이학박사 학위논문

# Role of cell wall-associated virulence factors of *Staphylococcus aureus* in biofilm formation and induction of inflammatory responses

# 황색포도상구균 바이오필름 형성과 염증반응 유도에서 세포벽 병독성인자들의 역할

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안기범

# Role of cell wall-associated virulence factors of *Staphylococcus aureus* in biofilm formation and induction of inflammatory responses

by

**Ki Bum Ahn**

Under the supervision of

**Professor Seung Hyun Han, Ph. D.**

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Department of Dental Science, Immunology and Molecular Microbiology, School of Dentistry, Seoul National University

# ABSTRACT

# Role of cell wall-associated virulence factors of *Staphylococcus aureus* in biofilm formation and induction of inflammatory responses

**Ki Bum Ahn**

Department of Dental Science, Immunology and Molecular Microbiology, School of Dentistry, Seoul National University

(Supervised by Professor **Seung Hyun Han**, Ph. D.)

## Objectives

*Staphylococcus aureus* is a Gram-positive pathogen that is frequently found in mucosal tissues of the respiratory and gastrointestinal tracts, and on the skin. *S. aureus* causes various infectious diseases such as pneumonia, septic shock, and endocarditis through induction of inflammatory responses and biofilm formation. Although cell wall-associated virulence factors of *S. aureus* are deeply involved in the pathogenesis of *S. aureus*, how they function in the induction of inflammatory responses and biofilm formation is unclear. Further,

it is also important to clarify the molecular mechanisms by which *S. aureus* induces inflammatory responses and biofilm formation to understand *S. aureus* pathogenesis, which is needed for developing effective therapeutic strategies against *S. aureus* infection. The aims of the present study were (1) to investigate the role of cell wall-associated virulence factors of *S. aureus* in the induction of inflammatory responses, (2) to determine the role of cell wall-associated virulence factors of *S. aureus* in the biofilm formation, and (3) to develop a method to regulate the biofilm formation.

## Methods

The mouse macrophage cell line RAW 264.7, bone marrow-derived macrophages (BMM) from the wild-type, Toll-like receptor 2 (TLR2)-deficient, or nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-deficient C57BL/6 mice, and human monocyte-derived macrophages were used to determine the abilities of the wild-type *S. aureus*, its mutant strains lacking cell wall-associated virulence factors such as lipoprotein (Sa.LPP), or lipoteichoic acid (Sa.LTA) to induce the production of inflammatory mediators. The prostaglandin E2 (PGE2) production in macrophages stimulated with Sa.LTA in the presence or absence of muramyl dipeptide (MDP) was determined by enzyme-linked immunosorbent assay (ELISA). Nitric oxide (NO) production in macrophages stimulated with the *S. aureus* wild-type or mutant strains lacking lipoteichoic acid (Δ*ltaS*), lipoproteins (Δ*lgt*), or D-

alanine (Δ*dltA*) was determined using Griess reagent. The expression of inducible NO synthase (iNOS) or cyclooxygenase-2 (COX-2) in macrophages stimulated with the *S. aureus* wild-type, mutant strains lacking cell wall-associated virulence factors, Sa.LTA, or MDP were determined by RT-PCR or Western blot analysis. Activation of transcription factors such as NF-κB, AP-1, or CRE in macrophages stimulated with *S. aureus* wild-type, mutant strains lacking cell wall-associated virulence factors, Sa.LTA, or MDP were determined by luciferase reporter gene assay. The ability of lipoprotein from *S. aureus* to activate TLR2 or TLR4 was determined using CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, a NF-κB reporter cell lines capable of inducing expression of membrane-bound CD25 in proportion to the degree of activation of TLR2 or TLR4, respectively. The cells were stimulated with the wild-type, Δ*ltaS*, Δ*lgt*, or Δ*dltA*, and the expression of CD25 was determined as an indication of TLR2 or TLR4 activation by flow cytometry. Biofilm of *S. aureus* was determined by crystal violet biofilm staining assay, confocal laser scanning microscopy (CLSM) using a LIVE/DEAD viability assay, or scanning electron microscopy (SEM). The release of autoinducer-2 (AI-2) from *S. aureus* in the presence or absence of LTA was determined by AI-2 reporter assay using *Vibrio harveyi* BB170 strain.

## Results

The wild-type, Δ*ltaS*, and Δ*dltA* strains of *S. aureus* induced NO production in the macrophages in a dose-dependent manner but this response was not observed when the cells were stimulated with the Δ*lgt* strain. Coincident with NO induction, the wild-type, Δ*ltaS*, and Δ*dltA* strains dose-dependently induced expression of iNOS at both mRNA and protein levels whereas Δ*lgt* failed to induce iNOS protein or mRNA. Transient transfection followed by a reporter gene assay and Western blotting experiments demonstrated that the wild-type, Δ*ltaS*, and Δ*dltA* strains, but not the Δ*lgt* strain, induced substantial activation of NF-κB and STAT-1 phosphorylation, both of which are known to be crucial for iNOS expression. Moreover, the wild-type, Δ*ltaS*, and Δ*dltA* strains increased TLR2 activation, which is known to mediate *S. aureus*-induced innate immunity, whereas the Δ*lgt* strain did not. These results suggest that lipoproteins are crucial for *S. aureus*-induced iNOS expression and NO production through TLR2.

*S. aureus* contains LTA and peptidoglycan (PGN) layers, both of which are considered as major virulence factors associated with inflammation. Since LTA and PGN are thought to cooperate in the establishment of inflammation, synergistic effects of Sa.LTA and MDP, the minimal structural unit of PGN, were examined on the induction of inflammatory mediators in macrophages. Treatment with MDP enhanced Sa.LTA-induced COX-2 and PGE2 production. The cooperative effect between Sa.LTA and MDP was not observed in COX-2

expression by macrophages derived from TLR2- or NOD2-deficient mice. In addition, MDP enhanced Sa.LTA-induced activation of the transcription factors NF-κB and CRE, which are known to modulate COX-2 gene transcription. These results suggest that Sa.LTA and MDP cooperatively induce COX-2 expression and PGE2 generation, which might contribute to the establishment of inflammation at sites of Gram-positive bacterial infection.

*S. aureus* culture supernatants containing high amount of LTA inhibited biofilm formation of *S. aureus*, but the Δ*ltaS* culture supernatants did not inhibit biofilm formation. Purified LTA from *S. aureus* inhibited biofilm formation of *S. aureus* in a dose-dependent manner whereas lipoproteins failed to inhibit biofilm formation. In addition, purified LTA from various bacterial species including *Streptococcus pneumoniae*, *Streptococcus gordonii*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Lactobacillus plantarum* also inhibited biofilm formation of *S. aureus* in a dose-dependent manner. Among these LTA, *L. plantarum* LTA (Lp.LTA) and *S. pneumoniae* LTA (Sp.LTA) showed the most potent inhibition of *S. aureus* biofilm formation.

Lp.LTA inhibited biofilm formation of *S. aureus* in a dose-dependent manner, whereas other cell wall components including *L. plantarum* lipoproteins (Lp.LPP) and *L. plantarum* PGN (Lp.PGN) did not have an influence on *S. aureus* biofilm formation. Lp.LTA also destroyed pre-formed biofilm of *S. aureus* in a dose-dependent manner, but did not alter bacterial growth. Interestingly, Lp.LTA induced increase of extracellular AI-2 in *S. aureus*, which partly contributed to the inhibition of *S. aureus* biofilm formation.

Moreover, Lp.LTA inhibited the production of poly-*N*-acetylglucosamine (PNAG) polysaccharide, which is a major component of biofilm in staphylococci, via inhibition of *ica* gene expression. However, D-alanine-removed Lp.LTA (Deala-Lp.LTA) did not inhibit *ica* gene expression and biofilm formation of *S. aureus*. Lp.LTA enhanced the efficacy of antibiotics that target the cell wall synthesis (penicillin and vancomycin) and protein synthesis (streptomycin and erythromycin) to inhibit biofilm formation of *S. aureus.* As expected, the biofilm formation of *S. aureus* clinical isolates was also inhibited by Lp.LTA. These results suggest that LTA could be an antibiofilm agent to be used for treatment of inflammatory diseases caused by *S. aureus* biofilm.

## Conclusion

Understanding the mechanisms of *S. aureus*-induced inflammation and biofilm formation is important for an efficient treatment or prevention of *S. aureus* causing infectious diseases. The present study demonstrates the roles of cell wall-associated virulence factors of *S. aureus* in inflammatory responses and biofilm formation. In *S. aureus*-mediated inflammatory responses, lipoprotein plays a crucial role in the induction of NO production in macrophages through activation of NF-κB and STAT1 triggered by TLR2. LTA and MDP synergistically induced inflammatory response by overproducing COX-2 through NOD2 and TLR2. In *S. aureus* biofilm formation, LTA, but

not lipoprotein, inhibited *S. aureus* biofilm by inhibiting the production of PNAG, which is an important component of biofilm formed by *S. aureus*. In particular, LTA from a beneficial bacterium *L. plantarum* substantially inhibited *S. aureus* biofilm formation. Collectively, lipoprotein in the cell wall of *S. aureus* plays a major role in the induction of inflammatory responses through activation of TLR2 and cooperative action between LTA and MDP might contribute to the establishment of inflammation. LTA acts as a negative regulator against *S. aureus* biofilm and Lp.LTA could be an anti-biofilm agent for prevention or treatment of chronic inflammatory diseases caused by *S. aureus* biofilm.

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**Keywords:** *Staphylococcus aureus*, Lipoprotein, Lipoteichoic acid, Inflammatory response, Biofilm formation, *Lactobacillus plantarum*

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# Abbreviations

| | |
|---|---|
| **AI-2** | autoinducer-2 |
| **AIP** | autoinducing peptides |
| **AP-1** | activator protein 1 |
| **BMM** | bone marrow-derived macrophage |
| **CD** | cluster of differentiation |
| **CFU** | colony-forming uint |
| **COX-2** | cyclooxygenase-2 |
| **CRE** | cAMP response elements |
| **DAG** | diacylglycerol |
| **DAMP** | danger-associated molecular patterns |
| **DAP** | diaminopimelic acid |
| **EPS** | extracellular polymeric substances |
| **EKSA** | ethanol-killed *S. aureus* |
| **ELISA** | enzyme-linked immunosorbent assay |
| **NAG** | α-D-*N*-acetylglucosamine |
| **IFN-γ** | interferon-gamma |
| **IL-1β** | interleukin-1β |
| **IL-6** | interleukin-6 |
| **IL-12** | interleukin-12 |
| **IP-10** | interferon gamma-inducible protein 10 |

| | |
|---|---|
| **LBP** | lipopolysaccharide-binding protein |
| **LPS** | lipopolysaccharide |
| **LPP** | lipoproteins |
| **LTA** | lipoteichoic acid |
| **MAMP** | microbe-associated molecular patterns |
| **MRSA** | methicillin-resistant *S. aureus* |
| **NF-κB** | nuclear factor-kappa B |
| **NO** | nitric oxide |
| **NOD** | nucleotide-binding oligomerization domain |
| **NOS** | nitric oxide synthase |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **MD-2** | differentiation factor 2 |
| **MDP** | muramyl dipeptide |
| **MIP-1α** | macrophage inflammatory protein-1 alpha |
| **MyD88** | myeloid differentiation primary response gene 88 |
| **PAFR** | platelet-activating factor receptor |
| **PGE2** | prostaglandin E2 |
| **PGN** | peptidoglycan |
| **PIR-B** | paired-Ig like receptor B |
| **PNAG** | poly-*N*-acetylglucosamine |
| **TAG** | triacylglycerol |
| **TLR** | Toll-like receptor |

| | |
|---|---|
| **TNF-α** | tumor necrosis factor-α |
| **VRSA** | vancomycin-resistant *S. aureus* |
| **WT** | wild-type |

# Chapter I. Introduction

## 1. General characteristics of *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive pathogen that commonly colonizes the nose, skin, and mucosal surfaces of even healthy individuals. Approximately 20-30% of healthy individuals are permanently colonized with *S. aureus*, while 30% are intermittently colonized [1, 2]. However, *S. aureus* may also cause a range of illnesses, from minor skin infection to life-threatening diseases such as septic shock, pneumonia, and endocarditis [3]. The emergence of antimicrobial resistance in *S. aureus* was first reported in the 1950s and its incidence has rapidly increased since then. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) are major causes of nosocomial and community acquired infections. In recent years, MRSA and VRSA have become a public health concern due to their resistance to multiple antibiotics that lead to limitation of treatment options and increase of morbidity and mortality [4, 5]. Thus, novel therapeutic strategies are needed to treat *S. aureus* infections. *S. aureus* can induce severe inflammatory responses and biofilm formation, which are closely associated with various infectious diseases. *S. aureus* induces inflammatory responses through interaction of microbe-associated molecular patterns (MAMPs) on *S. aureus* with pattern recognition receptors (PRRs) on host cells. In particular, cell wall-associated Toll-like receptor 2 (TLR2) ligands of *S. aureus* are predominantly involved in induction of inflammatory responses through activation of TLR2 on host cells [6]. *S.*

*aureus* biofilm has the ability to avoid phagocytosis by macrophages and neutrophils and 10-1,000 times more resistant to antibiotics or antimicrobial peptides than planktonic cells so that it is difficult to remove biofilm [7, 8].

## 2. Cell wall-associated virulence factors of *S. aureus*

### 2.1. Lipoprotein

#### 2.1.1. Physiological functions of lipoprotein

Bacterial lipoproteins are hydrophobic proteins that are anchored to a bacterial cell membrane by acyl moieties linked to N-terminal region [9]. These proteins have been shown to play important roles in a variety of bacterial physiological phenomena, including adhesion, sporulation, nutrient uptake, cell wall metabolism, signal transduction, resistance to antibiotics, and immune stimulation [10-12]. Particularly, more than 90% of lipoproteins in Gram-negative bacteria are localized to the inner leaflet of outer membrane, which are involved in production and maintenance of outer membrane [13]. Among them, some lipoproteins are associated with bacterial growth, transporting lipopolysaccharide, and peptidoglycan (PGN) synthesis [14-16]. In contrast, most Gram-positive bacterial lipoproteins are localized to the outer leaflet of cytoplasmic membrane that are involved in iron acquisition, adhesion, and antibiotic resistance [17-19]. Interestingly, *Bacillus subtilis* lipoprotein, PrsA, and *Mycobacterium tuberculosis* lipoproteins are associated with the cell growth [20, 21].

#### 2.1.2 Lipoprotein structure and biosynthesis

Bacterial lipoprotein precursors, preprolipoproteins, are inserted to the membrane by the Sec or Tat secretion pathway [22] and are modified by

sequential actions of two or three lipoprotein biosynthetic enzymes, preprolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase (Lsp), or lipoprotein N-acyl transferase (Lnt) [23] (Figure 1). Preprolipoproteins in bacterial membrane possess N-terminal signal peptide containing lipobox that is lipid-modified via attachment of a diacylglycerol moiety to the cysteine residue by Lgt, resulting in the formation of diacylglyceryl-prolipoproteins that can anchor to bacterial membrane. Then, Lsp cleaves the N-terminus signal peptide linked with lipidated cysteine residue of prolipoprotein, resulting in diacylated lipoproteins. Additionally, in the Gram-negative and some Gram-positive bacteria, Lnt adds an acyl chain to N-terminal cysteine residue, resulting in triacylated lipoprotein [22, 23].

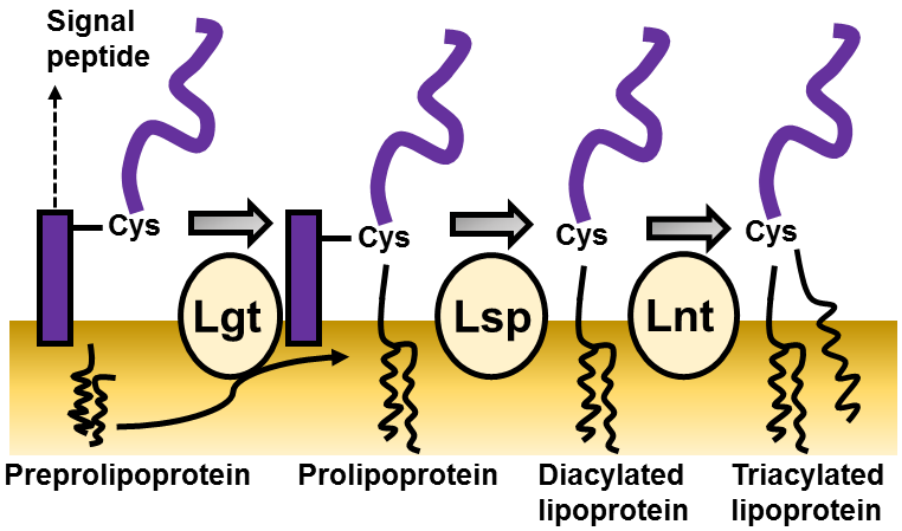


**Figure 1. Structure and biosynthesis of lipoprotein.** After preprolipoproteins are inserted to the membrane by the Sec or Tat secretion pathway, the Lgt transfers a diacylglyceryl moiety from a membrane phospholipid to the cysteine residue in lipobox motif. Then, Lsp cleaves the N-terminus signal peptide of prolipoproteins. Finally, the Lnt adds an acyl moiety to N-terminal cysteine residue, resulting in triacylated lipoprotein.

