Proinflammatory cytokine and chemokine modulation by *Streptococcus suis* in a whole-blood culture system

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Abstract

Streptococcus suis is an important swine and human pathogen. Inflammation, a hallmark of *S. suis* infection, is thought to be responsible for most clinical signs of meningitis, septicaemia and sudden death. In this work, using a porcine whole blood model, *S. suis* serotype 2 was shown to trigger the release of several pro-inflammatory cytokines as evaluated by reverse transcriptase-PCR and enzyme-linked immunosorbent assay. Although individual variations were observed among different *S. suis* strains, no correlations were observed between the strain origin/phenotype and cytokine levels. Live bacteria induced higher tumour necrosis factor alpha, interleukin-1 beta (IL-1β) and IL-6 levels than did heat-killed bacteria. In contrast, heat-killed bacteria stimulated higher levels of IL-8 and monocyte chemotactic protein one (MCP-1). The bacterial cell wall was observed to be the major cytokine-inducting components, whereas capsule expression was important for MCP-1 activation. The presence of specific antibodies suppressed bacterial growth resulting in significantly reduced levels of cytokine production. Thus, antibody-mediated bacterial phagocytosis combined with suppressed inflammation may be beneficial for infection control strategies. We provide first evidence of *S. suis*-induction of pro-inflammatory swine cytokines and demonstrate the strength and relevance of the whole blood culture systems in the investigation of *S. suis* modulation of cytokine production.

Introduction

Streptococcus suis is an important swine pathogen responsible for a wide variety of diseases, such as meningitis, endocarditis, arthritis and pneumonia (Higgins & Gottschalk, 1999). It is also recognized as a zoonotic agent that causes septicaemia, meningitis and/or endocarditis in humans, especially those occupationally exposed to pigs or swine by-products (Arends & Zanen, 1988; François *et al.*, 1998). Indeed, recent cases of human disease in Sichuan Province, China were directly linked to a concurrent outbreak of *S. suis* infection in pigs (http://www.wpro.who.int/media_centre/news/news_20050816.htm). Of the 33 serotypes officially described, *S. suis* serotype 2 is considered to be the most prevalent and virulent in diseased pigs and humans (Higgins & Gottschalk, 1999).

The clinical presentation of *S. suis* infection may vary from asymptomatic bacteraemia to fulminate systemic disease similar to Gram-negative sepsis. Meningitis is the most striking feature, and fibrin, oedema and cellular infiltrates in the meninges are typically observed (Gottschalk & Segura, 2000). The symptoms reported in the latest outbreak in China included high fever, malaise, nausea and vomiting, followed by meningitis, subcutaneous haemorrhage, toxic shock and coma in severe cases. The increased severity of *S. suis* infections in humans, such as a shorter incubation time, more rapid disease progression and a higher rate of mortality, underscores the critical need for a better understanding of the factors associated with the pathogenesis of *S. suis* infection. Indeed, the mechanisms underlying *S. suis* infection are poorly understood and are thought to involve multistep processes. It is generally accepted that the pathogen is transmitted via the respiratory route and remains within the palatine tonsils. From there, bacteria can travel either freely or bound to phagocytes in the circulation to reach the central nervous system (Gottschalk & Segura, 2000; Segura &

Gottschalk, 2002). The mechanisms used by *S. suis* to cross the blood–brain barrier are poorly understood. *Streptococcus suis* invasion of porcine brain microvascular endothelial cells (BMECs) has been reported recently (Vanier *et al.*, 2004). However, other mechanisms, such as the upregulation of proinflammatory mediators and increased leucocyte trafficking, may also contribute to the breakdown of the blood–brain barrier (Gottschalk & Segura, 2000).

The critical virulence factors for *S. suis* serotype 2 have not been clearly defined. The presence of a polysaccharide capsule (CPS) has been correlated with increased resistance to phagocytosis by murine and porcine phagocytes (Charland *et al.*, 1998; Segura *et al.*, 1998). Indeed, nonencapsulated mutants have been shown to be avirulent in the experimental inoculation of pigs (Charland *et al.*, 1998; Smith *et al.*, 1999). However, natural avirulent *S. suis* strains are well encapsulated, indicating that other important virulence factors are essential. Indeed, a haemolysin (suilysin), two functionally undefined proteins [muramidase-released protein (MRP) and extracellular factor (EF)] and the recently described fibrinonectin- and fibrinogen-binding protein have been proposed as putative virulence factors (Gottschalk *et al.*, 1995; Smith *et al.*, 1997; de Greeff *et al.*, 2002; Winterhoff *et al.*, 2002). As isogenic mutants lacking MRP and EF, and those lacking suilysin, are still pathogenic in young piglets, these proteins are not absolutely required for virulence (Smith *et al.*, 1996; Allen *et al.*, 2001; Lun *et al.*, 2003). Interestingly, important differences exist between European and North American strains of *S. suis* serotype 2, as the virulence 'markers' EF, MRP and suilysin are usually absent in North American strains (Gottschalk *et al.*, 1998). These two phenotypes (MRP^+, EF^+, $suilysin^+$ and MRP^-, EF^-, $suilysin^-$) are also genotypically different (Chatellier *et al.*, 1999). It has been proposed that the pathogenesis of the infection caused by these two groups of virulent *S. suis* strains may be different (Gottschalk & Segura, 2000).

Although robust proinflammatory cytokine responses from cells of the innate immune system are important for controlling acute infection, the potential for these responses to contribute to immune-mediated pathology has been well documented. Indeed, proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, as well as several chemokines, have been associated with bacteria-induced meningeal inflammation and septic shock with consequent clinical deterioration (van Furth *et al.*, 1996; Sprenger *et al.*, 1996; Lahrtz *et al.*, 1998).

Although fibrinopurulent histopathological lesions have been reported in *S. suis* infections, and septic shock conditions have been described in the past and recently in the China outbreak (Leelarasamee *et al.*, 1997; Madsen *et al.*, 2002), few studies have addressed the capacity of this pathogen to upregulate inflammatory mediators. It has been shown that *S. suis*, especially its cell wall components, is able to elicit the production of TNF-α and IL-6 from murine macrophages (Segura *et al.*, 1999). The upregulated expression of adhesion molecules, as well as the induction of TNF-α, IL-1β, IL-6 and the chemokines IL-8 and monocyte chemotactic protein-1 (MCP-1), has been reported in *S. suis*-infected human monocytes, suggesting a possible mechanism of the modulation of leucocyte infiltration (Gottschalk & Segura, 2000; Al-Numani *et al.*, 2002; Segura *et al.*, 2002). Finally, the production of IL-6, MCP-1 and IL-8 has been observed in *S. suis*-activated human BMECs (Vadeboncoeur *et al.*, 2003). However, no data are available on *S. suis*-induced cytokine production by cells of swine origin using swine-specific cytokine detection techniques. To this end, whole-blood systems have been used extensively to study the expression and regulation of cytokines of human (DeForge & Remick, 1991; Oliver *et al.*, 1993; Foca *et al.*, 1998) and, less frequently, animal (Yancy *et al.*, 2001) origin. This system contains both mononuclear and polymorphonuclear leucocytes, and therefore may represent a useful and relevant *in vitro* approach to study inflammatory events occurring *in vivo*. In the present study, we used the porcine whole-blood model to investigate the induction of proinflammatory cytokines and chemokines by *S. suis* and to determine the contribution of different bacterial components to cytokine activation.

