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Role of *Streptococcus gordonii* cell wall components in the bacterial biofilm formation and chemokine induction

*Streptococcus gordonii*에 의한 바이오필름 형성과 케모카인 유도에서 세균의 세포벽 인자의 역할

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ABSTRACT

Role of *Streptococcus gordonii* cell wall components in the bacterial biofilm formation and chemokine induction

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Objectives

Streptococcus gordonii, a Gram-positive, oral, and commensal bacterium, can be a notorious life-threatening pathogen causing endocarditis. It is frequently isolated from the periapical lesion of patients with apical periodontitis. Serine-rich repeat (SRR) adhesins of *S. gordonii* such as gordonii surface protein B (GspB) are associated with bacterial colonization and cell-associated virulence factors like lipoteichoic acid (LTA) and lipoprotein are representative virulence factors to induce pro-inflammatory cytokines and chemokines in host cells. Although they seemingly contribute to biofilm formation and the inflammatory responses during apical periodontitis, little is known about the pathogenic mechanisms by *S. gordonii* and key virulence factors of *S. gordonii* to be responsible for biofilm formation on root canal

dentin and the induction of inflammatory responses. The aim of this study was to investigate the role of *S. gordonii* cell wall-associated virulence factors on biofilm formation and interleukin (IL)-8 induction in human periodontal ligament (PDL) cells.

Methods

The effect of *S. gordonii* GspB on biofilm formation was investigated using wild-type and GspB-deficient mutant *S. gordonii* strains. Confocal microscopy and crystal violet assay were performed to determine biofilm formation. Bacterial growth was examined by measuring optical density at 600 nm with spectrometry. Bacterial adherence and biofilm formation on the culture plate and human dentin slices were visualized with a scanning electron microscope. The role of *S. gordonii* cell wall-associated virulence factors on IL-8 induction in human PDL cells was investigated using ethanol-inactivated *S. gordonii* wild-type, LTA-deficient mutant (Δ*ltaS*), and lipoprotein-deficient mutant (Δ*lgt*). IL-8 mRNA expression and production were determined by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Toll-like receptor 2 (TLR2) activation was determined using transient transfection followed by a reporter gene assay in the human embryonic kidney 293 (HEK293) cells overexpressing TLR2. The cells were pretreated with inhibitors of MAP kinases for 1 h followed by stimulation with *S. gordonii* or its lipoproteins to characterize the intracellular signaling pathways required for IL-8 induction by *S. gordonii*.

Results

The GspB-deficient *S. gordonii* mutant strain was less potent than the wild-type strain in the biofilm formation. Of note, there was no difference in bacterial growth rate between the mutant and the wild-type strains. Differences in biofilm-forming ability between the wild-type and mutant strains were more distinct in the sucrose-supplemented media. Furthermore,

the GspB-deficient mutant exhibited attenuated formation of aggregates on the surface of the culture plate and human dentin slices. On the other hands, *S. gordonii* wild-type induced IL-8 expression at both protein and mRNA levels in human PDLs in a dose- and time-dependent manner. Transient transfection followed by a reporter gene assay in the HEK293 cells overexpressing TLR2 demonstrated that *S. gordonii* wild-type and purified lipoproteins induced substantial activation of NF-κB, whereas purified LTA showed a minimal activation. Additionally, IL-8 production induced by *S. gordonii* wild-type or purified lipoproteins was significantly inhibited by anti-TLR2 neutralizing antibody. *S. gordonii* wild-type and Δ*ltaS* induced IL-8 production but such response was not observed when the cells were stimulated with the Δ*lgt*. Concordantly, lipoproteins purified from *S. gordonii* induced IL-8 production in PDLs in a dose-dependent manner whereas LTA purified from *S. gordonii* failed to induce it. Furthermore, *S. gordonii* lipoprotein-induced IL-8 production was decreased by inhibitors for p38, ERK, and JNK.

Conclusions

Taken together, the results in this study show that GspB is closely involved in the initial adherence and biofilm formation of *S. gordonii*, especially on human dentin. Furthermore, lipoprotein of *S. gordonii* plays a critical role in the IL-8 production in human PDL cells. Therefore, this current results suggest that GspB promotes *S. gordonii* biofilm formation and lipoprotein of *S. gordonii* leads to inflammatory responses by promoting IL-8 production of human PDL cells, which may cause apical periodontitis.

Keywords: *Streptococcus gordonii*, GspB, Biofilm, Human periodontal ligament cells, Lipoprotein, Interleukin-8

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1. INTRODUCTION

1.1. *Streptococcus gordonii*

Streptococcus gordonii is a Gram-positive facultative anaerobic bacterium that is frequently observed as a member of human oral microflora [1]. *S. gordonii* is a notorious life-threatening pathogen as it can enter blood vessels and cause infective endocarditis [2]. In the oral cavity, it is associated with various oral infectious diseases such as dental caries and periodontitis. It is involved in dental biofilm formation by acting as an early colonizer that facilitates incorporation of various oral pathogens [3]. Moreover, oral streptococcus species, including *S. gordonii*, have the ability to colonize on the dentin surfaces and deeply penetrate dentinal tubules [4]. Thus, it is difficult to remove the colonization of *S. gordonii* in the root canal so that *S. gordonii* is often isolated from the root canals with apical periodontitis receiving endodontic treatment [5]. In addition, *S. gordonii* can exchange genes with *Enterococcus faecalis* in root canal biofilm, thereby potentiating its virulence and antibiotic resistance [6]. Thus, *S. gordonii* is deeply involved in pathogenesis of apical periodontitis and refractory apical periodontitis.

1.2. Biofilm

Biofilm is a community of microorganisms, that attach to a surface and are embedded in their extracellular polymeric substances (EPSs) [7]. Bacteria that can form biofilm are responsible for approximately 80% of human bacterial

infections [8]. Bacterial biofilm is associated with various chronic inflammatory diseases such as dental caries, periodontitis, endocarditis, and cystic fibrosis pneumonia [9-12]. Bacteria that reside in biofilm are 10–1000 times more resistant to anti-microbial agents compared to planktonic bacteria [13, 14] because EPSs act as a physical shield [15]. In addition, bacteria are likely to grow slowly in the biofilm and thus they are not efficiently killed by antibiotics that target bacterial growth [16]. Therefore, it is difficult to remove bacteria embedded in biofilm on the root canal, so that biofilm has been considered to be one of the causes of endodontic failures and refractory apical periodontitis.

1.3. Apical periodontitis and IL-8

Apical periodontitis is an inflammatory disease in apical region, resulting from endodontic infection by bacteria existing in apical root canal [17, 18]. The bacteria can enter apical periodontal ligament through apical foramen, which causes local inflammation, destruction of periapical tissues, and resorption of alveolar bone in the lesion [17]. During apical periodontitis by bacterial infection, chemokines are involved in local inflammation by causing heavy infiltration of immune cells [19-21]. Particularly, interleukin (IL)-8 is detected at a high level in most of exudates from infected root canal with periapical lesions [19, 22], leading to heavy infiltration of neutrophils that play an important role in the inflammation [17, 23, 24]. Additionally, IL-8 can recruit and activate osteoclasts in infection site leading to resorption of hard

tissues related to apical abscess [19, 25]. Furthermore, IL-8 levels in the periapical lesion is known to be correlated with the pains of the patients with apical periodontitis [26].

1.4. Serine-rich repeat (SRR) adhesins

Serine-rich repeat (SRR) adhesins are cell-wall associated glycoproteins of Gram-positive bacteria [27, 28]. SRR adhesins have the ability to bind sialic acid on the host cells [29]. They consist of a signal peptide, short serine-rich repeat 1, binding region, long serine-rich repeat 2, and cell wall-anchoring domain [30]. SRR adhesins of *S. gordonii*, such as gordonii surface protein B (GspB), are essential for bacterial binding to sialic acid motifs on platelets [29], which is a crucial component of infective endocarditis [2]. Accumulating reports suggest that SRR adhesins mediate the adherence and biofilm of some Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus parasanguis*, and *Streptococcus cristatus* [31-34].

1.5. Lipoteichoic acid and lipoprotein

Gram-positive bacteria have various virulence factors. Among them, cell-associated virulence factors like lipoteichoic acid (LTA) and lipoprotein are representative virulence factors, which have been known to be recognized Toll-like receptor 2 (TLR2) [35, 36]. These cell-associated virulence factors stimulate a variety of host immune cells to induce pro-inflammatory cytokines

and chemokines [35, 37-39]. LTA of *Staphylococcus aureus* or *Streptococcus pyogenes* induced IL-8 production in human peripheral blood monocytes [39]. Lipoprotein of *S. aureus* induced IL-8 production in human intestinal epithelial cells [38]. Likewise, LTA, lipoprotein, and peptidoglycan from *S. grodonii* also induce pro-inflammatory cytokines production such as IL-6 and TNF in dendritic cells via TLR2 [40].

