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Streptococcus gordonii induces nitric oxide production through its lipoproteins stimulating Toll-like receptor 2 in murine macrophages

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ABSTRACT

Streptococcus gordonii, a Gram-positive commensal in the oral cavity, is an opportunistic pathogen that can cause endodontic and systemic infections resulting in infective endocarditis. Lipoteichoic acid (LTA) and lipoprotein are major virulence factors of Gram-positive bacteria that are preferentially recognized by Toll-like receptor 2 (TLR2) on immune cells. In the present study, we investigated the effect of *S. gordonii* LTA and lipoprotein on the production of the representative inflammatory mediator nitric oxide (NO) by the mouse macrophages. Heat-killed *S. gordonii* wild-type and an LTA-deficient mutant (Δ*ltaS*) but not a lipoprotein-deficient mutant (Δ*lgt*) induced NO production in mouse primary macrophages and the cell line, RAW 264.7. *S. gordonii* wild-type and Δ*ltaS* also induced the expression of inducible NO synthase (iNOS) at the mRNA and protein levels. In contrast, the Δ*lgt* mutant showed little effect under the same condition. Furthermore, *S. gordonii* wild-type and Δ*ltaS* induced NF-κB activation, STAT1 phosphorylation, and IFN-β expression, which are important for the induction of iNOS gene expression, with little activation by Δ*lgt*. *S. gordonii* wild-type and Δ*ltaS* showed an increased adherence and internalization to RAW 264.7 cells compared to Δ*lgt*. In addition, *S. gordonii* wild-type and Δ*ltaS*, but not Δ*lgt*, substantially increased TLR2 activation while none of these induced NO production in TLR2-deficient macrophages. Triton X-114-extracted lipoproteins from *S. gordonii* were sufficient to induce NO production. Collectively, we suggest that lipoprotein is an essential cell wall component of *S. gordonii* to induce NO production in macrophages through TLR2 triggering NF-κB and STAT1 activation.

1. Introduction

Streptococcus gordonii belongs to the viridans group of oral streptococci that are commonly found in the human oral cavity as normal flora (Garnier et al., 1997). *S. gordonii* is important for the development of dental plaque as an early colonizer (Rosan and Lamont, 2000). However, when *S. gordonii* enters the bloodstream through oral trauma or dental treatment including toothbrushing and single-tooth extraction, it can cause systemic infections such as infective endocarditis (Veloso et al., 2011). *S. gordonii* was reported to occupy 12.7% of 47 oral streptococci isolated from 42 confirmed cases of infective endocarditis (Douglas et al., 1993) and to be one of the most common viridans group streptococci together with *Streptococcus oralis* and *Streptococcus sanguinis* causing the disease (Westling et al., 2008). *S. gordonii* forms endocardial vegetation by obtaining carbohydrates from host glycoproteins via *N*-acetyl-β-D-glucosaminidase (Langley et al., 2008). However, the virulence factors and pathogenic mechanisms of *S. gordonii* and host immune responses against it are not fully understood.

In infection by Gram-positive bacteria, Toll-like receptor 2 (TLR2) is crucial for host immune responses because most Gram-positive bacteria preferentially activate TLR2 on host cells (Mogensen, 2009). TLR2-deficient mice are highly susceptible to infection by Gram-positive pathogens such as *Staphylococcus aureus* (Vidlak et al., 2011), *Streptococcus pneumoniae* (Lammers

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et al., 2012), and *Listeria monocytogenes* (Seki et al., 2002). Reports suggest that cell wall virulence factors of *S. gordonii* including lipoteichoic acid (LTA) and lipoproteins are responsible for pathogenesis through bacterial colonization (Nobbs et al., 2009), and inflammation (Chan et al., 2007; Segawa et al., 2013). X-ray crystallography demonstrates that both LTA and lipoproteins directly interact with TLR2 (Jin and Lee, 2008). LTA and lipoproteins induce pro-inflammatory mediators such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α) through similar but distinct signaling pathways. LTA and lipoproteins can stimulate TLR2 recruiting MyD88 leading to the activation of NF-κB, which is sufficient to induce TNF-α (Han et al., 2006; Kim et al., 2015). On the contrary, lipoprotein-induced NO production requires TLR2/MyD88/NF-κB and IFN-β/JAK/STAT1 signal transduction, while LTA-induced NO production requires TLR2/MyD88/NF-κB and PAFR/JAK2/STAT1 (Dietrich et al., 2010; Han et al., 2006).

NO is an amphiphilic radical gas that regulates the cardiovascular system, neurotransmission and inflammatory reaction by mammalian cells (Tuteja et al., 2004). NO is synthesized by nitric oxide synthases (NOSs) such as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Aktan, 2004). Among them, iNOS produces micromolar concentrations of NO in activated macrophages to elicit antibacterial activity during infections (Chakravortty and Hensel, 2003). The iNOS is also important in immune responses through modulating immune cell proliferation, activation, differentiation, and trafficking, and cytokine production (Bogdan, 2001). However, whether *S. gordonii* induces NO in macrophages is unknown and the cell wall components responsible for NO production are poorly studied. Thus, we investigated the induction of NO in the mouse macrophages and the macrophage cell line RAW 264.7 by LTA-deficient and lipoprotein-deficient *S. gordonii* mutant strains.

