Original article

Interactions of *Haemophilus parasuis* and its LOS with porcine brain microvascular endothelial cells

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Abstract – *Haemophilus parasuis* is a swine pathogen that causes Glässer's disease, which is characterized by polyserositis and meningitis. The pathogenesis of the *H. parasuis* infection is poorly understood. To cause meningitis, *H. parasuis* has to cross the blood-brain barrier (BBB) to gain access to the central nervous system (CNS). We recently showed that *H. parasuis* adheres to and invades porcine brain microvascular endothelial cells (PBMEC). The aim of this study was to evaluate the role of *H. parasuis* lipooligosaccharide (LOS) in the adhesion to PBMEC and to determine if *H. parasuis* (and/or its LOS) is able to induce apoptosis and activation of PBMEC. Results showed that adhesion of *H. parasuis* to PBMEC was partially mediated by LOS. Moreover, *H. parasuis* induces caspase-3-mediated apoptosis of PBMEC in a time – and dose – dependent manner, but its LOS did not seem to be involved in such a process. Furthermore, *H. parasuis* and, to a lesser extent, its LOS, was able to induce the release of IL-8 and IL-6 by PBMEC. Field strains of *H. parasuis* serotypes 4 and 5 induced similar levels of these inflammatory mediators. Our data suggest that *H. parasuis* uses cellular adhesion, induction of apoptosis and up-regulation of inflammatory mediators as mechanisms to invade the CNS via the BBB, and that LOS would play a certain but limited role in such pathological process.

Haemophilus parasuis / LOS / endothelial cells / BBB / meningitis

1. INTRODUCTION

Haemophilus parasuis is a commensal organism of the upper respiratory tract of swine. It is also the causative agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis, meningitis as well as other important conditions [2, 22, 26, 29]. To date, 15 serotypes have been identified and serotypes 5 and 4 are the most prevalent serotypes in North America [18, 37]. However, little is known about the real nature of autoclaved antigens involved in the serotyping, being the capsule, outer membrane and lipooligosaccharide (LOS) already suggested as playing a certain role [4, 37].

The pathogenesis of *H. parasuis* infection is poorly understood. The initial site of colonization is probably the nasal cavity and/or the tracheal mucosa [29], with subsequent dissemination throughout the host [29, 41]. To cause meningitis, bacteria have to reach the subarachnoid space. Hence, the passage of bacteria through the blood-brain barrier (BBB) to access the central nervous system (CNS) is thought to be a key step in the pathogenesis of meningitis [41]. A better understanding of the mechanisms involved in this critical step is therefore of considerable importance. It has been proposed that meningeal pathogens could reach the subarachnoid space by directly invading brain microvascular endothelial cells

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(BMEC) [14, 41] and/or by increasing BBB permeability [38]. We recently showed that *H. parasuis* can adhere to and invade porcine BMEC [41]. BMEC, the primary components of the BBB, are linked by intercellular tight junctions that control molecular exchanges between the blood and the brain [38]. Alterations of these tight junctions can lead to an increase in BBB permeability [38]. Bacteria-induced overexpression of immunomodulatory mediators may be deleterious to the host and have been linked to an increase in BBB permeability in meningitis [38]. Alterations of endothelial cells could also be the result of bacteria-induced cytotoxic effects or cell destruction by apoptosis [35, 38]. Research thus far has suggested that *H. parasuis* is not toxic to endothelial cells [41].

Our understanding of *H. parasuis* virulence factors is also very limited, with a few putative potential candidates [5, 10, 21, 29, 33]. Differential display RT-PCR [24] and microarray [23] analysis have also suggested virulence-related genes.

Several reports have proposed that lipopolysaccharide (LPS) or lipooligosaccharide (LOS) of several meningeal pathogens such as *Escherichia coli*, *Neisseria meningitidis* and *Haemophilus* sp. [17, 19, 25] are major virulence factors. *Haemophilus influenzae* LOS has been shown to cause marked cytotoxicity against bovine BMEC [38]. Moreover, *Histophilus* (*Haemophilus*) *somnus* LOS is known to induce apoptosis of bovine endothelial cells [35]. However, little is known about the actual biological activity of LOS from *H. parasuis*. Amano et al. [3] have proposed that *H. parasuis* endotoxins are linked to endotoxic shock, aggravation of clinical signs, and death of pigs with septicemia, suggesting that LOS might play an important role in pathogenesis. However, the specific role of *H. parasuis* LOS in the pathogenesis of infection, particularly with respect to meningitis, has yet to be investigated.

The aim of this study was to further characterize the pathogenesis of *H. parasuis*-induced meningitis by investigating the relative contribution of *H. parasuis* LOS in adhesion to porcine BMEC, and its ability to induce

PBMEC apoptosis and pro-inflammatory cytokine production.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

H. parasuis Nagasaki strain (reference strain of serotype 5) isolated from a septicemic pig with meningitis [26] (kindly provided by Dr R.F. Ross, Iowa State University, USA), was used as the reference strain in this study. Field strains of *H. parasuis* serotypes 4 ($n = 18$) and 5 ($n = 10$) isolated from cases of meningitis, septicemia, arthritis or pneumonia in swine, as well as seven strains of serotype 4 recovered from nasal swabs of clinically healthy animals (from farms considered free from endemic disease caused by *H. parasuis*) were also used in this study. Bacterial strains were grown to the late logarithmic phase of growth as described previously [41]. Bacteria were appropriately diluted in cell culture medium before infection. Heat-killed bacteria were prepared by incubation at 60 °C for 45 min, and lack of viable bacteria was confirmed by plating heat-killed bacteria suspension onto pleuropneumonia-like organisms (PPLO) agar (Difco Laboratories, Detroit, MI, USA). For most of the experiments, we used 1×10^7 CFU/mL of live bacteria and 5×10^8 CFU/mL of heat-killed bacteria as there is an increase of live bacteria from 1×10^7 CFU/mL to 5×10^8 CFU/mL during the 48 h incubation time (data not shown).

