Role of surface proteins SspA and SspB of *Streptococcus gordonii* in innate immunity

Elisoa Andrian,1,2,3 Gaofu Qi,2,3 Jun Wang,2,3,4 Scott A. Halperin2,3,4 and Song F. Lee1,2,3,4

1Department of Applied Oral Sciences, Faculty of Dentistry, Dalhousie University, Halifax, NS, Canada
2Department of Microbiology and Immunology, Dalhousie University, Halifax, NS, Canada
3Canadian Center for Vaccinology, Dalhousie University and the IWK Health Centre, Halifax, NS, Canada
4Department of Pediatrics, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada

*S. gordonii*, a normal inhabitant of the human oral cavity, is a potential live vaccine vehicle. Several pathogen-associated molecular patterns from *S. gordonii* that are recognized by antigen-presenting cells have recently been identified. In this study, we have identified that the cell-wall-anchored proteins SspA and SspB are immunostimulatory components of *S. gordonii*. SspA and SspB are members of the antigen I/II family of proteins widely expressed by viridans oral streptococci. The results showed that the mutant (OB219) lacking SspA and SspB had a reduced ability to induce cytokine/chemokine production in epithelial cells and bone-marrow-derived dendritic cells as compared with the parent strain (DL1). Purified SspA induced interleukin-6 and monocyte chemotactic protein-1 production from human lung epithelial A549 cells. The induction could be inhibited by a function-blocking anti-β1 integrin mAb and the purified SspA could bind to β1 integrin precoated on microtitre plates, suggesting that the induction was effected by SspA–β1 integrin interactions. The role of SspA and SspB in innate immunity was further demonstrated in a mouse intranasal challenge experiment, which showed that the clearance of OB219, the recruitment of neutrophils (as indicated by myeloperoxidase activity), and chemokine and cytokine production in the lungs of OB219-inoculated mice were delayed or reduced as compared with the DL1-inoculated mice. In addition to the above, *S. gordonii* OB219 was more sensitive to polymyxin, nisin and histatin-5 than DL1, suggesting that SspA and SspB also play a role in susceptibility to cationic antimicrobial peptides. Collectively, the results indicate that SspA and SspB are immunostimulatory components of *S. gordonii* and play an important role in modulating the host’s innate immunity.

INTRODUCTION

*Streptococcus gordonii* is a Gram-positive commensal bacterium of the human oral cavity. The bacterium has garnered interest as a potential live oral vaccine delivery vector due to its commensal nature and its ease in genetic manipulation (Lee, 2003; Oggioni et al., 1999). Although *S. gordonii* is a commensal bacterium, it is readily recognized by professional antigen-presenting cells, such as monocytes and dendritic cells (DCs), inducing both the production of cytokines and the upregulation of surface proteins indicative of cellular maturation (Chan et al., 2007; Ciabattini et al., 2006; Corinti et al., 1999). The activation of antigen-presenting cells by extracellular bacteria is thought to occur predominantly through Toll-like receptor (TLR) recognition of pathogen-associated molecular patterns (PAMPs) that are expressed by the bacteria (Kawai & Akira, 2005). We have recently demonstrated that lipoteichoic acid, lipoproteins, peptidoglycan and chromosomal DNA from *S. gordonii* are components of PAMPs that are recognized by DCs and can induce cytokine production and upregulation of cell surface markers (Chan et al., 2007; Mayer et al., 2009a, b). The activation of DCs by these PAMPs primarily involved TLR-2 and TLR-9 via a MyD88-dependent pathway.

