specific pathogen free piglets with French strains of Streptococcus suis capsular type 2.* Can J Vet Res, 2001. **65**(3): p. 196-200.
134. Galina, L., et al., *Interaction between Streptococcus suis serotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets.* Vet Rec, 1994. **134**(3): p. 60-64.
135. Vecht, U., et al., *Murine and pig models of Streptococcus suis type 2 infections are incompatible.* Adv Exp Med Biol, 1997. **418**: p. 827-829.
136. Gottschalk, M., R. Higgins, and S. Quessy, *Dilemma of the virulence of Streptococcus suis strains.* J. Clin. Microbiol., 1999. **37**: p. 4202-4203.
137. Gottschalk, M., R. Higgins, and S. Quessy, *Dilemma of the virulence of Strptococcus suis strains.* J Clin Microbiol, 1999. **37**(12): p. 4202-4203.
138. Staats, J.J., et al., *Presence of the Streptococcus suis suilysin gene and expression of MRP and EF correlates with high virulence in Streptococcus suis type 2 isolates.* Vet Microbiol, 1999. **70**(3-4): p. 201-211.
139. Jacques, M., et al., *Ultrastructural study of surface components of Streptococcus suis.* J Bacteriol, 1990. **172**(6): p. 2833-2838.
140. Smith, H.E., et al., *Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor.* Infect. Immun., 1999. **67**: p. 1750-1756.
141. Charland, N., et al., *Streptococcus suis serotype 2 mutants deficient in capsular expression.* Microbiology, 1998. **144**: p. 325-332.
142. Segura, M.A., P. Cléroux, and M. Gottschalk, *Streptococcus suis and group B Streptococcus differ in their interactions with murine macrophages.* FEMS Immunol. Med. Microbiol. , 1998. **21**: p. 189-195.
143. Elliott, S.D. and J.Y. Tai, *The type-specific polysaccharides of Streptococcus suis.* J Exp Med, 1978. **148**(6): p. 1699-1704.
144. Smith, H.E., et al., *Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor.* Infect Immun, 1999. **67**(4): p. 1750175-6.
145. Smith, H.E., et al., *The cps locus of Streptococcus suis serotype 2: genetic determinant for the synthesis of sialic acid.* Microb Pathog, 2000. **29**(2): p. 127-134.

146. Charland, N., et al., *Streptococcus suis serotype 2 mutants deficient in capsular expression.* Microbiology, 1998. **144 (Pt 2)**: p. 325-332.
147. Chabot-Roy, G., et al., *Phagocytosis and killing of Streptococcus suis by porcine neutrophils.* Microb Pathog, 2006. **41**(1): p. 21-32.
148. Segura, M., M. Gottschalk, and M. Olivier, *Encapsulated Streptococcus suis inhibits activation of signaling pathways involved in phagocytosis.* Infect. Immun., 2004. **72**: p. 5322-5330.
149. Segura, M. and M. Gottschalk, *Streptococcus suis interactions with the murine macrophage cell line J774: adhesion and cytotoxicity.* Infect. Immun., 2002. **70**: p. 4312-4322.
150. Charland, N., et al., *Role of capsular sialic acid in virulence and resistance to phagocytosis of Streptococcus suis capsular type 2.* FEMS Immunol. Med. Microbiol., 1996. **14**: p. 195-203.
151. Gottschalk, M., et al., *Characterization of six new capsular types (23 through 28) of Streptococcus suis.* J Clin Microbiol, 1991. **29**(11): p. 2590-2594.
152. Quessy, S., et al., *Increase of capsular material thickness following in vivo growth of virulent Streptococcus suis serotype 2 strains.* FEMS Microbiol Lett, 1994. **115**(1): p. 19-26.
153. Wessels, M.R., et al., *Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule.* Proc Natl Acad Sci U S A, 1989. **86**(22): p. 8983-8987.
154. Charland, N., et al., *Role of capsular sialic acid in virulence and resistance to phagocytosis of Streptococcus suis capsular type 2.* FEMS Immunol Med Microbiol, 1996. **14**(4): p. 195-203.
155. Kengatharan, K.M., et al., *Mechanism of Gram-positive Shock: Identification of Peptidoglycan and Lipoteichoic Acid Moieties Essential in the Induction of Nitric Oxide Synthase, Shock, and Multiple Organ Failure.* J. Exp. Med., 1998. **188**: p. 305-315.
156. Fischer, W., *Physiology of lipoteichoic acids in bacteria.* Adv. Microb. Physiol. , 1988. **29**: p. 233-302.
157. Poyart, C., et al., *Attenuated virulence of Streptococcus agalactiae deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells.* Molecular Microbiology, 2003. **49**(6): p. 1615-1625.
158. Perego, M., et al., *Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in Bacillus subtilis. Identification of genes and regulation.* J Biol Chem, 1995. **270**(26): p. 15598-15606.
159. Neuhaus, F.C., et al., *The dlt operon in the biosynthesis of D-alanyl-lipoteichoic acid in Lactobacillus casei.* Microb. Drug Resist., 1996. **2**: p. 77-84.
160. Kristian, S.A., et al., *D-Alanylation of Teichoic Acids Promotes Group A Streptococcus Antimicrobial Peptide Resistance, Neutrophil Survival, and Epithelial Cell Invasion.* J. Bacteriol., 2005. **187**(19): p. 6719-6725.
161. Kovács, M., et al., *A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in Streptococcus pneumoniae.* J. Bacteriol., 2006. **188**: p. 5797-5805.
162. Chan, K.G., et al., *Role of D-alanylation of Streptococcus gordonii lipoteichoic acid in innate and adaptive immunity.* Infect. Immun., 2007. **75**: p. 3033-3042.
163. Fittipaldi, N., et al., *D-alanylation of lipoteichoic acid contributes to the virulence of Streptococcus suis.* Infect. Immun., 2008. **76**: p. 587-594.

164. Vollmer, W., D. Blanot, and M.A. de Pedro, *Peptidoglycan structure and architecture.* FEMS Microbiol Rev, 2008. **32**(2): p. 149-167.
165. Mattsson, E., et al., *Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumor necrosis factor-alpha, interleukin-1ß and interleukin-6.* FEMS Immunol. Med. Microbiol., 1993. **7**: p. 281–287.
166. Boneca, I.G., *The role of peptidoglycan in pathogenesis.* Curr Opin Microbiol, 2005. **8**(1): p. 46-53.
167. Chaput, C. and I.G. Boneca, *Peptidoglycan detection by mammals and flies.* Microbes Infect, 2007. **9**(5): p. 637-647.
168. Vollmer, W. and A. Tomasz, *The pgdA gene encodes for a peptidoglycan N-acetylglucosamine deacetylase in Streptococcus pneumoniae.* J Biol Chem, 2000. **275**(27): p. 20496-501.
169. Boneca, I.G., et al., *A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 997-1002.
170. Baums, C.G., et al., *Identification of a novel virulence determinant with serum opacification activity in Streptococcus suis.* Infect. Immun., 2006. **74**: p. 6154-6162.
171. Baums, C.G., et al., *Identification of a novel virulence determinant with serum opacification activity in Streptococcus suis.* Infect Immun, 2006. **74**(11): p. 6154-62.
172. Takamatsu, D., et al., *Allelic variation and prevalence of serum opacity factor among the Streptococcus suis population.* J Med Microbiol, 2008. **57**(Pt 4): p. 488-94.
173. Vecht, U., et al., *Identification of two proteins associated with virulence of Streptococcus suis type 2.* Infect Immun, 1991. **59**(9): p. 3156-62.
174. Marraffini, L.A., A.C. Dedent, and O. Schneewind, *Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria.* Microbiol Mol Biol Rev, 2006. **70**(1): p. 192-221.
175. Vecht, U., et al., *Differences in virulence between two strains of Streptococcus suis type II after experimentally induced infection of newborn germ-free pigs.* Am J Vet Res, 1989. **50**(7): p. 1037-1043.
176. Smith, H.E., et al., *Repeats in an extracellular protein of weakly pathogenic strains of Streptococcus suis type 2 are absent in pathogenic strains.* Infect Immun, 1993. **61**(8): p. 3318-3326.
177. Smith, H.E., et al., *Mutants of Streptococcus suis types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs.* Infect Immun, 1996. **64**(10): p. 4409-4412.
178. Jacobs, A.A., et al., *Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of Streptococcus suis.* Infect Immun, 1994. **62**(5): p. 1742-1748.
179. Gottschalk, M.G., S. Lacouture, and J.D. Dubreuil, *Characterization of Streptococcus suis capsular type 2 haemolysin.* Microbiology, 1995. **141 (Pt 1)**: p. 189-195.
180. King, S.J., et al., *Distribution and genetic diversity of suilysin in Streptococcus suis isolated from different diseases of pigs and characterization of the genetic basis of suilysin absence.* Infect Immun, 2001. **69**(12): p. 7572-82.
181. Okwumabua, O., O. Abdelmagid, and M.M. Chengappa, *Hybridization analysis of the gene encoding a hemolysin (suilysin) of Streptococcus suis type 2: evidence for the absence of the gene in some isolates.* FEMS Microbiol Lett, 1999. **181**(1): p. 113-21.
182. Segers, R.P., et al., *Characterisation of the gene encoding suilysin from Streptococcus suis and expression in field strains.* FEMS Microbiol. Lett., 1998. **167**(2): p. 255-261.

183. Palmer, M., *The family of thiol-activated, cholesterol-binding cytolysins.* Toxicon, 2001. **39**(11): p. 1681-9.
184. Alouf, J.E., H. Knoll, and W. Kohler, *The family of mitogenic shock-inducing and superantigenic toxins from staphylococci and streptococci*, in *Sourcebook of bacterial protein toxins*, J.E. Alouf and J.H. Freer, Editors. 1991, Academic Press: London.
185. Charland, N., et al., *Streptococcus suis serotype 2 interactions with human brain microvascular endothelial cells.* Infect Immun, 2000. **68**(2): p. 637-43.
186. Lalonde, M., et al., *Interactions between Streptococcus suis serotype 2 and different epithelial cell lines.* Microbiology, 2000. **146 (Pt 8)**: p. 1913-21.
187. Norton, P.M., et al., *Epithelial invasion and cell lysis by virulent strains of Streptococcus suis is enhanced by the presence of suilysin.* FEMS Immunol Med Microbiol, 1999. **26**(1): p. 25-35.
188. Segura, M. and M. Gottschalk, *Streptococcus suis interactions with the murine macrophage cell line J774: adhesion and cytotoxicity.* Infect Immun, 2002. **70**(8): p. 4312-22.
189. Vadeboncoeur, N., et al., *Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2.* FEMS Immunol Med Microbiol, 2003. **35**(1): p. 49-58.
190. Vanier, G., et al., *Invasion of porcine brain microvascular endothelial cells by Streptococcus suis serotype 2.* Infect Immun, 2004. **72**(3): p. 1441-9.
191. Vanier, G., et al., *Porcine brain microvascular endothelial cell-derived interleukin-8 is first induced and then degraded by Streptococcus suis.* Microb Pathog, 2008.
192. Segura, M., et al., *Proinflammatory cytokine and chemokine modulation by Streptococcus suis in a whole-blood culture system.* FEMS Immunol Med Microbiol, 2006. **47**(1): p. 92-106.
193. Lun, S., et al., *Role of suilysin in pathogenesis of Streptococcus suis capsular serotype 2.* Microb Pathog, 2003. **34**(1): p. 27-37.
194. Al-Numani, D., et al., *Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by Streptococcus suis serotype 2.* Clin Exp Immunol, 2003. **133**(1): p. 67-77.
195. Jobin, M.C., et al., *Acquisition of plasmin activity and induction of arachidonic acid release by Streptococcus suis in contact with human brain microvascular endothelial cells.* FEMS Microbiol. Lett. , 2005. **252**: p. 105-111.
196. Feder, I., et al., *Partial characterization of Streptococcus suis type 2 hemolysin.* J Clin Microbiol, 1994. **32**(5): p. 1256-60.
197. Allen, A.G., et al., *Generation and characterization of a defined mutant of Streptococcus suis lacking suilysin.* Infect Immun, 2001. **69**(4): p. 2732-5.
198. Esgleas, M., S. Lacouture, and M. Gottschalk, *Streptococcus suis serotype 2 binding to extracellular matrix proteins.* FEMS Microbiol Lett, 2005. **244**(1): p. 33-40.
199. de Greeff, A., et al., *Contribution of fibronectin-binding protein to pathogenesis of Streptococcus suis serotype 2.* Infect Immun, 2002. **70**(3): p. 1319-25.
200. Quessy, S., et al., *Description of an albumin binding activity for Streptococcus suis serotype 2.* FEMS Microbiol Lett, 1997. **147**: p. 245-250.
201. Wang, K. and C. Lu, *Adhesion activity of glyceraldehyde-3-phosphate dehydrogenase in a Chinese Streptococcus suis type 2 strain.* Berl Munch Tierarztl Wochenschr, 2007. **120**(5-6): p. 207-9.
202. Jobin, M.C., et al., *Acquisition of host plasmin activity by the Swine pathogen Streptococcus suis serotype 2.* Infect Immun, 2004. **72**(1): p. 606-10.

203. Brassard, J., M. Gottschalk, and S. Quessy, *Cloning and purification of the Streptococcus suis serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin.* Vet Microbiol, 2004. **102**(1-2): p. 87-94.
204. Tan, C., et al., *The key virulence-associated genes of Streptococcus suis type 2 are upregulated and differentially expressed in vivo.* FEMS Microbiol Lett, 2008. **278**(1): p. 108-14.
205. Zhang, A., et al., *Identification of immunogenic cell wall-associated proteins of Streptococcus suis serotype 2.* Proteomics, 2008. **8**(17): p. 3506-15.
206. Serhir, B., et al., *Detection of immunoglobulin-G-binding proteins in Streptococcus suis.* J. Gen. Microbiol., 1993. **139**: p. 2953-2958.
207. Serhir, B., et al., *Purification and characterization of a 52-kilodalton immunoglobulin G-binding protein from Streptococcus suis capsular type 2.* J Bacteriol, 1995. **177**(13): p. 3830-6.
208. Benkirane, R., M.G. Gottschalk, and J.D. Dubreuil, *Identification of a Streptococcus suis 60-kDa heat-shock protein using western blotting.* FEMS Microbiol Lett, 1997. **153**(2): p. 379-85.
209. Benkirane, R., et al., *Immunohistochemical characterization of an IgG-binding protein of Streptococcus suis.* FEMS Immunol Med Microbiol, 1998. **20**: p. 121-127.
210. Esgleas, M., et al., *Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from Streptococcus suis.* Microbiology, 2008. **154**(Pt 9): p. 2668-79.
211. Pancholi, V., *Multifunctional alpha-enolase: its role in diseases.* Cell Mol Life Sci, 2001. **58**(7): p. 902-20.
212. Pancholi, V. and V.A. Fischetti, *alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci.* J Biol Chem, 1998. **273**(23): p. 14503-15.
213. Vanier, G., et al., *Disruption of srtA gene in Streptococcus suis results in decreased interactions with endothelial cells and extracellular matrix proteins.* Vet Microbiol, 2008. **127**(3-4): p. 417-24.
214. Geng, H., et al., *Identification and characterization of novel immunogenic proteins of Streptococcus suis serotype 2.* J Proteome Res, 2008. **7**(9): p. 4132-42.
215. Fittipaldi, N., et al., *Mutations in the gene encoding the ancillary pilin subunit of the Streptococcus suis srtF cluster result in pili formed by the major subunit only.* PLoS ONE, 2010. **5**: p. e8426.
216. Gottschalk, M., et al., *Hemagglutination properties of Streptococcus suis.* J Clin Microbiol, 1990. **28**(9): p. 2156-8.
217. Wang, C., et al., *The involvement of sortase A in high virulence of STSS-causing Streptococcus suis serotype 2.* Arch Microbiol, 2008.
218. Vanier, G., et al., *New putative virulence factors of Streptococcus suis involved in invasion of porcine brain microvascular endothelial cells.* Microb Pathog, 2008.
219. Fittipaldi, N., et al., *Mutations in the gene encoding the ancillary pilin subunit of the Streptococcus suis srtF cluster result in pili formed by the major subunit only.* PLoS ONE, 2010. **5**: p. e8426.
220. Supuran, T.C., A. Scozzafava, and A. Mastrolorenzo, *Bacterial proteases: current therapeutic use and future prospects for the development of new antibiotics.* Expert Opin. Ther. Pat., 2001. **11**: p. 221-259.
221. Allen, A.G., et al., *Identification and characterisation of hyaluronate lyase from Streptococcus suis.* Microb Pathog, 2004. **36**(6): p. 327-35.

222. Jobin, M.C. and D. Grenier, *Identification and characterization of four proteases produced by Streptococcus suis.* FEMS Microbiol. Lett., 2003. **220** p. 113-119.
223. Jobin, M.C. and D. Grenier, *Identification and characterization of four proteases produced by Streptococcus suis.* FEMS Microbiol Lett, 2003. **220**(1): p. 113-9.
224. Jobin, M.C., et al., *Cloning, purification, and enzymatic properties of dipeptidyl peptidase IV from the swine pathogen Streptococcus suis.* J. Bacteriol., 2005 **187**: p. 795-799.
225. Mentlein, R., *Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides.* Regul. Pept., 1999. **85**: p. 9–24.
226. Ge, J., et al., *Inactivation of dipeptidyl peptidase IV attenuates the virulence of Streptococcus suis serotype 2 that causes streptococcal toxic shock syndrome.* Curr. Microbiol. , 2009. **59**: p. 248-255.
227. King, S.J., et al., *Distribution, genetic diversity, and variable expression of the gene encoding hyaluronate lyase within the Streptococcus suis population.* J Bacteriol, 2004. **186**(14): p. 4740-7.
228. Vanier, G., et al., *Porcine brain microvascular endothelial cell-derived interleukin-8 is first induced and then degraded by Streptococcus suis.* Microb. Pathog. , 2009. **46**: p. 135-143.
229. Vanier, G., et al. *Up-regulation of IL-6 and IL-8 secretion by porcine brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2*. in *57th Annual Conference from the Canadian Society of Microbiologists*. 2007. Quebec, QC, Canada.: Université Laval.
230. Zhang, A., et al., *Identification and characterization of IgA1 protease from Streptococcus suis.* Vet. Microbiol., 2010. **6**: p. 171-175.
231. Bonifait, L., et al., *The cell envelope subtilisin-like proteinase is a critical virulence determinant for Streptococcus suis.* BMC Microbiology, 2010. **10**: p. 42.
232. Hu, Q., et al., *Identification of a cell wall-associated subtilisin-like serine protease involved in the pathogenesis of Streptococcus suis serotype 2.* Microb. Pathog., 2010. **48**(103-109).
233. Osaki, M., et al., *Characterization of Streptococcus suis genes encoding proteins homologous to sortase of gram-positive bacteria.* J Bacteriol, 2002. **184**(4): p. 971-82.
234. Navarre, W.W. and O. Schneewind, *Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope.* Microbiol Mol Biol Rev, 1999. **63**(1): p. 174-229.
235. Dramsi, S., P. Trieu-Cuot, and H. Bierne, *Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria.* Res Microbiol, 2005. **156**(3): p. 289-97.
236. Fittipaldi, N., et al., *Use of selective capture of transcribed sequences to identify genes preferentially expressed by Streptococcus suis upon interaction with porcine brain microvascular endothelial cells.* Appl. Environ. Microbiol. , 2007. **73**: p. 4359-4364.
237. Okwumabua, O., J.S. Persaud, and P.G. Reddy, *Cloning and characterization of the gene encoding the glutamate dehydrogenase of Streptococcus suis serotype 2.* Clin Diagn Lab Immunol, 2001. **8**(2): p. 251-7.
238. Kutz, R. and O. Okwumabua, *Differentiation of highly virulent strains of Streptococcus suis serotype 2 according to glutamate dehydrogenase electrophoretic and sequence type.* J. Clin. Microbiol., 2008. **46**: p. 3201-3217.
239. De Greeff, A., et al., *Lipoprotein signal peptidase of Streptococcus suis serotype 2.* Microbiology, 2003. **149**(Pt 6): p. 1399-407.

240. Tu, A.H., et al., *Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae.* Infect. Immun., 1999. **67**: p. 4720-4724.
241. Okwumabua, O. and S. Chinnapapakkagari, *Identification of the gene encoding a 38-kilodalton immunogenic and protective antigen of Streptococcus suis.* Clin Diagn Lab Immunol, 2005. **12**(4): p. 484-90.
242. Poyart, C., et al., *Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of Streptococcus agalactiae.* Infect. Immun., 2001. **69**: p. 5098-5106.
243. Poyart, C., et al., *Identification of Streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase.* J. Clin. Microbiol., 1998. **36**: p. 41-47.
244. Langford, P., A.E. Williams, and J.S. Kroll, *Superoxide dismutases of pathogenic and non-pathogenic Streptococcus suis type 2 isolates.* FEMS Microbiol Lett, 1991. **61**(2-3): p. 347-50.
245. Fontaine, M.C., J. Perez-Casal, and P.J. Willson, *Investigation of a novel DNase of Streptococcus suis serotype 2.* Infect Immun, 2004. **72**(2): p. 774-81.
246. Gottschalk, M., et al., *Production and characterization of two Streptococcus suis capsular type 2 mutants.* Vet Microbiol, 1992. **30**(1): p. 59-71.
247. Winterhoff, N., et al., *Identification and characterization of two temperature-induced surface-associated proteins of Streptococcus suis with high homologies to members of the Arginine Deiminase system of Streptococcus pyogenes.* J Bacteriol, 2002. **184**(24): p. 6768-76.
248. Casiano-Colon, A. and R.E. Marquis, *Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance.* Appl. Environ. Microbiol., 1988. **54**(6): p. 1318-1324.
249. Degnan, B.A., et al., *Characterization of an Isogenic Mutant of Streptococcus pyogenes Manfredo Lacking the Ability To Make Streptococcal Acid Glycoprotein.* Infect. Immun., 2000. **68**(5): p. 2441-2448.
250. Gruening, P., et al., *Structure, regulation, and putative function of the arginine deiminase system of Streptococcus suis.* J Bacteriol, 2006. **188**(2): p. 361-9.
251. Preissner, K.T. and G.S. Chhatwal, *Extracellular matrix and host cell surfaces: potential sites of pathogen interaction*, in *Cellular Microbiology*, P. Cossart, et al., Editors. 2000, ASM Press: Washington. p. 49-65.
252. Wu, Z., W. Zhang, and C. Lu, *Comparative proteome analysis of secreted proteins of Streptococcus suis serotype 9 isolates from diseased and healthy pigs.* Microb. Pathog., 2008. **45**: p. 159-166.
253. Wilson, S.M., et al., *Interactions between Streptococcus suis serotype 2 and cells of the myeloid lineage in the palatine tonsil of the pig.* Vet Immunol Immunopathol, 2007. **117**(1-2): p. 116-23.
254. Madsen, L.W., et al., *Bacterial colonization and invasion in pigs experimentally exposed to Streptococcus suis serotype 2 in aerosol.* J Vet Med B Infect Dis Vet Public Health, 2002. **49**(5): p. 211-5.
255. Williams, D.M., G.H. Lawson, and A.C. Rowlaind, *Streptococcal infectionin piglets: the palatine tonsils as portals of entry for Streptococcus suis.* Res. Vet. Sci., 1973. **15**: p. 352-362.
256. Belz, G.T. and T.J. Heath, *Tonsils of the soft palate of young pigs: crypt structure and lymphoepithelium.* Anat. Rec., 1996. **245**: p. 102-113.
257. Fedorka-Cray, P.J., et al., *Alternate routes of invasion may affect pathogenesis of Salmonella typhimurium in swine.* Infect. Immun., 1995. **63**: p. 2658-2664.

258. Salles, M.W., et al., *Changes in the leucocyte subpopulations of the palatine tonsillar crypt epithelium of pigs in response to Streptococcus suis type 2 infection.* Vet. Immunol. Immunopathol., 2002. **87**: p. 51-63.
259. Gottschalk, M., et al., *Adherence of Streptococcus suis capsular type 2 to porcine lung sections.* Can. J. Vet. Research, 1991. **55**: p. 302–304.
260. Wu, T., et al., *The orphan response regulator RevSC21 controls the attachment of Streptococcus suis serotype-2 to human laryngeal epithelial cells and the expression of virulence genes.* FEMS Microbiol. Lett., 2009. **292**: p. 170-178.
261. Si, Y., et al., *Contribution of glutamine synthetase to the virulence of Streptococcus suis serotype 2.* Vet. Microbiol., 2009. **139**: p. 80-88.
262. Gottschalk, M. and M. Segura, *The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions.* Vet Microbiol, 2000. **76**(3): p. 259-72.
263. Benga, L., et al., *Non-encapsulated strains reveal novel insights in invasion and survival of Streptococcus suis in epithelial cells.* Cell Microbiol, 2004. **6**(9): p. 867-81.
264. Chabot-Roy, G., et al., *Phagocytosis and killing of Streptococcus suis by porcine neutrophils.* Microb. Pathog., 2006. **41**: p. 21-32.
265. Williams, A.E. and W.E. Blakemore, *Pathogenesis of meningitis caused by Streptococcus suis type 2.* J. Infect. Dis., 1990. **162** p. 474-481.
266. Busque, P., et al., *Simultaneous flow cytometric measurement of Streptococcus suis phagocytosis by polymorphonuclear and mononuclear blood leukocytes.* Vet Microbiol, 1998. **63**(2-4): p. 229-38.
267. Al-Numani, D., et al., *Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by Streptococcus suis serotype 2.* Clin. Exp. Immunol., 2003. **133**: p. 67-77.
268. Segura, M., J. Stankova, and M. Gottschalk, *Heat-killed Streptococcus suis capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages.* Infect. Immun., 1999. **67**: p. 4646-4654.
269. Segura, M., N. Vadeboncoeur, and M. Gottschalk, *CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by Streptococcus suis capsular type 2.* Clin. Exp. Immunol., 2002. **127**: p. 243-254.
270. De Greeff, A., et al., *Involvement of NF-κB and MAP-kinases in the transcriptional response of alveolar macrophages to Streptococcus suis.* Vet. Microbiol., 2009. **141**: p. 59-67.
271. Graveline, R., et al., *TLR2-dependent recognition of Streptococcus suis is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness.* Int. Immunol., 2007. **19**: p. 375-389.
272. Vanier, G., et al., *New putative virulence factors of Streptococcus suis involved in invasion of porcine brain microvascular endothelial cells.* Microbial Pathog., 2009. **46**: p. 13-20.
273. Cavaillon, J.M., et al., *Cytokine cascade in sepsis.* Scand. J. Infect. Dis., 2003. **35**: p. 535-544.
274. Kim, K.S., *Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury.* Nat. Rev. Neurosci., 2003. **4**: p. 376-385.
275. van der Flier, M., et al., *Reprogramming the host response in bacterial meningitis: how best to improve outcome?* Clin. Microbiol. Rev. , 2003. **16**: p. 415-429.
276. Nassif, X., et al., *How do extracellular pathogens cross the blood-brain barrier?* Trends Microbiol., 2002. **10**: p. 227-232.

277. Rubin, L.L. and J.M. Staddon, *The Cell Biology Of The Blood-Brain Barrier.* Annual Review of Neuroscience, 1999. **22**(1): p. 11-28.
278. Francis, K., et al., *Innate immunity and brain inflammation: the key role of complement.* Expert. Rev. Mol. Med. , 2003. **23**: p. 1-19.
279. Balabanov, R. and P. Dore-Duffy, *Role of the CNS microvascular pericyte in the blood-brain barrier.* J. Neurosci. Res., 1998. **15**: p. 637-644.
280. Abbott, N.J., L. Rönnbäck, and E. Hansson, *Astrocyte–endothelial interactions at the blood–brain barrier.* Nat. Rev. Neurosci., 2006. **7**: p. 41-53
281. Charland, N., et al., *Streptococcus suis serotype 2 interactions with human brain microvascular endothelial cells.* Infect. Immun., 2000. **68**: p. 637-643.
282. Vadeboncoeur, N., et al., *Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2.* FEMS Immunol. Med. Microbiol., 2003. **35**: p. 49-58.
283. Grenier, D. and C. Bodet, *Streptococcus suis stimulates ICAM-1 shedding from microvascular endothelial cells.* FEMS Immunol. Med. Microbiol. , 2008. **54**: p. 271-276.
284. Tanabe, S. and D. Grenier, *Endothelial cell/macrophage cocultures as a model to study Streptococcus suis-induced inflammatory responses.* FEMS Immunol. Med. Microbiol., 2009. **55**: p. 100-106.
285. Stanimirovic, D. and K. Satoh, *Inflammatory mediators of cerebral endothelium: a role in ischemic brain inflammation.* Brain Pathol., 2000. **10**: p. 113-126.
286. Benga, L., P. Friedl, and P. Valentin-Weigand, *Adherence of Streptococcus suis to porcine endothelial cells.* J. Vet. Med. B Infect. Dis. Vet. Public Health, 2005. **52**: p. 392-395.
287. Vanier, G., et al., *Disruption of srtA gene in Streptococcus suis results in decreased interactions with endothelial cells and extracellular matrix proteins.* Vet. Microbiol., 2008. **127**: p. 417-424.
288. Engelhardt, B. and L. Sorokin, *The blood–brain and the blood–cerebrospinal fluid barriers: function and dysfunction.* Semin. Immunopathol., 2009. **31**: p. 497–511.
289. Adam, R.A., et al., *Porcine choroid plexus epithelial cells induce Streptococcus suis bacteriostasis in vitro.* Infect Immun, 2004. **72**(5): p. 3084-7.
290. Tenenbaum, T., et al., *Strain-dependent disruption of blood-cerebrospinal fluid barrier by Streptoccocus suis in vitro.* FEMS Immunol Med Microbiol, 2005. **44**(1): p. 25-34.
291. Tenenbaum, T., et al., *Cell death, caspase activation, and HMGB1 release of porcine choroid plexus epithelial cells during Streptococcus suis infection in vitro.* Brain Res, 2006. **1100**(1): p. 1-12.
292. Haselbach, M., et al., *Porcine Choroid plexus epithelial cells in culture: regulation of barrier properties and transport processes.* Microsc. Res. Tech., 2001. **52**: p. 137-152.
293. Tenenbaum, T., et al., *Polar bacterial invasion and translocation of Streptococcus suis across the blood-cerebrospinal fluid barrier in vitro.* Cell Microbiol., 2009. **11**: p. 323-336.
294. Williams, A.E. and W.F. Blakemore, *Pathology of Streptococcal meningitis following intravenous intracisternal and natural routes of infection.* Neuropathol. Appl. Neurobiol. , 1990. **16**: p. 345-356.
295. Gray, D.L. and D.P. Fedorko, *Laboratory Diagnosis of Bacterial Meningitis.* Clin. Microbiol. Rev., 1992. **5**: p. 130-145.
296. Chiavolini, D., G. Pozzi, and S. Ricci, *Animal models of Streptococcus pneumoniae disease.* Clin. Microbiol. Rev. , 2008. **21**: p. 666-685.

297. Thanawongnuwech, R., et al., *Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to Streptococcus suis infection.* Vet. Pathol., 2000. **37**: p. 143-152.
298. Robertson, I.D. and D.K. Blackmore, *Experimental studies on the comparative infectivity and pathogenicity of Streptococcus suis type 2. I. Porcine and human isolates in pigs.* Epidemiol. Infect., 1990. **105**: p. 469-478.
299. Pallarés, F.J., et al., *Comparison of experimental models for Streptococcus suis infection of conventional pigs.* Can. J. Vet. Research, 2003. **67**: p. 225-228.
300. Madsen, L.W., et al., *Aerogenous infection of microbiologically defined minipigs with Streptococcus suis serotype 2. A new model.* APMIS, 2001. **109**: p. 412-418.
301. Berthelot-Herault, F., et al., *Dilemma of virulence of Streptococcus suis: Canadian isolate 89-1591 characterized as a virulent strain using a standardized experimental model in pigs.* Can J Vet Res, 2005. **69**(3): p. 236-40.
302. Gu, H., H. Zhu, and C. Lu, *Use of in vivo-induced antigen technology (IVIAT) for the identification of Streptococcus suis serotype 2 in vivo-induced bacterial protein antigens.* BMC Microbiol., 2009. **18**.
303. Quessy, S., et al., *Discrimination of virulent and avirulent Streptococcus suis capsular type 2 isolates from different geographical origins.* Infect Immun, 1995. **63**(5): p. 1975-9.
304. Liu, L., et al., *Identification and Experimental Verification of Protective Antigens Against Streptococcus suis Serotype 2 Based on Genome Sequence Analysis.* Curr Microbiol, 2009. **58**: p.11-17
305. Quessy, S., et al., *Comparison of pig, rabbit and mouse IgG response to Streptococcus suis serotype 2 proteins and active immunization of mice against the infection.* Can. J. Vet. Research, 1994. **58**: p. 220-223.
306. Jacobs, A.A., et al., *Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of Streptococcus suis.* Infect. Immun., 1994. **62**(5): p. 1742-1748.
307. Williams, A.E., W.F. Blakemore, and T.J.L. Alexander, *A murine model of Streptococcus suis type 2 meningitis in the pig.* Research Vet. Science, 1988. **45**: p. 394-399.
308. Charland, N., et al., *Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by Streptococcus suis serotypes 1, 2 and 1/2.* Microbiology, 1997. **143**: p. 3607-3614.
309. Chen, Q., et al., *Enhancement of immunity and resistance in mice by pig IL-6 gene and CpG motifs encapsulated in chitosan nanoparticle.* Biotechol. J., 2008. **3**: p. 264-273.
310. Kay, R., *The sit of the lesion causing hearing loss in bacterial meningitis: a study of experimental streptococcal meningitis in guinea-pigs.* Neuropathol. Appl. Neurobiol. , 1991. **17**: p. 485-493.
311. Kawai, T. and S. Akira, *TLR Signaling.* Cell Death Differ., 2006. **13**: p. 816-825.
312. Janeway, C. and R. Medzhirov, *Innate Immune Recognition.* Ann. Rev. Immunol., 2002. **20**: p. 197-216.
313. Uematsu, S. and S. Akira, *Toll-like receptors and innate immunity.* J. Mol. Med. , 2006. **9**: p. 712-725.
314. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell 2006. **124**: p. 783-801.
315. Mogensen, T.H., *Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses.* Clin. Microbiol. Rev., 2009. **22**: p. 140-273.
316. Medzhitov, R. and C. Janeway, *Innate Immunity.* N. Engl. J. Med., 2000. **343**: p. 338-344.

317. Basset, C., et al., *Innate immunity and pathogen-host interaction.* Vaccine, 2003. **21**: p. S12-23.
318. Fukataa, M., A.S. Vamadevana, and M.T. Abreu, *Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders* Semin. Immunol., 2009. **21**: p. 242-253.
319. Leurier, F. and B. Lemaitre, *Toll-like receptors — taking an evolutionary approach.* Nature Reviews Genetics, 2008. **9**: p. 165-178.
320. Uenishi, H. and H. Shinkai, *Porcine Toll-like receptors: the front line of pathogen monitoring and possible implications for disease resistance.* Dev. Comp. Immunol. , 2009. **33**: p. 353-361.
321. Akira, S. and H. Hemmi, *Recognition of pathogen-associated molecular patterns by TLR family* Immunol. Letters, 2003. **85**: p. 85-95.
322. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat. Immunol., 2001. **2**: p. 675-680.
323. Dziarski, R., et al., *MD-2 Enables Toll-Like Receptor 2 (TLR2)-Mediated Responses to Lipopolysaccharide and Enhances TLR2-Mediated Responses to Gram-Positive and Gram-Negative Bacteria and Their Cell Wall Components.* J. Immunol., 2001. **166**: p. 1938-1944.
324. Gantner, B.N., et al., *Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2* J. Exp. Med. , 2003. **197**: p. 1107-1117.
325. Echchannaoui, H., et al., *Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammmation.* J. Infect. Dis., 2002. **186**: p. 798-806.
326. Malley, R., et al., *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.* Proc. Natl. Acad. Sci., 2003. **100**: p. 1966-1971.
327. Lehnardt, S., et al., *A mechanism for neurodegeneration induced by group B streptococci through activation of the TLR2/MyD88 pathway in microglia.* J. Immunol., 2006. **177**(583-592).
328. Lehnardt, S., et al., *TLR2 and Caspase-8 Are Essential for Group B Streptococcus-Induced Apoptosis in Microglia.* J. Immunol., 2007. **179**: p. 6134-6143.
329. Henneke, P., et al., *Novel engagement of CD14 and multiple toll-like receptors by group B streptococci.* J. Immunol. , 2001. **167**: p. 7069-7076.
330. Henneke, P., et al., *Cellular activation, phagocytosis, and bactericidal activity against group B streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways.* J. Immunol. , 2002 **169**: p. 3970-3977.
331. Travassos, L.H., et al., *Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition.* EMBO Rep., 2004. **5**: p. 1000-1006.
332. Schaaf, B., et al., *Mortality in human sepsis is associated with downregulation of Toll-like receptor 2 and CD14 expression on blood monocytes.* Diagn. Pathol., 2009. **4**: p. 12.
333. Takeda, K. and S. Akira, *TLR signaling pathways* Semin. Immunol., 2004. **16**: p. 3-9.
334. Tato, C.M. and C.A. Hunter, *Host pathogen interactions: subversion and utilisation of the NF-κB pathway during infection.* Infect. Immunol. , 2002. **70**: p. 3311-3317.
335. Caamaño, J. and C.A. Hunter, *NF-{kappa}B Family of Transcription Factors: Central Regulators of Innate and Adaptive Immune Functions* Clin. Microbiol. Rev., 2002. **15**: p. 414-429.
336. Schumann, R.R., et al., *Lipopolysaccharide and pneumococcal cell wall components activate the mitogen activated protein kinases (MAPK) erk-1, erk-2, and p38 in astrocytes.* Glia, 1998. **22**: p. 295-305.

337. Schindler, J.F., J.B. Monahan, and W.G. Smith, *p38 Pathway Kinases as Anti-inflammatory Drug Targets.* J. Dent. Res. , 2007. **86**: p. 800-811.
338. Kaminska, B., *MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits.* Biochim. Biophys. Acta, 2005 **1754**: p. 253-262.
339. Tsaia, P.J., et al., *Streptococcus pyogenes induces epithelial inflammatory responses through NF-κB/MAPK signaling pathways.* Microbes Infect. , 2006. **8**: p. 1440-1449.
340. Watters, J.J., et al., *A differential role for the mitogen-activated protein kinases in lipopolysaccharide signaling: the MEK/ERK pathway is not essential for nitric oxide and interleukin 1beta production.* J. Biol. Chem. , 2002. **15**: p. 9077-9087.
341. Kong, P.-J., et al., *c-Jun N-terminal kinase and p38 mitogen-activated protein kinase mediate double-strand RNA-induced inducible nitric oxide synthase expression in microglial cells.* Neurosci. Lett., 2008. **433**: p. 215-218.
342. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**: p. 37-40.
343. Blasi, E., A., et al., *NF-kB activation and p38 phosphorilation in microglial cells infected with Leptospira or exposed to partially purified leptospiral lipoprotein.* Microb. Pathogen., 2007. **42**: p. 80-87.
344. Hanisch, U.K., et al., *The protein tyrosine kinase inhibitor AG126 prevents the massive microglial cytokine induction by pneumococcal cell walls.* Eur. J. Immunol., 2001. **31**: p. 2104-2115.
345. Chien, H., et al., *Signal transduction pathways of nitric oxide release in primary microglial culture challenged with gram-positive bacterial constituent, lipoteichoic acid.* Neuroscience, 2005. **133**: p. 423-436.
346. Lappin, E. and A.J. Ferguson, *Gram-positive toxic shock syndromes.* Lancet Infect. Dis., 2009. **9**: p. 281-290.
347. Annane, D., E. Bellissant, and J.M. Cavaillon, *Septic shock.* Lancet, 2005. **365**: p. 63-78.
348. Nguyen, H.B., et al., *Severe sepsis and septic shock: review of the literature and emergency department management guidelines.* Ann. Emerg. Med., 2006. **48**: p. 28-54.
349. Paterson, R.L. and N.R. Webster, *Sepsis and the systemic inflammatory response syndrome.* J.R.Coll.Surg.Edinb., 2000. **45**: p. 178-182
350. Qureshi, K. and A. Rajah, *Septic shock: A review article.* Br. J. Med. Practic., 2008. **1**: p. 7-12.
351. Ackermann, M.R., *Acute Inflammation*, in *Pathologic basis of veterinary disease*, M.D. McGavin and J.F. Zachary, Editors. 2007, Mosby Elsevier: Philadelphia, PA.
352. Ramnath, R.D., et al., *Role of MCP-1 in endotoxemia and sepsis.* Int. Immunopharmacol., 2008. **8**: p. 810-818.
353. Thijs, L.G. and C.E. Hack, *Time course of cytokine levels in sepsis.* Intensive Care Med., 1995. **21**(Suppl. 2): p. S258-263.
354. Blackwell, T.S. and W. Christman, *Sepsis and cytokines: current status.* Br. J. Anaesth., 1996. **77**: p. 110-117.
355. Walley, K.R., et al., *Balance of inflammatory cytokines related to severity and mortality of murine sepsis.* Infect. Immun., 1996. **11**: p. 4733-4738.
356. Spooner, C.E., N.P. Markowitz, and L.D. Saravolatz, *The role of tumor necrosis factor in sepsis.* Clin. Immunol. Immunopath., 1992. **62**: p. S11-S17.
357. Tracey, K.J. and A. Cerami, *Tumor necrosis factor: an update review of its biology.* Crit. Care Med., 1993. **21**: p. S415-S422.
358. Dinarello, C.A., *Interleukin-1beta.* Crit. Care Med., 2005. **33**: p. S460-462.