2.2. LOS purification

LOS was purified from lyophilized *H. parasuis* Nagasaki strain, as previously described [9]. Briefly, bacteria (4 g) were subjected to two enzymatic treatments with 100 μg/mL of pancreatic DNase I (Sigma-Aldrich, Saint Louis, MO, USA), 25 μg/mL of pancreatic RNase A (Sigma-Aldrich) and 200 μg/mL of proteinase K (Roche, Laval, QC, Canada). Then, the bacterial suspension was passed through a French Press at $20\,000\,\text{lb/in}^2$ and was sonicated at maximum intensity to ensure complete bacterial breakage. After precipitation with MgCl$_2$ in 95% ethanol, LOS was obtained by differential centrifugation, lyophilized (4 g of lyophilized bacteria resulted in approximately 175 mg of purified lyophilized LOS) and stored at room temperature. Protein contamination of the LOS preparation was low as determined

by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver and Coomassie Blue stainings. Purified LOS was also examined for the presence of proteins by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), and was shown to have less than 5% protein contamination (w/w). Endotoxins from *H. parasuis* LOS and positive control *Escherichia coli* LPS O127:B8 (Sigma-Aldrich) were quantified by a *Limulus* Amebocyte Lysate gel-clot test (Pyrotell, Associates of Cape Cod, Falmouth, MA, USA) (sensitivity limit of 0.5 endotoxin units (EU)/mL), according to the manufacturer's instructions. A similar concentration of endotoxin of 4 EU/ng was obtained for both *H. parasuis* LOS and *E. coli* LPS.

2.3. PBMEC adhesion assay

The PBMEC/C1-2 cell line was cultivated as previously described [41]. Cells were cultured at 37 °C with 5% CO_2 in a humid atmosphere in T75 flasks (Falcon; Becton Dickinson, Bedford, MA, USA) and Primaria 24-well tissue culture plates (Falcon) precoated with 1% (v/v) type A gelatin from porcine skin (Sigma-Aldrich). For assays, cells were trypsinized by adding 0.05% trypsin – 0.03% EDTA solution (Invitrogen, Burlington, ON, Canada) and diluted in culture medium to obtain a concentration of 8×10^4 cells/mL. Then, the cellular suspension was distributed onto tissue culture plates which were incubated until cell confluence was reached. Before assays, culture medium was removed from the wells and replaced with complete medium without antibiotics.

The adhesion assay was performed as previously described [41]. Briefly, PBMEC were infected with *H. parasuis* Nagasaki strain at 1×10^6 CFU/mL in culture medium (1 mL per well) and incubated at 37 °C with 5% CO_2. After 90 min of incubation, cells were washed five times with pyrogen-free phosphate-buffered saline (PBS, 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) to remove non-adherent bacteria. Then, cells were incubated with 200 μL of 0.05% trypsin – 0.03% EDTA (Invitrogen) for 10 min at 37 °C. Thereafter, 800 μL of cold water was added and cells were disrupted by scrapping the bottom of the well and by repeated pipetting. Serial dilutions of the lysate were plated onto PPLO agar (Difco Laboratories) and incubated for 48 h at 37 °C to determine the number of cell-associated bacteria. To test the role of *H. parasuis* LOS on bacterial

adhesion to PBMEC, assay was performed as described above with some modifications. Briefly, purified LOS (100 μg/mL) from Nagasaki strain was added to the cell monolayer along with Nagasaki strain (1×10^6 CFU/mL). Results are expressed as percentage of adhesion compared with the adhesion level of Nagasaki strain alone (considered as 100%). Results are expressed as mean of duplicates from three independent experiments.

2.4. Cytotoxicity assay

The cytotoxic effect of bacteria or purified LOS was evaluated by measurement of lactate dehydrogenase (LDH) release using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA). Briefly, PBMEC were treated with live (up to 1×10^9 CFU/mL) or heat-killed (5×10^8 CFU/mL) *H. parasuis* Nagasaki strain, or purified LOS (up to 10 μg/mL). Monolayers were incubated with either live bacteria, heat-killed bacteria or purified LOS, and plates were centrifuged at $800 \times g$ for 10 min to increase contact of bacteria or LOS with the cells. Then, the plates were incubated at 37 °C for different incubation times up to 48 h. Non-treated cells were used as negative control, whereas cells lysed in 9% Triton X-100 (Sigma-Aldrich) were used as positive control (100% toxicity). Results are expressed as mean of duplicates from three independent experiments.

