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Immunological properties of lipoteichoic acid isolated from *Streptococcus mutans* and its binding proteins in human saliva

Streptococcus mutans 로부터 정제한 lipoteichoic acid 의 면역학적 특성 탐구와 인체 타액에서의 부착 단백질 동정

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Immunological properties of lipoteichoic acid isolated from *Streptococcus mutans* and its binding proteins in human saliva

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ABSTRACT

Immunological properties of lipoteichoic acid isolated from *Streptococcus mutans* and its binding proteins in human saliva

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Streptococcus mutans is a pathogenic Gram-positive bacterium that is closely associated with dental caries and subsequent pulpal inflammation. Although lipoteichoic acid (LTA) is considered a major virulence factor of Gram-positive bacteria, little is known about the innate immunity to *S. mutans* LTA and its binding proteins in human saliva. In this study, LTA was purified from *S. mutans* (Sm.LTA) through *n*-butanol extraction, hydrophobic-interaction column chromatography, and ion-exchange column chromatography to investigate its immunological properties using murine macrophages. Furthermore, its binding proteins (Sm.LTA-BPs) were identified in the saliva from caries-free and caries-positive human subjects using Sm.LTA-conjugated beads and LTQ-Orbitrap hybrid Fourier transform mass

spectrometry. The Sm.LTA preparation had no detectable contamination with endotoxins, proteins, or nucleic acids. Upon exposure to Sm.LTA, the murine macrophage cell-line RAW 264.7 cells produced tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) in a dose-dependent manner. Sm.LTA preferentially bound to and activated CHO/CD14/TLR2 cells rather than CHO/CD14/TLR4 cells, which are stable transfectants expressing CD14 together with TLR2 or TLR4. Sm.LTA could not induce TNF-α or NO production in macrophages derived from TLR2-deficient mice whereas it dose-dependently induced those inflammatory mediators in wild-type macrophages. Furthermore, Sm.LTA deacylated by alkaline hydrolysis neither stimulated TLR2 nor induced TNF-α or NO production, suggesting that lipid moieties are crucial for the immuno-stimulatory activity of Sm.LTA. Unlike *Staphylococcus aureus* LTA, which has a potent immuno-stimulating activity, Sm.LTA showed a modest induction of NO production comparable to LTAs of *Enterococcus faecalis* and *Lactobacillus plantarum*. To further identify the binding proteins in saliva from caries-free and caries-positive human subjects, Sm.LTA was conjugated onto *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow beads (Sm.LTA-beads). Sm.LTA retained its biological properties during conjugation as determined by the expression of NO and interferon gamma-inducible protein 10 (IP-10) in RAW 264.7 cells and activation of TLR2 in CHO/CD14/TLR2 cells. Then, Sm.LTA-BPs were isolated from the salivary pools prepared from each of ten caries-free and caries-positive human subjects followed by electrophoresis to separate distinctively-expressed proteins for further identification using the high-resolution mass spectrometry. A total of 9 and 12 LTA-BPs were identified with statistical significance in each salivary pool from caries-

free and caries- positive human subjects, respectively. Sm.LTA-BPs found in caries-free saliva included histone H4, profilin-1, and neutrophil defensin-1 and those in caries- positive saliva included cystatin-C, cystatin-SN, cystain-S, cystain-D, lysozyme C, calmodulin-like protein 3, and actin. Sm.LTA-BPs found in both groups were hemoglobins, prolactin-inducible protein, protein S100-A9, and SPLUNC2. In conclusion, the present study suggest that the Sm.LTA contributes to the inflammatory responses induced by *S. mutans* in which its lipid moiety is essential for TLR2 binding and activation.

Keywords: *Streptococcus mutans*, Lipoteichoic acid, Innate immunity, Toll-like receptor, Inflammatory responses, Salivary proteins

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CONTENTS

1. INTRODUCTION

1.1. Dental caries and *Streptococcus mutans*

Dental caries is one of the most common infectious disease that causes in irreversible destruction of tooth resulting in tooth loss and pain in the oral cavity [1]. Dental caries occurs in consequence of complex interactions among oral microorganisms, dietary factors, salivary components, and genetic influences. Among these factors, oral microorganisms that are responsible for acid formation on tooth surface is a major etiological agent in the progress of dental caries. After intaking high sucrose diet, certain acid-producing bacteria are tightly adhered on tooth surface followed by breaking down of teeth by conducting the acidic environment [2]. Among various microorganisms, *Streptococcus mutans* has been considered as a crucial cariogenic bacteria due to their abilities to produce large amount of acid and glucan as well as, to suppress the salivary buffering capacities, and to be resistant to low pH environment [3].

Streptococcus mutans, a Gram-positive facultative anaerobe, is a dominant etiological agent of dental caries [4]. Invasion of *S. mutans* into dental pulp contributes to the development of chronic pulpitis [5]. *S. mutans* produces various virulence factors including lipoteichoic acid (LTA), antigen I/II, glucosyltransferase, glucan-binding proteins, and sugar transport systems [4, 6]. Among these, LTA has been recognized as one of the major bacterial cell wall components (Fig. 1) involved in the adhesion of *S. mutans* to hydroxyapatite, a major component of dentin [7], and for the

inflammatory responses leading to tissue damage [8].

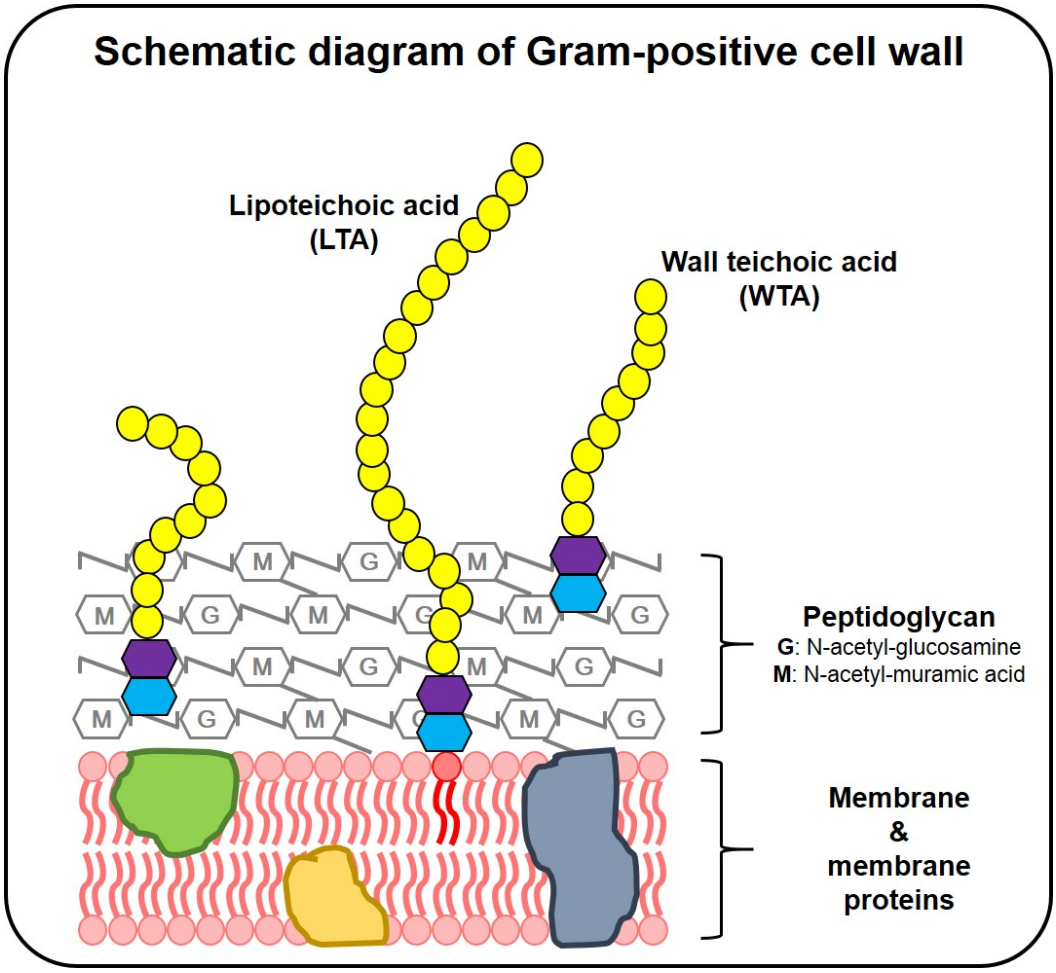

Figure 1. Schematic diagram of Gram-positive bacterial cell wall. Gram-positive bacterial cell wall is consisted of single cytoplasmic membrane with membrane proteins. It was surrounded with thick peptidoglycan (PGN). Two types of polysaccharide called teichoic acids (TA) are exposed to outside. These TA are covalently linked to PGN (wall teichoic acid; WTA) or cytoplasmic membrane (lipoteichoic acid; LTA).

1.2. LTA and LTA-binding proteins

LTA is an amphiphile composed of a hydrophilic polysaccharide and a hydrophobic glycolipid (Fig.2). So far, two classes of LTA (*i.e.,* type I and II) have been identified [9]. Type I LTA, to which most LTAs including *S. mutans* LTA (Sm.LTA) belong, is comprised of a diacylated glycolipid anchor linked to repeating polyglycerolphosphate units whose *sn*-2 position is esterified with D-alanine, α-D-*N*-acetylglucosamine, or hydroxyl substituent [10]. Type II LTA, which includes those of *Streptococcus pneumoniae* and *Streptococcus oralis*, are comprised of polyribitolphosphate repeating units linked to a positively-charged glycolipid [11]. Unlike type I LTA, type II LTA contains phosphocholine (PC) moiety in its repeating units, which plays a crucial roles in bacterial growth and pathogenesis [12]. Accumulating reports suggest that the immuno-stimulating activity of LTA is dependent on the composition and length of the repeating units and the type of glycolipid [13, 14].

Despite the pathological importance of LTA, many of the studies to have analyzed its immuno-stimulating activity have been controversial due to the use of improper purification methods that result in structural damage to the LTA [14] and contamination with other immunologically-active components such as endotoxins, lipoproteins, and nucleic acids [15]. Recently, an improved purification method has been introduced that uses extraction with *n*-butanol as a mild organic solvent followed by hydrophobic interaction column chromatography and anion exchange column chromatography, which allows for the preparation of highly-pure and

structurally-intact LTA [16, 17].

During initial stage of immune response by LTA, interaction of hydrophobic glycolipid and anionic polysaccharide with LTA-binding proteins (LTA-BPs) were responsible. For example, the glycolipid of LTA is essential for the interaction with Toll-like receptor 2 (TLR2) leading to activation of immune responses whereas polysaccharide of LTA is recognized by carbohydrate-recognizing proteins such as CD14, lipopolysaccharide-binding proteins, L-ficolin, and mannose-binding protein [18-20]. LTA-BPs are considered to be involved in both bacterial pathogenesis and host immune responses at infection by, for example, delivery of LTA to TLR2, induction of inflammation [20], activation of complements [18], or neutralization of LTA [19]. Thus, identification and characterization of LTA-BPs are crucial for understanding of bacterial pathogenesis and host immune responses.

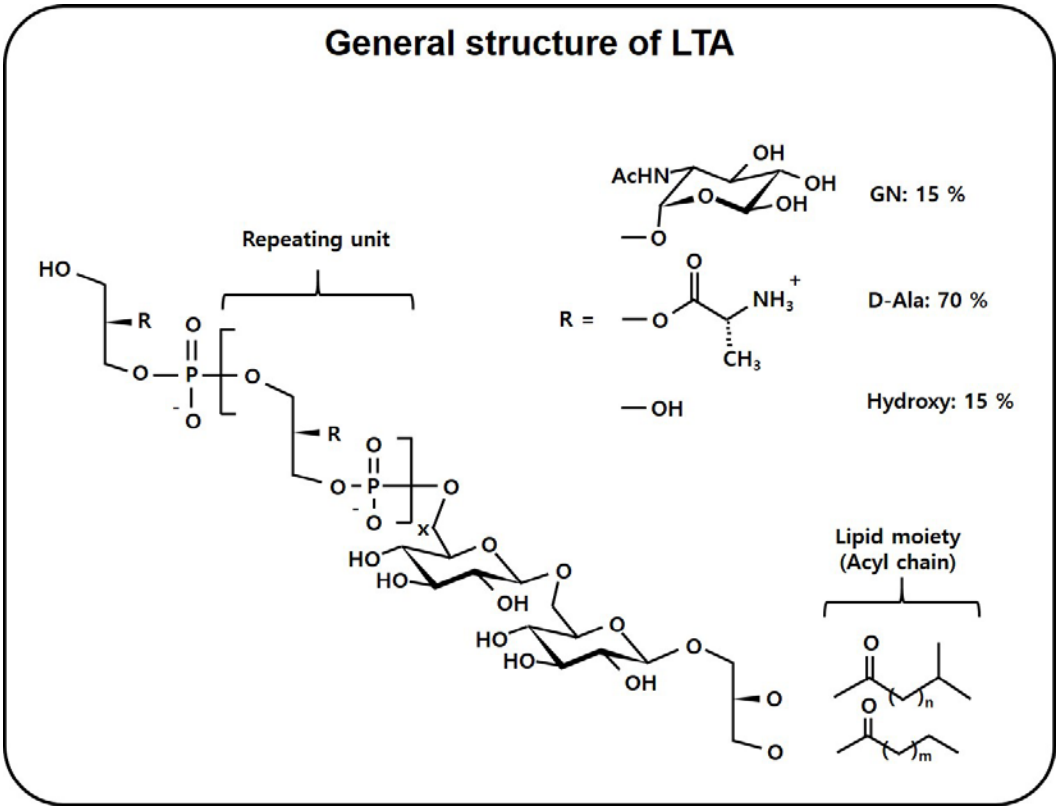

Figure 2. General structure of LTA. The general structure of LTA was proposed by Morath, S., et al [13, 14]. There are two structural types of LTAs are found. This figure shows an abundant type (polyglycerolphosphate-type LTA; type 1 LTA) of LTA. Type 1 LTA is expressed in most Gram-positive bacteria such as *S. aureus*, *B. subtilis*, and *S. mutans*. These LTA is comprised of a diacyl-containing glycolipid anchor linked to repeating polyglycerolphosphate units. And its *sn*-2 position is esterified with D-alanine, α-D-*N*-acetylglucosamine, or hydroxyl substituent.

1.3. Saliva and salivary proteins

Saliva plays an important role in the homeostatic regulation of pH, antimicrobial function, food digestion, and formation of acquired pellicle [21, 22]. Previous reports showed that the characteristics of saliva including pH and profile of salivary protein remarkably altered during the progress of oral disease such as dental caries, periodontitis, and oral cancer [23-25]. For example, the saliva from caries-active subjects exhibits relatively acidic pH [26] and different expression levels of proline-rich proteins, staterins, histatin, cystatin, lysozyme and lipocalins [25, 27]. Although these alteration of saliva might be responsible for the progress of dental caries by affecting the function of proteins involved in bacterial killing, bacterial adhesion or host immune responses [21, 28, 29], its molecular mechanism has not been fully characterized.

1.4. Aim of the present study

The aim of present study was (i) to purify LTA from *S. mutans* using the improved purification method using mild organic solvent, (ii) to investigate its immuno-stimulating activity with murine macrophages, and (iii) to identify the Sm.LTA-BPs in two different types of saliva obtained from caries-free or caries-positive subjects.

