Evaluation of the pathogenesis of meningitis caused by *Streptococcus suis* sequence type 7 using the infection of BV2 microglial cells

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*Streptococcus suis* is an important agent of swine and human meningitis. Among several sequence types (STs) characterized within the *S. suis* strain population, ST7 has emerged only in China and has been reported to be the cause of the human outbreak caused by *S. suis* in 2005. *S. suis* ST7 was shown to be derived from *S. suis* ST1 through a single-nucleotide change in the housekeeping gene thyA. The virulence potential of *S. suis* ST7 is reported to be higher than that of the worldwide-studied pathogenic *S. suis* ST1. The pathogenesis of ST1 infection has been partially elucidated, but information on the pathogenesis of ST7 infections remains scarce. To improve our understanding of the mechanisms involved in the development of meningitis caused by ST7, this study compared the microglial inflammatory response induced by ST1 and ST7 strains. The data showed that *S. suis* ST7 possessed a higher ability to induce pro-inflammatory cytokine production and to activate mitogen-activated protein kinase pathways and several transcription factors. The stimulation of microglial cells by *S. suis* increased the expression levels of the nucleotide oligomerization domain 2 (Nod2) gene. Finally, the results indicated that signal transducer and activator of transcription 3 (STAT-3) was involved in the development of meningitis induced by *S. suis* ST7 infection.

INTRODUCTION

A large outbreak of 215 human cases emerged in 2005 in Sichuan Province, PR China, with 61 cases presenting a streptococcal toxic shock-like syndrome and 154 cases with meningitis not previously reported with *Streptococcus suis* (Ye *et al.*, 2006). Using multilocus sequence typing, *S. suis* sequence type (ST) 7 was recognized as the causative agent of the Sichuan outbreak (King *et al.*, 2002; Ye *et al.*, 2006). When a phylogenetic tree of *S. suis* was constructed using the concatenated sequences from seven housekeeping genes included in the multilocus sequence typing analysis, *S. suis* ST25 (which includes most North-American strains) was located at an early stage in the evolution of *S. suis*, whereas *S. suis* ST7 was shown to be derived from *S. suis* ST1 through a single-nucleotide change in the housekeeping gene thyA (Ye *et al.*, 2008). The pathogenesis of ST1 infection has been partially elucidated (Domínguez-Punaro *et al.*, 2007, 2010), but information on the pathogenesis of infection caused by ST7 remains scarce.

Meningitis is the most striking feature of *S. suis* infections. Using an *S. suis* mouse model of meningitis/encephalitis, an important inflammatory response in the central nervous system (CNS), with expression of different pro-inflammatory genes, was observed. Interestingly, the expression of these genes and bacterial antigens were found probably to be associated with microglia and, to a lesser extent, with astrocytes (Domínguez-Punaro *et al.*, 2007). Microglia, the macrophage-like population within the CNS, represents the first line of defence against invading pathogens and has pro-inflammatory effector functions (Domínguez-Punaro *et al.*, 2010; Olson & Miller, 2004). Microglial cells can sense pathogens using a panoply of pattern-recognition receptors, including Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors...
(NLRs). The earliest-identified and best-characterized NLRs are NOD1 and NOD2. The role of NLRs in S. suis infection and modulation of CNS inflammation remains to be elucidated.

Here, we compared S. suis ST7 and ST1 infections of the microglial cell line BV2 and analysed the level of bacterial-induced cell cytotoxicity, cytokine production, Nod2 expression, phosphorylation levels of the mitogen-activated protein kinase (MAPK) pathways and activation of a number of transcription factors (TFs) in cultured microglial cells to understand better the pathogenesis of meningitis caused by S. suis ST7.

**METHODS**

**Cell culture.** BV2 cells were purchased from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, PR China). This cell line exhibits morphology and functional characteristics of microglia and has been shown to be a valid substitute for primary microglia cells (Bocchini et al., 1992; Henn et al., 2009). The cells were cultured in Dulbecco’s minimal essential medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (10,000 U ml⁻¹). To harvest the BV2 cells, cells were treated with trypsin (Invitrogen), centrifuged at 300 g for 10 min and resuspended in complete medium. Cell viability was assessed using trypan blue staining (Invitrogen). For each experiment, 1 ml cells per well was plated into a 24-well plate at a density of 3 × 10⁵ cells ml⁻¹ and maintained in 5% CO₂ at 37°C for 48 h to allow the cells to grow to confluency (1.5 × 10⁶ cells ml⁻¹) before the infection assays. The medium was changed every 24 h.