2.5. Detection of cell apoptosis

The ability of *H. parasuis* to induce apoptosis of PBMEC was determined by measuring DNA fragmentation using the Cell Death Detection ELISA kit (Roche). This immunoassay specifically detects histone regions of mono- and oligonucleosomes of fragmented DNA that are released during apoptosis. PBMEC were treated with live (1×10^8 CFU/mL) or heat-killed (5×10^8 CFU/mL) *H. parasuis* Nagasaki strain, or purified *H. parasuis* LOS (10 μg/mL). To inhibit stimulation due to LOS, polymyxin B (PmB, Sigma-Aldrich), a cationic antibiotic that binds to the lipid A region from LOS, was used. Heat-killed whole bacteria were pre-incubated with 10 μg/mL of PmB (Sigma-Aldrich) in cell culture medium with agitation for 30 min at room temperature, before incubation with PBMEC. The proapoptotic topoisomerase-I inhibitor campothecin (CAM, 40 μg/mL, Sigma-Aldrich) was

used as positive control. Apoptosis was measured at 6 h, 12 h and 24 h after stimulation. In selected experiments, cell monolayers were incubated for 24 h with different concentrations of heat-killed *H. parasuis* Nagasaki strain (1×10^6 to 1×10^9 CFU/mL). Monolayers were processed according to manufacturer's instructions. Plates were read in a Molecular Devices UVmax (Molecular Devices Corp, Sunnyvale, CA, USA) microplate reader. Samples from at least three individual assays were measured in duplicates. Results are expressed as absorbance of the experimental cell lysates read at 405 nm after 30 min which corresponds to the level of DNA fragmentation.

Apoptosis was confirmed using the Apoptosis Marker: Cleaved Caspase-3 (Asp 175) Western Detection Kit (Cell Signaling Technology, Danvers, MA, USA) which detects specifically activated caspase-3 and its inactive zymogen procaspase-3. Activation of caspase-3 requires proteolytic processing of its inactive zymogen, procaspase-3 (35 kDa), into activated p17 and p12 fragments. CAM (40 μg/mL, Sigma-Aldrich) was used as positive control. PBMEC were incubated with heat-killed *H. parasuis* Nagasaki strain (5×10^8 CFU/mL), CAM (Sigma-Aldrich), or medium alone for 24 h. Then, monolayers were processed according to manufacturer's instructions. After Western blotting, bands were visualized by adding LumiGLO substrate (Cell Signaling Technology). Results shown are representative immunoblots of three independent experiments.

2.6. Cytokine stimulation assays

Cells were treated with live or heat-killed *H. parasuis* Nagasaki strain at a concentration of 1×10^7 or 5×10^8 CFU/mL, respectively. Moreover, purified *H. parasuis* LOS (10 μg/mL) from Nagasaki strain was used to stimulate PBMEC, while *E. coli* LPS (10 μg/mL) was used as positive control. PBMEC were incubated for different times up to 48 h as described for the cytotoxicity assay. After each incubation time, plates were centrifuged at $800 \times g$ for 10 min and cell supernatants were harvested and frozen at $-20\,°$C.

To inhibit stimulation due to LOS, PmB (Sigma-Aldrich) was used. Whole bacteria or purified LOS were pre-incubated with 10 μg/mL of PmB (Sigma-Aldrich) in cell culture medium with agitation for 30 min at room temperature before cell infection. In selected experiments, the monolayers were incubated with heat-killed *H. parasuis* Nagasaki strain corresponding to different concentrations (1×10^6 to 1×10^9 CFU/mL), or with field strains of *H. parasuis* serotypes 4 and 5 for 24 h.

Cytokines and chemokines were detected by direct sandwich enzyme linked immunosorbent assays (ELISA) using porcine specific pair-matched antibodies for tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) (R&D Systems, Minneapolis, MN, USA) and transforming growth factor-β1 (TGF-β1) (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's recommendations. Two-fold dilutions of recombinant porcine TNF-α, IL-1β, IL-6, IL-8, and TGF-β1 were used to generate standard curves, and sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine. Plates were read in a Molecular Devices UVmax (Molecular Devices Corp) microplate reader. Standard and sample dilutions were measured in duplicates and analysis were performed at least three times for each individual stimulation assay. Unstimulated PBMEC provided basal levels of cytokine expression. These values were subtracted from data, thus all results obtained throughout this study represent up-regulated expression of cytokines after bacterial or LOS stimulation.

2.7. Statistical analysis

All data are expressed as mean ± standard deviation (SD) (error bars). Data were analysed by two-tailed, unpaired *t* test. For comparison of cytokine release induced by different *H. parasuis* strains, the Dunnett post-hoc test was used. A $P < 0.05$ was considered as significant. All assays were repeated at least three times in independent experiments.

3. RESULTS

3.1. LOS is partially involved in adhesion of *H. parasuis* to PBMEC

To determine if LOS is involved in adhesion of *H. parasuis* to PBMEC, purified LOS and live *H. parasuis* Nagasaki strain were used in a competitive binding assay. Results showed that adhesion of *H. parasuis* to PBMEC after 90 min of incubation was reduced up to

$62\% \pm 27$ ($P < 0.05$) in the presence of $100\,\mu g/mL$ of purified LOS. A positive correlation between LOS concentration and inhibition was observed, although a similar level of decrease was observed with concentrations of LOS higher than $100\,\mu g/mL$ (up to $500\,\mu g/mL$). Similar results were obtained when cells were treated first with LOS and then incubated with live *H. parasuis* (data not shown).

3.2. *H. parasuis* and its LOS are not toxic to PBMEC

High concentrations of live (up to $1 \times 10^9\,CFU/mL$) or heat-killed ($5 \times 10^8\,CFU/mL$) *H. parasuis* Nagasaki strain were used to stimulate PBMEC. During the period of incubation of 48 h, both live and heat-killed bacteria caused no cytotoxicity to PBMEC, with maximum values of 17% which were not significantly different than those observed in non-treated cells. Purified *H. parasuis* LOS was also used to stimulate PBMEC and caused no detectable cytotoxicity even at concentrations up to $10\,\mu g/mL$ (data not shown).