SspA (172 kDa) and SspB (164 kDa) are two major cell-wall-anchored proteins of *S. gordonii*. The proteins belong to a class of structurally, immunologically and functionally...
related proteins, named the antigen I/II family, produced by most species of oral viridans streptococci (Jenkinson & Demuth, 1997; Ma et al., 1991). The antigen I/II family proteins consist of between 1310 and 1653 amino acid residues and are all anchored to peptidoglycan via a C-terminal sortase motif LPXTG (Homonylo-McGavin & Lee, 1996; Jenkinson & Demuth, 1997). SspA and SspB are encoded by tandemly arranged genes but are independently expressed (Demuth et al., 1996). The two proteins are >96% identical except for the middle V domain which is 26% identical. SspA and SspB mediate S. gordonii binding to a high-molecular-mass salivary agglutinin glycoprotein (Demuth et al., 1996; Jakubovics et al., 2005), to type I collagen (Heddle et al., 2003) and to other oral bacteria (Demuth et al., 2001; Egland et al., 2001; Jakubovics et al., 2005). Recently, SspA and SspB were shown to facilitate the binding of S. gordonii to HEp-2 cells (Nobbs et al., 2007). The closely related antigen I/II protein of Streptococcus mutans, protein I/IIf, was reported to have ‘modulin’ properties, inducing interleukin (IL)-8 in epithelial cells and proinflammatory cytokines (IL-1β, IL-6 and tumour necrosis factor, TNF) in monocytes (Vernier-Geronthem et al., 1998). The response to protein I/II stimulation was independent of TLR-2, TLR-4 and TLR-6 but involved focal adhesion kinase (Al-Okla et al., 1999; Neff et al., 2003; Zeisel et al., 2005).

In contrast with the S. mutans protein I/IIf, very little is known of the immunostimulatory properties of S. gordonii SspA and SspB. The present study was aimed at understanding the role of SspA and SspB in the host’s response. Our results showed that SspA and SspB modulated the host’s innate immunity and contributed to bacterial clearance in an animal model.

METHODS

Bacteria and growth conditions. S. gordonii DL1 and its isogenic mutant OB219 (spA’spB::ermAM, Demuth et al., 1996) were grown in either brain heart infusion broth (BHI; Becton Dickinson), Todd–Hewitt broth (Becton Dickinson) or TYG broth (1% K2HPO4, 1% tryptone, 0.5% yeast extract, 1%, w/v, glucose) at 37°C and 5% CO2. Erythromycin, kanamycin and tetracycline were included in the medium at 10 μg ml−1, 250 μg ml−1 and 10 μg ml−1, respectively, when needed. Recombinant Escherichia coli was grown aerobically with vigorous shaking at 37°C in Luria–Bertani broth (1% tryptone, 0.5% yeast extract and 1%, w/v, NaCl) containing 50 μg kanamycin ml−1 and 10 μg tetracycline ml−1.

Mammalian cell cultures. Human lung epithelial A549 cells and human larynx epithelial HEp-2 cells (ATCC CCL-23) were cultured in modified Eagle’s medium (MEM) (Invitrogen Life Technologies) containing 1% (v/v) penicillin, 1% (v/v) streptomycin and 10% (v/v) fetal bovine serum (Invitrogen). Bone-marrow-derived DCs were cultured from the femur and tibia of 6-week-old female BALB/c mice (Charles River Laboratory) as previously described (Chan et al., 2007). Cells were incubated at 37°C in a 5% CO2 incubator. The use of mice for DC cultures and intranasal challenge experiments (see below) was approved by the University Committee on Laboratory Animals of Dalhousie University.

Cell stimulation. A549 or HEp-2 cells were seeded at 5 × 105 per well in 12-well plates and grown to 90% confluence. The monolayers were washed once with PBS and stimulated with 1 × 105 c.f.u. of S. gordonii (m.o.i. of ca 2) or the indicated amount of purified SspA for 24 h. In the antibody inhibition experiments, A549 cells were reacted with 1 μg antihuman β integrin mAb ml−1 (clone P4C10, Millipore) for 30 min prior to SspA addition. Eight-day-old immature DCs (1 × 105) were stimulated with S. gordonii at the ratios of 5, 10 or 20 bacterial cells to DCs (Mayer et al., 2009a, b). For controls, cells were stimulated with 1 μg LPS (E. coli, Sigma-Aldrich) ml−1, 20 ng TNF ml−1 (Sigma-Aldrich) or left unstimulated. Following 24 h of stimulation, the culture supernatants were collected and analysed for selected cytokines or chemokines by ELISA using reagents from R&D Systems or ebiosciences.