### 2.1.3 Host innate immunity against bacterial lipoproteins

Lipoprotein is a major virulence factor in some bacteria mediating adhesion and stimulating immune responses. To investigate the virulence effects of lipoproteins, pathogenesis of *lgt* or *lsp* mutant strains have been studied. Most of the studies indicate that these mutations attenuate virulence in animal infection models. For example, an *lgt* mutant reduces virulence of *Streptococcus pneumoniae* and an *lsp* mutant of *Mycobacterium tuberculosis* and *Listeria monocytogenes* exhibited reduced virulence [24-26]. Recently, it has been reported that the innate immune system recognizes lipoprotein and lipopeptides by TLR2. Moreover, some reports have revealed that TLR2 recognizes diacylated or triacylated lipoproteins through heterodimerization with TLR1 or TLR6, respectively [27, 28]. In *S. aureus*, lipoprotein also acts as a major virulence factor stimulating the activation of various cell types such as monocytes, epithelial cells, and osteoclasts via TLR2 to produce cytokines and chemokines [29-31]. Moreover, mice infected with lipoprotein-deficient *S. aureus* (Δ*lgt*) show lower inflammatory responses and mortality than mice infected with wild-type *S. aureus* [32]. However, other TLR2 ligand of *S. aureus* such as lipoteichoic acid (LTA) has also been reported as a major virulence factor of *S. aureus* to induce inflammatory responses [33-35]. Thus, it is currently unclear which TLR2 ligand of *S. aureus* predominantly contribute to inflammatory responses.

## 2.2. Lipoteichoic acid (LTA)

### 2.2.1. Physiological functions of LTA

LTA is an amphiphile of glycolipids linked with polyglycerol phosphates in Gram-positive bacteria and is considered to be a counterpart of lipopolysaccharide (LPS) in Gram-negative bacteria [36]. LTA plays important roles in various bacterial physiological functions including bacterial division, growth, interaction with host receptors or abiotic surfaces, and resistance to antimicrobial peptides [37]. It has been reported that LTA-deficient strains, such as *S. aureus*, *L. monocytogenes*, and *B. subtilis* show cell enlargement, abnormal cell division and septum formation suggesting that LTA is involved in cell-division machinery [38-40]. Interestingly, LTA synthesis enzymes interact with numerous enzymes involved in cell division or PGN synthesis, leading to formation of multi-enzyme complex which affect cell division processes [40]. In addition, LTA is associated with resistance to charged antibiotics or antimicrobial peptides [37]. The addition of D-alanine to the LTA contributes to a net positive charge on the bacterial cell surface, which decreases effectiveness of cationic antimicrobial substances. According to previous studies using D-alanine-deficient mutant strain, lacking D-alanine from LTA results in high susceptibility to cationic host antimicrobial peptides and antibiotics [41, 42]. Interestingly, the sensor kinase GraS of *S. aureus* and *Staphylococcus epidermidis* recognizes antimicrobial peptides resulting in regulation of D-alanylation in LTA to protect bacteria against antimicrobial challenges [43, 44]. LTA is also associated with bacterial adhesion to host cells

or abiotic surfaces. For example, D-alanine-deficient strains of *S. aureus*, *Enterococcus faecalis*, and *L. monocytogenes* show reduced bacterial adherence and biofilm formation [45-47].

### 2.2.2. LTA structure and biosynthesis

So far, five types of LTA (i.e., type I-V) have been identified (Figure 2) [48]. Among them, type I LTA has been best characterized and is found in most Gram-positive bacteria including *S. aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes, Bacillus subtilis,* and *Lactobacillus plantarum* [48]. Type I LTA contains polyglycerophosphate backbone linked to glycolipid anchor, dihexosyl-diacylglycerol, but a tetrahexosylglycerol with either two or three acyl chains is observed in *Lactobacillus gasseri* [49, 50]. Various enzymes are involved in LTA synthesis including YpfP, LtaA, and LtaS. The glycolipid anchor is produced in the bacterial cytoplasm by glycosyltransferase, YpfP, which transfers two glucose moieties from UDP-glucose to membrane lipid diacylglycerol, and flippase, LtaA, translocates the glycolipid from the inner to the outer leaflet of the membrane. Subsequently, LTA synthase, LtaS, polymerlizes the polyglycerol phosphates backbone on the glycolipid. Additionally, glycerolphosphate subunits are decorated with D-alanine ester by DltA, DltB, DltC, and DltD [51] (Figure 3).

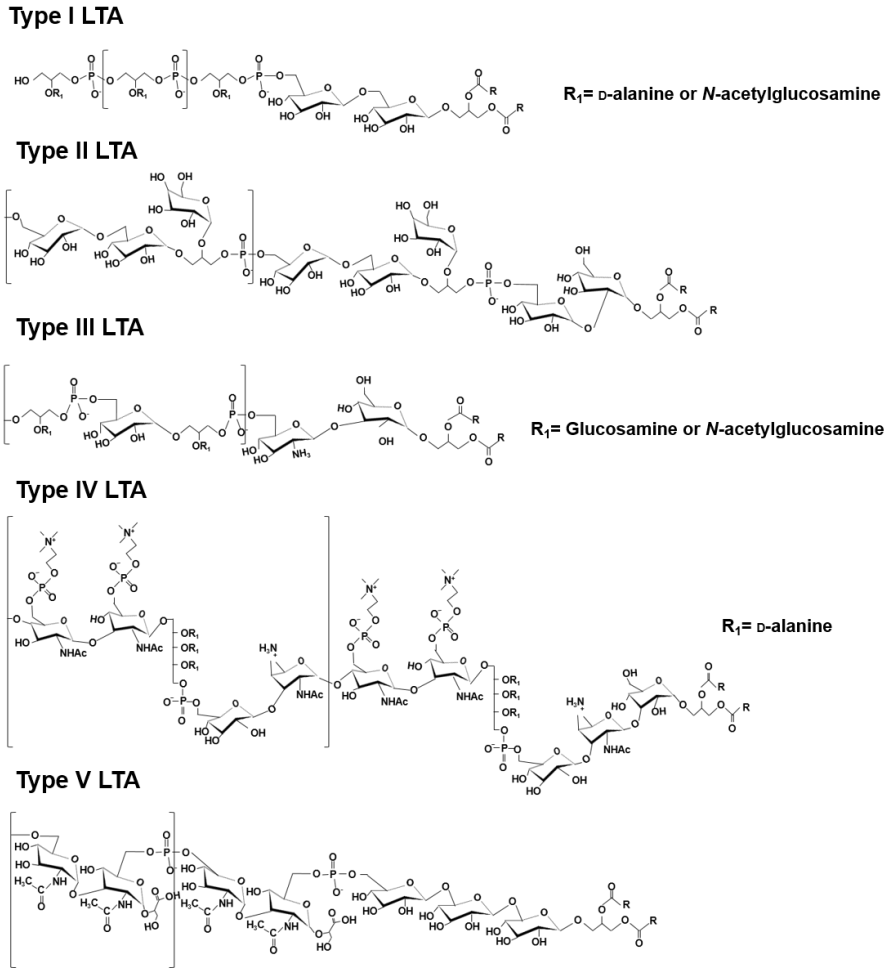


**Figure 2. Structure of different LTA types (modified from Kang, SS. et al. [48]).** Chemical formula for type I LTA found in *S. aureus*, type II LTA found in *Lactobacillus garvieae*, type III LTA found in *Clostridium innocuum*, type IV LTA found in *Streptococcus pneumoniae*, and type V LTA found in *Clostridium difficile*. $R_1$ denotes backbone substitutions and R indicates a fatty acid.

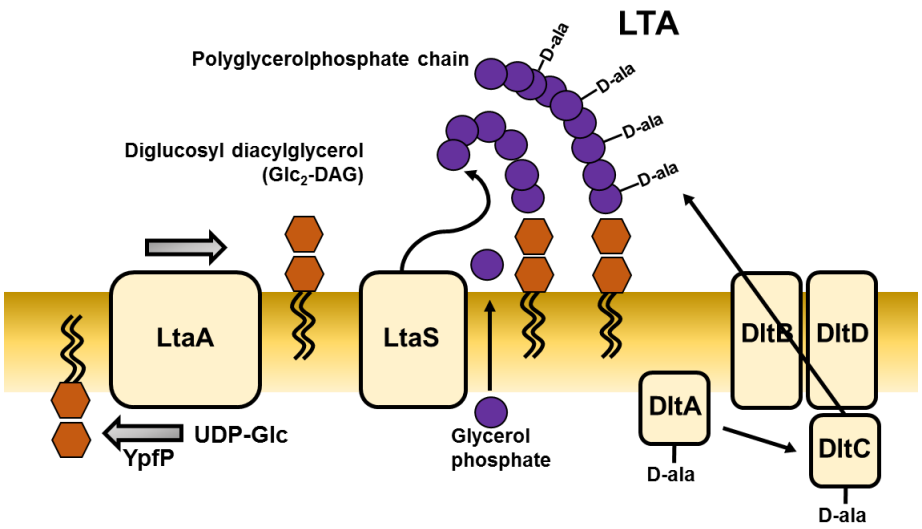


**Figure 3. Type I LTA biosynthesis.** YpfP synthesizes the glycolipid, which takes place in the bacterial cytoplasm. After glycolipid synthesis, it is transferred from the inner leaflet of the membrane to the outer leaflet by LtaA. Subsequently, LtaS polymerizes the polyglycerol phosphate backbone of LTA. Next, DltA ligates D-alanine onto the carrier protein DltC. The D-alanine is transported across the membrane and incorporated into polyglycerolphosphate of LTA by DltB and DltD.

### 2.2.3 Host innate immunity against LTA

LTA is spontaneously released from Gram-positive bacteria during growth or bacterial lysis induced by exogenous factors, such as lysozymes, cationic peptides, and antibiotics [52, 53]. LTA is recognized by various peripheral molecules including lipopolysaccharide-binding protein (LBP), cluster of differentiation (CD) 14, mannose-binding protein (MBP), L-ficolin, CD36, and soluble TLR2, which can promote innate immune responses to LTA or act as a scavenger for LTA [54-57]. In addition, LTA is recognized by TLR2 that recruits myeloid differentiation primary response gene 88 (MyD88) [58-60]. Although, lipoproteins have been reported a predominant TLR2 ligand of Gram-positive bacteria, such as *S. aureus*, several reports demonstrated that LTA is involved in the initiation and development of infectious diseases such as sepsis, pneumonia and meningitis [61, 62]. Indeed, LTA is associated with diverse inflammatory responses. *S. aureus* LTA induces the production of inflammatory mediators including IL-1β, IL-6, TNF-α, MCP-1, and MIP-1α in monocytes and macrophages [63-67]. Moreover, *S. pneumoniae* LTA also induces TNF-α and nitric oxide (NO) production [34, 35]. Similarly, LTA from oral pathogens including *E. faecalis* and *Streptococcus mutans* also induces production of TNF-α and NO in macrophages [68-71]. Interestingly, combination of LTA and PGN exhibits synergistic induction of inflammation [72, 73]. These data have recently been confirmed with muramyl dipeptide (MDP), minimal structural unit of PGN required for inflammation. MDP enhances the *S. aureus* LTA-induced TNF-α and IL-12 production in dendritic

cells and enhancement of dendritic cell maturation [74]. Recently, however, LTA is known to have anti-inflammatory effects. *S. aureus* LTA binds to paired-Ig like receptor B (PIR-B), negative regulator for PRR signaling, leading to decrease of IL-1β and IL-6 production in macrophages [75, 76]. Moreover, *E. faecalis* LTA inhibits *Aggregatibacter actinomycetemcomitans* LPS-induced IL-8 production via IRAK-M induction in human periodontal ligament cells [77]. Thus, the exact role of LTA in inflammatory or anti-inflammatory responses is still controversial.

## 2.3. PGN

Although Gram-positive and Gram-negative bacteria are surrounded by multi-layers of PGN, Gram-positive bacterial PGN approximately 5- to 10-fold thicker than that of Gram-negative bacteria [78]. PGN has alternating *N*-acetylglucosamine and *N*-acetylmuramic acid polymers interconnected through peptide bridges [79]. PGN has been divided into two main types, *meso*-diaminopimelic acid type (DAP-type) and L-lysine type (Lys-type) [80]. DAP-type and Lys-type PGN are mainly present in Gram-negative and Gram-positive bacteria, respectively. PGN is associated with cell division, physical barrier to the cell, antibiotic resistance, and structural strength of the cell. In addition, PGN is considered to be a major virulence factor and is closely associated with inflammation [3]. PGN is recognized by the intracellular receptors nucleotide-binding oligomerization domain 1 (NOD1) and NOD2. NOD1 appears to sense only Gram-negative bacterial PGN, specifically containing DAP [81]. In contrast, NOD2 senses both Gram-positive and Gram-negative bacterial PGN by recognizing MDP, a common structural motif of all PGNs that is the minimal structural unit with immuno-stimulating potential [82]. Accumulating studies suggest that LTA and PGN synergistically induce inflammatory responses [73, 74]. Remarkably, this was seen in the induction of NO when murine macrophages were co-treated with PGN from *S. aureus* or *B. subtilis* in the presence of *S. aureus* LTA, but not *Bacillus subtilis* LTA [73]. This implies that LTA is a determinant and far more important than PGN in the NO induction. Despite the evidence, cooperative action of LTA and PGN is still debatable since the LTA and PGN used in the early studies were contaminated and/or structurally damaged [83, 84].

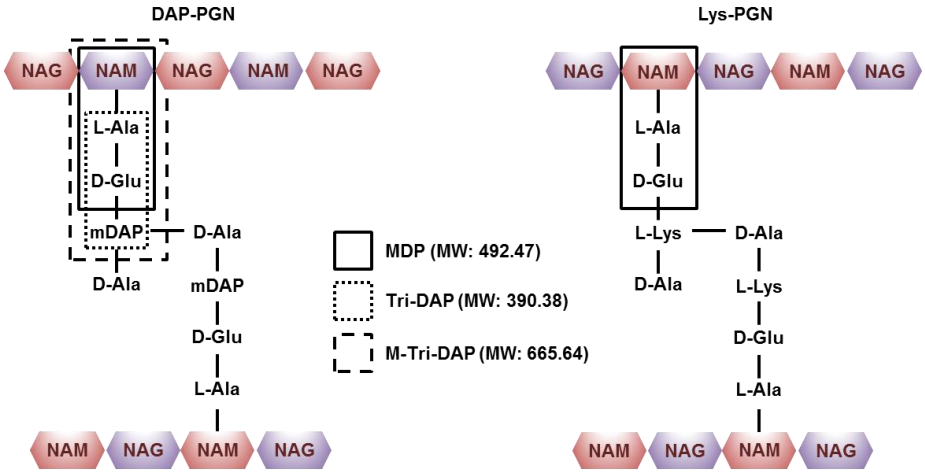


**Figure 4. Schematic illustration of the structures of PGN.** Two types of PGN (DAP-PGN and Lys-PGN) are shown. PGN is composed of a polymer consisting *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) with a short stem peptide. Tri-DAP and MDP act as ligands for NOD1 and NOD2, respectively, and M-Tri-DAP is considered as a ligand for both NOD1 and NOD2.

# 3. Inflammatory mediators

## 3.1. Nitric oxide (NO)

During microbial infection, host cells are activated by the recognition of MAMPs via PRRs and release various inflammatory mediators such as cytokines, chemokines, and NO [85]. These molecules primarily have the potential to protect against microbial infection but can also cause tissue damage and organ failure [86]. In particular, NO, which is synthesized from L-arginine by NO synthases (NOS) including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), has diverse functions in the cardiovascular, nervous, and immune systems, respectively [87]. nNOS and eNOS are constitutively expressed to produce nanomolar concentrations of NO regulating neuronal cell differentiation and microvascular permeability, respectively [88, 89]. In contrast, expression of iNOS is induced by inflammatory stimuli such as MAMPs and produces micromolar levels of NO [90]. High levels of NO induced by persistent activation of iNOS could have serious harmful effects on the host [91, 92].

## 3.2. Cyclooxygenase-2 (COX-2)

During bacterial infection, phospholipase A2 hydrolyzes membrane phospholipids to generate arachidonic acids, which are subsequently modified to various prostaglandins [93]. Two isoforms of cyclooxygenase (COX), COX-1 and COX-2, generate prostaglandins from arachidonic acid. COX-1 is

constitutively expressed in most tissues and cells in the body and is involved in homeostatic regulation. In contrast, COX-2 is expressed at very low steady-state levels and is inducible by pro-inflammatory stimuli including MAMPs [94-96] and danger-associated molecular patterns (DAMPs) [97]. COX-2 promotes the release of prostaglandin E2 (PGE2), which is an important modulator during systemic inflammation. PGE2 has been shown to cause detrimental effects including vasodilation and increased vascular permeability in sepsis [98].

# 4. Biofilm formation

## 4.1. General characteristics of biofilm

Biofilm is a community of microorganisms that is attached to biological and non-biological surfaces, including host tissues such as lung, intestine, heart valves, or tooth, and indwelling medical devices such as catheters. Attached microorganisms grow on a surface and produce extracellular polymeric substances (EPS), in which microorganisms are embedded [99]. The biofilm has been implicated in more than 80% of human microbial infections and involved in various infectious diseases such as endocarditis, sinusitis, gingivitis, cystic fibrosis, urinary tract infections, and osteomyelitis [100]. For these reasons, it is difficult to remove biofilm and this can lead to recurrent infections or chronic inflammation. There are five stages in the biofilm development [101]. Firstly, planktonic bacteria adhere to the biotic or abiotic surfaces, and form micro colonies which produce EPS resulting in irreversible attachment. A biofilm is formed and matured to multi-layered clusters. The biofilm subsequently further matured and exhibits resistance to host defense mechanisms or antibiotics. Finally, the biofilm disperses single bacteria or part of biofilm that are ready to colonize to other surfaces (Figure 5). Bacteria in biofilm are 10-1,000 times more resistant to antibiotics or antimicrobial peptides than planktonic cells. In addition, the biofilm has the ability to avoid phagocytosis by macrophages and neutrophils [7, 8].