1.6. Aim of this study

Little is known about the virulence factors of *S. gordonii* involved in the biofilm formation and the induction of inflammatory responses in the periapical areas. Therefore, in this study, the role of *S. gordonii* cell wall-associated virulence factors on biofilm formation and IL-8 induction in human periodontal ligament (PDL) cells was investigated.

2. MATERIAL AND METHODS

2.1. Bacteria, reagents, and chemicals

S. gordonii M99 wild-type and GspB-deficient strains [29] were kindly provided by Paul M. Sullam (University of California, San Francisco). *S. gordonii* CH1 wild-type, *ltaS*-deficient strains, and *lgt*-deficient strains were kindly provided by Dr. Ho Seong Seo (Korea Atomic Energy Research Institute). Crystal violet dye and ethanol were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Acetic acid was purchased from EMD Chemicals (Gibbstown, NJ, USA). Live/Dead BacLight™ Bacterial viability kit was purchased from Molecular Probes (OR, USA). Triton X-114 and octyl β-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha Modification of Eagle's Minimum Essential Media (α-MEM), Dulbecco's modified Eagle's medium (DMEM), and penicillin-streptomycin solution were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS), trypsin-EDTA, and Opti-MEM were purchased from Gibco-BRL (Carlsbad, CA, USA). Anti-human TLR2 antibody, its isotype control, and blasticidin S were purchased from InvivoGen (San Diego, CA, USA). TRIzol reagent was purchased from Invitrogen (Grand Island, NY, USA). Random hexamers and reverse transcriptase were purchased from Promega Corporation (Madison, WI, USA). Inhibitors for MAP kinases were purchased from Calbiochem (Darmstadt, Germany). EmeraldAmp GT PCR Master Mix was purchased from TaKaRa (Otsu, Shiga, Japan).

2.2. Bacteria culture and inactivation

S. gordonii M99 wild-type and GspB-deficient strain were cultured in Todd-Hewitt broth with 0.5% yeast extract (THY) (BD Biosciences, CA, USA) at 37^{o}C to mid-log phase. *S. gordonii* CH1 wild-type, *ltaS*-deficient strains, and *lgt*-deficient strains were cultured in Todd-Hewitt broth (Kisan Bio Co., Ltd, Seoul, Korea) with 0.5% yeast extract at 37^{o}C to mid-log phase. For Preparation of ethanol-inactivated *S. gordonii*, bacteria were inactivated by 70% ethanol with shacking for 3 h. After washing with phosphate-buffered saline (PBS), no bacterial colony was observed on the THY agar plate for 2 days (data not shown).

2.3. Cell culture

Human periodontal ligament cells (human PDL cells) were kindly provided by Prof. Joo-Cheol Park (Seoul National University) and grown in the completed α-MEM containing 10% FBS and 1% penicillin and streptomycin at 37 ^{o}C in 5% CO_2 incubator. Human embryonic kidney (HEK) 293 cells expressing TLR2 (HEK293-TLR2) were purchased from InvivoGen. The cell were cultured in DMEM

2.4. Preparation of human dentin slices

The preparation and use of human dentin slices in the experiments were approved by the Institutional Review Board of Seoul National University

Dental Hospital, Seoul, Korea (CRI 15007). Single-rooted premolars were obtained from patients ongoing orthodontic extractions in the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital. The root surfaces were cleaned by an ultrasonic scaler and sliced in cross section to a thickness of 500 μm with an Isomet precision saw (Buehler, IL, USA). These dentin slices were treated with 17% ethylenediaminetetraacetic acid (Sigma-Aldrich, MO, USA) for 5 min and then treated with 2.5% sodium hypochlorite (Sigma-Aldrich, MO, USA) for 5 min. After being neutralized with 5% sodium thiosulfate (Sigma-Aldrich, MO, USA) for 5 min, dentin slices were autoclaved for 15 min at 121℃. In order to confirm the sterility, the dentin slices were soaked and incubated in THY broth at 37^{o}C overnight and the incubation media were plated on THY agar plates and incubated at 37^{o}C overnight. No bacterial colonies were observed at the end of the incubation period (data not shown).

2.5. Cell transfection

HEK293-TLR2 cell (2.5×10^5 cells/ml, 5 ml, 60 mm dishes) were transfected with a NF-κB (nuclear factor-κB) reporter gene (pNF-κB-Luc, Clontech, Mountain View, CA, USA) in Opti MEM using Attractene transfection reagent for 16 h (Qiagen, Germantown, MD, USA). After collected, the cells (2.5×10^5 cells/ml, 200 μl, 96-well plates) were plated in completed DMEM. The cells were stimulated with *S. gordonii*, lipoprotein, LTA, or Pam2CSK4 for 16 h and lysed with GloLysis Buffer (Promega, Madison, WI, USA) and

luciferase activity in the cytoplasmic extracts was assayed using a luminometer (Molecular Devices, Sunnyvale, CA, USA).

2.6. Preparation of LTA of *S. gordonii*

Purified LTA was prepared from *S. gordonii* as previously described [41]. Briefly, bacteria were suspended in 0.1 M sodium citrate buffer (400 ml, pH 4.7) followed by ultrasonication. After mixed with an equal volume of n-butanol for 30 min, it was centrifuged to separate phase. The aqueous phase was dialyzed with a semi-permeable dialysis membrane (Spectra/Por 6;Spectrum® laboratories Inc., Ranch Dominquez, CA, USA) from endotoxin-free distilled water (Dai Han Pharm Co. Ltd., Seoul, Korea) followed by liquefied in 15% n-propanol in 0.1 M sodium acetate buffer (pH 4.7). After hydrophobic-interaction chromatography with an octyl-Sepharose column CL-4B (2.5 cm×10 cm) (Sigma-Aldrich, MO, USA), unbound molecules were washed with 15% n-propanol in 0.1 M sodium acetate buffer (200 ml, pH 4.7). After elution of bound molecules using 35% n-propanol in 0.1 M of the sodium acetate buffer (300 ml), it was performed inorganic phosphate assay. And then, column fractions including LTA were dialyzed from endotoxin-free water, followed by DEAE-Sepharose ion-exchange chromatography (FastFlow, Sigma-Aldrich,1.5×10 cm) in 0.1 M sodium acetate buffer (pH 4.7) including 30% n-propanol. After eluted with 0.1 M NaCl in sodium acetate buffer (300 ml), and it was collected, pooled, and lyophilized. The purified LTA quantity was determined through dry weight measurement.

2.7. Preparation of lipoprotein of *S. gordonii*

Purified and putative lipoprotein was isolated from *S. gordonii* as previously described [42]. Briefly, bacterial pellets was collected and suspended in Tris-buffered saline (TBS) with protease inhibitors. After sonication, the bacterial lysates were suspended in a final concentration of 2% Triton X-114 at 4^{o}C for 2.5 h. The cell debris was discarded using centrifuge. The supernatant was incubated at 37^{o}C for 15 min and centrifuged at 37^{o}C to separate into aqueous phase and Triton X-114 phase. After discarding aqueous phase, the equal volume of TBS was added into the Triton X-114 phase, and incubated at 37^{o}C for 15 min. After centrifugation, the Triton X-114 phase was incubated with methanol at -20^{o}C for overnight. The precipitated lipoproteins were dissolved in 10 mM octyl-beta-glucopyranoside in PBS.

2.8. Crystal violet assay

To compare the biofilm-forming ability of *S. gordonii* M99 wild-type and GspB-deficient strain, 1×10^{7}, 1×10^{8}, or 1×10^{9} CFU/ml of *S. gordonii* strains were cultured in THY media on 96-well plates (Nunc, Roskilde, Denmark) at 37^{o}C for 1, 3, 6, 12, or 24 h. To examine the biofilm-forming ability in the presence of sucrose, *S. gordonii* M99 wild-type and GspB-deficient strain at 1×10^{8} CFU/ml were cultured in THY media containing 0.01, 0.1, 1, or 3% of sucrose on 96-well plates at 37^{o}C for 24 h. The supernatant was removed and the wells were rinsed with phosphate-buffered

saline (PBS). Then, the biofilm was stained with 0.1% crystal violet for 30 min and solubilized in a mixture of 0.2% acetic acid and 95% ethanol. The optical density was measured at 600 nm with the VERSAmax plate reader (Molecular Devices, CA, USA).

2.9. Confocal laser scanning microscopy

S. gordonii M99 wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were cultured on cover-glass bottom dishes (SPL, Gyeonggido, Korea) for 24 h. After the removal of supernatant, the biofilm was stained with SYTO9 and propidium iodide to distinguish live and dead bacteria, respectively. After rinsing with PBS, the biofilm was observed using a confocal scanning laser microscope (LSM 5 Pascal, Carl Zeiss MicroImaging GmbH, Thuringen, Germany) at 400× magnification.