359. Dinarello, C.A., *Role of interleukin-1 in infectious diseases.* Immunol. Rev., 1992. **127**: p. 119-146.
360. Whicher, J.T. and S.W. Evans, *Cytokines in disease.* Clin. Chem., 1990. **36**: p. 1269-1281.
361. Hack, C.E., et al., *Increased plasma levels of interleukin-6 in sepsis.* Blood, 1989. **74**: p. 1704-1710.
362. Remick, D.G., et al., *Role of Interleukin-6 in Mortality from and Physiologic Response to Sepsis.* Infect. Immun., 2005. **73**: p. 2751-2757.
363. Luster, A.D., *Chemokines—chemotactic cytokines that mediate inflammation.* N. Engl. J. Med. , 1998. **338**: p. 436-445.
364. Hack, C.E., et al., *Interleukin-8 in Sepsis: Relation to Shock and Inflammatory Mediators.* Infect. Immun., 1992. **60**: p. 2835-2842.
365. Mukaida, N., T. Harada, and K. Matsushima, *Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions.* Cytokine and Growth Factor Rev., 1998. **9**: p. 9-23.
366. Cavaillon, J.M. and M. Adib-Conquy, *Determining the degree of immunodysregulation in sepsis.* Contrib. Nephrol., 2007. **156**: p. 101-111.
367. Oberholzer, A., C. Oberholzer, and L.L. Moldawer, *Interleukin-10: A complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug.* Crit. Care Med., 2002. **30**: p. S58-S63.
368. Wu, H.P., et al., *The interleukin-4 expression in patients with severe sepsis.* J. Crit. Care 2008. **23**: p. 519-524.
369. Vannier, E., L.C. Miller, and C.A. Dinarello, *Coordinated antiinflammatory effects of interleukin 4: Interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist.* Proc. Natl. Acad. Sci. , 1992. **89**: p. 4076-4080.
370. Smith, W.B., et al., *Transforming growth factor-beta 1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium.* J. Immunol., 1996. **157**: p. 360-368.
371. Dinarello, C.A., *Biologic basis for Interleukin-1 in disease.* Blood, 1996. **87**(6).
372. Arend, W.P. and C.J. Guthridge, *Biological role of interleukin 1 receptor antagonist isoforms.* Ann. Rheum. Dis., 2000. **59**: p. i60-i64.
373. Schröder, J., et al., *Pattern of soluble TNF receptors I and II in sepsis.* Infection, 1995. **3**: p. 143-148.
374. Aderka, D., et al., *Shedding Kinetics of Soluble Tumor Necrosis Factor (TNF) Receptors after Systemic TNF Leaking during Isolated Limb Perfusion.* J. Clin. Invest., 1998. **101**: p. 650–659.
375. Glezer, I. and S. Rivest, *Glucocorticoids: protectors of the brain during innate immune responses.* Neuroscientist, 2004. **10**: p. 538-52.
376. Alouf, J.E. and H. Müller-Alouf, *Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects.* Int. J. Med. Microbiol., 2003. **292**: p. 429-440.
377. Wood, T.F., M.A. Potter, and O. Jonasson, *Streptococcal Toxic Shock-Like Syndrome.* Ann. Surg., 1993. **217**: p. 109-114.
378. Wolf, J.E. and L.G. Rabinowitz, *Streptococcal toxic shock-like syndrome.* Arch. Dermatol., 1995. **131**: p. 73-77.
379. Holmes, C.L., J.A. Russell, and K.R. Walley, *Genetic Polymorphisms in Sepsis and Septic Shock: Role in Prognosis and Potential for Therapy.* Chest, 2003. **24**: p. 1103-1115

380. Sutherland, A., K. Walley, and J.A. Russell, *Polymorphisms in CD14, mannose-binding lectin, and Toll-like receptor-2 are associated with increased prevalence of infection in critically ill adults.* Crit. Care Med., 2005. **33**: p. 638-644.
381. Agnese, D.M., et al., *Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections.* J. Infect. Dis., 2002 **186**: p. 1522-1525.
382. Jessen, K.M., et al., *Common TNF-alpha, IL-1 beta, PAI-1, uPA, CD14 and TLR4 polymorphisms are not associated with disease severity or outcome from Gram negative sepsis.* BMC Infect. Dis., 2007. **18**: p. 108.
383. Aziz, R.K., et al., *Susceptibility to severe streptococcal sepsis: use of a large set of isogenic mouse lines to study genetic and environmental factors.* Genes Immun. , 2007. **8**: p. 404-415.
384. Emonts, M., et al., *Host genetic determinants of Neisseria meningitidis infections.* Lancet Infect. Dis., 2003. **3**: p. 565-577.
385. Roy, S., et al., *MBL genotype and risk of invasive pneumococcal disease: a case-control study.* Lancet, 2002. **359**: p. 1569-1573.
386. Yuan, F.F., et al., *Clinical relevance of TLR2, TLR4, CD14 and FcgammaRIIA gene polymorphisms in Streptococcus pneumoniae infection.* Immunol. Cell Biol. , 2008. **86**: p. 268-270.
387. Anthony, D.C. and S.J. Campbell, *Developmental influences on inflammation in the brain*, in *Immune and inflammatory responses in the nervous system*, N.J. Rothwell and S. Loddick, Editors. 2002, Oxford University Press: Oxford. p. 1-13.
388. Rivest, S., *Molecular insights on the cerebral innate immune system.* Brain Behav. Immun., 2003. **17**: p. 13-19.
389. Laflamme, N. and S. Rivest, *Effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor kappaB alpha within specific cellular populations of the rat brain.* J. Neurochem., 1999. **73**: p. 309-321.
390. Laflamme, N. and S. Rivest, *Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components.* FASEB J., 2001. **15**: p. 155-163.
391. Nadeau, S. and S. Rivest, *Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor kappa B activity in the brain during endotoxemia.* J. Neurosci., 2000. **20**: p. 3456-3468.
392. Laflamme, N., G. Soucy, and S. Rivest, *Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding Toll-like receptor 2 in the CNS.* J. Neurochem., 2001. **79**: p. 648-657.
393. Thibeault, I., N. Laflamme, and S. Rivest, *Regulation of the gene encoding the monocyte chemoattractant protein 1 (MCP-1) in the mouse and rat brain in response to circulating LPS and proinflammatory cytokines.* J. Comp. Neurol., 2001. **434**: p. 461-477.
394. Streit, W.J. and G.W. Kreutzberg, *Lectin binding by resting and reactive microglia.* J. Neurocytol., 1987. **16**: p. 249-260.
395. Stollg, G. and S. Jander, *The role of microglia and macrophages in the pathophysiology of the CNS* Progress Neurobiol., 1999. **58**: p. 233-247.
396. Rock, R.B., et al., *Role of Microglia in Central Nervous System Infections* Clin. Microbiol. Rev., 2004. **17**: p. 942-964.
397. Kreutzberg, G.W., *Microglia: a sensor for pathological events in the CNS.* Trends Neurosci., 1996. **19**: p. 312-318.

398. Chew, L.J., A. Takanohashi, and J. Bell, *Microglia and inflammation: impact on developmental brain injuries.* Ment. Retard. Dev. Disabil. Res. Rev., 2006. **12**: p. 105-112.
399. Bsibsi, M., et al., *Broad expression of Toll-like receptors in the human central nervous system.* J. Neuropathol. Exp. Neurol., 2002. **61**: p. 1013-1021.
400. Olson, J.K. and S.D. Miller, *Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs.* J. Immunol., 2004. **173**: p. 3916-3924.
401. Ebert, S., et al., *Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination.* J. Neuroimmunol., 2005. **159**: p. 87-96.
402. Prinz, M., et al., *Microglial activation by components of gram-positive and -negative bacteria: distinct and common routes to the induction of ion channels and cytokines.* J. Neuropathol. Exp. Neurol. , 1999. **10**: p. 1078-1089.
403. Braun, J.S., et al., *Apoptosis-Inducing Factor Mediates Microglial and Neuronal Apoptosis Caused by Pneumococcus.* J. Infect. Dis., 2001. **184**: p. 1300–1309.
404. Braun, J.S., et al., *Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis.* J. Clin. Invest. , 2002. **109**: p. 19-27.
405. Ribes, S., et al., *Toll-like receptor stimulation enhances phagocytosis intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia.* Infect Immun, 2009. **23**: p. ahead of printing.
406. Peppoloni, S., et al., *The lack of Pneumococcal surface protein C (PspC) increases the susceptibility of Streptococcus pneumoniae to the killing by microglia* Med. Microbiol. Immunol., 2006. **195**: p. 21-28.
407. Kielian, T., P. Mayes, and M. Kielian, *Characterization or microglial responses to Staphylococcus aureus: effects on cytokine, costimulatory molecule and Toll-like receptor expression.* J. Neuroimmunol., 2002. **130**: p. 86-99.
408. Kielian, T., et al., *Toll-like receptor 2 modulates the proinflammatory milieu in Staphylococcus aureus-induced brain abscess.* Infect. Immun., 2005. **73**: p. 7428-7435.
409. Kielian, T., et al., *S. aureus-dependent microglial activation is selectively attenuated by the cyclopentenone prostaglandin 15-deoxy-Delta12,14- prostaglandin J2 (15d-PGJ2).* J. Neurochem. , 2004. **90**: p. 1163-1172.
410. Rasley, A., J. Anguita, and I. Marriott, *Borrelia burgdorferi induces inflammatory mediator production by murine microglia.* Neuroimmunol., 2002. **130**: p. 22-31.
411. Rasley, A., et al., *Murine glia express the immunosuppressive cytokine, interleukin-10, following exposure to Borrelia burgdorferi or Neisseria meningitidis.* Glia, 2006. **53**: p. 583-592.
412. Sterka, D.J. and I. Marriott, *Characterization of nucleotide-binding oligomerization domain (NOD) protein expression in primary murine microglia.* J. Neuroimmunol., 2006. **179**: p. 65-75.
413. Chauhan, V.S., et al., *NOD2 plays an important role in the inflammatory responses of microglia and astrocytes to bacterial CNS pathogens.* Glia, 2009. **57**: p. 414-423.
414. Virgintino, D., et al., *Immunohistochemical and morphometric study on astrocytes and microvasculature in the human cerebral cortex.* Histochem J., 1997. **29**: p. 655-660.
415. Dhandapani, K.M., V.B. Mahesh, and D.W. Brann, *Astrocytes and Brain Function: Implications for Reproduction.* Exp. Biol. Med., 2003. **228**: p. 253-260.
416. Halliday, G.M., et al., *Glial fibrillary acidic protein (GFAP) immunohistochemistry in human cortex: a quantitative study using different antisera.* Neurosci. Lett. , 1996. **209**: p. 29-32.

417. Moynagh, P.N., D.C. Williams, and L.A. O'Neill, *Activation of NF-kappa B and induction of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 expression in human glial cells by IL-1. Modulation by antioxidants.* J. Immunol. , 1994. **153**: p. 2681-2690.
418. Bourke, E. and P.N. Moynagh, *Antiinflammatory effects of glucocorticoids in brain cells, independent of NF-kappa B.* J. Immunol., 1999. **163**: p. 2113-2119.
419. Bowman, C.C., et al., *Cultured astrocytes express toll-like receptors for bacterial products.* Glia, 2003. **43**: p. 281-291.
420. Falsig, J., et al., *Molecular basis for detection of invading pathogens in the brain.* J. Neurosc. Res., 2007. **86**: p. 1434-1447.
421. Hsieh, H.L., et al., *Lipoteichoic Acid Induces Matrix Metalloproteinase-9 Expression via Transactivation of PDGF Receptors and NF-kappaB Activation in Rat Brain Astrocytes.* Neurotox. Res., 2010. **17**: 344-359.
422. Esen, N., et al., *Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium Staphylococcus aureus.* J. Neurochem., 2004. **88**: p. 746-758.
423. Stenzel, W., et al., *The intermediate filament GFAP is important for the control of experimental murine Staphylococcus aureus-induced brain abscess and Toxoplasma encephalitis.* J. Neuropathol. Exp. Neurol., 2004. **63**: p. 631-640.
424. Esen, N., et al., *Modulation of connexin expression and gap junction communication in astrocytes by the gram-positive bacterium S. aureus.* Glia, 2007. **55**: p. 104-117.
425. Borysiewicz, E., D. Fil, and G.W. Konat, *Rho proteins are negative regulators of TLR2, TLR3, and TLR4 signaling in astrocytes.* J. Neurosci. Res., 2009. **87**: p. 1565-1572.
426. Lehnardt, S., et al., *The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS.* J. Neurosci., 2002. **22**: p. 2478-2786.
427. Buer, J. and R. Balling, *Mice, microbes and models of infection.* Nature Reviews Genetics, 2003. **4**: p. 195-205.
428. Obermaier, B., et al., *Disease models of acute bacterial meningitis.* Drug Discov. Today, 2006. **3**: p. 105-112.
429. Koedel, U. and H.W. Pfister, *Models of experimental bacterial meningitis. Role and Limitations.* Infect. Dis. Clin. North. Am., 1999. **13**(3): p. 549-577.
430. Scheld, W.M. and M.A. Sande, *Bactericidal versus bacteriostatic antibiotic therapy of experimental pneumococcal meningitis in rabbits* J. Clin. Invest., 1983. **71**: p. 411-419.
431. Schmidt, T. and M.G. Tauber, *Pharmacodynamics of antibiotics in the therapy of meningitis: Infection model observations* J. Antimicrob. Chemother., 1993. **31**: p. 61-70.
432. Koedel, U. and H.W. Pfister, *Models of experimental bacterial meningitis. Role and limitations.* Infect. Dis. Clin. North. Am., 1999. **13**: p. 549-577.
433. Quagliarello, V.J., W.J. Long, and W.M. Scheld, *Morphologic alterations of the blood-brain barrier with experimental meningitis in the rat. Temporal sequence and role of encapsulation.* J. Clin. Invest. , 1986. **77**: p. 1084-1095.
434. Pfister, H.W., et al., *Microvascular changes during the early phase of experimental bacterial meningitis.* J. Cereb. Blood Flow Metab. , 1990. **10**: p. 914-922.
435. Tsai, Y.H., R.S. Hirth, and F. Leitner, *A murine model for listerial meningitis and meningoencephalomyelitis: Therapeutic evaluation of drugs in mice* Chemotherapy 1080. **26**: p. 196-206.
436. Tang, T., et al., *Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins.* J. Clin. Invest., 1996. **97**: p. 2485-2490.

437. Klein, M., et al., *Protein expression pattern in experimental pneumococcal meningitis.* Microbes Infect., 2006 **8**: p. 974-983.
438. Klein, M., et al., *Morphological correlates of acute and permanent hearing loss during experimental pneumococcal meningitis.* Brain Pathol., 2003. **13**: p. 1-10.
439. Klein, M., et al., *MyD88-dependent immune response contributes to hearing loss in experimental pneumococcal meningitis.* J. Infect. Dis., 2007. **2007**(195): p. 1189-1193.
440. Gerber, J., et al., *A mouse model of Streptococcus pneumoniae meningitis mimicking several features of human disease.* Acta Neuropathol. (Berl), 2001. **101**: p. 499-508.
441. Gerber, J., et al., *Increased mortality and spatial memory deficits in TNF-alpha-deficient mice in ceftriaxone-treated experimental pneumococcal meningitis.* Neurobiol. Dis., 2004. **16**: p. 133-138.
442. Chiavolini, D., et al., *Method for inducing experimental pneumococcal meningitis in outbred mice. .* BMC Microbiol., 2004. **4**: p. 36-45.
443. Marks, M.I., et al., *Lethal Haemophilus influenzae type b infection in mice.* Infection, 1982. **10**: p. 261-266.
444. Holbein, B.E., *Iron-Controlled Infection with Neisseria meningitidis in Mice.* Infect Immun, 1980. **29**: p. 886-891.
445. Tsao, N., et al., *Development of hematogenous pneumococcal meningitis in adult mice: the role of TNF-alpha.* FEMS Immunol. Med. Microbiol., 2002. **32**: p. 133-140.
446. Moxon, E.R., et al., *Haemophilus influenzae meningitis in infant rats after intranasal inoculation.* J. Infect. Dis., 1974. **129**: p. 154-162.
447. Grandgirard, D., et al., *An infant mouse model of brain damage in pneumococcal meningitis.* Acta Neuropathol. (Berl), 2007. **114**: p. 609-617.
448. Mackinnon, F.G., et al., *Intranasal infection of infant mice with Neisseria meningitidis.* Microb. Pathog., 1992. **12**: p. 415-420.
449. Paul, R., U. Koedel, and H.W. Pfister, *Using knockout mice to study experimental meningitis.* Arch. Immunol. Ther. Exp. (Warsz), 2003. **51**: p. 315-326.
450. L., v.D.W., D.J. Adams, and A. Bradley, *Tools for targeted manipulation of the mouse genome* Physiol.Genomics, 2002. **11**: p. 133-164.
451. Kastenbauer, S. and H.W. Pfister, *Pneumococcal meningitis in adults: spectrum of complications and prognostic factors in a series of 87 cases.* Brain Behav. Immun., 2003. **126**: p. 1015-1025.
452. Koedel, U., et al., *Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis.* J. Immunol., 2003. **170**: p. 438-444.
453. Srivastava, A., et al., *The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease.* Infect. Immun. , 2005. **73**: p. 6479-6487.
454. Albiger, B., et al., *Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection.* Cell Microbiol, 2007. **9**: p. 633-644.
455. Koedel, U., et al., *MyD88 is required for mounting a robust host immune response to Streptococcus pneumoniae in the CNS.* Brain Behav. Immun., 2004: p. 1437-4145.
456. Kastenbauer, S., et al., *Protective role of NF-kappaB1 (p50) in experimental pneumococcal meningitis.* Eur. J. Pharmacol., 2004. **498**: p. 315-318.
457. Wellmer, A., et al., *Effect of deficiency of tumor necrosis factor α or both of its receptors on Streptococcus pneumoniae central nervous system infection and peritonitis.* Infect. Immun., 2001. **69**: p. 6881-6886.
458. Paul, R., et al., *Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis* Brain Behav. Immun., 2003. **126**: p. 1873-1882.

459. Wrinkler, F., et al., *Differential expression of nitric oxide synthases in bacterial meningitis: role of the inducible isoform for blood-brain barrier breakdown.* J. Infect. Dis., 2001. **183**: p. 1749-1759.
460. Lu, J., et al., *Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system.* Biochim. Biophys. Acta, 2002. **1572** p. 387-400.
461. Gel, P. and G. Ambrus, *Structure and function of complement activating enzyme complexes: C1 and MBL-MASPs.* Curr. Protein Pept. Sci. , 2001. **2**: p. 43-59.
462. Garlanda, C., et al., *Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility.* Ann. Rev. Immunol., 2005. **23**: p. 337-366
463. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J. Exp. Med. , 1999. **189:** 1777-1782.
464. Peiser, L., S. Mukhopadhay, and S. Gordon, *Scavenger receptors in innate immunity.* Curr. Opin. Immunol., 2002. **14**: p. 123-128.
465. Fraser, I.P., H. Koziel, and R.A. Ezekowitz, *The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity.* Semin. Immunol. , 1998. **10**: p. 363-72
466. Lea, Y., P.M. Murphy, and J.M. Wang, *Formyl-peptide receptors revisited* Trends Immunol., 2002. **23**: p. 541-548
467. Hasan, U., et al., *Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88.* J. Immunol., 2005. **174**: p. 2942-2950.
468. Jordan, R.W. and J.M. Roe, *An experimental mouse model of progressive atrophic rhinitis of swine.* Vet. Microbiol., 2004. **103**: p. 201-207.
469. Ojha, S., et al., *An experimental model of Actinobacillus suis infection in mice.* Comp. Med., 2007. **57**: p. 340-348.
470. Ono, E., et al., *Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock.* Proc. Natl. Acad. Sci. USA, 2004. **101**: p. 16150-16155.
471. Vidal, S.M., et al., *Forward Genetic Dissection of Immunity to Infection in the Mouse.* Annu. Rev. Immunol., 2008. **26**: p. 81-132.
472. Rosati, E., et al., *Cytokine response to Group B Streptococcus infection in mice.* Scand. J. Immunol., 1998. **47**: p. 314-323.
473. Beaudoin, M., et al., *Studies on a murine model for evaluation of virulence of Streptococcus suis capsular type 2 isolates.* FEMS Microbiol. Lett., 1992. **99**: p. 111-116.
474. Kataoka, Y., et al., *Experimental infections of mice and pigs with Streptococcus suis type 2.* J. Vet. Med. Sci. , 1991. **53**: p. 1043-1049.
475. Bonifait, L., et al., *The cell envelope subtilisin-like proteinase is a critical virulence determinant for Streptococcus suis.* BMC Microbiology, 2010. **10**: 42
476. Benga, L., et al., *Polysaccharide capsule and suilysin contribute to extracellular survival of Streptococcus suis co-cultivated with primary porcine phagocytes.* Vet. Microbiol., 2008. **132**: p. 211-219.
477. Lecours, M.P., et al., *Critical role for Streptococcus suis cell wall modifications and suilysin in resistance to Complement-dependent killing by dendritic cells.* J. Immunol., 2010. **Submitted**.
478. Hyams, C.J., et al., *Streptococcus pneumoniae resistance to complement-mediated immunity is dependent on capsular serotype.* Infect. Immun., 2010. **78** : 716-725.

479. van Amersfoort, E.S., T.J.C.v. Berkel, and J. Kuiper, *Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock.* Clin. Microbiol. Rev., 2003. **16**: p. 379-414.
480. Clatworthy, M.R. and K.G. Smith, *FcgammaRIIb balances efficient pathogen clearance and the cytokine-mediated consequences of sepsis.* J. Exp. Med, 2004. **199**: p. 717-723.
481. Wood, A.C. and I. Todd, *Staphylococcal enterotoxin B toxicity in BALB/c mice: effect on T-cells, plasma cytokine levels and biochemical markers.* FEMS Immunol. Med. Microbiol. , 1995. **11**: p. 91-97.
482. Teti, G., G. Mancuso, and F. Tomasello, *Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection.* Infect. Immun., 1993. **61**: p. 227-235.
483. Medina, E., et al., *Genetic control of susceptibility to group A streptococcal infection in mice.* J. Infect. Dis., 2001. **184**: p. 846-852.
484. Medina, E. and A. Lengeling, *Genetic regulation of host responses to group A streptococcus in mice.* Brief Funct. Genomic Proteomic, 2005. **4**: p. 248-257.
485. duPont, N.C., et al., *Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: Determinations of a panel of nine cytokines in clinical sample culture supernatants.* J. Reproduct. Immunol., 2005. **66**: p. 175-191.
486. Segura, M., J. Stankova, and M. Gottschalk, *Heat-killed Streptococcus suis capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages.* Infect. Immun. , 1999. **67**: p. 4646-4654.
487. Vadeboncoeur, N., et al., *Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2.* FEMS Immunol. Med. Microbiol. , 2003. **35**: p. 49-58.
488. Segura, M., et al., *Proinflammatory cytokine and chemokine modulation by Streptococcus suis in a whole-blood culture system.* FEMS Immunol. Med. Microbiol. , 2006. **47**: p. 92-106.
489. Mancuso, G., et al., *Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease.* J. Immunol. , 2004. **172**: p. 6324-6329.
490. Teti, G., G. Mancuso, and F. Tomasello, *Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection.* Infect. Immun. , 1993. **61**: p. 227-235.
491. Tsao, N., et al., *Tumor necrosis factor-alpha causes an increase in blood-brain barrier permeability during sepsis.* J. Med. Microbiol., 2001. **50**: p. 812-821.
492. Geiger, T., et al., *Induction of rat acute-phase proteins by interleukin 6 in vivo.* Eur. J. Immunol., 1988. **18**: p. 717-721.
493. Damas, P., et al., *Cytokine serum level during severe sepsis in human IL-6 as a marker of severity.* Ann. Surg., 1992. **215**: p. 356-362.
494. von Köckritz-Blickwede, M., et al., *Immunological Mechanisms Underlying the Genetic Predisposition to Severe Staphylococcus aureus Infection in the Mouse Model.* Am. J. Pathol. , 2008. **173**: p. 1657-1668.
495. Ashman, R.B., A. Fulurija, and J.M. Papadimitriou, *Strain-dependent differences in host response to Candida albicans infection in mice are related to organ susceptibility and infectious load.* Infect. Immun., 1996. **64**: p. 1866-1869.
496. Mullick, A., et al., *Dysregulated inflammatory response to Candida albicans in a C5-deficient mouse strain.* Infect. Immun., 2004. **72**: p. 5868-5876.
497. O'Malley, J., et al., *Comparison of acute endotoxin-induced lesions in A/J and C57BL/6 mice.* J. Hered., 1998. **89**: p. 525-530.

498. Stewart, D., et al., *Genetic contribution to the septic response in a mouse model.* Shock, 2002. **18**: p. 342-347.
499. Hollingsworth, J.W., et al., *Genetic basis of murine antibacterial defense to streptococcal lung infection.* Immunogenetics, 2007. **59**: p. 713-724.
500. Nilsson, U.R. and H.J. Muller-Eberhard, *Deficiency of the fifth component of complement in mice with an inherited complement defect.* J. Exp. Med., 1967. **125**: p. 1-16.
501. De Kimpe, S.J., et al., *The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure.* Proc Natl Acad Sci U S A, 1995. **92**: p. 10359-10363.
502. Pie, S., et al., *Th1 response in Salmonella typhimurium-infected mice with a high or low rate of bacterial clearance.* Infect. Immun., 1997. **65**: p. 4509-4514
503. Watanabe, H., et al., *Innate Immune Response in Th1- and Th2-Dominant Mouse Strains.* Shock, 2004. **22**: p. 460-466.
504. Remick, D.G., et al., *Combination immunotheraphy with soluble tumor necrosis factor receptors plus interleukin 1 receptor antagonist decreases sepsis mortality.* Crit Care Med, 2001. **29**: p. 473-481.
505. Ye, C., et al., *The clinical, experimental and genomic differences between intermediate pathogenic, highly pathogenic and epidemic Streptococcus suis.* J. Infect. Dis., 2009. **199**: p. 97-107.
506. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* J. Immunol., 1991. **147**: p. 3815-3822.
507. de Waal Malefyt, R., et al., *Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J. Exp. Med., 1991. **174**: p. 1209-1220.
508. Dennis, V.A., et al., *Interleukin-10 Anti-Inflammatory Response to Borrelia burgdorferi, the Agent of Lyme Disease: a Possible Role for Suppressors of Cytokine Signaling 1 and 3* Infect. Immun., 2006. **74**: p. 5780-5789.
509. Cassatella, M.A., et al., *Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide.* J. Exp. Med., 1993 **178**: p. 2207-2211.
510. van der Poll, T., et al., *Endogenous IL-10 protects mice from death during septic peritonitis.* J. Immunol., 1995. **155**: p. 5397-5401.
511. Gérard, C., et al., *Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia.* J. Exp. Med., 1993. **177**: p. 547-550.
512. Standiford, T.J., et al., *Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor.* J. Immunol., 1995. **155**: p. 2222-2229.
513. Kawai, S., et al., *The Role of Interleukin-10 in Systemic Inflammatory Response Syndrome with Sepsis.* J. Infect. Chemother., 1998. **4**: p. 121-127.
514. Puliti, M., et al., *Regulatory Role of Interleukin-10 in Experimental Group B Streptococcal Arthritis.* Infect. Immun., 2002. **70**: p. 2862-2868.
515. Cusumano, V., et al., *Interleukin-10 protects neonatal mice from lethal Group B Streptococcal infection.* Infect. Immun., 1996. **64**: p. 2850-2852.
516. Howard, M., et al., *Interleukin 10 protects mice from lethal endotoxemia.* J. Exp. Res., 1993. **177**: p. 1205-1208.

517. Latifi, S.Q., M.A. O'Riordan, and A.D. Levine, *Interleukin-10 controls the onset of irreversible septic shock.* Infect. Immun., 2002. **70**: p. 4441-4446.
518. Holmes, C.L., J.A. Russell, and K.R. Walley, *Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy.* Chest, 2003. **124**: p. 1103-1115.
519. Fantuzzi, G., M.T. Demitri, and P. Ghezzi, *Differential effect of glucocorticoids on tumour necrosis factor production in mice: up-regulation by early pretreatment with dexamethasone.* Clin. Exp. Immunol., 1994. **96**: p. 166-169.
520. Thaete, L.G., M.N. Nesbitt, and A.M. Malkinson, *Genetic analysis of the distribution of corticosterone-containing cells in mouse adrenal cortex.* Proc. Soc. Exp. Biol. Med., 1990. **194**: p. 97-102.
521. Droms, K.A. and A.M. Malkinson, *Mechanisms of Glucocorticoid Involvement in Mouse Lung Tumorigenesis.* Exp. Lung Res., 1991. **17**: p. 359-370.
522. Rodewald, A.K., et al., *Neonatal mouse model of group B streptococcal infection.* J. Infect. Dis., 1992. **166**: p. 635-639.
523. Gorchetchnikov, A. and S. Grossberg, *Space, time and learning in the hippocampus: how fine spatial and temporal scales are expanded into population codes for behavioral control.* Neural Netw., 2007. **20**: p. 182-193.
524. Bohr, V., O.B. Paulson, and N. Rasmussen, *Pneumococcal meningitis. Late neurologic sequelae and features of prognostic impact.* Arch Neurol., 1984. **10**: p. 1045-1049.
525. Morris, R.G.M., et al., *Place navigation impaired in rats with hippocampal lesions.* Nature, 1982. **297**: p. 681–683.
526. Riedel, G., et al., *Reversible neural inactivation reveals hippocampal participation in several memory processes.* Nat. Neurosci., 1999. **2**: p. 898-905.
527. Wellmer, A., et al., *Spatial memory and learning deficits after experimental pneumococcal meningitis in mice.* Neurosci. Lett., 2000. **296**: p. 137-140.
528. Ostergaard, C., et al., *Bacteremia causes hippocampal neural apoptosis in experimental pneumococcal meningitis.* BMC. Infect. Dis., 2010. **10**: p. [Epub ahead of print].
529. Kannan, S., et al., *Microglial Activation in Perinatal Rabbit Brain Induced by Intrauterine Inflammation: Detection with 11C-(R)-PK11195 and Small-Animal PET.* J. Nucl. Med., 2007. **48**: p. 946-954.
530. Zeni, P., et al., *MMPs contribute to TNF-alpha-induced alteration of the blood-cerebrospinal fluid barrier in vitro.* Am. J. Physiol. Cell Physiol. , 2007. **293**: p. C855-C864.
531. Tascioglu, A.B., *Brief review of vestibular system anatomy and its higher order projections.* Neuroanatomy, 2005. **4**: p. 24-27.
532. Chrisman, C.L., *Vestibular diseases.* Vet. Clin. North Am. Small Anim. Pract., 1980. **10**: p. 103-130.
533. Radlinsky, M.G. and D.E. Mason, *Diseases of the middle and inner ear*, in *Textbook of Veterinary Internal Medicine*, S.J. Ettinger, Editor. 2005, WB Saunders: Philadelphia. p. 1180-1186.
534. Rusmeechan, S. and P. Sribusara, *Streptococcus suis meningitis: the newest serious infectious disease.* J. Med. Assoc. Thai. , 2008. **91**: p. 654-658.
535. Madsen, L.W., et al., *Otitis Interna Is a Frequent Sequela to Streptococcus suis Meningitis in Pigs.* Vet. Pathol., 2001. **38**: p. 190-195.
536. Veselinović, D. and A. Veselinović, *Endoftalmitis.* Acta Medica Medianae . 2009. **48**: p. 56-62.
537. Coolen, L., et al., *Streptococcus suis meningitis, permanent perceptive deafness and endophthalmitis.* Intensive Care Med., 1989;. **15**: p. 545.

538. Heidt, M.C., et al., *Human Infective Endocarditis Caused by Streptococcus suis Serotype 2.* J. Clin. Microbiol. , 2005. **43**: p. 4898-4901.
539. Katsumi, M., et al., *Bacterial isolation from slaughtered pigs associated with endocarditis, especially the isolation of Streptococcus suis.* J. Vet. Med. Sci., 1997 **59**: p. 75-78.
540. Jin, L. and R.V. Lloyd, *In situ hybridization: methods and applications.* J. Clin. Lab. Anal., 1997. **11**: p. 2-9.
541. Tenenbaum, T., et al., *Polar bacterial invasion and translocation of Streptococcus suis across the blood-cerebrospinal fluid barrier in vitro.* Cell Microbiol., 2009. **11**: p. 323-336.
542. Pugin, J., et al., *CD14 is a pattern recognition receptor.* Immunity 1994. **1**: p. 509-516.
543. Yoshimura, A., et al., *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J. Immunol., 1999. **163**: p. 1-5.
544. Schröder, N.W., et al., *Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved.* J. Biol. Chem., 2003. **278**: p. 15587-15594.
545. Esen, N. and T. Kielian, *Central role of MyD88 in the response of microglia to pathogen-associated molecular patterns.* J. Immunol., 2006. **176**: p. 6802-6811.
546. Diab, A., et al., *Haemophilus influenzae and Streptococcus pneumoniae induce different intracerebral mRNA cytokine patterns during the course of experimental bacterial meningitis.* Clin. Exp. Immunol., 1997. **109**: p. 233-241.
547. Kielian, T. and W.F. Hickey, *Proinflammatory Cytokine, Chemokine, and Cellular Adhesion Molecule Expression during the Acute Phase of Experimental Brain Abscess Development* Am. J. Pathol., 2000. **157**: p. 647-658.
548. Nguyen, M.D., J.P. Julien, and S. Rivest, *Innate immunity: the missing link in neuroprotection and neurodegeneration?* Nat. Rev. Neurosci., 2002. **3**: p. 216-227.
549. Sebastiani, G., et al., *Host immune response to Salmonella enterica serovar Typhimurium infection in mice derived from wild strains.* Infect. Immun., 2002. **70**: p. 1997-2009.
550. Letiembre, M., et al., *Toll-like receptor 2 deficiency delays pneumococcal phagocytosis and impairs oxidative killing by granulocytes.* Infect. Immun., 2005. **73**: p. 8397-8401.
551. Bottcher, T., et al., *Differential regulation of Toll-like receptor mRNAs in experimental murine central nervous system infections.* Neurosci. Lett., 2003. **344**: p. 17-20.
552. Sajjan, U.S., et al., *H. influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression.* FASEB J., 2006. **20**: p. 2121-2123.
553. Teng, F., et al., *Toll-like receptor 3 is involved in airway epithelial cell response to nontypeable Haemophilus influenzae.* Cell Immunol. , 2010. **260**: p. 98-104.
554. Yamamoto, M., et al., *Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway.* Science, 2003. **301**: p. 640-643.
555. Brazeau, B., et al., *In vitro phagocytosis and survival of Streptococcus suis capsular type 2 inside murine macrophages.* Microbiology, 1996. **142**: p. 1231-1237.
556. Liu, X., et al., *NOD2 mediates inflammatory responses of primary murine glia to Streptococcus pneumoniae.* Glia, 2010. **58**: p. 839-847.
557. Wichgers Schreur, P.J., et al., *Differential activation of the Toll-like receptor 2/6 complex by lipoproteins of Streptococcus suis serotypes 2 and 9.* Vet. Microbiol., 2010. **143** : 363-370.

558. Koedel, U., et al. *Pharmacological Interference with NF-kB Activation Attenuates Central Nervous System (CNS) Complications in Experimental Pneumococcal Meningitis*. in *Intersci. Conf. Antimicrob. Agents Chemother.* 1999: Abstr. Intersci. Conf. Antimicrob. Agents Chemother.
559. Phulwani, N.K., et al., *TLR2 expression in astrocytes is induced by TNF-alpha- and NF-kappa B-dependent pathways.* J. Immunol., 2008. **181**: p. 3841-3849.
560. Tanabe, S., M. Gottschalk, and D. Grenier, *Hemoglobin and Streptococcus suis cell wall in synergy to potentiate the inflammatory response of monocyte-derived macrophages.* Innate Immun., 2008. **14**: p. 357-363.
561. Kang, E.H., et al., *EstA protein, a novel virulence factor of Streptococcus pneumoniae, induces nitric oxide and pro-inflammatory cytokine production in RAW 264.7 macrophages through NF-κB/MAPK.* Microb. Pathog., 2009. **47**: p. 196-201.
562. Kim, M.H., et al., *The crystal structure of the estA protein, a virulence factor from Streptococcus pneumoniae.* Proteins 2008. **70**: p. 578-583.
563. Waetzig, V., et al., *c-Jun N-terminal kinases (JNKs) mediate pro-inflammatory actions of microglia.* Glia, 2005. **50**: p. 235-246.
564. Jeohn, G.H., et al., *Gö6976 inhibits LPS-induced microglial TNFalpha release by suppressing p38 MAP kinase activation.* Neuroscience, 2002. **114**: p. 689-697.
565. Lee, C.W., C.S. Chien, and C.-M. Yang, *Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-like receptor 2 in tracheal smooth muscle cells.* Am. J. Physiol. Lung Cell Mol. Physiol., 2004. **286**: p. L921-L930.

I. ANNEXES

ANNEX 1. ARTICLE IV

« Immunisation with SsEno fails to protect mice against challenge with *Streptococcus suis* serotype 2 »

Esgleas, M., DomínguezPunaro, M. C., Li, Y., Harel, J., Dubreuill, D., Gottschalk, M.

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada.

Published in: *FEMS Microbiol. Lett.* 2009 May; 294(1):82-8

Details on the role of the candidate in the conception of the article: I actively participated in the experimental design concerning all animal studies; I substantially performed research, analyzed data and participated in writing the paper.