Binding assays. HEp-2 or A549 cells were seeded at a concentration of 106 cells per well in 24-well plates. Cells were grown to confluent monolayers. Monolayers were then washed with PBS, and S. gordonii cells (105 c.f.u.) in serum-free MEM were added. The plates were incubated for 90 min at 37°C. Following incubation, cells were washed with PBS to remove unbound bacteria. Cells were then detached with trypsin/express (Invitrogen) and lysed with 0.4 ml 0.025% (v/v) Triton X-100 for 5 min at 37°C. The lysates were serially diluted in PBS and plated in triplicate on BHI agar plates for viable cell counts. To determine the number of bacteria internalized, the monolayers were incubated with serum-free MEM containing 1% penicillin/streptomycin and 100 μg gentamicin ml−1 for 2 h prior to lysis and plating.

Production and purification of the SspA protein. The DNA coding for the mature SspA protein without the C-terminal LPXTG cell wall domain was amplified from the genome of S. gordonii DL1 by PCR using the primers SL629 (5’-TGGCCCGAGCCGGCGGATGAAATGCAGAAAACAAAT-3’) and SL593 (5’-CCTGGCCGGCCTGGCCCTGTGTTAATCTTTACCAAC-3’), SfiI sites underlined in both sequences) and long PCR enzyme mix (Fermentas Life Sciences). The 5 kb PCR product was cloned into the SfiI sites of pSecCR1 (Knight et al., 2008), putting the sspA gene behind the tetracycline-inducible promoter P tokay and the S. mutans spaP ribosome-binding site and signal sequence (Kelly et al., 1989; Mallaley et al., 2006). The cloning also created an in-frame fusion of a C-terminal histidine and an HA tag to SspA. To facilitate the delivery of the recombinant sspA gene to the chromosome, the 4.5 kb DNA fragment carrying the streptococcal replication origin on the plasmid was deleted. This was achieved by digesting the plasmid with Scal and EcoRI, followed by Klenow treatment. The resulting 9 kb DNA fragment was ligated to obtain the plasmid pSecSpA-1 and maintained in E. coli XL-blue. pSecSpA-1 was transformed into S. gordonii SL3 (hppC::tet) by natural transformation (Lee et al., 2002). The transformant obtained was named S. gordonii SecSpA.

To produce the recombinant SspA, S. gordonii SecSpA was grown in 2 l endotoxin-free TYG containing 10 μg tetracycline ml−1 and 400 μg kanamycin ml−1 until the late exponential phase of growth. The culture was centrifuged and the supernatant was mixed with an equal volume of 100% ethanol and incubated at 4°C for 16 h to precipitate the proteins. Following incubation, the precipitate was collected by centrifugation and dissolved in 20 mM sodium phosphate buffer (pH 6.9). The proteins were applied to a DEAE–Sephrose column (14 × 1.5 cm, GE Healthcare) and eluted with a linear (0–0.3 M) NaCl gradient in sodium phosphate buffer. Fractions containing the SspA protein were identified by Western blotting using the anti-HA mAb as the probe (1/8000, Sigma-Aldrich). Fractions containing the recombinant protein were pooled, concentrated by ultrafiltration (Amicon Ultra-Free-CL centrifugal filter devices, 100 kDa molecular mass cut off, Millipore) and stored at −70°C. The isolated SspA protein showed a single 175 kDa band on a 7.5% SDS-polyacrylamide gel, previously described by Laemmli (1970) (data not shown). The purified SspA contained <0.0125 ng of...
endotoxin per 100 μg SspA using the Limulus amoebocyte lysate assay (BioWhittaker).

β1 integrin binding. A 96-well polystyrene plate was coated with 100 ng human β1 integrin (Millipore) in PBS at 4 °C for 16 h. The plate was blocked with 1% (w/v) BSA and then incubated with purified SspA in Tris-buffered saline (pH 7.8) supplemented with 2 mM CaCl2 or the anti-β1 integrin mAb (1/10000 dilution) at room temperature for 5 h. The bound SspA was detected using the anti-HA mAb and the goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1/8000, Sigma-Aldrich). The bound anti-β1 mAb was detected using the goat anti-mouse IgG alkaline phosphatase-conjugate.