3.3. *H. parasuis* induces PBMEC apoptosis

To measure DNA fragmentation, PBMEC were incubated with heat-killed *H. parasuis* Nagasaki strain ($5 \times 10^8\,CFU/mL$) for different lengths of time up to 24 h. CAM induced time-dependent apoptosis, as indicated by the DNA fragmentation level observed (Fig. 1A). A time-dependent increase in PBMEC apoptosis level was also observed following incubation with heat-killed *H. parasuis* ($P < 0.05$). Apoptosis was detected as early as 6 h, remained constant at 12 h, and peaked at 24 h (Fig. 1A). A high concentration of live *H. parasuis* ($1 \times 10^8\,CFU/mL$) was required to induce a significant level of PBMEC apoptosis at 24 h ($P < 0.05$) (Fig. 1A) and this level was similar to that observed after 24 h stimulation with heat-killed *H. parasuis*.

Incubation of PBMEC with different concentrations of heat-killed *H. parasuis* revealed that induction of apoptosis was dose-dependent (Fig. 1B). In addition, results

showed that heat-killed bacteria were potent inducers of apoptosis at high concentrations (higher than $5 \times 10^8\,CFU/mL$), but apoptosis decreased rapidly to nondetectable levels at bacterial concentrations lower than $1 \times 10^7\,CFU/mL$ (Fig. 1B).

To determine if LOS is involved in the induction of PBMEC apoptosis, cells were incubated with purified *H. parasuis* LOS ($10\,\mu g/mL$). No DNA fragmentation was observed after stimulation with LOS (Fig. 1A) even at higher concentrations of up to $100\,\mu g/mL$ (data not shown). Moreover, levels of PBMEC apoptosis induced by PmB-treated heat-killed *H. parasuis* were similar to those observed with untreated heat-killed *H. parasuis* (Fig. 1A).

Apoptosis was confirmed using an anti-caspase-3 polyclonal antibody recognizing full length caspase-3 (procaspase-3, 35 kDa) and the fragment that results from its cleavage (17 kDa). We detected a 35 kDa band corresponding to procaspase-3 in each samples (Fig. 2). After CAM (positive control) and *H. parasuis* treatment, a 17 kDa band corresponding to the active form of caspase-3 appeared (Fig. 2), suggesting that this pathogen triggered caspase-3 dependent apoptosis of PBMEC.

3.4. *H. parasuis* induces time-dependent release of IL-6 and IL-8 by PBMEC

Stimulation of PBMEC with live ($1 \times 10^7\,CFU/mL$) or heat-killed ($5 \times 10^8\,CFU/mL$) *H. parasuis* Nagasaki strain up-regulated the expression of IL-6 and IL-8 in a time-dependent manner as shown in Figure 3. Moreover, levels of IL-8 secretion were significantly higher than those of IL-6 ($P < 0.05$). For both IL-6 and IL-8 production, heat-killed *H. parasuis* appeared to be a more potent stimulus than live *H. parasuis* ($P < 0.05$) (Fig. 3), which can be explained by the different bacterial concentrations used. Heat-killed *H. parasuis*-induced IL-6 and IL-8 productions gradually increased throughout the incubation period ($P < 0.05$) (Fig. 3). IL-6 production reached a plateau at 24 h (Fig. 3A), while IL-8 secretion peaked at 48 h ($P < 0.05$)

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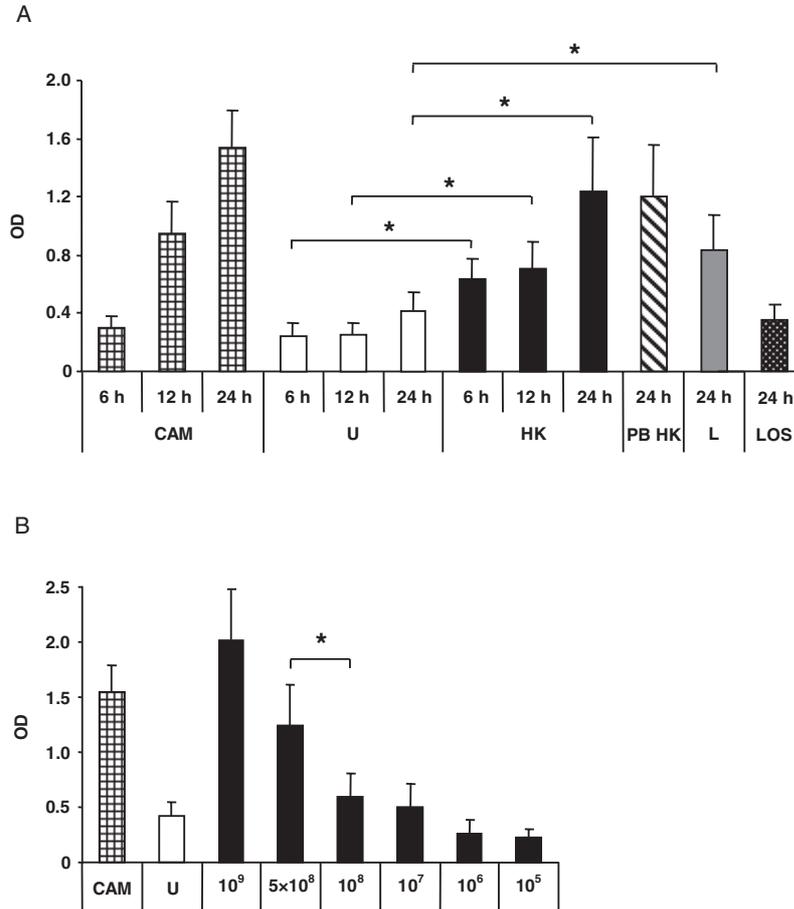
Figure 1. (A) Time course of PBMEC apoptosis after stimulation with heat-killed (HK) (5×10^8 CFU/mL), PmB-treated heat-killed (PB HK) (5×10^8 CFU/mL), live (L) (1×10^8 CFU/mL) *H. parasuis*, or LOS (10 μg/mL). (B) Effect of varying bacterial concentrations on induction of PBMEC apoptosis by heat-killed bacteria at 24 h. Unstimulated cells (U) and CAM (40 μg/mL) were used as negative and positive controls, respectively. Apoptosis was detected by measuring DNA fragmentation. Data are expressed as mean ± SD. * $P < 0.05$.