Materials and methods

Bacterial strains and growth conditions

The *Streptococcus suis* serotype 2 European virulent strain 31533 (MRP^+, EF^+, $suilysin^+$), originally isolated from a case of porcine meningitis and previously used for cytokine induction studies with murine and human cells (Segura *et al.*, 1999, 2002; Vadeboncoeur *et al.*, 2003), and the Canadian virulent strain 89–1591 (MRP^-, EF^-, $suilysin^-$) were used as reference strains. In addition, two isogenic mutants derived from strain 31533 were used: the nonencapsulated mutant B218 and the suilysin-negative mutant SX-911. Mutant B218 was produced in our laboratory by allelic exchange, and corresponds to a previously reported transposon-derived mutant (Charland *et al.*, 1998). Mutant B218 was confirmed to be nonencapsulated by electron microscopy and has been genetically characterized (N. Fittipaldi *et al.*, unpublished data). Mutant SX-911 was kindly provided by Dr P. Willson (Vaccine Infectious Disease Organization, Saskatoon, Canada), and was produced as reported previously (Lun *et al.*, 2003). For comparative purposes, several *S. suis* serotype 2 representative strains of known origin and phenotype (Table 1) were also tested for their capacity to induce proinflammatory cytokines.

Bacteria, maintained as stock cultures in 50% glycerol–Todd–Hewitt broth (THB; Difco, Detroit, MI) at −80 °C,

were grown overnight on sheep blood agar plates at 37 °C, and isolated colonies were used as inocula for THB, which were incubated for 8 h at 37 °C. Working cultures for blood stimulation were produced by inoculating 10 μL from a 1 : 1000 dilution of the 8 h culture into 30 mL of THB at 37 °C for 16 h with agitation. Bacteria were then washed twice in phosphate-buffered saline (PBS) and resuspended to 10^6 CFU mL^{-1} in cell culture medium. This concentration was sufficient to induce significant levels of cytokine production without any cytotoxic effects (data not shown). The final suspension was plated onto THB agar to accurately determine the CFU mL^{-1}.

For comparative purposes, in selected experiments, heat-killed bacterial suspensions were used for the stimulation of blood cells. Heat-killed bacteria were produced as described previously by incubating organisms at 60 °C for 45 min, which is the minimal condition required to kill *S. suis* cultures (Segura *et al.*, 1999). Killed bacterial preparations were stored in PBS at 4 °C, and the required number of bacteria were resuspended in cell culture medium immediately before stimulation assays.

Bacterial components

Lipopolysaccharide (LPS) from *Escherichia coli* O127 : B8 (Sigma-Aldrich, Oakville, ON, Canada) was used at 1 μg mL^{-1} as a positive control. Purified CPS of *S. suis* serotype 2 was prepared as reported previously (Sepulveda *et al.*, 1996), and used at concentrations in the range 1–50 μg mL^{-1}. Purified cell wall was produced as described previously (Segura *et al.*, 1999), and used at 1–250 μg mL^{-1}. Purified suilysin was kindly provided by A. Jacobs (Intervet International, Boxmeer, the Netherlands), and was activated by the addition of 0.1% 2β-mercaptoethanol before use at concentrations in the range 1–100 ng mL^{-1}.

Bacterial opsonization

In selected experiments, bacterial opsonization was performed by incubating bacteria in the presence of 20% of homologous anti-*S. suis* pig serum during the stimulation assay (see below). The serum tested positive for the presence of anti-*S. suis* antibodies by enzyme-linked immunosorbent assay (ELISA), and was obtained from an experimentally infected convalescent animal (data not shown). Normal pig serum was used as control.

Blood collection and cell stimulation

Blood from four normal Yorkshire pigs, aged 6–12 weeks, was collected from the jugular vein using Vacutainer Heparin blood collection tubes and mixed at equal proportions. Another tube was also collected to obtain a sample for haematological analysis. Animals originated from a specific pathogen-free herd which had not presented any isolation of *S. suis* from diseased animals for at least the last 2 years. Blood was then diluted 1 : 3 in RPMI 1640 culture medium (Invitrogen, Burlington, ON, Canada), and 6 mL of this diluted blood was plated per well into six-well tissue culture plates (Becton Dickinson, Bedford, MA). This blood suspension corresponded to 4×10^6 leucocytes mL^{-1}. Bacteria or purified components were then added as indicated in the 'Results' section, and plates were incubated at 37 °C in 5% CO_2. Stimulated or unstimulated (control) samples were collected at different time intervals up to 24 h. Cells were

Table 1. *Streptococcus suis* type 2 strains used in this study

			Presence of virulence markers[a]		
Strain	Geographic origin	Isolated from	Suilysin	MRP	EF
31533[b]	France	Diseased pig, meningitis	+	+	+
166′	France	Diseased pig, meningitis	+	+	+
24	France	Diseased pig, septicaemia	+	+	+
S735[c]	The Netherlands	Diseased pig, pneumonia	+	+	*
D282	The Netherlands	Diseased pig, meningitis	+	+	+
4005	The Netherlands	Diseased pig, meningitis	+	+	+
89-1591[b]	Canada	Diseased pig, septicaemia	−	−	−
95-8242	Canada	Diseased pig, meningitis	+	+	+
98-B575	Canada	Diseased pig, septicaemia	−	+s	−
98-8993	Canada	Diseased pig, septicaemia	−	+s	−
98-B099	Canada	Diseased pig, septicaemia	−	−	−
90-1330	Canada	Diseased pig, pneumonia	−	+	−

*indicates larger molecular weight variants of EF; '+s' indicates lower molecular weight variants of the MRP protein.

[a]EF, extracellular factor (110 kDa); MRP, muramidase-released protein (136 kDa).

[b]Strains used as reference in the present work, as well as in previous studies on *S. suis*-induced cytokine production (Segura *et al*. 1999, 2002; Vadeboncoeur *et al*. 2003).

[c]ATCC 43765 *S. suis* type 2 reference strain.

harvested for mRNA extraction, and supernatants were kept frozen until analysis. Unstimulated (time 0) samples were immediately processed to assess basal cytokine expression at the time of blood collection. All samples were processed within 2 h after their collection from the animals to avoid modulation of cytokine expression (Duvigneau *et al.*, 2003). In selected experiments, blood cultures were infected with *S. suis* strains, and bacterial growth after different incubation times was determined by plating serial dilutions of this culture onto THB agar to accurately determine the CFU mL^{-1}.

Total RNA extraction

At each sampling time (0, 1, 2, 4, 8, 12 and 24 h), culture plates were centrifuged for 5 min at 600 ***g***. Supernatants were collected, divided into aliquots and stored at −80 °C for cytokine analysis by ELISA. The cell pellet was treated with 0.83% ammonium chloride to lyse red blood cells, and washed twice with cell culture medium. The white blood cell pellet was resuspended and lysed by repeated pipetting in 1 mL of Trizol reagent (Invitrogen). Total RNA was extracted as specified by the manufacturer. The final RNA pellet was resuspended in 20 μL of diethyl pyrocarbonate-treated water, and the RNA concentration and purity were measured using an Ultrospec 2100 pro UV/visible spectrophotometer (Biochrom Ltd, Cambridge, UK). RNA was stored at −80 °C until analysis.