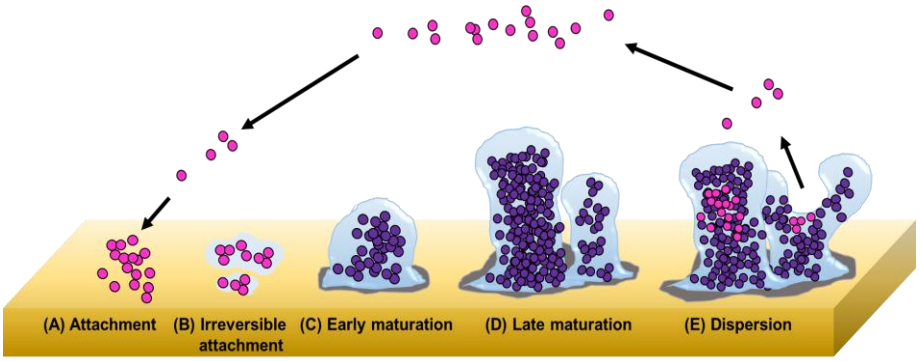


**Figure 5. The five stages of biofilm development.** (A) Initial attachment of planktonic bacteria to surfaces. (B) Irreversible attachment excreting extracellular polymeric substances. (C) Early maturation of biofilm-forming multi-layered clusters. (D) Late maturation of biofilm providing protection against host immune system and antibiotics. (E) The biofilm reaching maximum mass disperses single cells which are ready to colonize to other sites.

### 4.2. *S. aureus* biofilm

Approximately 20-25% of healthy humans are permanently colonized with *S. aureus*, while 75-80% are intermittently colonized or never colonized, which has been associated with increased risk of infection [2]. Invaded *S. aureus* adheres to host tissues and forms a biofilm. *S. aureus* can bind to host extracellular matrix proteins, tissues, or indwelling devices and form a biofilm, which is associated with *S. aureus* causing chronic infectious diseases such as endocarditis, osteomyelitis, and cystic fibrosis [102]. Biofilm increases the emergence of antimicrobial resistance in *S. aureus*. For example, several antibiotics including oxacillin, vancomycin, and cefotaxime decrease penetration throughout *S. aureus* biofilm [103]. Indeed, multidrug-resistant *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), major cause of nosocomial and community acquired infections, has become a public health concern due to limitation of treatment options and increase of morbidity and mortality [4, 5, 104]. Therefore, development of effective therapeutic strategies that can eradicate or atteuate *S. aureus* biofilm formation are required.

#### 4.2.1. Poly-*N*-acetylglucosamine (PNAG) in *S. aureus* biofilm

Although various bacterial or exogenous factors affect staphylococcal biofilm

formation, PNAG-dependent biofilm mechanism is currently the best understood in staphylococci [105, 106]. PNAG is composed of β-1,6-linked *N*-acetylglucosamine residues (80-85%) and 15-20% of the residues are deacetylated and are thus positively charged. Previous reports have demonstrated that the proteins IcaA, IcaB, IcaC, and IcaD are encoded by the intercellular adhesion (*ica*) locus and synthesize PNAG [107]. Firstly, *N*-acetylglucosamine oligomers are synthesized by IcaA and IcaD. Next, IcaC produces longer oligomers and induces translocation of the polysaccharide to the cell surface. The surface-attached protein IcaB is involved in deacetylation of the PNAG [106]. Some environmental factors including glucose, osmolarity, temperature, antibiotics, and ethanol, regulate *ica* gene expression [108, 109]. Moreover, quorum sensing molecule in staphylococci, autoinducer-2, has recently reported as a negative regulator of *ica* gene expression [110, 111].

### 4.2.2. Quorum sensing molecules in *S. aureus* biofilm

Quorum sensing system has been shown to be involved in biofilm formation by several bacteria including *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Burkholderia cepacia*, and others [112-115]. *S. aureus* has two types of quorum sensing system, accessory gene regulator (agr) and LuxS system which have been reported to influence the biofilm development. The *agr* locus has two operons, P2 and P3. The P2 operon contains *agr*BDCA which synthesizes autoinducing peptides (AIP). Accumulating studies suggest that AIP act as a negative regulator of *S. aureus* biofilm. For example, AIP induce detachment

of biofilm via induction of serine proteases in *S. aureus* [116]. The LuxS system is required for production of autoinducer-2 (AI-2). AI-2 is known to be involved in physiological functions in various bacterial species. For example, AI-2 influences motility of *Escherichia coli*, virulence of *Vibrio cholera, S. aureus,* and *S. epidermidis,* and antibiotics susceptibility in *Streptococcus anginosus* [117-121]. Moreover, the LuxS system regulates biofilm development of staphylococci. For example, the luxS-deficient strain of *S. aureus* and *S. epidermidis* showed increased biofilm formation and AI-2 inhibits the biofilm development of these strains [110, 111].

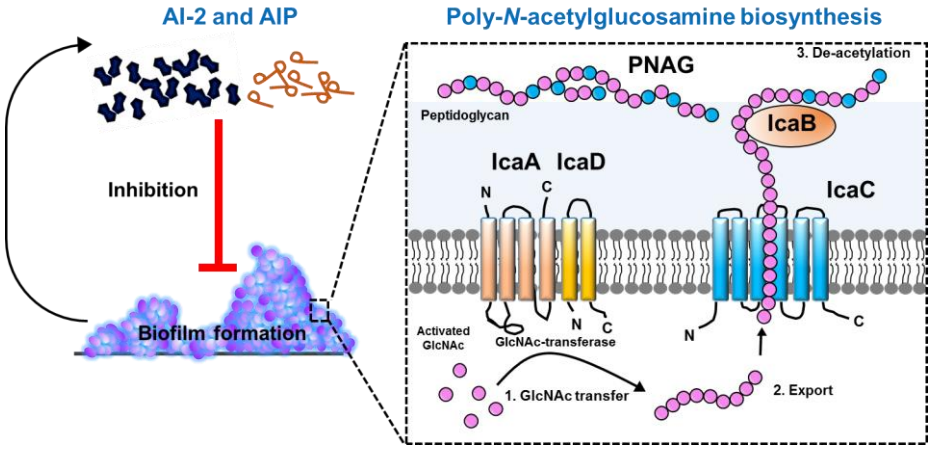


**Figure 6. The role of poly-*N*-acetylglucosamine and quorum sensing molecules in the biofilm formation of *S. aureus*.** *N*-acetylglucosamine oligomers are synthesized by IcaA and IcaD. Next, IcaC produces longer oligomers and induces translocation of the polysaccharide to the cell surface. The surface-attached protein IcaB is involved in deacetylation of the PNAG that is major component of staphylococal biofilm. In contrast, AI-2 and AIP act as negative regulators of *S. aureus* biofilm.

## 5. Aim of the present study

Understanding of the molecular mechanisms by which *S. aureus* induces inflammatory responses and biofilm formation is important for developing effective therapeutic strategies against *S. aureus* infection. The aim of the present study is to investigate the role of cell wall-associated virulence factors of *S. aureus* in the induction of inflammatory responses and biofilm formation. Under the research aim, (i) the role of lipoprotein and LTA in innate immune responses to *S. aureus*, (ii) the role of lipoprotein and LTA in the biofilm formation of *S. aureus*, (iii) molecular mechanisms of lipoprotein or LTA involved inflammatory responses and biofilm formation were investigated.

# Chapter II. Materials and Methods

## 1. Reagents and chemicals

Rabbit polyclonal antibody against iNOS was purchased from Upstate Biotechnology (Lake Placid, NY, USA) and rabbit polyclonal antibodies against STAT-1 or phosphorylated STAT-1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat polyclonal antibody against COX-2, Rabbit polyclonal antibodies against p65, Rabbit polyclonal antibodies TLR2, and anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies against β-actin, Triton X-114, lysostaphin, octyl β-D-glucopyranoside, control rabbit IgG, sulfanilamide, *S. aureus* PGN, Proteinase K and octyl-sepharose beads, and naphthylethylenediamine dihydrochloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Mouse monoclonal antibodies against phosphotyrosine, protein G agarose/salmon sperm DNA, and protein G agarose were purchased from Millipore Co. (Bedford, MA, USA). MDP, Pam2CSK4, and *Escherichia coli* LPS were purchased from InvivoGen (San Diego, CA, USA). Tri-DAP was from Anaspec, Inc. (San Jose, CA, USA). DNase I was purchased from Roche Molecular Biochemicals (Laval, Canada). Texas red-concanavalin A and LIVE/DEAD Bacterial Viability Kit were purchased from Molecular Probes (Eugene, OR, USA).

## 2. Cell culture

The mouse macrophage cell line RAW 264.7 (ATCC TIB-71) was obtained from the American Type Culture Collection (Manassas, VA, USA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml) and cultured at 37℃ in a humidified incubator with 5% $CO_2$. Human embryonic kidney (HEK) 293 cells expressing TLR2 or TLR2/6 were purchased from InvivoGen (San Diego, CA, USA). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of blasticidin (InvivoGen) and maintained at 37℃ in a humidified incubator with 5% $CO_2$.

## 3. Preparation of primary macrophages

Animal ethics approval for all experiments was obtained from the Institutional Animal Care and Use Committee of Seoul National University. C57BL/6 mice were from Orient Bio (Seongnam, Korea), TLR2-deficient C57BL/6 mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) and NOD2-deficient C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Bone marrow cells were isolated by flushing tibiae and femora with phosphate-buffered saline (PBS). Red blood cells were removed using red blood cell lysing buffer (Sigma-Aldrich Chemical Co.). Cells were

differentiated into macrophages in the presence of 10% L929-conditioned medium in complete DMEM containing 50 μM 2-mercaptoethanol. Adherent cells were harvested, resuspended, and plated ($1 \times 10^6$ cells/ml) in cell culture plates and stimulated with various MAMPs for the analysis of COX-2 or NO production. To prepare human monocyte-derived macrophages, peripheral blood mononuclear cells (PBMC) were obtained from the heparinized peripheral blood of healthy donors by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) under the approval by the Institutional Review Board of the Seoul National University. Monocytes were then isolated using I Mag™ anti-human CD14 magnetic beads (BD Biosciences, San Diego, CA, USA). For the generation of macrophages, CD14+ monocytes were cultured for 10 days in RPMI-1640 supplemented with 10% FBS and appropriate antibiotics in the presence 25 ng/ml of GM-CSF.

## 4. Bacterial strains and culture condition

*S. aureus* parental strain RN4220 (wild-type) was grown in Luria-Bertani (LB) broth at 37℃. Strains deficient in *ltaS*, *dltA*, and *lgt* genes coding for LTA synthase, D-alanine ligase, and lipoprotein diacylglycerol transferase, respectively, were used in this study [122-124]. LTA-deficient *S. aureus* (Δ*ltaS*) was grown in LB broth containing 50 μg/ml kanamycin at 30°C. D-alanine-deficient *S. aureus* (Δ*dltA*) and lipoprotein-deficient *S. aureus* (Δ*lgt*) were grown in LB broth containing 10 μg/ml erythromycin at 37°C [122-124]. To

prepare ethanol-killed *S. aureus* (EKSA), the cultured bacteria were incubated with 70% ethanol for 2 h at 4℃ and washed with phosphate-buffered saline (PBS). Complete killing was confirmed by plating on an LB-agar plate overnight. *L. plantarum* KCTC10887BP was obtained from Korean Collection for Type Culture (Daejeon, Korea) and grown in MRS broth at 37℃. Clinical isolates of *S. aureus* ATCC29213, was obtained from American Collection for Type Culture (VA, USA), *S. aureus* NCCP14780 and MRSA NCCP14769 were obtained from the National Culture Collection for Pathogens (Osong, Korea), which were grown in Tryptic Soy Broth (TSB) broth at 37℃. MRSA USA300 wild-type, Δ*agr,* and Δ*luxS* strains were obtained from Nebraska Transposon Mutant Library (Omaha, NE, USA).

## 5. Purification of LTA

LTA was prepared from *S. aureus*, *S. pneumoniae*, *S. gordonii*, *E. faecalis*, *B. subtilis*, and *L. plantarum* as previously described [125]. Bacterial pellets were suspended in 0.1 M sodium citrate buffer (pH 4.7) and lysed by ultrasonication followed by butanol extraction for 30 min. After centrifugation, the aqueous phase was dialyzed against pyrogen-free distilled water and equilibrated with 0.1M sodium acetate buffer containing 15% 1-propanol (pH 4.7) that was subjected to hydrophobic interaction chromatography on an octyl-sepharose CL-4B. The column was eluted with 35% 1-propanol in 0.1 M of the sodium acetate buffer. The fractions containing LTA were collected by phosphate assay, and the pool was subjected to DEAE-sepharose ion-exchange chromatography.

The column was eluted with a linear salt gradient (0 to 1 M NaCl in 0.1 M sodium acetate buffer containing 30% 1-propanol). The fractions containing LTA were collected by phosphate assay, and the pool was subjected to dialysis against pyrogen-free distilled water. Biologically-active molecules such as endotoxins, nucleic acids, or proteins were not detected in the purified LTA preparations. Structural intactness of LTA was confirmed with high-field nuclear magnetic resonance and matrix-assisted laser desorption ionization-time of flight mass spectrometry as previously described [126]. D-alanine-removed Lp.LTA and both of D-alanine and acyl chain-removed Lp.LTA were prepared by incubating intact Lp.LTA with 0.1 M Tris-HCl at pH 8.5 for 24 h and in 0.5 N NaOH for 2 h, respectively.

## 6. Isolation of lipoproteins

Isolation of lipoproteins from *S. aureus* RN4220 (Sa.LPP) was carried out as described previously [127]. *S. aureus* was grown in LB broth for 12 h at 37℃, then bacterial pellets were harvested and suspended in TBS (20 mM Tris-HCl, and 150 mM NaCl, pH 7.6) containing proteinase inhibitors. The lysates of bacteria was re-suspended in a final concentration of 2% Triton X-114 for 2 h at 4℃. After centrifugation, the supernatants were further incubated for 15 min at 37℃. The aqueous phase was discarded and the equal volume of TBS was added to the Triton X-114 phase. After centrifugation, the Triton X-114 phase was mixed with methanol for -20℃ overnight. The precipitated Sa.LPP was dissolved in 10 mM Octyl β-D-glucopyranoside.

## 7. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells ($3 \times 10^5$ cells/ml) were plated on 6-well plates. Cells were stimulated with 20 μg/ml of ethanol-killed wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt S. aureus* or 0.3, 1, or 3 μg/ml of Sa.LTA in the presence or absence of MDP for the indicated time periods. Total RNA was prepared using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. *S. aureus* ($1.5 \times 10^8$ CFU/ml) was stimulated with 10, 30, or 50 μg/ml of Lp.LTA for 3 h. Total RNA was prepared using easy-RED™ BYF Total RNA Extraction Kit (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA with random hexamers and reverse transcriptase (Promega Corporation, Madison, WI, USA). Amplification of cDNA using PCR was in 20 μl containing 0.5 unit of rTaq and 10 picomole of primers specific for iNOS (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), COX-2 (forward primer: 5'-CCCCCACAGTCAAAGACACT-3', reverse primer: 5'-GAGTCCATGTTCCAGGAGGA-3'), β-actin (forward primer: 5'-GTGGGGCGCCCCAGGCACCA-3', reverse primer: 5'-CTCCTTAATGTCACGCACGATTTC-3'), icaA (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), icaB (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-

AATGAGGATGCAAGGCTGG-3'), icaC (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), icaD (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), clfA (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), clfB (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), cna (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), eno (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'), and gyrB (forward primer: 5'-GGATAGGCAGAGATTGGAGG-3', reverse primer: 5'-AATGAGGATGCAAGGCTGG-3'). Amplified PCR products (after 32 cycles for iNOS, 30 cycles for COX-2, 28 cycles for β-actin, and 35 cycles for the remaining genes) were separated on 1.5% agarose gels and visualized by staining with ethidium bromide.

## 8. Western blot analysis

RAW 264.7 cells ($3 \times 10^5$ cells/ml) were treated with indicated stimuli for indicated time periods to examine COX-2 or iNOS expression. In separate experiments, mouse bone marrow-derived macrophages and human monocyte-

derived macrophages ($1 \times 10^6$ cells/ml) were treated with the indicated stimuli for 12 h. Cells were lysed with lysis buffer (20 mM HEPES, 300 mM NaCl, 100 mM KCl, 10 mM EDTA, and 1% Nonidet P-40) and the lysates were obtained by centrifugation at 13,000 × *g* for 10 min. The lystaes were separated by 10% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA) using a tank transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skim milk in TBS (20 mM Tris, and 150 mM NaCl, pH 7.6) at room temperature for 1 h and incubated with primary antibodies specific to COX-2 or iNOS at 4℃ for overnight. After washing three times with TBS containing 0.05% tween 20 (TBST), membranes were incubated with HRP-conjugated secondary antibody in the blocking buffer at room temperature for 1 h. Membranes were washed three times with TBST and immuno-reactive bands were detected with SUPEX ECL solution (Neuronex, Pohang, Korea).

## 9. Measurement of PGE2

RAW 264.7 cells were plated on 12-well plates ($3 \times 10^5$ cells/ml) in 1 ml of media. Cells were stimulated with Sa.LTA (1 μg/ml) in the presence or absence of MDP (1 μg/ml) for 24 h. After incubation, the culture media were collected and analyzed for production of PGE2 by a commercial PGE2 assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

## 10. Measurement of NO

RAW 264.7 cells ($3 \times 10^5$ cells/ml) and bone marrow-derived macrophages ($1 \times 10^6$ cells/ml) were treated with 10, 20, or 50 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 24 h. The culture supernatants were collected, mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated for 5 min at room temperature. The optical density was then measured at 540 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). NO production was determined using a standard curve of $NaNO_2$ optical density.