(Fig. 3B). Live bacteria induced a low and gradual production of IL-6 from 6 h to 48 h ($P < 0.05$) (Fig. 3A), whereas a high level of IL-8 release was observed as soon as 6 h ($P < 0.05$) (Fig. 3B).

We also investigated the ability of live (1×10^7 CFU/mL) and heat-killed (5×10^8 CFU/mL) *H. parasuis* Nagasaki strain to induce the release of TNF-α, IL-1β and TGF-β1. No cytokine production was detected after 48 h incubation with either *H. parasuis* Nagasaki

strain or with LPS positive control (data not shown).

3.5. *H. parasuis* induces dose-dependent cytokine production by PBMEC

To evaluate induction of IL-6 and IL-8 production, PBMEC were stimulated with different concentrations of heat-killed *H. parasuis* Nagasaki strain (from 1×10^6 to 1×10^9 CFU/mL) for 24 h. As shown in Figure 4,
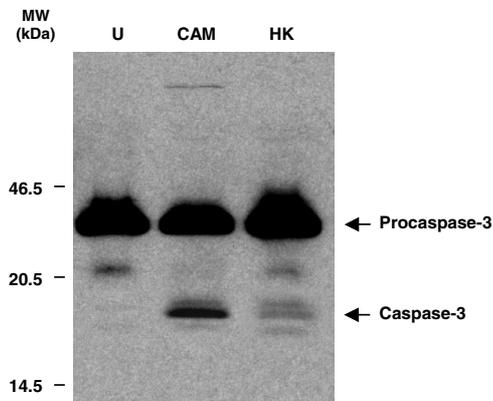
Figure 2. Western blot analysis of procaspase-3 processing in PBMEC stimulated with heat-killed *H. parasuis* (HK) (5×10^8 CFU/mL). Unstimulated cells (U) and CAM ($40 \mu g/mL$) were used as negative and positive controls, respectively. Cells treated with controls and heat-killed *H. parasuis* were harvested and cell extracts were analysed by Western blot. Procaspase-3 and caspase-3 were detected by anti-caspase-3 antibody. Arrows indicate inactive $35 kDa$ procaspase-3 and active $17 kDa$ caspase-3.

lower amounts of cytokines are produced when cells are stimulated with lower numbers of bacteria. No production of IL-6 was observed at bacterial concentrations lower than 1×10^6 CFU/mL (Fig. 4A). IL-8 production was more sustained than IL-6 production. Thus, levels of IL-8 release began to decrease at 1×10^8 CFU/mL, and then remained almost constant until 1×10^6 CFU/mL, which was the lowest concentration that was able to induce detectable IL-8 production (Fig. 4B).

### 3.6. Partial involvement of LOS for cytokine release by PBMEC

It was of interest to determine whether LOS was responsible for the pro-inflammatory cytokine production induced by whole bacteria. To address this, experiments were performed using the LOS inhibitor PmB. No change in basal cytokine secretion was observed after incubation with PmB alone (data not shown). Heat-killed *H. parasuis* (5×10^8 CFU/mL) treated with PmB was still

able to stimulate PBMEC to produce IL-6 and IL-8 (Fig. 3), although levels of cytokine release were significantly lower (reduction of about 50%; $P < 0.05$) than those induced by untreated heat-killed *H. parasuis.*

To further characterize the involvement of *H. parasuis* LOS in the induction of cytokine production, PBMEC were stimulated with LOS ($10 \mu g/mL$) purified from *H. parasuis* Nagasaki strain. LOS stimulated PBMEC to produce IL-6 and IL-8 ($P < 0.05$) (Fig. 5), although to a lesser extent compared with heat-killed or live bacteria. Stimulation with LOS, however, yielded similar results as with *E. coli* LPS. Cytokine production induced by *H. parasuis* LOS was time-dependent: IL-6 and IL-8 were gradually up-regulated ($P < 0.05$) and reached a plateau at $24 h$ (Fig. 5A and B). To confirm the role of *H. parasuis* LOS in cytokine induction, LOS was also treated with PmB before stimulation of PBMEC. As shown in Figure 5, IL-6 production was not detected ($P < 0.05$) with PmB-treated LOS at any time point tested (Fig. 5A). Induction of IL-8 secretion by PmB-treated LOS was not completely abolished but was significantly lower ($P < 0.05$) compared with that stimulated by untreated LOS (Fig. 5B).

3.7. Field strains of *H. parasuis* induce IL-6 and IL-8 production by PBMEC

Several field strains of *H. parasuis* were compared for their capacity to induce the release of IL-6 and IL-8 (Fig. 6). Results showed that all the field strains tested (5×10^8 CFU/mL) were able to stimulate PBMEC to produce IL-6 and IL-8, although individual differences were observed among the strains, especially those of serotype 4 (Fig. 6). Moreover, the mean capacity to induce IL-6 and IL-8 was similar between *H. parasuis* serotypes 4 and 5 strains isolated from diseased pigs. Similar levels of cytokines were also observed with strains recovered from healthy animals (Fig. 6). No correlation between the origin of the strains (meningitis, pneumonia, etc.) and their capacity to induce cytokines could be observed (results not shown).