2. Materials and methods

2.1. Bacteria, reagents and chemicals

Todd-Hewitt broth (THB) and yeast extract were from BD Biosciences (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and HyClone (Logan, UT, USA), respectively. Anti-iNOS rabbit polyclonal IgG antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal antibodies against STAT1 or phosphorylated STAT1 (P-STAT1) were from Cell Signaling Technology (Beverly, MA, USA). Recombinant murine macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ). Triton X-114 and octyl β-D-glucopyranoside were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were from Sigma-Aldrich unless otherwise indicated.

2.2. Generation of S. gordonii mutant strains

LTA-deficient (Δ*ltaS*) and lipoprotein-deficient (Δ*lgt*) mutants were prepared from the *S. gordonii* CH1 wild-type strain as described previously (Bensing et al., 2004). The upstream flanking region of *ltaS* was amplified by polymerase chain reaction (PCR) using primers F3110 (5′-ATGGTACCAAGAAAAGAGAGCATAGTCCC-3′) and R5116 (5′-CTCTCGAGTTTTTTCACAAAAGTACTTCCTTG-3′), followed by digestion with *Kpn*I and *Xho*I. The downstream flanking region of *ltaS* was also amplified by PCR using F3116 (5′-TCGGATCCAGCAATAACTTTGTCACACC-3′) and R5110 (5′-TAGCGGCCGCTATACGAATTTATCCAAAAAAC-3′), then digested with *Bam*HI and *Not*I. PCR products were ligated into corresponding restriction enzyme sites of pC326. The resulting suicide plasmid (pC-Δ*ltaS*) was introduced into *S. gordonii* wild-type by natural transformation (Bensing and Sullam, 2002). CH1 was diluted 100-fold with fresh THB containing 20% heat-inactivated horse serum, 100 ng/ml competent-stimulating peptide (CSP-CH1, DVRSNKIRLWWENIFFNKK) and 1 μg pC-Δ*ltaS* plasmid and incubated for 2 h at 37 °C. Transformation mixtures were plated on TH agar plates containing 5 μg/ml chloramphenicol after additional incubation at 37 °C overnight. Deletion of *ltaS* was confirmed by PCR using specific primers F3110 and R5155 (5′-AAGCAATTGGAATAAAGAAGCG-3′), and primers F3155 (5′-AATTTGTTTGATTTTTAATGG-3′) and R5110.

2.3. Preparation of heat-killed S. gordonii

S. gordonii CH1 wild-type, Δ*ltaS*, and Δ*lgt* strains were cultured in THB supplemented with 5% yeast extract (THY) at 37 °C to mid-log phase. After washing with phosphate-buffered saline (PBS) three times, bacterial cells were incubated at 80 °C for 2 h. Complete killing of bacteria was confirmed by plating on THY-agar at 37 °C for 48 h. No bacterial colonies were observed.

2.4. Culture of RAW 264.7 cells

The murine macrophage cell-line RAW 264.7 (TIB-71) was from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

2.5. Preparation of primary macrophages

Animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of Seoul National University (SNU-140512-6-2). Balb/c mice were from Orient Bio (Seongnam, Korea) and TLR2-deficient mice were from Dr. Shizuo Akira (Osaka University, Osaka, Japan). Bone marrow-derived macrophages were prepared from 6- to 8-week old mice as previously described (Castrillo et al., 2003). Briefly, bone marrow cells were obtained from tibiae and femurs by flushing with complete DMEM and red blood cells were removed by suspension with a red blood cell lysing buffer (Sigma–Aldrich). The cells were differentiated into macrophages by incubation with DMEM containing 20 ng/ml of M-CSF, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol for five days. The cells were plated at 1×10^6 cells/ml on 96-well plates and treated with heat-killed *S. gordonii* wild-type, Δ*ltaS*, or Δ*lgt* for 24 h for analysis of NO production.

2.6. Determination of NO production

RAW 264.7 cells or mouse primary macrophages were plated at 1×10^6 cells/ml on 96-well plates and stimulated with various concentrations of heat-killed *S. gordonii* wild-type, Δ*ltaS*, or Δ*lgt* for various time periods. Nitrite accumulation of culture supernatants was measured as previously described (Lee et al., 2015). Briefly, equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) was incubated with culture supernatants for 5 min at room temperature. After incubation, the quantity of NO was measured at 540 nm with a microtiter plate reader (Versamax, Molecular Devices Corporation, Sunnyvale, CA, USA) using $NaNO_2$ as a standard.

2.7. Cell viability assay

Cell viability was measured with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (Sigma-