Reverse transcriptase-PCR (RT-PCR) for porcine cytokines

Total RNA (2 μg) was mixed with 400 ng of random primers (Roche Molecular Biochemicals, Indianapolis, IN), 2 μL of 10 mM dNTP mixture (Pharmacia Biosciences, Baie D'urfe, QC, Canada) and DEPC-treated water up to 24 μL, heated at 65 °C for 5 min and cooled at 4 °C in a Biometra T-Gradient thermocycler (Biometra GmbH, Goettingen, Germany). A second mixture (14 μL), consisting of 8 μL of 5× First-strand buffer, 4 L of 0.1 M dithiothreitol (DTT) and 2 μL of RNA-guard ribonuclease inhibitor (Pharmacia Biosciences), was added and the reaction sample was incubated for 10 min at 25 °C, and then for 2 min at 42 °C. Superscript II reverse transcriptase (2 μL of 200 U L^{-1}; Invitrogen) was added, and the final mixture was incubated for 50 min at 42 °C, and then for 15 min at 70 °C, and held at 4 °C. Samples were stored at −20 °C until future use.

Primers for the cytokines (Invitrogen) used in this study are listed in Table 2. The PCR mixture was composed of 4 μL of cDNA, 1 μL of 10 mM dNTP (DNA polymerization mixture, Amersham Biosciences), 2.5 μL of 10 × Taq Buffer (Roche Molecular Biochemicals), 0.4 μL of each cytokine and housekeeping gene primers, 1 μL Taq DNA polymerase (Roche Molecular Biochemicals) and water up to a total volume of 25 μL. For IL-8 primers, the commercial Roche buffer was substituted with a home-made buffer (10 × 100 mM Tris-HCl, 17.5 mM $MgCl_2$, 500 mM KCl, pH 8.3). The PCR cycling consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min (57 °C for IL-8), elongation at 72 °C for 1 min and a final elongation at 72 °C for 7 min. The number of cycles used for each cytokine is listed in Table 2. An amount of 15 μL of PCR product was separated in a 1.8% Tris-acetate EDTA buffer (TAE) agarose gel, stained with ethidium bromide and photographed. The intensity of the bands was quantified by densitometry with an AlphaImager 2000 Multimage camera (Alpha Innotech Corp., San Leandro, CA) and software (AlphaEase 3.2). To compare the relative mRNA expression levels in each of the samples, the values are presented as the ratio of the band intensities of the cytokine RT-PCR product over the

Table 2. Reverse transcriptase-PCR (RT-PCR) conditions and cytokine primers used in this study

Primer pair	Sequence 5′–3′	bp	*T* (°C)	Cycles	HK gene
IL1β-F	TCAGGCAGATGGTGTCTGTC	430	55	32	GAPDH
IL1β-R	GGTCTATATCCTCCAGCTGC				
IL6-F	GGAACGCCTGGAAGAAGATG	470	55	35	β2M
IL6-R	ATCCACTCGTTCTGTGACTG				
IL8-F	TGCAGCTTCATGGACCAG	350	57	36	GAPDH
IL8-R	TGTTGCTTCTCAGTTCTCTTC				
TNF-α-F	CACTGAGAGCATGATCCGAG	470	55	35	β2M
TNF-α-R	GGCTGATGGTGTGAGTGAGG				
MCP-1-F	ATTAATTCTCCAGTCACCTG	420	55	35	β2M
MCP-1-R	AACACCAGTAGTCATGGAGG				
β2M-F	CTGCTCTCACTGTCTGG	295	55–60	NA	NA
β2M-R	ATCGAGAGTCACGTGCT				
GAPDH-F	CACTGGTGTCTTCACGAC	295	55–60	NA	NA
GAPDH-R	GCCATCCACAGTCTTCTG				

β2M, β2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HK, housekeeping; IL, interleukin; MCP-1, monocyte chemotactic protein-1; NA, not applicable; *T*, annealing temperature; TNF-α, tumor necrosis factor-α.

corresponding housekeeping RT-PCR product run simultaneously. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β2-microglobulin were both tested as housekeeping genes to ensure that there was no competition between the cytokine and housekeeping primers, and the gene giving the best result for each cytokine is presented (see Table 2).

Cytokine quantification by ELISA

IL-1β was quantified using the Immunoassay kit Swine IL-1β (Biosource International, Camarillo, CA), as specified by the manufacturer. TNF-α, IL-6 and IL-8 were measured by sandwich ELISA using porcine-specific pair-matched antibodies from R&D Systems (Minneapolis, MN), according to the manufacturer's recommendations. MCP-1 was measured using monoclonal antihuman MCP-1 and biotinylated antihuman MCP-1 antibody (R&D Systems). Twofold dilutions of recombinant porcine TNF-α and IL-6 (78–5 000 pg mL^{-1}, R&D Systems), porcine IL-8 (18–600 pg mL^{-1}, R&D Systems) and human MCP-1 (7–1000 pg mL^{-1}, R&D Systems) were used to generate standard curves. Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. Standard and sample dilutions were added in duplicate wells to each ELISA plate (Nunc, VWR, Ville Mont Royal, QC, Canada), and all analyses were performed at least four times for each individual stimulation assay. Plates were read in a Molecular Devices UVmax (Molecular Devices Corp., Sunnyvale, CA) microplate reader.

Endotoxin contamination

All solutions and bacterial preparations used in these experiments were tested for the presence of endotoxin using a *Limulus* amoebocyte lysate (LAL) gel-clot test (Pyrotell, STV, Cape Cod, MA) with a sensitivity limit of 0.03 EU mL^{-1}. In some experiments, endotoxin contamination during the stimulation of blood was controlled by parallel assays with polymixin B at 10 μg mL^{-1} (data not shown). Cell culture medium and solutions contained less than 0.03 EU mL^{-1}, a concentration below that which is known to cause cell activation (Martin & Dorf, 1991).

Statistics

Each test of blood culture activation was performed at least in triplicate. Cytokine mRNA semiquantitative values for RT-PCR are expressed as the mean cytokine/housekeeping ratios pooled from three independent experiments, and quantitative values obtained by ELISA are expressed as the means ± standard deviation (SD) of pg mL^{-1} values. Differences were analysed for significance using Student's unpaired *t*-test (two-tailed *P* value). $P < 0.05$ was used as the threshold for significance.

Results

Kinetics of porcine cytokine gene expression

The incubation of blood without stimuli yielded low and constant basal levels of cytokine mRNA expression (Figs 1a–d), with the exception of MCP-1 which started to increase at 4 h poststimulation and reached a plateau at 12 h (Fig. 1e). The stimulation of blood cells with LPS (positive control), heat-killed bacteria or live *Streptococcus suis* resulted in time-dependent cytokine production. Live bacteria induced higher and more sustained levels of TNF-α, IL-1β and IL-6 mRNA expression than did heat-killed bacteria, but the latter induced higher levels of both chemokines (MCP-1 and IL-8). Stimulation with heat-killed (10^6 or 10^8 CFU mL^{-1}) or live (10^6 or 10^8 CFU mL^{-1}) *S. suis* was dose dependent (data not shown). Interestingly, the kinetics of cytokine production induced by European and Canadian virulent strains were similar (data not shown), and only the results obtained with the European strain 31533 are presented in Fig. 1.

Stimulation with LPS or heat-killed *S. suis* induced similar time-dependent TNF-α and IL-1β mRNA expression, with values peaking at 1–2 h poststimulation and decreasing thereafter. Live bacteria induced a delayed peak at 4 h poststimulation, but the expression levels were sustained considerably longer throughout the incubation time for both cytokines (Figs 1a and b). For IL-6, LPS-induced mRNA expression occurred at an earlier time than that observed with heat-killed or live bacteria (at 2 and 4 h poststimulation, respectively). However, as was the case for TNF-α and IL-1β, only live bacteria induced high and sustained levels of mRNA expression (Fig. 1c).