2.10. Examination of bacterial growth

S. gordonii M99 wild-type and GspB-deficient mutant strains were cultured in the fresh THY media for 0, 1, 3, 6, 9, 12, 15, 24, 36, and 48 h with shaking. The optical density was measured at 600 nm by using a spectrophotometer (GeneSpec III, Hitachi Genetics Corporation, CA, USA).

2.11. Scanning electron microscope (SEM) analysis

S. gordonii M99 wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were cultured on 48-well plates (Nunc, Roskilde, Denmark) or human dentin slices for 24 h. The supernatant was removed and the biofilm was fixed with a primary fixing solution (2% paraformaldehyde and 2.5% glutaraldehyde in PBS). The biofilm was washed with PBS, and fixed again with 1% osmium tetroxide for 1.5 h [43]. Then, the biofilm was rinsed with distilled water, and dehydrated in serially-graded ethanol solutions (70, 80, 90, and 95% for 15 min each and 100% for 15 min three times). The biofilm was dried using a critical point dryer (HCP-2, Hitachi, Tokyo, Japan), coated with platinum using an ion sputter (Quorum Q150T S, Quorum Technologies Ltd, East Grinstead, UK) and observed with a SEM (S-4700, Hitachi, Tokyo, Japan) at 5,000 and 10,000× magnifications with an acceleration voltage of 15 kV. The SEM images were processed to measure the area of the *S. gordonii* aggregate by using open-source software ImageJ (National Institutes of Health, USA). Then, the area of *S. gordonii* aggregates was converted to the percentage of the total area.

2.12. Reverse transcription-polymerase chain reaction

Human PDL cells were plated at 3×10^5 cells/ml in a 6-well plate. After stimulation with various concentrations of 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type, Δ*ltaS*, or Δ*lgt* for various time periods, total RNA was extracted using TRIzol reagent according to the manufacturer's

instructions. Complementary DNA (cDNA) was synthesized from 4 μg of total RNA using random hexamers and reverse transcriptase (Promega Corporation, Madison, WI, USA). Amplification of cDNA by PCR was performed in a total reaction volume of 26 μl containing EmeraldAmp GT PCR Master Mix and 10 picomoles of primers specific for human IL-8 (forward primer: 5'-TCT GCA GCT CTG TGT GAA GG-30, reverse primer: 5'-TGA ATT CTC AGC CCT CTT CAA-3') or β-actin (forward primer: 5'-GTG GGG CGC CCC AGG CAC CA-3', reverse primer: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3') for 32 cycles for IL-8 and 25 cycles for β-actin. Amplified PCR products were separated and then visualized by staining with ethidium bromide. The data were analyzed with an image analyzer (Gel Documentation system, Vilber Lourmat, Torcy, France).

2.13. Enzyme-linked immunosorbent assay

Human PDL cells were plated at 3×10^5 cells/ml in a 96-well plate. The cells were then stimulated with ethanol-inactivated *S. gordonii*, its lipoprotein, or LTA. To analysis of TLR2 activation using TLR2 neutralizing antibody, the cells were pre-treated with anti-human TLR2 (10 μg/ml) or human IgA2 control (10 μg/ml) for 1 h followed by stimulation with ethanol-inactivated *S. gordonii* (3×10^8 CFU/ml), lipoprotein (50 μg/ml), or LTA (50 μg/ml) for 24 h. For analysis of MAP kinase activation using MAP kinase inhibitors, the cells were pre-treated with 2 or 20 μM of SB203580 (p38 kinase inhibitor), U0126 (ERK inhibitor), or SP600125 (JNK inhibitor) for 1 h, followed by stimulation with ethanol-inactivated *S. gordonii* (3×10^8 CFU/ml) or its

lipoprotein (100 μg/ml) for 24 h. After incubation, IL-8 levels of culture supernatants were analyzed using a IL-8 ELISA kit (Biolegend, CA, USA) according to the manufacturer's instructions.

2.14. Statistical analysis

The mean value ± standard deviation (S.D.) of all experiments was obtained from triplicate samples. Statistical significance was examined with a *t*-test. An asterisk (*) indicates that there was significantly difference at $P < 0.05$.

3. RESULTS

3.1. GspB plays an important role in *S. gordonii* biofilm formation

To examine the role of GspB in the biofilm formation of *S. gordonii*, the biofilm-forming abilities of *S. gordonii* GspB-deficient mutant and wild-type strains were compared. When the bacteria were cultured on the cover-glass bottom culture plate followed by confocal microscopic analysis, the GspB-deficient mutant showed decreased biofilm formation compared with the wild-type bacteria (Fig. 1A). Quantitative analysis of biofilm formation using crystal violet assay also showed that biofilm formation of the *S. gordonii* GspB-deficient strain was lower than that of the wild-type strain (Fig. 1B). These results imply that GspB is important in *S. gordonii* biofilm formation.

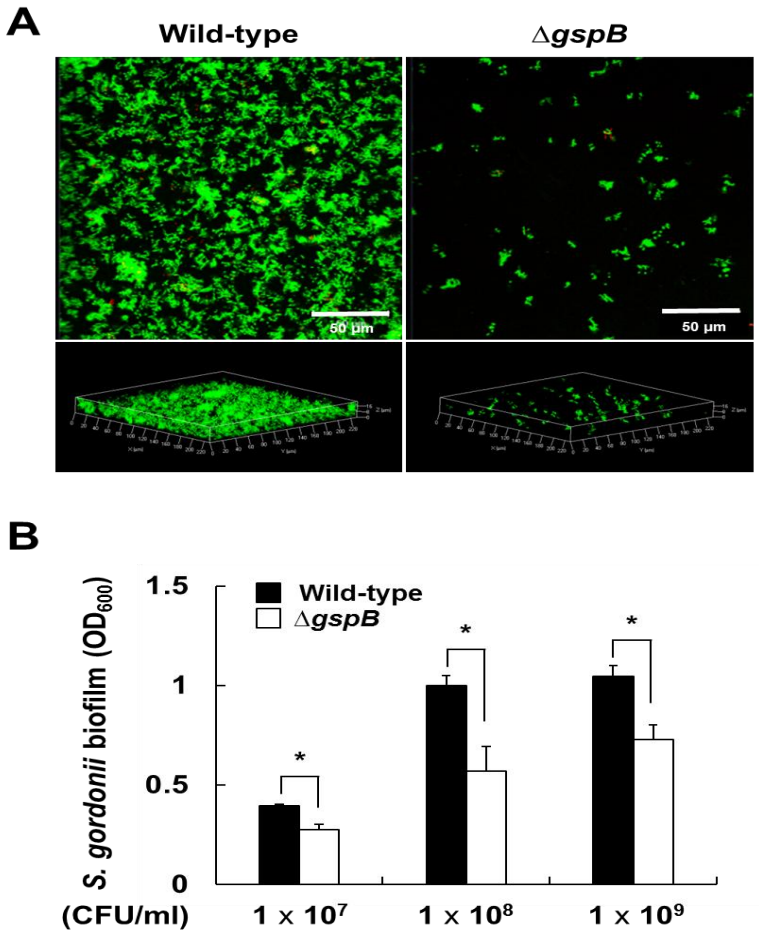

Figure 1. GspB-deficient *S. gordonii* forms biofilms less potently than the wild-type bacteria. (A) *S. gordonii* wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were grown on glass bottom dishes for 24 h. The bacterial biofilm formation was determined through SYTO9 and PI staining followed by confocal microscopic analysis. (B) *S. gordonii* wild-type and GspB-deficient mutant strains (1×10^7, 1×10^8, and 1×10^9 CFU/ml) were grown on 96-well culture plates for 24 h. The bacterial biofilm formation was determined by crystal violet staining followed by spectrometric analysis at 600 nm with a microplate reader. Data are presented as the mean value ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the wild-type. One of the three representative results is shown.

3.2. GspB of *S. gordonii* participates in the early stage of biofilm development without interfering with bacterial growth

In time-course experiments, the biofilm-forming ability of *S. gordonii* wild-type and GspB-deficient strain at 1, 3, 6, 12, and 24 h was compared. As shown in Fig. 2A, reduced biofilm formation of the GspB-deficient strain was observed as early as at 3 h, which is considered to be the early stage of biofilm development. To test whether a differential bacterial growth rate would be responsible for the decreased biofilm formation of the *S. gordonii* GspB-deficient strain, the growth rates were examined. The bacterial growth of the GspB-deficient strain was not different from that of the wild-type (Fig. 2B). Thus, these results indicate that the decreased biofilm formation of the GspB-deficient mutant is initiated in the early stage of biofilm development and bacterial growth is not related to this phenomenon.