2. MATERIALS AND METHODS

2.1. Bacteria, reagents, and chemicals

S. mutans KCTC 3065 and *Lactobacillus plantarum* KCTC 10887BP were obtained from the Korean Collection for Type Culture (Daejon, Korea). *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Octyl-Sepharose CL-4B, DEAE-Sepharose, lipopolysaccharide (LPS) of *E. coli* O111:B4, polymyxin B and *N*-hydroxysuccinimidyl (NHS)-Sepharose® 4 Fast Flow beads were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pam_2CSK_4, anti-mouse TLR2-blockoing antibody and the isotype control were obtained from InvivoGen (San Diego, CA, USA). Recombinant mouse IFN-γ was purchased from R&D Systems (Minneapolis, MS, USA). All other reagents were obtained from Sigma-Aldrich unless otherwise indicated.

2.2. Bacterial culture and purification of LTA

S. mutans and *E. faecalis* were cultured in Brain Heart Infusion medium (BD Biosciences, San Jose, CA, USA) and *S. aureus* and *L. plantarum* were cultured in Tryptic Soy Broth (BD Biosciences) and MRS media (Neogen, Lansing, MI, USA), respectively. LTA was prepared from *S. mutans*, *S. aureus*, *E. faecalis*, and *L. plantarum*. Approximately 100 g (wet weight) of *S. mutans, S. aureus, E. faecalis, and L. plantarum* were washed with phosphate-buffered saline (PBS), resuspended in 1000 ml of 0.1 M sodium citrate buffer (pH4.7) and were disrupted using

ultrasonication followed by organic solvent extraction with *n*-butanol. Subsequently, hydrophobic interaction chromatography and ion-exchange chromatography were performed using Octyl-Sepharose CL-4B and DEAE-Sepharose, respectively. The purified LTA was quantified by measurement of dry weight. In order to detect residual proteins in Sm.LTA preparation, Sm.LTA (1, 5, 10, or 20 μg) was subjected to SDS-PAGE followed by silver staining.

2.3. Phosphate assay

Measurement of phosphates was used to quantify LTA. Each eluted LTA sample or lyophilized LTA was treated with a nitric acid-sulfuric acid mixture to hydrolyze phosphate diester linkages. Samples were then reacted with molybdate and stannous chloride. Using sodium phosphate solution as a standard, the amount of phosphate was quantified by measuring the optical density at 690 nm using a microtiter-plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. *Limulus* amebocyte lysate (LAL) assay

The level of endotoxin in the purified LTA was measured using a commercially-available LAL test kit (QCL-1000; Cambrex Bio Science, Walkersville, MD, USA).

2.5. SDS-PAGE and silver staining

The samples were mixed with 4× sample buffer (8% Sodium dodecyl sulfate, 2-mercaptoethanol, 30% glycerol, 0.25 M Tris-HCl containing 0.02% bromophenol

blue, pH 6.8) and incubated at 100°C for 10 min. Then, the solutions were subjected into 12 or 15% SDS-PAGE gel and run at 120 V for 2 h. The gels were treated with fixation solution (50% methanol/12% acetic acid/0.0185% CH_2O) for 1 h then washed with 50% ethanol for 20 min twice. Next, the gels were treated with sensitizing solution [0.02% (w/v) $Na_2S_2O_3 \cdot 5H_2O$] for 1 min. After washing with non-pyrogenic water for 2 s three times, the gels were soaked in silver reaction solution (0.2% $AgNO_3$/0.027% CH_20) for 1 h and washed twice with non-pyrogenic water for 20 s. Finally, the gels were treated with developing solution [6% Na_2CO_3/0.00004% (w/v) $Na_2S_2O_3 \cdot 5H_2O$/0.0185% CH_2O] for 5-10 min to obtain the desired band intensity. When the bands were successfully appears, the gels were washed and incubated with stopping solution (50% methanol/12% acetic acid) to cease the silver reaction.

2.6. Preparation of heat-killed bacteria

S. mutans was grown in BHI medium at 37°C without shaking to mid-log phase (OD_{600}, 1). Collected cells were washed with PBS three times and killed by heat at 60°C for 60 min. To confirm the bacteria was killed completely, the killed bacteria was plated on BHI media with 1.5% agar and incubated overnight at 37°C. No bacterial colonies on agar plate were observed.

2.7. RAW 264.7 cell culture

The murine macrophage cell line, RAW 264.7 from the ATCC was cultured in

Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2.

2.8. Determination of inflammatory mediators

A commercial tumor necrosis factor-alpha (TNF-α) and interferon gamma-inducible protein-10 (IP-10) ELISA kits (R&D Systems) were used according to the manufacturer's instructions to determine the concentration of TNF-α and IP-10. The production of nitric oxide (NO) was determined by measuring the accumulation of nitrite in the culture supernatants [30]. A standard curve of nitrite concentration was constructed using $NaNO_2$. The optical density was measured using a microtiter-plate reader (Molecular Devices).

2.9. Measurement of TLR binding and activation

CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, which constitutively express human CD14 together with human TLR2 or TLR4, can express membrane-bound CD25 in proportion to the activation of TLR2 and TLR4, respectively [31]. For the binding assay, 2×10^5 CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were treated with Sm.LTA (1 or 3 μg/ml) or Sm.LTA-biotin (1 or 3 μg/ml) at 4°C for 30 min. After washing with PBS, the cells were treated with streptavidin-FITC (BioLegend, San Diego, CA) for 15 min at room temperature. After washing with PBS, Sm.LTA-biotin binding to the cells was analyzed by flow cytometry using a FACSCalibur flow

cytometer with CellQuest software (BD Biosciences). For the activation assay by Sm.LTA or Sm.LTA-beads, 2 or 3 × 10^5 CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were treated with various stimuli for 24 h, and the expression of CD25 was examined by flow cytometry. To determine the expression of TLR2 and TLR4, CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were stained with FITC-conjugated mouse monoclonal anti-human TLR2 or PE-conjugated mouse monoclonal anti-human TLR4 antibodies (BioLegend) for 30 min at 4°C and then, the expression was analyzed by flow cytometry as described above.

2.10. Preparation of bone marrow-derived macrophages

The Institutional Animal Care and Use Committee of Seoul National University (Seoul, Korea) approved all animal experiments. Dr. Shizuo Akira (Osaka University, Osaka, Japan) kindly provided TLR2-deficient mice, and wild-type C57BL/6 mice were purchased from Orient Bio Inc. (Seoul, Republic of Korea). Bone marrow cells were isolated from the femurs and tibiae of 6 week-old mice, and red blood cells were subsequently removed using red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO, USA). The cells were differentiated to macrophages by incubation with 10% L929-cultured medium in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol for six days. Adherent cells were collected and plated in 96-well plates for 12 h. The cells were then stimulated with Sm.LTA (0, 1, 3, 10, or 30 μg/ml), Pam_2CSK_4 (0.1 μg/ml) in the presence of IFN-γ (0.1 ng/ml) for 24 h. At the end of stimulation, the culture supernatants were collected and analyzed for TNF-α and NO production.

2.11. Conjugation of Sm.LTA with biotin or NHS-beads

For the conjugation of biotin, one milligram of Sm.LTA was conjugated with 50 μl of 10 mg/ml biotin-3-sulfo-*N*-hydroxysuccinimide ester sodium salt (biotin-NHS) by mild vortexing at room temperature for 4 h. After the reaction, unconjugated biotin-NHS was removed using the PD-10 Desalting column (GE Healthcare Life Sciences, Buckinghamshire, UK). For the conjugation of NHS-beads, five hundred milligrams of NHS-Sepharose® 4 Fast Flow beads were washed with pyrogen-free water and then incubated with 2.5 mg of *S. mutans* LTA (Sm.LTA) with gentle agitation at 4°C for 4 h followed by further reaction with 0.5 M ethanolamine (pH 8.0) to block remained reactive sites on NHS-beads at 4°C for 1 h. After collecting the supernatant, the beads were washed with pyrogen-free water five times and the conjugation of Sm.LTA onto bead (Sm.LTA-beads) was confirmed by conducting Kaiser's test [32]. Finally, the amount of biotinylated Sm.LTA (Sm.LTA-biotin) was directly quantified using phosphate assay and Sm.LTA onto beads were calculated by measuring the quantity of remained LTA in supernatant using phosphate assay as described above.

2.12. Preparation of deacylated *S. mutans* LTA

Sm.LTA was incubated in 0.5 N NaOH for 2 h. Then, NaOH and free fatty acids in the mixture were removed using the PD-10 Desalting column (GE Healthcare Life Sciences). Deacylation was confirmed using thin-layer chromatography. The quantity of deacylated LTA was determined by measuring the amount of phosphate.

2.13. Collection of saliva from human

Collection of saliva sample was conducted by the approval of the Institutional Review Board of the Seoul National University Dental Hospital (IRB No. CRI11008). Ten each of caries-free and caries-active subjects were selected outpatients. Outpatients showing good oral hygiene with no current caries or no more than two restorations for previous caries were categorized as caries-free subjects. Conversely, outpatients showing poor oral hygiene with more than three current caries or more than 10 restorations for previous caries were categorized as caries-positive subjects. The mean value ± standard deviation (SD) for the Decayed-Missing-Filled Teeth (DMFT) index for caries-free and caries-positive subjects are 1.3 ± 0.8 and 14 ± 9.1, respectively. Ten caries-free and 10 caries-positive subjects fasted at least 2 h until saliva collection and tooth brushed their teeth for 2 min without toothpaste. All individuals were rinsed their mouth with water for 10 min and then 10 ml of saliva were collected in 50 ml conical tube. Proteins in the saliva were protected from degradation by adding Complete Mini Protease Inhibitor Cocktail EDTA-free tablets (Roche, Mannheim, Germany). Other components without proteins such as cells, debris, and insoluble materials were removed by centrifugation at 7000 × g at 4°C for 15 min. After centrifugation, the supernatant was collected and stored at - 80°C until use. To compare the protein profiles in saliva from caries-free and caries-positive subjects, the quantity of proteins in saliva samples were determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) and 10 μg of proteins from each sample were mixed with sample buffer and boiled at 100°C for 10 min. The samples were loaded in 15% SDS-PAGE gels, electrophoresed, and stained with

Coomassie blue..

2.14. Isolation and identification of Sm.LTA-binding proteins (Sm.LTA-BPs)

One milligram of saliva proteins from ten subjects each from the caries-free or caries-positive group were pooled separately. After eliminating nonspecifically-bound proteins from each salivary pool by pre-incubation with native beads (300 mg) at 4^{o}C for 1 h, the pre-cleared saliva was incubated with native beads (30 mg) or Sm.LTA-beads (30 mg) with gentle agitation at 4^{o}C for 4 h. After washing the beads three times with phosphate-buffered saline, the bound proteins were eluted by boiling at 100^{o}C for 10 min in the sample buffer and separated on 15% SDS-PAGE by electrophoresis at 120 V for 3 h. After visualizing the proteins by silver straining, the whole lanes containing visible and non-visible bands were cautiously cut off and subsequently, subjected to 7-Tesla Finnigan LTQ-Orbitrap hybrid Fourier transform mass spectrometry as previously described [33]. The obtained mass and tandem mass spectra were analyzed with Mascot Daemon (Matrix Science, London, UK) using the IPI human database (IPI.HUMAN.v.3.73). The peptide score was - 10 x Log (P), where P indicates the probability that the observed match was a random event. Individual peptide scores over 36 were considered as identity or extensive homology (P<0.05). The mass spectrometric analysis was performed three times independently, and proteins comprising more than one peptide with over 36 peptide scores in at least two independent analyses were identified as Sm.LTA-BPs

2.15. Statistical analysis

The mean values ± standard deviations were determined from triplicated samples and two-tailed *t*-test was used determine statistical significance. Difference with statistical significance between test group and non-treatment control group were determined when $P < 0.05$. All experiments including mass spectrometric analysis were conducted in triplicate under the similar conditions.

3. RESULT

3.1. LTA purified from *S. mutans* is immunostimulatory.

Sm.LTA was purified using the sequential application of *n*-butanol extraction, hydrophobic-interaction column chromatography, and ion-exchange column chromatography. Column fractions with high phosphate content were selected and pooled (fractions 12 to 15 from the Octyl-Sepharose column and fractions 9 to 14 from the DEAE-Sepharose column) (Fig. 3A and 3B) as previously described [16]. The LAL assay demonstrated that the Sm.LTA preparation contained endotoxins at a level of 19 pg per milligram of Sm.LTA, which is an amount unlikely to stimulate macrophages [34]. In addition, there was no detectable nucleic acids observed at 260 and 280 nm of UV absorption (data not shown) and protein contamination assessed by SDS-PAGE followed by silver staining (Fig. 4) in Sm.LTA preparation. Next, the immuno-stimulating activity of the Sm.LTA in RAW 264.7 cells was determined by measuring the proinflammatory mediators NO and TNF-α. As shown in Figs. 5A and 5B, the production of NO and TNF-α was induced in a concentration-dependent manner upon stimulation in the presence of IFN-γ for 24 h as previously described [35]. This activity was not inhibited by the potent endotoxin antagonist polymyxin B, while the production of NO and TNF-α by *E. coli* LPS was abrogated in the presence of polymyxin B (Figs. 5A and 5B). These results imply that high-purity Sm.LTA is immunostimulatory in macrophages.

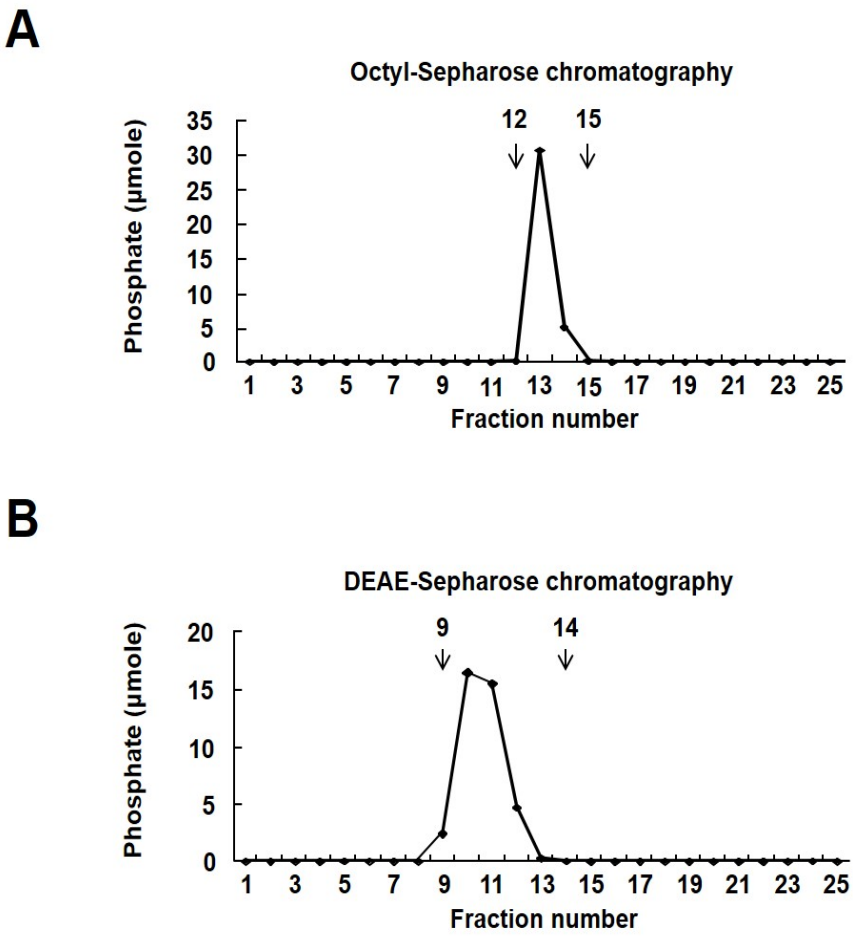

Figure 3. Two sequential column chromatography. *S. mutans* was lysed by ultrasonication, and Sm.LTA was purified from the lysate by *n*-butanol extraction, Octyl-Sepharose column chromatography, and DEAE-Sepharose column chromatography. Phosphates in the eluents were quantified in order to identify Sm.LTA-containing fractions after (A) Octyl-Sepharose column chromatography and (B) DEAE-Sepharose column chromatography. Results shown are representative of three independent experiments.