## 11. Transient transfection and reporter gene assay

RAW 264.7 ($3 \times 10^5$ cells/ml), HEK293-TLR2 ($5 \times 10^5$ cells/ml), or HEK293-TLR2/6 ($5 \times 10^5$ cells/ml) cells were plated in 12-well plates overnight. The cells were transfected with pNF-κB-Luc, pAP-1-Luc, or pCRE-Luc (Clontech, Mountain View, CA, USA) together with the pRL-TK *Renilla* luciferase plasmids (Promega) using a Lipofectamine and Plus reagent (Invitrogen) in serum-free medium for 3 h. The culture medium was replaced with the fresh medium and the cells were further incubated for 21 h, and then, the cells were stimulated with the indicated stimuli for an additional 16 h. For reporter gene assays, the cells were lysed with reporter lysis buffer (Promega). After centrifugation at 13,000 × *g* for 10 min at 4℃, the culture supernatant was transferred to new tubes and firefly and *Renilla* luciferase activities were

analyzed with the Dual Luciferase Reporter Assay System (Promega) using a Victor 1420 Multilabel counter (Perkin Elmer, Waltham, MA, USA). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

## 12. Chromatin immunoprecipitation (ChIP) assay

RAW 264.7 cells ($5 \times 10^5$ cells/ml) were plated on 100-mm dishes and were stimulated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* for 4 h and fixed with 1% formaldehyde for 5 min at 37℃. The cells were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) for 10 min at 4℃, and then DNA was sheared by sonication. 10% of the lysates used for DNA input control and the remaining lysates were diluted 5-fold with a dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, and 167 mM NaCl). Diluted lysates were pre-cleared with protein G agarose/ salmon sperm DNA beads and incubated with anti-p65 antibody, anti-phospho-STAT1 antibody, or control rabbit IgG overnight at 4℃. Immune complexes were collected by adding protein G agarose/ salmon sperm DNA beads and washed five times. Beads were incubated with elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 min at room temperature to obtain chromatin complexes, and then cross-linking of protein-DNA was reversed by incubating with 5 M NaCl for 4 h at 65 ℃. DNA was purified with DNA Clean and Concentrator-5 kit (Zymo Research, CA, USA) and subjected to PCR using specific primers for iNOS promoter (5’ primer: 5’-CTG CCC AAG CTG ACT TAC TAC-3’, 3’ primer: 5’-GAC CCT GGC AGC AGC CAT CAG-3’).

## 13. Immunoprecipitation

RAW 264.7 cells ($5 \times 10^5$ cells/ml) were plated on 100-mm dishes, and were stimulated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* for 30 min and lysed with lysis buffer (20 mM HEPES, 300 mM NaCl, 100 mM KCl, 10 mM EDTA, and 1% Nonidet P-40). The lysates were immunoprecipitated with anti-TLR2 antibody for 16 h, and then protein G-agarose beads were added and incubated for 2 h. The beads were washed three times using the lysis buffer and boiled at 95℃ for 5 min in the sample loading buffer. Immunoprecipitated lysates were separated by 10% SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris and 150 mM NaCl, pH 7.6) at room temperature for 1 h and immunoblotted with anti-phosphotyrosine antibody at 4℃ overnight. After three washes with TBS containing 0.05% Tween 20 (TBST), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer at room temperature for 1 h. The membranes were washed three times with TBST and immunoreactive bands were detected with SUPEX ECL solution (Neuronex, Pohang, Korea).

## 14. Crystal violet staining

*S. aureus* ($5 \times 10^7$ CFU/ml) was grown in 96-well plates at 37℃ for 24 h, planktonic bacteria were removed by gently washing with PBS, and biofilms were stained with 0.1% crystal violet solution for 30 min at room temperature, followed by washes with PBS to remove nonspecific stain. The adhering dye was dissolved with solution (95% ethanol and 0.1% acetic acid), and the absorbance was measured at 600 nm in a microplate reader (Molecular Devices, CA, USA).

## 15. Confocal laser scanning microscopy

*S. aureus* ($5 \times 10^7$ CFU/ml) was grown in coverglass bottom dishes at 37℃ for 24 h in the presence of Lp.LTA (10, 30, or 50 μg/ml), planktonic bacteria were removed by gently washing with PBS, and the adherent bacteria were stained using LIVE/DEAD Bacterial Viability Kit for 5 min at room temperature in the dark, followed by washes with PBS. Biofilms were visualized by LSM700 confocal laser scanning microscope (Zeiss, Jena, Germany).

## 16. Scanning electron microscopy

*S. aureus* ($5 \times 10^7$ CFU/ml) was grown in 24-well plate at 37℃ for 24 h in the presence of Lp.LTA (10 or 30 μg/ml) and then gently washed with PBS to remove planktonic bacteria. The adherent bacteria were prefixed with a PBS containing 2.5% glutaraldehyde and 2% paraformaldehyde (pH 7) at 4 ℃

overnight and washed with PBS. The samples were subsequently fixed with 1% osmium tetroxide for 1.5 h then washed three times with distilled water and dehydrated by replacing the buffer with increasing concentrations of ethanol (70%, 80%, 90%, 95%, 100% each for 15 min and 100% for 15 min). After drying with hexamethyldisilazane and coating with gold sputter, samples were examined using a scanning electron microscope (S-4700, Hitachi, Tokyo, Japan)

## 17. Autoinducer-2 (AI-2) measurement

AI-2 reporter assay was performed according to previously described. *S. aureus* ($1 \times 10^7$ CFU/ml) was grown at 37℃ for 24 h in the presence of Lp.LTA (10, 30, or 50 μg/ml), then culture supernatants were obtained using centrifugation and filtered with 0.2 μm pore size filter (Millipore, MA, USA). *S. aureus* culture supernatants (20 μl) were added into white 96-well plates. To examine bioluminescence, *V. harveyi* BB170 strain grown in AB medium for 16 h at 30℃, was diluted 1:5,000 in fresh AB medium and 180 μl of *V. harveyi* BB170 was added to each sample. Luminescence was measured by GloMax microplate luminometer (promega, WI, USA).

## 18. Statistical analysis

All experiments were performed at least three times. The mean value ± standard deviation (S.D.) was obtained from triplicate samples for each treatment group. Statistical significance was examined with a *t*-test. Asterisks (*) indicate treatment groups that were significantly different from the control group at $P < 0.05$.

# Chapter III. Results

## 1. Role of cell wall-associated virulence factors of *S. aureus* in the inflammation

## 1.1. Role of lipoproteins of *S. aureus* on the production of nitric oxide

### 1.1.1. Lipoprotein-deficient *S. aureus* does not induce NO production in macrophages

To determine which cell wall virulence factors are involved in *S. aureus*-induced NO production in macrophages, RAW 264.7 cells were treated for 24 h with 10, 20, 50, or 100 μg/ml of ethanol-killed wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt S. aureus*. As shown in Figure 7A, wild-type, Δ*ltaS*, and Δ*dltA* strains induced an increase in NO in a dose-dependent manner, whereas Δ*lgt* completely failed to do so. Similar results were observed in mouse bone marrow-derived macrophages (Figure 7B). Then, to examine the effects of isolated lipoproteins from *S. aureus* (Sa.LPP) on NO production, RAW264.7 cells were treated with 1, 10, or 100 μg/ml of *S. aureus* lipoproteins for 24 h and the culture supernatants were collected to determine NO production. As shown in Figure 7C, isolated lipoproteins significantly induced NO production in a dose-dependent manner. These results suggest that lipoproteins are an essential component of *S. aureus* in the induction of NO production by macrophages.

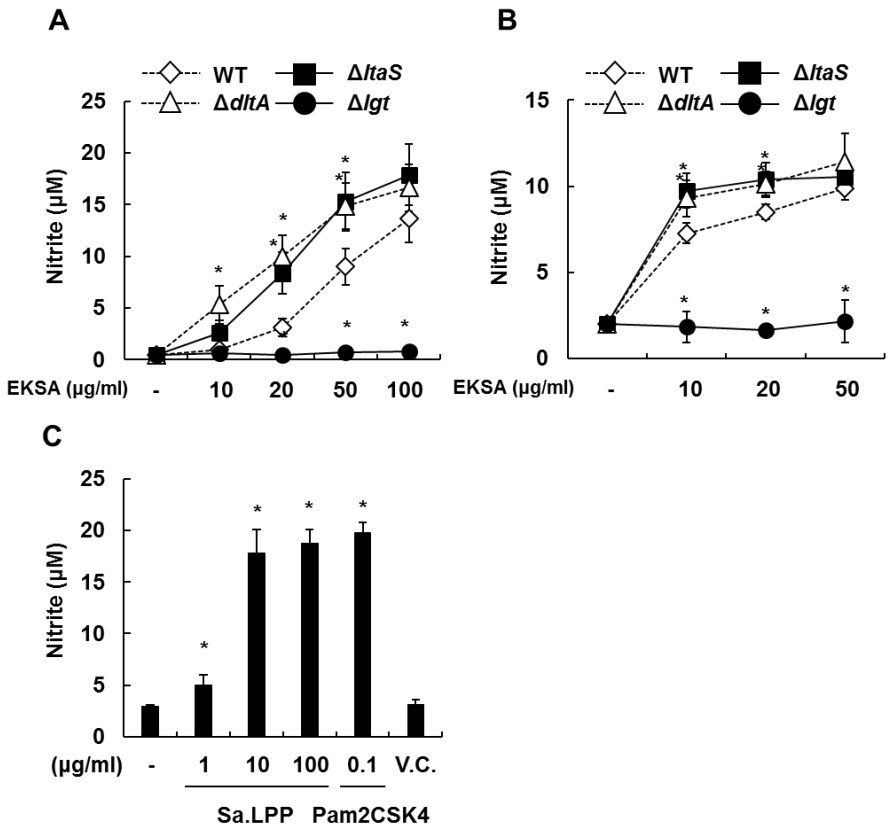


**Figure 7. Lipoprotein-deficient *S. aureus* does not induce NO production in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 10, 20, 50, or 100 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 24 h. (B) Mouse bone marrow-derived macrophages ($1 \times 10^6$ cells/ml) were stimulated with 10, 20, or 50 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 24 h. (C) RAW 264.7 cells ($3\times 10^5$ cells/ml) were treated with 1, 10, or 100 μg/ml of *S. aureus* lipoproteins or 0.1 μg/ml of Pam2CSK4 as a positive control for 24 h. PBS containing Octyl β-D-glucopyranoside was used as a vehicle control (V.C.). At the end of the incubation period, culture supernatants were collected to determine NO production. Data are the mean ± S.D. of triplicate results. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type *S. aureus* treatment group.

### 1.1.2. Lipoprotein-deficient *S. aureus* does not induce iNOS protein or mRNA expression in RAW 264.7 cells

Next, the expression of iNOS as an enzyme catalyzing the micromolar production of NO was examined. RAW 264.7 cells were stimulated with 20 μg/ml of ethanol-killed *S. aureus* (wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt*) and the level of iNOS protein was examined. iNOS protein expression was increased in cells treated with wild-type, Δ*ltaS*, and Δ*dltA*, but not in those treated with Δ*lgt* (Figure 8A). The *iNOS* mRNA level was also evaluated to determine whether *S. aureus*-induced iNOS protein expression was due to an increase in expression of *iNOS* mRNA. *iNOS* mRNA expression was increased by treatment with wild-type, Δ*ltaS*, and Δ*dltA* strains, but not by Δ*lgt* (Figure 8B). These results further confirmed that lipoproteins are an essential virulence factor in *S. aureus*-induced iNOS expression in macrophages.

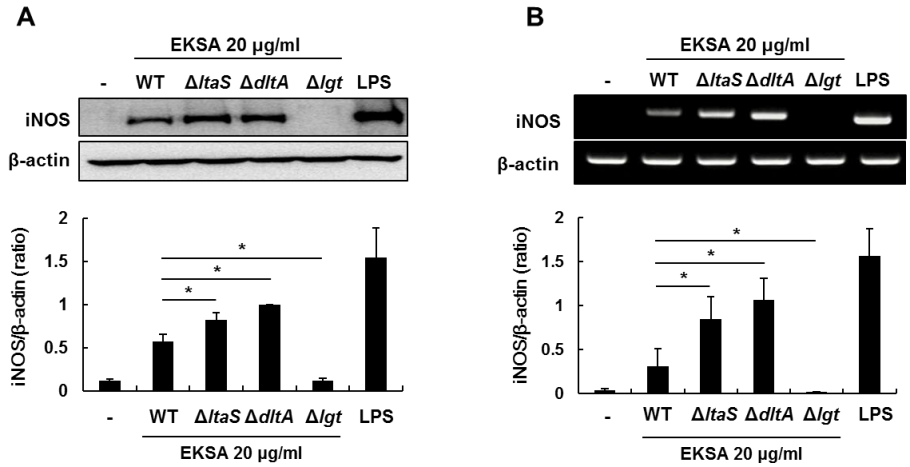


**Figure 8. Lipoprotein-deficient *S. aureus* does not induce iNOS protein and mRNA expression in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 20 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 12 h. The cells were lysed and subjected to Western blotting to measure iNOS protein. One of three similar results is shown. (B) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 20 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 4 h. Total RNA was isolated and the expression of *iNOS* and *β-actin* mRNA was examined by RT-PCR. One of three similar results is shown. The bottom panel shows quantification of iNOS and β-actin protein and mRNA bands using a densitometer. The ratio of iNOS to β-actin represents the relative level of iNOS normalized to that of β-actin in individual groups. Data are the mean ± S.D. of triplicate results. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type *S. aureus* treatment group.

### 1.1.3. Lipoprotein-deficient *S. aureus* does not induce NF-κB activation

NF-κB is known to be important transcription factors for iNOS expression in various cell types including intestinal epithelial cells and macrophages [128, 129]. To identify which cell wall component is involved in *S. aureus*-induced NF-κB activation, RAW 264.7 cells were treated with ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* and the transcriptional activity of NF-κB was measured by luciferase reporter gene assay. As shown in Figure 9A, NF-κB transcriptional activity was significantly increased in cells treated with wild-type, Δ*ltaS*, or Δ*dltA* whereas no change was observed after treatment with Δ*lgt*. Furthermore, in order to determine whether NF-κB was recruited to the iNOS promoter, ChIP assay was performed in RAW 264.7 cells treated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* for 4 h. As shown in Figure 9B, ethanol-killed *S. aureus* wild-type induce p65 binding to the iNOS promoter whereas Δ*lgt* failed to recruit these transcription factors to the iNOS promoter, indicating that *S. aureus* lipoproteins induce direct binding of NF-κB to iNOS promoter in RAW 264.7 cells.

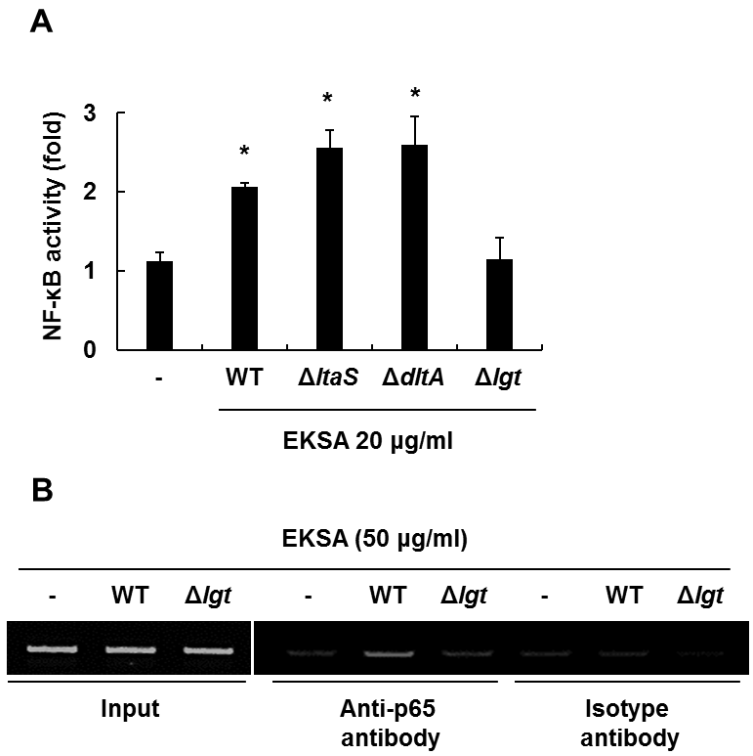


**Figure 9. Lipoprotein-deficient *S. aureus* does not induce NF-κB activation in macrophages.** (A) RAW 264.7 cells ($5 \times 10^5$ cells/ml) were co-transfected with firefly luciferase reporter plasmid regulated by NF-κB transcription factor and pRL-TK renilla luciferase plasmid as an internal control of transfection efficiency for 24 h. The cells were stimulated with 20 μg/ml of ethanol-killed wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 15 h. After stimulation, the cells were lysed and dual luciferase activities were measured. Firefly luciferase activity was normalized to renilla luciferase activity for each sample. Data are the mean ± S.D. of triplicate results. Asterisks indicate significant induction at $P < 0.05$ compared with wild-type *S. aureus* treatment group. (B) RAW 264.7 cells ($5 \times 10^5$ cells/ml) were treated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* strains for 4 h. Then, the cells were fixed and harvested to perform ChIP assay. The cells were lysed and subjected to chromatin immunoprecipitation with anti-p65 antibody. PCR amplification was performed using primers specific for iNOS promoter.

### 1.1.4. Lipoprotein-deficient *S. aureus* does not induce STAT-1 phosphorylation and IFN-β expression

STAT-1 is known to be important transcription factors for iNOS expression in various cell types including intestinal epithelial cells and macrophages. To examine STAT-1 activation, RAW 264.7 cells were treated with ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 120 min and STAT-1 phosphorylation was analyzed by Western blotting. Treatment with wild-type, Δ*ltaS*, and Δ*dlt S. aureus* increased phosphorylation of STAT-1, whereas Δ*lgt S. aureus* had no effect (Figure 10A). Furthermore, in order to determine whether phosphorylated STAT-1 (pSTAT-1) were recruited to the iNOS promoter, ChIP assay was performed in RAW 264.7 cells treated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* for 4 h. As shown in Figure 10B, ethanol-killed *S. aureus* wild-type induce pSTAT-1 binding to the iNOS promoter whereas Δ*lgt* failed to recruit these transcription factors to the iNOS promoter, indicating that *S. aureus* lipoproteins induce direct binding of pSTAT-1 to iNOS promoter in RAW 264.7 cells. The STAT-1 pathway is activated by IFN-β during LPS-induced NO production [130]. IFN-β mRNA expression in RAW 264.7 cells was increased by wild-type, Δ*ltaS*, and Δ*dltA*, but not by Δ*lgt S. aureus* (Figure 10C).