By contrast with the results obtained for the proinflammatory cytokines, heat-killed bacteria induced higher mRNA expression of the chemokines IL-8 and MCP-1 than did live bacteria (Figs 1d and e). For IL-8, mRNA expression peaked at similar times after stimulation with LPS, heat-killed or live bacteria (between 2 and 4 h). However, LPS and heat-killed bacteria induced a higher expression of IL-8 mRNA that gradually decreased, whereas live bacteria induced lower levels that were sustained up to 24 h (Fig. 1d). Opposite results were observed for MCP-1; LPS and heat-killed bacteria induced continuous increases in mRNA expression, whereas the levels induced by live bacteria gradually decreased to baseline between 12 and 24 h poststimulation (Fig. 1e).

As a significant peak of mRNA expression was generally observed after 4 h of incubation, this time was used as the endpoint for the following experiments.

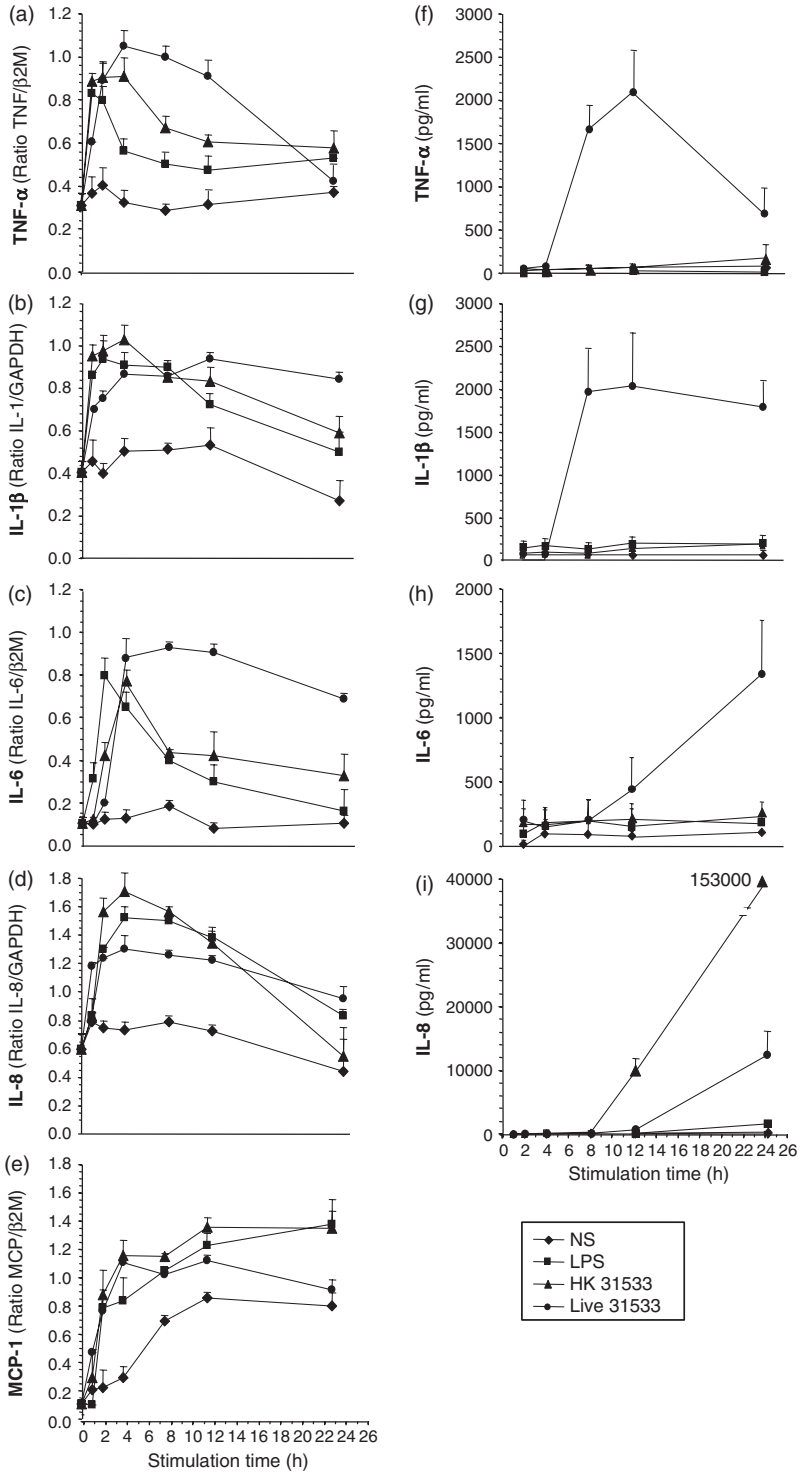

Fig. 1. Kinetics of production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) by whole-blood cells not stimulated (NS) or stimulated with lipopolysaccharide (LPS) (1 μg mL^{-1}), heat-killed (HK, 10^8 CFU mL^{-1}) or live (10^6 CFU mL^{-1}) *Streptococcus suis* strain 31533. (a–e) Kinetics of mRNA expression. Leucocytes were lysed in Trizol reagent before RNA extraction and reverse transcriptase-PCR (RT-PCR) analysis. Data are expressed as the mean ratio of cytokine over housekeeping gene expression pooled from three independent experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β2-microglobulin (β2M) was used as housekeeping gene. (f–i) Kinetics of protein secretion. Blood cell culture supernatants were harvested after stimulation and assayed for cytokine production by enzyme-linked immunosorbent assay (ELISA). Data were pooled from three independent experiments and are expressed as the mean ± SD in pg mL^{-1}.

Kinetics of cytokine secretion

The secretion of cytokines in blood cultures was also evaluated by ELISA (Figs 1f–i). In general, LPS and heat-killed bacteria stimulated low production of the proinflammatory cytokines TNF-α, IL-1β and IL-6. On the other hand, live bacteria induced a significantly higher secretion of these cytokines, a finding that correlated with the higher and

more sustained levels of mRNA expression shown in Figs 1a–c. High levels of TNF-α and IL-1β were detected after 6 h of incubation, peaking between 8 and 12 h poststimulation, whereas IL-6 release reached significant levels between 12 and 24 h.

Different results were obtained for the chemokine IL-8. Most strikingly, elevated IL-8 protein levels were observed when cells were activated with heat-killed *S. suis*, but not with LPS, although both stimulants induced similar patterns of mRNA expression. Heat-killed bacteria stimulated an increase in IL-8 secretion at 8 h, and high levels ($>150\ ng\ mL^{-1}$) were observed at 24 h poststimulation (Fig. 1i). Live bacteria induced lower and more delayed IL-8 secretion (at 12 h poststimulation) than did heat-killed bacteria. This delayed IL-8 secretion detected in the supernatants may reflect the low but sustained levels of mRNA expression observed in live *S. suis*-activated blood cultures (Fig. 1d).

Although human antibodies have been used previously to detect porcine MCP-1 (Ekekezie *et al.*, 2001), in our hands, the sensitivity of the human ELISA test was not sufficiently adequate to detect MCP-1 in the porcine blood culture supernatants (data not shown). As antibodies against porcine MCP-1 were not commercially available at the time the experiments were performed, detection of porcine MCP-1 protein was abandoned in subsequent experiments.