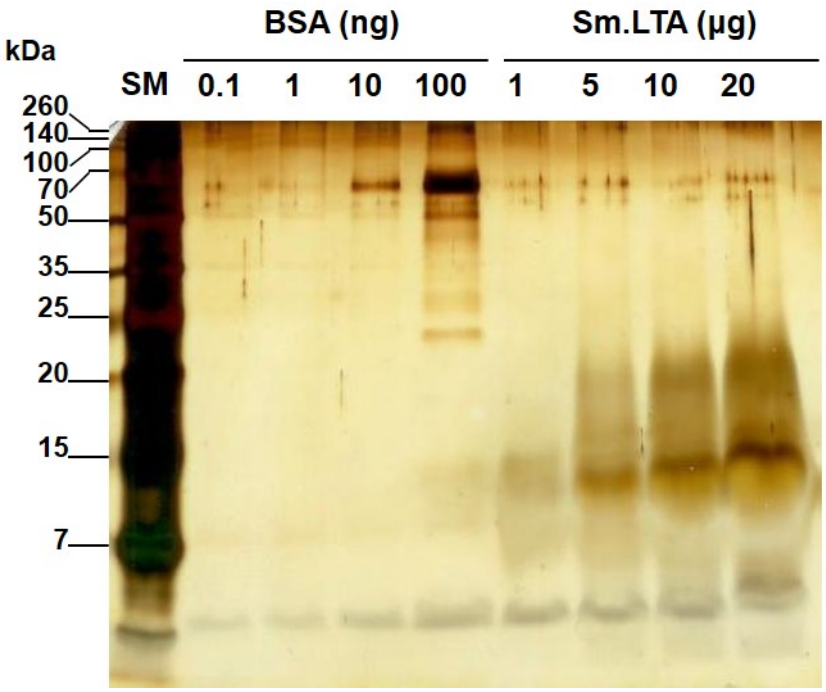

Figure 4. Silver staining of Sm.LTA. Sm.LTA preparation was separated using SDS-PAGE and visualized by silver staining to examine residual proteins in Sm.LTA preparation. Bovine serum albumin (0 to 100 ng) was used as a standard. SM; size marker.

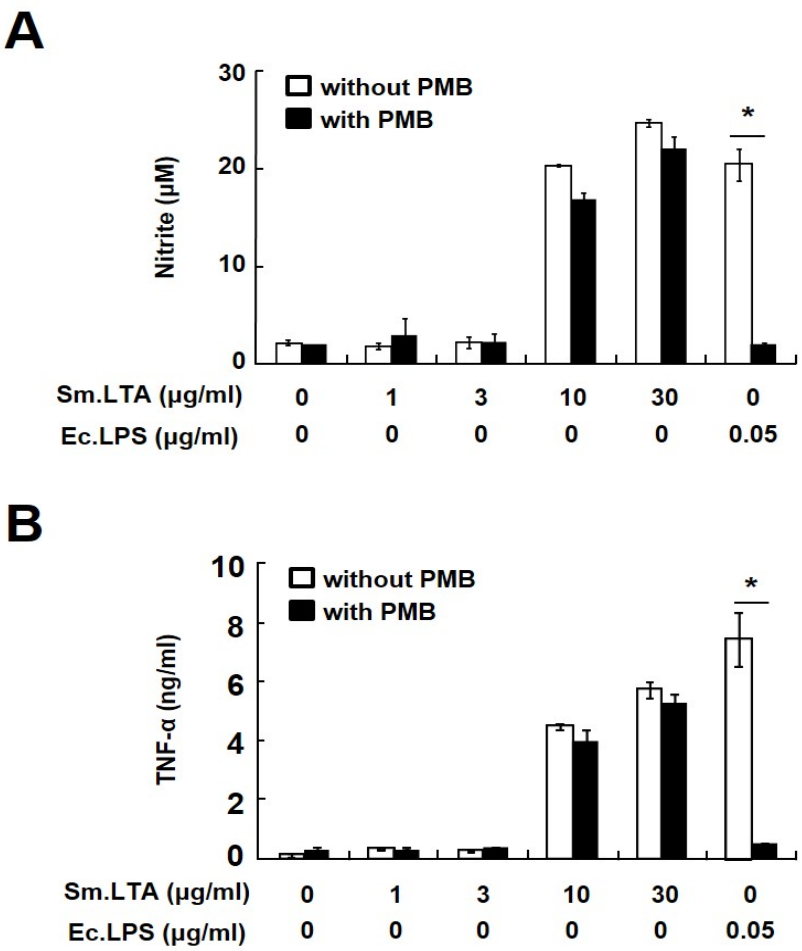

Figure 5. LTA purified from *S. mutans* is immunostimulatory. RAW 264.7 cells (1 × 10^6 cells/ml) were pretreated with IFN-γ (0.1 ng/ml) for 1 h, followed by stimulation with Sm.LTA (1, 3, 10 or 30 μg/ml) and *E. coli* LPS (Ec.LPS; 0.05 μg/ml) for 24 h in the presence or absence of polymyxin B (PMB; 25 μg/ml). At the end of the culture period, the culture supernatants were analyzed to determine (A) NO production using Griess agent and (B) TNF-α production using ELISA. Data represent means ± S.D obtained from triplicate samples. One of three similar results is shown. An asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with the non-treatment groups of polymyxin B.

3.2. *S. mutans* LTA preferentially binds to and activates TLR2 rather than TLR4.

The immune response to LTAs from various Gram-positive bacteria tends to be mediated via TLR2 but not via TLR4 [13]. Prior to determine the binding of Sm.LTA to TLR2 or TLR4, the expression of TLR2 and TLR4 on CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were analyzed by flow cytometry. Fig. 6 indicated that a significant expression of TLR2, but not TLR4, was observed on CHO/CD14/TLR2 cells, in contrast, a significant expression of TLR4, but not TLR2, was found on CHO/CD14/TLR4 cells. The expression ratio of TLR2 to TLR4 was 90:6 and 6:51 on CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, respectively (Fig. 6). To determine the binding of Sm.LTA to TLR2 or TLR4, CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were treated with biotinylated Sm.LTA followed by staining with streptavidin-FITC and the amount of Sm.LTA bound to the cells was assessed using flow cytometric analysis. Sm.LTA in the Sm.LTA-biotin retained its biological activity, as determined by NO and TNF-α assays (data not shown). Although Sm.LTA-biotin bound to both CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, it preferentially bound to CHO/CD14/TLR2 cells (Fig. 7). Furthermore, to determine if Sm.LTA could activate cells via TLR2 in the manner of other LTAs, CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were treated with Sm.LTA (0 or 30 μg/ml) for 24 h. Flow cytometric analysis demonstrated that an increase in CD25 expression occurred only on CHO/CD14/TLR2 cells treated with Sm.LTA, which was similar to the results obtained with the representative TLR2 ligand Pam_2CSK_4 (Fig. 8). Such expression was not observed in CHO/CD14/TLR4 cells under the same conditions, whereas *E. coli* LPS, which is a well-known TLR4-specific ligand, potently induced CD25

expression (Fig. 8). Since a previous study reported that CHO cells express endogenous hamster TLR4 [36], the endotoxin contamination was further confirmed using CHO/CD14/TLR2 cells whether Sm.LTA alters expression of CD25 on cell surface. As shown in Figs. 7 and 8, the Sm.LTA-induced expression of CD25 on CHO/CD14/TLR2 cells was not altered in the presence of polymyxin B, which completely inhibited *E. coli* LPS-induced CD25 expression on CHO/CD14/TLR4 cells. These results indicate that Sm.LTA was pure and that it activated cells via TLR2 rather than TLR4.

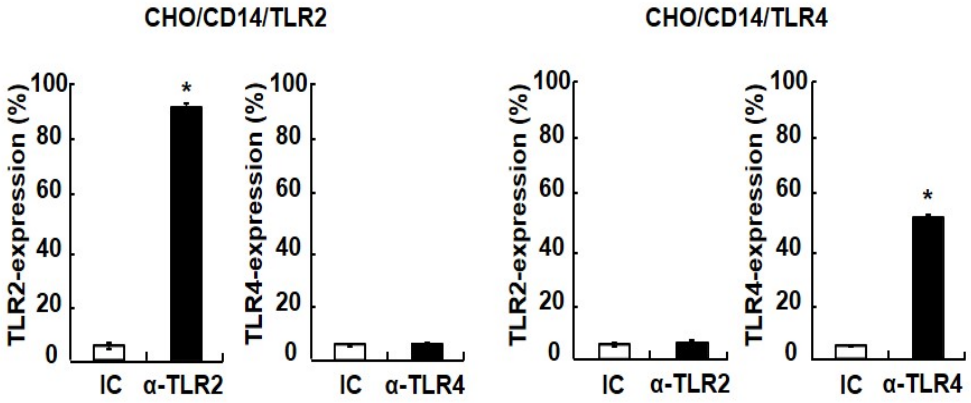

Figure 6. Expression of TLR2 and TLR4 on CHO reporter cells. CHO/CD14/TLR2 and CHO/CD14/TLR4 cells (2×10^5 cells/ml) were stained with anti-TLR2 or anti-TLR4 antibodies for 30 min at 4°C. The level of TLR2- and TLR4-expression were measured by flow cytometry. Data are means ± S.D. obtained from three independent experiments. An asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with the isotype control (IC)-treatment group.

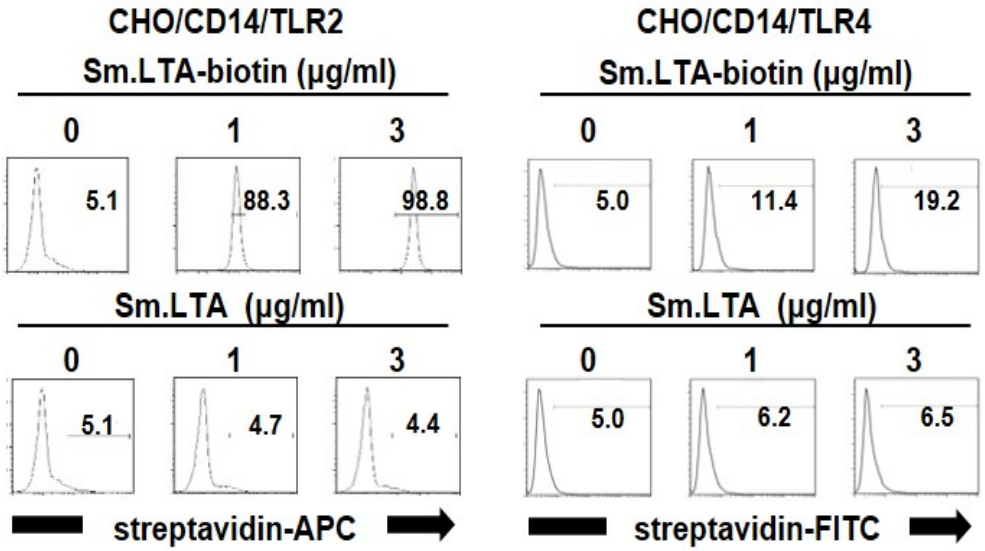

Figure 7. Sm.LTA preferentially binds to TLR2. CHO/CD14/TLR2 and CHO/CD14/TLR4 cells (2×10^5 cells/ml) were treated with Sm.LTA or Sm.LTA-biotin at 4°C for 30 min, followed by staining with streptavidin-APC and streptavidin-FITC, respectively. Binding activity was analyzed by flow cytometric analysis. One of three similar results is shown.

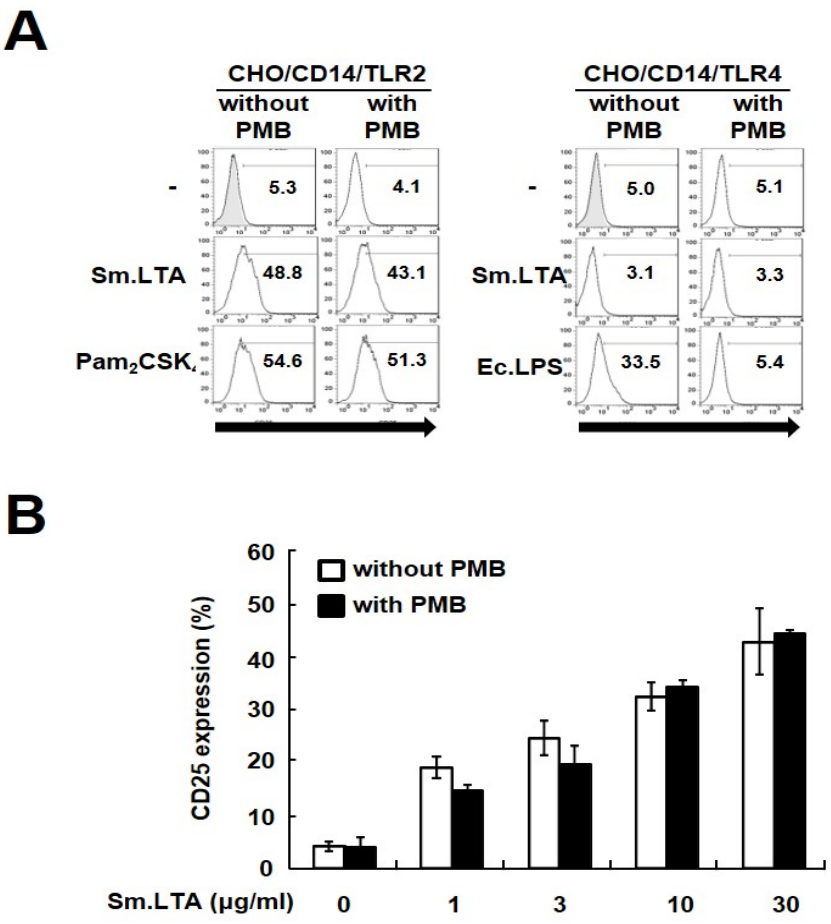

Figure 8. Sm.LTA activates TLR2 but not TLR4. (A) CHO/CD14/TLR2 and CHO/CD14/TLR4 cells at 3 × 10^5 cells/ml were treated with Sm.LTA (30 μg/ml), Pam_2CSK_4 (0.1 μg/ml), or *E. coli* LPS (Ec.LPS; 0.01 μg/ml). CD25 expression by TLR2- or TLR4-dependent NF-κB activation was analyzed using flow cytometric analysis. The percentage of CD25-positive cells is shown in the upper right of each histogram. One of three similar results is shown. (B) CHO/CD14/TLR2 cells were treated with Sm.LTA (0, 1, 3, 10, or 30 μg/ml) in the presence or absence of polymyxin B (PMB; 25 μg/ml) for 24 h. CD25 expression was determined by flow cytometry and mean values ± S.D. were obtained from three independent experiments.

3.3. TLR2 is necessary for the production of NO and TNF-α in macrophages induced by *S. mutans* LTA.

To further examine if TLR2 is necessary for the immunostimulating activity of Sm.LTA, bone marrow-derived macrophages from TLR2-deficient or wild-type mice were stimulated with various concentrations of Sm.LTA (0, 1, 3, 10, or 30 μg/ml) in the presence of IFN-γ (0.1 ng/ml) for 24 h, and the production of NO and TNF-α was measured. As shown in Fig. 9A and 9B, NO and TNF-α productions were not induced by Sm.LTA or Pam_2CSK_4 in TLR2-deficient macrophages, whereas NO and TNF-α were increased in wild-type macrophages under the same conditions. In order to further confirm whether Sm.LTA induced the inflammatory mediators via TLR2, NO production was determined in RAW 264.7 cells in the presence or absence of anti-TLR2 blocking antibody. Fig. 10 indicated that a significant reduction of NO production was observed in the presence of anti-TLR2 blocking antibody. As shown in Fig. 11, the whole bacteria of *S. mutans* induced remarkably increased NO production in a concentration-dependent manner in bone marrow-derived macrophages from wild-type mice. Although the increase was also observed in bone marrow-derived macrophages from TLR2-deficient mice, the levels were significantly ($P < 0.05$) less than those in macrophages from wild-type mice. These results suggest that TLR2 signaling pathways are essentially required for the immuno-stimulating activity of Sm.LTA.