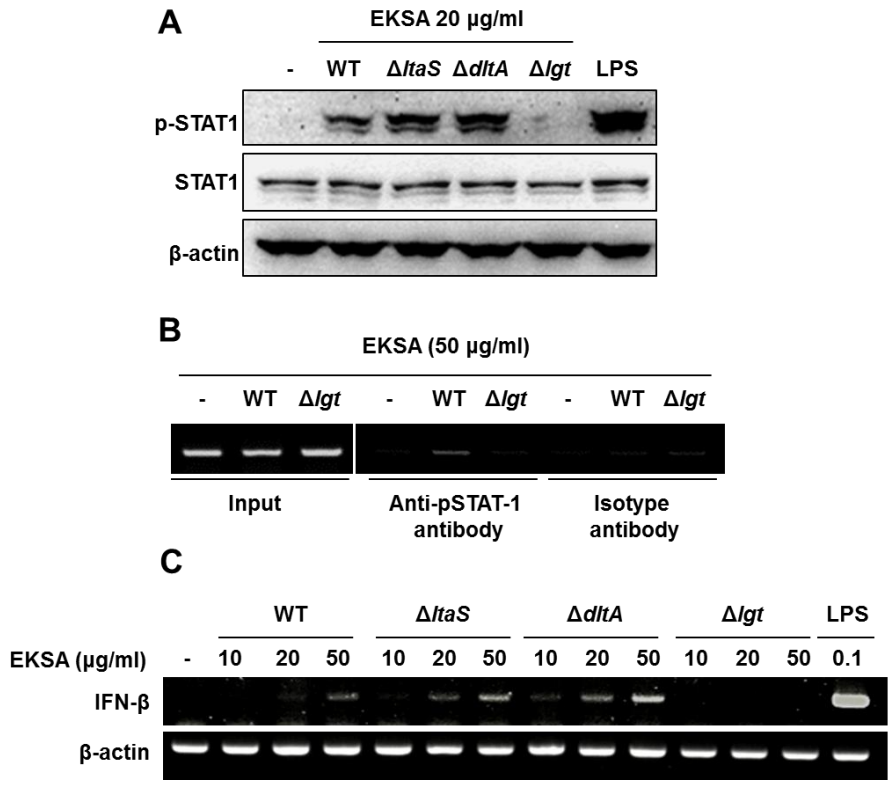


**Figure 10. Lipoprotein-deficient *S. aureus* does not induce NF-κB or STAT-1 activation in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 20 μg/ml ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 120 min. The cells were lysed and subjected to Western blotting to measure phosphorylated STAT-1 and total STAT-1. (B) RAW 264.7 cells ($5 \times 10^5$ cells/ml) were treated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* strains for 4 h. Then, the cells were fixed and harvested to perform ChIP assay. The cells were lysed and subjected to chromatin immunoprecipitation with anti-pSTAT-1 antibody. PCR amplification was performed using primers specific for iNOS promoter. (C) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 10, 20, or 50 μg/ml of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 4 h. Total RNA was isolated and the expression of IFN-β and β-actin mRNA was examined by RT-PCR. One of three similar results is shown.

### 1.1.5. Lipoprotein-deficient *S. aureus* does not induce TLR2 activation

Both LTA and lipoproteins from *S. aureus* are sensed by TLR2, but not by TLR4, receptors on the host immune cells to elicit inflammation [3, 131]. To identify which cell wall virulence factors of *S. aureus* are predominantly recognized by TLR2, CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were treated with ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 24 h. Treatment with wild-type, Δ*ltaS*, and Δ*dltA* increased TLR2 activation, whereas Δ*lgt* did not (Figure 11A). Moreover, TLR4 activation was not observed in CHO/CD14/TLR4 cells treated with any of the *S. aureus* strains (Figure 11B). Next, it was examined whether ethanol-killed *S. aureus* wild-type-induced TLR2 activation is attributed to either homodimerization of TLR2 or heterodimerization of TLR2 with TLR6. HEK293-TLR2 and -TLR2/6 cells were transiently transfected with NF-κB luciferase reporter plasmids and then, stimulated with 5, 20, or 50 μg/ml of *S. aureus* wild-type or Δ*lgt* for 16 h. As shown in Figure 11C, treatment with ethanol-killed *S. aureus* wild-type enhanced NF-κB activation in both HEK293-TLR2 and -TLR2/6 cells, but *S. aureus* Δ*lgt* failed to induce NF-κB activation in both HEK293-TLR2 and HEK293-TLR2/6 cells. Moreover, *S. aureus* wild-type more strongly induced NF-κB activation in HEK293-TLR2/6 cells than in HEK293-TLR2 cells, indicating that *S. aureus* lipoproteins induce TLR2 activation through TLR2/6 heterodimerization. Additionally, it was examined whether stimulation with *S.*

*aureus* lipoproteins induce the TLR2 phosphorylation. RAW264.7 cells were treated with *S. aureus* wild-type or Δ*lgt* (50 μg/ml) for 30 min and then, cell lysates were immunoprecipitated with anti-TLR2 antibody followed by Western blotting with anti-phosphotyrosine antibody. As shown in Figure 11D, *S. aureus* wild-type induced TLR2 tyrosine phosphorylation, whereas *S. aureus* Δ*lgt* had no effect, suggesting that lipoproteins of *S. aureus* can induce TLR2 tyrosine phosphorylation in RAW264.7 cells. These results imply that lipoproteins are the major cell wall component of *S. aureus* that induces activation of TLR2.

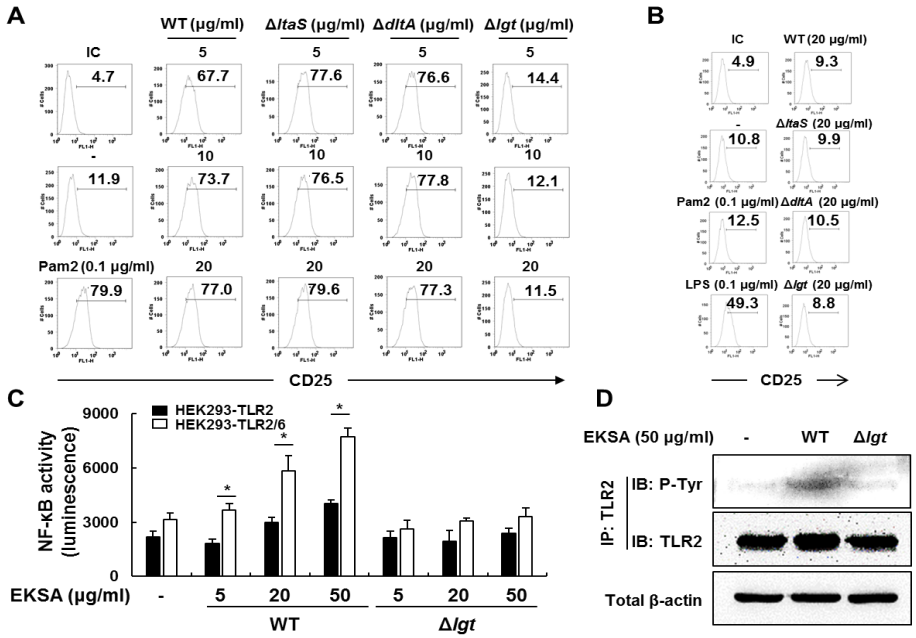


**Figure 11. Lipoprotein-deficient *S. aureus* does not induce TLR2 activation.** (A) CHO/CD14/TLR2 or (B) CHO/CD14/TLR4 cells at 2 × $10^5$ cells/ml were stimulated with the indicated concentrations of ethanol-killed *S. aureus* wild-type, Δ*ltaS*, Δ*dltA*, or Δ*lgt* for 24 h. CD25 expression resulting from TLR2- or TLR4-dependent NF-κB activation was analyzed using flow cytometric analysis. Pam2CSK4 and *E. coli* LPS were used as positive controls for TLR2 and TLR4 activation, respectively. The percentage of CD25-positive cells is shown in each histogram. One of three similar results is shown. (C) HEK-TLR2 and HEK-TLR2/6 cells (5× $10^5$ cells/ml) were transiently transfected with luciferase reporter plasmids regulated by NF-κB together with pRL-TK *Renilla* lucifease plasmids. Then, the cells were treated with *S. aureus* wild-type or Δ*lgt* (5, 20, or 50 μg/ml) for 16 h. The cells were lysed and subject to dual luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are the mean values ± S.D. of triplicated samples. An asterisk indicates a significant difference at $P < 0.05$ compared with *S. aureus*-stimulated cells. One of three similar results is shown. (D) RAW 264.7 cells (5 × $10^5$ cells/ml) were treated with 50 μg/ml of ethanol-killed *S. aureus* wild-type or Δ*lgt* strains for 30 min. Then, the cells were lysed and immunoprecipitated with anti-TLR2 antibody followed by Western blotting with anti-phosphotyrosine antibody. β-actin was used for loading control.

## 1.2. Role of *S. aureus* LTA and PGN on the production of COX-2

### 1.2.1. *S. aureus* LTA with staphylococcal PGN synergistically induces COX-2 expression

To determine if Sa.LTA-induced COX-2 expression is enhanced by Sa.PGN, RAW 264.7 cells, mouse primary macrophages, and human primary macrophages were treated with LTA together with the Sa.PGN. As shown in Figure 12A-C, synergistic induction of COX-2 was observed when cells were co-treated with Sa.LTA and Sa.PGN. These results suggest that Sa.PGN enhances the ability of Sa.LTA to induce the production of COX-2 in macropahges.

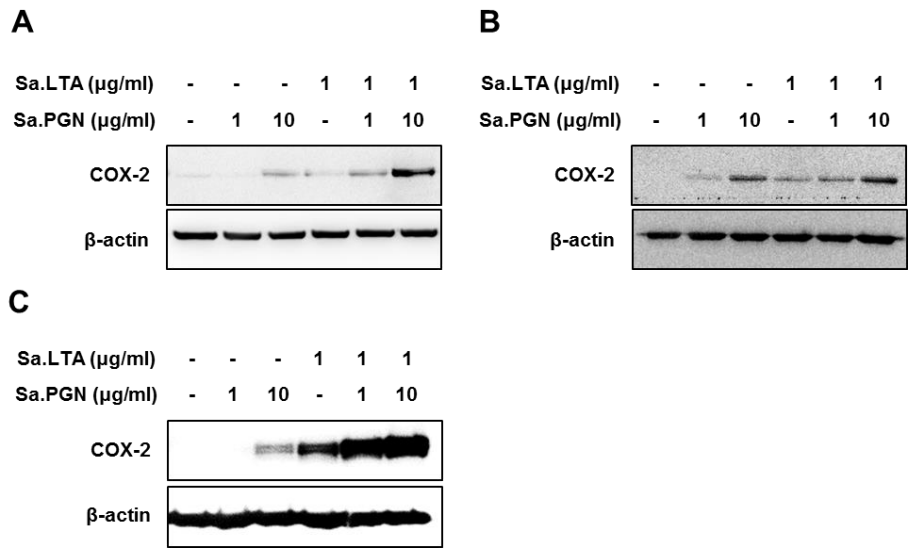


**Figure 12. *S. aureus* LTA with PGN synergistically induces COX-2 expression in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml), (B) Mouse bone marrow-derived macrophages ($1 \times 10^6$ cells/ml), and (C) Human monocyte-derived macrophages ($1 \times 10^6$ cells/ml) were stimulated with Sa.LTA (1 μg/ml) in the presence or absence of Sa.PGN (1 or 10 μg/ml) for 12 h. Cells were lysed and subjected to Western blotting for the determination of COX-2.

### 1.2.2. NOD2 ligand, but not NOD1 ligand, enhances *S. aureus* LTA-induced COX-2 expression

PGN is recognized by the intracellular receptors NOD1 and NOD2. NOD2 senses MDP in the PGN of both Gram-positive and Gram-negative bacteria whereas NOD1 senses Tri-DAP, which is exclusively found in Gram-negative bacteria. To determine if Sa.LTA-induced COX-2 expression is enhanced by NOD1 ligand or NOD2 ligand, RAW 264.7 cells were treated with LTA together with the representative NOD1 ligand Tri-DAP, the NOD2 ligand MDP, or a NOD1/2 ligand M-Tri DAP. Interestingly, a cooperative effect was observed only with MDP or M-Tri-DAP but not with Tri-DAP as determined by Western blotting (Figure 13B) and RT-PCR (Figure 13C). Next, it was further examined whether Sa.LTA-induced COX-2 expression is enhanced by the NOD2 ligand MDP in mouse and human primary macrophages. Sa.LTA induced-COX-2 expression was synergistically enhanced by MDP or M-Tri-DAP, but not by Tri-DAP in mouse primary macrophages (Figure 13D). Similarly, both MDP and M-Tri-DAP enhanced Sa.LTA-induced COX-2 expression in human primary macrophages (Figure 13E). These results suggest that NOD2, but not NOD1, signaling enhances Sa.LTA-induced COX-2 expression.

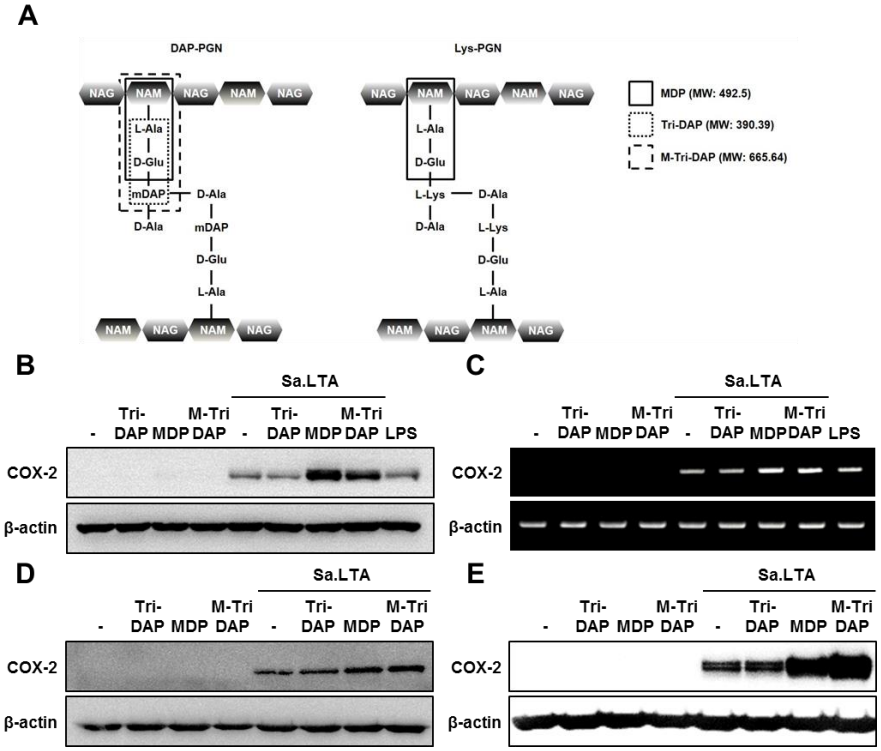


**Figure 13. NOD2 ligand enhances *S. aureus* LTA-induced COX-2 expression.** (A) Schematic illustration of the structures of PGN. Two types of PGN (DAP-PGN and Lys-PGN) are shown. PGN is composed of a polymer consisting *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) with a short stem peptide. Tri-DAP and MDP act as ligands for NOD1 and NOD2, respectively, and M-Tri-DAP is considered as a ligand for both NOD1 and NOD2. (B, C) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with Sa.LTA (1 μg/ml) in the presence or absence of Tri-DAP (10 μg/ml), MDP (10 μg/ml), or M-Tri-DAP (10 μg/ml) for 12 h. (B) Cells were lysed and subjected to Western blotting for determination of COX-2 or (C) total RNA was isolated from cells for analysis of COX-2 mRNA expression. One of three similar results is shown. (D) Mouse bone marrow-derived macrophages ($1 \times 10^6$ cells/ml) or (E) human monocyte-derived macrophages ($1 \times 10^6$ cells/ml) were stimulated as described above and COX-2 expression was determined using Western blot analysis. One of three similar results is shown.

### 1.2.3. MDP enhances *S. aureus* LTA-induced COX-2 and PGE2 production

To examine if MDP potentiates the ability of Sa.LTA to induce COX-2 expression, RAW 264.7 cells were stimulated with Sa.LTA in the presence or absence of MDP. As shown in Figure 14A, Sa.LTA, but not MDP, alone was sufficient to induce COX-2 protein expression. MDP, however, further enhanced the COX-2 expression induced by Sa.LTA. Time-course experiments demonstrated that COX-2 protein reached a peak at 12 h post stimulation (Figure 14B). Next, PGE2 expression as an indication of the functional activity of COX-2 was examined in the cells treated with LTA and MDP. Figure 14C shows that MDP augmented the release of PGE2 induced by Sa.LTA. These results suggest that MDP enhances the ability of Sa.LTA to induce the production of COX-2 and PGE2 in RAW 264.7 cells.