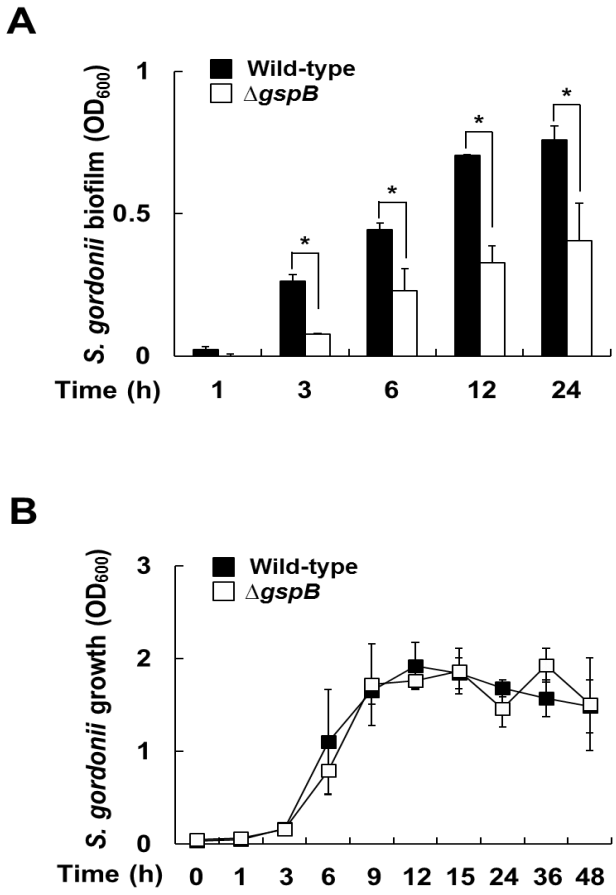

Figure 2. Reduced biofilm formation of the GspB-deficient *S. gordonii* is not due to an attenuation of bacterial growth. (A) *S. gordonii* wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were grown on 96-well culture plates for 1, 3, 6, 12, and 24 h. The extent of biofilm formation was quantified by crystal violet staining followed by spectrometric analysis at 600 nm with a microplate reader. (B) *S. gordonii* wild-type and GspB-deficient mutant strains were grown at 37℃ for 1, 3, 6, 9, 12, 15, 24, 36, and 48 h with shaking. Bacterial growth was monitored at 600 nm using a spectrophotometer at the indicated time points. Data are presented as the mean value ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the wild-type. One of three similar results is shown.

3.3. Difference in biofilm-forming ability is more distinct in sucrose-supplemented media

Biofilm formation is influenced by various environmental factors such as carbohydrates, sodium chloride, and temperature [44]. In particular, *S. gordonii* uses sugars such as sucrose, glucose, and fructose to produce glucans that enhance its biofilm formation [45]. Thus, it was investigated whether the two *S. gordonii* strains differ in biofilm formation in the presence of sucrose. The *S. gordonii* wild-type biofilm was significantly increased by the addition of sucrose in a dose-dependent manner (Fig. 3). However, the biofilm made by the *S. gordonii* GspB-deficient mutant strain was barely increased by the addition of sucrose to the culture media (Fig. 3). These results suggest that the difference in biofilm-forming capacity between the wild-type and GspB-deficient mutant *S. gordonii* strains is substantial in the presence of sugars where dental biofilm can easily form.

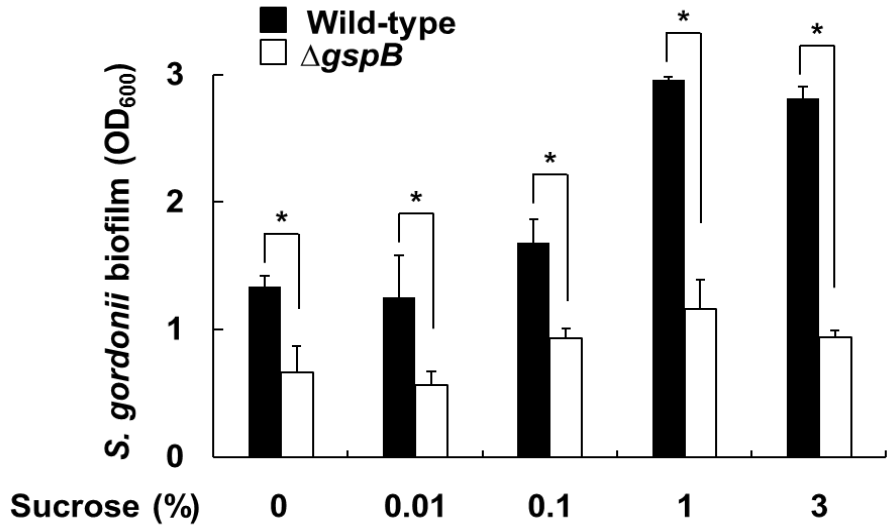

Figure 3. Difference in biofilm-forming ability is more distinct in sucrose-supplemented media. *S. gordonii* wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were grown in the presence of sucrose at various concentrations (0.01, 0.1, 1, and 3%) on 96-well culture plates for 24 h. The bacterial biofilm formation was determined by crystal violet staining followed by spectrometric analysis at 600 nm with a microplate reader. Data are presented as the mean value ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the wild-type. One of the three similar results is shown.

3.4. GspB is involved in *S. gordonii* biofilm formation on human dentin slices

Next, the SEM analysis to visualize the biofilm formed by *S. gordonii* wild-type and GspB-deficient mutant bacteria was performed. Concomitant with the confocal microscopic and biochemical analyses, GspB-deficient bacteria had less biofilm formation compared with the wild-type bacteria on the polystyrene cell-culture plate (Fig. 4A). Next, the *in vivo* relevance of the current results using a human dentin biofilm model were examined. SEM analysis results showed that the biofilm formation of the GspB-deficient strain was less than that of the wild-type on the human dentin slices (Fig. 4B). Notably, any morphological changes between *S. gordonii* wild-type and GspB-deficient strains could not be observed. The area of *S. gordonii* aggregate on the human dentin slices was quantitated using ImageJ software. As shown in Fig. 4C, the aggregated area of GspB-deficient strain was significantly decreased compared with that of the wild-type. These results imply that GspB contributes to *S. gordonii* biofilm formation on human dentin.

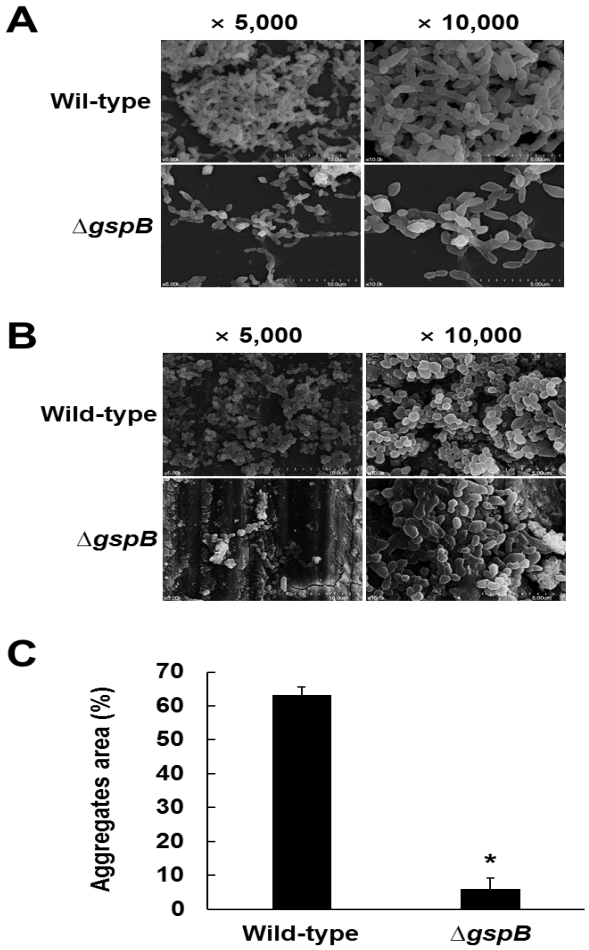

Figure 4. Reduced biofilm formation of GspB-deficient *S. gordonii* occurs not only on plastic surfaces but also on human dentin slices. (A, B) *S. gordonii* wild-type and GspB-deficient mutant strains (1×10^8 CFU/ml) were grown (A) on 96-well plastic culture plates or (B) on human dentin slices at 37℃ for 24 h. Then, SEM analysis was performed at 5,000× and 10,000× magnifications. One of three representative results is shown. (C) The area of *S. gordonii* aggregate on the human dentin slices was measured by using software ImageJ and converted to the percentage of the total area. Data are presented as the mean value ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the wild-type.