Intranasal challenge. Two groups of 6-week-old BALB/c mice (female, n = 20) were sedated with ketamine and xylazine and given either 1 × 109 c.f.u. S. gordonii DL1 or OB219 intranasally. The inoculation was achieved by slowly pipetting 25 μl suspension into the nostrils (12.5 μl per nostril). At 5, 10, 24 and 72 h post-inoculation, five mice from each group were euthanized. The lungs, spleens and bronchoalveolar lavage (BAL) fluids were collected. The lungs and the spleens were homogenized in 3 and 1 ml PBS, respectively, serially diluted and plated on BH agar. Colonies were counted after 48 h of incubation. Aliquots of the lung homogenates were centrifuged (14000 g, 20 min) and the supernatants were assayed for CXCL1/keratinocyte-derived chemokine and CXCL2/macrophage inflammatory protein 2 (R&D Systems). The cell pellets were assayed for myeloperoxidase (MPO) activity following the previously described protocol (Zhou et al., 2009). The BAL fluids were centrifuged (500 g, 5 min) and the supernatant fluids were saved for the measurement of monocyte chemotactic protein (MCP)-1 and IL-6 by ELISA (eBioscience).

Antibacterial susceptibility assay. S. gordonii cells (1 × 107 c.f.u. in 100 μl Todd–Hewitt broth supplemented with 3% glucose) at mid-exponential phase of growth were added to an equal volume of Todd–Hewitt glucose broth containing 250 μg human β defensin-2 (Peptides International) ml−1, 140 μg polymyxin B sulfate (Fluka Buchs) ml−1, 200 μg histatin-5 (Sigma-Aldrich) ml−1 or 200 μg nisin (Sigma-Aldrich) ml−1. For the histatin-5 assay, 50 μM Zn2+ was included to enhance the antibacterial activity (Rydgård et al., 2006). Preliminary experiments showed that Zn2+ at this concentration was not inhibitory to S. gordonii (data not shown). For controls, bacterial suspensions were added to broth without cationic antimicrobial peptides (CAMPs). The bacterial suspensions were incubated at 37 °C for the indicated length of time. Following incubation, samples were serially diluted and plated. The plates were counted after 48 h of incubation. For the per cent survival was calculated as (c.f.u. in treatment groups/c.f.u. in controls) × 100.

RNA isolation and reverse-transcriptase PCR (RT-PCR). Total RNA was extracted from OB219 and parent DL1 cells using the hot acid phenol method of Peterson et al. (2000) as previously described (Tremblay et al., 2009). Contaminating DNA was removed by digestion with amplification grade DNase I (Invitrogen Life Technologies) and the resulting RNA was verified to be free of DNA by performing PCR experiments for 16S rRNA using primers SL525 (5′-GAATTAAACCACTGCTCCACCCG-3′) and SL697 (5′-ATTTATGGGCGTAAACCGGC-3′). Complementary DNA was synthesized using random primers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer’s instructions. The transcript for the SGO_0212 gene (www.oralgen.lanl.gov), which is immediately downstream to sspA–sspB, was detected by PCR with Taq DNA polymerase and primers 5′-TGCGGATTGTGATCTCATGTG-3′ and 5′-TGACCTCTAGTGGCCAGCTG-3′. The amplification of the 16S rRNA transcript was used as a control. The PCR mixtures were incubated at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final 5 min extension at 72 °C. The PCR products (200 bp for SGO_0212 and 400 bp for 16S rRNA) were analysed by gel electrophoresis.

Statistical analysis. The results were analysed with the paired Student’s t-test; P ≤ 0.05 was considered significant.

RESULTS

S. gordonii OB219 mutant adheres to mammalian cells better than the parent strain

Since SspA and SspB are involved in cell binding (Nobbs et al., 2007), the adherence ability of S. gordonii DL1 and its isogenic mutant OB219 to mammalian cells was compared. OB219 was constructed by the deletion of a 4 kb fragment of the sspA and sspB sequence with the ermAM cassette (Demuth et al., 1996). To verify that the deletion did not result in a polar effect, the expression of the downstream gene SGO_0212 was analysed by RT-PCR. The results showed that SGO_0212 was expressed in OB219 similarly to the parent DL1 (data not shown).