On the basis of the kinetics of protein secretion described above, an incubation time of 12 h was selected for the evaluation of TNF-α and IL-1β production, whereas IL-6 and IL-8 secretion was measured at 24 h in subsequent experiments.

Induction of cytokine mRNA expression by different *Streptococcus suis* strains

Several strains of different origins (Table 1) were compared for their capacity to elicit cytokine gene expression from blood cells. As mentioned above, cytokine mRNA was measured at 4 h because this time point represented the peak or the beginning of the plateau phase of mRNA expression for most cytokines evaluated here. An initial concentration of $10^6\ CFU\ mL^{-1}$ of each bacterial strain was used and similar bacterial growth rates were observed for all strains after 4 h of incubation in blood (final concentration of $10^8\ CFU\ mL^{-1}$; data not shown). No consistent associations were observed between cytokine production and the geographical origin or phenotype of the strains studied (Fig. 2). These results suggest that there were only minor individual differences in the capacity of different strains of *S. suis* type 2 to induce cytokine mRNA expression. As no significant differences between strains were observed at the mRNA level, which was demonstrated to be a more sensitive technique than ELISA, proteins levels were not measured in this experiment.

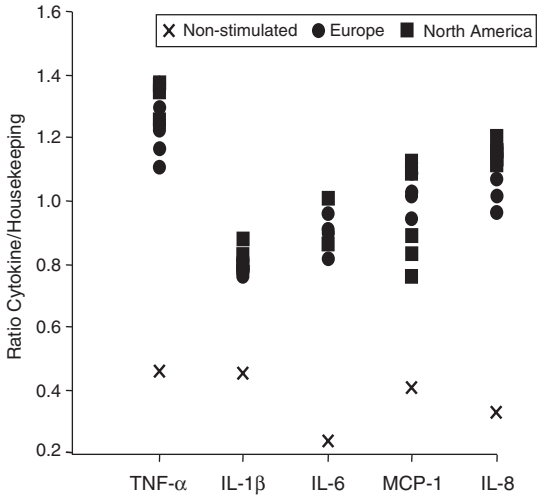

Fig. 2. Comparative reverse transcriptase-PCR (RT-PCR) analysis of cytokine mRNA expression in whole-blood cell culture stimulated by live *Streptococcus suis* serotype 2 strains ($10^6\ CFU\ mL^{-1}$) of different phenotypes and origins (as listed in Table 1). Points represent the mean cytokine over housekeeping gene expression ratio for each European or North American strain at 4 h poststimulation.

Role of bacterial components on cytokine production

The ability of different bacterial components to induce cytokine production is shown in Figs 3a–e (panels I and II show the mRNA levels and the corresponding gel photographs, and panel III shows the protein levels). The relative contribution of the CPS, *S. suis* cell wall and suilysin to cytokine induction was evaluated using purified components, as well as the nonencapsulated mutant strain B218 and the suilysin-negative mutant strain SX-911, both derived from the wild-type strain 31533. As indicated, an incubation time of 4 h was used for mRNA analysis, whereas the supernatants were analyzed at 12 h (TNF-α and IL-1β) or 24 h (IL-6 and IL-8) poststimulation for protein levels.

Comparison between the encapsulated strain 31533 and its nonencapsulated isogenic mutant B218 revealed no significant differences in the ability to induce the expression of TNF-α, IL-1β, IL-6 and IL-8 at both the mRNA ($P = 0.8$, 0.9, 0.5 and 0.3, respectively) and protein ($P = 0.3$, 0.5, 0.2 and 0.1, respectively; Figs 3a–d) level. In contrast, the nonencapsulated mutant induced a significantly lower expression of MCP-1 mRNA than did the parent strain ($P < 0.05$; Fig. 3e). It has been reported previously that nonencapsulated *S. suis* strains are rapidly eliminated by professional phagocytes (Charland *et al.*, 1998; Segura *et al.*, 1998; Smith *et al.*, 1999). Accordingly, a significantly lower bacterial growth and survival in blood culture was observed for the mutant B218 compared with the parent strain (Fig. 3f).

To further delineate the role of *S. suis* serotype 2 capsule in the induction of cytokine production, different concentrations