3.5. *S. gordonii* induces IL-8 mRNA expression and its protein production in human PDL cells

To examine the capability of *S. gordonii* in IL-8 mRNA expression, human PDL cells were stimulated with 0.3, 1, or 3 × 10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type for 3 h. *S. gordonii* significantly induced IL-8 mRNA expression in human PDL cells in a dose-dependent manner (Fig. 5A). When the cells were stimulated with ethanol-inactivated *S. gordonii* wild-type for 1, 3, 6, 12, or 24 h, IL-8 mRNA expression was increased in a time-dependent manner (Fig. 5B). To examine whether *S. gordonii* can induce IL-8 protein in human PDL cells, the cells were stimulated by 0.3, 1, 3, or 10 × 10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type for 24 h. *S. gordonii* significantly induced IL-8 production in human PDL cells in a dose-dependent manner (Fig. 5C). Furthermore, when human PDL cells were stimulated with 3 × 10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type for 1, 3, 6, 12, 24, or 48 h, IL-8 production was increased by *S. gordonii* in a time-dependent manner (Fig. 5D). These results imply that *S. gordonii* could induce the IL-8 chemokine expression in human PDL cells.

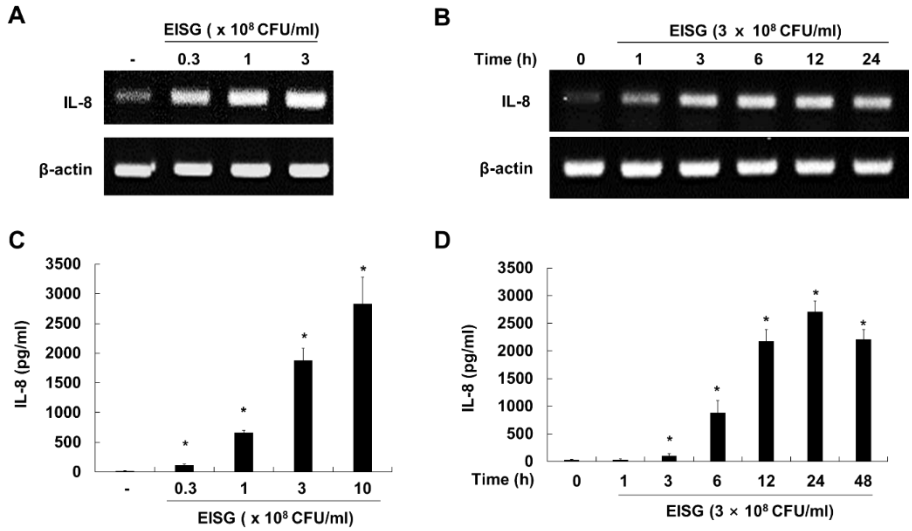

Figure 5. *S. gordonii* induces IL-8 production in human periodontal ligament (PDL) cells. (A, B) Human PDL cells (3×10^5 cells/ml) were stimulated (A) with 0, 0.3, 1, or 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type for 3 h, or (B) with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* for 0, 1, 3, 6, 12, or 24 h. After total RNA was isolated from the cells with TRIzol reagent, IL-8 mRNA expression was examined by RT-PCR. (C, D) Human PDL cells (3×10^5 cells/ml) were stimulated (C) with 0, 0.3, 1, 3, or 10×10^8 CFU/ml of ethanol-inactivated *S. gordonii* for 24 h, or (D) with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* for 0, 1, 3, 6, 12, 24 or 48 h. After the cell culture media were collected, levels of IL-8 production were measured by ELISA. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the non-treatment control group. EISG; ethanol-inactivated *S. gordonii*.

3.6. *S. gordonii*-induced NF-κB activity and IL-8 production are mediated by TLR2

Although TLR2 is known to recognize Gram-positive bacteria and their cell wall components, TLR2 participation in the recognition of *S. gordonii* has not been reported in human PDL cells. Therefore, whether *S. gordonii* was recognized by TLR2 using HEK293-TLR2 cells and human TLR2-neutralizing antibody was examined. Fig. 6A showed that *S. gordonii* wild-type induced substantial activation of NF-κB in the HEK293-TLR2 cells. Additionally, as shown in Fig.6B, *S. gordonii*-induced IL-8 production was decreased by human TLR2-neutralizing antibody in human PDL cells. Therefore, these results imply that *S. gordonii*-induced IL-8 production is mediated by TLR2 in human PDL cells.

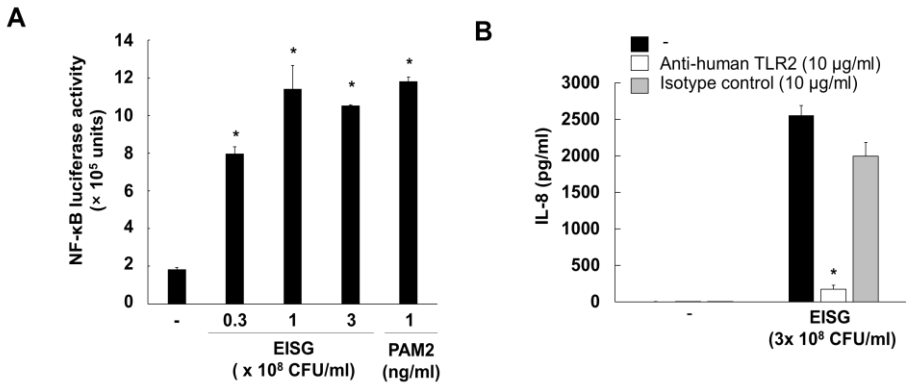

Figure 6. *S. gordonii*-induced NF-κB activity and IL-8 production are mediated by TLR2. (A) HEK-TLR2 cells (2.5×10^5 cells/ml) were transfected with an NF-κB luciferase reporter plasmid using Attractene transfection reagent. After 16 h, the cells were stimulated with 0.3, 1, or 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type or 1 ng/ml of Pam2CSK4 for 16 h. After the cells were lysed, luciferase assay was conducted. (B) Human PDL cells (3×10^5 cells/ml) were pre-treated with anti-human TLR2 antibody or its isotype control antibody for 1 h followed by stimulation with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii*. After the cell culture media were collected, levels of IL-8 production were measured by ELISA. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the control group stimulated with the bacteria in the presence or absence of isotype control antibody. EISG; ethanol-inactivated *S. gordonii*, PAM2; Pam2CSK4.

3.7. Lipoprotein-deficient *S. gordonii* does not induce IL-8 production in human PDL cells

To determine which cell wall components of *S. gordonii* are involved in IL-8 production in human PDL cells, human PDL cells were stimulated with ethanol-inactivated *S. gordonii* wild-ype, LTA-deficient strain (Δ*ltaS*), or lipoprotein-deficient strain (Δ*lgt*). As shown in Fig. 7A and B, *S. gordonii* wild-type and Δ*ltaS* strain induced IL-8 mRNA and protein expression in human PDL cells, while Δ*lgt* did not. These results indicate that lipoprotein could play a key role in *S. gordonii*-induced IL-8 production in human PDL cells.

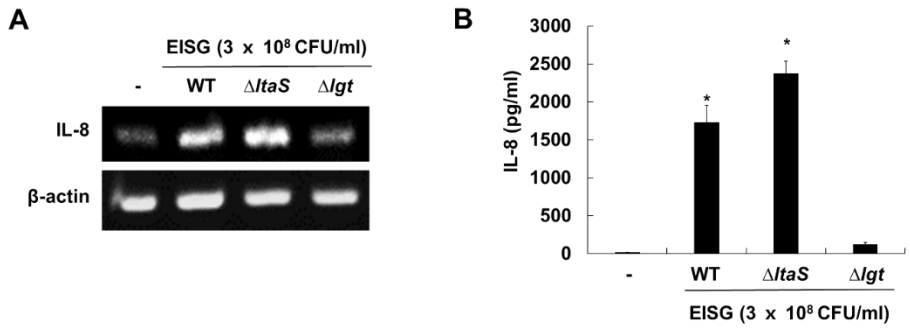

Figure 7. Lipoprotein-deficient, but not LTA-deficient, *S. gordonii* loses the ability to induce IL-8 production in human PDL cells. (A) Human PDL cells (3×10^5 cells/ml) were stimulated with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type, Δ*ltaS* or Δ*lgt* for 3 h. After total RNA was isolated from the cells with TRIzol reagent, IL-8 mRNA expression was determined by RT-PCR. (B) Human PDL cells (3×10^5 cells/ml) were stimulated with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type, Δ*ltaS*, or Δ*lgt* for 24 h. After the cell culture media were collected, IL-8 levels were measured by ELISA. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the non-treatment control group. EISG; ethanol-inactivated *S. gordonii*, WT; wild-type, Δ*ltaS*; LTA-deficient strain, Δ*lgt*; lipoprotein-deficient strain.