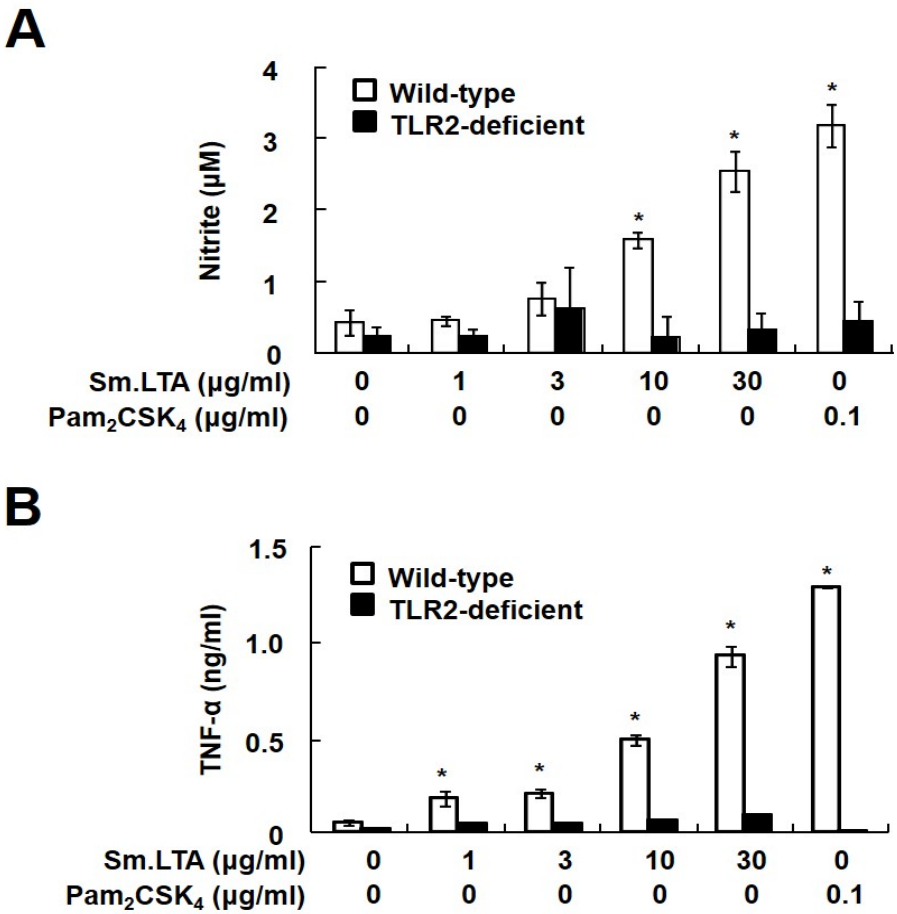

Figure 9. TLR2 deficiency is critical for the production of NO and TNF-α in macrophages stimulated with *S. mutans* LTA. Bone marrow-derived macrophages were prepared from wild-type and TLR2-deficient mice. Cells (1×10^6 cells/ml) were pretreated with IFN-γ (0.1 ng/ml) for 1 h followed by stimulation with Sm.LTA (0, 1, 3, 10 or 30 μg/ml) or Pam_2CSK_4 (0.1 μg/ml) for an additional 24 h. At the end of the culture period, the culture supernatants were collected for the analysis of (A) NO and (B) TNF-α production. Data are means ± S.D. and an asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with non-treatment groups (A and B). One of three similar results is shown.

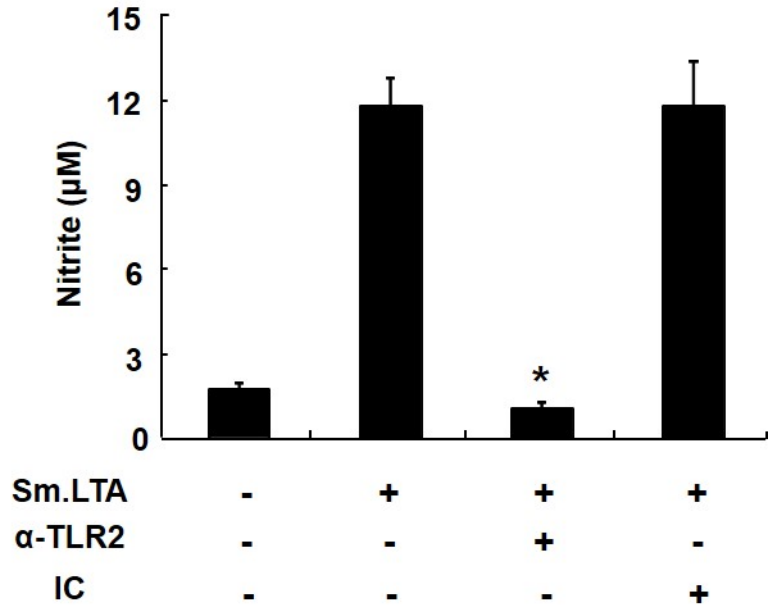

Figure 10. Blocking of TLR2 abrogates the production of NO. RAW 264.7 cells (1 × 10^6 cells/ml) were pre-treated with IFN-γ (0.1 ng/ml) and TLR2-blocking antibody (α-TLR2; 20 μg/ml) or isotype control (IC; 20 μg/ml) for 1 h followed by Sm.LTA treatment (30 μg/ml) for an additional 24 h and NO production was determined. Data are means ± S.D. and an asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with isotype control treatment group. One of three similar results is shown.

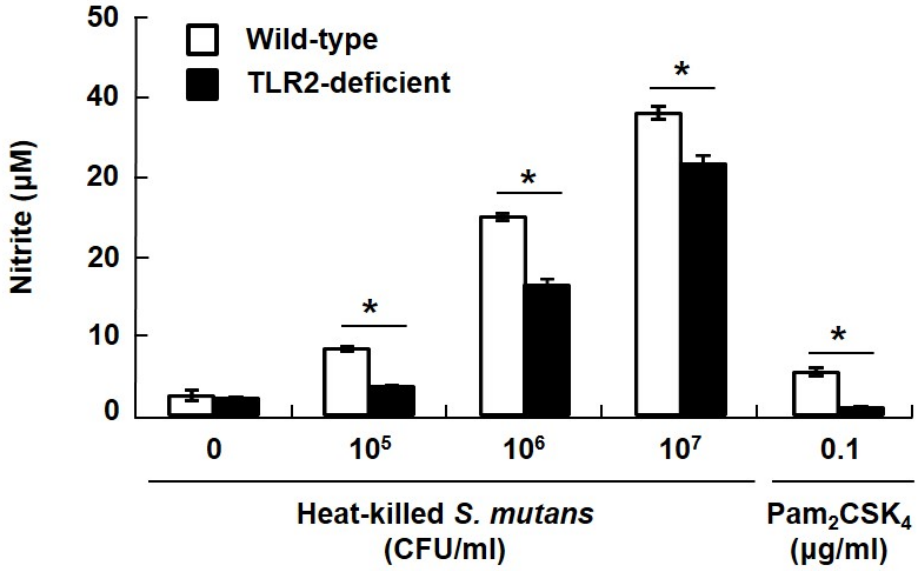

Figure 11. TLR2-deficiency alleviates the production of NO stimulated with heat-killed *S. mutans*. Bone marrow-derived macrophages (1×10^6 cells/ml) were treated with heat-killed *S. mutans* (10^5, 10^6 or 10^7 CFU/ml) or Pam_2CSK_4 (0.1 μg/ml) for 24 h. At the end of the culture, the supernatants were collected for the analysis of NO. Data are means ± S.D. and an asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with treatment group in wild-type. One of three similar results is shown.

3.4. The lipid moiety of *S. mutans* LTA is responsible for immuno-stimulating activity.

Accumulating reports suggest that the lipid moiety of LTA is important in TLR2 binding and downstream signaling [13, 17, 37]. In order to examine whether the lipid moiety of Sm.LTA is necessary for immunostimulating activity, Sm.LTA was deacylated and examined for its ability to activate TLR2 and to induce inflammatory mediators. As shown in Figs. 12, the deacylated LTA did not stimulate TLR2 or induce the production of NO or TNF-α. These results suggest that the lipid moiety of Sm.LTA is essential for immunostimulatory activity.

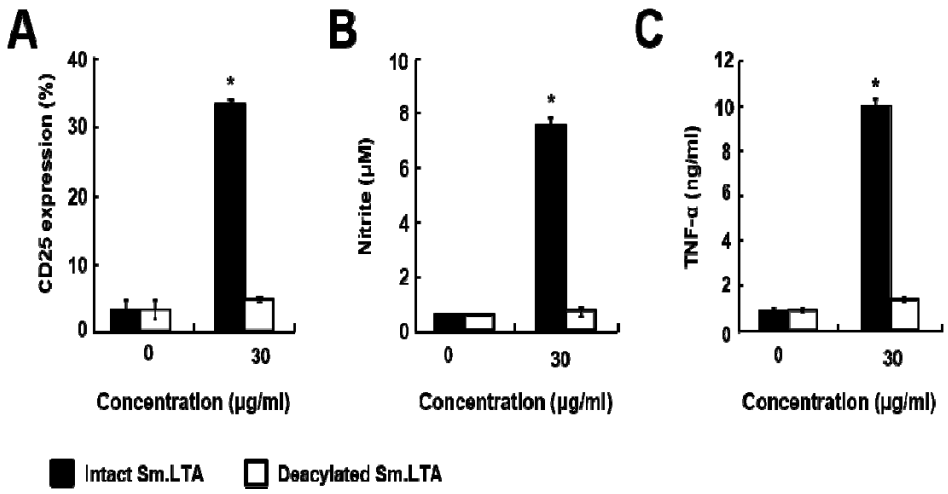

Figure 12. The lipid moiety of *S. mutans* LTA is essential for TLR2 binding and the induction of NO and TNF-α production. (A) CHO/CD14/TLR2 cells were treated with Sm.LTA (Intact Sm.LTA) or its deacylated form (Deacylated Sm.LTA) for 24 h. CD25 expression was determined by flow cytometry and the means ± S.D. were obtained from three independent experiments. (B and C) RAW 264.7 cells (1×10^6 cells/ml) were pretreated with IFN-γ (0.1 ng/ml) for 1 h followed by stimulation with Sm.LTA or its deacylated form for 24 h. At the end of the incubation period, culture supernatants were collected for the analysis of (B) NO and (C) TNF-α production. One of three similar results is shown. An asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with non-treatment group of Sm.LTA.

3.5. *S. mutans* LTA is comparable to LTAs of other oral bacteria in immuno-stimulatory potential.

Previous study reported that LTAs from different Gram-positive bacteria have differential immunostimulating potentials [17] and that some LTAs, including *L. plantarum* LTA, are unable to induce NO production alone but can stimulate with the addition of IFN-γ [35]. In order to compare immuno-stimulating potential, LTAs were purified from *S. aureus*, *E. faecalis*, *L. plantarum*, and *S. mutans* under the same conditions and were used to treat RAW 264.7 cells in the presence or absence of IFN-γ. As shown in Fig. 13, *S. aureus* LTA was able to dose-dependently induce NO production, even in the absence of IFN-γ. However, *E. faecalis* .LTA, *L. plantarum* LTA, and *S. mutans* LTA alone showed little NO production at concentrations up to 10 μg/ml. However, all LTAs showed a dose-dependent induction of NO production in the presence of IFN-γ, although Sa.LTA induced the highest NO production (Fig. 13B). These results suggest that Sm.LTA has a modest immunostimulatory potential comparable to those of the LTAs of other oral bacteria.

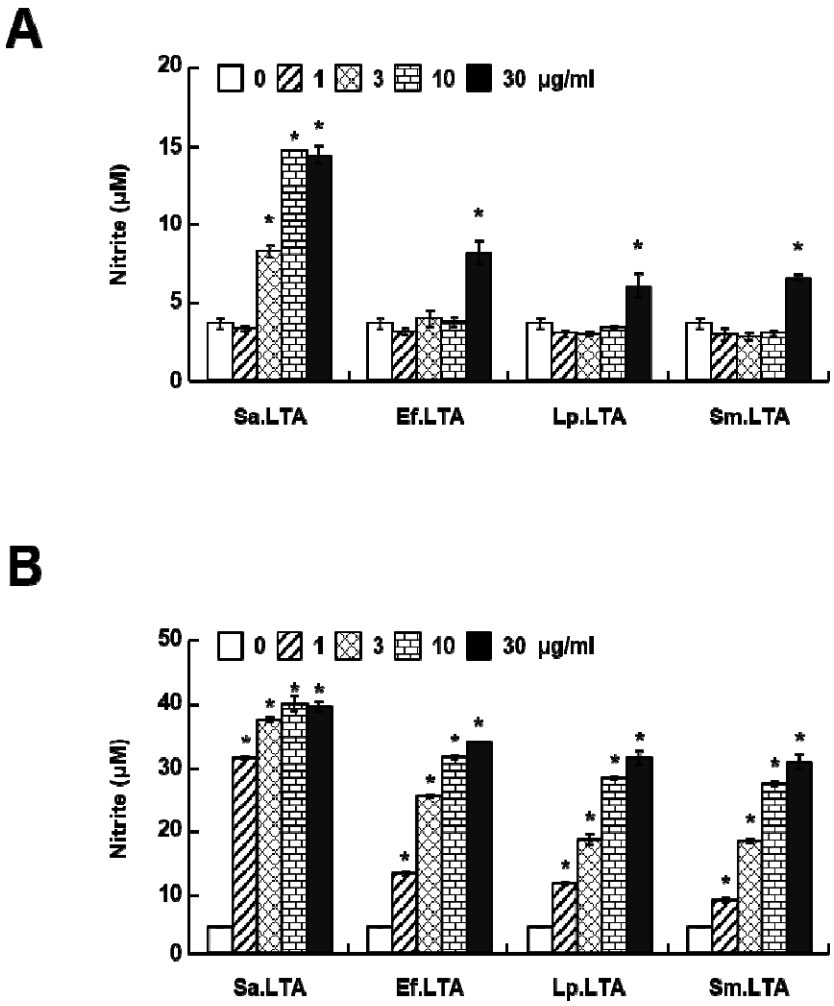

Figure 13. *S. mutans* LTA is comparable to LTAs of other oral bacteria in immuno-stimulating potential. RAW 264.7 cells (1×10^6 cells/ml) were stimulated with LTAs (0, 1, 3, 10, or 30 μg/ml) from *S. aureus* (Sa.LTA), *E. faecalis* (Ef.LTA), *L. plantarum* (Lp.LTA), or *S. mutans* (Sm.LTA) pretreated (A) without or (B) with 0.1 ng/ml IFN-γ for 24 h. At the end of the incubation period, the culture supernatants were collected and analyzed for NO production. Data are means ± S.D. and an asterisk (*) indicates experimental groups with statistical significant at $P < 0.05$ compared with non-treatment group of LTA. One of three similar results is shown.

3.6. Sm.LTA-conjugated beads were prepared by using NHS-Sepharose beads.

In prior to capturing Sm.LTA-binding proteins (Sm.LTA-BPs) in human saliva, Sm.LTA-beads were prepared. LTA purified from *S. mutans* was conjugated to NHS-beads, forming stable bonds with the primary amines of the D-alanine substituents of Sm.LTA. The phosphate assay revealed that one milligram of beads contained approximately 6 μg of Sm.LTA after the conjugation.