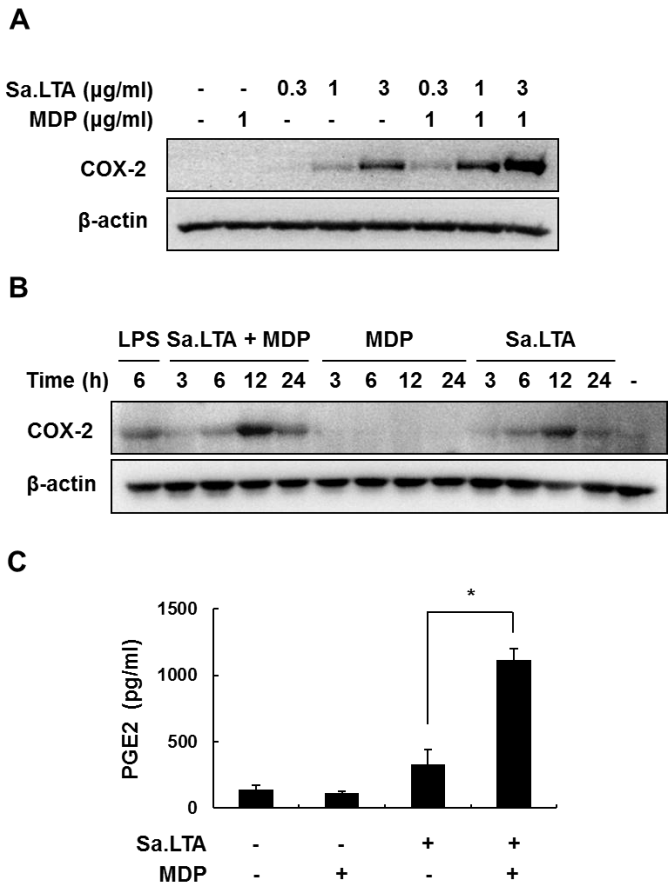


**Figure 14. MDP enhances *S. aureus* LTA-induced COX-2 expression in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with 0, 0.3, 1, or 3 μg/ml of Sa.LTA in the presence or absence of MDP (1 μg/ml) for 12 h. Cells were lysed and subjected to Western blotting for the determination of COX-2. One of three similar results is shown. (B) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with Sa.LTA (1 μg/ml) and/or MDP (1 μg/ml) for 3, 6, 12, or 24 h. The cells were lysed and subjected to Western blotting for the determination of COX-2. One of three similar results is shown. (C) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with LTA (1 μg/ml) and/or MDP (1 μg/ml) for 24 h. PGE2 release was measured in the culture media by ELISA. Data are the mean values ± S.D. of triplicated samples. Asterisk indicates a significant induction at $P < 0.05$ compared with Sa.LTA-treated group.

### 1.2.4. MDP enhances *S. aureus* LTA-induced COX-2 mRNA expression

Next, it was examined whether the enhanced induction of COX-2 protein expression was due to an increase in COX-2 mRNA. Figure 15A shows that LTA alone enhanced COX-2 mRNA levels whereas MDP alone did not induce COX-2 mRNA. However, treatment with LTA plus MDP markedly enhanced COX-2 mRNA levels. Stimulating the cells with LTA (1 μg/ml) in the presence or absence of MDP (1 μg/ml) for various time periods showed that the Sa.LTA-induction of COX-2 mRNA was enhanced by MDP in a time-dependent manner (Figure 15B). These results indicate that the increase in COX-2 occurred at the transcriptional level.

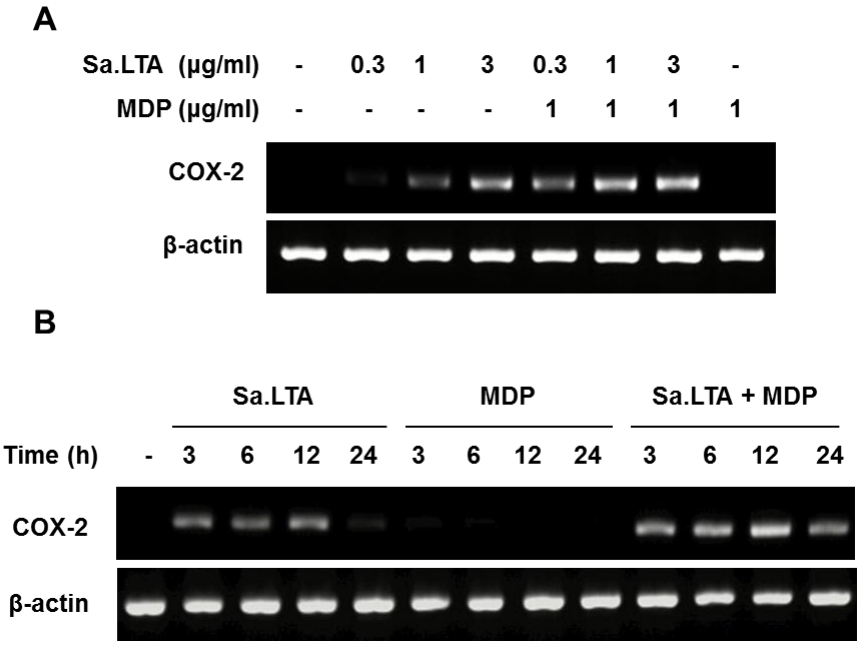


**Figure 15. MDP enhances *S. aureus* LTA-induced COX-2 mRNA expression in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with Sa.LTA (0, 0.3, 1, or 3 μg/ml) in the presence or absence of MDP (1 μg/ml) for 6 h. One of three similar results is shown. (B) RAW 264.7 cells ($3 \times 10^5$ cells/ml) were stimulated with Sa.LTA (1 μg/ml) and/or MDP (1 μg/ml) for 3, 6, 12, or 24 h. Total RNA was isolated and COX-2 mRNA levels were examined by RT-PCR. One of three similar results is shown.

### 1.2.5. TLR2 and NOD2 are necessary for the synergistic induction of COX-2 in macrophages treated with LTA and MDP

LTA and MDP are primarily recognized by the TLR2 and NOD2, respectively. To determine whether TLR2 and NOD2 are necessary for the LTA/MDP-induced production of COX-2, bone marrow macrophages (BMMs) derived from wild-type, TLR2-deficient, or NOD2-deficient mice were treated with Sa.LTA in the presence or absence of MDP for 12 h. As shown in Figure 16, MDP enhanced Sa.LTA-induced COX-2 protein expression in wild-type BMMs, a pattern similar to that observed in RAW 264.7 cells. However, MDP failed to increase the Sa.LTA-induced COX-2 expression in NOD2-deficient and TLR2-deficient BMMs. These data suggest that TLR2 and NOD2 are essential for LTA/MDP induction of COX-2 expression.

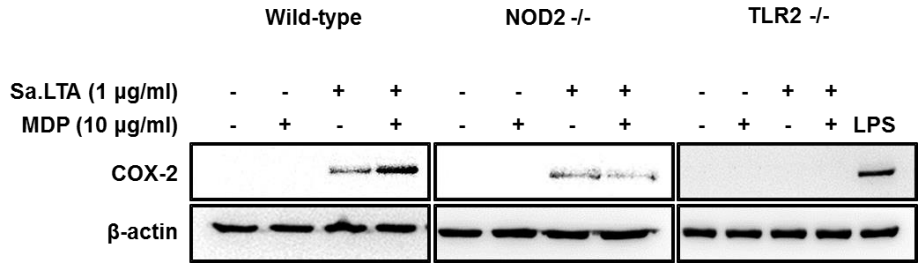


**Figure 16. TLR2 and NOD2 are essential for LTA/MDP-induced COX-2 expression.** Bone marrow macrophages ($1 \times 10^6$ cells/ml) were prepared from wild-type, NOD2-deficient, and TLR2-deficient mice and stimulated with Sa.LTA (1 μg/ml) in the presence or absence of MDP (10 μg/ml) for 12 h. Then, the cells were lysed and Western blotting was performed for the determination of COX-2. One of three similar results is shown.

### 1.2.6. MDP enhances the activation of *S. aureus* LTA-induced NF-kB and CRE transcription factors

The promoter region of the COX-2 gene contains *cis*-acting binding sequences for NF-κB, CRE, and AP-1, which are known to modulate COX-2 gene expression [132]. The transcriptional activity of NF-κB, CRE, and AP-1 was examined in RAW 264.7 cells treated with Sa.LTA and MDP using a transient transfection and luciferase reporter gene assay. As shown in Figure 17A and 17B, Sa.LTA increased the activity of NF-κB and CRE transcription factors, and MDP further enhanced this effect. In addition, cooperative effects between Sa.LTA and MDP were seen for the AP-1 transcription factor (Figure 17C), statistically no significant ($P > 0.05$). These results suggest that the activation of NF-κB and CRE contributes to the enhanced expression of COX-2 in Sa.LTA/MDP-stimulated cells.

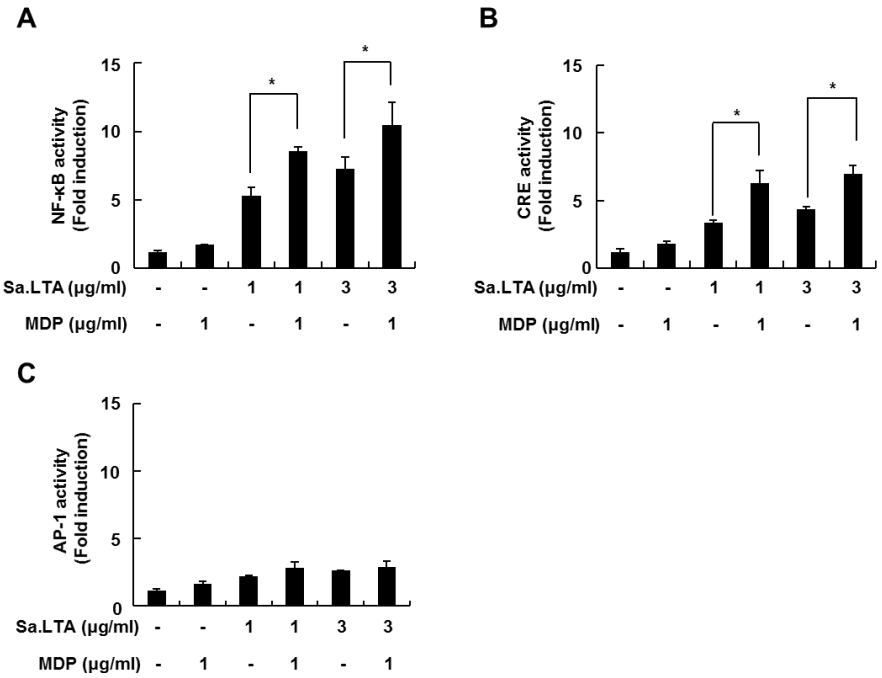


**Figure 17. MDP enhances *S. aureus* LTA-induced NF-κB and CRE activation in macrophages.** RAW 264.7 cells ($3 \times 10^5$ cells/ml) were transiently transfected with reporter plasmids encoding luciferase regulated by (A) NF-κB, (B) CRE, or (C) AP-1 together with pRL-TK *Renilla* luciferase plasmid. Then, the cells were stimulated with Sa.LTA (1 or 3 μg/ml) and/or MDP (1 μg/ml) for 16 h. The cells were lysed and subjected to dual luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are the mean values ± S.D. of triplicated samples. Asterisk, significant induction at $P < 0.05$ compared with Sa.LTA-treated groups. One of three similar results is shown.

### 1.2.7. *S. aureus* LTA, but not *Bacillus subtilis* LTA has no synergistic effects with MDP on COX-2 expression

Next, the synergistic effect of Sa.LTA and MDP with Bs.LTA and MDP were compared on the COX-2 expression in macrophages. As shown in Figure 18A-C, Synergistic induction of COX-2 by MDP was observed in the treatment with Sa.LTA but not with Bs.LTA in macrophages. These results suggest that the synergistic effect of LTA and MDP is dependent on the bacterial strains producing LTA. Moreover, the differential immuno-stimulating activity between Sa.LTA and Bs.LTA may be due to their structural differences.

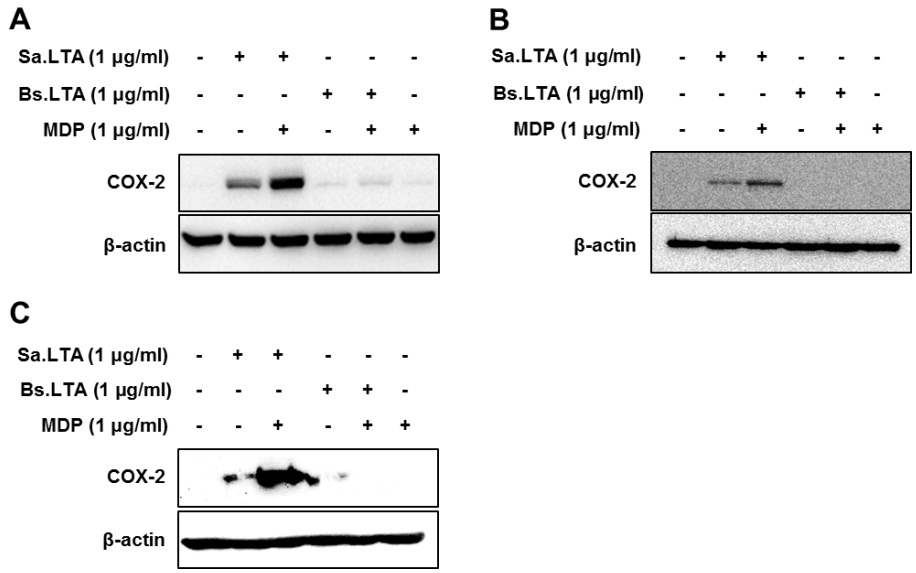


**Figure 18. *S. aureus* LTA, but not *B. subtilis* LTA has synergistic effects with MDP on COX-2 expression in macrophages.** (A) RAW 264.7 cells ($3 \times 10^5$ cells/ml), (B) Mouse bone marrow-derived macrophages ($1 \times 10^6$ cells/ml), and (C) Human monocyte-derived macrophages ($1 \times 10^6$ cells/ml) were stimulated with Sa.LTA (1 μg/ml) or *B. subtilis* LTA (Bs.LTA) together with MDP (1 μg/ml) for 12 h. Then, the cells were lysed and subjected to Western blotting for the determination of COX-2.

# 2. Role of cell wall components in the *S. aureus* biofilm formation

## 2.1. Role of cell wall components of *S. aureus* in the regulation of biofilm formation

### 2.1.1. Time-dependent release of LTA from *S. aureus* differentially regulate the biofilm formation of *S. aureus*

To determine how much LTA is released from *S. aureus* to the culture supernatants in the planktonic or biofilm state, the presence of LTA was examined in the harvested culture supernatants at various times. For LTA detection, 20 μl of each culture supernatant was used. LTA was highly found from culture supernatants at 24 h to culture supernatants at 72 h under shaking culture condition, whereas LTA was slightly found in culture supernatants at 24 h and was increased in culture supernatants at 48 h and 72 h under static culture condition (Figure 19A). Next, the effect of the culture supernatants collected from each time point were investigated on the biofilm formation of *S. aureus*. Interestingly, the culture supernatants containing LTA, but not culture supernatants without LTA inhibited biofilm formation of *S. aureus* (Figure 19B). Thus, LTA might be a major key regulatory molecule in these culture supernatants for inhibition of biofilm formation.

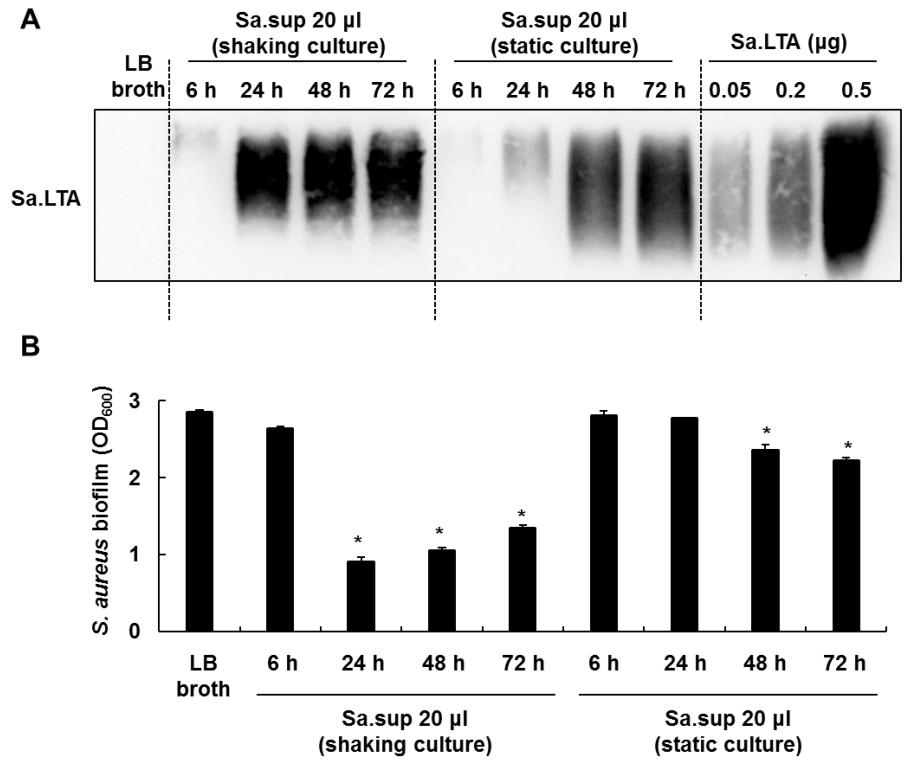


**Figure 19. Time-dependent release of LTA from *S. aureus* differentially regulate the biofilm formation of *S. aureus*.** (A) The culture supernatants of *S. aureus* were obtained at 6, 24, 48, or 48 h in shaking or static culture condition and 20 μl of culture supernatants were subjected to Western blotting to measure LTA in the culture supernatants. One of three similar results is shown. (B) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown in polystyrene plates at 37℃ for 24 h in the presence of the culture supernatants of *S. aureus* obtained at 6, 24, 48, or 48 h in shaking or static culture condition. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with the non-treatment group.

### 2.1.2. LTA is an important molecule in the culture supernatants of *S. aureus* for inhibiting *S. aureus* biofilm formation

To determine if endogenous LTA released from *S. aureus* affects the bacterial biofilm formation, the culture supernatants were harvested from wild-type *S. aureus* and Δ*ltaS S. aureus* at 24 h incubation to detect the presence of LTA. LTA was highly detected in 24 h culture supernatants of wild-type *S. aureus*, whereas LTA was not detected in the culture supernatants of Δ*ltaS S. aureus* under the same condition (Figure 20A). Next, the effect of the 6 h and 24 h culture supernatants of wild-type *S. aureus* and Δ*ltaS S. aureus* were examined on the biofilm formation of *S. aureus*. The wild-type *S. aureus* 24 h culture supernatants inhibited biofilm formation, but the Δ*ltaS S. aureus* culture supernatants did not inhibit biofilm formation (Figure 20B). These results indicate that released LTA interferes with the development of biofilm of *S. aureus*. In addition, the role of the D-alanine in the inhibitory effects of LTA against biofilm of *S. aureus* was investigated via comparison of the effects of culture supernatants of wild-type and Δ*dltA S. aureus*. As shown in Figure 20D, culture supernatants of Δ*dltA S. aureus* did not show the inhibitory effect on the biofilm formation indicating that D-alanine residues of LTA are crucial for the inhibitory effects of LTA on the biofilm formation.