The adherence of the mutant to the two human epithelial cell lines, HEP-2 and A549, was five- and sevenfold higher than the parent strain (Table 1). The level of internalization of the two bacteria by the epithelial cells was marginal. Interestingly, despite the increased adherence, OB219 induced less IL-6, IL-8 and MCP-1 in these epithelial cells than the parent strain (Table 1).

Induction of cytokine production by DCs

To further examine the response to S. gordonii, the professional antigen-presenting cells DCs were stimulated by the parent and mutant strains and the amounts of selected cytokines produced were quantified. S. gordonii DL1 induced IL-6, IL-10, IL-12 and TNF production by DCs as compared with the unstimulated control (Fig. 1). OB219 also induced the production of these four cytokines by DCs, but the cytokine levels were significantly lower than those induced by DL1. These results are consistent with those of the epithelial cells in that OB219 has a reduced ability to induce cytokine or chemokine production.

SspA induces IL-6 and MCP-1 production by A549 cells

The above results suggest that SspA and SspB are responsible for the different responses elicited by the parent and mutant bacteria. To help to confirm this, SspA was produced as a secreted protein by S. gordonii and purified from the culture supernatant. The purified SspA induced the production of MCP-1 and IL-6 by A549 cells in a dose-dependent manner (Fig. 2a, c). The production of MCP-1 and IL-6 was reduced by a function-blocking anti-β1 integrin mAb (Fig. 2b, d), suggesting that SspA mediated its stimulation via binding to β1 integrin. To support this notion, the purified SspA was tested for β1

http://mic.sgmjournals.org
integrin binding. The results showed that the purified SspA bound to β1 integrin precoated on microtitre plates (Fig. 3).

Susceptibility to CAMPs

CAMPs are part of the host’s innate immunity. Since SspA and SspB are surface proteins, it is conceivable that they may play a role in protecting S. gordonii from CAMPs. To test this, the S. gordonii parent and OB219 cells were incubated with polymyxin B, nisin, human β defensin-2 or histatin-5. The results showed that both strains were susceptible to these CAMPs, but OB219 was significantly more susceptible to polymyxin B, nisin and histatin-5 than the parent strain (Fig. 4). The two strains showed no difference in susceptibility to human β defensin-2.

Intranasal challenge in mice

The above results indicate that SspA and SspB are immunostimulatory and play a role in modulating the host’s innate immune responses. To examine the role of SspA and SspB in vivo, we employed a mouse model of intranasal challenge. The animals were given a single dose of bacteria intranasally; bacterial clearance, MPO enzyme levels and chemokine and cytokine production over a 72 h time course were used as indicators of the outcome of the challenge. The results showed that S. gordonii DL1 was cleared quickly from the lungs while the mutant strain OB219 was cleared more slowly (Fig. 5a). At 72 h post-challenge, there was still a substantial number of OB219 cells present in the lungs. No difference in clearance of the two strains from the spleen was observed (Fig. 5b).

The levels of MPO enzyme and the neutrophil-attracting chemokines CXCL1 and CXCL2 in the DL1-inoculated mice peaked at 10 h post-inoculation and declined rapidly (Fig. 5c–e). In contrast, MPO, CXCL1 and CXCL2 levels peaked at a lower level and decreased more slowly in the OB219-inoculated mice. In addition, the level of MCP-1 in the OB219-inoculated mice was lower than in the DL1-inoculated mice (Fig. 5f). The proinflammatory cytokine IL-6 production in the DL1-inoculated mice was rapid and peaked early whereas it was delayed in the OB219-inoculated mice (Fig. 5g). There was no difference in the production of IL-12, TNF, IL-10 and IL-1β between the two groups of mice (data not shown).