3.7. The conjugated Sm.LTA retained immunostimulatory activity similar to that of free Sm.LTA.

To examine whether Sm.LTA retained its immunostimulatory activity after conjugation, RAW 264.7 cells were stimulated with Sm.LTA-beads or native beads. The production of NO and IP-10 was significantly (P<0.05) increased by stimulation with Sm.LTA or Sm.LTA-beads, whereas no such an induction occurred upon treatment with native beads (Figs. 14A and 14B). To further confirm the ability of Sm.LTA-beads to activate TLR2, CHO/CD14/TLR2 cells were stimulated with Sm.LTA-beads and various control groups including beads, Sm.LTA, and Pam_2CSK_4, and TLR2-dependent CD25 expression was measured by flow cytometry. The induction of CD25 expression was observed upon treatment with Sm.LTA-beads, Sm.LTA, or Pam_2CSK_4, but not with native beads (Fig. 15). These results suggest that Sm.LTA-beads retained its TLR2 dependent-immunostimulatory activity.

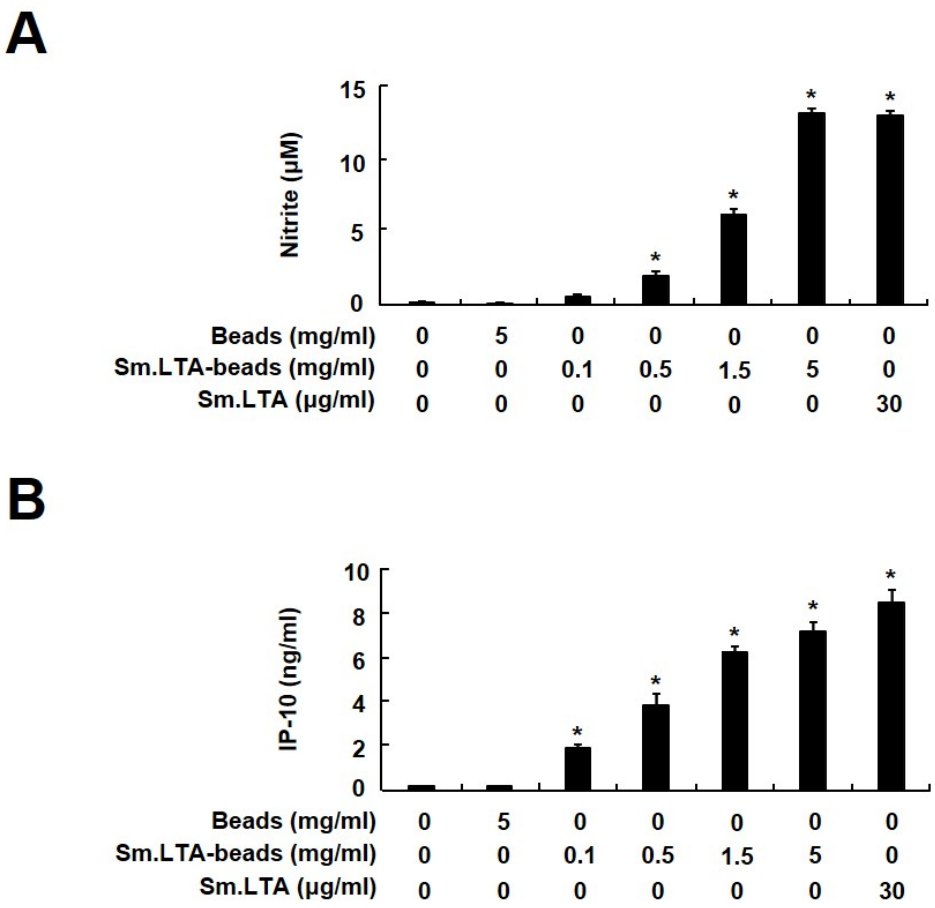

Figure 14. Conjugated Sm.LTA induces inflammatory mediators. RAW 264.7 cells (1×10^6 cells/ml) were pretreated with IFN-γ (0.1 ng/ml) for 1 h followed by stimulation with Sm.LTA-beads (0.15, 0.5, 1.5, or 5 mg/ml), control-beads (Beads; 5 mg/ml) or Sm.LTA (30 μg/ml) for 24 h. At the end of the incubation period, culture supernatants were obtained for the analysis of (A) NO and (B) IP-10 production. Data are means ± S.D. and an asterisk (*) indicates experimental groups with statistical significance at $P < 0.05$ compared with non-treatment group.

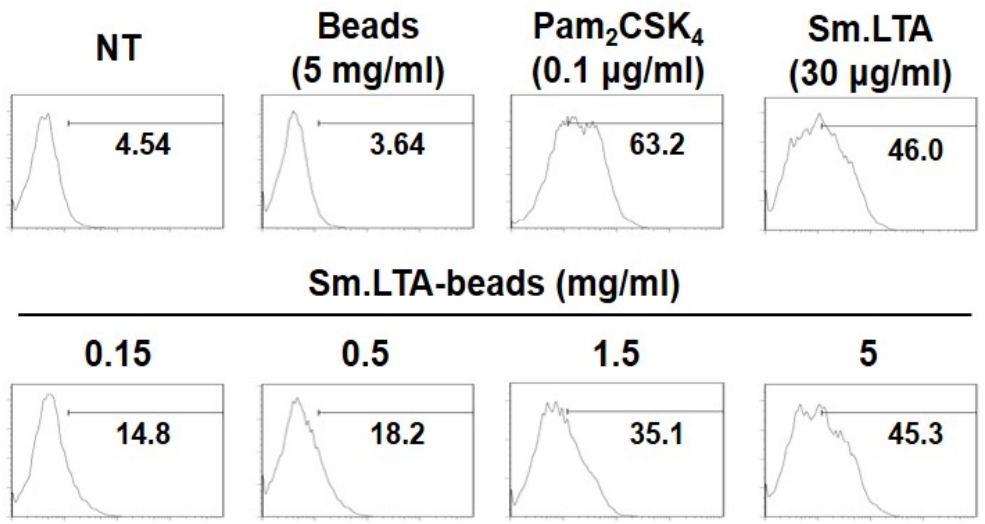

Figure 15. Conjugated Sm.LTA activates TLR2. Conjugated Sm.LTA stimulated cells via TLR2. CHO/CD14/TLR2 cells at 2×10^5 cells/ml were treated with Sm.LTA-beads (0.15, 0.5, 1.5, or 5 mg/ml), control-beads (Beads; 5 mg/ml), Sm.LTA (30 μg/ml) or Pam_2CSK_4 (0.1 μg/ml) for 24 h. After the incubation, beads and other stimuli removed by washing with PBS. CD25 expression by TLR2-dependent NF-κB activation was analyzed by flow cytometric analysis. Histogram shows the percentage of CD25-positive cells. One of three similar result is shown.

3.8. Protein profiles of caries-free vs. caries-positive saliva were modestly different.

To compare the protein contents of caries-free and caries-positive saliva, 10 μg of saliva from each subject was separated on 15% SDS-PAGE gel and stained with Coomassie blue to visualize the proteins. As shown in Fig. 14, a similar pattern of salivary contents among caries-free individuals was observed (Fig. 16A), however, relatively irregular and different protein profiles were found for each individual saliva sample of the caries-positive group (Fig. 16B). Remarkably, protein concentration in saliva was 1.7-fold higher in caries-positive subjects than in that from caries-free subjects.

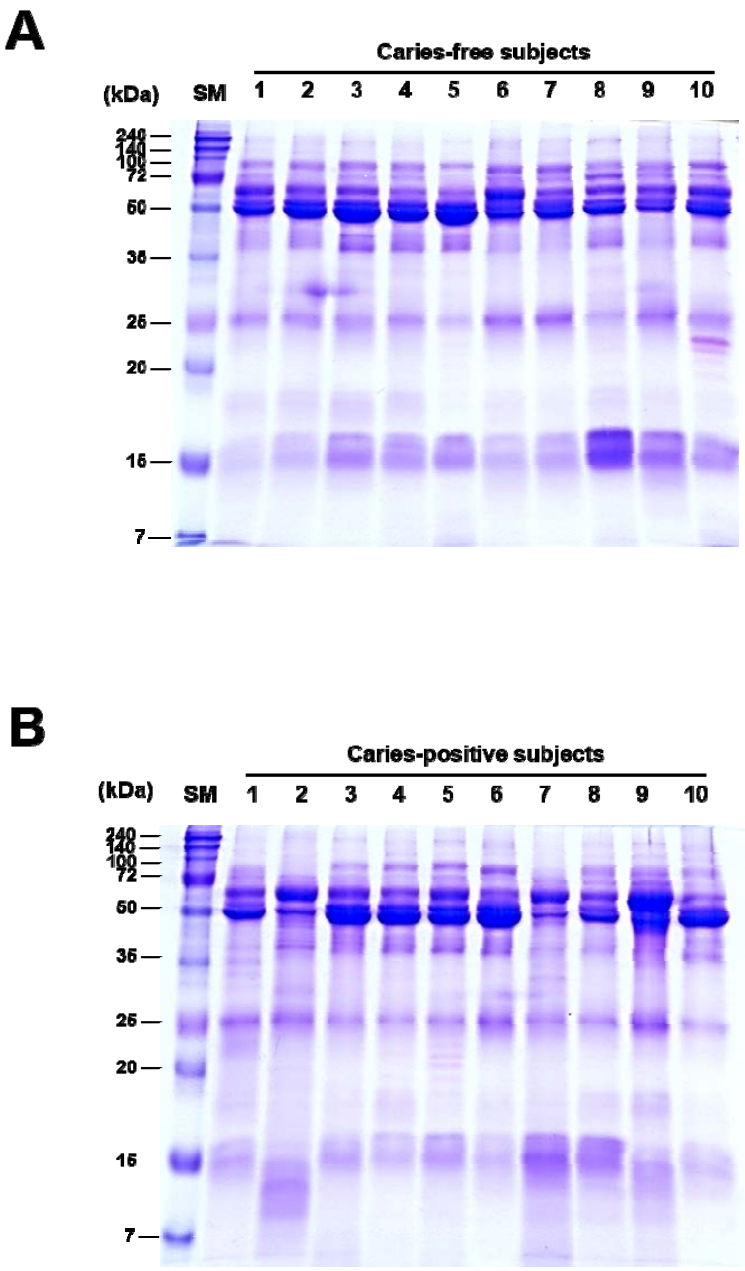

Fig. 16. The protein profiles in human saliva. Ten saliva samples (# 1-10) from caries-free and 10 samples (# 1-10) from caries-active subjects were load into 15% SDS-PAGE gels and separated by electrophoresis. Separated proteins were visualized by coomassie blue staining. SM; size marker.

3.9. Sm.LTA-BP profiles differ between caries-free and caries-positive saliva.

In order to isolate Sm.LTA-BPs, saliva from ten individuals each from the caries-free and the caries-positive group were pooled by group, and non-specifically binding proteins were removed by pre-incubation with native beads. After precipitating the Sm.LTA-BPs from each salivary pool, the Sm.LTA-BPs were separated by 15% SDS-PAGE and visualized by silver staining to see the distinct bands from each salivary pool (Fig. 17). Then, Sm.LTA-BPs in the whole lanes containing visible and non-visible bands were identified using LTQ-Orbitrap hybrid Fourier transform mass spectrometry. From the independently performed experiments, a total 77 and 57 proteins with statically significant ($P < 0.05$) were initially identified in the saliva pool from caries-free and carries-positive subjects, respectively. Among these proteins, 8 and 12 proteins that repetitively identified were selected and considered as Sm.LTA-BPs in caries-free (Table 1) and caries positive saliva (Table 2), respectively. Sm.LTA-BPs found in caries-free saliva included histone H4, profilin-1, and neutrophil defensin-1, and those in caries-positive saliva included cystatin-C, cystatin-SN, cystatin-S, cystatin-D, lysozyme C, calmodulin-like protein 3, and β-actin. The Sm.LTA-BPs found in both groups were hemoglobin subunits α and β, prolactin-inducible protein, protein S100-A9, and short palate, lung and nasal epithelium clone 2 (SPLUNC2).

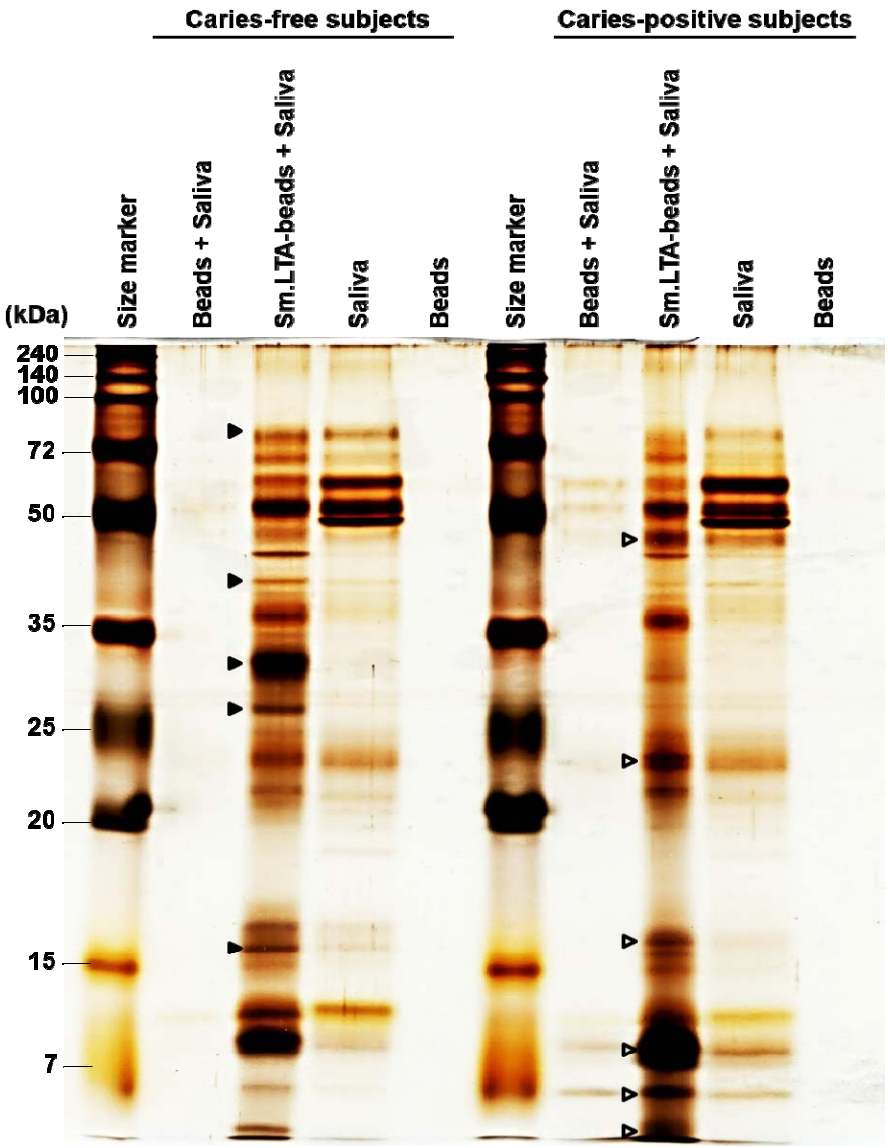

Figure 17. The protein profiles in Sm.LTA-binding proteins. Eluted samples from Sm.LTA-beads or control-beads (Beads) were subjected into SDS-PAGE and visualized using silver staining. One of three similar results is shown. The filled triangle (►) indicates protein bands increased in caries-free samples compared with caries-active samples. The arrow (▷) indicates protein bands increased in caries-active samples compared with caries-free samples.