**Bacterial strains and growth conditions.** The strains used in this study were S. suis strains 31533 and GZ1, which were typed as ST1, and strains SC84 and SC49, two representative strains of ST7. Strain 31533 was originally isolated from a case of porcine meningitis and has been widely used in previous studies (Dominguez-Punaro et al., 2007, 2010; Segura et al., 2002, 2006). Strain GZ1 was originally isolated from a patient with streptococcal toxic shock-like syndrome in combination with meningitis during the Sichuan outbreak in 2005 (Ye et al., 2009; Zheng et al., 2012). Strain SC84 was isolated from a patient with streptococcal toxic shock-like syndrome in combination with meningitis during the Sichuan outbreak in 2005 (Ye et al., 2009; Zheng et al., 2012). Strain SC49 was isolated from a diseased pig whose owner was the host of the SC84 strain (Zheng et al., 2012). SC84 and GZ1 were selected because their whole genomes have been sequenced by our laboratory (GenBank accession nos FM252031 and CP000837).

The S. suis strains were grown overnight on Columbia blood base agar plates (Detgerm Microbiological Science) at 37°C, and isolated colonies were inoculated into 10 ml Todd–Hewitt broth (THB; Oxoid). The culture was incubated without shaking for 8 h at 37°C. Working cultures were prepared by transferring 10 μl 1:1000 diluted 8 h cultures into 30 ml THB and incubating without shaking for 16 h at 37°C with 5% CO₂. The bacteria were washed twice in PBS (pH 7.4; Invitrogen). The pellet was then resuspended in 10 ml PBS. Serial dilutions of the suspension were plated onto THB agar plates to determine the number of c.f.u. ml⁻¹.

**Infection of BV2 microglial cells.** S. suis (1 × 10⁶ c.f.u. per well) was added to BV2 cells and incubated for 2, 4, 6, 8 and 12 h, and for 15 h (for interleukin-1 (IL-1) and IL-23 assays only) in 24-well flat-bottomed plates (Becton Dickinson). The supernatant was collected for measuring cytokine concentrations and lactate dehydrogenase (LDH) release, as described previously (Zheng et al., 2011). The remaining cells were washed three times with PBS and then lysed by repeated pipetting with Trizol (1 ml per well; Invitrogen). The Trizol lysate was transferred to nuclease-free tubes for RNA extraction. Each test was repeated at least three times in independent experiments. In pro-inflammation cytokine analysis, cells with medium alone (unstimulated cells) served as negative controls. Cells activated with 0.1 μg synthetic triacylated lipoprotein Pam3CSK4 ml⁻¹ (InvivoGen), a known TLR2 agonist, were used as positive controls. Pam3CSK4 was used as a negative control in the IL-23 test. Pam3CSK4 was diluted in cell-culture medium before the assay. Each test was repeated at least three times in independent experiments.

**Cytokine analysis and cytotoxicity tests.** IL-1α, IL-1β, IL-6, tumour necrosis factor α (TNF-α), and monocyte chemotactic protein 1 (MCP-1) concentrations were measured using a Bio-Plex mouse cytokine group 1 reagent kit (Bio-Rad). Cytokine levels were analysed with Bio-Plex Manager version 6.0 software. Levels of IL-23 in cell-culture supernatants were measured using an ELISA kit (R&D Systems). Bacterial-induced cell cytotoxicity was evaluated by measuring LDH release using a Cyto-Tox 96 cytotoxicity kit (Promega) according to the manufacturer’s instructions. The percentage cytotoxicity was calculated after measuring sample absorbance at 490 nm (A₄₉₀ nm) as [([A₄₉₀ nm−A₈₅₀ nm]/A₁₀₀₀ nm−A₈₅₀ nm)] × 100, where A₈₅₀ nm represents the A₈₅₀ of non-infected cells and A₁₀₀₀ nm represents the A₈₅₀ of cells treated with lysis buffer, as described by the manufacturer. Empty wells with cell-culture medium alone served as control blanks. All data were expressed as means ± SD.