DISCUSSION

The results from the present study indicate that the cell wall proteins SspA and SspB are immunostimulatory components of S. gordonii. This statement is supported by the results that the mutant lacking SspA and SspB had a reduced ability to induce cytokines in epithelial cells and DCs as compared with the parent strain. The immunostimulatory characteristic of SspA was confirmed by IL-6 and MCP-1 induction in A549 cells using purified SspA. Vernier-Georgenthum et al. (1998) previously showed that the S. mutans antigen I/II protein

| Table 1: Adherence to epithelial cells and induction of cytokines by S. gordonii DL1 and OB219 |
|-----------------------------------------------|------------------------|-----------------|-----------------|-----------------|-----------------|
|                                      | A549                  | HEP-2           | D1              | OB219            |
| Bound bacteria (×10⁶)                  | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
| Internalized bacteria                  | 45±11                 | 70±14           | 97±11.5         | 97±11.5         |
| Cytokine production (pg ml⁻¹)          | IL-6                  | IL-8            | MCP-1           | IL-6            |
| Unstimulated control                   | 2.08±0.11             | 67.5±11.3       | 13.6±4.3        | 54.2±9.2        |
|                                        | (P<0.001)             | (P=0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 1000.2±283.2          | 1080.2±197.1    | 1202.2±281.2    | 1202.2±281.2    |
|                                        | 1392.3±283.2          | 1392.3±283.2    | 1633.2±262.2    | 1633.2±262.2    |
|                                        | 22.1±11.4             | 22.1±11.4       | 22.1±11.4       | 22.1±11.4       |
|                                        | (P=0.001)             | (P=0.001)       | (P=0.001)       | (P=0.001)       |
|                                        | 54.3±2.8              | 92.4±2.8        | 239.4±61.6      | 239.4±61.6      |
|                                        | (P=0.001)             | (P=0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 51.7±2.8              | 49.2±2.8        | 161.9±14.4      | 161.9±14.4      |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 95.4±3.8              | 95.4±3.8        | 3.94±0.51       | 3.94±0.51       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 95.4±3.8              | 95.4±3.8        | 3.94±0.51       | 3.94±0.51       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
|                                        | 3.41±0.51             | 2.27±0.42       | 2.08±0.11       | 2.08±0.11       |
|                                        | (P<0.001)             | (P<0.001)       | (P<0.001)       | (P<0.001)       |
promotes the induction of IL-8 in epithelial cells, IL-8 and IL-6 in endothelial cells, and IL-1β, TNF and IL-6 in monocytes. Thus, our findings are in agreement with those of Vernier-Georgenthum et al. (1998) and suggest that the antigen I/II family proteins of oral viridans streptococci are immunostimulatory and can be considered as a class of immunostimulatory proteins separate from lipoproteins, a well-known bacterial PAMP recognized by TLRs (Kawai & Akira, 2005; Mayer et al., 2009b).

The ability of SspA to induce IL-6 and MCP-1 production appears to involve β1 integrin binding. This is supported by the observation that IL-6 and MCP-1 induction could be inhibited by the function-blocking anti-β1 mAb and

Fig. 1. Cytokine production by bone-marrow-derived DCs following stimulation by S. gordonii DL1 (white bars) and mutant OB219 (black bars). Controls (grey bars): LPS-stimulated DCs (LPS) and unstimulated cells (Unstim). The ratio of S. gordonii to DCs is indicated. The concentration of LPS used was 1 μg ml⁻¹. Results reported are means ± SD of triplicate experiments. The experiment was repeated twice and similar trends were observed. *P<0.05; **P<0.01.

Fig. 2. MCP-1 and IL-6 production by A549 cells following stimulation with purified SspA. (a, c) A549 cells stimulated with an increasing amount of SspA, as indicated. (b, d) A549 cells stimulated with 5 μg SspA or incubated with 1 μg human anti-β1 integrin antibody ml⁻¹ prior to stimulation with 5 μg SspA (SspA+Ab). TNF, cells stimulated with 20 ng TNF ml⁻¹ as a positive control (Standiford et al., 1991). Unstim, unstimulated. Results reported are means ± SD of triplicate experiments. *P<0.05.
that the purified SspA could bind β1 integrin. Our results support previous observations that IL-8 induction in endothelial cells by the S. mutans antigen I/IIf protein involved β1 integrin binding (Al-Okla et al., 1999). Our results also advance the observations by Nobbs et al. (2007) that SspA and SspB are involved in S. gordonii binding to HEp-2 cells and the ability of S. gordonii to induce IL-8 production in the epithelial cells. Cytokine/chemokine induction by the S. mutans antigen I/IIf protein was shown to involve focal adhesion kinase signalling (Al-Okla et al., 1999; Neff et al., 2003; Zeisel et al., 2005). Whether this is also the case for SspA remains to be determined.