Table 1. Sm.LTA-BPs in caries-free saliva identified by high-resolution LTQ-Orbitrap hybrid Fourier transform mass spectrometry.

Protein description (Gene symbol)	Accession number	Peptide sequence	Peptide score
Hemoglobin subunit beta (HBB)	IPI00654755	VLGAFSDGLAHLDNLK	98.16
		VNVDEVGGEALGR	94.26
		SAVTALWGKVNVDEVGGEALGR	84.98
		VVAGVANALAHKYH	84.54
		FFESFGDLSTPDAVMGNPK	82.84
		VVAGVANALAHKYH	55.67
		EFTPPVQAAYQK	55.60
		SAVTALWGK	55.29
		LLVVYPWTQR	52.38
		EFTPPVQAAYQK	39.72
		LHVDPENFR	36.29
Prolactin-inducible protein (PIP)	IPI00022974	TYLISSIPLQGAFNYK	79.21
		ELGICPDDAAVIPIK	71.01
		FYTIEILKVE	65.81
		IIIKNFDIPK	54.44
Hemoglobin subunit alpha (HBA2;HBA1)	IPI00410714	VGAHAGEYGAEALER	82.57
		TYFPHFDLSHGSAQVK	67.22
		MFLSFPTTK	44.90
		VLSPADKTNVK	43.63
Histone H4 (HIST2H4B;HIST1H4C;HIST1H4I; HIST1H4E;HIST1H4F;HIST1H4H)	IPI00453473	ISGLIYEETR	74.56
		VFLENVIR	48.13
		TLYGFGG	43.13
Protein S100-A9 (S100A9)	IPI00027462	NIETIINTFHQYSVK	63.42
		LGHPDTLNQGEFK	52.34
		KDLQNFLK	44.92
Profilin-1 (PFN1)	IPI00216691	DSLLQDGEFSMDLR	83.48
		TFVNITPAEVGVLVGKDR	40.12
Neutrophil defensin-1 (DEFA1;DEFA1B)	IPI00005721	IPACIAGER	66.39
		YGTCIYQGR	44.20
Short palate, lung and nasal epithelium carcinoma-associated protein 2 (BPIFA2, SPLUNC2)	IPI00304557	LKVDLGVLQK	54.61
		LLPTNTDIFGLK	51.95
		LLNNVISK	42.35

Table 2. Sm.LTA-BPs in caries-active saliva identified by high-resolution LTQ-Orbitrap hybrid Fourier transform mass spectrometry.

Protein description (Gene symbol)	Accession number	Peptide sequence	Peptide score
Prolactin-inducible protein (PIP)	IPI00022974	TYLISSIPLQGAFNYK	90.85
		TVQIAAVVDVIR	83.59
		ELGICPDDAAVIPIK	68.51
		FYTIEILKVE	65.62
		TFYWDFYTNR	55.73
		FYTIEILK	48.07
		IIIKNFDIPK	47.91
Hemoglobin subunit beta (HBB)	IPI00654755	VNVDEVGGEALGR	94.22
		VLGAFSDGLAHLDNLK	86.96
		SAVTALWGKVNVDEVGGEALGR	69.36
		VVAGVANALAHKYH	55.95
Short palate, lung and nasal epithelium carcinoma-associated protein 2 (BPIFA2, SPLUNC2)	IPI00304557	ISNSLILDVK	82.80
		AQEAEKLLNNVISK	80.00
		STVSSLLQK	58.00
		LKVDLGVLQK	56.56
		LLPTNTDIFGLK	54.31
		FVNSVINTLK	53.33
		VDLGVLQK	43.16
		LLNNVISK	42.07
		TQLQTLI	37.40
Cystatin-C (CST3)	IPI00032293	LVGGPMDASVEEEGVR	111.73
		ALDFAVGEYNK	61.50
		RALDFAVGEYNK	51.47
Cystatin-SN (CST1)	IPI00305477	IIPGGIYNADLNDEWVQR	87.30
		SQPNLDTCAFHEQPELQK	77.60
		EEDRIIPGGIYNADLNDEWVQR	65.36
		ALHFAISEYNK	58.24
		QQTVGGVNYFFDVEVGR	49.30
		ARQQTVGGVNYFFDVEVGR	48.26
Hemoglobin subunit alpha (HBA2;HBA1)	IPI00410714	VGAHAGEYGAEALER	80.84
		TYFPHFDLSHGSAQVK	70.13
		MFLSFPTTK	48.22
Cystatin-S (CST4)	IPI00032294	SQPNLDTCAFHEQPELQK	64.67
		EENRIIPGGIYDADLNDEWVQR	60.76
		IIPGGIYDADLNDEWVQR	68.51
		ALHFAISEYNK	58.24
Lysozyme C (LYZ)	IPI00019038	STDYGIFQINSR	85.52
		STDYGIFQINSR	82.38
		RLGMDGYR	52.25
		ATNYNAGDRSTDYGIFQINSR	46.00
		LGMDGYR	37.20
		QYVQGCGV	36.84
Cystatin-D (CST5)	IPI00002851	TLAGGIHATDLNDK	105.36
		SQPNLDNCPFNDQPK	61.94
Calmodulin-like protein 3 (CALML3)	IPI00216984	AADTDGDGQVNYEEFVR	85.86
		LSDEEVDEMIR	58.63
		SLGQNPTEAELR	48.10
		VFDKDGNGFVSAAELR	41.58
Actin, cytoplasmic 1 (ACTB)	IPI00021439	SYELPDGQVITIGNER	100.04
		VAPEEHPVLLTEAPLNPK	51.31
		LDLAGRDLTDYLMK	36.18
		QEYDESGPSIVHR	47.90
Protein S100-A9 (S100A9)	IPI00027462	NIETIINTFHQYSVK	76.89
		LGHPDTLNQGEFK	53.68

4. DISCUSSION

In order to develop effective preventives or therapeutics for dental caries and subsequent pulpitis, it is important to identify bacterial factor(s) capable of inducing inflammatory mediators and to understand the mechanisms at the molecular level. Previously, a commercial LTA purified from *S. mutans* was reported to induce TNF-α and NO production in RAW 264.7 cells [38], as these mediators are known to be closely associated with inflammatory responses [39, 40]. However, the reproducibility of this report has been in doubt since polymyxin B inhibited, at least partially, the production of TNF-α and NO in cells stimulated with Sm.LTA [38]. Contamination of LTAs by endotoxins due to the use of improper purification methods was also reported [15]. Hence, the need for pure LTA has become a prerequisite to addressing questions regarding the role of LTA in bacterial pathogenesis and host immunity. In this study, Sm.LTA was successfully obtained as high-pure and it able to induce the expression of inflammatory mediators. These findings concluded that the obtained Sm.LTA was in high-purity as (i) the Sm.LTA preparation had no detectable contamination with endotoxin or other biologically-active impurities such as proteins or nucleic acids, (ii) polymyxin B did not interfere with the production of TNF-α and NO induced by Sm.LTA, (iii) the Sm.LTA failed to stimulate TLR4, which specifically responds to endotoxin, and (iv) the induction of TNF-α and NO by Sm.LTA did not occur in TLR2-deficienct macrophages or at deacylation of Sm.LTA.

Since Sm.LTA directly interacted with TLR2 to produce TNF-α and NO, which did not occur in TLR2-deficient macrophages, TLR2 appears to be crucial for Sm.LTA-induced innate immunity. The need for TLR2 might be a typical characteristic of LTA,

as such TLR2-dependency has been identified not only with Sm.LTA but also with LTAs from other Gram-positive bacteria, including *S. aureus*, *B. subtilis* [41], *E. faecalis* [42], and *S. pneumoniae* [43]. Recently reported study showed that staphylococcal LTA [44] and pneumococcal LTA [37] directly bind to TLR2. Notably, the lipid moiety of pneumococcal LTA anchors into a pocket in TLR2 [37]. Deacylated LTA neither stimulated TLR2 activation nor induced the production of pro-inflammatory mediators [13, 17, 45]. In agreement with the previous studies, present study also characterized that the chemical deacylation of Sm.LTA with alkaline hydrolysis failed to activate TLR2 and to induce NO and TNF-α, indicating that the acyl chain of Sm.LTA is critical for the induction of inflammatory mediators.

The immuno-stimulating activity of Sm.LTA appears to be modest, and is comparable to the activity of Ef.LTA and Lp.LTA but is less potent than that of Sa.LTA. The differential immuno-stimulating potentials of LTAs might be due to subtle differences in their molecular structures. For example, LTAs possessing unsaturated fatty acids seem to be weaker in immuno-stimulatory activity than LTAs with saturated fatty acids. Indeed, Lp.LTA, which has very weak immuno-stimulating potential, has a unique structure of unsaturated fatty acid glycolipids instead of the saturated diacyl chains that are present in normal type I LTAs [46]. Moreover, pneumococcal LTA, which has unsaturated fatty acids, is 100 times less potent in terms of immuno-stimulating activity than staphylococcal LTA, which has saturated fatty acids [13]. This may be because LTAs with saturated fatty acids easily interact with membrane lipid rafts, which are enriched with highly saturated fatty acids [47], and are therefore more likely to be involved in the initiation of TLR2 signaling [48]. On the other hand,

LTAs with a lower number of repeating units and lower D-alanine content tend to be less potent in terms of immuno-stimulatory activity [14]. Nevertheless, the precise composition and structure of Sm.LTA remains to be characterized.

Such data in the present study further provide specific and additive information from the previous finding [35] for LTA-induced inflammatory responses in murine macrophages. And present study supported following results that (i) Sm.LTA directly binds to TLR2. (ii) The inflammatory responses are mediated by direct Sm.LTA-TLR2 interaction. (iii) The lipid moiety of Sm.LTA is a critical factor to mediate the inflammatory responses through TLR2 binding and activation. Therefore, the present study further elucidates that Sm.LTA, as a TLR2 ligand, induced inflammatory responses in macrophages and the lipid moiety of Sm.LTA is critical for the binding and activation of macrophages in the presence of IFN-γ.

Although Sm.LTA alone showed relatively weak induction of NO in macrophages, the presence of IFN-γ remarkably enhanced induction by Sm.LTA. These results provide an important clue to understanding the pathogenic mechanism of pulpitis. At the site of a pupal inflammation, a large number of immune cells infiltrate into the inflamed pulp [49] and IFN-γ levels are remarkably elevated [5]. It has been shown that *S. mutans* acts as a good inducer of IFN-γ in natural killer cells in an IL-12-dependant manner [50]. The production of NO is known to be concomitantly increased in the inflamed dental pulp compared to the normal conditions [51]. Therefore, such findings suggest that the concerted action of LTA and IFN-γ might be important in the NO production that leads to pulp inflammation and tissue damage.

In the initiation and progression of dental caries, the interaction of salivary proteins with *S. mutans* or its virulence factor plays a crucial role in either host immune responses or bacterial pathogenesis. Thus, the identification of Sm.LTA-BPs in the saliva is important for understanding the pathogenesis of dental caries and the host defense mechanisms and further developing preventives and therapeutics against dental caries. Therefore, present study identified several proteins as Sm.LTA-BPs and categorized into three types which are related with caries-free, caries-active, or both human subjects (Table. 3). These results implies that (i) the conjugated Sm.LTA retained their immunostimulating activity, (ii) the protein profile of saliva from caries-free and caries-positive are differ, and (iii) the identified Sm.LTA-BPs were comparable between caries-free and caries-positive.

Histone H4, profilin-1, and neutrophil defensin-1 were identified in large quantity in the saliva from caries-free subjects suggesting that they are likely to be involved in antimicrobial and host defense. Histone, an important component of chromatin, can directly bind to bacterial virulence factors LTA and LPS resulting in the disruption of bacterial membrane and the reduction of TNF-α and NO production, respectively [52, 53]. Neutrophil defensin-1 is a member of alpha-defensin which has anti-microbial function to broad spectrum of microorganisms such as bacteria, fungi and enveloped viruses by disrupting the negative charged membranes of microbes [54]. Profilin-1 is a small ubiquitous protein involved in actin polymerization [55]. Upon the infection with *Listeria monocytogenes*, the profilin-1 contributes to the host defense by interfering bacterial motility in the cytosol due to its affinity to bacterial surface proteins [56].

On the other hand, cystatins, lysozyme C, calmodulin-like protein 3, and actin were identified in the saliva from caries-active subjects. These caries-active-related proteins are likely associated with antimicrobial activity and cytoskeletal modulation. Cystatins, a cysteine protease inhibitor, which have antimicrobial effect against oral pathogens such as *A. actinomycetemcomitants* and *P. gingivalis* leading to a decrease in their colonization [29]. And it is also involved in the formation of acquired pellicle by interacting with hydroxyapatite [57]. Lysozyme C hydrolyzing bacterial peptidoglycan also has an anti-inflammatory activity against LPS by inducing the conformational change of LPS [58]. Actin is a major cytoskeletal protein composing actin filaments. It has been reported that the interaction of actin with LTA regulates bacterial entry into cytosol [59]. Calmodulin-like protein-3 is an epithelial-specific protein which is involved in migration of the cells by stabilizing the myosin-10 in oral epithelial cells contributing to wound healing [60].

Hemoglobin subunit α and β, protein S100-A9, SPLUNC2, and prolactin-inducible protein were found in both caries-free and caries-active saliva. Hemoglobin mainly exists in the blood but also possible to exist in the oral cavity at leakage of gingival crevicular fluid or oral wound [61, 62]. Hemoglobin is known to synergistically up-regulate the immuno-stimulating activity of LTA of various Gram-positive bacteria by forming complex with LTA in macrophage and monocyte [63]. In addition, its bactericidal activity against Gram-negative bacteria such as *Escherichia* and *Salmonella* species was also reported [64]. Protein S100-A9 is an immuno-modulative protein frequently found in cytoplasm and/or nucleus of a wide range of cells and the heterodimeric complex of protein S100-A9/S100-A8 has affinity to hydrophobic

molecules [65]. Recently, protein S100-A9 has been known as TLR4 agonist leading to inflammation via nuclear factor-kappa B (NF-κB) activation [66]. The SPLUNC2 belongs to a member of bactericidal/permeability-increasing proteins and LPS-binding protein (LBP) exhibiting anti-inflammatory, antimicrobial, and endotoxin neutralizing activity [67]. Prolactin-inducible protein is known as an LBP capable of binding to many oral bacteria including *A. actinomycetemcomitans* and *P. gingivalis* [68] and *Gemella haemolysans*, *Gemella morbillorium*, *Streptococcus acidominimus*, *Streptococcus oralis*, *Streptococcus salivarius*, and *Streptococcus parasanguinis* [69] that may play a role in blockage of bacterial adhesion. Therefore, Hemoglobin, protein S100-A9, SPLUNC2, and prolactin-inducible protein are supposed to contribute the host innate immunity in the oral cavity.

In conclusion, these results suggest that Sm.LTA has inflammatory potential through the stimulation of the TLR2 signaling pathway and that the lipid moiety of Sm.LTA plays a crucial role in this mechanism. And the Sm.LTA-BPs were identified and compared that which salivary proteins interact with LTA of *S. mutans* responsible for dental caries using the saliva from caries-free and caries-positive subjects (Fig. 18). Based on this mechanism, accumulating data suggests that therapeutic use by interfering with the Sm.LTA-TLR2 interaction should be developed. For instance, the development of a decoy receptor for TLR2 could be a good therapeutic candidate, as demonstrated by the ability of a TLR4 decoy receptor to block LPS-TLR4 binding [70]. On the other hand, the deacylation of Sm.LTA could also be effective for the prevention and/or treatment of dental caries and subsequent pulpitis, as calcium hydroxide inactivates Ef.LTA by delipidation [45]. Moreover, saliva is important to

maintain the homeostatic regulation of oral environment, salivary proteins interacting with microbial virulence factors might play a crucial role in the host innate immunity. The Sm.LTA-BP profiles might provide important clues to better understand the molecular interaction between the host and *S. mutans* during progress of dental caries in the oral cavity.