**RNA extraction.** Total RNA was purified using an RNA extraction kit (Invitrogen). The final RNA pellet was resuspended in 50 μl nuclelease-free water (Invitrogen). RNA concentration and purity were measured using a GeneQuant spectrophotometer ND-1000 (NanoDrop). The RNA was stored at −80°C until used.

**RT-PCR and real-time PCR for Nod2.** Total RNA (1 μg) was mixed with 4 μl iScript reaction mix (5 × 1), 1 μl iScript reverse transcriptase (Bio-Rad) and 20 μl nuclelease-free water, and heated at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. One microtitre cDNA (20 ng μl⁻¹) was mixed with 5 μl 2× master mix (Bio-Rad), 1 μl primers (250 nM final concentration of forward and reverse primer) and 3 μl nuclelease-free water. The primers used for amplification of the different target cDNA were all tested to achieve an amplification efficiency between 90 and 110%. The primer sequences were designed from GenBank mRNA sequences using web-based software Primerquest from Integrated DNA technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/): Nod2 forward primer 5'-CTCGAGATCCGCCGCGACG-3' and reverse 5'-TTGCTCTCC-ACCGGAGCCAG-3'; and β-actin forward primer 5'-CAACGGTG- GAAAGATGACC-3' and reverse 5'-AGCATAGCCTCTGATAGTG-3'. 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 denaturation for 2 s at 98°C and annealing/extension for 5 s at 56°C (melt curve 65–95°C, increment 0.5°C per 10 s). The levels of Nod2 gene expression after S. suis infection were calculated after normalizing the cycle threshold (Cₜ) against the housekeeping β-actin gene using the 2^ΔΔCt method. The results are presented as fold induction relative to non-infected BV2 cells. Results are means ± SD of three independent experiments. Cells activated with 0.1 μg L18-muramyl dipeptide (MDP; InvivoGen) ml⁻¹, a known NOD2 agonist, were used as a positive control. Because Pam3CSK4 (0.1 μg ml⁻¹) should not activate Nod2, it was included as a negative control in these assays.

**In vitro cytokine production and TLR2 blockade.** Blocking experiments for TLR2 were performed using blocking antibodies (InvivoGen). Briefly, BV2 cells were incubated with 5 μg mouse TLR2 neutralizing antibody (clone C9A12; mouse IgG2a isotype) ml⁻¹ for
1 h to ensure complete blocking prior to incubation at 37 °C with S. suis (1 × 10^6 c.f.u. per well) for 8 and 12 h. The blocking efficacy of the antibody was assessed using the TLR2-specific ligand Pam3CSK4 (0.1 µg ml⁻¹), as described previously (Zheng et al., 2012). In order to exclude non-specific inhibition, an isotype control of mouse TLR2 neutralizing antibody (5 µg ml⁻¹; SouthernBiotec) was also included in the study.

**Phosphoprotein detection of MAPK pathways.** BV2 microglial cells were incubated with S. suis (1 × 10^6 c.f.u. per well) for 0.5, 1, 2, 3, 4, 5 and 6 h in 24-well flat-bottomed plates. The lysates derived from cell cultures were prepared using a cell lysis kit (Bio-Rad) according to the protocol provided by the manufacturer. Protein concentrations were determined using a Quick Start Bradford protein assay (Bio-Rad). The protein concentration of all samples was adjusted to 200 µg ml⁻¹. Phosphoprotein assays for stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), extracellular signal-regulated kinase (ERK1/2) and p38 were determined using a phosphoprotein detection reagent kit (Bio-Rad) according to the manufacturer’s instructions. The values were determined as mean fluorescence intensity (MFI) using Bio-Plex Manager version 6.0 software, and results are shown as means ± SD of three independent experiments.

**Analysis of the DNA-binding activity of 40 TFs.** BV2 microglial cells were incubated with S. suis (1 × 10^6 c.f.u. per well) for 4, 6, 8 and 12 h in 24-well flat-bottomed plates. Nuclear extracts of BV2 cells were prepared according to the Panomics protocol below. Protein concentrations were determined using a Quick Start Bradford protein assay. The total protein of each 5 µl sample was adjusted to 2 µg. DNA-binding activity assays for 40 TFs were determined using panel 1 of the Procarta transcription factor plex kit (Panomics) according to the manufacturer’s directions. The values are presented as fluorescence intensity using Bio-Plex Manager version 6.0 software. Results are shown as means ± SD of three independent experiments.