3.8. Lipoprotein of *S. gordonii* is importantly involved in the IL-8 production in human PDL cells

To confirm whether purified lipoprotein can also induce the IL-8 production, human PDL cells were stimulated with purified lipoprotein or LTA for 24 h. As shown in Fig. 8A, lipoprotein of *S. gordonii* induced IL-8 production in human PDL cells in a dose-dependent manner, while purified LTA of *S. gordonii* did not. Furthermore, lipoprotein of *S. gordonii* induced significant activation of NF-κB in the HEK293-TLR2 cells, whereas purified LTA showed a minimal activation (Fig, 8B). Additionally, lipoprotein-induced IL-8 production was decreased by human TLR2 neutralizing antibody in human PDL cells (Fig. 8C). These results indicate that lipoprotein of *S. gordonii* induces IL-8 production in human PDL cells via TLR2.

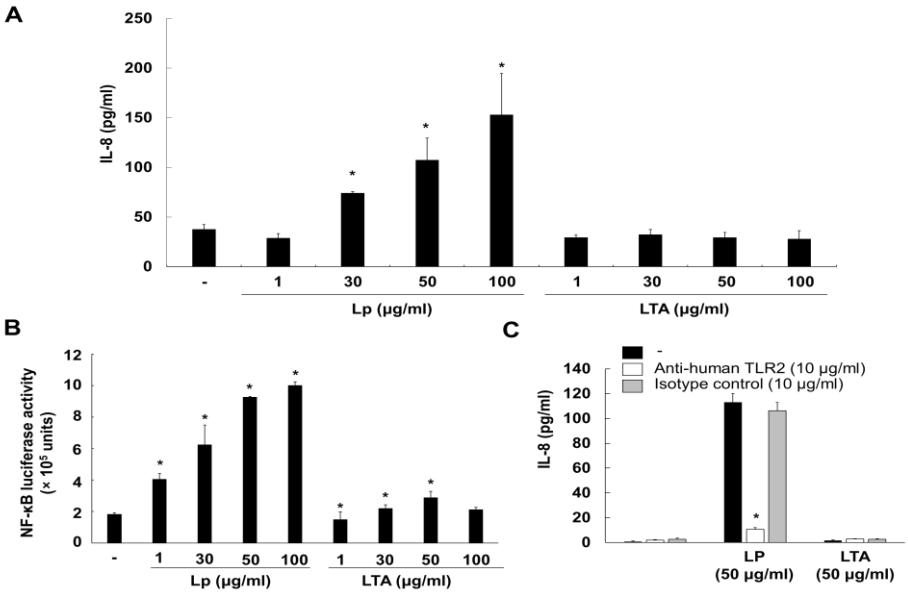

Figure 8. Lipoprotein, but not LTA, of *S. gordonii* is able to induce IL-8 production through TLR2 activation in human PDL cells. (A) Human PDL cells (3×10^5 cells/ml) were stimulated with 1, 30, 50, or 100 μg/ml of lipoprotein or LTA purified from *S. gordonii* for 24 h. After the cell culture media were collected, IL-8 levels were measured by ELISA. (B) HEK-TLR2 cells (2.5×10^5 cells/ml) were transfected with an NF-κB luciferase reporter plasmid using Attractene transfection reagent. After 16 h, the cells were stimulated with 1, 30, 50, or 100 μg/ml of lipoprotein or LTA purified from *S. gordonii* for 16 h. After the cells were lysed, luciferase assay was conducted. (C) Human PDL cells (3×10^5 cells/ml) was pre-treated with anti-human TLR2 antibody or its isotype control antibody for 1 h followed by stimulation with 50 μg/ml of lipoprotein or LTA purified from *S. gordonii* for 24 h. After the cell culture media were collected, levels of IL-8 production were measured by ELISA. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the control group stimulated with the bacteria in the presence or absence of isotype control antibody.

3.9. *S. gordonii* induces IL-8 production through p38 kinase, while its lipoprotein through p38 kinase, ERK, and JNK

To demonstrate whether p38 kinase, ERK, or JNK, which is known to be responsible for IL-8 production, are involved in IL-8 production by *S. gordonii* and its lipoprotein, inhibitors of p38 kinase (SB203580), ERK (U0126), or JNK (SP600125) were used. Fig. 9A shows that ethanol-inactivated *S. gordonii*-induced IL-8 production was significantly decreased by p38 kinase inhibitor, but not by ERK or JNK inhibitors. Interestingly, pretreatment with p38 kinase, ERK, or JNK inhibitors have lipoprotein-induced IL-8 production attenuated (Fig. 9B). These results imply that *S. gordonii* induces high level of IL-8 production via p38 kinase although its lipoprotein induces IL-8 production via phosphorylation of p38 kinase, ERK, and JNK in human PDL cells.

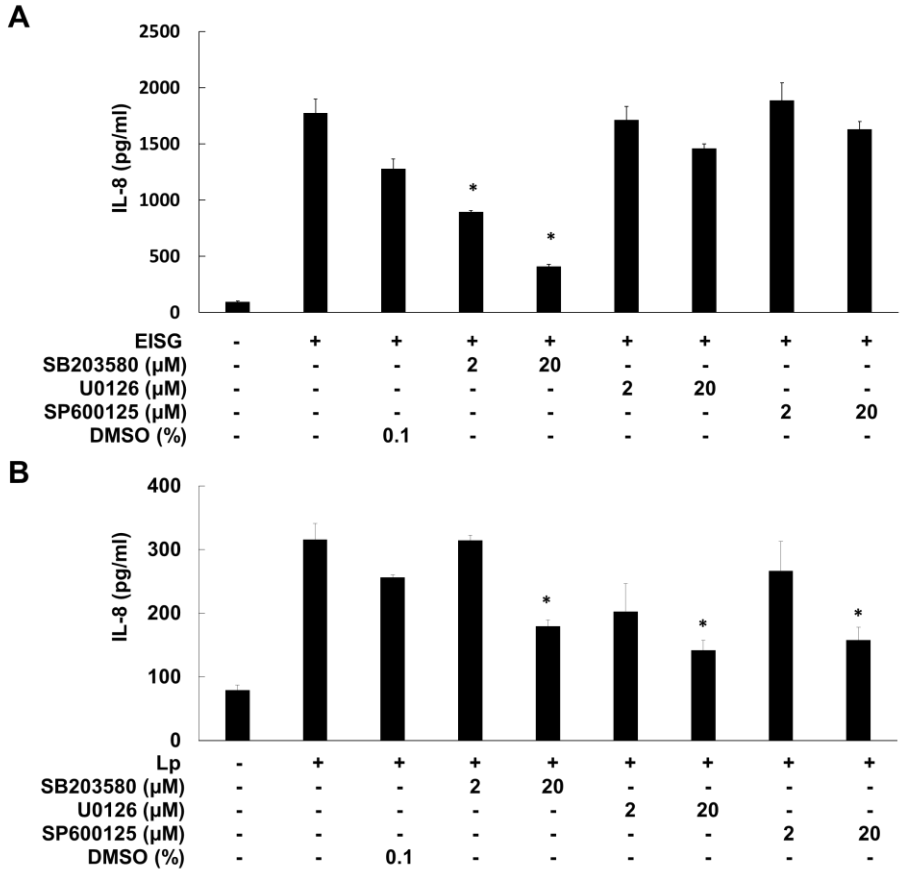

Figure 9. MAP kinase subtypes are differentially involved in IL-8 production induced by *S. gordonii* and its lipoprotein. (A, B) Human PDL cells (3×10^5 cells/ml) were pretreated with the indicated concentrations of p38 kinase inhibitor (SB203580), ERK inhibitor (U0126), or JNK inhibitor (SP600125) for 1 h. Then, the cells were stimulated (A) with 3×10^8 CFU/ml of ethanol-inactivated *S. gordonii* wild-type or (B) with 100 μg/ml of *S. gordonii* lipoprotein for an additional 24 h. After the cell culture media were collected, IL-8 levels were measured by ELISA. Data are presented as the mean value ± S.D. of triplicate samples. Asterisks indicate significant differences at $P < 0.05$ compared with the vehicle control group. EISG; ethanol-inactivated *S. gordonii*, LP; lipoprotein.