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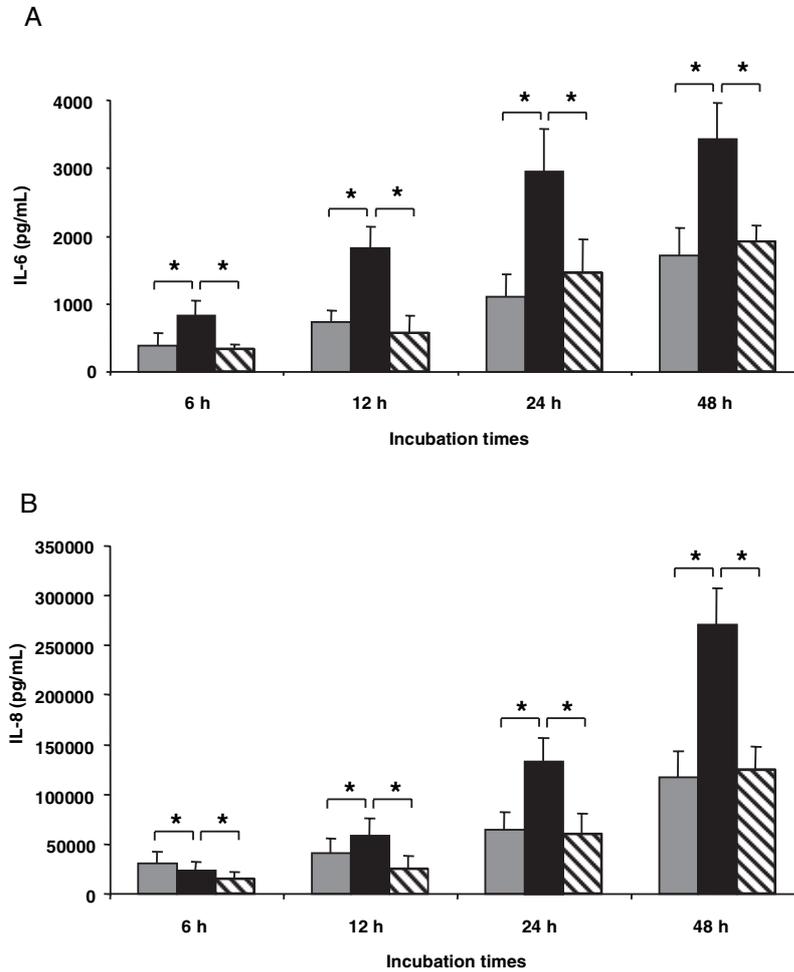
Figure 3. Kinetics of pro-inflammatory cytokines IL-6 (A) and IL-8 (B) production by PBMEC stimulated with live (1×10^7 CFU/mL, grey bars), heat-killed *H. parasuis* (5×10^8 CFU/mL, black bars), or PmB-treated heat-killed *H. parasuis* (5×10^8 CFU/mL, hatched bars). Data are expressed as mean ± SD. * $P < 0.05$.

4. DISCUSSION

To cause meningitis, bacterial pathogens must reach the CNS by traversing the BBB. It is generally accepted that bacterial interactions with BMEC, one of the primary components of the BBB, are mainly characterized by specific bacterial attachment with consequent invasion, toxicity and/or increase of permeability. We have previously shown that *H. parasuis* is able to adhere to and to invade

PBMEC [41]. We now demonstrated that LOS significantly impaired the adhesion of *H. parasuis* to PBMEC, suggesting a role for this bacterial component as an adhesin. Likewise, LPS from other bacterial species, including swine pathogens of the same family, such as *Actinobacillus pleuropneumoniae* have been shown to be important adhesins [30, 32]. Nevertheless, other adhesins may also contribute to bacteria-cell interactions based on our finding that the addition of LOS to a competitive

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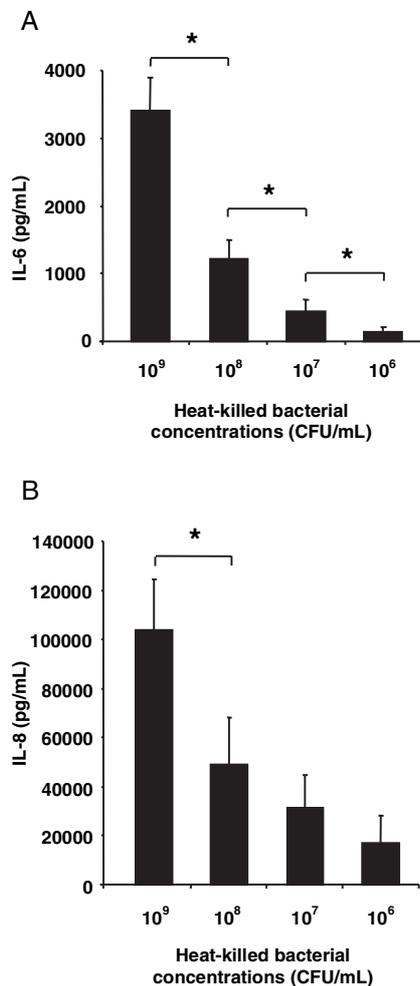
Figure 4. Effect of different concentrations of heat-killed bacteria (1×10^6 to 1×10^9 CFU/mL) on the release of IL-6 (A) and IL-8 (B) by PBMEC after 24 h. Data are expressed as mean ± SD. * $P < 0.05$.

assay did not completely abolish the adhesion of *H. parasuis* to PBMEC. In addition, the presence of a contaminant protein that might play a certain role as adhesin in the LOS preparation can not be completely ruled out. Bacterial attachment is often mediated by more than one component in order to multiply contact sites and to ensure strong attachment [1].