SUMMARY

In our ongoing efforts to develop a vaccine against *Streptococcus suis* infection, we tested the potential of *Streptococcus suis* enolase (SsEno), a recently described *S. suis* adhesin with fibronectin-binding activity, as a vaccine candidate in a mouse model of *S. suis*- induced septicemia and meningitis. Here, we show that SsEno is highly recognized by sera from convalescent pigs and is highly immunogenic in mice. Subcutaneous immunization of mice with SsEno elicited strong immunoglobulin G (IgG) antibody responses. All four IgG subclasses were induced, with IgG1, IgG2a and IgG2b representing the highest titers followed by IgG3. However, SsEno-vaccinated and non-vaccinated control groups showed similar mortality rates after challenge infection with the highly virulent *S. suis* strain 166'. Similar results were obtained upon passive immunization of mice with hyperimmunized rabbit IgG anti-SsEno. We also showed that anti-SsEno antibodies did not increase the ability of mouse phagocytes to kill *S. suis* in vitro. In conclusion, these data demonstrate that although recombinant SsEno formulated with Quil A triggers a strong antibody response, it does not confer effective protection against infection with *S. suis* serotype 2 in mice.

INTRODUCTION

Despite increasing research in recent years, *Streptococcus suis* continues to cause a variety of diseases in pigs worldwide, including septicemia, meningitis, arthritis and endocarditis [1]. Among the 35 serotypes described, serotype 2 is considered the most virulent and is most frequently isolated from diseased pigs [1]. In addition, *S. suis* has also been described as an important zoonotic agent, especially in Europe and Asia [2]. Human infections with *S. suis* are most frequently manifested as purulent meningitis, but septic shock with multiple organ failure, endocarditis, pneumonia, arthritis, and peritonitis have also been reported [2].

The pathogenesis of *S. suis* infection is not fully understood. In swine, the potential portals of entry for *S. suis* are the palatine and pharyngeal tonsils and thereafter bacteria can spread via the hematogenous or lymphogenous route [3]. Once in the bloodstream, *S. suis* has to resist phagocytosis and killing by phagocytic cells to cause acute septicemia that may lead to septic shock. Bacteria can reach different organs, including the central nervous system (CNS), via different mechanisms that are only partially elucidated to date [4].

Several potential virulence factors have been implicated in the infection process. The most promising virulence factors to date are the capsule polysaccharide (CPS) and serum opacity factor (OFS) [5], as isogenic mutants lacking either of these factors are rapidly cleared and eliminated from circulation [5-7]. However, some non-virulent strains are also encapsulated or have allelic variations of OFS [8], indicating that virulence of *S. suis* likely involves multiple factors [4]. Other virulence candidates have been proposed, but most of them are not present in all virulent strains or are present in non-virulent strains [9]. So far, whole cell vaccines or bacterins (commercial and autogenous) have been used in the field to prevent *S. suis* disease, however, with disappointing results [10, 11]. Vaccination with these bacterins does not induce high levels of antibodies and causes, at most, serotype-specific responses [1]. We recently identified SsEno, a new *S. suis* surface fibronectin-binding protein [12] that might participate in the pathogenesis of *S. suis* infection by mediating bacterial attachment to and internalization into brain microvascular endothelial cells (BMEC) [12]. SsEno might be an

attractive vaccine candidate against *S. suis* infections as it possesses highly conserved epitopes [13] and is expressed at the surface of all *S. suis* serotypes described to date [12]. In addition, a recent study identified *S. suis* enolase as an important antigenic protein that contributes to the virulence of *S. suis* [14]. The objective of this study was to determine whether the immune response induced by immunization with purified SsEno can confer protection against challenge with the homologous strain of *S. suis* serotype 2 in a mouse model of infection.

METHODS

Bacterial strains

S. suis serotype 2 highly virulent strain 166' [15] was kindly provided by Dr. M. Kobisch, AFSSA, Ploufragan, France. Working log-phase cultures were prepared in Todd Hewitt broth (THB) (BD, Sparks, MD) as previously described [16]. Growth was allowed until the suspension reached approximately 5 x 10^8 CFU ml^{-1}. Final inoculum corresponded to 10^7 CFU ml^{-1} (for pigs) and 10^8 CFU ml^{-1} (for mice). *Escherichia coli* strain BL21DE3 (Novagen, Madison, WI) was used for expression experiments as described elsewhere [12].

Cloning and expression of the α-enolase gene

Cloning and purification of SsEno were performed as previously described [12]. Briefly, the coding sequence of SsEno (*S. suis* Enolase) was amplified by PCR using chromosomal DNA from *S. suis* SS166 as template and the complete gene was cloned into pET-32a vector (Novagen, Madison, WI). The plasmid pET-32a-SsEno was introduced into *E. coli* Bl21DE3 for an IPTG-inducible expression of recombinant *S. suis* enolase (rSsEno). The His-tagged fusion protein was purified by chromatography under native conditions on HisTrap according to the manufacturer's instructions (Amersham Biosciences AB, Uppsala, Sweden). Protein concentrations were determined by the Lowry method [17]. Prior to vaccination assay, the purity (>95%) of recombinant SsEno preparations was determined by scanning densitometry of the protein on an SDS-PAGE gel stained with Coomassie blue and with a silver-stained SDS-PAGE gel (data not shown) as previously reported [12].

Presence of anti-SsEno antibodies in convalescent animals

All animal experiments were conducted according to the guidelines and protocols set forth by the Canadian Council on Animal Care and approved by the Université de Montréal committee on animal care. Seven 4-week-old piglets from a herd free of *S. suis* serotype 2 disease were infected intravenously with 10^7 CFU from a log-phase culture of *S. suis* strain 166'.

Animals were monitored for clinical signs and treated with antibiotics if needed to avoid death. Serum samples were taken befor and three weeks post-infection. Antibody titers against rSsEno from these convalescent pig sera were measured by direct ELISA. Maxisorp® flat-bottom microtiter 96-well plates (Nunc, VWR, Mississauga, ON, Canada) were coated overnight at 4°C with 5 µg ml^{-1} of purified recombinant SsEno. The plates were further incubated with 1/1000 dilution of pig sera and bound antibodies were detected by incubation with peroxydase-conjugated goat anti-swine IgG (Jackson Immunoresearch Laboratories West Grove, PA) for 1 h at room temperature. The plates were developed with TMB substrate (Zymed, S. San Francisco, CA, USA) and absorbance was measured at 450 nm.

Active protection assay of mice with rSsEno

Six-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were immunized subcutaneously twice, one week apart, with either 20 µg of purified SsEno mixed with 20 µg of Quil-A adjuvant (Brenntag Biosector, Frederikssund, Denmark) or 20 µg of Quil-A only as a control in 100 µl of phosphate-buffered saline (PBS) per mouse. Ten days after the second vaccination, animals were challenged intraperitoneally with 10^8 CFU per mouse of log-phase *S. suis*, strain 166' in 1 ml of THB. Sera collected from each mouse before immunization, before the second dose and before challenge infection were assayed for anti-SsEno antibody titers by ELISA, as described below. Mice were monitored daily for clinical signs such as abnormal behavior (hyperexcitation, episthotonus, opisthotonus, bending of the head toward one side, walking in circles or strong locomotive problems), rough hair coat, ataxia and mortality until day 10 post-infection [16]. This mouse model of infection was recently used to reproduce septic shock and meningitis that might be consider to be similar to those induced by *S. suis* in pigs [1, 16].

Passive protection of mice with rabbit antibodies against rSsEno

Groups of 12 female CD-1 mice (Charles River, 6 week old) were injected intraperitoneally with 0.5 ml of rabbit anti-rSsEno serum [12] or 0.5 ml of normal rabbit serum as a control. Hyperimmune sera against rSsEno was produced as previously described [18] and

the titer (<1/100 000) evaluated by ELISA [19]. Three hours later, 10 mice per group were injected intraperitoneally with 10^8 CFU per mouse of log-phase *S. suis*, strain 166' in 1 ml of THB. Sera were collected 24 h after the serum administration to measure anti-SsEno antibodies by ELISA assay as described below. Mice were monitored daily for weight loss, clinical signs and mortality.

Determination of active and passive antibody titers in mice by ELISA

Titers of SsEno-specific total IgG in mice sera were determined by ELISA as previously described [18]. Briefly, Polysorb plates (Nunc-Immunoplates, Rochester, NY, USA) were coated overnight at 4°C with purified recombinant SsEno. Due to an extremely high antibody response (see below), and comparing to what was used to measure antibodies in swine, a significant reduced concentration of SsEno (0.3 μg ml^{-1}) was used to coat the plates. After incubation with serial dilutions of test, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 antisera (Serotec, Kidlington, Oxford, UK) for 1 h at room temperature. For determination of antibodies titers in the passive protection assay, the same protocol was used but with peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a secondary antibody. Plates were then developed as described above. The antibody titer was considered as the serum dilution that resulted in an OD_{450} reading of 0.1 after subtracting background.

Killing of S. suis serotype 2 strain 166' by mouse phagocytes in the presence of anti-SsEno antibodies

In vitro killing of *S. suis* serotype 2 strain 166' by mouse phagocytes in the presence of polyclonal anti-SsEno antibodies was measured as described previously [20, 21] with some modifications. Briefly, 0.3 ml (from 5 different animals) of freshly heparinised blood was mixed with 0.5 ml of appropriately THB diluted bacteria (200-500 CFU ml^{-1}) in the presence of 25 □g ml^{-1} of protein G purified rabbit anti-SsEno antibodies [12]. Similar concentrations of purified normal rabbit antibodies were used as negative control and of purified hyperimmune rabbit anti-*S. suis* (whole cell) antibodies as a positive control, as previously described [22]. Mixtures

were incubated at 37ºC for 3 h with constant slow rotation [20, 21]. At the end of the incubation, an appropriately diluted aliquot was plated onto THB agar and incubated overnight at 37ºC to count surviving bacteria. Results are expressed as mean percentage ± standard deviation of bacterial survival, with survival of bacteria opsonised with normal rabbit antibodies considered as 100%. Data are representative of four independent experiments. An internal control for the growth of *S. suis* serotype 2 strain 166' in the presence of the different conditions was also included. Briefly, the same concentration of diluted bacteria (200-500 CFU ml^{-1}) were grown in THB in the presence of 25 □g ml^{-1} of the different purified antibodies or no antibodies for 3 h in stationary conditions and the number of CFU in each condition were counted.

Statistics

Antibody titers and percentage of killed bacteria of experimental groups were compared using student's *t*-test (*** $p<0.0005$,** $p<0.005$, * $p<0.05$). Survival curves were evaluated using the Kaplan-Meier method and the significance of the difference was tested using the Log-rank test.

RESULTS

Immunogenicity of SsEno in pigs

None of the seven animals used in this study had SsEno-specific antibodies before the experimental infection (Fig. 1), as only background values were detected in these animals. In contrast, pigs infected with a log-phase culture of *S. suis* strain 166' showed significant anti-SsEno-IgG responses at 3 weeks post-infection (Fig. 1).

SsEno-specific IgG and IgG subclasses in active protection assay

There were no SsEno-specific antibodies in sera from any of the animals before the first vaccination (Fig 2(a)). Immunization of mice with SsEno elicited a strong antigen-specific

response. At 14 days after the first vaccination, SsEno elicited a significant IgG response that was further increased after the second immunization (Fig. 2(a)). Analysis of sera demonstrated that although SsEno-immunized animals produced all IgG subclasses, IgG1, IgG2a and IgG2b responses were predominant followed by IgG3 (Fig. 2(b)). In contrast, animals vaccinated only with adjuvant did not show any antibody responses (Fig. 2(a) and 2(b)).

Clinical signs and mortality in the active protection assay

A few hours after challenge infection with *S. suis* serotype 2 strain 166', all mice (control and SsEno immunized group) exhibited clinical signs, such as ruffled hair coat suggesting fever and slow response to stimuli. From day 0 to 3 post-infection, 25 % of immunized animals died from septicemia (Fig. 3(a)). After day 3 post-infection, 58 % of the immunized animals that survived to septicemia developed severe CNS signs such as running in circles and opisthotonos and died or met criteria for euthanasia due to the severity of their condition (Fig. 3(a)). Similar results were observed in the control group; although all the animals resisted the septicemic phase, 33 % of animals died after day 3 post-infection due to meningitis (Fig. 3(a)). In all dead animals, *S. suis* serotype 2 was isolated from different organs (data not shown).

During the septicemic phase, the immunized group lost ~15 % of their body weight (Fig. 3(b)). Similar results were seen in the control group (Fig. 3(b)). In both groups, animals that survived the septicemic phase were able to recover their initial body weight at the end of the experiment (Fig. 3(b)).

Clinical signs and mortality in the passive protection assay

A few hours after infection, passively immunized animals presented similar clinical signs as those vaccinated in the active protection assay. During the first 12 h, 30% of animals immunized with anti-SsEno antibody died from septicemia compared with 70% of the control group. However, all passively immunized animals as well as control mice died within the first 6 days post-infection from either septicemia or meningitis (Fig. 4). Analysis of sera clearly

indicated that there was a high titer of rabbit anti-SsEno only in the passive immunized group (data not shown).

Killing of *S. suis* serotype 2 strain 166' by mouse phagocytes

To further evaluate the bactericidal/opsonic capacity of anti-rSsEno antibodies, a killing assay was performed using mouse whole blood. As shown in Fig. 5, *S. suis* treated with normal rabbit IgGs grew well in mouse blood (negative control). In contrast, *S. suis* treated with rabbit anti-whole *S. suis* IgGs was rapidly killed as expected (positive control). Incubation of *S. suis* with affinity purified anti-SsEno IgG antibodies did not enhance bacterial killing, confirming results obtained in the passive protection assay.

DISCUSSION

Several approaches have been used to develop vaccines for *S. suis*. However, little success has been achieved thus far because the protection elicited was either serotype or strain dependent, and results in most instances have been equivocal [23]. For example, killed whole cells or live avirulent vaccines can provide partial protection but only with repeated immunization [24, 25]. Subunits protein-based vaccines have also been tested. using virulence markers such the hemolysin [26], the muramidase-released protein and extracellular protein factor [25] that have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, their use is hindered by the fact that a substantial number of virulent strains in some geographical regions do not express these proteins [4]. More recently, a surface expressed protein (SAO) has been observed to confer protection against experimental infection in mice and pigs [27]. However, as this protection is not complete, other proteins could be combined with SAO to optimize protection.

Fibronectin-binding proteins have been suggested as potential vaccine targets for preventing bacterial infections because antibodies directed against such adhesins may prevent

bacterial attachment and also enhance opsonization and killing by leukocytes [28, 29]. In this study, we tested the potential of the recently described fibronectin-binding protein, SsEno, as a vaccine candidate in a mouse model of *S. suis*-induced septicemia and meningitis [16]. SsEno is a good vaccine candidate based on the following characteristics: i) it is present at the surface of all 35 different *S. suis* serotypes [12]; ii) it contributes to *S. suis* adhesion to and invasion of host cells [12]; and iii) it is a highly conserved protein [13]. In addition, we further showed that sera from convalescent animals strongly recognize this protein, suggesting expression of SsEno in vivo. Very recently, another study also suggested an important role of enolase in the virulence of *S. suis* [14]. However, the potential of this protein to confer protection against other pathogens is controversial. Mice immunized with antibodies against *Plasmodium falciparum* enolase are protected from challenge infection with a lethal mouse malaria strain [30]. In contrast, recombinant enolase from *Candida albicans* induces only modest protection against disseminated candidiasis [31]. To our knowledge, a protection test against streptococci using enolase as an antigen to elicit an immune response has not been reported yet.

To evaluate SsEno as a vaccine candidate against *S. suis* infection, we used a highly virulent strain for swine in a well standardized mouse model, which presents two different phases: a) a septicemic phase, (24-48h post-infection) and, b) a meningitis/encephalitis phase, where animals that survive septicemia will subsequently die from a serious infection of the CNS [16]. Our results indicated that the antibodies actively elicited by SsEno at the concentration used in combination with Quil-A adjuvant was not protective from either phase of challenge infection with *S. suis*. The Th1-type responses and IgG subclasses induced by the SAO protein are two main components of host immunity against *S. suis* infection [27]. In this study, all SsEno-specific IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were induced, with IgG1 and the Th1-type antibodies, IgG2a and IgG2b, as the predominant subclasses. Therefore, other components of the host immune response besides non-opsonic antibodies may be necessary for effective protection against *S. suis* infection. We attributed the fact that the vaccinated group presented a higher level of mortality than the control group to a simple variation in an animal assay. Although it would have been tempting to interpret the results as a possible role of antibodies against enolase playing a certain role in autoimmunity as described [32], animals from both groups were perfectly healthy at the moment of challenge.

To confirm these results, we tested whether rabbit anti-SsEno serum provides passive protection against challenge infection with the same strain of *S. suis* in mice. As expected, and although a slight delay in the appearance of severe clinical signs, passive immunization with anti-SsEno serum did not confer significant protection against *S. suis* infection. As it has been shown that antibodies derived from hyperimmune sera against whole bacteria (including antibodies against the capsule) can protect mice against infection [33] and induce bacteria killing [22], it is possible that anti-SsEno antibodies are not opsonic, allowing bacteria to reproduce in high numbers to cause disease. On the other hand, antibodies against other fibronectin-binding proteins have already been described to enhance phagocyte killing in other bacterial species [28]. To test this, we evaluated the capacity of the purified anti-SsEno IgGs to opsonize bacteria and promote their killing by murine phagocytes. Although most of the produced antibodies were of the Th1 type, results obtained in this study showed that the anti-SsEno IgG subclasses were not able to induce *S. suis* killing by mouse phagocytes.

Since anti-SsEno has been shown to reduce bacterial adhesion and invasion to BMEC in vitro [12], we had expected a certain degree of protection against the CNS phase of infection. However, SsEno is not the only receptor involved in such process [34]. It is possible that the high concentration of *S. suis* in blood, as a consequence of a lack of bacterial killing by leukocytes, may overcome the partial inhibition of bacterial-BMEC interactions mediated by anti-SsEno antibodies.

In summary, although we demonstrated that SsEno elicits an important antibody response in convalescent pigs and immunized mice, this response as evaluated in the present study is inadequate for effective protection against *S. suis* infection.

ACKNOWLEDGEMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant 0680154280.

ANNEX 1. ARTICLE IV. FIGURES

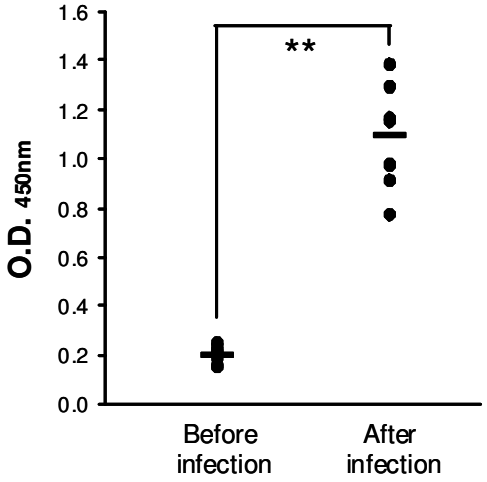


Fig. 1. ELISA detection of antibodies against SsEno in sera from pigs before and after challenge with *S. suis* serotype 2. Plates were coated with rSsEno and then incubated for 1.5 h with a dilution of 1/1000 of swine sera (before and after challenge). Mean values (line) comparing sera from non-infected (n = 7) and infected pigs (n = 7) are shown. **, P<0.005.

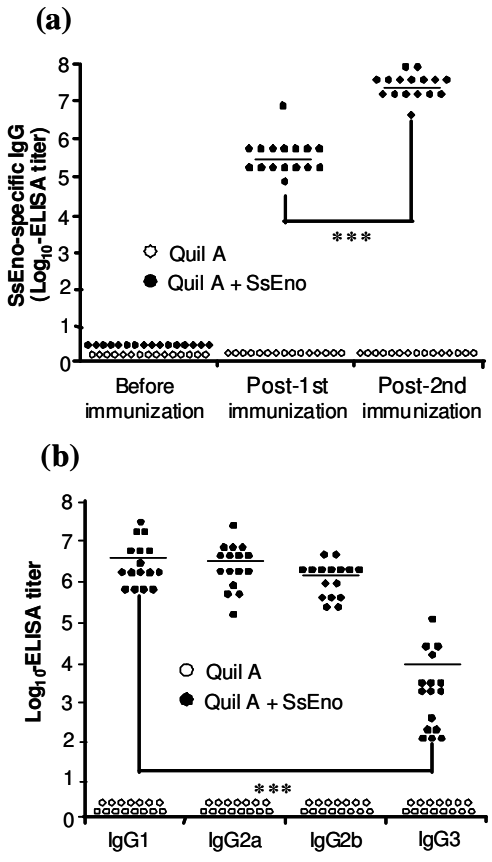


Fig. 2. Serum antibody responses in mice immunized with Quil A (open cycles) alone or Quil A plus recombinant SsEno (solid cycles). (a) Total SsEno-specific serum IgG. (b) IgG subclasses in sera at 14 days after the second immunization (before challenge). Antibody titers of individual mice are shown, with the average titer represented as a bar. (n=16 for SsEno-immunized animals; n=15 for control animals). ***, P<0.0005

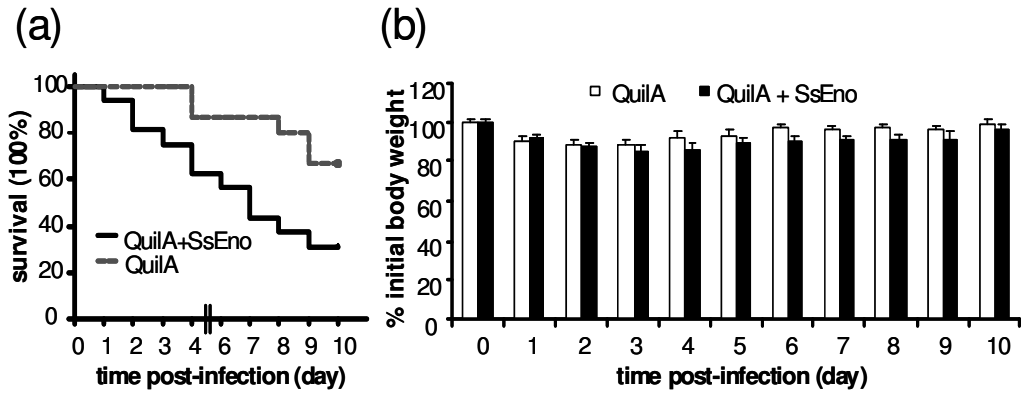


Fig. 3. Survival (a) and weight lost (b) of mice in the active protection assay. Mice were first immunized with Quil A (n=14, white) or Quil A-rSsEno (n=15, black) and then challenged with 10^8 CFU ml^{-1} of *S. suis* 166'. Survival rate (a) and % of initial body weight (b) were monitored for 10 days. Body weight of each mouse was measured every day.

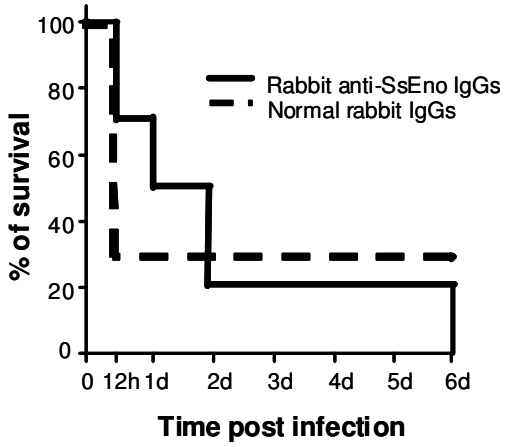


Fig. 4. Survival of mice in the passive protection assay. Mice were immunized with rabbit anti-SsEno antibodies (n=12) or control rabbit antibodies (n=12). Mice were then challenged intraperitoneally with 10^8 CFU ml^{-1} of *S. suis* serotype 2 strain 166' and survival was monitored for 10 days.

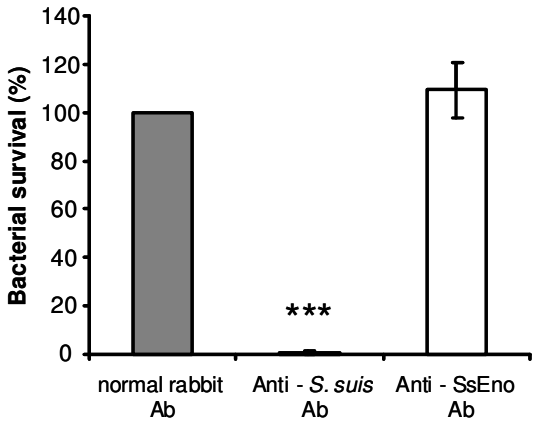


Fig. 5. Effect of antibodies on opsonophagocytic killing of *S. suis* strain 166' by mouse phagocytes. Antibodies were purified from sera of a rabbit immunized with SsEno. Antibodies from a normal rabbit and from a rabbit hyperimmunized with whole *S. suis* serotype 2 were used as negative and positive controls, respectively. Data are expressed as mean percentage ± standard deviation of bacterial survival, with survival of bacteria opsonised with normal rabbit antibodies considered as 100%. Data are representative of four independent experiments. ***, $P<0.0005$.

REFERENCES

1. Higgins, R. and M. Gottschalk, *Streptococcal diseases*, in *Diseases of swine*, S.D.A. B. E. Straw, W. L. Mengeling, and D. J. Taylor, Editor. 2005, Iowa State University Press: Ames. p. 769-783.

2. Lun, Z.R., et al., *Streptococcus suis: an emerging zoonotic pathogen.* Lancet Infect Dis, 2007. **7**(3): p. 201-9.

3. Madsen, L.W., et al., *Streptococcus suis serotype 2 infection in pigs: new diagnostic and pathogenetic aspects.* J Comp Pathol, 2002. **126**(1): p. 57-65.

4. Gottschalk, M. and M. Segura, *The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions.* Vet Microbiol, 2000. **76**(3): p. 259-72.

5. Baums, C.G., et al., *Identification of a novel virulence determinant with serum opacification activity in Streptococcus suis.* Infect Immun, 2006. **74**(11): p. 6154-62.

6. Charland, N., et al., *Streptococcus suis serotype 2 interactions with human brain microvascular endothelial cells.* Infect Immun, 2000. **68**(2): p. 637-43.

7. Smith, H.E., et al., *Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor.* Infect Immun, 1999. **67**(4): p. 1750-6.

8. Takamatsu, D., et al., *Allelic variation and prevalence of serum opacity factor among the Streptococcus suis population.* J Med Microbiol, 2008. **57**(4): p. 488-494.

9. Gottschalk, M., M. Segura, and J. Xu, *Streptococcus suis infections in humans: the Chinese experience and the situation in North America.* Anim Health Res Rev, 2007. **8**(1): p. 29-45.

10. Halbur, P., et al., *Efficacy of antimicrobial treatments and vaccination regimens for control of porcine reproductive and respiratory syndrome virus and Streptococcus suis coinfection of nursery pigs.* J Clin Microbiol, 2000. **38**(3): p. 1156-60.

11. Lapointe, L., et al., *Antibody response to an autogenous vaccine and serologic profile for Streptococcus suis capsular type 1/2.* Can J Vet Res, 2002. **66**(1): p. 8-14.

12. Esgleas, M., et al., *Isolation and characterization of α-enolase, a new fibronectin-binding protein from Streptococcus suis.* Microbiology, 2008. **154**: p. 2668-2679.

13. Ehinger, S., et al., *Plasmin(ogen)-binding alpha-enolase from Streptococcus pneumoniae: crystal structure and evaluation of plasmin(ogen)-binding sites.* J Mol Biol, 2004. **343**(4): p. 997-1005.

14. Jing, H.B., et al., *Proteome analysis of Streptococcus suis serotype 2.* Proteomics, 2008. **8**(2): p. 333-349.

15. Berthelot-Herault, F., et al., *Dilemma of virulence of Streptococcus suis: Canadian isolate 89-1591 characterized as a virulent strain using a standardized experimental model in pigs.* Can J Vet Res, 2005. **69**(3): p. 236-40.

16. Fittipaldi, N., et al. *Reduced Virulence of Streptococcus suis in a Murine Model of Infection After Inactivation of the dltA Gene*. in *107th General Meeting of the American Society for Microbiology*. 2007. Toronto, Canada.

17. Lowry, O.H., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 267-75.

18. Li, Y., et al., *Identification of a surface protein of Streptococcus suis and evaluation of its immunogenic and protective capacity in pigs.* Infect Immun, 2006. **74**(1): p. 305-12.

19. Shah, P. and E. Swiatlo, *Immunization with polyamine transport protein PotPD protects mice against systemic infection with Streptococcus pneumoniae.* Infect Immun, 2006. **74**(10): p. 5888-5892.

20. Lancefield, R.C., *Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test.* J Exp Med, 1957. **106**(4): p. 525-44.

21. Pancholi, V. and V.A. Fischetti, *alpha -Enolase, a Novel Strong Plasmin(ogen) Binding Protein on the Surface of Pathogenic Streptococci.* J. Biol. Chem., 1998. **273**(23): p. 14503-14515.

22. Chabot-Roy, G., et al., *Phagocytosis and killing of Streptococcus suis by porcine neutrophils.* Microb Pathog, 2006. **41**(1): p. 21-32.

23. Haesebrouck, F., et al., *Efficacy of vaccines against bacterial diseases in swine: what can we expect?* Vet Microbiol, 2004. **100**(3-4): p. 255-68.

24. Busque, P., et al., *Immunization of pigs against Streptococcus suis serotype 2 infection using a live avirulent strain.* Can J Vet Res, 1997. **61**(4): p. 275-9.

25. Wisselink, H.J., et al., *Protection of pigs against challenge with virulent Streptococcus suis serotype 2 strains by a muramidase-released protein and extracellular factor vaccine.* Vet Rec, 2001. **148**(15): p. 473-7.

26. Jacobs, A.A., A.J. van den Berg, and P.L. Loeffen, *Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of Streptococcus suis.* Vet Rec, 1996. **139**(10): p. 225-8.

27. Li, Y., et al., *Immunization with recombinant Sao protein confers protection against Streptococcus suis infection.* Clin Vaccine Immunol, 2007. **14**(8): p. 937-43.

28. Rennermalm, A., et al., *Antibodies against a truncated Staphylococcus aureus fibronectin-binding protein protect against dissemination of infection in the rat.* Vaccine, 2001. **19**(25-26): p. 3376-83.

29. Wizemann, T.M., J.E. Adamou, and S. Langermann, *Adhesins as targets for vaccine development.* Emerg Infect Dis, 1999. **5**(3): p. 395-403.

30. Pal-Bhowmick, I., et al., *Protective Properties and Surface Localization of Plasmodium falciparum Enolase.* Infect Immun, 2007. **75**(11): p. 5500-8.

31. Montagnoli, C., et al., *Immunogenicity and protective effect of recombinant enolase of Candida albicans in a murine model of systemic candidiasis.* Med Mycol, 2004. **42**(4): p. 319-24.

32. Terrier, B., et al., *Alpha-enolase: a target of antibodies in infectious and autoimmune diseases.* Autoimmun Rev, 2007. **6**(3): p. 176-82.

33. Charland, N., et al., *Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by Streptococcus suis serotypes 1, 2 and 1/2.* Microbiology, 1997. **143 (Pt 11)**: p. 3607-14.

34. Vanier, G., M. Segura, and M. Gottschalk, *Characterization of the invasion of porcine endothelial cells by Streptococcus suis serotype 2.* Can J Vet Res, 2007. **71**(2): p. 81-9.

ANNEX 2. ARTICLE V

« D-Alanylation of the Lipoteichoic Acid Contributes to the Virulence of *Streptococcus suis*»

Nahuel Fittipaldi[1], Tsutomu Sekizaki[2,3], Daisuke Takamatsu[2], Josée Harel[1], María de la Cruz Domínguez-Punaro[1], Sonja Von Aulock[4], Christian Draing[4], Corinne Marois[5], Marylène Kobisch[5] and Marcelo Gottschalk[1]*

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada; [2]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; [3]United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; [4]Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; [5]Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d'Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France.

Published in: *Infect. Immun*. 2008 Aug;76(8):3587-94.

Details on the role of the candidate in the conception of the article: I actively participated in the experimental design concerning mouse studies; I substantially performed research, analyzed data and participated in writing the paper.

Abstract

We generated by allelic replacement a Δ*dltA* mutant in a virulent *Streptococcus suis* serotype 2 field strain and evaluated the contribution of lipoteichoic acid (LTA) D-alanylation to the virulence traits of this swine pathogen and zoonotic agent. Absence of LTA D-alanylation resulted in increased susceptibility to the action of cationic antimicrobial peptides. In addition, and in contrast to the wild type strain, the Δ*dltA* mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Finally, the Δ*dltA* mutant was attenuated in both the CD1 mouse and porcine models of infection, probably reflecting decreased ability to escape immune clearance mechanisms and impaired capacity to traverse across host barriers. The results of this study suggest that LTA D-alanylation is an important factor in *S. suis* virulence.

Introduction

Streptococcus suis is a major swine pathogen and a zoonotic agent responsible for, among other diseases, meningitis and septicemia (15). In swine, *S. suis* causes severe losses to the industry (15) while human *S. suis* infection is emerging as an important public health issue (13). Very recently, more than 200 cases of human *S. suis* infection were reported during an outbreak in China, 38 of which resulted in death (39). *S. suis* is considered the primary cause of adult meningitis in Vietnam (20) and human *S. suis* infection resulting in death or in severe post-infection sequelae has already been reported in different Asian and European countries as well as in New Zealand, Australia, Argentina, Canada and the United States (13). Among *S. suis* serotypes, serotype 2 is responsible for most cases of disease in both swine and humans, and almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype (13, 15). Despite the increasing number of studies, our understanding of the pathogenesis of *S. suis* infection remains limited. The polysaccharide capsule is known to play a critical role in the pathogenesis of *S. suis* infection (15). It has been shown that unencapsulation of *S. suis* correlated with increased phagocytosis by porcine macrophages and killing by porcine neutrophils (4, 6, 29) and that it severely impaired virulence in a porcine model of infection (29). Recently, an isogenic mutant for a serum opacity-like factor was found to be highly attenuated in pigs (2). Other proposed putative virulence factors such as the suilysin, the extracellular protein factor, the muramidase-released protein and a fibronectin/fibrinogen-

binding protein were found to be associated with and/or partially involved in, but not essential for, virulence (7, 15).

S. suis can affect the viability of porcine blood brain barrier (BBB)-forming cells such as porcine choroid plexus epithelial cells (CPEC) through necrotic and apoptotic mechanisms (34). It also can adhere to and invade *in vitro*-cultured porcine brain microvascular endothelial cells (porcine BMEC), another type of BBB-forming cells (35). The ability of *S. suis* to interact with these cells is thought to be important for attaining the central nervous system (CNS) and causing meningitis in swine (13). In a recent study (11), the selective capture of transcribed sequences (SCOTS) approach was used to elucidate genes that this pathogen preferentially upregulates upon its interactions with porcine BMEC. Among other genes, the study identified a gene member of a putative *S. suis dlt* operon (11). In all bacteria where this operon has been studied, it has been found to be responsible for the incorporation of D-alanine residues into lipoteichoic acids (LTA), surface-associated amphiphilic molecules found in most Gram positive bacteria (23).

The cell wall of *S. suis* has been proposed as an important virulence factor. Several studies have shown that the cell wall or its purified components such as the LTA contribute to exacerbate the host inflammatory response to infection (13, 15). However, the structure and composition of *S. suis* LTA are poorly known. It has been proposed that LTA from *S. suis* may have a backbone structure similar to that of Group A streptococcal teichoic acid, but with differences in the attachment of glucosyl substituents (9). Besides its involvement in inflammation, LTA may also play a direct role in *S. suis* virulence. Indeed, a recent study has shown that inhibition of the adherence of *S. suis* to porcine BMEC can be obtained by pre-incubation of the cells with purified LTA (36). In addition, it has been proposed that *S. suis* may D-alanylate its LTA and that a high ratio of D-alanine to glycerol phosphate in this molecule may be important for the interaction of this pathogen with host cells (11). It is known from previous reports that D-alanylated LTA is important for the virulence of Gram-positive pathogens based on findings that it enables these organisms to modulate their surface charge, to regulate ligand binding and to control the electromechanical properties of the cell wall (23). In addition, formation of D-alanyl-LTA is required to resist the action of cationic antimicrobial peptides (CAMPs) (1, 17, 18, 26). The D-alanylation of *S. suis* LTA and its contribution to the pathogenesis

of infection have yet to be documented. In this study, we demonstrate that *S. suis* D-alanylates its LTA and that this modification is important for the virulence traits of this pathogen.

Materials and methods

Bacterial strains, plasmids, media, culture conditions and chemicals.

Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *S. suis* strains were grown in Todd-Hewitt (Becton Dickinson, Sparks, MD) broth (THB) or agar (THA) at 37°C under 5% CO_2. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or agar medium (Becton Dickinson) at 37°C. When needed, antibiotics (Sigma, Oakville, Ontario, Canada) were added to the culture media at the following concentrations: for *S. suis*, chloramphenicol (Cm) at 5 µg/ml and spectinomycin (Sp) at 100 µg/ml; for *E. coli*, kanamycin (Km) and Sp at 50 µg/ml; Cm at 10 µg/ml. Unless otherwise indicated, all chemicals were purchased from Sigma.

DNA manipulations.

Restriction enzymes, DNA-modifying enzymes and Taq and Pwo DNA polymerases were purchased from GE Healthcare (Piscataway, NJ, USA) or Takara Bio (Otsu, Shiga, Japan) and used according to the manufacturers' recommendations. *S. suis* genomic DNA was prepared by the guanidium thiocyanate method (24). Mini-preparations of recombinant plasmids from *E. coli* and transformation of *E. coli* were performed by standard procedures (27). Southern hybridizations were performed by the procedures described previously (28), except that hybridization was carried out at 68°C. For preparation of probes, DNA fragments were labelled with digoxigenin (DIG) using the DIG-PCR labeling mixture (Roche Diagnostics, Laval, Qc, Canada) according to the manufacturer's instructions. Oligonucleotide primers were from Invitrogen (Burlington, Ontario, Canada).

Allelic replacement.

(i) Construction of the knockout vector. DNA fragments corresponding to regions upstream and downstream the *dltA* gene (Fig. 1A) were amplified from genomic DNA of *S. suis* strain 31533 by PCR using the primer sets 2872F plus 3765R (left arm) and 5250F plus 5809R

(right arm). A Sp resistance cassette (gene *aad9*) was amplified from plasmid pSmall with primers specF3 and specR. All three primers sets introduce unique restriction sites (Table 2). PCR amplicons were digested using the appropriate restriction enzymes, and sequentially ligated in the order left arm-Sp cassette-right arm using T4 DNA ligase. The resulting fragment was amplified by PCR using primers 2872F and 5809R, cloned into vector pCR4 (TOPO TA PCR cloning kit; Invitrogen), excised with HindIII and BamHI and recloned into the HindIII and BamHI sites of the temperature sensitive *S. suis*-*E. coli* shuttle vector pSET5s, which carries the gene *cat* conferring Cm resistance (32), giving rise to knockout vector p5ΔdltA (Fig. 1B). (ii) Generation of *S. suis* Δ*dltA*. Procedures for selection of mutants by allelic exchange via double crossover have been described previously (32). Briefly, *S. suis* strain 31533 was transformed with p5ΔdltA by electroporation as previously described (31). The cells were grown at 28°C in the presence of Cm and Sp selection. Bacteria at mid-logarithmic growth phase were diluted with THB containing Sp and grown at 28°C to early logarithmic phase. The cultures were then shifted to 37°C and incubated for 4 h. Subsequently, the cells were spread onto THA agar containing Sp and incubated at 28°C. Temperature-resistant, Sp-resistant colonies were screened for loss of vector-mediated Cm resistance to detect putative mutants which had exchanged their wild-type allele for a genetic segment containing the *aad9* gene as a consequence of homologous recombination via a double crossover. Allelic replacement in candidate clones was verified by PCR and Southern hybridization, which confirmed the expected genotype (data not shown).

Transmission electron microscopy.