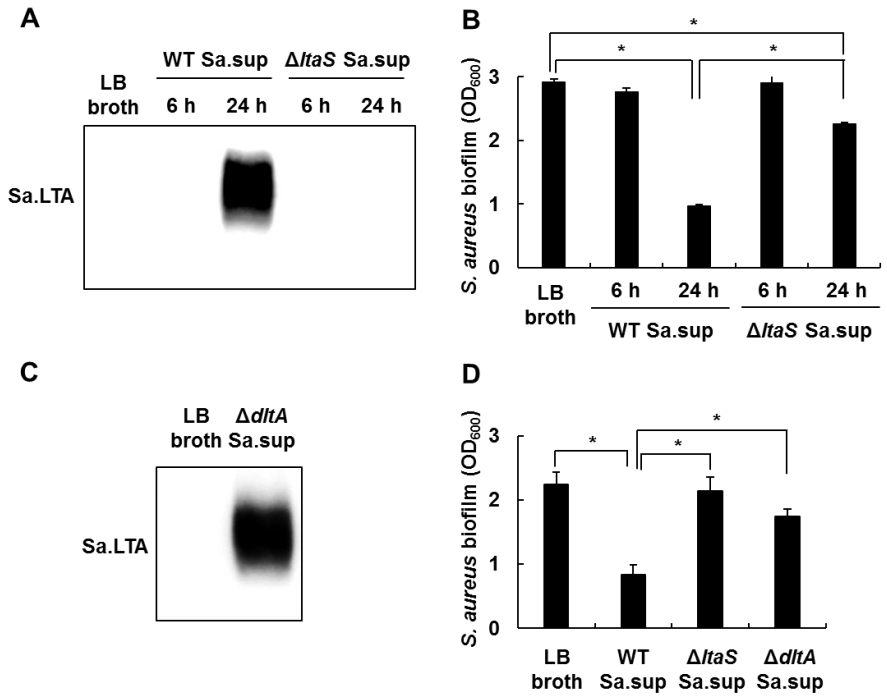


**Figure 20. LTA is an important molecule in the culture supernatants of *S. aureus* for inhibiting *S. aureus* biofilm formation.** (A) The culture supernatants of wild-type or Δ*ltaS S. aureus* were obtained at 6 or 24 h in shaking incubation and 20 μl of culture supernatants were subjected to Western blotting to measure LTA in the culture supernatants. (B) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of the culture supernatants of wild-type or Δ*ltaS S. aureus* obtained at 6 or 24 h. The extent of biofilm formation was determined by a crystal violet assay. (C) The culture supernatants of Δ*dltA S. aureus* were obtained at 24 h under shaking culture condition and LTA was determined in 20 μl of the culture supernatants using Western blot analysis. (D) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of the culture supernatants of wild-type, Δ*ltaS,* or Δ*dltA S. aureus* obtained at 24 h and biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with the LB broth treatment or wild-type Sa.sup treatment group.

### 2.1.3. Purified LTA from *S. aureus* inhibits biofilm formation of *S. aureus*

To confirm the inhibitory effect of LTA on the biofilm formation of *S. aureus*, LTA was purified from *S. aureus* using butanol extraction, octyl-sepharose chromatography, and ion-exchange chromatography, as previously described [125] and regulatory effect of LTA was examined on the biofilm formation. LTA inhibited biofilm formation of *S. aureus* in a dose-dependent manner (Figure 21A). Next, LTA from Δ*lgt S. aureus*, lipoprotein-deficient strain, was purified to exclude contamination of lipoprotein from LTA and the effect of LTA of Δ*lgt* strain was examined on the biofilm of *S. aureus*. LTA of Δ*lgt S. aureus* also inhibited biofilm formation of *S. aureus* in a dose-dependent manner (Figure 21B). In addition, LTA from Δ*dltA S. aureus*, D-alanine-deficient strain, was purified to examine the role of D-alanine in the inhibitory effect of LTA on the biofilm formation of *S. aureus* and the effect of LTA of Δ*dltA* strain was examined on the biofilm of *S. aureus*. LTA of Δ*dltA S. aureus* did not inhibit biofilm formation of *S. aureus* (Figure 21C). Lipoproteins from Δ*ltaS S. aureus* were also purified and the effect of the lipoproteins was examined on the biofilm of *S. aureus*. Lipoproteins did not inhibit biofilm formation of *S. aureus* (Figure 21D). Thus, these results suggest that LTA is a regulatory molecule to inhibit biofilm formation of *S. aureus* and D-alanine moieties are critical for the inhibitory function of LTA.

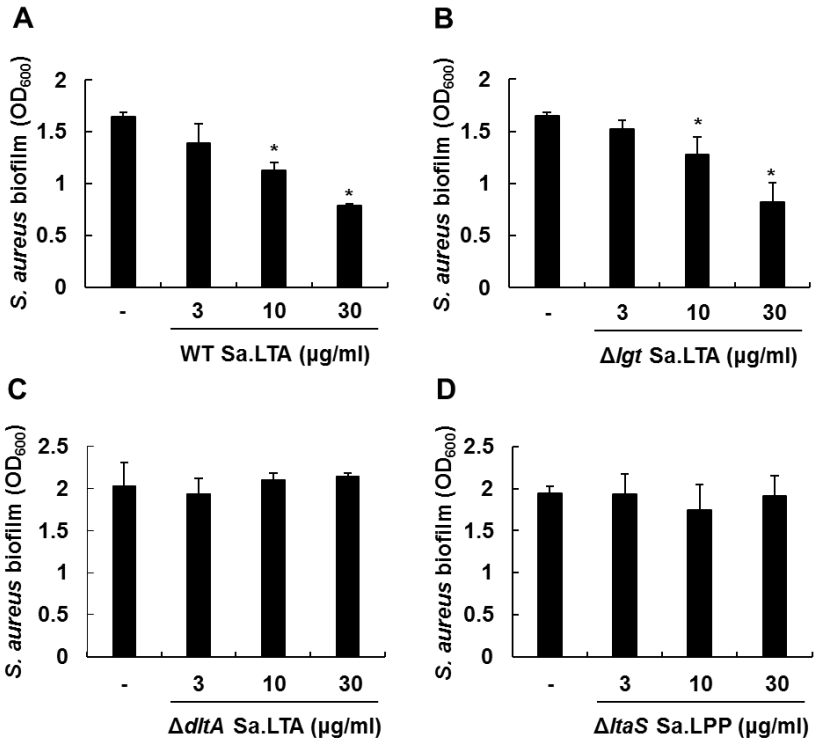


**Figure 21. Purified LTA from *S. aureus* inhibits biofilm formation of *S. aureus*.** (A-D) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of purified LTA from (A) wild-type (3, 10, or 30 μg/ml), (B) Δ*lgt S. aureus* (3, 10, or 30 μg/ml), or (C) Δ*dltA S. aureus* (3, 10, or 30 μg/ml), or (D) lipoproteins purified from Δ*ltaS S. aureus* (3, 10, or 30 μg/ml). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with the non-treatment group.

### 2.1.4. Purified LTA from pathogenic or non-pathogenic bacterial species inhibits biofilm formation of *S. aureus*

LTA is present in almost all of Gram-positive bacterial cell wall and its functions are dependent on structural differences from each bacterial strain [125]. To investigate whether the inhibitory effect of LTA on the biofilm formation of *S. aureus* is a shared characteristic of LTAs or specific effects depending on bacterial species, LTA was purified from various bacterial species such as *Streptococcus pneumoniae*, *S. gordonii*, *E. faecalis*, *B. subtilis*, and *L. plantarum* and the effects of these LTAs on the *S. aureus* biofilm formation were examined. As shown in Figure 22A-E, LTAs obtained from all bacterial species inhibited biofilm formation of *S. aureus* in a dose-dependent manner. Especially, biofilm formation was considerably inhibited by *S. pneumoniae* LTA (Sp.LTA) and *L. plantarum* LTA (Lp.LTA) (Figure 22A and E). These results suggest the possibility that Lp.LTA can be used as an anti-biofilm agent because safety of Lactobacilli has been verified. Thus, Lp.LTA among these LTAs was chosen to further study the mechanisms for inhibition of biofilm by LTA.

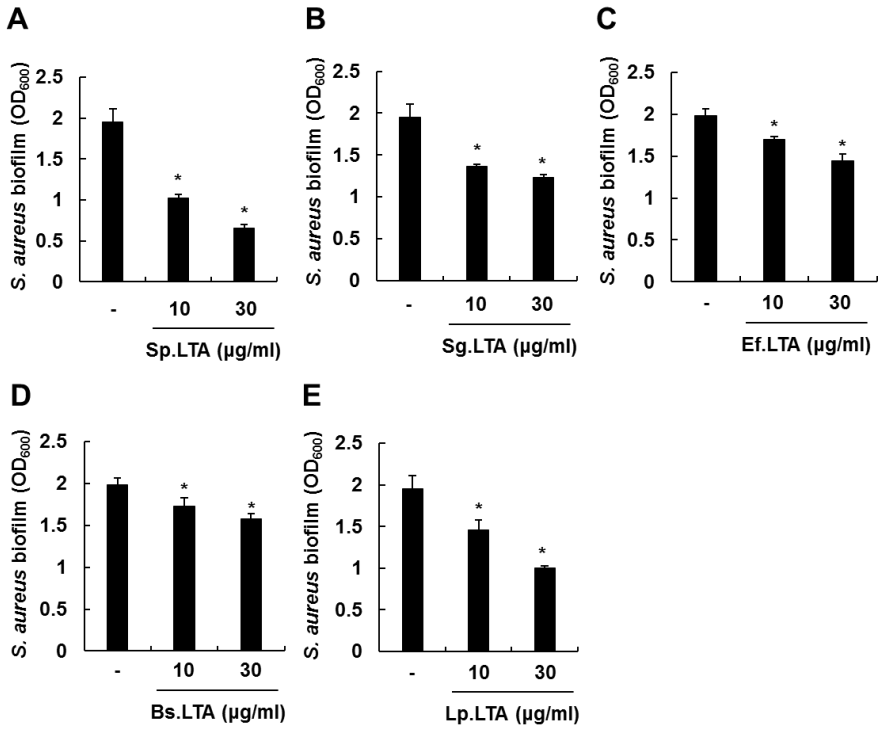


**Figure 22. LTA purified from various Gram-positive bacteria inhibits biofilm formation of *S. aureus*.** *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of purified LTA from (A) *S. pneumoniae*, (B) *S. gordonii*, (C) *E. faecalis*, (D) *B. subtilis*, or (E) *L. plantarum*. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with the non-treatment group.

## 2.2. Regulatory effect of cell wall components of *Lactobacillus plantarum* on biofilm of *S. aureus*

### 2.2.1. *L. plantarum* LTA, but not other cell wall-associated molecules, has an inhibitory effect on *S. aureus* biofilm formation

To identify *L. plantarum* cell wall-associated molecules responsible for the inhibition of *S. aureus* biofilm formation, the biofilm formation of *S. aureus* was examined after stimulation with various cell wall-associated molecules such as Lp.LTA, *L. plantarum* PGN (Lp.PGN), and *L. plantarum* lipoproteins (Lp.LPP). As shown in Figure 23A, Lp.LTA inhibited biofilm formation of *S. aureus* in a dose-dependent manner, whereas Lp.PGN and Lp.LPP did not have an influence on *S. aureus* biofilm formation. These results suggest that Lp.LTA acts as an inhibitory molecule against *S. aureus* biofilm, but not other cell wall-associated molecule of *L. plantarum*.

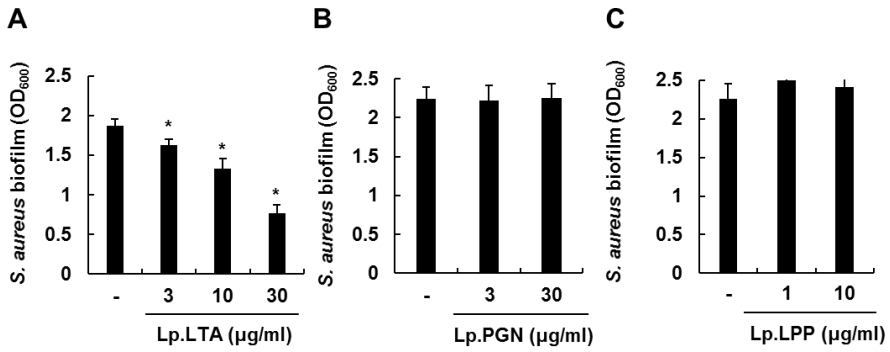


**Figure 23. *L. plantarum* LTA, but not other cell wall-associated molecules, has an inhibitory effect on *S. aureus* biofilm formation.** *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of (A) Lp.LTA (3 or 30 μg/ml), (B) Lp.PGN (3 or 30 μg/ml), or (C) Lp.LPP (1 or 10 μg/ml). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

### 2.2.2. *L. plantarum* LTA inhibits biofilm formation and aggregation of *S. aureus*

Next, biofilm of *S. aureus* formed in the presence of Lp.LTA was evaluated visually and quantitatively using confocal microscopy. *S. aureus* biofilm formation was inhibited by Lp.LTA in a dose-dependent manner, whereas dead cell population (i.e. PI-stained) was hardly observed in the presence of Lp.LTA (Figure 24A). SEM analysis indicated that Lp.LTA inhibited aggregation of *S. aureus* but did not induce any morphological changes on *S. aureus* (Figure 24B). In addition, in order to examine the effect of Lp.LTA on aggregation of *S. aureus*, changes in *S. aureus* aggregation were examined in the presence of Lp.LTA. As shown in Figure 24C, Lp.LTA inhibited the aggregation of *S. aureus* in a dose-dependent manner.

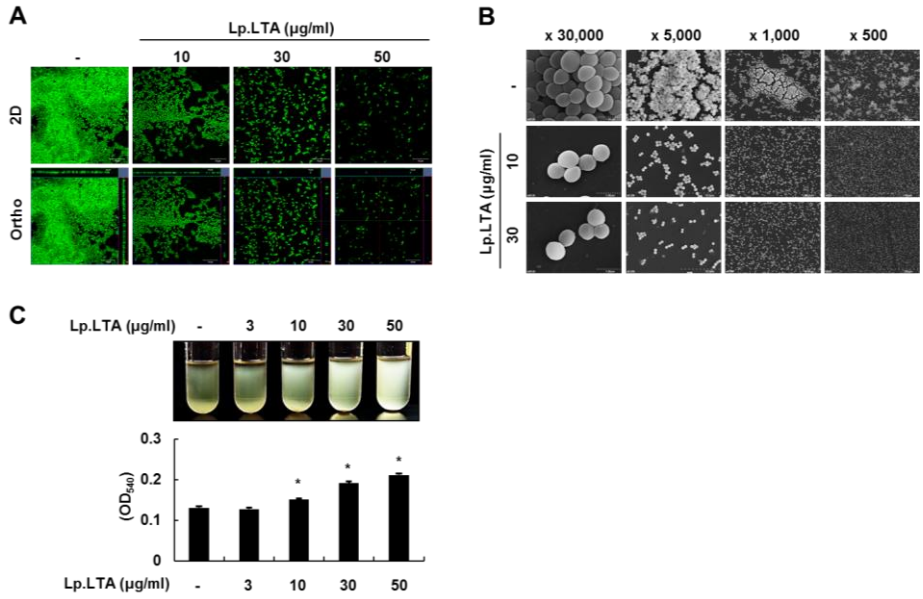


**Figure 24. *L. plantarum* LTA inhibits biofilm formation and aggregation of *S. aureus*.** (A, B) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of Lp.LTA (10, 30, or 50 μg/ml). Lp.LTA-treated *S. aurueus* biofilms were visualized by (A) a confocal laser scanning microscope (green from SYTO9 and red from propidium iodide) or (B) a scanning electron microscope (Magnification: ×500, ×1,000, ×5,000, and ×30,000). (C) *S. aureus* ($2 \times 10^8$ CFU/ml) was grown for 24 h in the presence of 3, 10, 30, or 50 μg/ml of Lp.LTA. Aggregation of *S. aureus* was determined by optical density (O.D.) at 540 nm. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with the non-treatment group.

### 2.2.3. *L. plantarum* LTA inhibits biofilm development at early stage and can disrupt pre-formed biofilms.

To examine if the inhibitory activity of Lp.LTA occurs at early and late phases of biofilm development, the *S. aureus* biofilm formation at 3, 6, 12, 24, and 48 h were measured after treated with Lp.LTA. Lp.LTA inhibited the biofilm formation at all time points examined (Figure 25A), without alteration of the bacterial growth (Figure 25D). Next the destructive effects of Lp.LTA on pre-formed biofilm of *S. aureus* were determined. Pre-formed biofilm (24 h) of *S. aureus* was treated with Lp.LTA for 6 h and the degree of destruction of the pre-formed biofilm was examined. As shown in Figure 25B, Lp.LTA destroyed pre-formed biofilm of *S. aureus* in a dose-dependent manner. In addition, to determine the time point for inhibitory effect of Lp.LTA during biofilm development of *S. aureus*, *S. aureus* biofilm was treated with Lp.LTA at various time points and biofilm formations was measured at the time point (24 h). Figure 25C shows that Lp.LTA has ability to inhibit *S. aureus* biofilm formation at 1 h to 12 h. These results suggest that Lp.LTA inhibits *S. aureus* biofilm formation at early stage without affect the bacterial growth and even disrupts the pre-existing biofilm.