4. DISCUSSION

S. gordonii is an early colonizer for biofilm formation and recruits other periodontal pathogens causing various dental diseases [3]. If *S. gordonii* enters the blood stream, it can cause detrimental systemic diseases including endocarditis [2]. Furthermore, it is frequently isolated from the root canal even after treatment of apical periodontitis, called refractory apical periodontitis [5]. Apical periodontitis is an inflammatory disease caused by bacteria colonizing root canal. If these bacteria enter the periodontal ligament through apical foramens, they encounter PDL cells, prominent cells in periodontal ligament [17, 46, 47]. Human PDL cells are largely fibroblastic cells and can produce chemokines and pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα [48-50]. Although *S. gordonii* is associated with apical periodontitis and refractory apical periodontitis, the pathogenic mechanism of *S. gordonii* on apical periodontitis and refractory apical periodontitis is still not clear. Here, it is demonstrated that GspB, which is a major SRR adhesin of *S. gordonii*, is essential for biofilm formation of *S. gordonii* on dentin. Furthermore, it is demonstrated that *S. gordonii* induces IL-8 production in human PDL cells and its lipoprotein play a critical role in IL-8 production through activation of TLR2.

The current finding of GspB being important for *S. gordonii* biofilm formation is in keeping with other reports. Pneumococcal SRR protein (PsrP), known to mediate bacterial attachment to the surface of lung cells, promotes

biofilm formation by the aggregation of *Streptococcus pneumoniae* in the nasopharynx and lungs of infected mice [32]. They also showed that SraP and GspB, the SRR proteins of *Staphylococcus aureus* and *S. gordonii*, respectively, augment bacterial aggregation [32]. On the other hand, *Streptococcus parasanguis,* a primary colonizer of the tooth surface and essential for the formation of multi-species biofilm, expresses fimbriae-associated protein (Fap1) that is known to be required for biofilm formation [33]. Recently, Fap1 was determined to be a SRR adhesin [51]. In addition, an oral biofilm-forming bacterium, *Streotococcus cristatus*, expresses a high molecular weight serine-rich protein (SrpA), which mediates biofilm formation, in particular, with heterologous bacterial species such as *Fusobacterium nucleatum* [34]. Therefore, the present study's observations in conjunction with the results of prior research suggest that SRR adhesins are important cell wall components of Gram-positive bacteria in mediating biofilm formation.

Biofilm development can be divided into four stages: (i) initial contact of the bacteria to a solid surface, (ii) irreversible attachment, (iii) biofilm maturation, and (iv) dispersion of bacteria from the biofilm [52]. In this study, the results showed that the attenuated biofilm formation of GspB-deficient *S. gordonii* mutant was initially observed in the early stages of the bacterial culture even though no difference in bacterial growth was noted until 48 h. This implies that GspB is essential for the initial attachment of *S. gordonii* during biofilm

development. Similarly, the biofilm-forming ability of Fap1-deficient *S. parasanguis* noticeably declined at 2 h after inoculation compared with the wild-type [33]. In contrast, biofilm formation of the PsrP-deficient *S. pneumoniae* was not different from that of the wild-type until 24 h [32]. The existence of a polysaccharide capsule could be a possible explanation for the difference in light of the fact that SRR adhesins of *S. pneumoniae* are hindered by the thick polysaccharide capsule, which prevents the bacteria from attaching to the surface and forming a biofilm [32, 53, 54]. Nonetheless, PsrP may be involved in *S. pneumoniae* biofilm formation in the human body because the length of the polysaccharide capsule is shortened after infection [55]. Notably, *S. gordonii* [56] and *S. parasanguis* [57] do not have capsules. It has been reported that SRR adhesins from clinical isolates are associated with pathogenesis of oral infectious diseases, infective endocarditis, pneumonia, meningitis, and neonatal sepsis [31]. GspB and Hsa, for instance, are SRR adhesins of *S. gordonii* that mediate binding of the bacteria to platelets in the host due to its ability to bind to sialic acid residues on the host cell [2]. In addition, the binding ability of GspB to sialic acid motif is important in the dental biofilm formation, as *S. gordonii* is a predominant member of the human oral microflora contributing to the biofilm formation [1]. Indeed, GspB can bind in a sialic acid-dependent manner to salivary proteins such as low-molecular-weight salivary mucin MG2 and salivary agglutinin coated on the enamel or dentin surfaces [58]. Remarkably, salivary mucin MG2 has O-linked oligosaccharides carrying sialyl-T antigen, which is selectively recognized by GspB [59]. In light of the fact that dentin

sialoprotein is a sialic acid-rich glycoprotein, found in dentin [60], the current results showing GspB-deficient *S. gordonii* exhibited weaker biofilm formation on human dentin slices compared to the wild-type bacteria might be due to decreased interaction with sialic acid.

Biofilm formation is a complex process and is coordinated by the interaction of multi-species bacteria. Thus, the current study using a single bacterial species *S. gordonii* may not be sufficient to completely understand the oral biofilm formation and, thus, further studies using multi-species bacteria and fungi will shed a light to the field. Nevertheless, *S. gordonii* seems to be one of the key microorganisms in the oral biofilm by interacting with other microorganisms. Indeed, *S. gordonii* supports the invasion of *Porphyromonas gingivalis* to dentinal tubules via interspecies interaction [61] and also enhances the biofilm formation of *Candida albicans* [62]. In addition, it is known that the surface of many bacteria and fungi are sialylated [63, 64] and thereby those microbes can interact with SRR adhesins of other bacteria. Thus, an important role of SRR adhesins including GspB of *S. gordonii* in the multispecies microbial interactions is expected.

This study shows that *S. gordonii* induces IL-8 production in human PDL cells. These results are coincident with other reports. *S. gordonii* induced IL-8 production in HOK-18A cells, which are human gingival epithelial cells [65]. Furthermore, OKF4 cells, oral epithelial cell line, produced IL-8 by *S.*

gordonii biofilm or planktonic bacteria [66]. In addition, human epithelial KB cells and endothelial cells produced IL-8 by *S. gordonii* [67]. A549 and HEp-2, which are epithelial cells of human lung or larynx, respectively, produced IL-8 when they were stimulated with *S. gordonii* [68]. However, *S. gordonii* did not induce IL-8 production in HGF-1 (human gingival fibroblast) [65]. It may be due to few TLR2 expression in gingival fibroblasts, but human PDL cells have higher TLR2 expression [69]. Indeed, in this study, *S. gordonii*-induced IL-8 production is dependent on TLR2-mediated recognition. Therefore, the current results together with the other accumulated reports suggest that TLR2 activation is important for inducing host immune responses to *S. gordonii*.

The current data shows that *S. gordonii* wild-type and Δ*ltaS* induced IL-8 production, but such response was not observed when the cells were stimulated with the Δ*lgt*. Besides, purified lipoproteins from *S. gordonii* induced IL-8 production via TLR2. The results are concordant with the previous report on other Gram-positive bacteria. Lipoprotein of *Staphylococcus aureus* is critical in IL-8 production in Caco-2 cell via TLR2 [38]. *Streptococcus suis* lipoprotein also induced IL-8 production in porcine peripheral blood mononucleated cell (PBMC) by activating TLR2 [70]. In addition to IL-8, *Streptococcus agalactiae* lipoprotein also induced TNF production in RAW 264.7 macrophage cell-line through activation of TLR2 [35]. *S. aureus* lipoprotein also plays a key role in nitric oxide production in RAW 264.7 cells via TLR2 [71]. In dendritic cells, lipoprotein of *S. gordonii* activated TLR2, resulting in IL-6, TNF, and IL-10 production [40]. Therefore,

the current study together with the other previous reports indicates that TLR2 activation by lipoprotein is important for the induction of pro-inflammatory cytokines and chemokines by *S. gordonii*.

Although LTA has been known as TLR2 ligand, Δ*ltaS* induced IL-8 production in human PDL cells. These results are consistent with the previous report showing that LTA of *Enterococcus faecalis* hardly induced IL-8 production in human PDL cells [72]. Accumulating evidence suggests that lipoproteins have higher potency than LTA in TLR2 stimulation and pro-inflammatory cytokines productions than LTA [73]. Indeed, Pam2CSK4 mimicking Gram-positive bacterial lipoprotein induced 2-fold more IL-8 production in human odontoblast [74] and 100-fold more pro-inflammatory cytokines including IL-6, and TNFα in murine macrophage than LTA [75]. In this study, *S. gordonii* LTA was minimally recognized by TLR2. Moreover, LTA of *S. gordonii* did not induce IL-8 production in human PDL cells. Thus, TLR2 activation by *S. gordonii* LTA might be insufficient to induce IL-8 production in human PDL cells.