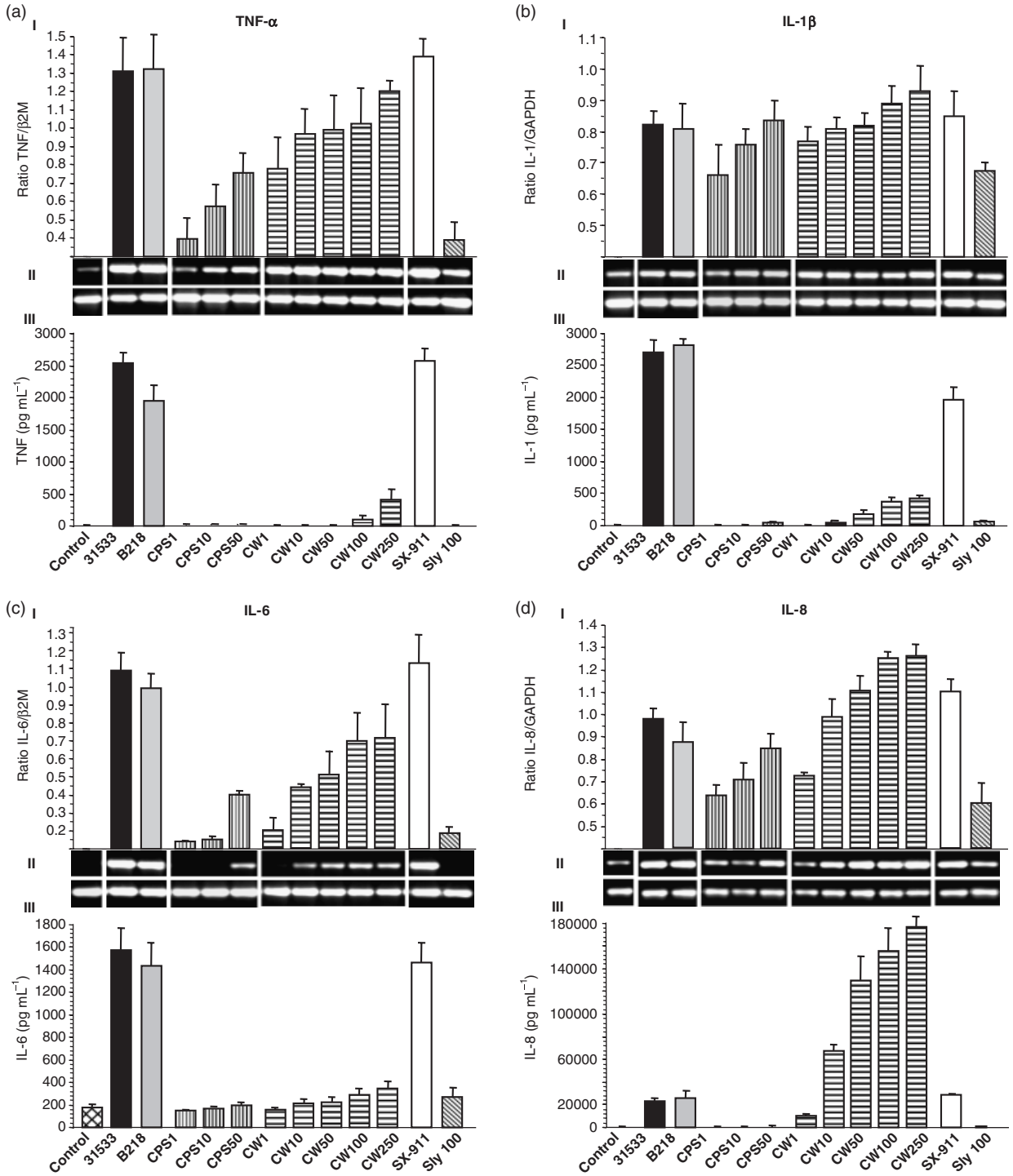

Fig. 3. Contribution of different bacterial components to cytokine production. Expression of tumor necrosis factor-α (TNF-α) (a), interleukin-1β (IL-1β) (b), IL-6 (c), IL-8 (d) and monocyte chemotactic protein-1 (MCP-1) (e) by whole-blood cells stimulated with live *Streptococcus suis* strain 31533, nonencapsulated mutant B218 or suilysin-negative mutant SX-911 at 10^6 CFU mL^{-1}, or stimulated with purified capsular polysaccharide (CPS; at 1, 10 and 50 μg mL^{-1}), purified cell wall (CW; at 1, 10, 50, 100 and 250 μg mL^{-1}) or suilysin (Sly; at 100 ng mL^{-1}). Unstimulated cells were used as control. Panel I: mRNA expression at 4 h poststimulation. Data represent the mean ratio of cytokine over housekeeping gene expression pooled from three independent experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β2-microglobulin (β2M) was used as housekeeping gene. Panel II: corresponding agarose gel from PCR. Upper gel panel: specific cytokine gene bands. Lower gel panel: housekeeping gene bands. Panel III: cytokine secretion in culture supernatants as measured by enzyme-linked immunosorbent assay (ELISA) at 12 h (for TNF-α and IL-1β) or 24 h (for IL-6 and IL-8) poststimulation. Data are expressed as the mean ± SD in pg mL^{-1}. (f) Bacterial growth during the 4 h stimulation period expressed as CFU mL^{-1}. *$P < 0.05$.

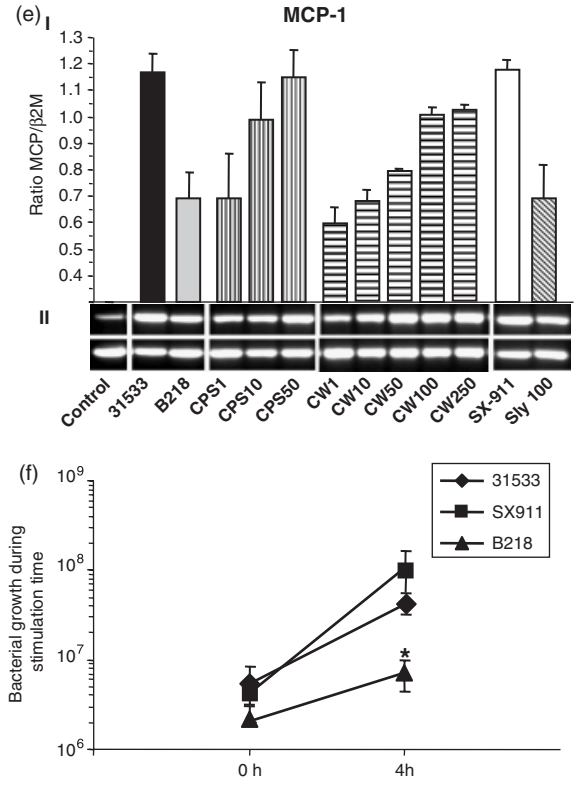

Fig. 3. Continued.

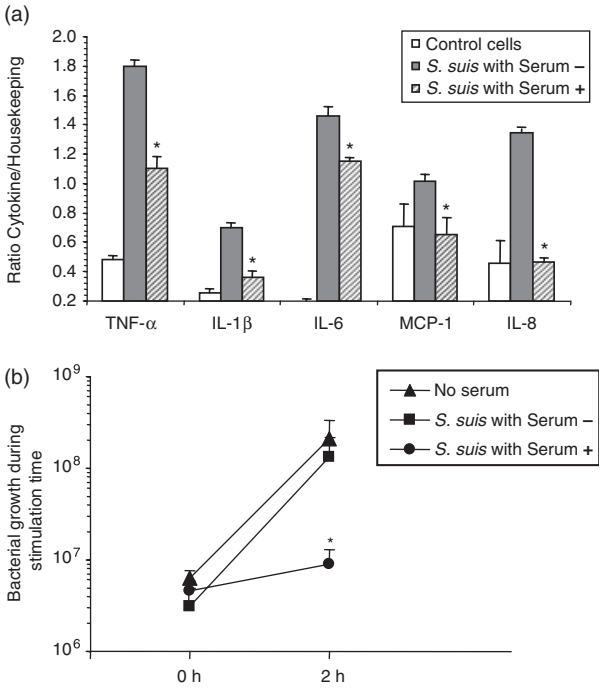

Fig. 4. Effect of antibody-mediated opsonization on *Streptococcus suis*-induced cytokine production. Blood cells were stimulated with 20% of homologous anti-*S. suis* serum (Serum+) or with negative serum as control (Serum–). (a) mRNA expression at 4 h poststimulation as measured by reverse transcriptase-PCR (RT-PCR). Data represent the mean ratio of cytokine over housekeeping gene expression pooled from three independent experiments. Unstimulated cells were used as control of basal cytokine expression. (b) Bacterial growth during the 4 h stimulation period expressed as CFU mL^{-1}. $^*P < 0.05$.

of CPS, ranging from 1 to 50 $\mu g\,mL^{-1}$, were tested. Purified CPS induced moderate (TNF-α, IL-6 and IL-8) to high (IL-1β and MCP-1) levels of mRNA expression in a dose-dependent manner, but corresponding protein secretion was not observed above the limits of detection by ELISA.

Purified cell wall activated the production of most cytokines. Less than 10 $\mu g\,mL^{-1}$ of purified cell wall induced high levels of TNF-α, IL-1β, IL-8 and MCP-1 mRNA expression, whereas IL-6 mRNA expression increased with higher concentrations and peaked at a dose of 100 $\mu g\,mL^{-1}$. For protein secretion, high levels of IL-8 but low levels of IL-1β, IL-6 and TNF-α were detected (Figs 3a–d).

Furthermore, the nonhaemolytic mutant SX-911 induced similar mRNA and protein levels of the proinflammatory cytokines as the parent 31533 strain. It should be noted that both strains showed similar bacterial growth rates in the blood culture system (Fig. 3f). Purified haemolysin induced relatively high mRNA levels of IL-1β and MCP-1, intermediate levels of IL-8 and low levels of TNF-α and IL-6. However, purified haemolysin stimulated very low protein levels of these cytokines.

Effect of antibody opsonization on *Streptococcus suis*-induced cytokine production

To evaluate the effect of bacterial opsonization on cytokine induction, cell stimulation was performed in the presence of homologous anti-*S. suis* serum or negative serum as a control. As shown in Fig. 4a, bacteria opsonized with *S. suis*-specific immune serum induced significantly lower levels of cytokine mRNA expression than did bacteria opsonized with negative control serum ($P = 0.003$, 0.02, 0.005, 0.03 and 0.001 for TNF-α, IL-1β, IL-6, MCP-1 and IL-8, respectively). The lower expression of cytokine mRNA correlated with lower bacterial growth and survival in the blood culture following bacterial opsonization ($P < 0.05$; Fig. 4b).