**Statistics.** In order to reduce the quantity of graphs, we merged the data from the 31533 and GZ1 strains, and the SC84 and SC49 strains, into ST1 and ST7 strain groups, respectively. In the graphs, data are expressed as the means of the two ST1 and two ST7 strains. Statistical analysis was performed using Student’s unpaired t-test. Differences between the groups were considered significant at P<0.05.

**RESULTS**

**Cytotoxicity induced by ST7 is higher than that induced by ST1**

Cytotoxicity levels induced by S. suis were time dependent. Live bacterial cell suspensions of S. suis ST7 strains were significantly more toxic to microglial cells than ST1 strains from 6–12 h incubation (Fig. 1e).

**ST7 induces higher cytokine production levels by microglial cells than ST1**

Stimulation with live S. suis induced time-dependent pro-inflammatory cytokine and chemokine production, levels of which were significantly higher than that of unstimulated cells. The positive control, Pam3CSK4, induced high levels of all these cytokines. The kinetics and levels of IL-6, TNF-α, MCP-1 and IL-1α secreted by microglial cells in response to S. suis were different (Fig. 1). At 8 and 12 h incubation, the levels of IL-6 and TNF-α in BV2 cells infected with the ST7 strains were significantly higher than those infected with the ST1 strains (Fig. 1a, b). In contrast to IL-6 and TNF-α, no obvious differences in MCP-1 levels were observed between the two STs at 8 h of incubation. However, microglial cells infected with ST7 strains produced significantly higher levels of MCP-1 at 12 h incubation than those infected with ST1 strains (Fig. 1c). IL-1 is another important pro-inflammatory cytokine that has been found to be involved in the pathogenesis of S. suis (De Greeff et al., 2010; Vadeboncoeur et al., 2003). Significant levels of IL-1α were observed only after 12 h of incubation. Microglial cells infected with ST7 strains produced significantly higher levels of IL-1α at 15 h incubation than the ST1 strains (Fig. 1d). We did not find differences in IL-1β levels between the two STs at any incubation time (data not shown).

**S. suis ST7 possesses a higher capacity to activate MAPK phosphorylation in microglial cells than ST1**

Domínguez-Punaro et al. (2010) showed that ERK1/2, JNK and p38 are involved in the regulation of microglial production of pro-inflammatory cytokines and chemokines in response to S. suis infection (Domínguez-Punaro et al., 2010). To evaluate further whether ST1 and ST7 strains differed in their capacity to activate MAPK pathways, the phosphorylation patterns of ERK1/2, JNK and p38 were compared in microglial cells infected with the two STs. In general, phosphorylation levels of these MAPK kinases were found to increase at 30 min post-infection (p.i.), reaching maximal levels at 4 h of bacterial cell contact. Downregulation of MAPK phosphorylation was observed at longer incubation times (data not shown). The levels of phosphorylation of ERK1/2, JNK and p38 were significantly higher when cells were infected with ST7 strains at 4 h p.i. compared with those observed with ST1 strains (Fig. 2).

**Role of TLR2 in cytokine production by microglial cells infected with S. suis ST1 and ST7**

To determine the role of TLR2 in release of the pro-inflammatory cytokines IL-6, TNF-α and MCP-1 by microglial cells, blocking experiments were performed using an anti-TLR2 neutralizing antibody. The efficacy of the antibody to block TLR2 was evaluated using Pam3CSK4 as a positive control. Cytokine production induced by Pam3CSK4 was significantly reduced, although not completely eliminated, by treatment of the cells with anti-TLR2 antibody (Fig. 3). Treatment with an isotype control did not affect cytokine production by microglial cells in response to S. suis ST1 or ST7 infection (data not shown). Pre-treatment of microglial cells with anti-TLR2 antibody significantly reduced the production of IL-6 and TNF-α but not that of MCP-1 induced by either S. suis ST1 or ST7 at 8 h (data not shown) and 12 h p.i. (Fig. 3). Interestingly, the production of MCP-1 by microglial cells

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stimulated with Pam3CSK4 was significantly reduced at 8 and 12 h post-stimulation when cells were pre-treated with the anti-TLR2 antibody (Fig. 3).