Transmission electron microscopy was performed as previously described (12). Briefly, overnight (ON) cultures of *S. suis* wild type (WT) or mutant Δ*dltA* strains were mixed with rabbit anti-*S. suis* serotype 2 polyclonal serum and incubated at room temperature for 1 h. Cells were then fixed in cacodylate buffer (0.1M cacodylate, 5% glutaraldehyde, 0.15% ruthenium red, pH 7.2) for 2 h. After fixation, cells were immobilized in 4% agar, washed in cacodylate buffer and post-fixed ON at 4°C in 2% osmium tetraoxyde. Samples were dehydrated in graded series of ethanol and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Model 420, Philips Electronics, The Netherlands).

Determination of LTA D-alanine content.

S. suis WT and Δ*dltA* mutant strains were cultured in tryptic soy broth (Becton Dickinson) containing beef extract (5 g/l) and glucose (8 g/l) at 37°C for 18 h with shaking. After incubation, bacteria were harvested by centrifugation at 4225 x g for 20 min. Integrity of bacteria and potential contamination by Gram-negative species were checked by Gram staining and microscopy. LTA were prepared by butanol extraction (which preserves the integrity of the D-alanine substitutions) and hydrophobic interaction chromatography as previously described (22). Nuclear magnetic resonance (NMR) spectra from LTA were recorded on a Bruker Avance DRX 600 spectrometer (Bruker BioSpin, Ettlingen, Germany) equipped with an inverse TXI-H/C/N triple resonance probe at 300 K using 3 mm Bruker Match sample tubes. Spectra were measured in D_2O using sodium 3-trimethylsilyl- 3,3,2,2-tetradeutero-propanoate as an internal standard for ^1H NMR (δ_H 0.00 ppm).

Antimicrobial peptides sensitivity.

Assays were carried out in sterile 96-well microtiter plates. Logarithmic-phase *S. suis* cells were adjusted to approximately 10^4 CFU/ml in 100 µl THB containing one of the following antimicrobial compounds in serial dilutions: Colistin (0 to 200 µg/ml), polymyxin B (0 to 300 µg/ml) and magainin II (0 to 45 µg/ml). Plates were incubated for 24 h at 37°C. The MIC was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth by $OD_{600\ nm}$ measurement.

Killing by porcine neutrophils.

Experiments were carried out as described previously (4). Briefly, blood samples were collected by venous puncture from high health status pigs which tested negative by ELISA (19) for *S. suis* serotype 2. Cell populations were separated by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation and neutrophils isolated by sedimentation in 6% dextran. Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Neutrophils were resuspended at a final concentration of 5×10^6 cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated porcine serum. Bacteria (WT or Δ*dltA* mutant strains at approx. 1×10^4 CFU/ml) were opsonized with complete normal porcine serum for 30 min at 37°C and then mixed in microtubes with neutrophils at 5×10^6 cells/ml. The

mixture was incubated for 90 min at 37°C under 5% CO_2. Under these conditions bacteria are not toxic to neutrophils (4). After incubation cells were lysed with sterile water and viable bacterial counts on THA were performed.

Experimental infections.

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. (i) Pigs: A total of 20, 4-week-old, second-generation of caesarean-derived pigs were used in this study. Strict biosecurity measures were implemented to avoid undesirable contamination of the pigs; these included an air filtration system and airlocks for each unit. Pigs were divided in 3 groups. Six out of 7 animals in groups 1 and 2 were inoculated by intravenous injection of 10^8 CFU of the *S. suis* WT 31533 or mutant Δ*dltA* strains, respectively. The remaining animal in both the WT and Δ*dltA* mutant groups was not inoculated although it was housed with inoculated animals and served as sentinel. Group 3 (N=6) were sham-inoculated animals. Clinical signs and presence of *S. suis* in blood were monitored during the trial. Surviving animals in all 3 groups were sacrificed 7 days pi and examined for pathological lesions. Bacteriological isolation from different organs (liver, spleen, lungs, heart and articulations) was performed as described below for mice. (ii) CD1 mice: A recently described murine model for *S. suis* infection was used (8). A total of 60 female CD1 mice aged 6 weeks (Charles River laboratories, Wilmington, MA) were used for virulence assessment. At day 0, animals were divided in 4 groups of 15 mice. Group 1 was inoculated by intraperitoneal injection of 1 ml of *S. suis* strain 31533 suspension at 5 x 10^7 CFU/ml, while group 2 received the same dose of mutant strain Δ*dltA*. Groups 3 and 4 received 1 ml of a 5 x 10^6 CFU/ml suspension of the WT and the mutant strains, respectively, using the same route of inoculation. Mice were monitored 3 times/day for 10 days for clinical signs and assigned clinical scores as previously described (8). Blood samples (5 µl) were collected daily (from the tail vein) and at euthanasia (by cardiac puncture) and used to evaluate bacterial load by plating onto sheep blood agar plates. Isolated tiny α-hemolytic colonies were counted and assigned to *S. suis* by serotyping as previously described (16). Surviving animals in both groups were sacrificed at day 10 and macroscopic examination was performed. Bacterial colonization of the liver, spleen and brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500 µl of PBS and homogenized. Thereafter, 50 µl of the suspensions were plated as described above. In addition, an enrichment of the samples was

carried out by inoculation of 300 µl of homogenized organ samples or 100 µl of blood into THB, followed by ON incubation at 37°C and subsequent dilution and plating onto sheep blood agar plates as described above.

Adherence to and invasion of porcine BMEC.

The porcine BMEC cell line PBMEC/C1-2 (33) was grown in Primaria 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) using IF culture medium (a mixture of 1:1 Iscove's modified Dulbecco's and Ham's F-12 media, Invitrogen) supplemented as previously described (35). *S. suis* was grown in THB for 16 h at 37°C, harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), pH 7.3, and resuspended in fresh IF culture medium. The invasion assays were performed as described previously (35). Briefly, confluent monolayers of porcine BMEC at 10^5 cells/well were infected with 1-ml aliquots of bacterial suspensions at 10^5 CFU/ml (multiplicity of infection (MOI) of 1). The plates were centrifuged at 800 x *g* for 10 min and incubated for 2 h at 37°C with 5% CO_2. The monolayers were then washed twice with PBS. A 1 ml volume of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G was added to each well, and incubation continued for 1 h. After incubation, monolayers were washed 3 times with PBS, trypsinized and disrupted by repeated pipetting. Serial dilutions of the cell lysates were plated onto THA and incubated ON at 37°C. To confirm that 100% of the extracellular bacteria were killed after the antibiotic treatment, a 100-µl sample of the last PBS wash was plated onto THA (results not shown). Adherence assays were performed essentially as described for invasion, but neither antibiotic treatment nor extended incubation was performed. After 2 h incubation, cells were vigorously washed 5 times with PBS, trypsinized, disrupted, and serial dilutions of the cell lysates were plated as described above.

Results and Discussion

The *dlt* operon is responsible for LTA D-alanylation in *S. suis*.

The genetic organization of the *S. suis dlt* operon is depicted in Fig. 1A. Sequence comparison at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), as well as previous reports (17, 18, 25), showed that the *S. suis dlt* operon is organized in a fashion similar to that of all *dlt* operons reported in pathogenic streptococci so far, with the exception of *S. agalactiae*, which also includes 2

regulatory genes upstream of the *dltA* gene (25). Accordingly, the deduced proteins showed a high degree of similarity to streptococcal Dlt proteins (data not shown). To assess the contribution of the *dlt* operon to LTA D-alanylation, we constructed by allelic replacement a Δ*dltA* mutant strain and analyzed the content of D-alanine in purified LTA of the WT and Δ*dltA* mutant strains by NMR. Fig. 2 shows the NMR spectra for LTA of the two strains. Both LTA showed the expected peaks for fatty acids (0.85 and 1.3 ppm) and sugars (3.5 to 4.5 ppm). However, peaks for D-alanine (1.65, 4.3 and 5.4 ppm) were absent in the Δ*dltA* mutant spectrum, suggesting that the LTA of the mutant is devoid of this amino acid substitution. *In vitro* growth of the Δ*dltA* mutant was comparable to that of the WT strain (Fig. 3A) and no other major phenotypic changes were observed. In contrast to previous reports on *S. agalactiae* and *Streptococcus pyogenes* Δ*dltA* mutants, which were either poorly separated or multiseptated in the stationary phase of growth (18, 26), the *S. suis* Δ*dltA* mutant was encapsulated, well separated and presented normal septation (Fig. 3B).

***S. suis* LTA D-alanylation contributes to antimicrobial peptide resistance and decreases susceptibility to neutrophil killing.**

CAMPs kill bacteria by forming pores in the cytoplasmic membrane (30). Introduction of positively charged D-alanine residues into the LTA would reduce the global negative charge of the *S. suis* envelope, thus providing the bacterium with a physical mechanism of resistance against the action of CAMPs (23). To assess this hypothesis, we performed an evaluation of the sensitivity of the WT and Δ*dltA* mutant to selected CAMPs. The *S. suis* Δ*dltA* mutant was more sensitive than the WT strain against the bacteria-derived cationic peptide polymyxin B and colistin and the frog-derived peptide magainin II (Table 3). These results were in agreement with previous reports of inactivation of the *dltA* gene in streptococcal species (17, 18, 26) and indicate that D-alanylation of LTA is an important component of the intrinsic resistance of *S. suis* to CAMPs killing. On the other hand, the WT and *dltA* mutant strains were equivalent in their susceptibility to the antibiotics gentamicin and penicillin G and to lysozyme (data not shown). Functional homologues of the CAMPs tested in this study are secreted by neutrophils both into the phagosome as well as extracellularly. (21). When we compared killing of WT and Δ*dltA* mutant strains by purified porcine neutrophils, in agreement with a previous study (4), the WT strain avoided killing by neutrophils when opsonized with normal complete porcine sera. On the other hand, 20% of the Δ*dltA* mutant bacteria were killed by neutrophils (Fig. 4).

This level of killing was similar to that of the unencapsulated mutant strain BD102, despite the fact that the Δ*dltA* mutant does not have altered capsule expression (Fig. 4). This was surprising, since encapsulated WT *S. suis* has been shown to prevent phagocytosis by porcine neutrophils (unless opsonized by specific antibodies) (4). However, it is known that neutrophils are also able to destroy infecting microorganisms in the absence of phagocytosis in the so-called neutrophil extracellular traps (NETs) (3). Interestingly, it has recently been shown in *Streptococcus pneumoniae* that absence of LTA D-alanylation results in enhanced extracellular killing in NETs by neutrophils but not in an increased phagocytosis of this organism by these polymorphonuclear cells (37). Although our killing assay is not able to discriminate between intra and extracellular killing, taking all these findings together, it might be proposed that the encapsulated *S. suis* Δ*dltA* mutant is killed by porcine neutrophils extracellularly, perhaps after being trapped in NETs. In addition, we speculate that the enhanced killing of the *S. suis* Δ*dltA* mutant might be the consequence of the absence of LTA D-alanylation, which results in an increased susceptibility to CAMPs released by neutrophils. Further experiments are needed to evaluate this hypothesis.

Virulence of the Δ*dltA* mutant is attenuated in pigs.

Several Δ*dltA* mutants in different Gram positive pathogens have been reported and almost all of these mutants were highly susceptible to CAMPs and killing by neutrophils and/or macrophages (1, 5, 10, 14, 17, 18, 26, 37, 38). However, only a limited number of studies have analyzed *in vivo* the contribution of LTA D-alanylation to the virulence of these pathogens. In these cases, virulence of the tested Δ*dltA* mutants greatly varied between bacterial species preventing the drawing of conclusions regarding the contribution of LTA D-alanylation to virulence traits of pathogens from previous studies (1, 26, 37, 38). Finally, for various valid reasons, previous studies on the virulence of Δ*dltA* mutants in Gram positive species have used surrogated models of infection instead of the natural hosts (1, 26, 37, 38). *S. suis* shares certain characteristics with pathogens for which Δ*dltA* mutants have been described. However, its pathogenesis of infection is essentially different (15). In this study, we have for the first time evaluated the virulence of a Gram positive Δ*dltA* mutant in the context of its natural host by intravenous inoculation of pigs. Animals in the sham-inoculated group did not present any clinical signs during the entire duration of the trial. In contrast, severe clinical signs were recorded in 5 out of the 6 animals inoculated with the WT strain during the first 4 days of the

trial. These 5 pigs died or were sacrificed for ethical reasons at day 2 pi (3 animals) and at day 4 pi (2 pigs). The remaining inoculated animal and the sentinel pig in this group survived until the end of the trial (Fig. 5). Animals infected with the Δ*dltA* mutant presented, on average, less severe clinical signs during the 4 first days pi. However, 2 animals died and an additional was euthanized by ethical reasons in this group (Fig. 5). Nevertheless, the remaining inoculated animals noticeably recovered starting at day 4 pi, and, along with the sentinel pig in the Δ*dltA* group, survived until the end of the trial. Hyperthermia (> 40.5°C) was observed in all pigs infected with either WT or Δ*dltA* strain at 24 h pi. Temperatures returned to normal values after day 4 in both groups. However, in the WT group, the sentinel pig developed hyperthermia from day 6 pi. *S. suis* serotype 2 could be isolated from the blood of all inoculated pigs in both groups and the sentinel animal in the WT group. Pigs in the latter group had higher bacterial counts (as high as 1 x 10^{10} CFU/ml in some cases) than those infected with the Δ*dltA* mutant (average of 1 x 10^{8} CFU/ml) during the first 4 days pi. Similar to the blood, bacterial titers in organs were slightly lower in pigs inoculated with the Δ*dltA* mutant compared with animals inoculated with the WT strain. However, examination at necropsy did not find major differences between the WT and Δ*dltA* mutant groups regarding damage to tissues or organs. At euthanasia, macroscopic lesions typical of *S. suis* infection were found in most animals infected with the WT or Δ*dltA* strains, especially at the pleura, pericardium and peritoneum. Fibrin deposits were observed in the liver and spleen of most animals in both groups. Pneumonia and fibrinal pleurisy were also observed in some animals. Additionally, the meninges showed inflammation consistent with meningitis. Lameness was observed in all pigs infected with the WT or Δ*dltA* strain. At necropsy, articulations showed inflammation, with fibrin deposits and excess of synovial liquid. Results from the experimental infection showed that the Δ*dltA* mutant is attenuated in the pig and suggest that LTA D-alanylation provides an advantage to the WT strain. However, this conclusion is mitigated by the facts that dissemination of the bacterium was not prevented and mortality was observed among animals inoculated with the Δ*dltA* mutant. Since clearance of the mutant from circulation might primarily rely on neutrophil activity, the high dose used to inoculate the animals may explain, at least in part, the mortality observed. Indeed, it has been proposed that suilysin may affect complement activity and suilysin-producing *S. suis* strains, such as the WT and mutant strains used in this study, have been shown to be toxic to neutrophils at high titers (4). In addition, since CAMPs activity primarily occurs at mucosal surfaces, the extremely aggressive

intravenous route of administration may have also influenced the clinical onset observed in pigs.

Absence of LTA D-alanylation impairs *S. suis* virulence in mice.

To better evaluate the attenuation of the *S. suis* Δ*dltA* mutant observed in the pig we performed additional *in vivo* trials using the CD1 mouse model of infection that uses the intraperitoneal route of inoculation (8). We performed two different trials at high and intermediate doses of infection. At the high dose (5 x 10^7 CFU per animal) most mice in both the WT and Δ*dltA* mutant groups presented severe clinical signs associated with septicemia, such as depression, swollen eyes, weakness and prostration during the first 72 h pi. At this dose we did not find a clear reduction in the ability of the Δ*dltA* mutant to successfully initiate infection and induce septicemia in mice. In fact, several mice died from septicemia in both groups during the first 3 days of the trial (Fig. 6A). *S. suis* was isolated at high titers (> 1 x 10^7 CFU/ml) from blood samples and organs such as the liver and spleen of septicemic animals (> 1 x 10^7 CFU/0.5 g of tissue in some animals). From day 5 pi, some mice in both WT and Δ*dltA* groups developed clinical signs associated with *S. suis* meningitis in the mouse (8), such as hyperexcitation, episthotonus, opisthotonus, bending of the head and walking in circles. It has been proposed that maintaining a high level of bacteremia is essential for CNS disease to appear at later stages of the infection (13). Interestingly, the number of meningitis-presenting mice was lower in the Δ*dltA* group (N=1) than in the WT group (N=6), and this observation was consistent with the reduction in the bacterial load in blood among animals inoculated with the Δ*dltA* mutant compared to those having received the WT strain (data not shown). Therefore, we performed a second trial in the mouse using an intermediate dose of infection (5 x 10^6 CFU per animal) in order to avoid development of septicemia. Mice in both groups presented moderate clinical signs during the first 72 h pi, but no animals died from septicemia in either group. However, from day 7 pi, several mice in the WT group developed clinical signs associated with meningitis. *S. suis* was isolated from the brain of these animals at high titers (>1 x 10^6 CFU/0.5 g of tissue). In strong contrast, no clinical signs of meningitis were observed in the Δ*dltA* group nor was *S. suis* isolated from the brain of any animal infected with the Δ*dltA* mutant. Significant differences in the mortality rate were noted between mice inoculated with the WT and those with the Δ*dltA* mutant strain (Kaplan-Meyer, $P < 0.05$) at the intermediate dose of infection (Fig. 6B).

***S. suis* LTA D-alanylation promotes adherence to and invasion of porcine BMEC.**

Experimental infection of mice at the intermediate dose of infection clearly demonstrated that the Δ*dltA* mutant is less fit to induce CNS disease. A recent study of *S. suis* meningitis in the mouse showed that cells lining the choroid plexus and the brain endothelium are potential entry sites for this pathogen into the CNS (8). In addition, previous studies demonstrated the ability of *S. suis* to adhere to and invade immortalized porcine BMEC (35, 36). Recently, it has been shown that expression of the *dlt* operon is upregulated upon interaction of *S. suis* with porcine BMEC (11). Therefore, to assess the contribution of the LTA D-alanyl modification to adherence to and invasion of porcine BMEC, we compared the interactions of WT and Δ*dltA* mutant strains with cultured monolayers of these cells. After 2 h-incubation of *S. suis* with porcine BMEC at a MOI of 1, followed by vigorous washing, we found a marked decrease in the total number of cell-associated Δ*dltA* mutant bacteria compared with the WT parent strain (Fig. 7). Using antibiotic protection to quantify bacteria which had invaded the intracellular compartment, a similar reduction in internalization of the Δ*dltA* mutant was observed (Fig. 7). Therefore, LTA D-alanylation itself plays a role in facilitating *S. suis* adherence to and invasion of porcine BMEC and we speculate that this occurs, mainly, through cell envelope charge stabilization that allows efficient display of proteinaceous adhesins and/or invasins (23). Porcine BMEC are one of the main cellular types forming the porcine BBB, a structure that successful meningitis-causing pathogens must cross in order to develop meningitis. Interestingly, a previous report proposed that the diminished resistance to killing by leucocytes was responsible for the impairment in its ability to induce meningitis in the mouse of a *S. agalactiae* Δ*dltA* mutant (26). Taking together our results with porcine BMEC and the observed outcome of the experimental infections in both the murine and porcine models of infection, we speculate that in addition to failure of the Δ*dltA* mutant to maintain a high level of bacteremia, impaired interaction with BMEC are also responsible for the reduced ability of the Δ*dltA* mutant to induce meningitis.

In summary, *S. suis* LTA D-alanylation mediated by the *dlt* operon contributes phenotypically to resistance to CAMPs, likely through an increased net positive surface charge. It also enhances the resistance of *S. suis* to neutrophil killing as well as its capacity for adherence to and invasion of porcine BMEC. In addition, LTA D-alanylation contributes to *S. suis* virulence in both the murine and the porcine models of infection, probably through

interference with innate immune clearance mechanisms and by facilitating penetration of host barriers. The results of this study strongly suggest that LTA D-alanylation is an important virulence factor of this swine pathogen and zoonotic agent.

Acknowledgments

We are indebted to P. Willson for the generous gift of plasmid pSmall and to P. Friedl for kindly providing the porcine BMEC/C1-2 cell line. We thank D. Montpetit for performing the electron microscopy and G. Vanier for help with the porcine BMEC tests. We also thank S. Lacouture for useful suggestions and M. Takahashi and MP Lecours for assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and by the Centre de Recherche en Infectiologie Porcine (CRIP-FQRNT). NF and MCDP are recipients of NSERC postgraduate scholarships.

ANNEX 2. ARTICLE V. TABLES

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

	Relevant characteristics	Source
Bacterial strains		
E. coli Top 10	General strain for cloning. F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. suis 31533	Serotype 2 field strain. Highly virulent	(35)
S. suis Δ*dltA*	Δ*dltA* mutant strain derived from strain 31533	This work
S. suis BD102	Unencapsulated mutant strain derived from strain 31533	(12)
Plasmids		
pCR4	*E. coli* vector for cloning of PCR fragments	Invitrogen
pSmall	*E. coli-S. suis* shuttle vector. Sp^R. Source of *aad9*	Willson, P (unpublished results)
pSET5s	Temperature sensitive suicide vector for *S. suis* mutagenesis. Cm^R (*cat*)	(32)
p5ΔpgdA	pSET5s carrying the construction for allelic exchange	This work

Table 2. Oligonucleotide primers used in this study. Restriction sites are in bold

Primer name	Sequence (5' – 3')	Restriction site
2872F	GCAGTTACCTCTAAGCTTGCGACAACGG	HindIII
3765R	CTGCTAATCATTTGGATCCTCTCCTC	BamHI
5250F	CTTCCTTTGACTGCAGATGGGAAGATT	PstI
5809R	CGTCTATAAGGATCCATAGGG	BamHI
specF3	GCCAATGAGATCTATAAATAAAC	BglII
specR	AAAGTGTTTCCTGCAGTTTTTCAA	PstI

Table 3. Sensitivity of the *S. suis* WT and Δ*dltA* mutant strains to the action of selected antimicrobial peptides.

		MICs (μg/ml)	
Peptide (origin)	Net charge	31533 (WT)	Δ*dltA*
Colistin (*Bacillus colistinus)*	+5	50	25
Polymyxin B (*Bacillus polymyxa*)	+5	75	18.75
Magainin II (claw frog skin)	+4	45	5.6

ANNEX 2. ARTICLE V. FIGURES

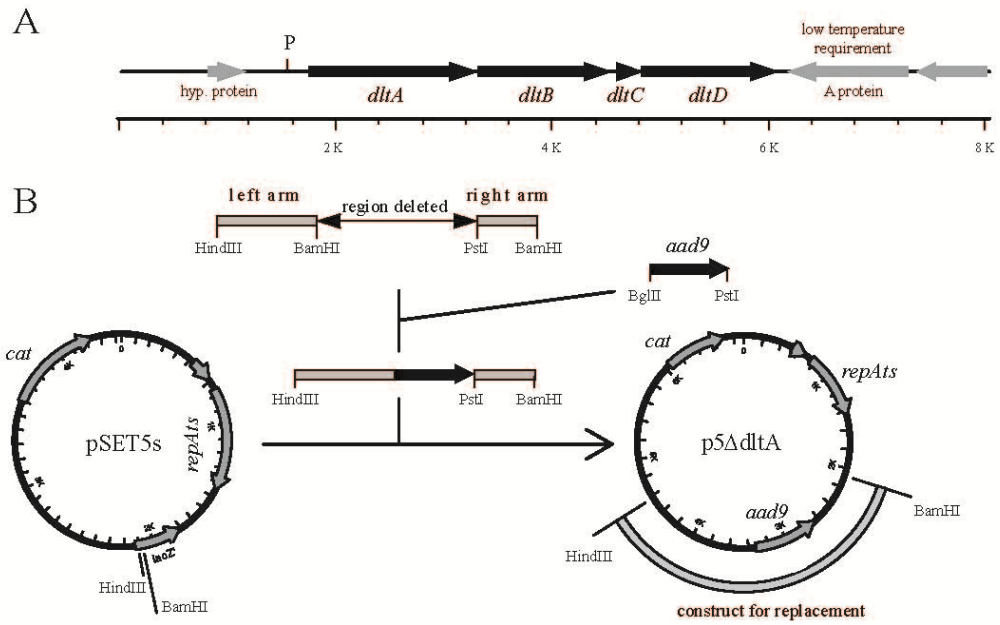


Fig. 1. Genetic organization of the *S. suis dlt* operon and strategy for the construction of the Δ*dltA* mutant.

(A) Genetic organization of the *S. suis dlt* operon as determined by sequencing of the region in strain 31533 and comparison with data from sequenced strain P1/7 available at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/). The *S. suis dlt* operon comprises 4 genes, *dltABCD*, and extends for 4340 bp. A putative strong promoter (indicated by P) was predicted upstream of the *dltA* gene. The operon comprises 4 genes, *dltA* (1563 bp), *dltB* (1242 bp), *dltC* (240 bp) and *dltD* (1266 bp). A putative strong promoter was predicted 228 bp upstream of the start codon for *dltA* using the software package Softberry BProm (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb).

(B). Strategy followed in this study to construct the knock-out vector used to generate the Δ*dltA* mutant. See Materials and Methods for details.

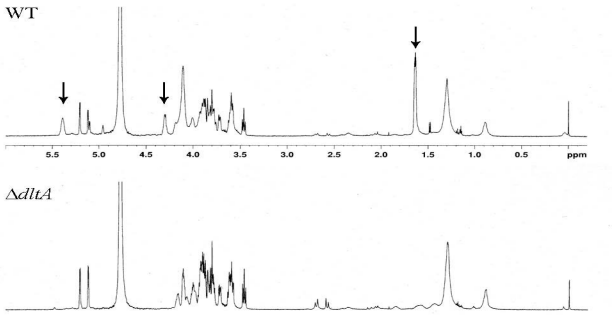


Fig. 2. Nuclear magnetic resonance spectra of WT (upper panel) and Δ*dltA* mutant (bottom panel) LTA analysis.
The arrows show the peaks for D-alanine residues in the WT strain spectrum. These peaks are missing in the Δ*dltA* mutant spectra. No other differences between LTA of the two strains were found.

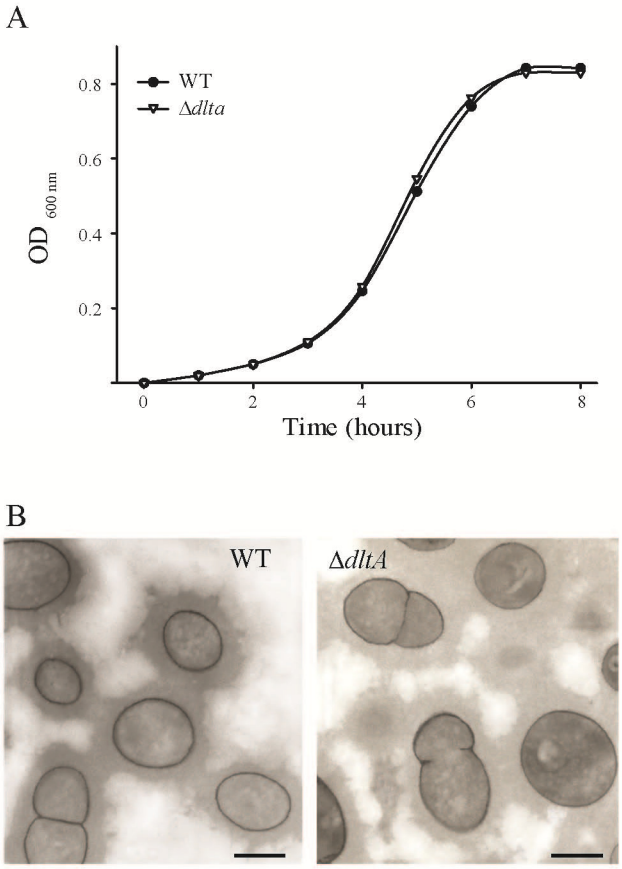


Fig. 3. Growth curves and morphology of the WT and Δ*dltA* mutant strains.
A) Growth curves of *S. suis* WT and Δ*dltA* mutant strains. The Δ*dltA* mutant grew similarly as the WT parent strain under normal laboratory conditions. **B) Morphology of the Δ*dltA* mutant (left) and the WT (right) strains.** Transmission electron microscopy showed that both strains were well separated, presented normal septation and were surrounded by a thick polysaccharide capsule. Bar = 0.5 µm.

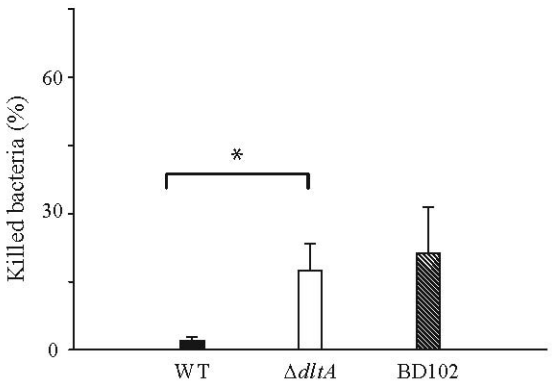


Fig. 4. Percentage of bacteria killed after 90 min incubation with porcine neutrophils.

The different strains were opsonized with complete porcine sera before incubation. The level of killing of the Δ*dltA* mutant was similar to that observed for the unencapsulated mutant BD102 and significantly higher than that of the WT strain. Data are from at least 3 independent experiments. Error bars show standard deviation. Asterisks indicate significant differences, t-test, P<0.05.

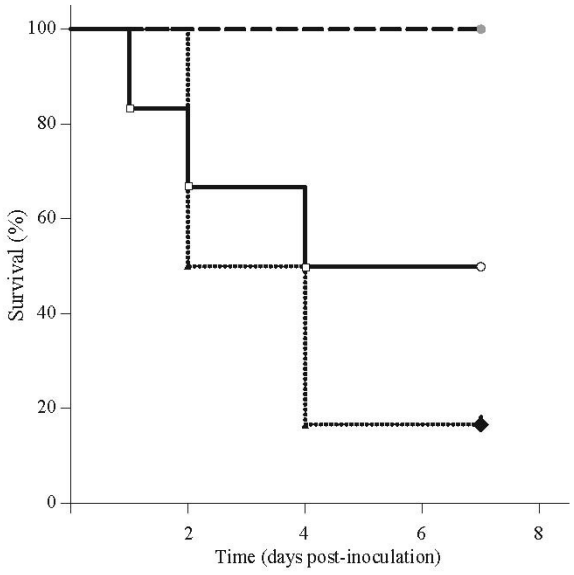


Fig. 5. Survival of pigs inoculated with the WT (dotted line) or the Δ*dltA* mutant (solid line) strains and pigs that were sham-inoculated (dashed line).

All the sham-inoculated animals survived the trial. The survival rate of pigs in the Δ*dltA* mutant group was 50%, while in the WT group most animals died from septicemia in the first days of the trial (survival rate of 17%). The sentinel animals were not considered in this analysis. See text for details. deviation. Asterisks indicate significant differences, t-test, P<0.05.

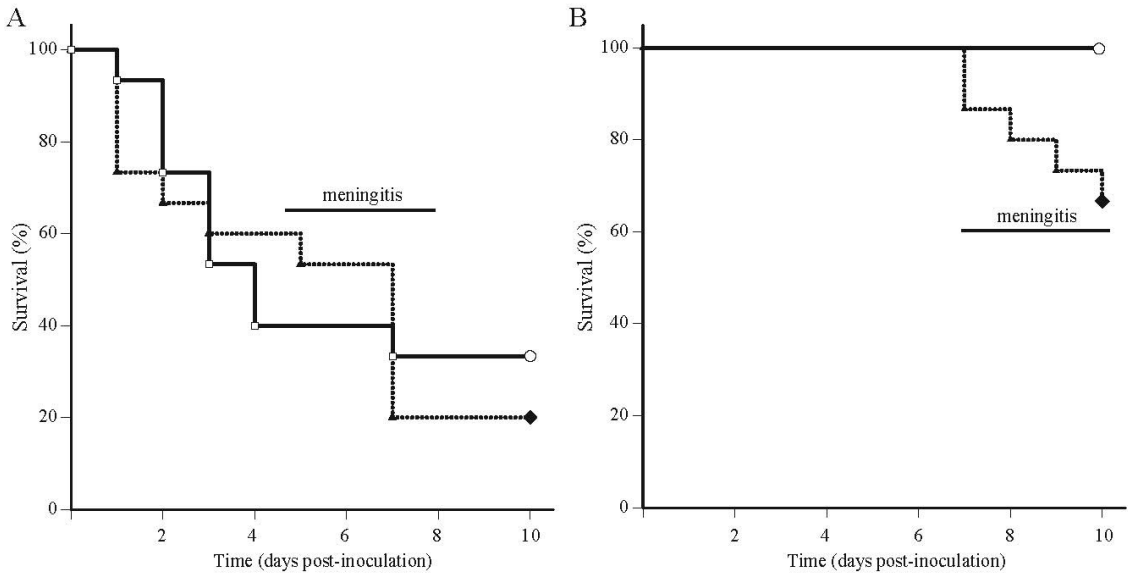


Fig. 6. Survival of mice inoculated with the WT (dotted line) or the Δ*dltA* mutant (solid line) strains.

A)No significant differences in survival were observed between groups at the high dose of inoculation. However, fewer animals in the mutant group died from meningitis. (B) At the intermediate dose of inoculation all mice in the Δ*dltA* mutant group survived, while 35% of the WT group died from meningitis. Significant differences in survival, Kaplan-Meyer, P<0.05.

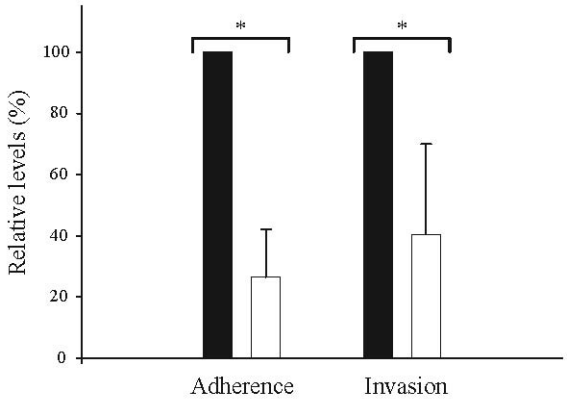


Fig. 7. Interactions of the Δ*dltA* mutant and the WT strains with porcine BMEC.

The Δ*dltA* mutant showed reduced levels of adherence to and invasion of porcine BMEC. Data for the WT strain has been normalized to 100%. Data are from at least 4 independent experiments. Error bars show standard deviation. Asterisks indicate significant differences, t-test, P<0.05.

References

1. Abachin, E., C. Poyart, E. Pellegrini, E. Milohanic, F. Fiedler, P. Berche, and P. Trieu-Cuot. 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol Microbiol 43:1-14.

2. Baums, C. G., U. Kaim, M. Fulde, G. Ramachandran, R. Goethe, and P. Valentin-Weigand. 2006. Identification of a Novel Virulence Determinant with Serum Opacification Activity in *Streptococcus suis*. Infect. Immun. 74:6154-6162.

3. Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil Extracellular Traps Kill Bacteria. Science 303:1532-1535.

4. Chabot-Roy, G., P. Willson, M. Segura, S. Lacouture, and M. Gottschalk. 2006. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. Microb Pathog 41:21-32.

5. Chan, K. G., M. Mayer, E. M. Davis, S. A. Halperin, T. J. Lin, and S. F. Lee. 2007. Role of D-alanylation of *Streptococcus gordonii* lipoteichoic acid in innate and adaptive immunity. Infect Immun 75:3033-3042.

6. Charland, N., J. Harel, M. Kobisch, S. Lacasse, and M. Gottschalk. 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology 144 :325-332.

7. de Greeff, A., H. Buys, R. Verhaar, J. Dijkstra, L. van Alphen, and H. E. Smith. 2002. Contribution of fibronectin-binding protein to pathogenesis of *Streptococcus suis* serotype 2. Infect Immun 70:1319-1325.

8. Dominguez-Punaro, M. C., M. Segura, M.-M. Plante, S. Lacouture, S. Rivest, and M. Gottschalk. 2007. *Streptococcus suis* Serotype 2, an Important Swine and Human Pathogen, Induces Strong Systemic and Cerebral Inflammatory Responses in a Mouse Model of Infection. J Immunol 179:1842-1854.

9. Elliott, S. D., M. McCarty, and R. C. Lancefield. 1977. Teichoic acids of group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). J Exp Med 145:490-499.

10. Fabretti, F., C. Theilacker, L. Baldassarri, Z. Kaczynski, A. Kropec, O. Holst, and J. Huebner. 2006. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect Immun 74:4164-4171.

11. Fittipaldi, N., M. Gottschalk, G. Vanier, F. Daigle, and J. Harel. 2007. Use of selective capture of transcribed sequences to identify genes preferentially expressed by

Streptococcus suis upon interaction with porcine brain microvascular endothelial cells. Appl Environ Microbiol 73:4359-4364.

12. Fittipaldi, N., J. Harel, B. D'Amours, S. Lacouture, M. Kobisch, and M. Gottschalk. 2007. Potential use of an unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant as a live attenuated vaccine in swine. Vaccine 25:3524-3535.

13. Gottschalk, M., M. Segura, and J. Xu. 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim Health Res Rev **8**:29-45.

14. Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung, and F. Gotz. 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog 3:e102.

15. Higgins, R., and M. Gottschalk. 2005. Streptococcocal Diseases, p. 769-783. *In* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames, Iowa.

16. Higgins, R., and M. Gottschalk. 1990. An update on *Streptococcus suis* identification. J Vet Diagn Invest 2:249-252.

17. Kovacs, M., A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck, and R. Bruckner. 2006. A functional *dlt* operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. J Bacteriol 188:5797-5805.

18. Kristian, S. A., V. Datta, C. Weidenmaier, R. Kansal, I. Fedtke, A. Peschel, R. L. Gallo, and V. Nizet. 2005. D-alanylation of teichoic acids promotes group A streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. J Bacteriol 187:6719-6725.

19. Lapointe, L., S. D'Allaire, A. Lebrun, S. Lacouture, and M. Gottschalk. 2002. Antibody response to an autogenous vaccine and serologic profile for *Streptococcus suis* capsular type 1/2. Can J Vet Res 66:8-14.

20. Mai, N. T., N. T. Hoa, T. V. Nga, L. D. Linh, T. T. Chau, D. X. Sinh, N. H. Phu, L. V. Chuong, T. S. Diep, J. Campbell, H. D. Nghia, T. N. Minh, N. V. Chau, M. D. de Jong, N. T. Chinh, T. T. Hien, J. Farrar, and C. Schultsz. 2008. *Streptococcus suis* Meningitis in Adults in Vietnam. Clin Infect Dis 46:659–667.

21. Mollinedo, F., J. Calafat, H. Janssen, B. Martin-Martin, J. Canchado, S. M. Nabokina, and C. Gajate. 2006. Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. J Immunol 177:2831-2841.

22. Morath, S., A. Geyer, and T. Hartung. 2001. Structure-Function Relationship of Cytokine Induction by Lipoteichoic Acid from *Staphylococcus aureus*. J. Exp. Med. 193:393-398.

23. Neuhaus, F. C., and J. Baddiley. 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67:686-723.

24. Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8:151-156.

25. Poyart, C., M. C. Lamy, C. Boumaila, F. Fiedler, and P. Trieu-Cuot. 2001. Regulation of D-alanyl-lipoteichoic acid biosynthesis in *Streptococcus agalactiae* involves a novel two-component regulatory system. J Bacteriol 183:6324-6334.

26. Poyart, C., E. Pellegrini, M. Marceau, M. Baptista, F. Jaubert, M. C. Lamy, and P. Trieu-Cuot. 2003. Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol Microbiol 49:1615-1625.

27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

28. Sekizaki, T., Y. Otani, M. Osaki, D. Takamatsu, and Y. Shimoji. 2001. Evidence for horizontal transfer of SsuDAT1I restriction-modification genes to the *Streptococcus suis* genome. J Bacteriol 183:500-511.

29. Smith, H. E., M. Damman, J. van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits. 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect Immun 67:1750-1756.

30. Taheri-Araghi, S., and B. Y. Ha. 2007. Physical basis for membrane-charge selectivity of cationic antimicrobial peptides. Physical review letters 98:168101.