Previous studies in our laboratory [41] indicated that cytotoxicity is likely not a

mechanism used by *H. parasuis* to increase BBB permeability. In this study, we confirmed that *H. parasuis* as well as its purified LOS are not toxic to PBMEC. These results differ from those with *H. influenzae* and *H. somnus* [16, 35, 38]. Although not toxic, we demonstrated that both live and heat-killed *H. parasuis* are able to induce PBMEC apoptosis in caspase-3 dependent fashion as previously demonstrated for *H. somnus* [35, 36]. This observation also provides new insights into the role of apoptosis in the regulation of BBB permeability, particularly given that caspases are able to cleave cytoskeletal structures including tight and adherence junctional components [15]. More specifically, it has been demonstrated that tight junctional proteins, ZO-1 and ZO-2 and occludin, are cleaved by caspase-3 [15]. The role of apoptosis in the potential increase of the BBB permeability by *H. parasuis* remains to be investigated, with the use, for example, of transwells as previously described [6].

Since washed heat-killed *H. parasuis* induced similar levels of apoptosis as live bacteria, we hypothesized that cell-associated and heat-resistant bacterial components are involved in this process. Hence, we investigated whether *H. parasuis* LOS plays a role in the apoptosis of PBMEC. Differently from what has been observed for *H. somnus* [35], the absence of detectable DNA fragmentation following incubation of *H. parasuis* LOS with PBMEC suggests that *H. parasuis* LOS did not induce apoptosis. Moreover, we observed no decrease in apoptosis level induced by PmB-treated heat-killed bacteria. Further studies are needed to identify other surface structures that might be involved in *H. parasuis*-mediated PBMEC apoptosis.

As mentioned above, bacterial pathogens may also increase BBB permeability by stimulating the release of inflammatory mediators, which may increase not only local inflammatory mediators that contribute to the development of clinical signs but also barrier permeability [14, 35]. Notably, bacterial meningitis is by definition associated with inflammatory responses in the subarachnoid space, and TNF-α, IL-1β, IL-6, and IL-8 are

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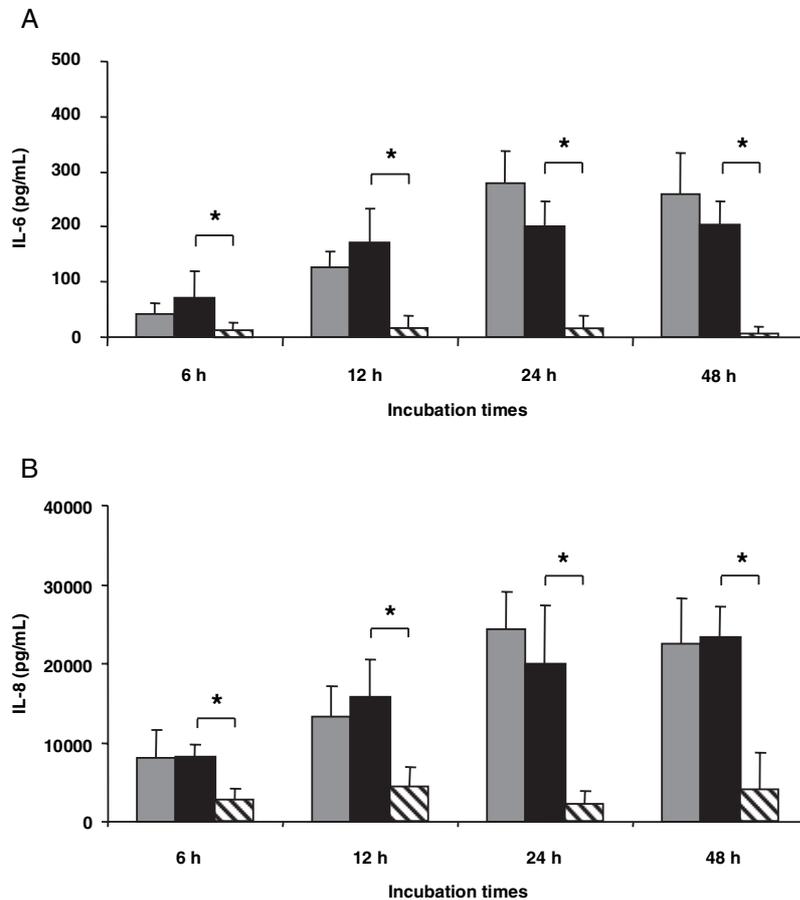
Figure 5. Kinetics of pro-inflammatory cytokines IL-6 (A) and IL-8 (B) production by PBMEC stimulated with *H. parasuis* LOS (10 µg/mL, black bars) or PmB-treated LOS (10 µg/mL, hatched bars). Purified *E. coli* LPS (10 µg/mL, grey bars) was used as a positive control. Data are expressed as mean ± SD. * $P < 0.05$.

the most frequently detected cytokines and chemokines in the cerebrospinal fluid (CSF) [11, 14]. Along with other cells, BMEC can produce pro-inflammatory cytokines as a result of stimulation by other cytokines (e.g. TNF-α, IL-1β) [7,40] or direct interaction with microbial pathogens [7, 39]. The present study demonstrates for the first time that *H. parasuis* induces the production of IL-8 and IL-6 by PBMEC. Chemokines are chemotactic molecules that mediate the recruitment of inflammatory cells such as neutrophils and activated T cells to sites of infection or injury [20]. The expression of IL-8 by cerebral endothelial

cells is thought to be a key event that initiates the translocation of adhering leukocytes across the BBB [34]. Since leukocytes themselves may cause injury by releasing potentially harmful products such as free radicals, metal-loproteases, perchloric acid, and eicosanoids, interactions between leukocytes and endothelial cells may also contribute to CNS damage during bacterial meningitis [11, 34]. IL-6, a key mediator of the inflammatory response in swine infections [13], is considered to be one of the best markers of meningitis [12,27]. IL-6 release could also lead to an increase in BBB permeability, since this cytokine has been