Accumulating evidence suggests that when gingival epithelial cell is infected by *S. gordonii*, MAP kinase pathway, including p38 kinase, ERK, and JNK, is the most impacted at transcriptional level in the cell [76] and activation of MAP kinase regulates IL-8 mRNA expression at the transcriptional levels [77]. In this study, is was investigated whether MAP kinase is involved in IL-8 production by *S. gordonii* and its lipoprotein in human PDL cells. The current

results showed that *S. gordonii* lipoprotein-induced IL-8 production in human PDL cells is regulated by all MAP kinase tested, whereas *S. gordonii*-induced IL-8 production involves only p38 kinase. One possible explanation for these differential results might be cooperative effect of lipoprotein together with other components in *S. gordonii* because *S. gordonii* has not only lipoprotein, but also other components such as peptidoglycan. Previous reports have demonstrated that TLR2 ligand and NOD2 ligand have synergistic effect on IL-8 production in human PDL cells [78]. The other reports showed that TLR2 ligand, *S. aureus* LTA, and NOD2 ligand, muramyl dipeptide, have synergistic effect on cyclooxygenase-2 (COX-2) expression [79], which is expressed via p38 kinase [80-82], and IL-8 expression would be mediated by COX-2 [80, 83]. Therefore, *S. gordonii*-induced IL-8 production may result from complicated stimulation by lipoprotein together with other components in human PDL cells.

Taken together, the results in this study show that GspB is closely involved in the initial adherence and biofilm formation of *S. gordonii*, especially on human dentin. Furthermore, lipoprotein of *S. gordonii* plays a critical role in the IL-8 production in human PDL cells. Therefore, this current results suggest that GspB promotes *S. gordonii* biofilm formation and lipoprotein of *S. gordonii* leads to inflammatory responses by promoting IL-8 production of human PDL cells, which may cause apical periodontitis and refractory apical periodontitis (Fig. 10).

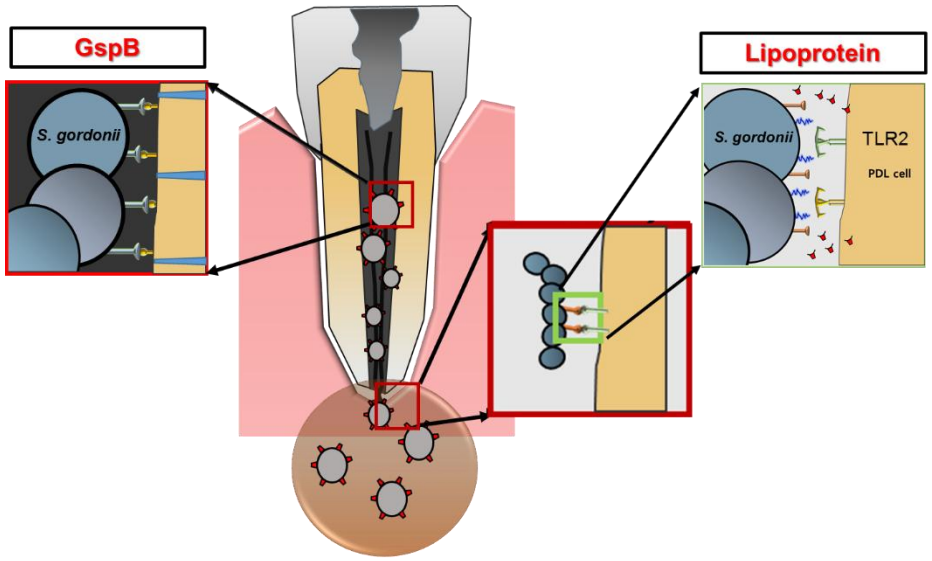

Figure 10. Proposed pathogenic mechanisms of *S. gordonii* in apical periodontitis. GspB promotes *S. gordonii* biofilm formation and lipoprotein of *S. gordonii* leads to inflammatory responses by promoting IL-8 production of human PDL cells, which may cause apical periodontitis and refractory apical periodontitis.

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국문 초록

1. 연구목적

*Streptococcus gordonii*는 통성혐기성 그람양성균으로 일반적으로 구강 내 정상 세균총으로 존재하는 세균으로 알려져 있지만 기회감염을 통해 난치성 치근단 치주염, 심내막염 등과 같은 감염질환을 유발하는 대표적인 원인균으로 보고되고 있다. *S. gordonii*는 인체 감염 후 바이오필름을 형성하고 염증반응을 강하게 유도함으로써 감염 질환을 유발한다고 알려져 있다. 하지만 현재까지 염증성 질환에 대한 *S. gordonii*의 명확한 병인기전과 이에 관여하는 핵심 세포벽 인자는 규명된 바가 없다. 따라서 본 연구에서는 *S. gordonii*의 바이오필름 형성과 대표적 염증매개인자인 IL-8 (Interleukin-8)의 생성 유도에 관여하는 핵심 세포벽 인자 및 작용기전을 규명하는 연구를 진행하였다.

2. 연구방법

*S. gordonii*의 세린풍부반복체 부착소 (serine-rich-repeat adhesin)인 gordonii surface protein B (GspB)가 바이오필름 형성에 미치는 영향을 보기 위해 *S. gordonii* 야생형 균주와 GspB 결손 돌연변이주를 사용하였으며 공초점 레이저 주사현미경 및 크리스탈 바이올렛 염색기법을 이용하여 바이오필름 형성 정도를 측정하였다. 또한 *S. gordonii* 야생형 균주와 GspB 결손 돌연변이주의 성장은 분광광도계를 이용하여 흡광도를 측정하였다. 세포배양판 및 상아질 절편에서의 박테리아 부착과 바이오필름형성은 주사전자현미경을 이용하여 확인하였다. *S. gordonii* 세포벽 인자의 염증매개인자 발현 유발 능력을 비교하기 위해 인체유래 치주인대세포에 *S. gordonii* 야생형 균주와 리포테이코익산 결손 돌연변이주 및 지질단백질 결손 돌연변이주를 처리하여 IL-8 발현양상을 역전사효소 중합연쇄반응 (Reverse Transcription-Polymerase Chain Reaction, RT-PCR)과 효소결합면역분석법 (Enzyme-Linked ImmunoSorbent Assay, ELISA)을 이용하여 확인하였다. 세포벽 인자의 톨유사수용체 (Toll-like receptor) 활성 정도를 확인하기 위해 TLR2를 과발

현하고 있는 인간배아신장유래세포 (human embryonic kidney 293, HEK293)에 *S. gordonii* 야생형 균주 또는 *S. gordonii*에서 분리한 지질단백질을 처리하여 NF-κB활성정도를 확인하였으며 또한 TLR2 중화항체를 한 시간 전처리하고 *S. gordonii* 야생형 균주 또는 *S. gordonii*에서 분리한 지질단백질을 처리하여 IL-8 발현정도를 확인하였다. 숙주염증유발에 관련된 신호전달체계를 알아보기 위해 p38과 ERK 및 JNK 억제제를 한 시간 전 처리 한 후 *S. gordonii* 야생형 균주 또는 지질단백질을 처리 하여 IL-8 생성 정도를 ELISA를 이용하여 확인하였다.

3. 연구결과

S. gordonii 야생형 균주에 비해 GspB 결손 돌연변이주의 바이오필름 형성정도가 초기 단계인 3시간 이후 부터 떨어졌으며, 이 때 각 균주의 성장 속도 차이는 없음을 확인하였다. 또한, *S. gordonii* 야생형 균주와 GspB 결손 돌연변이주 간 바이오필름 형성 능력 차이는 자당 첨가 배지에서 더 명확하게 나타났다. 세포배양판 및 상아질 절편에서의 바이오필름 형성 능력 또한 *S. gordonii* 야생형 균주에 비해 GspB 결손 돌연변이주에서 떨어짐을 확인하였다. *S. gordonii* 야생형 균주를 치주인대세포에 처리하였을 때 IL-8 생성 및 TLR2 활성을 유도하였다. 또한, 치주인대세포에서 *S. gordonii* 야생형 균주와 리포테이코익산 결손 돌연변이주가 IL-8 생성을 유도하는 반면 지질단백질 결손 돌연변이주는 IL-8의 생성을 유도하지 못하였다. *S. gordonii*에서 분리한 지질단백질을 처리하였을 때 농도 의존적으로 IL-8 생성이 유도되었으나 리포테이코익산을 처리하였을 때에는 IL-8 생성이 유도되지 않았다. 또한, 지질단백질에 의해 유도된 IL-8 생성에 p38과 ERK 및 JNK의 활성이 관여됨을 확인하였다.

4. 결 론

본 연구 결과를 종합해보면 *S. gordonii*의 GspB는 세균 부착 초기부터 관여함으로써 바이오필름 형성을 조절하는 핵심 세포벽 인자로 작용하며 지질단백질은 치주인대세포에서 *S. gordonii*에 의한

IL-8 생성에 핵심적으로 관여하여 염증반응을 매개하는 주요세포 벽인자로 작용하는 것을 알 수 있었다.

주요어 : *Streptococcus gordonii*, GspB, 바이오필름, 치주인대세포, 지질단백질, Interleukin-8

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