**S. suis enhances Nod2 mRNA expression levels in microglial cells**

We have demonstrated previously that TLR2 expression is required for some, but not all, of the inflammatory responses induced by *S. suis* infection of primary murine astrocytes (Zheng et al., 2011). Similarly, only a partial involvement of TLR2 in microglial cell activation by ST1 and ST7 strains was observed in Fig. 3. Liu et al. (2010) demonstrated that NOD2 plays a critical role in the establishment of the lethal inflammation associated with streptococcal meningitis. Thus, in the present study, mRNA expression levels of *Nod2* were tested using real-time PCR at 6, 8 and 12 h p.i. with live *S. suis* ST7 and ST1 strains. At 8 h p.i., expression of *Nod2* was significantly higher compared with unstimulated microglial cells. The positive-control MDP induced significant levels of *Nod2* expression, whilst Pam3CSK4 failed to activate transcription of this gene.
However, no significant differences were observed in Nod2 expression levels between ST7- and ST1-infected cells at any incubation time (Fig. 4). As is the case with TLR2, NOD2 seems to be involved in the inflammatory response induced by \textit{S. suis} independently of the ST.

\textbf{\textit{S. suis} ST7 possesses a higher capacity to activate TF DNA-binding activity in microglial cells than ST1}

To elucidate further the mechanism(s) by which \textit{S. suis} induced the production of cytokines in microglial cells, we analysed the binding activities of 40 TFs in \textit{S. suis}-exposed microglial cells. The two ST strains induced DNA binding of nuclear factor (NF)-E2, activator protein-1 (AP-1), NF-κB, NF-Y and cAMP-responsive element binding protein (CREB) with similar kinetics (data not shown). The intensity of the DNA-binding complex increased with time. \textit{S. suis} ST7 and ST1 strains both induced significant activation of these TFs after 6 h incubation time compared with unstimulated cells. At 12 h p.i., ST7 strains possessed a higher capacity to activate the DNA-binding activity of NF-κB, AP-1, NF-E2 and CREB in microglial cells than ST1 strains (Fig. 5a–d). No significant differences in activation of the TF NF-Y were observed between the two ST strains at any incubation time (Fig. 5e). In contrast, to the best of our knowledge, this is the first study showing that ST7 strains are able to induce time-dependent activation of signal transducer and activator of transcription 3 (STAT-3) compared with unstimulated cells (Fig. 5f). The ST1 strain and Pam3CSK4 failed to induce significant activation of STAT-3 at any incubation time (Fig. 5f).

\textbf{\textit{S. suis} ST7 possesses a higher capacity to induce IL-23 production by microglial cells than ST1}

As STAT-3 is one of the major signal transducers of the IL-23 pathway (Lankford & Frucht, 2003), we next evaluated whether ST7 strains possessed a higher capacity to induce IL-23 production by microglial cells than ST1 strains. In general, IL-23 levels increased after 12 h of incubation compared with unstimulated cells. At 15 h p.i., the ST7 strains induced significantly higher levels of IL-23 secretion by microglial cells than the ST1 strains. Pam3CSK4 as a negative control did not induce IL-23 production by

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\textbf{Fig. 2.} \textit{S. suis}-induced phosphorylation of MAPK pathways in microglial cells. BV2 microglial cells were incubated in the presence of bacteria \((1\times10^6\text{ c.f.u. per well})\) for 4 h. The lysates derived from cell cultures were analysed for phosphoprotein levels using Bio-Plex Manager software. Data are expressed as MFI (± SD) from three independent experiments. Black bars, ST7; white bars, ST1; grey bars, blank. *\(P<0.05\), indicates significant differences between \textit{S. suis}-infected cells and control cells (blank); †\(P<0.05\), indicates significant differences between cells infected with ST7 and ST1 strains.