Discussion

The pathogenesis of *Streptococcus suis* infection, particularly concerning the aetiology of meningitis, is poorly understood (Gottschalk & Segura, 2000). There is evidence, however, that the immune system may play an important role in both the development of and protection against disease. In a mouse model of meningitis, a typical protective T-helper-1 (Th1) inflammatory response is observed following *S. suis* infection. Paradoxically, the failure to control this inflammatory cascade may be at the origin of clinical manifestations and poor outcome (our unpublished observations).

Indeed, several inflammatory and infectious diseases are associated with the overproduction of proinflammatory cytokines and chemokines, and the recruitment and activation of different leucocyte populations are a hallmark of acute inflammation (Saukkonen *et al.*, 1990; Sprenger *et al.*, 1996). These cytokines are believed to mediate responses associated with clinical deterioration, multiorgan system failure and death from septic shock (Verhoef & Mattsson, 1995). As these cytokines have been detected in the blood and cerebrospinal fluid during invasive meningeal infections, the ability of *S. suis* to induce cytokine production may have considerable biological relevance (Saukkonen *et al.*, 1990). We have previously reported that *S. suis* stimulates the release of proinflammatory cytokines from murine and human mononuclear cells, as well as from human BMECs (Segura *et al.*, 1999, 2002; Vadeboncoeur *et al.*, 2003). The present work provides novel evidence that *S. suis* serotype 2 is able to interact with leucocytes of porcine origin in a whole-blood system, inducing the upregulation of the proinflammatory mediators TNF-α, IL-1β, IL-6, IL-8 and MCP-1. Thus, the results obtained here probably reflect the interaction of *S. suis* with different cell types, including monocytes, neutrophils and lymphocytes, present in the whole-blood culture. Indeed, the use of the whole-blood system may help to preserve the integrity of cellular interactions, as well as the effect of lymphocyte-derived factors on proinflammatory cytokine production. Although the whole-blood system cannot completely mimic the complexity of the *in vivo* milieu, it is considered to be a relevant *ex vivo* model of cytokine production, particularly with respect to localized cytokine production in enclosed environments, such as arthritic joints, the alveolar space, the subarachnoid space or abscesses (DeForge *et al.*, 1992).

As reported previously (Dozois *et al.*, 1997), the incubation of blood cells in the absence of stimuli yielded low basal levels of cytokine mRNA expression, with the exception of MCP-1. This chemokine is normally present during steady state to allow the normal migration of mononuclear cells, and a relatively strong constitutive expression has been described previously (Sprenger *et al.*, 1997). However, the activation of blood cells with *S. suis* resulted in a significantly higher upregulation of cytokine mRNA in a time-dependent manner. In general, heat-killed bacteria and LPS induced similar kinetics of mRNA activation for TNF-α, IL-1β and IL-6. Comparable activities by these two types of stimulant have been reported previously with murine and human mononuclear cells (Segura *et al.*, 1999, 2002). However, the kinetics and magnitude of the upregulation elicited by live bacteria were unexpected. Previous studies have reported that heat-killed or viable bacteria stimulate similar levels of cytokine release from THP-1 human cells (Segura *et al.*, 2002). In the present study, live bacteria induced a delayed but more sustained increase of mRNA expression, especially for TNF-α and IL-6, and this upregulation corresponded with higher protein levels. The sequential onset and kinetics of cytokine production, in the order of TNF-α, IL-1β and IL-6, is consistent with those previously reported in cultured blood cells (DeForge *et al.*, 1992; Oliver *et al.*, 1993). In contrast, low levels of protein secretion were detected in blood cells stimulated by heat-killed bacteria or LPS. Cartensen *et al.* (2005) have recently shown significant TNF-α protein levels in pig whole-blood cultures only with doses of LPS higher than $10\,\mu g\,mL^{-1}$. Thus, the use of different doses of the stimulus may account for these divergent results.

The induction of cytokine production is mediated at the transcriptional level, at the posttranscriptional level, by the enhancement of mRNA stability or by posttranslational processing, as is IL-1β. Although most cytokines are transcriptionally regulated (Brorson *et al.*, 1991), the lack of correlation between mRNA expression and protein levels has been reported previously (Vezina *et al.*, 1995; Thanawongnuwech *et al.*, 2001). With *in vitro* culture systems, the secreted cytokines interact with their target cells, resulting in shorter lifetimes of the circulating proteins (Verfaillie *et al.*, 2001). Therefore, the level of a secreted cytokine, as measured by bioassays and ELISA, may reflect the net amount in the supernatant following consumption and degradation *in vitro*. In this study, it is possible that the TNF-α, IL-1β and IL-6 secreted by cells stimulated with heat-killed bacteria or LPS may have interacted with different cells in the whole-blood cell system, and therefore were not present in sufficient quantities to be detectable by ELISA. On the other hand, live bacteria induced a more sustained stimulation of mRNA expression, which may explain the higher protein levels detected by ELISA. Encapsulated live bacteria are resistant to phagocytosis (Charland *et al.*, 1998; Segura *et al.*, 2004) and thus are able to persist and multiply in the blood. Indeed, the level of bacteria increased from 10^6 to $10^8\,CFU\,mL^{-1}$ during the incubation period, resulting in robust and prolonged activation of host blood cells.

The patterns of upregulated mRNA expression for the chemokines IL-8 and MCP-1 in *S. suis*-activated blood cells differed from those obtained for TNF-α, IL-1β and IL-6. Heat-killed bacteria increased IL-8 and MCP-1 mRNA expression to a greater extent than did live bacteria. This difference was more markedly evident in the protein levels detected by ELISA, as demonstrated by the very high levels of the IL-8 protein stimulated by heat-killed bacteria compared with the delayed secretion induced by live bacteria. Accordingly, heat-killed *S. suis* has been reported to induce extremely high and sustained levels of IL-8 and MCP-1 secretion by human THP-1 monocytes (Segura *et al.*, 2002). The delayed induction of IL-8 in live bacteria-stimulated cultures may be related, in part, to the amplification loop mediated by IL-1β and TNF-α. These cytokines, highly

induced by live bacteria, have been reported to regulate IL-8 production during the inflammatory cascade (DeForge *et al.*, 1992; Segura *et al.*, 2002). Reduced expression of the chemokine MCP-1 was also observed in live bacteria-stimulated blood cultures. Thus, it could be hypothesized that lower levels of these chemokines may facilitate bacterial multiplication and dissemination in blood. Differences in the pattern of cytokine production elicited by killed or live organisms have been reported for other bacterial species after infection of phagocytic cells (Mitsuyama *et al.*, 1990; Cross *et al.*, 2004), although little information is available using the more complex system of whole-blood cell cultures. Differences in the persistence and numbers of bacterial cells in the whole-blood system, as well as in the amounts of exposed surface antigens, which are probably modified by heat treatment, may also account for the disparate cytokine patterns induced by these two bacterial preparations.