31. Takamatsu, D., M. Osaki, and T. Sekizaki. 2001. Construction and characterization of *Streptococcus suis-Escherichia coli* shuttle cloning vectors. Plasmid 45:101-113.

32. Takamatsu, D., M. Osaki, and T. Sekizaki. 2001. Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. Plasmid 46:140-148.

33. Teifel, M., and P. Friedl. 1996. Establishment of the Permanent Microvascular Endothelial Cell Line PBMEC/C1-2 from Porcine Brains. 228:50.

34. Tenenbaum, T., F. Essmann, R. Adam, A. Seibt, R. U. Janicke, G. E. Novotny, H. J. Galla, and H. Schroten. 2006. Cell death, caspase activation, and HMGB1 release of porcine choroid plexus epithelial cells during *Streptococcus suis* infection in vitro. Brain research 1100:1-12.

35. Vanier, G., M. Segura, P. Friedl, S. Lacouture, and M. Gottschalk. 2004. Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. Infect Immun 72:1441-1449.

36. Vanier, G., M. Segura, and M. Gottschalk. 2007. Characterization of the invasion of porcine endothelial cells by *Streptococcus suis* serotype 2. Can J Vet Res 71:81-89.

37. Wartha, F., K. Beiter, B. Albiger, J. Fernebro, A. Zychlinsky, S. Normark, and B. Henriques-Normark. 2007. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. Cell Microbiol 9:1162-1171.

38. Weidenmaier, C., A. Peschel, V. A. Kempf, N. Lucindo, M. R. Yeaman, and A. S. Bayer. 2005. DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. Infect Immun 73:8033-8038.

39. Yu, H., H. Jing, Z. Chen, H. Zheng, X. Zhu, H. Wang, S. Wang, L. Liu, R. Zu, L. Luo, N. Xiang, H. Liu, X. Liu, Y. Shu, S. S. Lee, S. K. Chuang, Y. Wang, J. Xu, and W. Yang. 2006. Human *Streptococcus suis* outbreak, Sichuan, China. Emerging infectious diseases 12:914-920.

ANNEX 3. ARTICLE VI

« Significant contribution of the *pgdA* gene to the virulence of *Streptococcus suis*»

Nahuel Fittipaldi[1], Tsutomu Sekizaki[2,3]†, Daisuke Takamatsu[2], María de la Cruz Domínguez-Punaro[1], Josée Harel[1], Nhat Khai Bui[4], Waldemar Vollmer[4] and Marcelo Gottschalk[1]*

[1]Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada. [2]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan, [3]United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan, [4]Institute for Cell and Molecular Biosciences, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom.

Published in: *Mol. Microbiol*. 2008 Dec;70(5):1120-35

Details on the role of the candidate in the conception of the article: I actively participated in the experimental design concerning animal studies and cytokine production; I substantially performed research, analyzed data and participated in writing the paper.

Summary

Streptococcus suis is a major swine pathogen and emerging zoonotic agent. In this study we have determined the muropeptide composition of *S. suis* peptidoglycan (PG) and found, among other modifications, N-deacetylated compounds. Comparison with an isogenic mutant showed that the product of the *pgdA* gene is responsible for this specific modification which occurred in very low amounts. Low level of PG N-deacetylation correlated with absence of significant lysozyme resistance when wild type *S. suis* was grown *in vitro*. On the other hand, expression of the *pgdA* gene was increased upon interaction of the bacterium with neutrophils *in vitro* as well as *in vivo* in experimentally inoculated mice, suggesting that *S. suis* may enhance PG N-deacetylation under these conditions. Evaluation of the Δ*pgdA* mutant in both the CD1 murine and the porcine models of infection revealed a significant contribution of the *pgdA* gene to the virulence traits of *S. suis*. Reflecting a severe impairment in its ability to persist in blood and decreased ability to escape immune clearance mechanisms mediated by neutrophils, the Δ*pgdA* mutant was severely attenuated in both models. The results of this study suggest that modification of PG by N-deacetylation is an important factor in *S. suis* virulence.

Introduction

Streptococcus suis is a major swine pathogen responsible for severe economic losses to the porcine industry (Higgins & Gottschalk, 2005). It is also the causative agent of serious infections in humans, especially in people in close contact with swine or pork by-products (Gottschalk *et al.*, 2007). In both swine and humans the main clinical manifestations of *S. suis* are meningitis and septicaemia. While *S. suis* outbreaks in swine are common and sometimes devastating, most reports in humans describe sporadic cases resulting in either death or severe post-infection sequelae (Gottschalk *et al.*, 2007). In recent times, however, *S. suis* has strongly emerged as an important public health issue in some Asian countries. For instance, it has very recently been shown that it is the primary cause of adult meningitis in Vietnam (Mai *et al.*, 2008). Moreover, during an episode in China, more than 200 human *S. suis* cases, 39 of which resulting in death, were reported in a single outbreak (Yu *et al.*, 2006). Most cases of disease in both swine and humans are caused by *S. suis* serotype 2 and, therefore, almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype (Higgins & Gottschalk, 2005). However, the current understanding of the *S. suis* pathogenesis of infection remains limited. It has been shown that the polysaccharide capsule is essential for the virulence of the bacterium (Chabot-Roy *et al.*, 2006, Charland *et al.*, 1998, Smith *et al.*, 1999). Recently, an isogenic mutant for a serum opacity-like factor was found to be attenuated in pigs (Baums *et al.*, 2006). Several other factors, such as a heamolysin (suilysin), the so-called extracellular protein factor and muramidase-released protein, as well as a fibronectin/fibrinogen-binding protein, have been shown to be linked to, but not essential for, the virulence of *S. suis* (de Greeff *et al.*, 2002, Higgins & Gottschalk, 2005).

Cell wall components of *S. suis* have also been proposed to be important virulence factors and several studies have shown that they contribute to exacerbate the host inflammatory response to infection (Gottschalk et al., 2007, Higgins & Gottschalk, 2005). The main cell wall component of Gram-positive bacteria is peptidoglycan (PG), which ensures the stability and rigidity of the cell wall. PG consists of glycan strands made of alternating *β*-1,4-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) which are cross-linked by short peptide chains (Vollmer *et al.*, 2008a). This rather invariant structure is exploited by the host for both recognition of bacteria, through the nucleotide-binding oligomerisation domain (Nod)1 and Nod2 proteins, which recognise muropeptides released during cell wall turnover, as

well as for destruction of the microorganisms through the hydrolytic activity of lysozyme (Boneca, 2005, Chaput & Boneca, 2007). However, some Gram positive bacteria have developed efficient mechanisms to circumvent these host defences. For instance, *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pneumoniae* modify their PG by O-acetylation of the C-6 atom of NAM through the action of the OatA protein (Crisostomo *et al.*, 2006, Hebert *et al.*, 2007, Herbert *et al.*, 2007). Some pathogens, such as *Streptococcus pneumoniae and Listeria monocytogenes,* deacetylate the NAG (mostly) and NAM (to a lesser extent) residues into glucosamine or muramic acid through the action of the *pgdA* gene product (Boneca *et al.*, 2007, Vollmer & Tomasz, 2000). These modifications have been shown to result in escaping Nod1 and Nod2 surveillance and/or increased resistance to lysozyme.

The structure of *S. suis* PG has not yet been reported. However, a recent study identified a *S. suis* homolog of the *S. pneumoniae pgdA* gene which was highly upregulated during the interactions of *S. suis* with porcine brain microvascular endothelial cells, suggesting that this bacterium might be able to modify its PG by N-deacetylation (Fittipaldi *et al.*, 2007a). In this study, we determined the *S. suis* PG composition and compared it to that of a Δ*pgdA* isogenic mutant. We demonstrate that *S. suis* N-deacetylates its PG through the action of the *pgdA* gene product and show that the deletion of this gene has a significant impact on the virulence traits of this pathogen.

Results

Fine structure of *S. suis* PG.

The fine structure of *S. suis* PG has never been reported. To determine it, we used the classical method, i.e. isolation and purification of the SDS-insoluble cell wall, removal of teichoic acids by hydrofluoric acid, release of muropeptides by the muramidase cellosyl, separation of muropeptides by high-performance liquid chromatography (HPLC), and matrix assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometry (MS) of the collected muropeptide fractions. Based on previous reports on the amino acids present in the PG of streptococci (Schleifer & Kandler, 1972) the chromatographic behaviour of the muropeptides and their molecular masses, the structures of 13 muropeptides isolated from *S. suis* PG could be proposed with high confidence. The PG of *S. suis* had mainly D-isoglutamine in

position 2 and L-lysine in position 3 of the peptide. The cross-links did not contain an interpeptide bridge frequently found in streptococci. Thus, the muropeptide profile of *S. suis* appeared relatively simple (Fig. 1A), with three major compounds accounting for >75% of the total material, the monomer Tri (the disaccharide tripeptide, compound 1) (Fig. 1B), the dimer TetraTri (bis-disaccharide tetratripeptide, compound 7), and the trimer TetraTetraTri (tris-disaccharide tetratetratripeptide, compound 15). Minor compounds identified had either non-amidated D-glutamate at position 2 (compounds 2 and 8) or a free tetrapeptide or pentapeptide instead of the prevailing tripeptide at the acceptor site of the peptide (compounds 5, 6, 13, 14). Other minor muropeptides had lost a GlcNAc residue (compounds 3, 10, 17), indicating the previous activitiy of a PG glucosaminidase, or a GlcNAcMurNAc moiety (compound 12) due to the activity of a PG amidase. These major and minor muropeptides accounted together for >93% of the total UV-absorbing material (Supplemental Table 1).

Strikingly, the classical method for PG analysis failed to detect any deacetylated muropeptides in the PG of *S. suis* wild-type (WT) field strain 31533, despite the presence of an intact *pgdA* N-deacetylase gene in its genome (see below). Thus, if present at all, deacetylated muropeptides were expected in very small quantities. Therefore, we analysed the muropeptide mixture on a linear ion trap-Fourier transform (LTQ-FT) mass spectrometer which has superior sensitivity and mass accuracy. LTQ-FT-MS detected all 13 major and minor muropeptides from the previous HPLC-MALDI-TOF analysis (Table 1). In addition, 7 new muropeptides were identified including the trimer TetraTetraTetra (compound 19), the tetramer TetraTetraTetraTri (compound 20) and two additional muropeptides with losses of two GlcNAc residues (compounds 11 and 18). Interestingly, LTQ-FT-MS identified the deacetylated versions of all three major muropeptides (compounds 4, 9, 16, Fig. 1B and Table 1). Deacetylated muropeptides were identified in very low quantities, probably lower than 1% of the total muropeptides, preventing determination of the site of deacetylation (NAG or NAM). However, the observed mass intensities of these deacetylated muropeptides were well above the signal noise (Table 1).

The *pgdA* gene product is responsible for N-deacetylation of *S. suis* PG.

N-deacetylation of PG has been shown to occur through the action of the *pgdA* gene product in some Gram-positive species (Boneca et al., 2007, Meyrand *et al.*, 2007, Vollmer &

Tomasz, 2000). A *S. suis pgdA* homolog has recently been identified (Fittipaldi et al., 2007a). Genomic searches at the 4 currently available *S. suis* sequenced genomes available at the NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/), and the DOE Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes/strsu/strsu.home.html) databases, as well as Southern hybridizations using specific probes revealed that *pgdA* is the only putative PG N-deacetylase encoded by *S. suis* (data not shown). The *S. suis pgdA* gene comprises 1398 bp and seems to be transcribed from a putative promoter located 30 bp upstream of the beginning of the coding sequence (identified using the Bprom software at http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (Fig. 2A). A region of dyad symmetry that might function as a Rho-independent transcription terminator was detected 11 bp downstream the stop codon using the Findterm program (http://www.softberry.ru/berry.phtml?group=programs&subgroup=gfindb&topic=findterm). The predicted translated sequence of the *S. suis pgdA* gene showed strong homology to previously reported PgdA proteins, including the presence of all the conserved catalytic residues and metal ligand amino acids previously reported for *S. pneumoniae* PgdA (Blair *et al.*, 2005) (Fig. 2B). In order to investigate the involvement of *S. suis pgdA* in PG N-deacetylation we inactivated the gene via a double crossover event, generating a mutant strain in which the *pgdA* gene was replaced by a spectinomycin (Sp) resistance cassette (*aad9* gene). Inactivation of *pgdA* had no major consequences on growth either in normal laboratory medium (Fig. 3A) or in the presence of 10 % porcine or foetal bovine sera (data not shown). In addition, the Δ*pgdA* mutant strain presented a normal morphology and was as encapsulated as the WT strain (Fig 3B). However, when we prepared PG from the Δ*pgdA* mutant and analysed it as described above for the parent strain, no N-deacetylated muropeptides were found (Table 1), showing that N-deacetylation of *S. suis* PG is achieved through the action of the *pgdA* gene product.

S. suis is susceptible to lysozyme _in vitro_ and inactivation of the _pgdA_ gene does not result in an increased susceptibility.

In some Gram positive species N-deacetylation of NAG and/or NAM residues has been shown to confer resistance to lysozyme (Boneca *et al*., 2007, Meyrand *et al*., 2007, Vollmer & Tomasz, 2000). Since *S. suis* modifies its PG by N-deacetylation, we sought to evaluate whether this modification results in resistance against this hydrolytic enzyme. We therefore performed

a comparison of the sensitivity to lysozyme of both the *S. suis* WT and Δ*pgdA* mutant strains. Growth of WT *S. suis* was found to be inhibited by lysozyme in a dose-dependent manner. This behaviour was observed both when lysozyme was added at the beginning of the stationary phase of growth (Fig. 4) or at the mid-log phase (data not shown). Growth of *S. suis* was inhibited at concentrations (5 µg/ml) which were much lower than those not affecting growth of several other Gram-positive species such as *S. pneumoniae* (80 µg/ml) and *L. monocytogenes* (10 µg/ml), probably reflecting the low percentage of N-deacetylated muropeptides in its PG. Although recorded values of $OD_{600\ nm}$ were slightly lower than those of the WT strain, the Δ*pgdA* mutant behaved essentially as the WT strain at all the concentrations tested. Since the $OD_{600\ nm}$ assay does not prove lysozyme-mediated killing, we performed plating and colony counts for every condition and time tested. Results showed a strong correlation between the reported decrease in $OD_{600\ nm}$ and loss of viability of *S. suis* (data not shown).

The virulence of the Δ*pgdA* mutant is severely impaired in the murine model of infection.

Components of the *S. suis* cell wall have been proposed to play an important role in the pathogenesis of infection of this pathogen (Higgins & Gottschalk, 2005). In order to evaluate if PG N-deacetylation contributes to the virulence of *S. suis* we performed an experimental infection of CD1 mice using a recently described model of infection (Dominguez-Punaro *et al.*, 2007). Results of the trial showed that most animals in the WT group presented severe clinical signs associated with septicaemia and septic shock, such as depression, swollen eyes, weakness and prostration during the first 72 h post-inoculation (pi). Several mice died from septicaemia in this group. From day 5 pi, some animals in the WT group developed clinical signs associated with *S. suis* meningitis in the mouse (Dominguez-Punaro *et al*., 2007), such as hyperexcitation, episthotonus, opisthotonus, bending of the head and walking in circles. In strong contrast, mice in the Δ*pgdA* group did not present any major clinical sign associated with *S. suis* infection during the trial, with the exception of slight depression following inoculation which subsided 48 h pi (24 h pi in some cases). No mouse died in this group and significant differences in the mortality rate were noted between the WT and Δ*pgdA* groups (LogRank test, $P < 0.05$) (Fig. 5A). *S. suis* was isolated at high titres for several days (> 1 x 10^7 CFU/ml in some cases) from blood samples (Fig. 5B) and, at euthanasia, from organs such as the liver and spleen of septicaemic animals in the WT group (data not shown). In addition, isolation from the brain and cerebrospinal fluid of animals showing clinical signs of meningitis demonstrated the presence

of *S. suis* at high titres in both samples (data not shown). In strong contrast, titres in blood following inoculation were lower on average in the Δ*pgdA* mutant group and decreased rapidly. After day 3 pi *S. suis* could was not isolated from this tissue, even if an enrichment of the sample was performed, suggesting that the bacterium had been cleared from circulation (Fig. 5B). In addition, at euthanasia, the Δ*pgdA* mutant could not be isolated from the liver, spleen or brain of any animal in the mutant group (data not shown). No macroscopic lesions associated with *S. suis* were observed in this group at post-mortem examination.

Induction of key cytokines involved in septic shock is abolished in mice inoculated with the Δ*pgdA* mutant.

Differences in PG composition between the WT and Δ*pgdA* mutant strains might result in a dissimilar ability to induce production of several cytokines, as demonstrated for *L. monocytogenes* (Boneca *et al*., 2007). On the other hand, it has been suggested that an exacerbated inflammatory response leading to septic shock is responsible for death at early stages of the infection of most mice infected with *S. suis* (Dominguez-Punaro *et al*., 2007). Since we did not observe clinical signs associated with septic shock in mice inoculated with the Δ*pgdA* mutant, we studied kinetically the ability of this strain to induce cytokine production in the mouse. Analysis of the response showed that production of both interferon-γ (IFN-γ) and interleukin (IL)-6 was severely impaired in animals which received the Δ*pgdA* mutant in comparison to those inoculated with the WT strain. In fact, mice that received the WT strain presented an important peak of both IFN-γ and IL-6 at 6 h pi followed by a gradual return to basal levels at 48 h pi. In contrast, animals in the mutant group did not show production of these cytokines above basal levels (Fig. 6A and 6B). Animals in the WT group also showed augmented production of IL-1β up to 48 h pi, whereas the Δ*pgdA* mutant induced very low amounts of this cytokine (Fig. 6C). On the other hand, production of tumour necrosis factor-α (TNF-α) was essentially similar in mice inoculated either with the WT or Δ*pgdA* mutant strains (data not shown). We also studied the chemokine response. Induction of both KC RANTES chemokines was abolished in the Δ*pgdA* mutant group while in the the WT group animals reached peak production between 6 and 12 h pi to return to baseline at 48 pi (Fig. 6D and 6E). Levels of monocyte chemotactic protein-1 (MCP-1) were similar in both groups during the 12 first hours pi, but, thereafter, this chemokine abruptly dropped in the mutant group while it persisted in the WT group up to 36 h pi (Fig. 6F). Overall, with the exception of TNF-α, the

Δ*pgdA* mutant failed to stimulate the production of pro-inflammatory cytokines and chemokines at all the time points tested. On the other hand, the observed production of these inflammatory mediators in the WT group was in perfect agreement with a previous report (Dominguez-Punaro *et al*., 2007).

The Δ*pgdA* mutant is killed by porcine neutrophils.

It has been shown that in the absence of a specific humoral response, well encapsulated *S. suis* can avoid killing by phagocytes and persist in blood (Chabot-Roy *et al*., 2006, Charland *et al*., 1998, Smith *et al*., 1999). However, our *in vivo* results suggested that the impaired virulence of the Δ*pgdA* mutant may result from its reduced ability to survive in this tissue. Based on previous results in *L. monocytogenes* that showed diminished ability of a Δ*pgdA* mutant to resist killing by phagocytic cells (Boneca et al., 2007), and taking into account that *S. suis* rapidly disappeared from blood, we postulated that clearance of the organism was primarily driven by neutrophils. Indeed, neutrophils are significantly increased in pigs infected with *S. suis* virulent strains and are usually predominant in lesions caused by this pathogen (Salles *et al.*, 2002, Sanford, 1987). When we compared *in vitro* killing of the WT and Δ*pgdA* mutant strains by porcine neutrophils, under our conditions, in the absence of opsonising antibodies but in the presence of normal complete porcine sera, the WT strain resisted the bactericidal effect of porcine neutrophils. On the other hand, suggesting a potential contribution of PG N-deacetylation to this resistance, the Δ*pgdA* mutant strain was efficiently eliminated at rates similar to those observed for an unencapsulated mutant. At the end of the assay, more than 20% of the Δ*pgdA* mutant bacteria were killed by neutrophils compared to less than 5% of the WT strain (Fig. 7).

The Δ*pgdA* mutant is attenuated in the porcine model of infection.

To confirm the observed impaired virulence of the Δ*pgdA* mutant, we conducted a trial in the pig, which is the natural host of *S. suis*. To this end, we used a well-standardised infection model that utilises the aggressive intravenous route of inoculation but provides repeatability (Berthelot-Herault *et al.*, 2001). Animals in the sham-inoculated group did not present any clinical signs during the entire extent of the trial. On the other hand, severe clinical signs such as depression, prostration, lameness and shaking were observed in all 10 animals inoculated

with the WT strain. Four pigs died or were sacrificed for ethical reasons within 24 h pi and 4 others at day 2 pi (Fig. 8). One of these pigs developed clinical signs of meningitis, including convulsions, episthotonus, opisthotonus and bending of the head. At euthanasia, macroscopic lesions typical of *S. suis* infection were found in most animals infected with the WT strain. Fibrin deposits were observed at the pleura and the pericardium of most animals in this group. In addition, all pigs inoculated with the WT group presented at least one swollen articulation with fibrin deposits and excess of synovial liquid. Additionally, some animals in the WT showed damage at the meninges consistent with meningitis. In comparison, animals inoculated with the Δ*pgdA* mutant strain showed less severe clinical signs during the trial. No animal presented shaking or convulsions in this group and no cases of meningitis were recorded. However, depression, prostration and lameness were common during the first 24 h pi. Mortality was also observed in this group, as 4 pigs were sacrificed for strict ethical reasons within the first 3 days pi (Fig. 8). Differences between groups regarding mortality were nonetheless significant (LogRank test, $p<0.05$). The remaining animals in the mutant group recovered starting from day 3 pi (day 2 pi in some cases) and survived until the end of the trial. Examination at necropsy did not reveal major damages to tissues or organs in this group, with the exception of swollen articulations in some animals. Hyperthermia was observed in all pigs infected with either the WT or Δ*pgdA* strains at 24 h pi and no major differences were observed between groups. Temperatures returned to normal values in surviving animals after day 3 in the Δ*pgdA* group and the 2 surviving inoculated animals in the WT group. *S. suis* could be isolated from the blood of all surviving pigs in both groups 48 h pi. However, pigs infected with the WT strain showed higher bacterial counts (1×10^9 CFU/ml in some cases) than those infected with the Δ*pgdA* mutant (average of 5×10^6 CFU/ml). Bacterial isolation from different organs revealed a high bacterial colonization of the liver, the spleen and (in some cases) the brain of pigs in the WT group. In contrast, isolation from the brain was negative in the Δ*pgdA* group, although the bacterium was found in the liver and spleen of most animals in this group. Similar to the blood, bacterial load in organs was lower in pigs inoculated with the Δ*pgdA* mutant compared with animals inoculated with the WT strain.

The *pgdA* gene is upregulated upon interaction with porcine neutrophils and within the host.

In vitro results obtained in this study showing a high sensitivity of the WT strain to lysozyme seem incompatible with the observed outcomes of the experimental infections of

both mice and swine as well as with the reported ability of *S. suis* to initiate and establish infections in other hosts (Gottschalk *et al*., 2007). However, absence of lysozyme resistance *in vitro* might be explained by a rather inactive *pgdA* gene and a subsequent low extent of PG deacetylation when *S. suis* grows in Todd-Hewitt broth (THB). We therefore hypothesised that *S. suis* might express the *pgdA* weakly in THB and upregulate its expression upon interaction with the host or host cells in order to resist the deleterious effects of lysozyme. To assess this hypothesis, we compared the relative expression levels of the *S. suis pgdA* gene under standard laboratory conditions to that observed upon interaction (as described above) with porcine neutrophils. After reverse transcription, the quantitative PCR (Q-PCR) assays showed that while the expression of the *pgdA* gene by *S. suis* remained essentially unchanged in the mock-infection sample (without neutrophils), it was highly upregulated (mean fold change of 6.7) after 90 min incubation with neutrophils (Fig. 9). We also performed the same analysis from *in vivo* samples (liver and spleen) collected after experimental infection of CD1 mice. Data obtained showed that *S. suis* consistently upregulated the expression of the *pgdA* gene in all the mice tested, with fold changes ranging from 3 to 13, depending on the animal and organ tested (Fig. 9).

Discussion

Even though it has been proposed as an important factor for *S. suis* virulence (Higgins & Gottschalk, 2005), knowledge on the cell wall composition of this pathogen is very limited. A partial structure of *S. suis* (lipo)teichoic acids (LTA) has been reported (Elliott *et al.*, 1977), and very recently it has been shown that *S. suis* modifies LTA by D-alanylation (Fittipaldi *et al.*, 2008). However, PG composition of this bacterium has largely remained elusive. In this study we provide for the first time evidence regarding the composition of this essential polymer in *S. suis,* which was found to be, in several aspects, typical of species within the low GC-content Gram-positive phylum *Lactobacillales*. Like in other streptococci, in *S. suis* mostly D-Gln is found at position 2 of the stem peptide, and an L-Lys residue is present at position 3, which can participate in 4-3 cross-links with neighbouring peptides (Schleifer and Kandler, 1972). However, unlike other streptococci, the PG of *S. suis* lacks an interpeptide bridge but contains direct L-Lys-D-Ala cross-links. Consistently, database searches revealed the absence in *S. suis* of homologues of genes encoding for known peptide branching enzymes of the Fem transferase or ATP-grasp families (Bellais *et al.*, 2006, Filipe & Tomasz, 2000) (data not shown). About 67%

of the peptides are present in dimeric or trimeric cross-links (Supplemental Table 1). *S. suis* PG has a low proportion of pentapeptides and tetrapeptides indicating high activities of DD- and LD-carboxypeptidases in this species. Some of the minor muropeptides correspond to modifications of canonical structures, suggesting the presence of glucosaminidase, amidase and N-deacetylase activities. While the enzymes responsible for the two former activities remain to be identified, N-deacetylation could be linked to the *S. suis pgdA* gene product. To fully ascribe this link, we tried to restore the WT phenotype by complementation of the Δ*pgdA* mutant strain with several different constructions and *E. coli-S. suis* shuttle vectors but, perhaps because it has deleterious effects in *E. coli,* we failed to obtain recombinant plasmids carrying the *S. suis pgdA* gene (data not shown). However, repeated generation of Δ*pgdA* mutants (3 additional independent mutants, Δ*pgdA* 2, 3 and 4, Supplemental Table 3) and consistent verification of a key phenotype such as neutrophil killing for each of them (Supplemental Figure 1) suggest that the occurrence of genetic events elsewhere in the genome other than inactivation of *pgdA* during the procedures for mutant isolation are unlikely. In addition, the genetic organization of the region (Fig. 2A) suggests that polar effects are highly improbable.

S. suis is an important swine pathogen and a zoonotic agent causing septicaemia and meningitis in both pigs and humans (Gottschalk et al., 2007). In order to establish an infection *S. suis* must first overcome the innate immune response of the host. One important mediator of this response is lysozyme. However, some bacterial species resist lysozyme activity by means of PG N-deacetylation and it has been proposed that the level of this modification may correlate with the level of resistance (Hebert et al., 2007, Vollmer *et al.*, 2008b). Consistently with this suggestion, the *S. suis* WT strain, which N-deacetylates PG during growth in normal laboratory culture medium to a very little extent, was sensitive to lysozyme at concentrations much lower than those allowing growth of *S. pneumoniae* and *L. monocytogenes* (Boneca et al., 2007, Vollmer & Tomasz, 2000). Furthermore, we did not observe any major differences between *S. suis* WT and Δ*pgdA* mutant strains regarding resistance to the action of this enzyme *in vitro*. In fact, these results may be explained by an already low degree (probably < 1 % of total muropeptide) of PG N-deacetylation of the WT strain. By comparison, resistance to lysozyme in *S. pneumoniae* correlated with over 80% of N-deacetylated glucosamine residues (Vollmer & Tomasz, 2000). In *L. monocytogenes* the extent of N-deacetylation was lower, but still very

important, with up to 50 % of N-deacetylated muropeptides (Boneca *et al*., 2007). In this regard, the demonstration that *S. suis* upregulates the expression of the *pgdA* gene upon interaction with neutrophils as well as during growth *in vivo* constitutes an interesting and unprecedented finding of this work. Since it is apparent that the *S. suis pgdA* gene product is responsible for PG N-deacetylation, it is tempting to speculate that this increased expression of the *pgdA* gene correlates with a higher degree of PG N-deacetylation under these conditions that, in turn, results in enhanced resistance to lysozyme produced by neutrophils. However, in view of the fact that it is technically impossible to prepare PG from these samples in amounts and purity suitable to perform HPLC and LTQ-FT mass spectrometry, we are unable to provide conclusive evidence for this hypothesis.

Results of the experimental infections carried out in this study using the Δ*pgdA* mutant suggest that PG N-deacetylation is a major factor in the virulence of *S. suis*. Overall, the infection outcome in the mouse clearly indicates that the Δ*pgdA* mutant is severely hampered in its ability to inflict damage to the host. The most important consequences of PG N-deacetylation are increased survival of *S. suis* at early stages of the infectious process and an increased ability of the pathogen to survive in blood. Interestingly, there was a complete absence of septic shock signs in mice inoculated with the Δ*pgdA* mutant and the production of inflammatory mediators was completely abrogated in mice inoculated with the Δ*pgdA* mutant, while, in perfect agreement with a previous study (Dominguez-Punaro *et al*., 2007), an exacerbated T helper-1 inflammatory response in mice inoculated with the WT strain was observed. Different results were obtained in a previous study with *L. monocytogenes* showing that the absence of PG N-deacetylation does not result in muropeptides unable to activate the NF-κB pathway, but, instead, in enhanced activation of that pathway in a Nod1- and Nod2-dependent manner (Boneca *et al*., 2007). With the results obtained in our study we thus propose that, rather than an impaired ability of the unmodified PG to induce exaggerated inflammation, the observed basal levels of pro-inflammatory cytokines in mice inoculated with the Δ*pgdA* mutant might result from the rapid clearance of the mutant by neutrophils (low levels of bacteraemia) along with an irrelevant activation of macrophages, lymphocytes and NK cells in this process.

In spite of the fact that the Δ*pgdA* mutant was able to induce clinical signs in some pigs, conclusions obtained for the mouse may be extended to the pig. Indeed, mortality observed in

the Δ*pgdA* mutant group may be explained, at least in part, by the fact that we have prioritised the use of the highly aggressive intravenous route of administration and a high dose of inocula in order to use a validated model of infection (Berthelot-Herault *et al*., 2001). Nevertheless, in contrast to the WT group, pigs that survived septic shock in the Δ*pgdA* mutant group noticeably recovered and it was apparent that the Δ*pgdA* mutant was less fit to survive in blood. This inability to persist in blood might also be important for later stages of the infection. Firstly, colonisation of organs was hampered in the mutant group in both the mouse and pig. In addition, it has been shown that if *S. suis* fails to induce acute fatal septicaemia but the level of bacteraemia remains high, CNS disease may appear afterwards (Dominguez-Punaro *et al*., 2007, Segura *et al.*, 2006). Interestingly, and in agreement with its observed rapid clearance from blood, we did not record any case of meningitis in neither model with the Δ*pgdA* mutant. On the other hand, meningitis was responsible for the death of 35 % of mice and 10 % of pigs in the respective WT groups.

Enhanced clearance of the Δ*pgdA* mutant strain from blood might be attributed to an increased susceptibility of the mutant to killing by neutrophils, as shown in this study. Interestingly, neither the WT or Δ*pgdA* mutant strains showed differences in resistance to the action of cationic antimicrobial peptides such as polymixin B, magainin II, colistin, human neutrophil peptide (HNP)-1 and HNP-2 (Supplemental Table 2), functional homologues of which are secreted by porcine neutrophils. We speculate that differences observed in killing by neutrophils between strains might primarily be explained by the enhanced resistance of the WT strain to lysozyme secreted by neutrophils, due to increased PG N-deacetylation after induction of the *pgdA* gene. On the other hand, since our killing assay does not differentiate between intra and extracellular killing it is difficult to estimate if increased killing of the Δ*pgdA* mutant correlate with increased phagocytosis. However, since the Δ*pgdA* mutant is as encapsulated as the WT strain and, on the other hand, it has been shown that capsular material interferes with the uptake of *S. suis* by phagocytes (Chabot-Roy *et al*., 2006, Charland *et al*., 1998, Segura *et al.*, 2004, Smith *et al*., 1999), enhanced phagocytosis of the mutant might be unlikely. Interestingly, it has been suggested, although not proved, that an encapsulated *S. suis* Δ*dltA* mutant might be killed by porcine neutrophils extracellularly in the so called neutrophil extracellular traps (NETs) (Fittipaldi *et al.*, 2008). Since neutrophils, in addition to delivering their complex antibiotic arsenal into the phagosome, also discharge their lysozyme-rich specific

granules extracellularly in the NETs (Cho *et al.*, 2005, Mollinedo *et al.*, 2006) it might be postulated that the Δ*pgdA* mutant may also be killed through the action of this enzyme after being trapped in these extracellular structures. Further experiments are needed to confirm this hypothesis.

In conclusion, we have determined the muropeptide composition of *S. suis* and showed that this bacterium, through the action of the *pgdA* gene product, modifies its PG by means of N-deacetylation. We showed that this PG modification, which is probably enhanced *in vivo*, greatly contributes to the virulence of *S. suis* in both the murine and porcine models of infection. The results of this study strongly suggest that PG N-deacetylation is a major virulence factor of this swine pathogen and zoonotic agent.

Experimental procedures

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Supplemental Table 3. *S. suis* strains were grown in THB or in Todd-Hewitt agar (THA) (Becton Dickinson, Sparks, MD, USA) at 37°C under 5% CO2. *Escherichia coli* strains were cultured in Luria-Bertani broth or agar medium (Becton Dickinson) at 37°C for 18 h. When necessary, antibiotics (purchased from Sigma-Aldrich, Oakville, On, Canada) were added to culture media at the following concentrations: for *E. coli*, kanamycin, 50 µg/ml; chloramphenicol (Cm), 10 µg/ml and Sp, 50 µg/ml; for *S. suis*, Cm, 5 µg/ml and Sp, 100 µg/ml.

Purification of PG.

S. suis cell wall was prepared from cultures in the exponential growth phase as described for *S. pneumoniae* (Garcia-Bustos & Tomasz, 1987, Severin *et al.*, 1997) Wall teichoic acid and acid-labile modifications (such as O-acetylation) were removed by treatment with hydrofluoric acid to obtain PG (Severin *et al*., 1997).

Preparation and HPLC-analysis of muropeptides.

PG (ca. 1.25 mg/ml) was stirred with 25 µg/ml cellosyl (kindly provided by Hoechst, Frankfurt, Germany) in 20 mM sodium phosphate pH 4.8 for 24 h at 37°C. Then, a second 25

µg/ml-aliquot of cellosyl was added and the incubation continued for another 24 h. The sample was boiled for 10 min and centrifuged at room temperature for 15 min at 16000 × g. The supernatant was recovered and reduced with sodium borohydride as described (Glauner, 1988). The reduced muropeptides were separated on a 250×4.6 mm, 3 µm Prontosil 120-3-C18 AQ reversed-phase column (Bischoff, Leonberg, Germany) as described (Glauner, 1988), with the exception of the running buffers. Buffer A was 10 mM sodium phosphate pH 6.0 supplemented with 13 µl/l of 10% sodium azide; buffer B was 10 mM sodium phosphate pH 6.0 with 30% methanol. The eluted muropeptides were detected at 205 nm.

MALDI-TOF analysis of muropeptides.

Muropeptides eluted from the C18 column were collected, concentrated in a SpeedVac to 10-20 µl, and acidified with 0.1% trifluor acetic acid (TFA). The sample was then zip-tipped (C-18 material, Millipore, UK) according to the manufacturer's standard protocol, eluted from the zip-tip with 3 µl of 50% acetonitrile, 0.1% TFA and mixed with α-cyano-4-hydroxycinnamic acid matrix prior to spotting on a target plate. MALDI-TOF analysis was done on a Voyager-DE STR mass spectrometer (Applied Biosystems, Warrington, UK) operating in positive ion reflectron mode over the mass range (m/z) of 500 to 3000.

Preparation of muropeptides and LTQ-FT-MS analysis.

Different conditions for muropeptide preparation were used for samples subjected to LTQ-FT-MS analysis. PG (ca. 0.8 mg/ml) was digested with 13 µg/ml cellosyl in 20 mM ammonium acetate buffer, pH 4.8, for 16 h at 37°C. The sample was boiled for 10 min and centrifuged at room temperature for 15 min at 16.000×g. The sample (1 µl) was applied on a C18-reversed phase self-packed 100×0.5 mm column and eluted with a linear gradient of acetonitrile in water, 0.1% formic acid. The eluted fractions were sprayed directly into an LTQ-FT mass spectrometer (Thermo, Bremen, Germany) operating in the positive ion mode. The details of the method will be published elsewhere (Bui *et al*., manuscript in preparation).

DNA manipulations.

Restriction enzymes, DNA-modifying enzymes and DNA polymerase were purchased from GE Healthcare (Piscataway, NJ, USA) or Takara Bio (Otsu, Shiga, Japan) and used

accordingly to the manufacturer's recommendations. Minipreparations of recombinant plasmids from, and transformation of *E. coli* were performed by standard procedures (Sambrook *et al.*, 1989). Transformation of *S. suis* was carried out as previously described (Takamatsu *et al.*, 2001a). *S. suis* genomic DNA was isolated by the guanidium thiocyanate method (Pitcher *et al.*, 1989). Southern hybridizations were performed by the procedures described previously (Sekizaki *et al.*, 2001). For preparation of probes, DNA fragments were labelled with digoxigenin (DIG) using the DIG-PCR labeling mixture (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. Oligonucleotide primers (Invitrogen, Burlington, ON, Canada) are listed in Supplemental Table 4.

Construction of the knockout vector for gene replacement.

DNA fragments were amplified from genomic DNA of *S. suis* strain 31533 by PCR using the primer sets 4197F-PG plus 4985R-PG (left arm) or 6276F-PG plus 6838R-PG (right arm) (Supplemental Table 4). A Sp resistance cassette (*aad9* gene) was amplified from pSmall with primers specF3 and specR. All three primer sets introduce unique restriction sites. PCR amplicons were digested using the appropriate restriction enzymes, and sequentially ligated in the order left arm-Sp cassette-right arm using T4 DNA ligase. The resulting fragment was amplified by PCR using primers 4197F-PG and 6838R-PG, cloned into pCR4 using the TOPO TA PCR cloning kit (Invitrogen) following the manufacturer's instructions and propagated in *E. coli*. The insert was then cut out with HindIII and BamHI and recloned into the HindIII and BamHI sites of the thermosensitive vector pSET-5s, which carries the Cm resistance gene *cat* (Takamatsu *et al.*, 2001b), generating knockout vector p5ΔpgdA.

Generation of *S. suis* Δ*pgdA*.

Procedures for selection of mutants by allelic exchange via double crossover were described previously (Takamatsu *et al*., 2001b). Briefly, *S. suis* strain 31533 was transformed with p5ΔpgdA, and the cells were grown at 28°C in the presence of Cm and Sp selection. Bacteria at the mid-logarithmic growth phase were diluted with THB containing Sp and grown at 28°C to early logarithmic phase. The cultures were then shifted to 37°C and incubated for 4 h. Subsequently, the cells were spread on THA containing Sp and incubated at 28°C. Temperature-resistant Sp-resistant colonies were screened for loss of vector-mediated Cm

resistance to detect putative mutants which had exchanged their WT allele for a genetic segment containing the *aad9* gene as a consequence of homologous recombination via a double crossover. Finally, the genetic organization of the resulting Δ*pgdA* mutant was verified by PCR and by Southern hybridisation (data not shown). Three additional independent Δ*pgdA* mutants (named Δ*pgdA* 2, 3, and 4 respectively, Supplemental Table 3) were generated using the same strategy.

Transmission electron microscopy.