One of the interesting findings from the present study is that S. gordonii OB219 was more susceptible to CAMPs than the parent strain. This result suggests that SspA and SspB play a protective role for the bacterium against CAMPs. It appears that the protection is not universal as OB219 was more susceptible to polymyxin, nisin and histatin-5 but not human β defensin-2. Both histatin-5 and β defensin-2 are naturally occurring peptides found in the oral cavity (Lamkin & Oppenheim, 1993; Mathews et al., 1999). Histatin-5 has anti-Candida and antibacterial activity (Helmerhorst et al., 2001; Rydengård et al., 2006), while β defensin-2 is a well-characterized antimicrobial peptide (Mathews et al., 1999). To the best of our knowledge, this is the first report demonstrating the potential role of cell-wall-anchored proteins in protecting the bacterium from CAMPs. The mechanism of protection remains unclear; however, one possibility is that in the absence of SspA and SspB, the cell membrane becomes more accessible to CAMPs.

The role of SspA and SspB in innate immunity is clearly demonstrated in the intranasal challenge experiment. OB219 was cleared from the lungs more slowly than the parent strain. The delay in clearance is probably due to a delay in neutrophil recruitment, which is indicated by the lower peak level of MPO, and CXCL1 and 2 chemokines and the prolonged presence of MPO and chemokines in these animals. It is not clear whether the increased adherence of OB219, as shown in the in vitro cell-binding experiment, plays any role in the delayed clearance. The in vitro cell-stimulation results indicate that OB219 has a reduced ability to induce MCP-1 and a number of cytokines including proinflammatory IL-6 and TNF. Although we did not

---

**Fig. 3.** Binding of SspA to β1 integrin. β1 Integrin coated on wells of a microtitre plate was reacted with the purified SspA, the anti-β1 integrin mAb or Tris-buffered saline (control) in ELISA. Results reported are means ± SD of triplicate experiments.

**Fig. 4.** Susceptibilities of S. gordonii DL1 and OB219 to antimicrobial peptides. S. gordonii DL1 (white bars) or OB219 (black bars) were incubated with polymyxin (a), nisin (b), human β defensin-2 (c) or histatin-5 (d) for the indicated time. The percentage survival was determined by viable cell counts and calculated as (c.f.u. in groups incubated with antimicrobials/c.f.u. in groups incubated in medium without antimicrobials)×100. Results reported are means ± SD from three independent experiments. *P ≤ 0.05; **P ≤ 0.01.
observe any changes in the level of IL-12, TNF, IL-10 and IL-1β in the lungs of OB219-inoculated mice as compared with the DL1-inoculated mice, we did detect a reduced MCP-1 and a delay in IL-6 induction for DL1-inoculated mice. The reason for the reduced cytokine induction despite better adherence of OB219 to epithelial cells remains unclear. A possible explanation is that the increased adherence is the result of non-specific interactions between the bacterium and host cell due to a change in cell surface charges. SspA and SspB are calcium-binding proteins (Duan et al., 1994) and in the absence of these two proteins, OB219 has a more negative cell surface charge than the parent strain (Davis et al., 2011). However, the interactions did not involve PAMPs and cellular receptors that lead to cytokine production.

In conclusion, SspA and SspB are immunostimulatory components of S. gordonii and play an important role in modulating host innate immunity.

ACKNOWLEDGEMENTS

We thank Yi-Jing Li and Bin-You Zheng of Dalhousie University for their technical assistance and Dr Howard Jenkinson, University of Bristol, for providing S. gordonii OB219. E.A. was a recipient of an IWK Postdoctoral Research Fellowship. This study was supported by the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada.

REFERENCES


Edited by: H. Ingmer