\textbf{Fig. 3.} Role of TLR2 in cytokine production by \textit{S. suis}-infected microglial cells. BV2 microglial cells \((1\times10^6\text{ per well})\) were pretreated with an anti-TLR2 neutralizing antibody \((5\mu\text{g ml}^{-1})\) and cultured with live \textit{S. suis} \((1\times10^6 \text{ cells per well})\) or Pam3CSK4 \((0.1\\mu\text{g ml}^{-1})\) for 12 h. Supernatants were collected for measuring IL-6, TNF-α and MCP-1 concentrations using a Bio-Plex mouse cytokine kit. Data are expressed as means ± SD (pg ml\(^{-1}\)) of pooled ST7 or ST1 from three independent experiments. *\(P<0.05\), indicates significant differences between the untreated and anti-TLR2-treated groups.
DISCUSSION

Microglial cells are major constituents of innate immunity within the CNS (Kreutzberg, 1996). Isolation of primary microglial cell cultures has helped to determine the many roles of these cells. In addition to primary microglial cells, several immortalized cell lines have been created to model primary microglia in vitro including murine-derived BV2 cells. Several studies use BV2 cells as surrogates for primary cells and report limited deviations (De Jong et al., 2008; Häusler et al., 2002). Here, we demonstrated that live S. suis ST7 strains induced higher levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-1α, and MCP-1 by BV2 microglial cells than ST1 strains. It should be noted that both ST strains are well encapsulated and highly resistant to phagocytosis by microglial cells (unpublished observations); thus, the observed differences in their microglial cells at any incubation time (Fig. 6). The higher capacity of ST7 strains to induce IL-23 correlated with the observed increased levels of STAT-3 activation.

**Fig. 4.** S. suis enhanced NOD2 expression levels in microglial cells. BV2 microglial cells (1×10⁶ per well) were infected with live S. suis (1×10⁶ per well) for 8 h. The levels of NOD2 gene expression after S. suis infection were calculated after normalizing C₅ values against the housekeeping gene β-actin using the 2⁻ΔΔC₅₀ method. The results are presented as fold induction values relative to non-infected microglial cells. Results are means ± SD of three independent experiments. MDP (0.1 μg ml⁻¹) and Pam3CSK4 (0.1 μg ml⁻¹) were used as positive and negative controls, respectively.

**Fig. 5.** S. suis enhanced DNA-binding activity of various TFs in microglial cells. BV2 microglial cells were incubated in the presence of bacteria (1×10⁶ c.f.u. per well) for 12 h. The cell nuclear extracts were analysed for the DNA-binding activity of 40 TFs using a Procarta transcription factor plex kit. Data were analysed using Bio-Plex Manager software and expressed as MFI (±SD) from three independent experiments. *P<0.05, indicates significant differences between S. suis-infected cells and control cells (blank); ††P<0.05, indicates significant differences between cells infected with ST7 and ST1.
inflammatory properties were not related to differences in bacterial killing.

In addition to the inflammatory properties, we also demonstrated that cytotoxicity levels induced by ST7 infection of microglial cells were higher than levels induced by ST1 strains. Our data suggested that the increased capacity to induce excessive pro-inflammatory cytokine production by microglia cells combined with the higher toxicity to these cells may contribute to the deterioration of clinical outcome during meningitis caused by ST7 strains.

The use of an MAPK inhibitor has been shown almost to eliminate cytokine release from microglia infected with an S. suis strain, showing the importance of ERK1/2, JNK and p38 in the inflammatory response against the pathogen (Dominguez-Punaro et al., 2010). Therefore, we postulated that the ST7 strains possess a higher capacity to activate these three MAPK intracellular signalling pathways than ST1 strains. As expected, the levels of phosphorylation obtained when microglial cells were infected with ST7 strains were stronger than those obtained with ST1 strains. In addition to the MAPK contribution to cytokine release, this pathway also regulates oxidative stress, which can in turn induce cellular injury (Kulebyakin et al., 2012; Park et al., 2012). Oxidative stress can cause overexpression of antioxidant responsive factors in cells. This was shown by our TF results. The two types of strain induced DNA binding of antioxidant Re (antioxidant responsive factor) with similar kinetics. At 12 h p.i., ST7 strains possessed a higher capacity to activate DNA-binding activity of antioxidant Re in microglial cells than ST1 strains (unpublished observations). These results indicated that oxidative stress injury was involved in the pathogenesis caused by S. suis.