ON cultures of *S. suis* WT strain 31533 or mutant strain Δ*pgdA* were mixed with specific polyclonal serum anti-*S. suis* serotype 2 and incubated at room temperature for 1 h, followed by fixation in cacodylate buffer (0.1M cacodylate, 5%, v/v, glutaraldehyde 0.15%, w/v, ruthenium red, pH 7.2) for 2 h. Cells were then immobilised in 4% (w/v) agar and post-fixed ON at 4°C in 2% (v/v) osmium tetraoxyde. Samples were dehydrated in graded series of ethanol and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Model 420, Philips Electronics, The Netherlands).

Lysozyme and cationic antimicrobial peptide sensitivity.

S. suis WT and Δ*pgdA* mutant were grown in THB to which was added hen egg lysozyme (final concentrations ranging from 2 to 50 µg/ml; Sigma-Aldrich). Growth was monitored by following the optical density at 600 nm and by plating serials dilutions onto THA followed by CFU enumeration after ON incubation. Assays for sensitivity to cationic antimicrobial peptides were carried out in sterile 96-well microtiter plates. Logarithmic-phase *S. suis* cells were adjusted to approximately 10^4 CFU/ml in 100 µl THB containing one of the following antimicrobial compounds (purchased from Sigma-Aldrich) in serial dilutions: Colistin (0 to 200 µg/ml), polymyxin B (0 to 300 µg/ml), magainin II (0 to 45 µg/ml), HNP-1 (0 to 15 µg/ml) and HNP-2 (0 to 15 µg/ml). Plates were incubated for 24 h at 37°C. The MIC was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth by $OD_{600\ nm}$ measurement.

Killing by porcine neutrophils.

Blood samples were collected by venous puncture from high health status pigs which tested negative by ELISA (Lapointe *et al.*, 2002) for *S. suis* serotype 2. Cell populations were separated by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation and neutrophils isolated by sedimentation in 6% dextran, as decribed (Chabot-Roy et al., 2006). Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Neutrophils were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% of heat-inactivated porcine serum at a final concentration of 5×10^6 cells/ml. Bacteria (WT or Δ*pgdA* mutant strains at approx. 1×10^4 CFU/ml) were opsonised with complete normal porcine serum for 30 min at 37°C and then mixed in microtubes with neutrophils at 5×10^6 cells/ml. The mixture was incubated for 90 min at 37°C under 5% CO_2. Under these conditions bacteria are not toxic to neutrophils (Chabot-Roy *et al.*, 2006). After incubation cells were lysed with sterile water and viable bacterial counts on THA were performed.

Experimental infection of mice.

All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. For virulence studies a total of 30 female CD1 mice aged 6 weeks old (Charles River laboratories, Wilmington, MA, USA) were used. At day 0, animals were divided in two groups. Group 1 was inoculated by intraperitoneal injection of 1 ml of a *S. suis* strain 31533 suspension at 5×10^7 CFU/ml, while group 2 received the same dose of the mutant strain Δ*pgdA* using the same route of administration. Mice were monitored 3 times a day during 10 days for clinical signs and assigned clinical scores as previously described (Dominguez-Punaro *et al.*, 2007). Surviving animals in both groups were sacrificed at day 10 pi. Blood was collected daily from the tail vein and at euthanasia by cardiac puncture and used to evaluate bacterial load in this tissue by plating onto sheep blood agar plates and enumeration after ON incubation. Bacterial colonisation of the liver, the spleen and the brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500 µl of phosphate buffered saline (PBS), pH 7.3, and homogenised. Thereafter, 50 µl of the suspensions were plated as described above. In addition, an enrichment of the samples was carried out by inoculation of 300 µl of homogenised organ

samples or 100 µl of blood into THB, followed by ON incubation at 37°C and subsequent dilution and plating onto sheep blood agar plates as described above.

Analysis of the systemic immune response in mice.

A total of 42 female CD1 mice aged 6weeks old (Charles River) were used to study the systemic immune response. The WT strain (1 ml of a 5 x 10^7 CFU/ml suspension) was inoculated as described above to 18 mice. The same number of mice received the Δ*pgdA* mutant (1 ml of a 5 x 10^7 CFU/ml suspension). Finally, 6 mice were sham-inoculated. At defined intervals (3h, 6h, 12 h, 24 h, 36 h and 48 h) pi infected mice were sacrificed (3 animals per time for those inoculated with the WT or mutant strains, and 1 mouse per time for those sham-inoculated), and blood was collected by cardiac puncture. The bacterial dose and time points used were those reported in a previous study (Dominguez-Punaro *et al*., 2007). For validation of the conditions, we previously performed an additional independent study with a limited number of animals and time points which showed results consistent with those obtained in the major experiments described in the manuscript (data not shown). Plasma was recovered from blood samples by centrifugation and the levels of IFN-γ, IL-6, IL-1β, TNF-α, MCP-1, KC and RANTES were determined using a liquid multiarray system (Luminex Molecular Diagnostics, Toronto, ON, Canada). Commercial Multiplex coated beads and biotinylated antibodies, as well as the Beadlyte microtiter 96 well filter plates were obtained from Millipore, (Billerica, MA, USA). Each multiplex assay was performed in duplicate following the manufacturer's specifications. Data were collected using the Luminex-100 system Version IS 2.2 and analysed by MasterPlex Quantitation Software (MiraiBio, San Francisco, CA, USA). Standard curves for immune response mediators were obtained using the standards supplied by the manufacturer.

Analysis of the *pgdA* gene expression.

Total RNA was prepared from 1 ml of an early stationary phase *S. suis* culture in THB ("untreated control", approx 10^7 CFU/ml) using RNAwiz (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To analyze expression of *pgdA* upon interaction of *S. suis* with neutrophils, the WT type strain was treated as described above in the killing section and allowed to interact with porcine neutrophils for 90 min. Five tubes of the mixture of neutrophils and bacteria were then pooled and total RNA was prepared as described above. In

addition, 5 tubes of a "killing mock-infection" (RPMI plus 10 % porcine serum without neutrophils, as described in the killing section) were pooled and total RNA prepared from using RNAwiz. For analysis of *in vivo* expression, 5 female CD1 mice aged 6weeks old were inoculated as described above with 1 ml of a *S. suis* strain 31533 suspension at 5 x 10^7 CFU/ml. Animals were sacrificed 24 h pi. Pieces of spleen and liver (weighing approx 0.2 g) were collected and disrupted with 0.1 mm glass beads using a mini bead beater (Biospec Products, Bartlesville, OK, USA) and total RNA prepared with RNAwiz, as described. All RNA samples were treated with TurboDNAse (Ambion), and absence of contaminating DNA was verified by PCR using primers AROA-F and BA9 (Supplemental Table 4), which target the *aroA* gene. RNAs were quantified by measurement of absorbance at 260 nm and their integrity verified by visualization on 1% denaturing agarose gels. Synthesis of cDNAs was performed in triplicate using SuperScript II (Invitrogen) with random hexamers (Roche, Laval, Qc, Canada). Q-PCR was carried out by using the QuantiTect SybrGreen PCR Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. For each sample, a no-reverse transcription reaction was run as a control. For each Q-PCR run, to normalize for the amount of sample cDNA added to each reaction, the Ct value of the *pgdA* gene was subtracted by the *Ct* value of the endogenous control *rpoD* gene (delta *Ct* = *Ct pgdA* –*Ct rpoD*), and then, for a comparison between the "untreated control" (bacteria grown in THB) group and the "treated" groups (upon interaction with neutrophils, "killing mock infection", *in vivo* samples), the delta *Ct* values of the treated groups were subtracted by the delta *Ct* value of the untreated control (delta delta *Ct* = delta *Ct* treated group − delta *Ct* untreated control). The fold changes were calculated by the formula of $2^{-\text{delta delta Ct}}$ (Livak & Schmittgen, 2001). Primers were SSU1448-FQ and SSU1448-RQ for *pgdA* and RPDO-F and RPDO-R for *rpoD* (Supplemental Table 4).

Experimental infection of pigs.

A total of 24 high-health-status pigs (ages 4 to 5 weeks) which tested negative by ELISA (Lapointe et al., 2002) for *S. suis* serotype 2 were used. Strict biosecurity measures were implemented to avoid undesirable contamination of the pigs; these included an air filtration system and airlocks for each unit. Pigs were divided in 3 groups. Animals in groups 1 (N=10) and 2 (N=10) were inoculated by intravenous injection of 1 ml of 1 x 10^8 CFU/ml of *S. suis* WT strain 31533 or mutant Δ*pgdA*, respectively. Group 3 (N=4) were sham-inoculated animals. Clinical signs and presence of *S. suis* in blood were monitored during the trial. Surviving animals in all 3

groups were sacrificed 5 days pi and examined for pathological lesions. Bacteriological isolation from different organs (liver, spleen, lungs, heart and articulations) was performed essentially as described above for the mice.

Statistics.

Unless otherwise specified, all the data were expressed as means ± standard deviations. Unless otherwise specified, data were analysed by two-tailed, unpaired *t* test and all assays were repeated at least three times. For data analysis of the systemic immune response and bacteremia in mice the Mann-Whitney test was used. For *in vivo* virulence experiments, survival was analysed with the LogRank test. For all tests, a value of $P < 0.05$ was considered as the threshold for significance.

Acknowledgments

We are indebted to P. Willson (Vaccine and Infectious Diseases Organization, Saskatoon, Canada) for the generous gift of plasmid pSmall. We are grateful to J. Grey (Pinnacle Proteomics Facility, Newcastle University, United Kingdom) for the MALDI-TOF-MS and LTQ-FT-MS analysis of muropeptides. We thank D. Montpetit (Centre de Recherche et Developpement sur les Aliments, St-Hyacinthe, Canada) for performing the electron microscopy. We also thank G. Vanier and S. Lacouture for useful suggestions and M. Takahashi and J. Aranda Rodríguez for assistance. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; 0680154280) and from the Centre de Recherche en Infectiologie Porcine (CRIP-FQRNT) to MG and from the European Commission through the EUR-INTAFAR program (LSHM-CT-2004-512138) to WV and NKB. NF and MCDP are recipients of NSERC postgraduate scholarships.

ANNEX 3. ARTICLE VI. TABLES

Table 1. Muropeptides detected by LTQ-FT mass spectrometry.

Muropeptide			WT		Δ*pgdA*	
No	proposed structure	theoretical neutral mass (Da)	neutral mass (Da)	signal intensity (A.U.)[1]	neutral mass (Da)	signal intensity (A.U.)[1]
1	Tri	823.3811	823.3793	157,566	823.3795	124,676
2	Tri (Glu)	824.3889	824.3644	n. q.[2]	824.3646	n. q.
3	Tri (-G)	620.3017	620.3012	5,961	620.3012	4,212
4	Tri (deAc)	781.3705	781.3708	450	n.d.[3]	n.d.
5	Tetra	894.4182	894.4171	17,168	894.4173	15,822
6	Penta	965.4553	965.4541	9,763	965.4549	4,835
7	TetraTri	1699.7887	1699.7874	108,961	1699.7871	59,454
8	TetraTri (Glu)	1700.7966	1700.7580	n.q.	1700.7598	n.q.
9	TetraTri (deAc)	1657.7781	1657.7766	5,488	n.d.	n.d.
10	TetraTri (-G)	1496.7093	1496.7084	5,551	1496.7083	3,095
11	TetraTri (-2G)	1293.6299	1293.6287	10,318	1293.6291	3,716
12	TetraTri (-GM)	1221.6088	1221.6080	3,021	1221.6086	903
13	TetraTetra	1770.8259	1770.8249	2,588	1770.8261	1,288
14	TetraPenta	1841.8630	1841.8620	3,834	1841.8626	2,013
15	TetraTetraTri	2576.1964	2576.1952	17,734	2576.1995	7,391
16	TetraTetraTri (deAc)	2534.1858	2534.1944	139	n.d.	n.d.
17	TetraTetraTri (-G)	2373.1170	2373.1174	1,813	2373.1226	544
18	TetraTetraTri (-2G)	2170.0376	2170.0369	2,191	2170.0407	780
19	TetraTetraTetra	2647.2335	2647.2275	84	2647.2398	19
20	TetraTetraTetraTri	3452.6040	3452.6084	1.972	3452.6113	708

[1] the noise of the signal is ≈10 A.U.

[2] not quantified because the signal partly overlaps with the isotope signal of the amidated compound

[3] n.d. not detected

Supplemental Table 1. Quantification and MALDI-TOF-MS analysis of the muropeptides separated by HPLC.

Muropeptide		Area %		Positive mass (Da) from collected peaks[2] reduced, Na^+ form	
No	proposed structure[1]	WT	Δ*pgdA*	measured	calculated
1	Tri	18.8	20.9	848.40	848.39
2	Tri (Glu)	2.2	2.4	849.38	849.40
3	Tri (-G)	n.q.[3]	n.q.	623.33[4]	623.33[4]
4	Tri (deAc)	n.d.[5]	n.d.	n.d.	806.38
5	Tetra	2.6	2.7	919.46	919.42
6	Penta	1.5	1.7	990.51	990.46
7	TetraTri	46.0	46.7	1726.86	1726.81
8	TetraTri (Glu)	2.5	1.9	1727.88	1727.82
9	TetraTri (deAc)	n.d.	n.d.	n.d.	1684.80
10	TetraTri (-G)	3.3	4.1	1523.77	1523.73
11	TetraTri (-2G)	n.d.	n.d.	n.d.	1320.65
12	TetraTri (-GM)	1.1	0.3	1246.67	1246.61
13	TetraTetra	1.7	1.3	1797.97	1797.85
14	TetraPenta	1.9	2.0	1869.02	1868.88
15	TetraTetraTri	11.7	10.2	2605.34	2605.23
16	TetraTetraTri (deAc)	n.d.	n.d.	n.d.	2563.22
17	TetraTetraTri (-G)	n.q.[6]	n.q.[6]	2402.27	2402.15
18	TetraTetraTri (-2G)	n.d.	n.d.	n.d.	2199.07
19	TetraTetraTetra	n.d.	n.d.	n.d.	2676.27
20	TetraTetraTetraTri	n.d.	n.d.	n.d.	3483.65

[1] see Fig. 1B
[2] peaks were collected from the WT sample
[3] not quantified, co-elutes with Tri
[4] mass of the H^+ form
[5] n.d., not detected
[6] **n.q., not quantified, co-elutes with TetraTetraTri**

Supplemental Table 2. Sensitivity of the *S. suis* WT and Δ*pgdA* mutant strains to the action of selected antimicrobial peptides.

		MICs (μg/ml)	
Peptide (origin)	Net charge	WT	Δ*pgdA*
Colistin (*Bacillus colistinus)*	+5	50	50
Polymyxin B (*Bacillus polymyxa*)	+5	75	75
Magainin II (claw frog skin)	+4	45	45
HNP-1 (human)	+3	6	6
HNP-2 (human)	+3	5	5

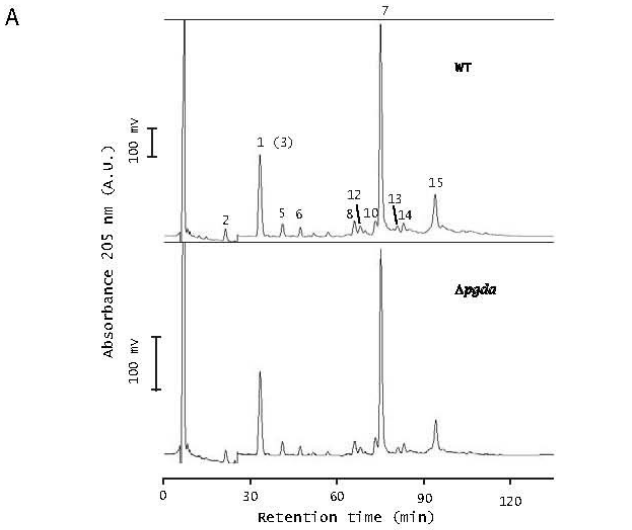
Supplemental Table 3. Bacterial strains and plasmids used in this study.

Bacterial strains	Relevant characteristics	Source
E. coli Top ten	General strain for cloning. F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. suis 31533	Serotype 2 field strain. Highly virulent	(Vanier, Segura et al. 2004)
S. suis Δ*pgdA*	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 2	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 3	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis Δ*pgdA* 4	Derived from strain 31533. Deletion of the *pgdA* gene	This work
S. suis BD102	Derived from strain 31533. Deletion of the promoter of the *aro* operon. Unencapsulated.	(Fittipaldi, Harel et al. 2007)
Plasmids		
pCR4	*E. coli* vector for cloning of PCR fragments	Invitrogen
pSmall	*E. coli-S. suis* shuttle vector. SpecR. Source of *aad9*	P. Willson (unpublished)
pSET5s	Thermosensitive suicide vector for *S. suis* mutagenesis. CmR (*cat*)	(Takamatsu, Osaki et al. 2001)
p5ΔpgdA	pSET5s carrying the construction for allelic exchange	This work

Supplemental Table 4. Oligonucleotide primers used in this study. Restriction sites are in bold.

Primer name	Sequence (5' – 3')	Restriction site
4197F-PG	GACGGGT**GGTCAA**GCTTTTGAATA	HindIII
4985R-PG	CCATTATGCCGAGCAAGG**AGATCT**TG	BglII
6276F-PG	GTAACAGACTTACT**CTGCAG**TCCTC	PstI
6838R-PG	GTGAACGTTT**GGATCC**TCTACGTAA	BamHI
SPECF2	TTATCAGGAT**AGATCT**TCGTTCGTGA	BglII
SPECR	AAAGTGTTTC**CTGCAG**TTTTTCAA	PstI
COMPPGDA-F3	GGATTTAAG**GGTACC**CGTCGCAAGGA	KpnI
COMPPGDA-R	ACCAG**GGATCC**TACTAACATGATTC	BamHI
AROA-F	AACGTGACCTACCTCCGTTG	
AROA-R	CGGTCATCGTAGAATTCGAGT	
SSU1448-FQ	TTCTCTCTGTACTTGCTCCC	
SSU1448-RQ	GGTCGCTCTAACCTTTGATG	
RPOD-F	TCTTTCAAATACATGCGGACTG	
RPOD-R	ATTCCATTTACGCTTGATGCTG	

ANNEX 3. ARTICLE VI. FIGURES



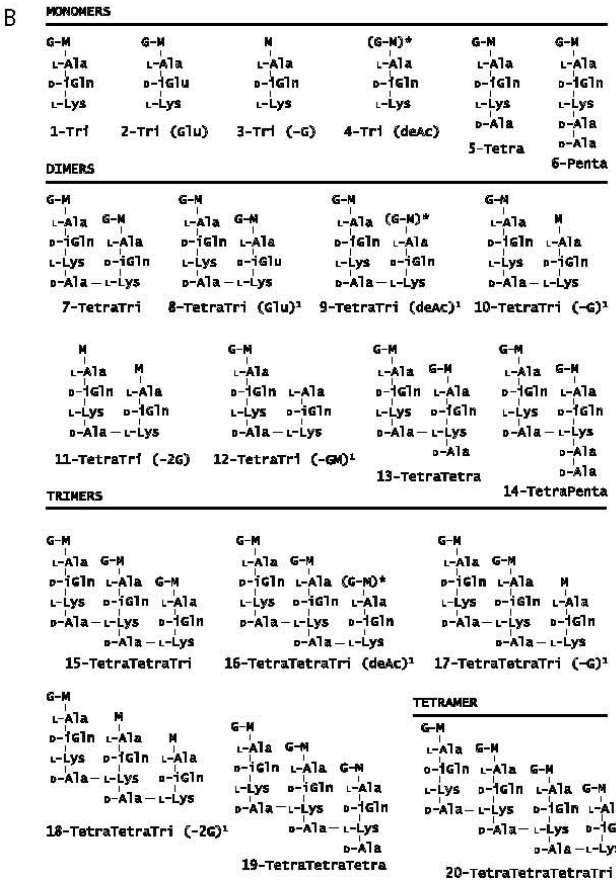


Fig. 1. *S. suis* muropeptide composition and proposed structures.

A. Muropeptide composition of *S. suis* WT and Δ*pgdA* mutant. The numbers correspond to the structures shown in B. The profiles of both strains showed similar muropeptide composition. However, see Table 1 and the text for differences between the WT and Δ*pgdA* mutant. B. Proposed structures for *S. suis* muropeptides. G indicates *N*-acetylglucosamine residue; M indicates *N*-acetylmuramic acid residue. Asterisks indicate deacetylation of G or M. In compounds marked with [1] it is not known which of the residues is modified.

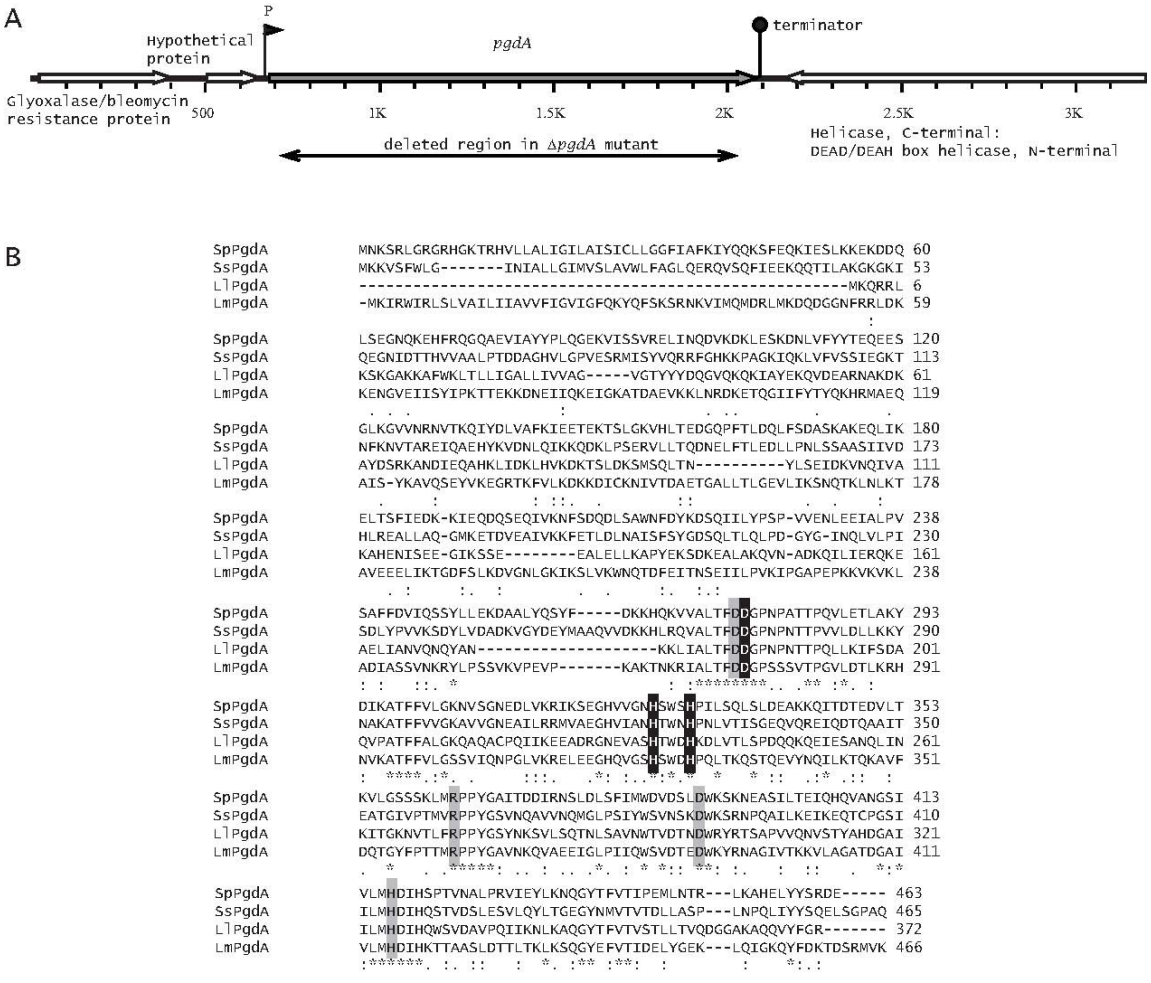


Fig.2. Genetic organization of the *pgdA* region and Clustal alignment of the predicted *S. suis* PgdA.

A. Genetic organization of the *S. suis pgdA gene* showing the predicted putative strong promoter (indicated by P) and the Rho-independent putative terminator. The region deleted in the Δ*pgdA* mutant (replaced by gene *aad9*) is also indicated. See the text for details B. Sequence alignment of *S. suis* (SsPgdA) and the described *N*-acetylglucosamine deacetylases from *S. pneumoniae* (SpPgdA) *L. lactis* (LlPgdA) and *L. monocytogenes* (LmPgdA). Sequences were aligned using the CLUSTALW software (http://www.ebi.ac.uk/Tools/clustalw/). Residues in grey and black boxes indicate the conserved catalytic residues and metal ligand amino acids characterised for *S. pneumoniae* PgdA (Blair et al., 2005), respectively. Asterisks below alignment indicate identical aminoacids, colons strongly conserved aminoacids and dots weakly conserved aminoacids.

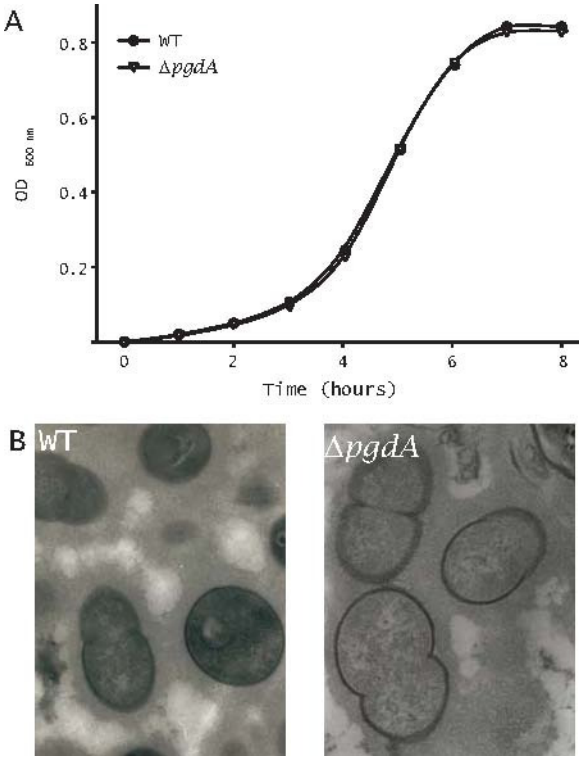


Fig. 3. Growth curves and morphology of the WT and Δ*pgdA* mutant. (A) Growth curves of *S. suis* WT and Δ*pgda* mutant strains. The Δ*pgdA* mutant (open triangles) grew similarly as the WT parent strain (closed circles) under normal laboratory conditions. (**B) Morphology of the WT (left) and the Δ*pgdA* mutant (right) strains.** Transmission electron microscopy showed that both strains were well separated and surrounded by a thick polysaccharide capsule. Original magnification : 30 000X.

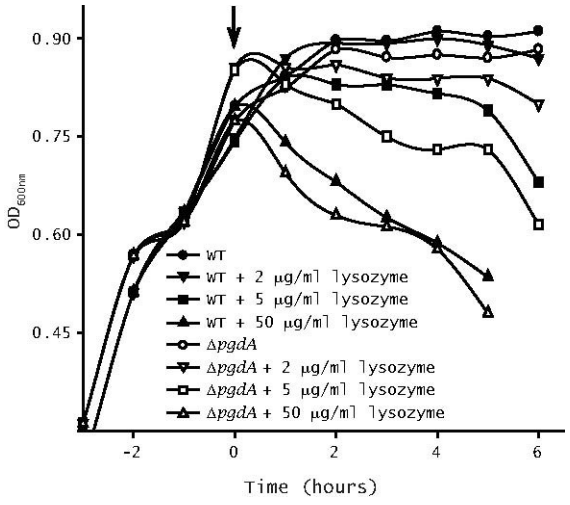


Fig. 4. Lysozyme sensitivity of *S. suis* WT and Δ*pgdA* mutant strains.

The WT and the Δ*pgdA* mutant strains were grown at 37 C, until the beginning of stationary phase of growth was reached, at which point, indicated by the arrow, lysozyme was added at different concentrations. Growth was monitored by measuring absorbance at 600_{nm}. Experiments were performed at least 3 times. Error bars are not shown for simplicity.

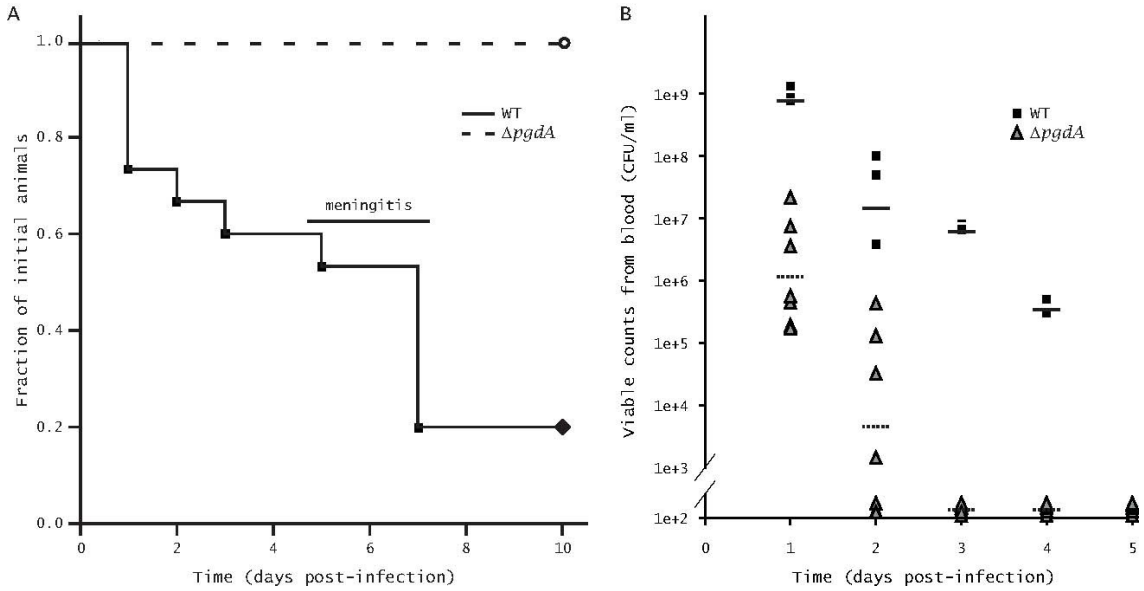


Fig. 5. Results of the WT and Δ*pgdA* mutant infection in mice.

A. Survival of mice inoculated with the WT (solid line) or the Δ*pgdA* mutant (dotted line) strains. All the mice in the Δ*pgdA* mutant (N=15) group survived, while 80% of the WT group (N=15) died from septicaemia or meningitis. Significant difference in survival were noted (LogRank test, P<0.05). B. Kinetics of bacterial clearance from blood in the WT and Δ*pgdA* mutant. The Δ*pgdA* mutant was isolated at lower titers than the WT strain following inoculation and was undetectable in this tissue from day 3 pi. Squares and triangles represent values from individual mice. The horizontal lines indicate the median for each group (WT: solid line; Δ*pgdA* mutant: dashed line). Significant differences in isolation from blood were noted between groups from day 1 to day 4 pi (Mann-Whitney, P<0.05).

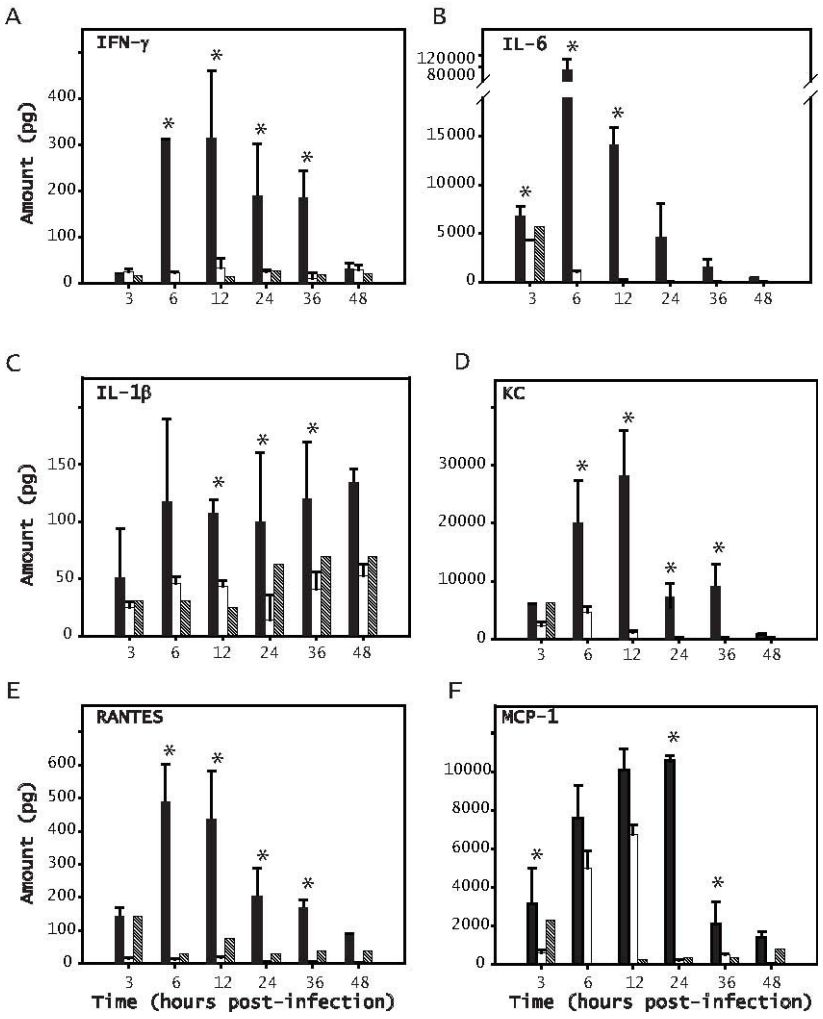


Fig. 6. Production of inflammatory mediators by mice inoculated with the WT strain (black bars, 3 animals per time), the Δ*pgdA* mutant (white bars, 3 animals per time) or sham-inoculated mice (dashed bars, 1 animal per time).

A. IFN-γ; B. IL-6; C. IL-1β; D. KC; E. RANTES; F. MCP-1. Significant differences (Mann-Whitney test $P<0.05$) were observed between the WT and the Δ*pgdA* mutant for all the inflammatory mediators at different time points (indicated by asterisks). Error bars indicate the standard errors of the median. See the text for further details.

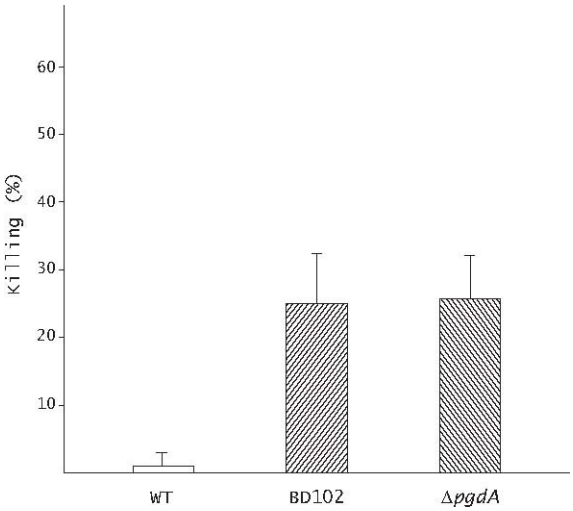


Fig. 7 Percentage of bacteria killed after 90 min incubation with porcine neutrophils.
The level of killing of the Δ*pgdA* mutant was similar to that observed for an unencapsulated mutant BD102 and significantly higher (t-test, P<0.05) than that of the WT strain. The different strains were opsonised with complete porcine sera before incubation. The experiment was repeated at least 5 times. Error bars indicate standard deviations calculated from biological repetitions.

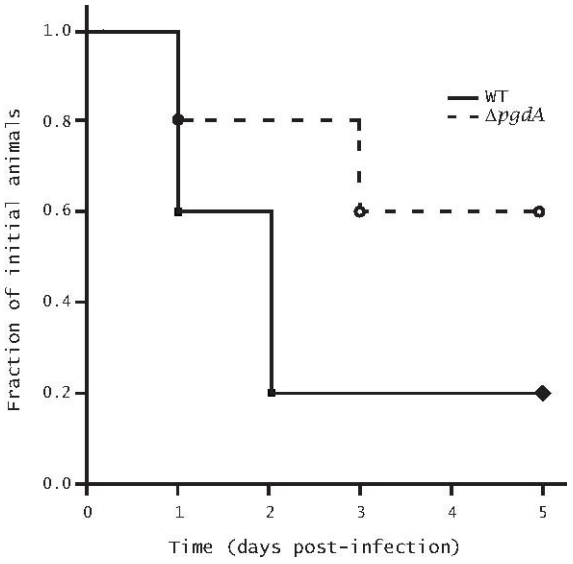


Fig. 8. Results of the WT and Δ*pgdA* mutant infection in swine.
Survival of pigs inoculated with the WT (solid line, N=10) or the Δ*pgdA* mutant (dotted line, N=10). Sham inoculated animals are not shown for simplicity. Significant differences in survival were noted, LogRank test, P<0.05. The survival rate of pigs in the Δ*pgdA* mutant group was 60%. In the WT group 80 % of the animals did not survive the trial. See the text for further details.

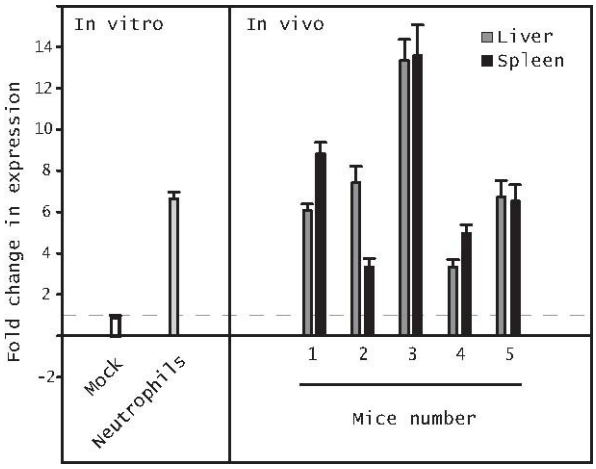


Fig. 9. Q-PCR analysis of the level of expression of the *S. suis pgdA* gene upon 90 min interaction of the bacteria with porcine neutrophils, or *in vivo* in the liver and spleen of experimentally infected mice.

The listed fold changes are relative to expression of *pgdA* when the bacteria were grown in THB (defined as 1 and shown by the dashed line). Fold changes were calculated using the delta-delta *Ct* method (Livak & Schmittgen, 2001). Please see the materials and methods section for further details. For *in vitro* samples (neutrophils), bars show the mean ± standard deviation of at least three biological repetitions. For *in vivo* samples, bars represent individual animals (mean ± standard deviation of three technical repetitions).

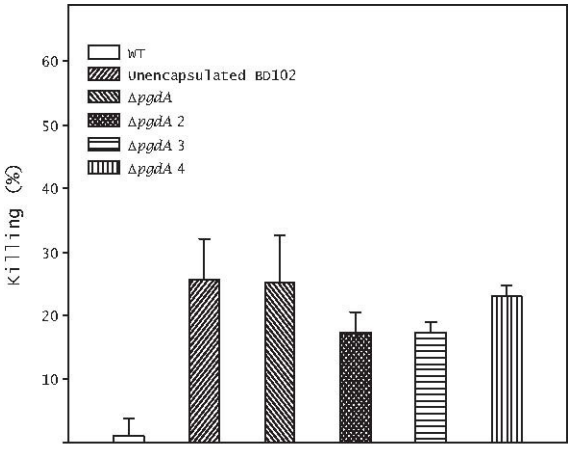


Fig. S1. Killing of the different Δ*pgdA* mutants by porcine neutrophils.