It is unclear which type of cell is predominantly responsible for cytokine production in the whole-blood culture system, and whether distinct cell populations contribute preferentially to the different patterns of induction of immune mediators by LPS, heat-killed bacterial antigen or live bacteria. Moreover, diverse cellular responses may account for the different cytokine responses induced by heat-killed bacteria vs. LPS, which failed to induce detectable levels of IL-8 protein secretion despite comparable mRNA expression kinetics. This result was unexpected, however, given that previous studies have shown LPS-induced IL-8 protein secretion in human whole-blood systems (DeForge *et al.*, 1992; Foca *et al.*, 1998). As discussed above, differences in the dose and type of LPS used may account for these conflicting findings. Interestingly, Yancy *et al.* (2001) also failed to detect significant IL-8 protein levels in a swine whole-blood system despite the high upregulation of its mRNA expression by concavalin A. A study by Burns *et al.* (1997) on IL-4 expression and protein production in stimulated human peripheral blood mononuclear cells, employing competitive PCR, flow cytometry and ELISA, showed that the use of only one of these methods produced misleading information. Thus, the results of the present study, obtained using a combined mRNA and protein detection system, demonstrate both the broad scope and strength of a whole-blood culture system to accurately represent the complexity of cytokine production *in vivo*.

Differences in the phenotype and genotype of *S. suis* isolates from North America or Europe have been widely reported (Gottschalk *et al.*, 1998; Chatellier *et al.*, 1999). However, in the present work, no association was observed between the cytokine responses and origin of the strains. Interestingly, a lack of relationship between the phenotype of *S. suis* strains and cytokine production has previously been reported in human monocytes and human BMECs (Segura *et al.*, 2002; Vadeboncoeur *et al.*, 2003). Unlike other important streptococcal species, information on *S. suis* virulence factors, as well as the molecules expressed at the bacterial surface, is limited (Gottschalk & Segura, 2000). Wilson *et al.* (1998) proposed that the capacity of bacteria or bacterial components to induce the overproduction of cytokines in host cells may be an important factor contributing to bacterial virulence and, in our model, may be directly related to the ability of bacteria to invade, survive and disseminate in blood during *in vivo* infection. The data presented here demonstrate that bacterial opsonization with serum containing *S. suis*-specific antibodies results not only in reduced numbers of multiplying organisms, but also in an important decrease in cytokine and chemokine production. Extensive studies by Mosser and coworkers have demonstrated that FcγR ligation can prevent inappropriate inflammatory cytokine responses (Anderson & Mosser, 2002; Mosser, 2003) and that immunoglobulin G (IgG)-opsonized *Haemophilus influenzae* induces significantly lower production of IL-12p40 protein and increased release of the anti-inflammatory cytokine IL-10, leading to reduced macrophage proinflammatory responses (Sutterwala *et al.*, 1998). Thus, vaccination approaches that induce the production of appropriate opsonizing antibodies may have a positive impact on the control of the infection and disease outcome. In this regard, we have recently demonstrated that antibody-mediated opsonization induces the killing of encapsulated *S. suis* by porcine neutrophils and monocytes, whereas complement plays a negligible role (Chabot-Roy *et al.*, 2004).

As discussed above, the CPS plays a critical role in bacterial resistance to phagocytosis. To elucidate its contribution to the activation of the inflammatory response, we compared the cytokine patterns induced by a nonencapsulated mutant strain with those produced by the encapsulated wild-type strain. The nonencapsulated mutant is easily eliminated by phagocytes present in the whole-blood cell system, resulting in reduced bacterial growth during the incubation period. Despite the lower numbers of bacteria in the culture, the nonencapsulated mutant induced similar levels of TNF-α, IL-1β, IL-6 and IL-8 mRNA expression and protein release as the wild-type strain. As purified cell wall material was observed to be a more potent inducer of cytokines than purified CPS, it is reasonable to suggest that increased exposure to cell wall components due to the absence of a capsule may account for the high capacity of the nonencapsulated mutant to induce cytokine production. Similarly, previous studies have indicated that *S. suis* cell wall components are the major stimuli for cytokine release by murine macrophages and human BMECs (Segura *et al.*, 1999; Vadeboncoeur *et al.*, 2003). Our results are also in agreement with the high inflammatory activity induced by lipoteichoic acids and peptidoglycans of Gram-positive bacteria (Timmerman *et al.*, 1993; Heumann *et al.*, 1994)

and the relatively low activation by streptococcal capsular polysaccharides (Tuomanen *et al*., 1985; Vallejo *et al*., 1996).

For MCP-1 production, however, the nonencapsulated mutant stimulated low levels of mRNA expression compared with the strong dose–response activation induced by both purified cell wall and CPS. Similar results were observed for MCP-1 production by human BMECs stimulated with a different nonencapsulated mutant strain and by human monocytes infected with strain Reims, a naturally poorly encapsulated *S. suis* isolate (Caumont *et al*., 1996; Segura *et al*., 2002; Vadeboncoeur *et al*., 2003). The reduced capacity of nonencapsulated *S. suis* strains to induce MCP-1 is difficult to explain, given that the cell wall components are highly exposed at the bacterial surface and that Gram-positive bacterial cell wall antigens are well known to induce MCP-1 production (von Hunolstein *et al*., 1997; Hausler *et al*., 2002; Moller *et al*., 2003). In the wild-type encapsulated strain, a balance between encapsulation and access to cell wall components, such that the cell wall is not completely covered by the capsular layer or components are released during bacterial growth (Tuomanen *et al*., 1985; Lalonde *et al*., 2000), may determine the relative extent of MCP-1 production. Alternatively, it is possible that the nonencapsulated mutant strain has altered levels of MCP-1-stimulating components on its bacterial surface compared with the wild-type strain, resulting in differential production of this chemokine. Experiments are ongoing in our laboratory to further investigate this issue.

In addition to cell wall components, it has been shown that several microbial toxins can also stimulate or modulate the inflammatory cytokine cascade (König *et al*., 1992; Rogers *et al*., 2003). However, in our whole-blood model, the nonhaemolytic mutant induced similar mRNA and protein levels of proinflammatory cytokines as the wild-type strain. Although purified haemolysin induced significant levels of cytokine mRNA expression, albeit to a lesser extent than the wild-type strain, low protein levels were observed. The low levels of protein secretion could either reflect reduced posttranscriptional activation or consumption/degradation of the protein *in vitro*. Nevertheless, as the nonhaemolytic mutant induced similar mRNA and protein levels of proinflammatory cytokines as the wild-type strain, it could be suggested that suilysin plays a limited role in the inflammatory response. Similarly, suilysin does not contribute significantly to cytokine production by mouse macrophages (Segura *et al*., 1999).

To conclude, our results show the complex patterns of cytokine activation induced by *S. suis*, and suggest that an equilibrium time- and dose-dependent production of immune mediators may directly influence whether bacteria will survive and disseminate or whether an exacerbated and potentially damaging inflammatory response will ensue in an attempt to control the infection. In addition, the different patterns of cytokine production induced by live vs. heat-killed bacteria may have important implications for the design of vaccination approaches. Indeed, to date, no successful vaccine against *S. suis* has been produced, and heat-killed bacterial preparations have failed to induce protection (Holt *et al*., 1990). The disappointing results from vaccine trials may be explained by observations that heat-killed bacteria are rapidly eliminated by chemokine-activated phagocytes and therefore are unable to activate protective long-lasting or memory immune responses. The results presented in this study suggest that a beneficial vaccination strategy may require the induction of antibody-mediated phagocytosis to effectively control the infection, as well as an appropriate level of proinflammatory cytokine response to minimize the risk of cytokine-mediated immunopathology.

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