A

B
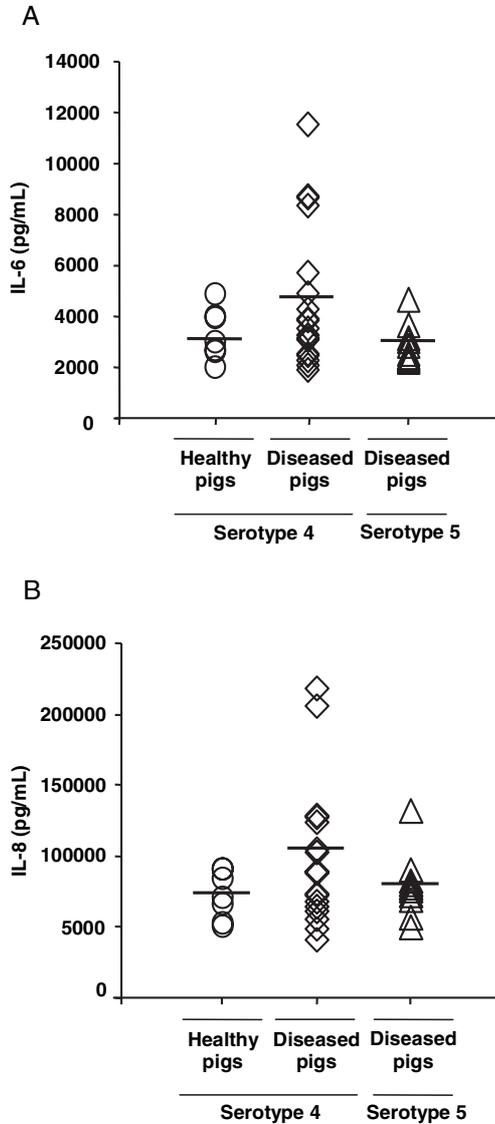
Figure 6. Comparative analysis of IL-6 (A) and IL-8 (B) production by different *H. parasuis* strains at 24 h post-infection. PBMEC were stimulated with heat-killed *H. parasuis* (5×10^8 CFU/mL) serotype 4 strains isolated from healthy ($n = 7$, circles) or diseased ($n = 18$, diamonds) pigs, or serotype 5 strains ($n = 10$, triangles) isolated from diseased pigs. Cytokine production in cell supernatants was measured by ELISA. Each point represents mean cytokine induction by one strain. Lines represent mean cytokine production by each serotype.

shown to modify transendothelial electrical resistance of cerebral endothelial cells, thereby reflecting alterations of BBB tight junctions [22].

In Gram-negative infections, cytokine-related disruption of the BBB is mainly mediated through LPS activity [42]. Previously, *H. parasuis* LOS has been shown to induce TNF-α production by a mouse macrophage cell line [28]. In the present study, PmB-treated heat-killed *H. parasuis* stimulated 50% less IL-6 and IL-8 release from PBMEC than did untreated heat-killed bacteria, indicating that LOS is only partially responsible for *H. parasuis*-induced cytokine production. Moreover, we observed that purified LOS was a relatively poor cytokine inducer suggesting that other cell-associated or heat-resistant bacterial component(s) are also involved in stimulating cytokine production. These results are consistent with other studies showing that pro-inflammatory responses elicited by *N. meningitidis* are mediated by LOS as well as non-LOS components [8, 31]. Further studies are needed to identify other surface structures involved in PBMEC stimulation by *H. parasuis*.

As mentioned above, 15 serotypes of *H. parasuis* have been identified using an immunodiffusion test [18, 37]. As serotype 5 strains are considered to be more virulent than those of serotype 4 [22], we compared several field strains of both serotypes for their capacity to induce pro-inflammatory cytokines. Although individual differences were observed, the mean capacity to induce cytokine release was similar between strains of serotypes 4 and 5 isolated from diseased pigs. Since the exact nature of the antigens involved in serotyping is not well know, it is difficult to establish a clear association between serotype, pathogenicity and inflammatory activator. In addition, strains isolated from healthy pigs (belonging to serotype 4) showed a similar capacity to induce cytokines. To induce high levels of pro-inflammatory cytokines from PBMEC, *H. parasuis* must be able to survive and replicate to reach high bacterial concentrations in the bloodstream. Recently, variability in serum

resistance of different strains of *H. parasuis* has been reported [5]. Thus, although most strains may have the potential to activate endothelial cells, only virulent and probably serum-resistant strains [5] may be able to achieve the high bacterial loads in the blood needed for effective cell activation.

To conclude, we demonstrated that *H. parasuis* LOS plays a contributing role in the adhesion of *H. parasuis* to PBMEC. We also demonstrated that *H. parasuis* is not toxic but can induce apoptosis of PBMEC while, surprisingly, LOS does not seem to play an important role in this process. In addition, we described the ability of *H. parasuis* to induce brain endothelial cells to release pro-inflammatory cytokines which in turn may play an important role in local inflammation as well as in increasing BBB permeability. However, in our model, LOS appeared to be a weak stimulator of cytokine production by PBMEC, suggesting that other bacterial components are responsible for the *H. parasuis*-induced cytokine response.

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