References

Baums, C. G., U. Kaim, M. Fulde, G. Ramachandran, R. Goethe & P. Valentin-Weigand, (2006) Identification of a novel virulence determinant with serum opacification activity in *Streptococcus suis*. *Infect Immun* 74: 6154-6162.

Bellais, S., M. Arthur, L. Dubost, J. E. Hugonnet, L. Gutmann, J. van Heijenoort, R. Legrand, J. P. Brouard, L. Rice & J. L. Mainardi, (2006) Aslfm, the D-aspartate ligase responsible for the addition of D-aspartic acid onto the peptidoglycan precursor of *Enterococcus faecium*. *J Biol Chem* 281: 11586-11594.

Berthelot-Herault, F., R. Cariolet, A. Labbe, M. Gottschalk, J. Y. Cardinal & M. Kobisch, (2001) Experimental infection of specific pathogen free piglets with French strains of *Streptococcus suis* capsular type 2. *Can J Vet Res* 65: 196-200.

Blair, D. E., A. W. Schuttelkopf, J. I. MacRae & D. M. van Aalten, (2005) Structure and metal-dependent mechanism of peptidoglycan deacetylase, a streptococcal virulence factor. *Proc Natl Acad Sci U S A* 102: 15429-15434.

Boneca, I. G., (2005) The role of peptidoglycan in pathogenesis. *Curr Opin Microbiol* 8: 46-53.

Boneca, I. G., O. Dussurget, D. Cabanes, M. A. Nahori, S. Sousa, M. Lecuit, E. Psylinakis, V. Bouriotis, J. P. Hugot, M. Giovannini, A. Coyle, J. Bertin, A. Namane, J. C. Rousselle, N. Cayet, M. C. Prevost, V. Balloy, M. Chignard, D. J. Philpott, P. Cossart & S. E. Girardin, (2007) A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. *Proc Natl Acad Sci U S A* 104: 997-1002.

Chabot-Roy, G., P. Willson, M. Segura, S. Lacouture & M. Gottschalk, (2006) Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. *Microb Pathog* 41: 21-32.

Chaput, C. & I. G. Boneca, (2007) Peptidoglycan detection by mammals and flies. *Microbes Infect* 9: 637-647.

Charland, N., J. Harel, M. Kobisch, S. Lacasse & M. Gottschalk, (1998) *Streptococcus suis* serotype 2 mutants deficient in capsular expression. *Microbiology* 144 (Pt 2): 325-332.

Cho, J. H., I. P. Fraser, K. Fukase, S. Kusumoto, Y. Fujimoto, G. L. Stahl & R. A. Ezekowitz, (2005) Human peptidoglycan recognition protein S is an effector of neutrophil-mediated innate immunity. *Blood* 106: 2551-2558.

Crisostomo, M. I., W. Vollmer, A. S. Kharat, S. Inhulsen, F. Gehre, S. Buckenmaier & A. Tomasz, (2006) Attenuation of penicillin resistance in a peptidoglycan O-acetyl transferase mutant of *Streptococcus pneumoniae*. *Mol Microbiol* 61: 1497-1509.

de Greeff, A., H. Buys, R. Verhaar, J. Dijkstra, L. van Alphen & H. E. Smith, (2002) Contribution of fibronectin-binding protein to pathogenesis of *Streptococcus suis* serotype 2. *Infect Immun* 70: 1319-1325.

Dominguez-Punaro, M. C., M. Segura, M. M. Plante, S. Lacouture, S. Rivest & M. Gottschalk, (2007) *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. *J Immunol* 179: 1842-1854.

Elliott, S. D., M. McCarty & R. C. Lancefield, (1977) Teichoic acids of group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). *J Exp Med* 145: 490-499.

Filipe, S. R. & A. Tomasz, (2000) Inhibition of the expression of penicillin resistance in *Streptococcus pneumoniae* by inactivation of cell wall muropeptide branching genes. *Proc Natl Acad Sci U S A* 97: 4891-4896.

Fittipaldi, N., M. Gottschalk, G. Vanier, F. Daigle & J. Harel, (2007a) Use of selective capture of transcribed sequences to identify genes preferentially expressed by *Streptococcus sui*s upon interaction with porcine brain microvascular endothelial cells. *Appl Environ Microbiol* 73: 4359-4364.

Fittipaldi, N., J. Harel, B. D'Amours, S. Lacouture, M. Kobisch & M. Gottschalk, (2007b) Potential use of an unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant as a live attenuated vaccine in swine. *Vaccine* 25: 3524-3535.

Fittipaldi, N., T. Sekizaki, D. Takamatsu, J. Harel, L. Dominguez-Punaro Mde, S. Von Aulock, C. Draing, C. Marois, M. Kobisch & M. Gottschalk, (2008) D-alanylation of lipoteichoic acid contributes to the virulence of *Streptococcus suis*. *Infect Immun* 76: 3587-3594.

Garcia-Bustos, J. F. & A. Tomasz, (1987) Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. *J. Bacteriol.* 169: 447-453.

Glauner, B., (1988) Separation and quantification of muropeptides with high-performance liquid chromatography. *Anal Biochem* 172: 451-464.

Gottschalk, M., M. Segura & J. Xu, (2007) *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. *Anim Health Res Rev* 8: 29-45.

Hebert, L., P. Courtin, R. Torelli, M. Sanguinetti, M. P. Chapot-Chartier, Y. Auffray & A. Benachour, (2007) *Enterococcus faecalis* constitutes an unusual bacterial model in lysozyme resistance. *Infect Immun* 75: 5390-5398.

Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung & F. Gotz, (2007) Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog* 3: e102.

Higgins, R. & M. Gottschalk, (2006) Streptococcocal Diseases. In: Diseases of swine. B. E. Straw, S. D'Allaire, W. L. Mengeling & D. J. Taylor (eds). Blackwell publishing, Ames Iowa, pp. 769-783.

Lapointe, L., S. D'Allaire, A. Lebrun, S. Lacouture & M. Gottschalk, (2002) Antibody response to an autogenous vaccine and serologic profile for *Streptococcus suis* capsular type 1/2. *Can J Vet Res* 66: 8-14.

Livak, K. J. & T. D. Schmittgen, (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. *Methods* 25: 402.

Mai, N. T., N. T. Hoa, T. V. Nga, L. D. Linh, T. T. Chau, D. X. Sinh, N. H. Phu, L. V. Chuong, T. S. Diep, J. Campbell, H. D. Nghia, T. N. Minh, N. V. Chau, M. D. de Jong, N. T. Chinh, T. T. Hien, J. Farrar & C. Schultsz, (2008) *Streptococcus suis* Meningitis in Adults in Vietnam. *Clin Infect Dis* [Epub ahead of print].

Meyrand, M., A. Boughammoura, P. Courtin, C. Mezange, A. Guillot & M. P. Chapot-Chartier, (2007) Peptidoglycan N-acetylglucosamine deacetylation decreases autolysis in *Lactococcus lactis*. *Microbiology* 153: 3275-3285.

Mollinedo, F., J. Calafat, H. Janssen, B. Martin-Martin, J. Canchado, S. M. Nabokina & C. Gajate, (2006) Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. *J Immunol* 177: 2831-2841.

Pitcher, D. G., N. A. Saunders & R. J. Owen, (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 8: 151-156.

Salles, M. W., J. Perez-Casal, P. Willson & D. M. Middleton, (2002) Changes in the leucocyte subpopulations of the palatine tonsillar crypt epithelium of pigs in response to *Streptococcus suis* type 2 infection. *Vet Immunol Immunopathol* 87: 51-63.

Sambrook, J., E. F. Fritsch & T. Maniatis, (1989) *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory Press, New York.

Sanford, S. E., (1987) Gross and histopathological findings in unusual lesions caused by *Streptococcus suis* in pigs. II. Central nervous system lesions. *Can J Vet Res* 51: 486-489.

Schleifer, K. H. & O. Kandler, (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36: 407-477.

Segura, M., M. Gottschalk & M. Olivier, (2004) Encapsulated *Streptococcus suis* inhibits activation of signaling pathways involved in phagocytosis. *Infect Immun* 72: 5322-5330.

Segura, M., G. Vanier, D. Al-Numani, S. Lacouture, M. Olivier & M. Gottschalk, (2006) Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system. *FEMS Immunol Med Microbiol* 47: 92-106.

Sekizaki, T., M. Osaki, D. Takamatsu & Y. Shimoji, (2001) Distribution of the SsuDAT1I restriction-modification system among different serotypes of *Streptococcus suis*. *J Bacteriol* 183: 5436-5440.

Severin, A., D. Horne & A. Tomasz, (1997) Autolysis and cell wall degradation in a choline-independent strain of *Streptococcus pneumoniae*. *Microb Drug Resist* 3: 391-400.

Smith, H. E., M. Damman, J. van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden & M. A. Smits, (1999) Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. *Infect Immun* 67: 1750-1756.

Takamatsu, D., M. Osaki & T. Sekizaki, (2001a) Construction and characterization of *Streptococcus suis-Escherichia coli* shuttle cloning vectors. *Plasmid* 45: 101-113.

Takamatsu, D., M. Osaki & T. Sekizaki, (2001b) Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. *Plasmid* 46: 140-148.

Vanier, G., M. Segura, P. Friedl, S. Lacouture & M. Gottschalk, (2004) Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. *Infect Immun* 72: 1441-1449.

Vollmer, W., D. Blanot & M. A. de Pedro, (2008a) Peptidoglycan structure and architecture. *FEMS Microbiol Rev* 32: 149-167.

Vollmer, W., B. Joris, P. Charlier & S. Foster, (2008b) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 32: 259-286.

Vollmer, W. & A. Tomasz, (2000) The *pgdA* gene encodes for a peptidoglycan N-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. *J Biol Chem* 275: 20496-20501.

Vollmer, W. & A. Tomasz, (2002) Peptidoglycan N-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. *Infect Immun* 70: 7176-7178.

Yu, H., H. Jing, Z. Chen, H. Zheng, X. Zhu, H. Wang, S. Wang, L. Liu, R. Zu, L. Luo, N. Xiang, H. Liu, X. Liu, Y. Shu, S. S. Lee, S. K. Chuang, Y. Wang, J. Xu & W. Yang, (2006) Human Streptococcus suis outbreak, Sichuan, China. Emerg Infect Dis 12: 914-920.

ANNEX 4. ARTICLE VII

"Disruption of *srtA* gene in *Streptococcus suis* results in decreased interactions with endothelial cells and extracellular matrix proteins"

Vanier G[a], Sekizaki T [bc], Domínguez-Punaro MC[a], Esgleas M[a], Osaki M[b], Takamatsu D[b], Segura M [d], Gottschalk M[a].

[a]Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, Saint-Hyacinthe, Qc, J2S 2M2, Canada. [b]Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. [c]United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagito, Gifu, Gifu 501-1193, Japan. [d]Centre for the Study of Host Resistance, McGill University Health Centre Research Institute, 1650 Cedar Avenue, Montreal, Qc, H3G 1A4, Canada

Published in: *Vet. Microbiol.* 2008 Mar 18;127(3-4):417-24

Details on the role of the candidate in the conception of the article: I actively participated in the experimental design concerning animal studies; I substantially performed research, analyzed data and participated in writing the paper.

ABSTRACT

Streptococcus suis, a major pathogen of swine, is an emerging zoonotic agent which causes meningitis and septic shock. In this study, we investigated the ability of *S. suis* mutant Strain (SRTΔA) lacking the sortase A gene (*srtA*) to interact with host cells and extracellular matrix (ECM) proteins, as well as its virulence in a mouse infection model. We demonstrated that mutant SRTΔA had reduced capacity to adhere to and invade porcine brain microvascular endothelial cells compared to the wild-type strain. In addition, mutant SRTΔA also showed significantly less adherence to plasma fibronectin, cellular fibronectin and collagen type I. However, disruption of *srtA* had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection. These results indicate that surface proteins anchored by sortase A are required for a normal level of bacterial binding. However, other factors may also be important for *S. suis* virulence and interaction with host tissues.

INTRODUCTION

Streptococcus suis serotype 2 is an important swine bacterial pathogen associated mainly with meningitis but also with other diseases such as endocarditis, arthritis, septicemia, and pneumonia [1]. Among 35 described serotypes, *S. suis* serotype 2 is the serotype most frequently recovered from diseased animals [1]. Recently, serotypes 32 and 34, unlike other serotypes, have been shown to be more related to *Streptococcus orisratti* [2]. As a zoonotic agent, *S. suis* has been isolated from human cases of meningitis, endocarditis, and toxic shock-like syndrome [3, 4]. A recent, unprecedented outbreak in China resulted in >200 human cases that were directly linked to a concurrent outbreak of *S. suis* infection in pigs. Of these human cases, 20% were fatal [4].

Our understanding of *S. suis* virulence factors remains limited. While a few factors have been shown to be critical for virulence, others are considered as putative although their precise role in *S. suis* related pathogenesis has yet to be clearly defined [5]. In addition, the multistep pathogenesis of meningitis caused by *S. suis* is poorly understood [5]. Most bacterial infections are initiated when bacteria adhere to host tissues. Likewise, initial interactions between *S. suis* and host tissues are also thought to be important for infection. In particular, interactions between *S. suis* and porcine brain microvascular endothelial cells (PBMEC) from the blood-brain barrier (BBB), which separates the central nervous system (CNS) from the bloodstream [6, 7], are thought to comprise a key step in the pathogenesis of *S. suis* induced meningitis [8].

The extracellular matrix (ECM) is a complex structural network beneath epithelial and endothelial cells and surrounding connective tissue cells [9]. Recently, it has been established that *S. suis* binds to several immobilized serum and ECM proteins such as plasma and cellular fibronectins and collagen types I, III, and V. Moreover, the adhesin(s) responsible for these interactions are proteinaceous in nature [10]. However, the mechanisms underlying interactions between these proteins and *S. suis* have not been elucidated. In other Gram positive bacteria, many cell surface proteins possessing a Leu-Pro-X-Thr-Gly (LPXTG) motif have

been reported to mediate bacterial interaction with host cells, namely ECM, and thus play an important role in virulence [11]. These specific proteins are covalently anchored to the cell wall by sortase A (SrtA), a membrane-bound thiol transpeptidase enzyme [11, 12]. Five sortases of *S. suis* have been described thus far: SrtA, SrtB, SrtC, SrtD and SrtE [13]. Of the five sortases, SrtA (recently classified as a class A sortase) has been shown to play a critical role in anchoring LPXTG proteins to *S. suis* cell wall [13, 14].

It is not clear whether SrtA and LPXTG proteins of *S. suis* are important for virulence and initial interactions between bacteria and host cells. In this study, we investigated the effects of a disruption of *srtA* gene on the capacity of *S. suis* to interact with PBMEC and ECM components and subsequently be virulent in an experimental mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. suis serotype 2 reference strain NCTC 10234 and its mutant SRTΔA, with targeted disruption of *srtA* as generated by allelic exchange via double crossover [13], were used in this study. Bacteria were grown overnight onto sheep blood agar plates at 37 °C and isolated colonies were incubated in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI, USA) for 8 h at 37 ºC with agitation. Working cultures were obtained by inoculating 10 µl of a 10^{-3} dilution of these cultures in 30 ml of THB and incubating for 16 h at 37 °C with agitation. Bacteria were washed twice in phosphate buffered saline (PBS) pH 7.3, and were appropriately diluted in cell culture medium before infection. An accurate determination of the number of colony forming unit per ml (CFU/ml) in the final suspension was made by plating onto THB agar using Autoplate® 4000 (Spiral Biotech, Norwood, MA, USA). *S. suis* mutant SRTΔA was grown in the presence of 5 µg ml-1 of chloramphenicol (Sigma-Aldrich, Oakville, ON, Canada). Both strains showed similar growth rates (data not shown).

Cell culture

The PBMEC/C1-2 cell line [15]was cultivated as previously described (Vanier *et al.*, 2004). Briefly, cells were grown in a 1:1 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 (Invitrogen, Burlington, ON, Canada) supplemented with 7.5% (v/v) heat inactivated fetal bovine serum, penicillin-streptomycin (Invitrogen), sodium bicarbonate, L-glutamine, human transferrin (MP Biomedicals, Solon, OH, USA), N-acetyl-cysteine, hypoxanthine, porcine heparin, human recombinant fibroblast growth factor-basic (Sigma-Aldrich), and β-mercaptoethanol (BioRad Laboratories, Mississauga, ON, Canada). Flasks (BD FalconTM; BD Biosciences, Mississauga, ON, Canada) and 24-well tissue culture plates (Primaria, BD FalconTM) were precoated with 1% (w/v) type A gelatin from porcine skin (Sigma-Aldrich). Cells were maintained at 37 °C with 5% CO2 in a humid atmosphere. For assays, PBMEC were trypsinized by adding a trypsin-EDTA solution (Invitrogen) and diluted in culture medium at 8 x 10^4 cells/ml. The cell suspension was distributed in tissue culture plates and incubated to confluence. Before the experiments, medium was removed from the plates and replaced by medium without antibiotics.

Adherence and invasion assays

For adhesion assay, bacteria were pelleted, washed twice with PBS, and resuspended at 10^6 CFU/ml in fresh cell culture medium without antibiotics as previously described [8, 16]. Confluent cell monolayers were infected with 1 ml aliquots of bacterial suspension. Plates were centrifuged at 800 × *g* for 10 min and incubated for 2 h at 37 °C with 5% CO2. The monolayers were washed five times with PBS, and incubated with 200 µl of 0.05% trypsin-0.03% EDTA for 10 min at 37 °C. After the addition of 800 µl of ice-cold deionised water, cells were detached and disrupted by scrapping the bottom of the well. Serial dilutions of this cell lysate were plated onto THB agars to enumerate viable bacteria. An invasion assay to quantify intracellular bacteria was performed in a similar manner as the adhesion assay, with one exception. Following the initial infection period, cells were washed twice with PBS and incubated with 1 ml of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G (Sigma-Aldrich) for 1 h at 37 °C with 5% CO2 (Vanier et al., 2004; Vanier et al., 2007). Levels of adhesion and invasion are expressed as the total number of CFU recovered per well.

Binding of *S. suis* to ECM proteins

Bacteria grown to a late exponential-phase were washed three times in PBS, resuspended at a concentration of 10^8 CFU/ml in 0.2% (v/v) formaldehyde, and incubated overnight at 4 °C. An enzyme linked immunosorbent assay (ELISA) was performed as previously described [10] using microtiter plates (Maxisorp, Nunc, VWR, Mississauga, ON, Canada) that had been coated overnight at 4 °C with 100 µl of plasma fibronectin (1 to 10 µg/ml) (Roche Diagnostics Corp., Indianapolis, IN, USA), cellular fibronectin (20 µg/ml) (Sigma-Aldrich), or collagen type I (1 to 12.5 µg/ml) (BD Biosciences) in 0.1 M carbonate coating buffer (pH 9.6). Wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST, pH 7.3), and blocked with 200 µl of 3% (w/v) non-fat dry milk in PBST. After 1 h at 37 °C, wells were washed three times with PBST and formaldehyde-killed bacterial suspensions (100 µl) were added. Plates were incubated for 2 h at 37 °C and washed three times. A 100 µl volume of *S. suis* serotype 2-specific rabbit antiserum [17] (diluted 1/3000 in PBST) was then added to each well. Plates were incubated for 1 h at 37 °C and were washed three times. A volume of 100 µl of

horseradish peroxidase-labelled anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) (diluted 1/8000 in PBST) was added and plates were incubated for 1 h at 37 °C. After washing three times with PBST, 3,3',5,5'-TetraMethylBenzidine (Zymed, Invitrogen) was used according to the manufacturer's instructions. Reactions were stopped by adding 25 □l of H2SO4 (1 N) and then read at 450 nm using a microplate reader (UVmax; Molecular Devices, Sunnyvale, CA, USA). Uncoated and casein-coated wells served as background and non-specific adhesion of *S. suis* to protein coated control wells, respectively. In addition, controls were performed to ensure that *S. suis* serotype 2- specific rabbit antiserum recognized wild-type strain and SRTΔA equally.

Intraperitoneal mouse virulence model

A total of 40 female CD1 6-week-old mice (Charles River Laboratories, Wilmington, MA, USA, 10 animals per group) were infected intraperitoneally with either 1 ml of *S. suis* strain NCTC 10234 or 1 ml of mutant SRTΔA (approximately 108 CFU in PBS). The CD1 mouse model was recently shown to be an excellent model of *S. suis* infection causing septic shock and meningitis [18]. Body weight changes (due to an excessive production of pro-inflammatory cytokines) were recorded daily post-infection (p.i.) [18]. Survival and development of clinical signs of meningitis were recorded over a 10-day period. In addition, bacterial numbers in blood and homogenized brain samples were determined by plating on sheep blood agar plates using Autoplate® 4000 at different timepoints p.i . Experiments involving mice were repeated twice (total number of 20 animals per group) and were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals, Animal Welfare Committee of the Université de Montréal.

Statistical analysis

All data are expressed as means □ standard deviations (error bars). Data were analyzed by twotailed, unpaired *t* test. Unless specified, all assays were repeated at least three times.

Statistical analysis of the survival data was performed with the LogRank test. A *P* value of ≤ 0.05 was accepted as the minimal level of significance.

RESULTS

***srtA* gene contributes to the interactions between S. suis and endothelial cells**

As shown in Fig. 1, adhesion and invasion levels of mutant SRTΔA were strongly reduced to 16 ± 24% and 17 ± 12%, respectively, compared to those of the wild-type strain (considered as 100%) when 10^6 CFU ml-1 bacterial suspensions were used. Similar results were obtained with different concentrations of bacteria (data not shown). The complemented mutant SRTΔA (Osaki et al., 2002) showed adhesion and invasion values of 88 ± 17% (*P* = 0.008 compared to the mutant SRTΔA) and 47 ± 4% (*P* = 0.004 compared to the mutant SRTΔA), respectively. These results confirm that sortase A is at least partially involved in *S. suis*-specific adhesion to and invasion of endothelial cells

***srtA* gene contributes to adherence of *S. suis* to ECM proteins**

An ELISA binding assay was used to determine whether the mutant SRTΔA had altered capacity to bind plasma and cellular fibronectins and collagen type I. As shown in Fig. 2 (a), SRTΔA had reduced binding to plasma fibronectin by (*P* < 0.05) compared to the wild-type strain. A similar decrease in adhesion to cellular fibronectin was also observed (data not shown). Binding to collagen type I by mutant SRTΔA was also diminished (*P* < 0.05; Fig. 2 (b)). Adhesion to fibronectin and collagen type I by both strains was dependent on the protein concentration (Fig. 2).

Effect of *srtA* gene disruption on virulence

Animals from both groups showed only limited weight loss (0 to 10% of initial weight) between day (d) 0 and d 5, with maximum loss observed at d 2 p.i. (data not shown). In addition, weight gain from d 6 to the end of the experiment was not significantly different between groups (*P* > 0.05; data not shown). With respect to cumulative survival, CD1 mice infected with SRTΔA had slightly longer median survival (2 d), although this was not statistically significant (*P* > 0.05) compared to mice infected with the wild-type strain (1 d) (Fig. 3). Both

strains displayed a similar mortality rate, with 80% of all animals dying within 10 d. Morbidity also did not differ between the groups; one mouse in each group developed clinical signs of meningitis (i.e. spatial disorientation). Blood and brain samples collected from infected animals during the first 2 d p.i. showed no significant differences in bacterial load between the two groups (data not shown)

DISCUSSION

In this study, we demonstrated that the mutant SRTΔA strain of *S. suis* has markedly impaired interactions with PBMEC *in vitro*. These findings provide evidence that sortase A of *S. suis* is involved in adhesion to and invasion of host cells and are consistent with those reported for other Gram-positive pathogens, such as *Streptococcus pneumoniae* [19], *Streptococcus sanguinis* [20], and *Streptococcus agalactiae* [21]. Our results also showed mutant SRTΔA has reduced binding to ECM proteins, suggesting that SrtA of *S. suis* may anchor LPXTG-containing microbial surface components which recognize adhesive matrix molecules (MSCRAMM) that mediate binding to plasma and cellular fibronectins and to collagen type I. These findings are in agreement with previous studies on *S. agalactiae* [21] and *S. gordonii* [22], showing that SrtA-expressing strains bind in significantly higher numbers to fibronectin compared to isogenic *srtA* mutant strains. Together these results suggest that LPXTG proteins anchored by SrtA are at least partially involved in the interactions between *S. suis* and host tissues.

Few LPXTG proteins of *S. suis* have been identified. Among these, the opacity factor of *S. suis* (OFS) and surface antigen one (Sao) have been recently described [23, 24]. OFS is an opacity factor involved in virulence but not in colonization [23]. Sao is an immunogenic protein that is expressed on the bacterial surface and confers protection to immunized animals [24]. Moreover, surface expression of some LPXTG proteins is downregulated after disruption of the *srtA* gene [13]. Indeed, mutant SRTΔA of *S. suis* has reduced expression of more than 15 major protein spots as visualized with two-dimensional polyacrylamide gel electrophoresis. From these protein spots, a previously described protein, the muramidase-released protein (MRP), as well as other newly described proteins similar to 5'-nucleotidase, and encoded by the genes *sntA*, *sntB*, and *sntC*, were identified. In European *S. suis* strains, MRP is considered to be a virulence marker [5]. However, MRP alone is not necessary for virulence in swine and is absent

in most North American virulent strains [1]. Other putative *S. suis* LPXTG proteins and their role in virulence have yet to be identified. Moreover, the precise LPXTG protein(s) involved in *S. suis*-host interactions are unclear and warrant further investigation.

It should be noted that a deficiency of sortase A did not completely abolish interactions between *S. suis* and PBMEC or ECM proteins, suggesting that other factors may also contribute to *S. suis* mediated pathogenesis. This is in agreement with our previous observations [16] demonstrating that non-proteinaceous cell wall components, such as lipoteichoic acid (LTA), are also involved in interactions between *S. suis* and PBMEC. Other pathogens, such as *S. pyogenes*, have been shown to possess multiple types of adhesins (LTA and proteins) that mediate interactions with host cells [25]. Non-LPXTG proteins (non-MSCRAMM) might also be involved in binding to ECM proteins. Notably, *S. suis* possesses non-MSCRAMM ECM-binding proteins such as FBPS [26], a 52-kDa IgG-binding protein [27], and a recently described Enolase [28]. In a previous study, bacteria coated with plasma fibronectin showed high levels of adhesion to and invasion of PBMEC, suggesting that *S. suis* uses plasma fibronectin as a bridge between bacteria and the host cell surface [16]. Results obtained in the present study suggest that non-MSCRAMM as well as LPXTG-containing MSCRAMM ECM-binding proteins may be involved in these host-pathogen interactions.

Disruption of *srtA* gene had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection. Our findings are consistent with those reported by Kharat and Tomasz (2003) showing that a *srtA* mutant of *S. pneumoniae* was as virulent as the wild-type strain in mice. Paterson and Mitchell (2006) demonstrated using a competitive infection model that *srtA* is a factor required for fitness rather than virulence of *S. pneumoniae*. It is not known whether a similar function can be attributed to *srtA* in *S. suis* infection. Indeed, there were no significant differences in levels of bacteremia between the wild-type and mutant strains. Survival of bacteria in blood is a critical step that enables the bacteria to disseminate throughout the host and ultimately gain access to the CNS. There is much evidence that the capsule is an important virulence factor that promotes bacterial resistance to phagocytic clearance [5]. Recent results in our laboratory obtained using a coagglutination assay showed

that the mutant SRTΔA is encapsulated (unpublished observation), which may explain its normal bacterial loads in blood. A high-grade bacteraemia was shown to be required for *S. suis* to subsequently reach the CNS, thus suggesting a critical role of the capsule for bacterial survival and dissemination in blood during *S. suis* invasion of the BBB [29].

Lastly, other class C sortases genes (*srtB*, *srtC*, *srtD* and *srtE*) [14] may also contribute to *S. suis*-host interactions. In *S. suis* NCTC 10234 strain, three class C sortases genes (*srtB*, *srtC*, *srtD*) cluster together and are associated with LPXTG proteins [13]. It is possible that these accessory class C sortases, which are present in about 50% of virulent *S. suis* strains (T. Sekizaki, unpublished observations), might specifically anchor the flanking LPXTG-containing proteins. Studies are ongoing in our laboratory to verify the role of other *S. suis* sortases in bacterial interactions with host cells and proteins.

We conclude that *S. suis* SrtA anchors LPXTG-containing surface proteins, thus playing an important role in bacterial colonization of host cells and adhesion to ECM proteins. However, anchorage of LPXTG proteins alone is not sufficient for virulence in mice. Together, these observations suggest that although LPXTG-containing surface proteins participate in multiple steps of the pathogenesis of *S. suis* infection, other non-LPXTG proteins, including secreted, membrane-bound and non-proteinaceous components, are likely to be involved in cell colonization, ECM binding, and virulence.

ACKNOWLEDGEMENTS

We thank M.-P. Lecours for excellent technical assistance. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant No. 0680154280. G.V. and M.C.D.P. are recipients of a NSERC scholarship. M. S. is recipient of a Canadian Institutes of Health Research fellowship.

ANNEX 4. ARTICLE VII. FIGURES

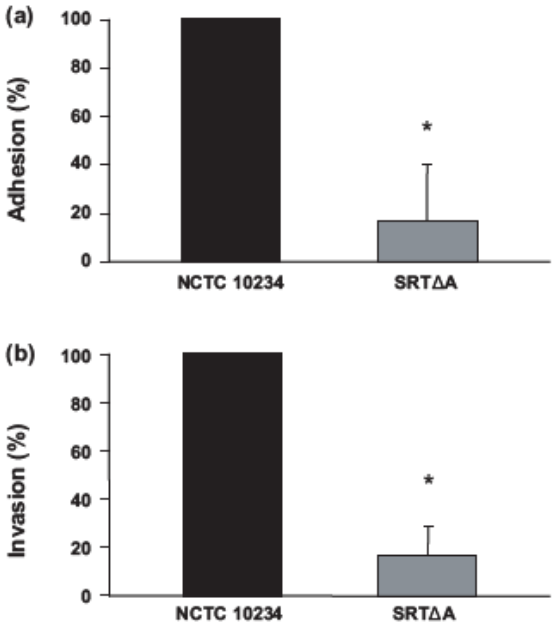


Fig. 1. (a) Adhesion to PBMEC by mutant SRTΔA compared to the wild-type strain NCTC 10234. (b) Invasion of PBMEC by mutant SRTΔA compared to the wild-type strain NCTC 10234. Adhesion and invasion are expressed as a percentage of the adhesion and invasion levels achieved by the wild-type strain NCTC 10234. Asterisk denotes values that are significantly different (P < 0.05) from those of NCTC 10234.

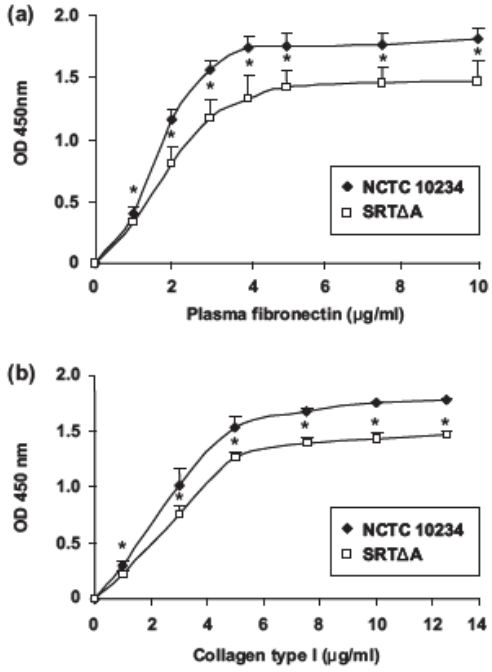


Fig. 2. Binding to different concentrations of plasma fibronectin (a) and collagen type I (b) by mutant SRTΔA compared to the wild-type strain NCTC 10234. Asterisk denotes values that are significantly different (P < 0.05) between the two strains.

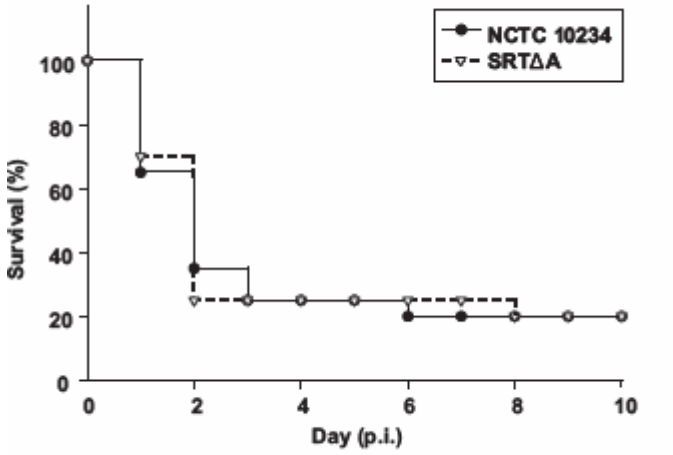


Fig. 3. Kaplan-Meier survival analysis of CD1 mice infected with the wild-type strain NCTC 10234 or the mutant SRTΔA (*P* = 0.56 for survival times between both groups). Six-week-old CD1 mice were inoculated i.p. with 108 bacteria, and survival was monitored over a 10-day period. Data are expressed as mean percentage of live animals in each group (n=20)

ANNEX 5. ARTICLE VIII

"New putative virulence factors of *Streptococcus suis* involved in invasion of porcine brain microvascular endothelial cells"

Ghyslaine Vanier[a], Nahuel Fittipaldi[a], Josh D. Slater[b], María de la Cruz Domínguez-Punaro[a], Andrew N. Rycroft[b], Mariela Segura[a], Duncan J. Maskell[c], Marcelo Gottschalk[a,*].

*Corresponding author. Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP) and Centre de Recherche en Infectiologie Porcine (CRIP), Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, Saint-Hyacinthe, Québec, J2S 2M2, Canada. Tel.: (450) 773-8521 ext. 1-8374, Fax : (450) 778-8108.

Published in: *Microb. Pathog.* 2009 Jan;46(1):13-20.

Details on the role of the candidate in the conception of the article: I actively participated in the experimental design concerning animal studies; I substantially performed research, analyzed data and participated in writing the paper.

1. Introduction

Streptococcus suis serotype 2 is an important bacterial pathogen of swine associated mainly with meningitis, but also with other infections such as septicemia, endocarditis, arthritis, and pneumonia [1]. Among the 35 serotypes described to date, *S. suis* serotype 2 is the one most frequently recovered from diseased animals [1]. As an emerging zoonotic agent, *S. suis* has been isolated from an increasing number of human cases of meningitis, endocarditis, and toxic shock-like syndrome [2, 3]. In 2005, an unprecedented outbreak in China resulted in >200 human cases that were directly linked to a concurrent outbreak of *S. suis* infection in pigs. Of these human cases, 20% were fatal [2].

Knowledge concerning virulence factors of *S. suis* is still limited. The capsular polysaccharide (CPS) and a recently described opacity factor (OFS) are critical for virulence since isogenic mutants lacking either of these factor were markedly less virulent [4-6]. Several other virulence candidates, such as a hemolysin (suilysin) [7], a fibronectin- and fibrinogen-binding protein (FBPS) [8], adhesins [9], proteases [10], and other proteins, such as muramidase-released protein (MRP) [11] and extracellular factor (EF) [12], have also been proposed. Recently, a study using a signature-tagged mutagenesis system identified a variety of genes potentially involved in virulence of *S. suis*; among them, genes related to capsule biosynthesis and regulation of suilysin expression were identified [13].

The multi-step pathogenesis of the infection caused by *S. suis* is poorly understood [14]. Most bacterial infections, including those caused by *S. suis*, are initiated by the attachment of bacteria to host tissues. In particular, interactions of *S. suis* with porcine brain microvascular endothelial cells (PBMEC) from the blood-brain

barrier (BBB), which separates the central nervous system (CNS) from the bloodstream [15, 16], are thought to be a key step in the pathogenesis of meningitis [14]. We have shown that *S. suis* is able to adhere to and invade PBMEC [17]. In fact, we have recently used this interaction model to demonstrate that *S. suis* preferentially expressed particular genes upon interactions with PBMEC using selective capture of transcribed sequences (SCOTS) [18]. These genes are involved in cell envelope modification, protein sorting, proteolytic activity, regulation, cell division/regulation, transport/binding, and metabolism/housekeeping [18]. The objective of this work was to find new putative virulence factors of *S. suis* by screening a Tn*917*-insertional mutant library using bacterial interactions with PBMEC as a reporter system.

2. Results

2.1 Identification of genes involved in S. suis *invasion of PBMEC*

A qualitative screening assay, adapted from a previously one using group B *Streptococcus* (GBS) [19], successfully allowed the identification of poorly invasive *S. suis* mutants. More than 1000 Tn*917* mutants were screened with PBMEC and 89 of these were qualitatively less invasive. Following a quantitative invasion assay with PBMEC, 19 of these mutants were confirmed to be significantly less invasive showing a reduction ranging from 97% to 70% ($P < 0.05$). Most of the other mutants also revealed a decreased adhesion level, but to a lesser extent (between 20 and 65%, data not shown). Only one Tn*917* insertion occurred in each of these mutants (Figure 1 and data not shown). Tn*917* insertion sites from these 19 poorly invasive mutants were sequenced and characterized, and interrupted genes were identified (Table 1). These genes can be divided into the following seven groups: surface protein, transport/binding, regulatory functions, metabolism, amino acid synthesis, protein

synthesis, and hypothetical proteins. Among them, a gene presenting homologies with a cell wall anchor domain of an unknown Leu-Pro-X-Thr-Gly (LPXTG) putative surface protein (mutant B8A) was identified. In addition, genes homologous to two genes from the same putative ABC-type multidrug transport system were found (mutants F8D and H4F). Moreover, a gene from a putative mercuric resistant regulatory protein (mutant I10B) downstream of the gene encoding sortase E, which was previously reported to be preferentially expressed by *S. suis* in contact with PBMEC [18], was also identified. Finally, a gene similar to a NUDIX hydrolase (mutant E5H) was also found. To the best of our knowledge, none of these genes has been previously associated with the pathogenesis of *S. suis* infection.

2.2 Virulence in mice

Five *S. suis* Tn*917* insertion mutants (A8E, B8A, E5H, F8D, I10B) were selected for further study on the basis of several considerations including reduced invasion (mutants A8E, B8A, E5H, F8D, I10B), previous association in the literature of similar genetic regions or genes with interactions with BMEC (mutants B8A, E5H, I10B) or biofilm formation (mutant F8D), or because two of the less invasive Tn*917* insertion mutants were interrupted in the same gene (mutant A8E). The virulence of the five selected mutants was assessed in a CD1 mouse model of infection. Median cumulative survival times from groups of CD1 mice infected with different mutant strains were markedly different from that for the wild-type strain P1/7. As shown in Fig. 2, compared to mice infected with the wild-type strain which gave a median survival time of 6 days, mice infected with some mutants gave similar (A8E, 5 days) or even lower (I10B and E5H, 1 day) survival times. In contrast, animals infected with mutants B8A and F8D gave markedly improved median survival times of 10 days. Moreover, mortality after 10 days was significantly lower in these two groups, compared to the wild-type infected mice ($P < 0.05$) (Table 2). Notably, zero mortality was recorded in

mice infected with mutant F8D. For all groups, mice showing severe clinical signs were sacrificed for welfare reasons.

Surviving animals from all groups showed variations in body weight. Animals from the group infected with wild-type P1/7 showed maximal mean weight loss of 15% on day 2, and recovered their initial body weight by day 7 p.i.. Animals from the group infected with I10B showed a constant decrease in body weight which correlated with the high mortality observed in this group. Animals from the groups infected by A8E and E5H showed maximal mean weight losses of 11% and 10% on days 2 and day 3 p.i., respectively ($P > 0.05$ vs P1/7 group), and recovery to normal body weight was delayed in animals infected with each of these mutants. In contrast, at day 2 p.i., animals infected with B8A showed less severe loss of body weight than animals infected with P1/7 wild-type (9% vs. 15%, $P < 0.05$). Similarly, animals infected with F8D showed only a 5% loss of body weight compared to 14% observed in those infected with wild-type P1/7 at day 3 p.i. ($P < 0.05$). Finally, mice infected with either B8A or F8D recovered their initial body weights by day 6 p.i., and showed a weight gain of 6% and 3% at day 10 p.i, respectively.