Table 3. Classification of Sm.LTA-BPs

State	Protein name	Functions	References
Caries-free	Histone H4	Binds with *S. aureus* LTA and disrupts bacterial membranes	[48]
	Profilin-1	Interferes bacterial motility by interacting with actin monomer	[52]
	Neutrophil defensin-1	Disrupts microorganism by interacting negative charged membrane	[50]
Caries-positive	Cystatins	Binds to hydroxyapatite, antimicrobial effect to *A. actinomycetencomitants*	[25, 71]
	Lysozyme C	Inhibits immuno-stimulating activity of LPS, hydrolyzes peptidoglycan,	[72]
	Calmodulin-like protein 3	Involves in cell migration, epithelial specific protein,	[60]
	Actin	Interacts with LTA further interferes bacterial entry	[59]
Both caries-free and -positive	Hemoglobin	Interacts with LTA, synergistically activates TLR2 and TLR4	[63, 73]
	Prolactin-inducible protein	Binds to various oral bacteria and it is likely to inhibits bacterial colonization	[69]
	Protein S100-A9	Regulates immune response via TLR4 and NF-κB pathway	[66]
	SPLUNC2	Shows antimicrobial and anti-inflammatory functions by interacting with LPS	[74]

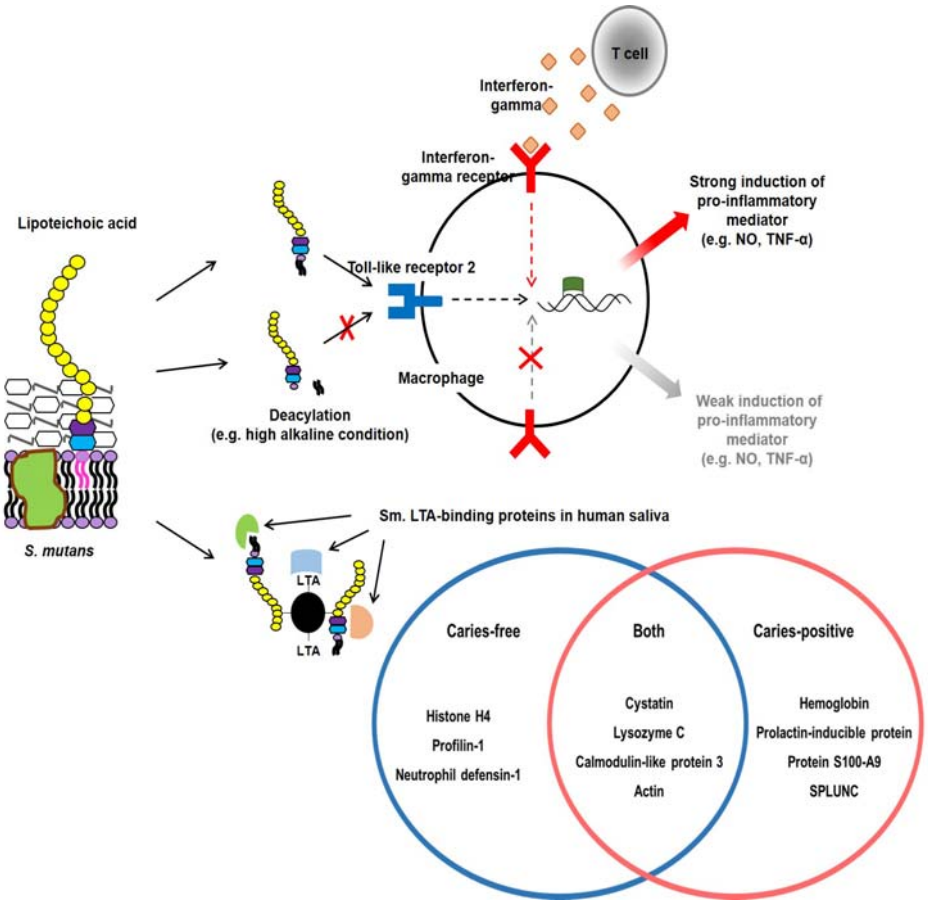

Figure 18. Proposed mechanism of immune response by Sm.LTA and its binding proteins. Sm.LTA stimulates macrophages to induce inflammatory mediators through TLR2, which can be enhanced in the presence of IFN-γ. The lipid moiety is crucial for the LTA-induced inflammatory mediators. Sm.LTA interacts with various salivary proteins that can be classified by the existence of dental caries.

5. REFERENCES

1. Struzycka, I. (2014) **The oral microbiome in dental caries**. *Pol J Microbiol* 63, 127-35
2. Catalan, M. A., Scott-Anne, K., Klein, M. I., Koo, H., Bowen, W. H., and Melvin, J. E. (2011) **Elevated incidence of dental caries in a mouse model of cystic fibrosis**. *PLoS One* 6, e16549
3. Klinke, T., Kneist, S., de Soet, J. J., Kuhlisch, E., Mauersberger, S., Forster, A., and Klimm, W. (2009) **Acid production by oral strains of *Candida albicans* and lactobacilli**. *Caries Res* 43, 83-91
4. Hamada, S. and Slade, H. D. (1980) **Biology, immunology, and cariogenicity of *Streptococcus mutans***. *Microbiol Rev* 44, 331-84
5. Hahn, C. L., Best, A. M., and Tew, J. G. (2000) **Cytokine induction by *Streptococcus mutans* and pulpal pathogenesis**. *Infect Immun* 68, 6785-9
6. Mitchell, T. J. (2003) **The pathogenesis of streptococcal infections: from tooth decay to meningitis**. *Nat Rev Microbiol* 1, 219-30
7. Ciardi, J. E., Rolla, G., Bowen, W. H., and Reilly, J. A. (1977) **Adsorption of *Streptococcus mutans* lipoteichoic acid to hydroxyapatite**. *Scand J Dent Res* 85, 387-91
8. Ginsburg, I. (2002) **Role of lipoteichoic acid in infection and inflammation**. *Lancet Infect Dis* 2, 171-9
9. Wicken, A. J. and Knox, K. W. (1975) **Lipoteichoic acids: a new class of bacterial antigen**. *Science* 187, 1161-7
10. Hermann, C., Spreitzer, I., Schroder, N. W., Morath, S., Lehner, M. D.,

Fischer, W., Schutt, C., Schumann, R. R., and Hartung, T. (2002) **Cytokine induction by purified lipoteichoic acids from various bacterial species--role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release.** *Eur J Immunol* 32, 541-51

11. Fischer, W. (1997) **Pneumococcal lipoteichoic and teichoic acid.** *Microb Drug Resist* 3, 309-25

12. Percy, M. G. and Grundling, A. (2014) **Lipoteichoic acid synthesis and function in gram-positive bacteria.** *Annu Rev Microbiol* 68, 81-100

13. Han, S. H., Kim, J. H., Martin, M., Michalek, S. M., and Nahm, M. H. (2003) **Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2.** *Infect Immun* 71, 5541-8

14. Morath, S., Geyer, A., Spreitzer, I., Hermann, C., and Hartung, T. (2002) **Structural decomposition and heterogeneity of commercial lipoteichoic Acid preparations.** *Infect Immun* 70, 938-44

15. Gao, J. J., Xue, Q., Zuvanich, E. G., Haghi, K. R., and Morrison, D. C. (2001) **Commercial preparations of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages in vitro.** *Infect Immun* 69, 751-7

16. Morath, S., Geyer, A., and Hartung, T. (2001) **Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*.** *J Exp Med* 193, 393-7

17. Ryu, Y. H., Baik, J. E., Yang, J. S., Kang, S. S., Im, J., Yun, C. H., Kim, D. W., Lee, K., Chung, D. K., Ju, H. R., and Han, S. H. (2009) **Differential immunostimulatory effects of Gram-positive bacteria due to their**

lipoteichoic acids. *Int Immunopharmacol* 9, 127-33

18. Lynch, N. J., Roscher, S., Hartung, T., Morath, S., Matsushita, M., Maennel, D. N., Kuraya, M., Fujita, T., and Schwaeble, W. J. (2004) **L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement**. *J Immunol* 172, 1198-202

19. Polotsky, V. Y., Fischer, W., Ezekowitz, R. A., and Joiner, K. A. (1996) **Interactions of human mannose-binding protein with lipoteichoic acids**. *Infect Immun* 64, 380-3

20. Schroder, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gobel, U. B., Weber, J. R., and Schumann, R. R. (2003) **Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved**. *J Biol Chem* 278, 15587-94

21. Bennick, A., Chau, G., Goodlin, R., Abrams, S., Tustian, D., and Madapallimattam, G. (1983) **The role of human salivary acidic proline-rich proteins in the formation of acquired dental pellicle in vivo and their fate after adsorption to the human enamel surface.** *Arch Oral Biol* 28, 19-27

22. Lenander-Lumikari, M. and Loimaranta, V. (2000) **Saliva and dental caries**. *Adv Dent Res* 14, 40-7

23. Henskens, Y. M., van den Keijbus, P. A., Veerman, E. C., Van der Weijden, G. A., Timmerman, M. F., Snoek, C. M., Van der Velden, U., and Nieuw Amerongen, A. V. (1996) **Protein composition of whole and parotid saliva**

in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J Periodontal Res* 31, 57-65

24. Jessie, K., Jayapalan, J. J., Ong, K. C., Abdul Rahim, Z. H., Zain, R. M., Wong, K. T., and Hashim, O. H. (2013) **Aberrant proteins in the saliva of patients with oral squamous cell carcinoma.** *Electrophoresis* 34, 2495-502

25. Koscielniak, D., Jurczak, A., Zygmunt, A., and Krzysciak, W. (2012) **Salivary proteins in health and disease.** *Acta Biochim Pol* 59, 451-7

26. Kuriakose, S., Sundaresan, C., Mathai, V., Khosla, E., and Gaffoor, F. M. (2013) **A comparative study of salivary buffering capacity, flow rate, resting pH, and salivary Immunoglobulin A in children with rampant caries and caries-resistant children.** *J Indian Soc Pedod Prev Dent* 31, 69-73

27. Vitorino, R., de Morais Guedes, S., Ferreira, R., Lobo, M. J., Duarte, J., Ferrer-Correia, A. J., Tomer, K. B., Domingues, P. M., and Amado, F. M. (2006) **Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility.** *Eur J Oral Sci* 114, 147-53

28. Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., and Aderem, A. (2004) **Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron.** *Nature* 432, 917-21

29. Ganeshnarayan, K., Velliyagounder, K., Furgang, D., and Fine, D. H. (2012) **Human salivary cystatin SA exhibits antimicrobial effect against *Aggregatibacter actinomycetemcomitans*.** *J Periodontal Res* 47, 661-73

30. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and

Tannenbaum, S. R. (1982) **Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids.** *Anal Biochem* 126, 131-8

31. Medvedev, A. E., Henneke, P., Schromm, A., Lien, E., Ingalls, R., Fenton, M. J., Golenbock, D. T., and Vogel, S. N. (2001) **Induction of tolerance to lipopolysaccharide and mycobacterial components in chinese hamster ovary/CD14 cells is not affected by overexpression of Toll-like receptors 2 or 4.** *J Immunol* 167, 2257-67

32. Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) **Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides.** *Anal Biochem* 34, 595-8

33. Choi, S., Baik, J. E., Jeon, J. H., Cho, K., Seo, D. G., Kum, K. Y., Yun, C. H., and Han, S. H. (2011) **Identification of *Porphyromonas gingivalis* lipopolysaccharide-binding proteins in human saliva.** *Mol Immunol* 48, 2207-13

34. Zidek, Z., Kmonickova, E., Kostecka, P., and Jansa, P. (2013) **Microfiltration method of removal of bacterial contaminants and their monitoring by nitric oxide and *Limulus* assays.** *Nitric Oxide* 28, 1-7

35. Kang, S. S., Ryu, Y. H., Baik, J. E., Yun, C. H., Lee, K., Chung, D. K., and Han, S. H. (2011) **Lipoteichoic acid from *Lactobacillus plantarum* induces nitric oxide production in the presence of interferon-gamma in murine macrophages.** *Mol Immunol* 48, 2170-7

36. Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M., and Golenbock, D. T. (1999) **Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin.** *J Immunol* 162,

6971-5

37. Kang, J. Y., Nan, X., Jin, M. S., Youn, S. J., Ryu, Y. H., Mah, S., Han, S. H., Lee, H., Paik, S. G., and Lee, J. O. (2009) **Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer.** *Immunity* 31, 873-84

38. English, B. K., Patrick, C. C., Orlicek, S. L., McCordic, R., and Shenep, J. L. (1996) **Lipoteichoic acid from viridans streptococci induces the production of tumor necrosis factor and nitric oxide by murine macrophages.** *J Infect Dis* 174, 1348-51

39. Coleman, J. W. (2001) **Nitric oxide in immunity and inflammation.** *Int Immunopharmacol* 1, 1397-406

40. Croft, M., Duan, W., Choi, H., Eun, S. Y., Madireddi, S., and Mehta, A. (2012) **TNF superfamily in inflammatory disease: translating basic insights.** *Trends Immunol* 33, 144-52

41. Michelsen, K. S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C. J., and Schumann, R. R. (2001) **The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2.** *J Biol Chem* 276, 25680-6

42. Baik, J. E., Ryu, Y. H., Han, J. Y., Im, J., Kum, K. Y., Yun, C. H., Lee, K., and Han, S. H. (2008) **Lipoteichoic acid partially contributes to the inflammatory responses to *Enterococcus faecalis*.** *J Endod* 34, 975-82

43. Draing, C., Pfitzenmaier, M., Zummo, S., Mancuso, G., Geyer, A., Hartung, T., and von Aulock, S. (2006) **Comparison of lipoteichoic acid from different**

serotypes of *Streptococcus pneumoniae*. *J Biol Chem* 281, 33849-59

44. Im, J., Choi, H. S., Kim, S. K., Woo, S. S., Ryu, Y. H., Kang, S. S., Yun, C. H., and Han, S. H. (2009) **A food-born heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), suppresses tumor necrosis factor-alpha expression in lipoteichoic acid-stimulated RAW 264.7 cells**. *Cancer Lett* 274, 109-17

45. Baik, J. E., Jang, K. S., Kang, S. S., Yun, C. H., Lee, K., Kim, B. G., Kum, K. Y., and Han, S. H. (2011) **Calcium hydroxide inactivates lipoteichoic acid from *Enterococcus faecalis* through deacylation of the lipid moiety**. *J Endod* 37, 191-6

46. Jang, K. S., Baik, J. E., Han, S. H., Chung, D. K., and Kim, B. G. (2011) **Multi-spectrometric analyses of lipoteichoic acids isolated from *Lactobacillus plantarum*.** *Biochem Biophys Res Commun* 407, 823-30

47. Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S. J., and Pierce, S. K. (2003) **Location is everything: lipid rafts and immune cell signaling**. *Annu Rev Immunol* 21, 457-81

48. Soong, G., Reddy, B., Sokol, S., Adamo, R., and Prince, A. (2004) **TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells.** *J Clin Invest* 113, 1482-9

49. Korkmaz, Y., Lang, H., Beikler, T., Cho, B., Behrends, S., Bloch, W., Addicks, K., and Raab, W. H. (2011) **Irreversible inflammation is associated with decreased levels of the alpha1-, beta1-, and alpha2-subunits of sGC in human odontoblasts**. *J Dent Res* 90, 517-22

50. Hahn, C. L. and Liewehr, F. R. (2007) **Innate immune responses of the**

dental pulp to caries. *J Endod* 33, 643-51

51. da Silva, L. P., Issa, J. P., and Del Bel, E. A. (2008) **Action of nitric oxide on healthy and inflamed human dental pulp tissue.** *Micron* 39, 797-801