The role of TLR2 in the recognition of S. suis by several types of host cell is well established (Dominguez-Punaro et al., 2010; Graveline et al., 2007; Zheng et al., 2011, 2012). To confirm the role of TLR2 in the S. suis-induced inflammatory response of microglial cells, we investigated the induction of cytokines after TLR2 neutralization of microglial cells infected with ST1 or ST7 strains. Interestingly, TLR2 neutralization only partially impaired TNF-α and IL-6 production, and did not affect MCP-1 release in response to S. suis, suggesting that alternative receptors are also involved in bacterial recognition. In this regard, we also observed that Nod2 expression levels were increased in S. suis-infected cells. Based on these results and previous observations (Zheng et al., 2012), we suggest that maximal microglia responses to live S. suis require the involvement of TLRs and cytosolic NOD2. However, no differences were observed in the TLR2 neutralizing effect or in Nod2 expression levels between the two types of strain, suggesting that differential engagement of these receptors is not sufficient to explain the observed differences in pathogenesis of ST1 and ST7.

Analysis of the activation of TFs involved in immune responses gave novel insights into the molecular mechanisms involved in S. suis interactions with microglial cells. In general, the intracellular transcription regulation initiated by S. suis was shown to involve the TFs NF-E2, AP-1, NF-κB, NF-Y and CREB. ST7 strains induced higher levels of activation of NF-E2, AP-1, NF-κB and CREB than ST1 strains, which is in agreement with the greater pro-inflammatory properties of ST7. Similarly, Tsai et al. (2006) demonstrated that the TFs NF-κB and AP-1, regulated by MAPKs, are activated after streptococcal infection. Gupta et al. (1999) demonstrated that bacterial cell-wall peptidoglycan induces phosphorylation of CREB through CD14 ligation. It can be hypothesized that differences in cell-wall composition between the two types of strain contribute to the capacity to activate the CREB pathway. The CCAAT motif is one of the common promoter elements present in the proximal promoter of numerous mammalian genes. The major CCAAT box-recognizing TF is NF-Y, which consists of three subunits: NF-YA, NF-YB and NF-YC. NF-Y factor contributes to the activation of microglia and participates in the regulation of major histocompatibility complex class II, enabling the microglia to present antigens efficiently to T-cells and providing the CNS with a means to respond rapidly and efficiently to a wide variety of pathogens (Olson & Miller, 2004; Ting & Baldwin, 1993). Despite the importance of this TF, no differences were observed in the capacity of ST1 and ST7 strains to modulate NF-Y. As such, the mechanisms underlying differential activation of all these TFs as well as their role in the pathogenesis of S. suis infection warrant further investigation.

We are, to the best of our knowledge, the first to report that ST7 strains can induce time-dependent activation of STAT-3. STAT-3 is the major signal transducer for IL-23, a cytokine shown to be upregulated in adult mouse microglia (Li et al., 2003). IL-6 has also been reported to play a role in bacterial-induced STAT-3 activation (O’Shea, 1997; Zhu et al., 2010). To confirm the role of TLR2 in the S. suis-induced inflammatory response of microglial cells, we investigated the induction of cytokines after TLR2 neutralization of microglial cells infected with ST1 or ST7 strains. Interestingly, TLR2 neutralization only partially impaired TNF-α and IL-6 production, and did not affect MCP-1 release in response to S. suis, suggesting that alternative receptors are also involved in bacterial recognition. In this regard, we also observed that Nod2 expression levels were increased in S. suis-infected cells. Based on these results and previous observations (Zheng et al., 2012), we suggest that maximal microglia responses to live S. suis require the involvement of TLRs and cytosolic NOD2. However, no differences were observed in the TLR2 neutralizing effect or in Nod2 expression levels between the two types of strain, suggesting that differential engagement of these receptors is not sufficient to explain the observed differences in pathogenesis of ST1 and ST7.

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& Paul, 2008). In this study, although ST1 strains possessed the capacity to induce the production of low levels of IL-23 and IL-6 by microglial cells, they were not able to significantly activate STAT-3. Interestingly, Miettinen et al. (2000) reported that neutralization of IL-6 did not have any effect on Streptococcus pyogenes-induced activation of STAT-3. These results suggest that ST7-dependent activation of STAT-3 may be related to a more efficient induction of IL-23 and/or IL-6 production than that of ST1 strains. It might also be possible that ST7 strains possess alternative mechanisms to induce STAT-3 pathways. Kawanokuchi et al. (2008) showed for the first time that primary microglia produce IL-17 in response to IL-23. Although we did not detect IL-17 in the supernatant of in vivo microglial cells stimulated by S. suis (unpublished observations), we cannot rule out the possibility that IL-17 is involved in the pathogenesis of meningitis caused by S. suis in vivo.

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