The percentages of morbidity and mortality were different among groups of mice infected with the mutant strains (Table 2). Mice infected with I10B or E5H showed severe clinical signs of disease, severe lethargy and sudden death. These findings explain the high levels of mortality observed in mice infected with these two mutants. On the other hand, overall morbidity and mortality in animals infected with A8E were similar to those infected with the wild-type strain. In contrast, most of the mice infected with B8A showed only mild signs of infection such as swollen eyes and rough hair coat. This is in agreement with the low mortality compared to relatively high morbidity percentages observed in this group (Table 2). Notably, mice infected with

F8D showed no mortality and only 20% of them showed mild clinical signs, such as rough hair coat.

S. suis were recovered from most organs from the majority of mice infected with P1/7, E5H, I10B and A8E between days 1 and 10 (Table 2). However, *S. suis* was detected in only a few mice infected with mutant B8A and F8D (Table 2). Interestingly, at the end of the experiment (day 10 p.i.), *S. sui*s was recovered from 60% of surviving mice infected with wild-type P1/7, while it was recovered from only 40% of surviving mice infected with B8A and 20% of mice infected with F8D. These findings suggest a clear reduction in the invasive and/or within-host survival capacities of these two mutant strains.

2.3 Virulence in swine

Based on these results, two *S. suis* Tn*917* insertion mutants, B8A and F8D, were selected for further evaluation in a pig model of infection. The percentage of morbidity of pigs infected with F8D was slightly lower compared to that observed in pigs infected with wild-type P1/7 strain and B8A (Figure 3A). In addition, at the end of the experiment (day 4), the percentage of surviving pigs was higher in the group infected with F8D (Figure 3A). All pigs infected with the wild-type P1/7 strain showed signs of infection such as severe depression and lameness (9/10) and severe joint stiffness (1/10). Although animals infected with F8D or B8A showed lameness (7/10 and 9/10, respectively) and depression (2/10 and 7/10, respectively), presentation of these clinical signs was delayed compared to the earlier development of clinical signs in pigs infected with the wild-type strain. Furthermore, no significant body weight loss was observed in pigs infected with the mutant strains during the experimental period (data not shown). According to United Kingdom legislation, the presentation of these clinical

signs was considered to be sufficient to require animal euthanasia. Hence, no clinical records could be performed after 4 days of infection for welfare reasons.

At the appearance of clinical signs, *S. suis* levels in blood were significantly different ($P < 0.05$) between groups, with $4.2 \pm 2.7 \times 10^3$, $1.1 \pm 1.8 \times 10^3$, and $0.6 \pm 0.7 \times 10^3$ CFU/ml of blood from pigs infected with P1/7, B8A and F8D, respectively. *S. suis* bacteria were recovered from most sampled organs from the majority of pigs irrespective of the infection group (data not shown). Unfortunately, no comparative quantitative analysis could be performed since animals were sampled during post-mortem examinations.

On the basis of previous observations with pig whole blood cells and in the mouse model of infection [20, 21], levels of pro-inflammatory cytokine IL-6, an endogenous pyrogen known as a marker for ongoing bacterial infections in pigs [22, 23], and the chemokine IL-8 were evaluated in plasma samples throughout the experiment. No differences were observed in IL-8 levels in plasma from pigs infected with wild-type strain P1/7 or either of the mutant strains (data not shown). On the other hand, markedly higher levels of pro-inflammatory IL-6 were observed in plasma from pigs infected with the P1/7 wild-type (Figure 3B). In contrast, lower (Figure 3B) and delayed (data not shown) levels of IL-6 were observed in plasma from pigs infected with B8A or F8D, which is in agreement with the lower fever indices [23] shown by animals infected with these mutants (Figure 3A).

3. Discussion

Despite its importance as a major swine pathogen and as an emerging life-threatening zoonotic agent, little is known about virulence factors of *S. suis* [14, 24]. In our continued effort to understand the pathogenesis of *S. suis* infection, we used a qualitative invasion assay using PBMEC to screen a mutant library, followed by assessment of virulence levels of selected less invasive mutants in mouse and pig models of infection. Two mutants, B8A and F8D, were less invasive for PBMEC, attenuated in mice as evidenced by decreased colonization of organs, reduced mortality and morbidity and, to a considerably lesser extent, also attenuated in pigs as indicated by decreased bacterial loads in blood, less severe and delayed clinical signs, and lower levels of the pyrogenic cytokine IL-6 in plasma.

In B8A, the Tn*917* insertion site is located in a LPXTG-motif cell wall anchor domain from an unknown LPXTG protein (Table 1). Many surface proteins possessing a LPXTG motif have been reported as mediating the interactions of Gram positive bacteria with host tissues, as they often confer the ability to bind to plasma components and host proteins from the extracellular matrix, and to adhere to and facilitate the invasion of host cells, thus playing important roles in virulence [25]. In *S. suis*, a few important proteins that contain the LPXTG motif have been reported. These include OFS, an opacity factor involved in virulence though not in colonization [5], surface antigen one (Sao), an immunogenic protein of unknown function conferring protection on immunized animals [26, 27], and the virulence marker MRP [11]. In B8A, the gene encoding the mutated LPXTG protein (*ssu1889*) is followed by 4 other genes encoding proteins with LPXTG motifs, flanking a cluster of three class C sortase genes: *srtB*, *srtC* and *srtD* [28, 29]. Interestingly, this region shows organizational similarity with the *rlrA* island from *Streptococcus pneumoniae* strain TIGR4 [30, 31]. Moreover, *ssu1889* gene from *S. suis* showed 49 % sequence homology to SP_0462 gene (GenBank

accession number NC_003028) from *rlrA* island from *S. pneumoniae* TIGR4. LPXTG proteins are covalently anchored to the cell wall by sortases [25]. In *S. suis*, sortase A (a class A sortase) plays a critical role in anchoring LPXTG proteins to the cell wall [28]. Moreover, we demonstrated recently that a mutant deficient for *srtA* had reduced capacity to adhere to and invade PBMEC and was less adherent to plasma fibronectin, cellular fibronectin and collagen type I compared to the wild-type strain [32]. The identities of the proteins anchored by class C sortases in *S. suis* are not yet known as their role in virulence.

Pili were very recently described in Gram positive pathogens such as group A, group B *Streptococcus* (GAS, GBS) and *S. pneumoniae* [33]. Although not completely elucidated, pilus assembly in GAS and GBS would occur through covalent polymerization by class C sortases of LPXTG pilin subunits encoded by genes within a pilus island [33]. Moreover, pili are important for GBS adhesion to and invasion of hBMEC [34]. In *S. suis*, Fittipaldi *et al.* [18] recently described a putative pilus island constituted by a signal peptidase which is followed downstream by the genes for a putative ancillary protein and a main pilus subunit and a previously undescribed class C sortase. Interestingly, expression of the signal peptidase gene from this island was upregulated in presence of PBMEC [18]. This *S. suis* island presents similar genetic organization to GBS pilus island 2b (PI-2b), one of the three identified pilus islands in this species [33]. Moreover, Jacques *et al*. [35] reported the presence of thin and short pilus-like structures on the surface of *S. suis* using electron microscopy. Therefore, it is tempting to speculate on a second *S. suis* putative pilus island, similar to the *rlrA* island from *S. pneumoniae*, that has been interrupted in B8A and that this region would play a role in the invasion of endothelial cells from swine BBB and thus in virulence.

In F8D, the Tn*917* insertion site was located in an ATPase component from an ABC-type multidrug transport system (Table 1) with homology to the BcrA subfamily of

ABC transporters involved in bacitracin resistance [36]. In bacteria, ABC transporters are important virulence factors because they play roles in secretion of toxins and antimicrobial agents and they are associated with many physiological processes (uptake of nutrients, non-classical secretion of signalling molecules, multidrug resistance) [37]. Moreover, roles for genes from ABC transport systems in cell-to-surface and/or cell-to-cell interactions and biofilm development have been proposed for different organisms such as *Streptococcus gordonii, Pseudomonas fluorescens,* and *Agrobacterium tumefaciens* [38-40]. More specifically, in *Streptococcus mutans*, the *glrA* gene encoding for a bacitracin transport ATP-binding protein plays a role in biofilm formation [41]. In *Burkholderia cepacia*, the multidrug-resistance efflux pump BcrA is an immunodominant antigen in cystic fibrosis patients [42]. It is worth noting that some strains of *S. suis* form biofilms [43].

The genomic region interrupted by Tn*917* in F8D seems to be required for *S. suis* interactions with host cells since a second mutant (H4F), in which Tn*917* was inserted in the second gene located downstream of the gene mutated in F8D (Table 1), was also less invasive for PBMEC. In H4F, a putative bacteriocin operon protein (*ssu0833*) was interrupted. Interestingly, *S. suis* was previously shown to produce bacteriocin-like inhibitory substances [44]. Both genes, *ssu0835* (mutant F8D) and *ssu0833* (mutant H4F) share sequence homologies with ABC-transporter forming genes from the GAS streptococcin A-FF22 lantibiotic regulon [45], where streptococcin A-FF22 is a bacteriocin. Overall, this genetic region seems to be important in *S. suis* interactions with host cells and virulence in *in vivo* models. Interestingly, a gene homologous to an ATP-binding protein from another multidrug ABC transporter was also reported to be preferentially expressed by *S. suis* in contact with PBMEC [18]. However, the actual function of the putative ABC transporter reported in the present study, as well as its substrate, remain to be determined.

The contribution to virulence of a hypothetical protein from mutant A8E was studied since two mutants (A8E and E12D, see Table 1) in which this gene was interrupted were less able to invade PBMEC. However, A8E showed no significant decrease in virulence in mice. In *E. coli* K1, a NUDIX hydrolase, *ygdP*, was associated with invasiveness of hBMEC [46]. Although the NUDIX hydrolase identified in the present study (in mutant E5H) seems also to be involved in *S. suis* invasiveness of PBMEC *in vitro*, no reduction in virulence was observed in mice. Finally, *srtE* encoding a class C sortase was reported to be preferentially expressed by *S. suis* in contact with PBMEC [18]. The transposon insertion in I10B was located in the *merR* gene located downstream of *srtE* [28]. However, I10B showed no decrease of virulence in mice. Although this region seems to be involved in interactions between *S. suis* and PBMEC, its actual role in *S. suis* pathogenesis remains to be determined. Hence, a reduction in cell invasion level does not always correlate with a significant reduction in virulence *in vivo.* This lack of direct relationship for some mutants tested in this study could be explained by the fact that *in vivo* models of virulence are much more complex and involve different parameters.

In conclusion, the qualitative screening PBMEC invasion assay is a good first step model to find new putative virulence factor for *S. suis* since genes coding for a LPXTG protein and an ATPase component from an ABC transporter were identified as being involved in invasion of swine endothelial cells and in *S. suis* virulence in mouse and pig models of infection in this study. It is worth noting that the PBMEC have been used in this study as a model and that these mutations could have also affected the bacterial ability to interact not only with these cells but also with different host cells and components, with the consequence of a reduced virulence. Finally, we acknowledge that transposon insertion polar effects on other genes may be present in some of these mutants. Further work using site-directed mutagenesis on each of the most interesting genes identified in this study is granted. In fact, studies focusing on the regions where

these two genes are located are under way in our laboratory.

4. Materials and methods

4.1 Bacterial strains

S. suis serotype 2 strain P1/7 [47], isolated from a field case of meningitis, harbouring virulence markers MRP, EF, and suilysin, and from which complete genome had been sequenced by the *S. suis* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_suis), was used as wild-type strain for this study. A mutant library was created using the pTV408 temperature-sensitive suicide vector to deliver Tn*917* randomly into the chromosomal DNA of *S. suis* strain P1/7 via electroporation [47]. *S. suis* Tn*917* insertion mutants were grown in the presence of 1 μg/ml of erythromycin (Sigma-Aldrich, Oakville, ON, Canada) and showed similar growth rate to wild-type strain P1/7 (data not shown).

4.2 PBMEC culture

The maintenance of the porcine brain microvascular endothelial cell line PBMEC/C1-2 [48] was previously described [17, 49]. A cell suspension of 8 x 10^4 cells/ml in culture medium was distributed in 24-well tissue culture plates (Primaria, BD Falcon™; BD Biosciences, Mississauga, ON, Canada) precoated with 1% (w/v) type A gelatin from porcine skin (Sigma-Aldrich, Oakville, ON, Canada) and incubated to confluence. Before the experiments, medium was removed from plates and replaced by medium without antibiotics.

4.3 Qualitative screening invasion assay

A qualitative screening assay, previously described [19], and with some modifications, was adapted to *S. suis*. Briefly, *S. suis* bacteria were grown in Todd-Hewitt broth (THB, Difco Laboratories, Detroit, MI, USA) for 8 h at 37 °C with agitation. Working cultures were obtained by inoculating 100 µl of a 10^{-1} dilution of these cultures in 5 ml of THB and incubating them for 16 h at 37 °C with agitation. Bacteria were washed in phosphate-buffered saline (PBS, pH 7.3) and 100 µl aliquots of bacterial suspension (approximately 1 x 10^8 CFU) were used to infect confluent cell monolayers from 24-well tissue culture plates containing 900 µl of cell culture medium (for an initial inoculum of 1 x 10^7 CFU/ml corresponding to a multiplicity of infection of approximately 40:1). Plates were centrifuged at 800 *g* for 10 min and incubated for 2 h at 37 °C with 5 % CO_2. The monolayers were washed twice with PBS, and incubated with 1 ml of cell culture medium containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin G (Sigma-Aldrich) for 1 h at 37 °C with 5 % CO_2. Then, cells were washed three times with PBS, and incubated with 200 µl of 0.05% trypsin-0.03% EDTA for 10 min at 37 °C. Cells were detached and disrupted by scraping the bottom of the well and repeated pipetting. This cell lysate was left in the well and mixed with 1 ml of THB containing a low concentration of agar (7.5 %, w/v) and incubated overnight at 37 °C. The number of *S. suis* colonies (representing intracellular invading *S. suis*) in the well were visually compared with the number of colonies obtained using the P1/7 wild-type strain (non-quantitative assay).

4.4 Quantitative invasion assay

The quantitative invasion assay was performed in essentially the same way as the qualitative invasion assay described above, but with some modifications. Bacteria were grown in THB for 8 h at 37 °C with agitation. Working cultures were obtained by inoculating 10 µl of a 10^{-3} dilution of these cultures into 30 ml of THB and incubating them for 16 h at 37 °C with agitation. Then, bacteria were pelleted, washed with PBS, and resuspended at 10^6 CFU/ml in fresh cell culture medium without antibiotics as previously described [17, 49]. An accurate determination of the number of colony forming units per ml (CFU/ml) in the final suspension was made by plating onto THB agar using Autoplate® 4000 (Spiral Biotech, Norwood, MA, USA). Confluent cell monolayers were infected with 1 ml aliquots of bacterial suspension for 2 h, as described above. After incubation with 0.05% trypsin-0.03% EDTA for 10 min at 37 °C, 800 µl of ice-cold deionised water was added and the cells were scraped from the bottom of the well. Serial dilutions of this cell lysate were plated onto THB agar and incubated overnight at 37 °C for counts of viable bacteria.

4.5 Plasmid rescue and sequencing of the insertion site

*Hind*III (Roche) digestions of chromosomal DNA were self-ligated using T4 DNA ligase (Quick Ligase kit from New England Biolabs, Pickering, ON, Canada) and then transformed into *Escherichia coli* XL1-Blue Supercompetent cells (Stratagene, La Jolla, CA) followed by selection of plasmids by plating onto Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml). Resultant colonies were picked and streaked out, and plasmid DNA was purified from liquid cultures using standard methods (QIAprep Spin Miniprep Kit, Qiagen, Mississauga, ON, Canada). The plasmids, containing inserts

of host chromosomal DNA flanking the transposon insertion site, were sequenced using a 373A DNA Sequencing System (Applied Biosystems, Foster City, CA) at the DNA Sequencing Facility of the University of Maine (Orono, ME). The primer tn*917* seq (5'-AGAGAGATGTCACCGTCAAGT-3'), designed to read out from the transposon, was used for sequencing. *S. suis* DNA sequences flanking the Tn*917* insertion sites. Sequences were analysed using the BLAST software package in the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparison was also performed against data produced by the *S. suis* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_suis) for European strain P1/7 and by the Joint Genome Institute Microbial Genomics **(**http://genome.jgi-psf.org/cgi-bin/runAlignment?db=strsu&advanced=1) for North American strain 89/1591.

Genomic DNA was isolated from the wild-type P1/7 and mutants strains by the guanidium thiocyanathe method [50]. DNA was digested with EcoRI restriction endonuclease (GE Healthcare Piscataway, NJ, USA). A digoxigenin (DIG)-labelled probe specific for the erm gene, (which is present in Tn917 but absent from the wild-type genome) was generated from pVT408 by PCR using primers ermF 5'-ACGAGTGAAAAAGTACTCAACC-3' and ermR 5'-ACCTCTGTTTGTTAGGGAATTG-3' (Invitrogen, Burlington, ON, Canada) and the DIG-PCRlabeling mixture (Roche Diagnostics, Laval, QC, Canada). Southern-blots were performed by the procedures described previously [51] except that hybridizations were carried out at 68°C.

4.6 Mouse infection

A total of 70 female CD1 6-week-old mice (Charles River Laboratories, Wilmington, MA, USA, 5 animals per group) were infected by intraperitoneal injection with 1 ml of either *S. suis* strain P1/7 or the Tn*917* insertion mutants A8E, B8A, E5H,

F8D, and I10B at approximately 2 x 10^7 CFU in THB. Mice infected with the vehicle solution (sterile THB) were used as controls [20]. This CD1 mouse model is useful to study the early septic shock-like syndrome leading to death and, the second late phase of *S. suis* infection that induces evident brain damage [20]. Body weight changes, clinical signs of infection (swollen eyes, rough hair coat, lethargy, and neurological signs) and survival were recorded daily post-infection (p.i.) over a 10-day (d) period as described [20]. In addition, the presence of *S. suis* in blood (collected by cardiac puncture) and homogenized brain and liver samples was determined by plating on sheep blood agar plates using Autoplate® 4000 at different times post-infection. Experiments involving mice were repeated twice (10 animals per group) and were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals, Animal Welfare Committee of the Université de Montréal.

4.7 Inoculation of pigs

Thirty Large White four-week-old piglets (from a high health status herd) were randomly grouped into 3 different pens one week before infection. Pigs were intravenously challenged via the ear vein with 1 ml of either *S. suis* strain P1/7, mutant B8A or mutant F8D at approximately 1 x 10^7 CFU in THB. Pigs were monitored daily p.i. for clinical signs of lameness, joint swelling, prostration, ataxia, recumbency, opisthotonus, paddling, and depression. Rectal temperature and body weight changes were also recorded. Results of rectal temperatures are expressed as fever index representing the percentage of observations for the experimental group of a body temperature >40°C. The presence of *S. suis* in blood samples was monitored when clinical signs appeared, and in organs (brain, meninges, liver) at post-mortem examinations [1]. Organs samples were homogenized with stainless steel beads (Qiagen), diluted in PBS and plated on Columbia agar (Oxoid Ltd., Basingstoke, UK)

plates with oxolinic acid (5 µg/ml), colistin sulphate (10 mµg/ml) and 5% (v/v) defibrinated horse blood. Pig experiments were conducted in the United Kingdom in accordance with the Animal Scientific Procedures Act 1986.

4.8 Determination of cytokine levels in plasma from experimentally infected pigs

Interleukin (IL)-6 and IL-8 were measured by ELISA using porcine-specific pair-matched antibodies from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's recommendations. Twofold dilutions of recombinant porcine IL-6 (78 to 5000 pg/ml, R&D Systems) and IL-8 (18 to 1200 pg/ml, R&D Systems) were used to generate standard curves. Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. Standard and sample dilutions were added in duplicate wells to each ELISA plate (Nunc, VWR, Ville Mont Royal, QC, Canada), and all analyses were performed at least three times for each plasma sample. Plates were read using a Molecular Devices UVmax (Molecular Devices Corp, Sunnyvale, CA, USA) microplate reader.

4.9 Statistical analysis

All data are expressed as means ± standard deviations (error bars). Unless specified, data were analyzed by two-tailed, unpaired *t* test and all assays were repeated at least three times. Statistical analysis of the survival and body weight data were performed with the LogRank test and the Mann-Whitney Rank Sum test, respectively. A *P* value of < 0.05 was considered as the threshold for significance.

Acknowledgments

We thank M.-P. Lecours, D. Meunier-Côté, C. A. Walker and M. Stubberfield for excellent technical assistance and Pr. T. Sekizaki (National Institute of Animal Health, Tsukuba, Ibaraki, Japan) for helpful discussions and advice. This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) and Centre de recherche en infectiologie porcine (CRIP-FQRNT) grants to M. Gottschalk, and by a Wellcome Trust Research Leave Fellowship for Clinical Academics to J. Slater. During these studies, G. Vanier was a recipient of both Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) and NSERC scholarships. M.C. Domínguez-Punaro and N. Fittipaldi are recipients of NSERC scholarships.

ANNEX 5. ARTICLE VIII. TABLES

Table 1: Genes identified from less invasive *S. suis* Tn*917* insertion mutants with PBMEC

Mutant	Gene [a]	Putative function (Organism)	Adhesion [b] (%)	Invasion [b] (%)	GenBank identification	Reference
Surface protein						
B8A	ssu1889	LPXTG-motif cell wall anchor domain (*Bacillus cereus subsp. cytotoxis* NVH 391-98)	66	21	YP_001376331	NA
Transport/binding						
F8D	ssu0835	ABC-type multidrug transport system, ATPase component (*S. suis* 05ZYH33)	37	18	ABP89860	[50]
H4F	ssu0833	Putative bacteriocin operon protein (*S. suis* 05ZYH33)	75	7	ABP89858	[50]
Regulatory functions						
B9B	ssu0828	Response regulator consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain (*S. suis* 98HAH33)	44	18	ABP92047	[50]
G7E	ssu1005	Regulatory protein, GntR (*S. suis* 89/1591)	37	18	ZP_00875100	NA
I10B	ssu0455	Putative mercuric resistant regulatory protein *merR* (*S. suis*)	50	27	BAB83973	[28]
Metabolism						
B1A	ssu0199	Phosphotransferase system cellobiose-specific component IIC (*S. suis* 05ZYH33)	13	29	ABP89180	[50]
B2D and C3A	ssu0890	Aldose 1-epimerase (*S. suis* 89/1591)	20	4	ZP_00874440	NA
D12D	ssu1175	Lactoylglutathione lyase and related lyases (*S. suis* 05ZYH33)	65	17	ABP90309	[50]
E5H	ssu0990	NUDIX hydrolase (*S. suis* 89/1591)	49	7	ZP_00875084	[46]
H3H	ssu0797	Short-chain alcohol dehydrogenase (*S. suis* 05ZYH33)	36	3	ABP89821	[50]
Amino acid synthesis						

E12F	ssu0838	Carbamoylphosphate synthase large subunit (*S. suis* 05ZYH33)	9	8	ABP89862	[50]
J7E	ssu1611	Aspartokinase (*S. suis* 05ZYH33)	48	7	ABP90777	[50]
H3F	ssu0411	Shikimate kinase (*S. suis* 05ZYH33)	73	25	ABP89424	[50]
Protein synthesis						
E7A	ssu0470	Lysyl-tRNA synthetase, class-II (*S. suis* 05ZYH33)	64	17	ABP89485	[50]
Hypothetical proteins						
A8E and E12D	ssu0906	Hypothetical protein (*S. suis* 05ZYH33)	13	30	YP_001198415	[50]
J2F	ssu0823	Hypothetical protein (*S. suis* 98HAH33)	59	7	YP_001200441	[50]

[a] Genes are named in accordance with the *S. suis* strain P1/7 sequencing project nomenclature.

NA: not available

[b] Levels of adhesion to and invasion of PBMEC obtained with parental strain P1/7 are considered as 100%.

Table 2: Virulence of *S. suis* wild-type strain P1/7 and five Tn*917* insertion mutants in CD1 mice

	No. of mice	Morbidity[a] (%)	Mortality[b] (%)	No. of mice in which *S. suis* was isolated from:		
				blood	brain	liver
P1/7	10	100	70	8	9	8
F8D	10	20	0	1	2	1
B8A	10	90	20	4	4	2
A8E	10	70	60	7	8	7
E5H	10	80	80	8	8	8
I10B	10	100	100	10	10	10
negative	10	0	0	0	0	0

[a]Percentage of mice with clinical symptoms.

[b]Percentage of mice that died due to infection or were sacrificed for animal welfare reasons.

These measurements were performed over a period of 10 day post-infection.

ANNEX 5. ARTICLE VIII. FIGURES

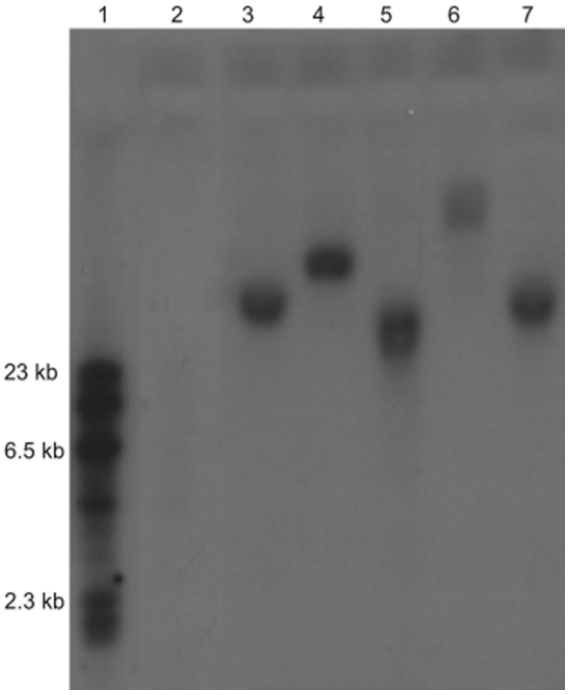


Fig. 1. Southern-blot of *S. suis* wild-type P1/7 and Tn*917* mutant chromosomal DNA digested with *EcoR*I restriction endonuclease and hybridized with a DIG-labelled-probe specific for the *erm* gene. Lane 1, DNA Molecular Weight Marker II, DIG-labelled (Roche); Lane 2, wild-type *S. suis* P1/7; Lane 3 to 7, 5 individual S. suis Tn917 mutants used in mouse *in vivo* experiments (A8E, B8A, E5H, F8D, and I10B, respectively). All the mutants presented only one insertion of the transposon.

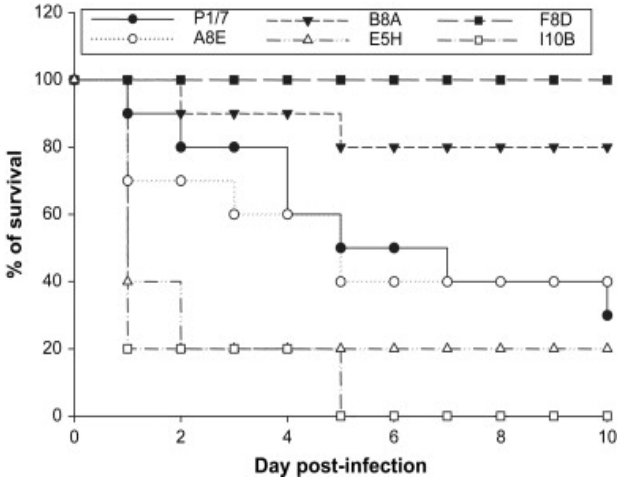


Fig. 2. Survival curves for CD1 mice infected with the wild-type strain P1/7 and 5 Tn*917* insertion mutants. Six-week-old CD1 mice were inoculated i.p. with 2 x 10^7 bacteria, and mice survival was monitored over a 10-day period. Data are expressed as mean percentage of live animals in each group (n=10).

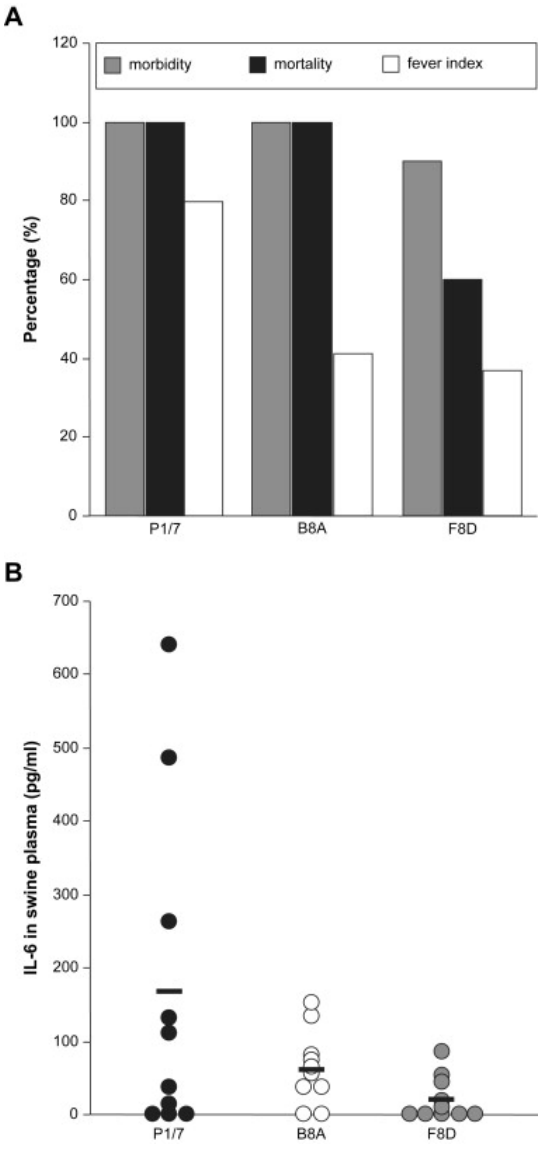


Fig. 3. (A) Virulence of *S. suis* wild-type strain P1/7 and two Tn*917* insertion mutants in pigs. Morbidity and mortality represent the percentage of pigs with clinical symptoms and the percentage of pigs that were sacrificed for animal welfare reasons, respectively. Fever indice represents the percentage of observations for the experimental group of a body temperature >40°C. **(B) Levels of IL-6 in swine plasma from pigs infected with *S. suis* wild-type strain P1/7 and two Tn*917* insertion mutants 24h post-infection.** Bars indicate mean IL-6 level for a group.

References

[1] Higgins R, Gottschalk M. Streptococcal Diseases, (Straw BE, Zimmerman JJ, D'Allaire S,Taylor DJ, ed.) Vol. Ames, Iowa: Blackwell Publishing; 2006, p. 769-83.

[2] Ye C, Zhu X, Jing H, Du H, Segura M, Zheng H, et al. *Streptococcus suis* sequence type 7 outbreak, Sichuan, China. Emerg Infect Dis 2006;12:1203-8.

[3] Willenburg KS, Sentochnik DE, Zadoks RN. Human *Streptococcus suis* meningitis in the United States. N Engl J Med 2006;354:1325.

[4] Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology 1998;144 (Pt 2):325-32.

[5] Baums CG, Kaim U, Fulde M, Ramachandran G, Goethe R, Valentin-Weigand P. Identification of a novel virulence determinant with serum opacification activity in *Streptococcus suis*. Infect Immun 2006;74:6154-62.

[6] Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, Stockhofe-Zurwieden N, et al. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect Immun 1999;67:1750-6.

[7] Gottschalk MG, Lacouture S, Dubreuil JD. Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiology 1995;141 (Pt 1):189-95.

[8] de Greeff A, Buys H, Verhaar R, Dijkstra J, van Alphen L, Smith HE. Contribution of fibronectin-binding protein to pathogenesis of *Streptococcus suis* serotype 2. Infect Immun 2002;70:1319-25.

[9] Brassard J, Gottschalk M, Quessy S. Cloning and purification of the *Streptococcus suis* serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin. Vet Microbiol 2004;102:87-94.

[10] Jobin MC, Grenier D. Identification and characterization of four proteases produced by *Streptococcus suis*. FEMS Microbiol Lett 2003;220:113-9.

[11] Vecht U, Arends JP, Van der Molen EJ, van Leengoed LAMG. Differences in virulence between two strains of *Streptococcus suis* type II after experimentally induced infection of newborn germ-free pigs. Am. J. Vet. Res. 1989;50:1037-43.

[12] Vecht U, Wisselink HJ, Jellema ML, Smith HE. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun 1991;59:3156-62.

[13] Wilson TL, Jeffers J, Rapp-Gabrielson VJ, Martin SM, Klein LK, Lowery DE, et al. A novel signature-tagged mutagenesis system for *Streptococcus suis* serotype 2. Vet Microbiol 2007;doi:10.1016/j.vetmic.2006.12.025.

[14] Gottschalk M, Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol 2000;76:259-72.

[15] Goldstein GW, Betz AL. The blood-brain barrier. Sci Am 1986;255:74-83.

[16] Kniesel U, Wolburg H. Tight junctions of the blood-brain barrier. Cell Mol Neurobiol 2000;20:57-76.

[17] Vanier G, Segura M, Friedl P, Lacouture S, Gottschalk M. Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. Infect Immun 2004;72:1441-9.

[18] Fittipaldi N, Gottschalk M, Vanier G, Daigle F, Harel J. Use of selective capture of transcribed sequences to identify genes preferentially expressed by *Streptococcus suis* upon interaction with porcine brain microvascular endothelial cells. Appl Environ Microbiol 2007;73:4359-64.

[19] Nizet V, Smith AL, Sullam PM, Rubens CE. A simple microtiter plate screening assay for bacterial invasion or adherence. Methods in Cell Science 1998;20:107-11.

[20] Dominguez-Punaro MC, Segura M, Plante MM, Lacouture S, Rivest S, Gottschalk M. *Streptococcus suis* serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J Immunol 2007;179:1842-54.

[21] Segura M, Vanier G, Al-Numani D, Lacouture S, Olivier M, Gottschalk M. Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system. FEMS Immunol Med Microbiol 2006;47:92-106.

[22] Fossum C, Wattrang E, Fuxler L, Jensen KT, Wallgren P. Evaluation of various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers for acute bacterial infection in swine--a possible role for serum interleukin-6. Vet Immunol Immunopathol 1998;64:161-72.

[23] Fossum C. Cytokines as markers for infections and their effect on growth performance and well-being in the pig. Domest Anim Endocrinol 1998;15:439-44.

[24] Gottschalk M, Segura M, Xu J. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Animal Health Research Reviews 2007;8:1-17.

[25] Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 1999;63:174-229.

[26] Li Y, Gottschalk M, Esgleas M, Lacouture S, Dubreuil JD, Willson P, et al. Immunization with recombinant Sao protein confers protection against *Streptococcus suis* infection. Clin Vaccine Immunol 2007;doi:10.1128/CVI.00046-07.

[27] Li Y, Martinez G, Gottschalk M, Lacouture S, Willson P, Dubreuil JD, et al. Identification of a surface protein of *Streptococcus suis* and evaluation of its immunogenic and protective capacity in pigs. Infect Immun 2006;74:305-12.

[28] Osaki M, Takamatsu D, Shimoji Y, Sekizaki T. Characterization of *Streptococcus suis* genes encoding proteins homologous to sortase of gram-positive bacteria. J Bacteriol 2002;184:971-82.

[29] Dramsi S, Trieu-Cuot P, Bierne H. Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res Microbiol 2005;156:289-97.

[30] Hava DL, Hemsley CJ, Camilli A. Transcriptional regulation in the *Streptococcus pneumoniae rlrA* pathogenicity islet by RlrA. J Bacteriol 2003;185:413-21.

[31] Hemsley C, Joyce E, Hava DL, Kawale A, Camilli A. MgrA, an orthologue of Mga, Acts as a transcriptional repressor of the genes within the *rlrA* pathogenicity islet in *Streptococcus pneumoniae*. J Bacteriol 2003;185:6640-7.

[32] Vanier G, Sekizaki T, Dominguez-Punaro MC, Esgleas M, Osaki M, Takamatsu D, et al. Disruption of *srtA* gene in *Streptococcus suis* results in decreased interactions with endothelial cells and extracellular matrix proteins. Vet Microbiol 2007;In press.

[33] Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol 2006;4:509-19.

[34] Maisey HC, Hensler M, Nizet V, Doran KS. Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. J Bacteriol 2007;189:1464-7.

[35] Jacques M, Gottschalk M, Foiry B, Higgins R. Ultrastructural study of surface components of *Streptococcus suis*. J Bacteriol 1990;172:2833-8.

[36] Podlesek Z, Comino A, Herzog-Velikonja B, Zgur-Bertok D, Komel R, Grabnar M. *Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance. Mol Microbiol 1995;16:969-76.

[37] Davidson AL, Chen J. ATP-binding cassette transporters in bacteria. Annu. Rev. Biochem. 2004;73:241-68.

[38] Mitrakul K, Loo CY, Gyurko C, Hughes CV, Ganeshkumar N. Mutational analysis of the *adcCBA* genes in *Streptococcus gordonii* biofilm formation. Oral Microbiol Immunol 2005;20:122-7.

[39] Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol 2003;49:905-18.

[40] Matthysse AG, Yarnall HA, Young N. Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. J Bacteriol 1996;178:5302-8.

[41] Motegi M, Takagi Y, Yonezawa H, Hanada N, Terajima J, Watanabe H, et al. Assessment of genes associated with *Streptococcus mutans* biofilm morphology. Appl Environ Microbiol 2006;72:6277-87.

[42] Wigfield SM, Rigg GP, Kavari M, Webb AK, Matthews RC, Burnie JP. Identification of an immunodominant drug efflux pump in *Burkholderia cepacia*. J Antimicrob Chemother 2002;49:619-24.

[43] Grenier D, Grignon L, Gottschalk M. Characterisation of biofilm formation by a *Streptococcus suis* meningitis isolate. Vet J 2007;doi:10.1016/j.tvjl.2007.09.005.

[44] Melancon D, Grenier D. Production and properties of bacteriocin-like inhibitory substances from the swine pathogen *Streptococcus suis* serotype 2. Appl Environ Microbiol 2003;69:4482-8.

[45] McLaughlin RE, Ferretti JJ, Hynes WL. Nucleotide sequence of the streptococcin A-FF22 lantibiotic regulon: model for production of the lantibiotic SA-FF22 by strains of *Streptococcus pyogenes*. FEMS Microbiol Lett 1999;175:171-7.

[46] Bessman MJ, Walsh JD, Dunn CA, Swaminathan J, Weldon JE, Shen J. The gene *ygdP*, associated with the invasiveness of *Escherichia coli* K1, designates a Nudix hydrolase, Orf176, active on adenosine (5')-pentaphospho-(5')-adenosine (Ap5A). J Biol Chem 2001;276:37834-8.

[47] Slater JD, Allen AG, May JP, Bolitho S, Lindsay H, Maskell DJ. Mutagenesis of *Streptococcus equi* and *Streptococcus suis* by transposon Tn*917*. Vet Microbiol 2003;93:197-206.

[48] Teifel M, Friedl P. Establishment of the permanent microvascular endothelial cell line PBMEC/C1-2 from porcine brains. Exp Cell Res 1996;228:50-7.

[49] Vanier G, Segura M, Gottschalk M. Characterization of the invasion of porcine endothelial cells by *Streptococcus suis* serotype 2. Can J Vet Res 2007;71:81-9.

[50] Chen C, Tang J, Dong W, Wang C, Feng Y, Wang J, et al. A glimpse of streptococcal toxic shock syndrome from comparative genomics of *S. suis* 2 Chinese isolates. PLoS ONE 2007;2:e315.