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Lipoproteins in *Streptococcus gordonii* are critical in the infection and inflammatory responses

Hyun Young Kim[a,1], A Reum Kim[a,1], Ho Seong Seo[b], Jung Eun Baik[a], Ki Bum Ahn[a,b], Cheol-Heui Yun[c], Seung Hyun Han[a,*]

[a] Department of Oral Microbiology and Immunology, DRI and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul 08826, Republic of Korea
[b] Research Division for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup 56212, Republic of Korea
[c] Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea

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ABSTRACT

Gram-positive bacteria such as *Streptococcus gordonii* causing life-threatening infective endocarditis are mainly recognized by Toll-like receptor 2 (TLR2). Lipoteichoic acid (LTA) and lipoproteins are representative TLR2 ligands that play important roles in bacterial infection and in host inflammatory responses. In the present study, we generated an LTA-deficient mutant (Δ*ltaS*) and a lipoprotein-deficient mutant (Δ*lgt*) and investigated the contributions of LTA and lipoproteins to bacterial morphology and their effect on induction of proinflammatory cytokines in THP-1 and mouse bone-marrow derived macrophages (BMDMs). Deletion of *ltaS* and *lgt* was confirmed by PCR analysis of genomic DNA from each mutant. The mutants with absence of LTA or lipoproteins were examined by SDS-PAGE followed by Western blotting with anti-LTA antibodies and silver staining, respectively. Interestingly, scanning and transmission electron microscopies showed no difference in the bacterial cell morphology or size between the wild-type and the mutants even though substantial changes in the cell size and/or morphology have been reported in other Gram-positive bacteria such as *Staphylococcus aureus, Listeria monocytogenes*, and *Bacillus subtilis*. However, *S. gordonii* wild-type and Δ*ltaS* potently induced the expression of proinflammatory cytokines including TNF-α, IL-8, and IL-1β at the mRNA and protein levels, while Δ*lgt* did not have these effects. Furthermore, lipoproteins purified from *S. gordonii* also induced the expression of the aforementioned cytokines more potently than the purified LTA. Neither LTA nor lipoprotein induced TNF-α, KC (IL-8 counterpart in mouse), and IL-1β in TLR2-deficient BMDMs. *S. gordonii* Δ*lgt* was less virulent than the wild-type or Δ*ltaS* in a mouse intraperitoneal infection model. Collectively, these results suggest that *S. gordonii* lipoproteins, but not LTA, are mainly responsible for the infection and inflammatory responses.

1. Introduction

Streptococcus gordonii is a Gram-positive commensal and facultative anaerobic bacterium that is commonly found in the human oral cavity (Abiko et al., 2010). *S. gordonii* plays an important role as an early colonizer in dental biofilm formation and binds to human dentin using gordonii surface protein B (GspB) (Kim et al., 2016). *S. gordonii* is also an opportunistic pathogen that can cause bacteremia and systemic infection by entering the bloodstream through damaged gingival crevicular tissue after tooth brushing and dental extraction (Lockhart et al., 2008). Moreover, *S. gordonii* can cause life-threatening infective endocarditis due to thrombosis by binding to platelets and forming vegetation on heart valves (Keane et al., 2010). Intravenous infection of *S. gordonii* induces production of TNF-α and proliferation of splenocytes through Toll-like receptor 2 (TLR2) signaling (Segawa et al., 2013). However, it is unclear which TLR2 ligands of *S. gordonii* are important for the induction of inflammatory mediators.

Lipoteichoic acid (LTA) and lipoproteins are representative TLR2 ligands of Gram-positive bacteria and are associated with the induction of inflammatory responses (Kang et al., 2016). LTA is an amphipathic molecule composed of a hydrophilic phosphate-containing polymer and a hydrophobic glycolipid anchor (Jang et al., 2011; Kang et al., 2016). Polymerization of the phosphate-containing groups is mediated by LTA synthase (LtaS), which is an essential enzyme for LTA synthesis

* Corresponding author at: Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Building 86, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea.
E-mail address: shhan-mi@snu.ac.kr (S.H. Han).
[1] Hyun Young Kim and A Reum Kim contributed equally to this work.

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(Grundling and Schneewind, 2007). LTA plays an important role in various aspects of bacterial physiology such as growth, division, and biofilm formation (Fedtke et al., 2007). Lipoproteins are also amphipathic molecules composed of a hydrophilic protein moiety and a hydrophobic diacylglycerol moiety (Nakayama et al., 2012a). In Gram-positive bacteria, lipoprotein synthesis is mediated by lipoprotein diacylglyceryl transferase (Lgt), which is an essential enzyme for formation of covalent bonds between diacylglycerol moieties and cysteine residues in prolipoproteins (Buddelmeijer, 2015). Bacterial lipoproteins also play an important role in bacterial growth and virulence (Hutchings et al., 2009).

TLRs are representative pattern recognition receptors in vertebrates which play important roles in recognition of microbe-associated molecular patterns. To date, 10 TLRs (TLRs1-10) have been reported in human and 12 TLRs (TLRs1-9, 11, 12 and 13) in mice (De Nardo, 2015). Among them, TLR2 and TLR4 are essential for host immunity against infection caused by Gram-positive and Gram-negative bacteria, respectively (Takeda and Akira, 2005). TLR2 activation acts as a homodimer or heterodimers such as TLR2/1 or TLR2/6 and induces translocation of NF-κB through MyD88-dependent signaling pathway (Kang et al., 2016). LTA and lipoproteins can activate NF-κB signaling pathway through TLR2 leading to inflammatory responses in the host cells, but they have distinct signaling pathways. For example, LTA induces inflammatory responses through TLR2 activation and JAK2/STAT1 signaling via platelet-activating factor receptor (Han et al., 2006; Park et al., 2013), whereas lipoproteins through TLR2-mediated NF-κB, IRF1 and IRF7 signaling pathways (Dietrich et al., 2010). In addition, lipoproteins are a well-known B cell mitogen (Moller, 1999), whereas LTA rather inhibits B cell proliferation induced by lipopolysaccharide (LPS) (Kang et al., 2018).