52. Augusto, L. A., Decottignies, P., Synguelakis, M., Nicaise, M., Le Marechal, P., and Chaby, R. (2003) **Histones: a novel class of lipopolysaccharide-binding molecules.** *Biochemistry* 42, 3929-38

53. Morita, S., Tagai, C., Shiraishi, T., Miyaji, K., and Iwamuro, S. (2013) **Differential mode of antimicrobial actions of arginine-rich and lysine-rich histones against Gram-positive *Staphylococcus aureus*.** *Peptides* 48, 75-82

54. White, S. H., Wimley, W. C., and Selsted, M. E. (1995) **Structure, function, and membrane integration of defensins.** *Curr Opin Struct Biol* 5, 521-7

55. Tobacman, L. S. and Korn, E. D. (1982) **The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by *Acanthamoeba* profilin.** *J Biol Chem* 257, 4166-70

56. Grenklo, S., Geese, M., Lindberg, U., Wehland, J., Karlsson, R., and Sechi, A. S. (2003) **A crucial role for profilin-actin in the intracellular motility of *Listeria monocytogenes*.** *EMBO Rep* 4, 523-9

57. Johnsson, M., Richardson, C. F., Bergey, E. J., Levine, M. J., and Nancollas, G. H. (1991) **The effects of human salivary cystatins and statherin on hydroxyapatite crystallization.** *Arch Oral Biol* 36, 631-6

58. Ohno, N. and Morrison, D. C. (1989) **Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity.** *J Biol Chem* 264, 4434-41

59. Sela, S., Marouni, M. J., Perry, R., and Barzilai, A. (2000) **Effect of lipoteichoic acid on the uptake of *Streptococcus pyogenes* by HEp-2 cells**. *FEMS Microbiol Lett* 193, 187-93

60. Bennett, R. D., Mauer, A. S., and Strehler, E. E. (2007) **Calmodulin-like protein increases filopodia-dependent cell motility via up-regulation of myosin-10**. *J Biol Chem* 282, 3205-12

61. Fabian, T. K., Fejerdy, P., and Csermely, P. (2008) **Salivary genomics, transcriptomics and proteomics: The emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases**. *Curr Genomics* 9, 11-21

62. Marti, G. A., Friedman, S. M., Cabrini, R. L., and Costa, O. R. (2002) **Role of crevice in the occurrence of hemoglobin in saliva**. *Acta Odontol Latinoam* 15, 11-3

63. Hasty, D. L., Meron-Sudai, S., Cox, K. H., Nagorna, T., Ruiz-Bustos, E., Losi, E., Courtney, H. S., Mahrous, E. A., Lee, R., and Ofek, I. (2006) **Monocyte and macrophage activation by lipoteichoic Acid is independent of alanine and is potentiated by hemoglobin**. *J Immunol* 176, 5567-76

64. Hodson, D. and Hirsch, J. G. (1958) **The antibacterial activity of hemoglobin**. *J Exp Med* 107, 167-83

65. Kerkhoff, C., Klempt, M., and Sorg, C. (1998) **Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9)**. *Biochim Biophys Acta* 1448, 200-11

66. Riva, M., Kallberg, E., Bjork, P., Hancz, D., Vogl, T., Roth, J., Ivars, F., and Leanderson, T. (2012) **Induction of nuclear factor-kappaB responses by**

the S100A9 protein is Toll-like receptor-4-dependent. *Immunology* 137, 172-82

67. Zhou, H. D., Li, G. Y., Yang, Y. X., Li, X. L., Sheng, S. R., Zhang, W. L., and Zhao, J. (2006) **Intracellular co-localization of SPLUNC1 protein with nanobacteria in nasopharyngeal carcinoma epithelia HNE1 cells depended on the bactericidal permeability increasing protein domain**. *Mol Immunol* 43, 1864-71

68. Baik, J. E., Hong, S. W., Choi, S., Jeon, J. H., Park, O. J., Cho, K., Seo, D. G., Kum, K. Y., Yun, C. H., and Han, S. H. (2013) **Alpha-amylase is a human salivary protein with affinity to lipopolysaccharide of *Aggregatibacter actinomycetemcomitans***. *Mol Oral Microbiol* 28, 142-53

69. Schenkels, L. C., Walgreen-Weterings, E., Oomen, L. C., Bolscher, J. G., Veerman, E. C., and Nieuw Amerongen, A. V. (1997) **In vivo binding of the salivary glycoprotein EP-GP (identical to GCDFP-15) to oral and non-oral bacteria detection and identification of EP-GP binding species**. *Biol Chem* 378, 83-8

70. Jung, K., Lee, J. E., Kim, H. Z., Kim, H. M., Park, B. S., Hwang, S. I., Lee, J. O., Kim, S. C., and Koh, G. Y. (2009) **Toll-like receptor 4 decoy, TOY, attenuates gram-negative bacterial sepsis**. *PLoS One* 4, e7403

71. Shomers, J. P., Tabak, L. A., Levine, M. J., Mandel, I. D., and Ellison, S. A. (1982) **The isolation of a family of cysteine-containing phosphoproteins from human submandibular-sublingual saliva**. *J Dent Res* 61, 973-7

72. Takada, K., Ohno, N., and Yadomae, T. (1994) **Lysozyme regulates LPS-induced interleukin-6 release in mice**. *Circ Shock* 44, 169-74

73. Cox, K. H., Cox, M. E., Woo-Rasberry, V., and Hasty, D. L. (2012) **Pathways involved in the synergistic activation of macrophages by lipoteichoic acid and hemoglobin**. *PLoS One* 7, e47333

74. Gorr, S. U., Abdolhosseini, M., Shelar, A., and Sotsky, J. (2011) **Dual host-defence functions of SPLUNC2/PSP and synthetic peptides derived from the protein**. *Biochem Soc Trans* 39, 1028-32

국문초록

*Streptococcus mutans*는 구강 내에서 다양한 인자들을 통하여 치아우식을 일으키는 것으로 알려진 그람양성균이다. 중증 치아우식 환자의 경우 이러한 세균이 치수 부분까지 침입하여 염증반응을 일으키는 것으로 알려져 있다. 그러나 *S. mutans*가 구강 내에서 염증을 일으키는 주된 인자는 명확하게 밝혀져 있지 않으며, 이러한 인자가 어떠한 경로를 통하여 면역반응을 일으키는지에 대한 연구 및 구강 내에서 부착하는 물질에 대한 연구는 아직 잘 알려져 있지 않다. 따라서 본 연구는 그람양성균의 주요 세포벽 구성 물질 중 하나인 lipoteichoic acid (LTA)를 순수 분리 정제하고 그것의 면역학적 특성을 알아본 뒤, 구강 내에서 LTA에 부착하는 단백질을 정상인과 치아우식 환자의 타액 내에서 분리 동정함으로써 이들의 차이를 비교 분석하였다.

*Streptococcus mutans*와 비교 군으로 사용할 균주-*Enterococcus faecalis*, *Staphylococcus aureus*, *Lactobacillus plantarum*-를 각각 적정 배지에서 배양하여 모은 뒤 초음파 처리하여 파쇄한 후 부틸알콜을 이용하여 세포벽 인자들만을 추출한다. 소수성 상호작용과 이온성 상호작용을 이용한 칼럼크로마토그래피를 순차적으로 수행하여 LTA를 순수 분리 정제한다. 분리 정제한 LTA는 분자구조 내에 존재하는 인산염의 양을 통하여 정량하였다. 정량 된 LTA는 *Limulus* amebocyte lysate assay, UV absorption, bicinchoninic acid assay 등의 방법을 이용하여 순도를 확인하였다. 이후, *S. mutans*의 LTA (Sm.LTA)의 면역학적 특성을 파악하기 위하여 마우스 대식세포인 RAW 264.7 세포에 Sm.LTA를 interferon-

gamma가 존재하는 조건에서 다양한 농도별로 처리하여 염증매개인자인 nitric oxide (NO)와 tumor necrosis factor-alpha (TNF-α)의 발현량을 NO assay와 enzyme-linked immunosorbent assay를 통하여 확인하였다. 또한 동일한 조건의 실험을 내독소의 길항제인 polymyxin B (PMB)의 존재하에 실험하여 내독소의 의한 오염 여부를 확인하였다. 이전까지의 연구에 의하면 다양한 LTA는 톨유사수용체-2 (TLR2)에 의하여 인식되어 면역 활성을 일으킨다고 알려져 있었기 때문에 Sm.LTA 또한 이러한 수용체에 의해 인식되는지 확인하고자 CD14 단백질과 TLR2 또는 TLR4가 과발현 되도록 형질 전환된 CHO/CD14/TLR2 또는 CHO/CD14/TLR4 세포 (CHO reporter cell)를 이용하였다. 우선적으로 Sm.LTA에 biotin을 결합시킨 Sm.LTA-biotin을 이용하여 TLR2 또는 TLR4가 과발현 된 세포 중 어떠한 세포에 우선적으로 부착하는지 알아보는 실험을 수행하고 이를 유세포분석기로 측정하였다. 또한 CHO reporter cell은 TLR2 또는 TLR4 자극에 반응하여 세포 표면에 CD25 단백질을 발현하도록 되어있기 때문에 다양한 농도의 Sm.LTA를 처리하여 세포 표면의 CD25의 발현량을 유세포분석기로 측정하였다. 추가적으로 이러한 면역반응에서 TLR2의 역할이 필수적임을 확인하기 위하여 정상 쥐와 TLR2가 결여된 쥐로부터 골수 유래 대식세포를 얻은 뒤 다양한 농도의 Sm.LTA 또는 열처리 사멸된 *S. mutans*를 처리하여 염증매개인자의 발현량을 관찰하였을 뿐만 아니라 TLR2 저지 항체를 처리한 후 염증매개인자의 발현량 차이 또한 확인하였다. 일반적으로 LTA와 같은 지질다당체와 TLR2와의 결합 및 하위 단계의 신호전달에서는 아실 사슬의 역할이 중요하다고 보고되어있기 때문에 Sm.LTA의 아실 사슬을 화학적인 방법을 통하여 제거한 뒤 염증매개인자의 발현량을 RAW 264.7세포를 통

하여 확인하였다. 또한 다양한 세균 (*E. faecalis, S. aureus, L. plantarum*)으로부터 얻은 LTA의 면역학적 활성 능력을 비교하기 위하여 각각의 LTA를 정제한 뒤, RAW 264.7세포에 농도별로 처리하여 염증매개인자의 발현량을 비교하였다. 이후, 구강 내에서 세균의 부착 및 염증반응에 중요할 것으로 예상되는 Sm.LTA-부착 단백질을 동정하고자 우선적으로 Sm.LTA를 NHS-Sepharose® 4 Fast Flow beads (NHS-비드)와 결합시켜 Sm.LTA-비드를 만들었다. 결합 후, Sm.LTA-비드에서 Sm.LTA의 면역 활성이 남아있는지 RAW 264.7세포에 처리하여 염증매개인자의 발현량을 통하여 확인하고, 추가적으로 CHO/CD14/TLR2세포에 처리하여 세포 표면의 CD25 단백질 발현량을 유세포분석기를 통해 확인하였다. 다른 한편으로 정상인과 치아우식 환자의 구강 내에 존재하는 단백질의 발현 양상의 차이를 알아보기 위하여 다양한 외래환자로부터 타액을 얻은 후, 그들의 현재 및 과거 치아우식의 수, 구강의 위생상태의 정도에 따라 각각 상위, 하위 10명씩을 선정하여 정상인과 치아우식 환자로 분류하였다. 그 후, 이들의 타액에서 단백질을 제외한 나머지 물질들을 원심분리를 통하여 제거한 뒤 남아 있는 단백질을 SDS-PAGE를 통하여 크기별로 분리하고 Coomassie blue염색법을 통해 가시화하여 비교하였다. 그 후에 Sm.LTA에 부착하는 단백질을 동정하기 위해 각각 10명의 정상인과 치아우식 환자의 타액을 혼합한 뒤, Sm.LTA-비드와 반응시켜 Sm.LTA-부착 단백질을 분리하였다. 분리 된 Sm.LTA-부착 단백질은 SDS-PAGE를 통하여 다시 크기 별로 분리하고 은 염색법을 통하여 가시화하였다. 이렇게 분리 되어진 정상인과 치아우식 환자의 타액 내 단백질은 LTQ-orbitrap mass spectrometry방법을 통하여 동정하였다.

분리 정제된 Sm.LTA는 면역학적 활성을 가지지 않는 범위의 오염도를

가지는 것을 확인할 수 있었으며 RAW 264.7 세포에서 NO와 TNF-α를 농도 의존적으로 발현하는 것을 확인하였다. 또한 이러한 염증매개인자 발현은 PMB에 의해 저해되지 않았다. 뿐만 아니라 Sm.LTA는 CHO reporter cell을 통한 실험에서 TLR2에 우선적으로 결합하고 활성화시켜 세포 표면의 CD25 발현을 유도하는 것을 확인하였다. 이러한 TLR2의 중요성은 TLR2가 결여된 쥐의 골수 유래 대식세포에 Sm.LTA를 처리하였을 때와 열처리 사멸된 *S. mutans*를 처리하였을 때 염증매개인자의 발현이 유의적으로 감소하는 것을 통하여 확인하였고, 추후 TLR2 억제 항체를 처리한 뒤 염증매개 인자발현이 감소하는 것을 추가적으로 확인하였다. 또한 Sm.LTA의 아실 사슬을 제거한 Sm.LTA를 처리했을 때, 염증매개인자의 발현뿐 만 아니라 CD25의 발현량이 현저히 감소하는 것을 관찰하였다. 그러나 다양한 균주에서 정제한 LTA들을 RAW 264.7 세포에 농도별로 처리했을 때, 염증매개인자의 발현량에서 각각의 LTA 간에 큰 차이를 보이는 것을 확인할 수 있었다. 이후 Sm.LTA를 NHS-비드에 부착시킨 뒤 면역학적 활성을 확인해 본 결과, 부착 전의 Sm.LTA와 염증매개인자의 발현 양상 및 TLR2 활성화 양상 모두 차이가 없는 것을 관찰하였다. 다른 한편으로 다양한 환자의 타액에서 단백질을 분리하여 발현 양상을 확인한 결과 정상인의 경우 유사한 패턴의 단백질 발현이 관찰된 것에 반해 치아우식 환자의 경우 불규칙적인 패턴을 보이는 것을 관찰할 수 있었다. 또한 같은 양의 타액 내 존재하는 단백질의 양 또한 치아우식 환자에서 1.7배 더 많은 것을 확인하였다. 이후 Sm.LTA-부착 단백질을 질량분석기를 통해 확인한 결과 정상인의 타액 내 부착 단백질로 histone H4, profilin-1, neutrophil defensin-1이 확인되었고 치아우식 환자의 경우 cystatin-C, cystatin-SN, cystain-S, cystain-D, lysozyme C,

calmodulin-like protein 3, actin이 확인되었으며 hemoglobins, prolactin-inducible protein, protein S100-A9, SPLUNC2는 두 그룹에서 공통적으로 발견됨을 확인하였다.

이상의 연구결과들을 통하여 다음과 같은 결론을 얻을 수 있었다. Sm.LTA는 TLR2에 우선적으로 부착한 후 활성화시켜 하위 신호 전달을 통해 면역 활성을 일으켜 염증매개인자의 발현을 유도하는 것을 알 수 있으며, Sm.LTA는 구강 내에서 다양한 단백질들과 부착하고 이러한 부착 단백질들은 정상인과 치아우식 환자에서 차이가 있음을 알 수 있다.

주요어: *Strepcococcus mutans*, Lipoteichoic acid, 톨유사수용체2, 선천성면역, 타액단백질

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