Clinical studies have reported that the concentrations of inflammatory cytokine such as TNF-α, IL-8, and IL-1β are significantly higher in the sera of infective endocarditis patients compared to those of healthy controls (Araujo et al., 2015). However, the specific components of *S. gordonii* that induce inflammatory cytokine expression in host cells have not been identified. To address this knowledge gap, we generated *S. gordonii* mutant strains lacking each of two major TLR2 ligands of Gram-positive bacteria, LTA and lipoprotein, to identify the key components responsible for these inflammatory responses.

2. Materials and methods

2.1. Bacteria, reagents, and chemicals

Todd-Hewitt broth (THB) and yeast extract were purchased from BD Biosciences (San Diego, CA, USA). RPMI-1640 medium supplemented with 2.05 mM $_{L}$-glutamine and penicillin/streptomycin was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Grand Island, NY, USA). Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Triton X-114 and octyl β-d-glucopyranoside were from Sigma-Aldrich Inc. All other reagents were purchased from Sigma-Aldrich Inc. unless otherwise stated.

2.2. Generation of S. gordonii mutant strains

S. gordonii Δ*ltaS* and Δ*lgt* mutant strains were generated as described previously (Bensing et al., 2004). Briefly, the upstream flanking region and downstream flanking region of each target gene were amplified by polymerase chain reaction (PCR), followed by digestion with the appropriate restriction enzymes. The PCR amplicons of the flanking regions were ligated into the corresponding restriction enzyme sites of pC326. The suicide plasmid (pC-Δ*ltaS* or pC-Δ*lgt*) was introduced into the *S. gordonii* CH1 strain by the natural transformation method (Bensing et al., 2004). Briefly, CH1 was diluted 100-fold in THB containing 20% heat-inactivated horse serum, 100 ng/ml of a competent-stimulating peptide (CSP-CH1, DVRSNKIRLWWENIFFNKK), and 1 μg of pC-Δ*ltaS* or pC-Δ*lgt*. The reaction was incubated at 37 °C for 2 h. Transformation mixtures were spread on TH agar plates containing 5 μg/ml chloramphenicol after an additional incubation at 37 °C overnight. Deletion of *ltaS* and *lgt* was confirmed by PCR using the specific primers F3110 (5′-ATG GTA CCA AGA AAA GAG AGC ATA GTC CC-3′) plus R5110 (5′-GCT TAT CGA TAC CGT CGA CCT CGA GTT TTT TCA CAA AAG TAC TTC CTT GTC T-3′) and F3104 (5′-ACG GTA CCT GTT GTC ATC ATT GCC AGA G-3′) plus R5104 (5′-ACG CGG CCG CTG AAT AGT CTA ACA TCT CTT TTG-3′). Bacterial 16S ribosomal RNA gene was confirmed by PCR using the specific primers 27f1 (5'-CIA GTG TAG AGG TGA AAT-3') plus 518r1 (5'-CCC CGT CAA TTC CTT TGA GTT-3').

2.3. Determination of bacterial growth

Wild-type *S. gordonii* was cultured at 37 °C in THB containing 5% yeast extract (THY) for 0, 1, 2, 3, 6, 9, 12, 15, 18, and 24 h. *S. gordonii* Δ*ltaS* and Δ*lgt* mutants were cultured under the same condition except supplementation with 5 μg/ml chloramphenicol. *S. gordonii* growth was measured by optical density at 600 nm using a spectrophotometer (Gene Spec III; Hitachi, Tokyo, Japan).

2.4. Preparation of ethanol-killed S. gordonii

Wild-type *S. gordonii* was cultured at 37 °C to mid-log phase in THY. The *S. gordonii* Δ*ltaS* and Δ*lgt* mutants were cultured at 37 °C to mid-log phase in THY supplemented with 5 μg/ml chloramphenicol. Bacterial pellets were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS). Next, the bacteria were incubated with 70% ethanol in PBS (Millipore, Bedford, MA, USA) at room temperature with shaking for 3 h. After ethanol-mediated killing, bacteria were washed three times with PBS to remove any residual ethanol. Complete killing of *S. gordonii* was confirmed by plating the killed bacterial preparation on THY agar plates and incubating the plates at 37 °C for 48 h. No bacterial colonies were observed at the end of incubation.

2.5. Culture of THP-1 and differentiation into macrophages

The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in complete RPMI-1640 medium (Hyclone) containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an incubator with 5% CO_2. For differentiation into macrophages, cells (1×10^6 cells/ml) were cultured in RPMI-1640 complete medium supplemented with PMA (100 nM) for 2 days. The culture medium was replaced with fresh culture medium, followed by an additional incubation for 24 h. Then, the cells were treated with indicated stimuli for 24 h.

2.6. Preparation of bone marrow-derived macrophages

All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of Seoul National University (SNU-171211-2). C57BL/6 mice were purchased from Orient Bio (Gyeonggi-do, Korea) and TLR2-deficient mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). Bone marrow-derived macrophages (BMDMs) were prepared as previously described (Kim et al., 2017). BMDMs (5×10^5 cells/ml) were stimulated with indicated stimuli for 24 h.

2.7. Intraperitoneal challenge of mice

Wild-type *S. gordonii* and mutants were cultured at 37 °C in THY to mid-log phase in the absence or presence of 5 μg/ml chloramphenicol. Bacterial pellets were harvested by centrifugation and washed three