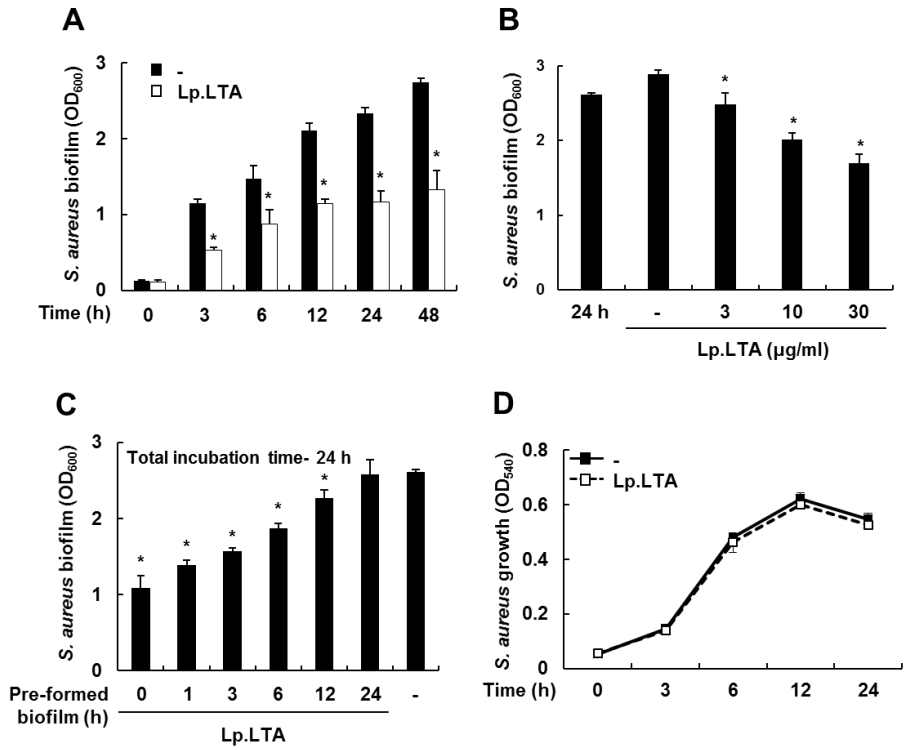


**Figure 25. *L. plantarum* LTA inhibits biofilm development at early stage and can disrupt pre-formed biofilms.** (A) *S. aureus* ($1 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 3, 6, 12, 24, or 48 h in the presence or absence of Lp.LTA (30 μg/ml). (B) *S. aureus* ($5 \times 10^7$ CFU/ml) grown on polystyrene plates at 37°C for 24 h was treated with Lp.LTA (3, 10, or 30 μg/ml) and further incubated at 37℃ for 6 h. (C) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 1, 3, 6, or 12 h, followed by treatment with Lp.LTA (30 μg/ml) at 37°C for up to 24 h. The biofilm formation was determined by a crystal violet assay. (D) *S. aureus* ($1 \times 10^7$ CFU/ml) was grown under shaking condition for 3, 6, 12, or 24 h in the presence or absence of Lp.LTA (30 μg/ml). The growth of *S. aureus* was determined by O.D. at 540 nm. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

### 2.2.4. *L. plantarum* LTA-induced AI-2 release contributes to the inhibition of *S. aureus* biofilm formation.

Quorum sensing molecules are deeply involved in bacterial biofilm formation [133]. In case of *S. aureus*, both AI-2 and AIP are known to inhibit biofilm formation of *S. aureus* through autocrine pathway [111, 116]. To determine if AI-2 and AIP are involved in the inhibitory effects of Lp.LTA against *S. aureus* biofilm, wild-type, Δ*luxS*, AI-2-deficient strain, and Δ*agrA*, AIP-deficient strain, were treated with Lp.LTA and the biofilm was measured by crystal violet assay. As shown in Figure 26A, biofilms of wild-type and Δ*agrA* strains were effectively inhibited up to 59.8% and 53.7% by Lp.LTA, respectively, whereas the biofilm formation of Δ*luxS* strain was inhibited up to 84.9% by Lp.LTA. To determine extracellular AI-2 production in *S. aureus* in the presence of Lp.LTA, *S. aureus* was treated with Lp.LTA and AI-2 from *S. aureus* culture supernatants was measured by using bioluminescent bacterial reporter strain, *Vibrio harveyi* BB170. Lp.LTA induceed increase of extracellular AI-2 in *S. aureus* in a dose-dependent manner (Figure 26B). When *S. aureus* was pretreated with D-ribose, AI-2 antagonist, inhibitory effect of Lp.LTA on biofilm formation was alleviated (Figure 26C). These results suggest that Lp.LTA-induced release of AI-2 is significantly involved in Lp.LTA-induced inhibition of *S. aureus* biofilm.

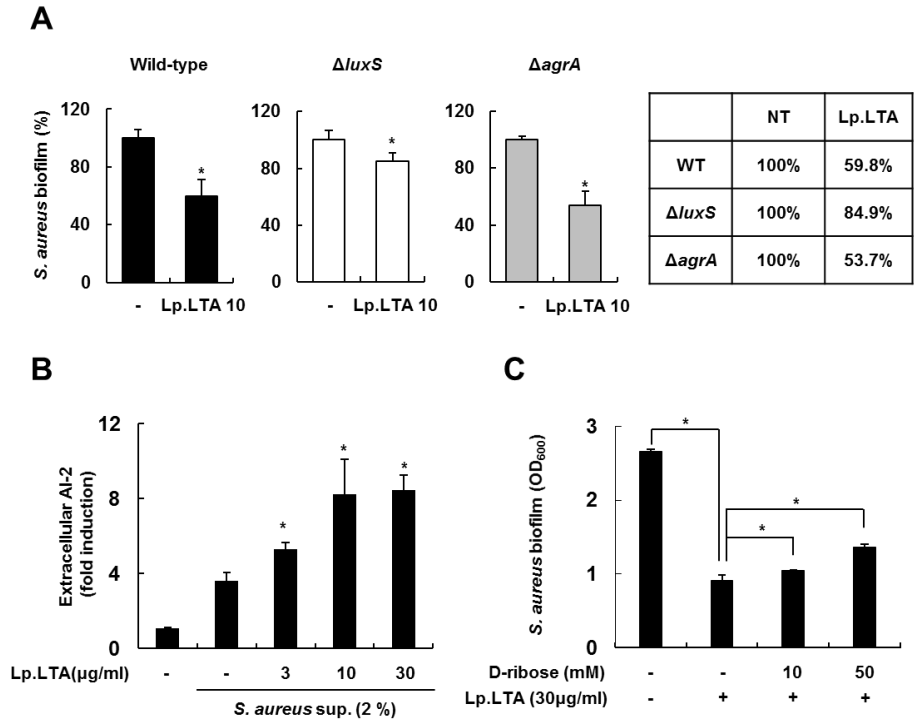


**Figure 26. *L. plantarum* LTA-induced AI-2 release contributes to the inhibition of *S. aureus* biofilm formation.** (A) *S. aureus* wild-type ($1 \times 10^7$ CFU/ml), AI-2-deficient strain (Δ*luxS*) ($1 \times 10^7$ CFU/ml), and AIP-deficient strain (Δ*agrA*) ($1 \times 10^7$ CFU/ml) were grown in the presence of Lp.LTA (10 μg/ml) at 37℃ for 24 h. (B) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown h in the presence of Lp.LTA (3, 10, or 30 μg/ml) on polystyrene plates at 37℃ for 24 h. The culture supernatants were collected to determine AI-2 release using a bioluminescent bacterial reporter strain, *Vibrio harveyi* BB170. (C) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37℃ for 24 h in the presence of Lp.LTA (30 μg/ml) with 10, 50, 100, or 500 mM of D-ribose. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

### 2.2.5. *L. plantarum* LTA inhibits the *ica* gene expression and exopolysaccharide production in *S. aureus*

To investigate whether Lp.LTA affect *S. aureus* biofilm-associated genes expression, firstly the ability of Lp.LTA to bind to *S. aureus* was examined. *S. aureus* was treated with biotinylated-LTA and binding of Lp.LTA to *S. aureus* was measured by flow cytometry using streptavidin-FITC. Figure 27A shows that binding of Lp.LTA to *S. aureus* was increased in a dose-dependent manner. Previous reports have demonstrated that the proteins IcaA, IcaB, IcaC, and IcaD are encoded by the *ica* locus and synthesize PNAG, a major component of staphylococci biofilms [105, 106]. The biofilm-associated gene expression including *icaA*, *icaB*, *icaC*, and *icaD* were examined in *S. aureus* stimulated with Lp.LTA. Lp.LTA inhibited mRNA expression of *icaA*, *icaB*, and *icaC* by *S. aureus* in a dose-dependent manner (Figure 27B). Moreover, Lp.LTA inhibited PNAG production by *S. aureus* in a dose-dependent manner (Figure 27C). These observations suggest that Lp.LTA inhibits *ica* gene expression and PNAG production, leading to the inhibition of *S. aureus* biofilm formation.

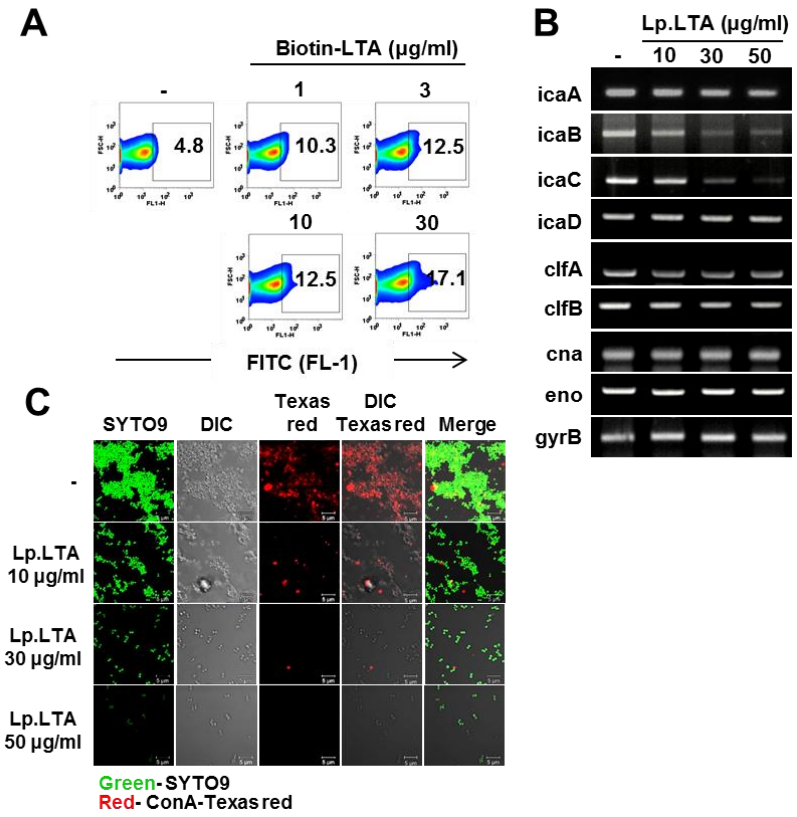


**Figure 27. *L. plantarum* LTA inhibits the *ica* gene expression and exopolysaccharide production in *S. aureus*.** (A) *S. aureus* ($5 \times 10^7$ CFU/ml) was treated with 1, 3, 10, or 30 μg/ml of biotinylated Lp.LTA (biotin-Lp.LTA) for 1 h. Binding of biotin-Lp.LTA with *S. aureus* was detected by streptavidin-FITC and analyzed by flow cytometry analysis. The percentage of biotin-Lp.LTA-positive *S. aureus* is shown in each histogram. One of three similar results is shown. (B) *S. aureus* ($1 \times 10^8$ CFU/ml) was treated with 10, 30, or 50 μg/ml of Lp.LTA for 3 h. Total RNA was isolated and the mRNA expression of *icaA, icaB, icaC, icaD, clfA, clfB, cna, eno,* or *gyrB* was examined by RT-PCR. (C) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown at 37℃ for 24 h in the presence of Lp.LTA (10, 30, or 50 μg/ml). Then, *S. aureus* biofilm formation and exopolysaccharide production were examined with SYTO9 and conA-texas red staining, respectively, followed by analysis with the confocal microscope (green from SYTO9 and red from conA-texas red).

### 2.2.6. D-alanine moieties of *L. plantarum* LTA are critical for the inhibitory effect on *S. aureus* biofilm formation

Initially, D-alanine-removed Lp.LTA (Deala-Lp.LTA) and Lp.LTA deficient in both D-alanine and acyl chain (Deala/Deacyl-Lp.LTA) were prepared by treatment with HCl (pH 8.5) and NaOH (pH 12), respectively (Figure 28A). To identify the functional moieties responsible for the inhibitory effects of Lp.LTA on biofilm formation of *S. aureus*, the biofilm formation of *S. aureus* was examined after treatment with Lp.LTA, Deala-Lp.LTA, and Deala/Deacyl-Lp.LTA. As shown in Figure 28B, Lp.LTA, but not Deala-Lp.LTA or Deala/Deacyl-Lp.LTA, inhibited biofilm formation of *S. aureus* in a dose-dependent manner. Electron microscopic analysis also indicated that *S. aureus* biofilm formation was inhibited by Lp.LTA, but not by Deala-Lp.LTA (Figure 28C). Furthermore, in order to determine whether D-alanine moieties were involved in the inhibitory effects of Lp.LTA on *ica* mRNA expression, RT-PCR was perfomed with *S. aureus* treated with Lp.LTA or Deala-Lp.LTA. As shown in Figure 26D, Lp.LTA inhibited *icaB* and *icaC* mRNA expression, whereas Deala-Lp.LTA failed to do so, indicating that D-alanine is a critical component of Lp.LTA for it to exert inhibitory effects on the biofilm formation of *S. aureus*.

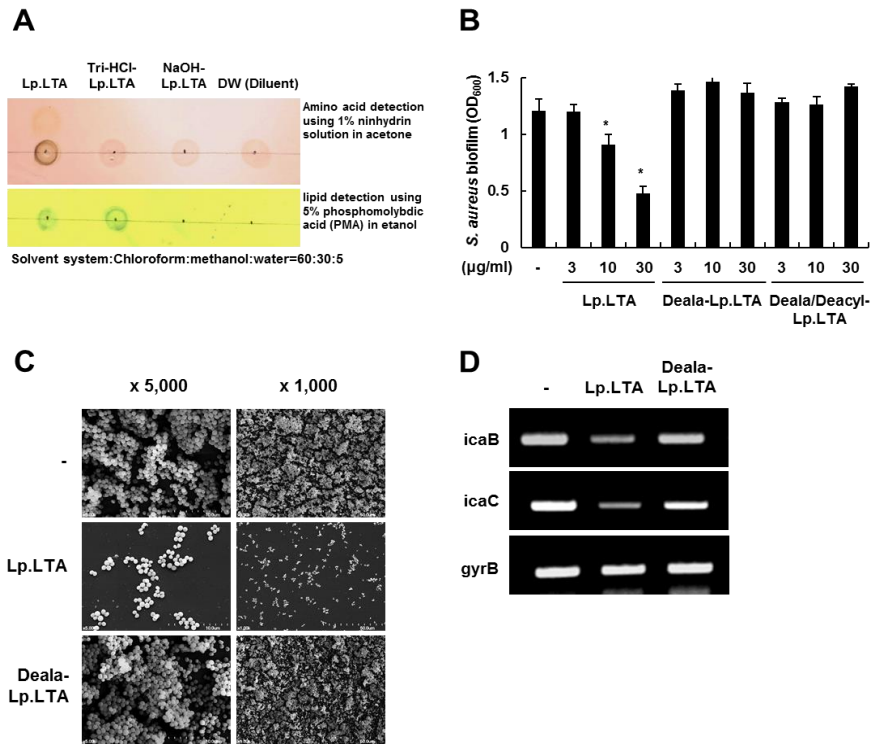


**Figure 28. D-alanine moieties of Lp.LTA are critical for the inhibitory effect on *S. aureus* biofilm formation.** (A) Deala-Lp.LTA and Deala/Deacyl-Lp.LTA were prepared by treatment of Lp.LTA with Tris-HCl (0.1 M, pH 8.5) or NaOH (0.5 N, pH 12). Then, D-alanine and acyl chain moieties of Lp.LTA were detected by 1% ninhydrin solution and 5% phosphomolybdic acid, respectively. (B) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates at 37°C for 24 h in the presence of 3, 10, or 30 μg/ml of Lp.LTA, Deala-Lp.LTA, or Deala/Deacyl-Lp.LTA. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group. (C) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown at 37℃ for 24 h in the presence of 30 μg/ml of Lp.LTA or Deala-Lp.LTA. *S. aureus* biofilm was analyzed with scanning electron microscope (magnification: ×1,000 and ×5,000). (D) *S. aureus* ($1 \times 10^8$ CFU/ml) was treated with 30 μg/ml of Lp.LTA or Deala-Lp.LTA for 3 h. Total RNA was isolated and the expression of *icaB, icaC,* or *gyrB* mRNA was examined by RT-PCR.

### 2.2.7. *L. plantarum* LTA enhances the ability of antibiotics to inhibit biofilm formation of *S. aureus*

To determine if Lp.LTA can enhance the bactericidal activity of antibiotics on biofilm formation of *S. aureus*, *S. aureus* was co-treated with Lp.LTA and various antibiotics targeting the cell wall synthesis (penicillin and vancomycin) or protein synthesis (streptomycin and erythromycin) followed by measurement of the biofilm formation. As shown in Figure 29, 13% inhibition by penicillin treatment, 20% inhibition by vancomycin treatment, 6% inhibition by streptomycin treatment, and 25% inhibition by erythromycin treatment were increased to 50%, 60%, 40%, and 56% inhibition in the presence of Lp.LTA, respectively. These results suggest that Lp.LTA can enhance the antimicrobial effect of antibiotics against *S. aureus* biofilm formation.

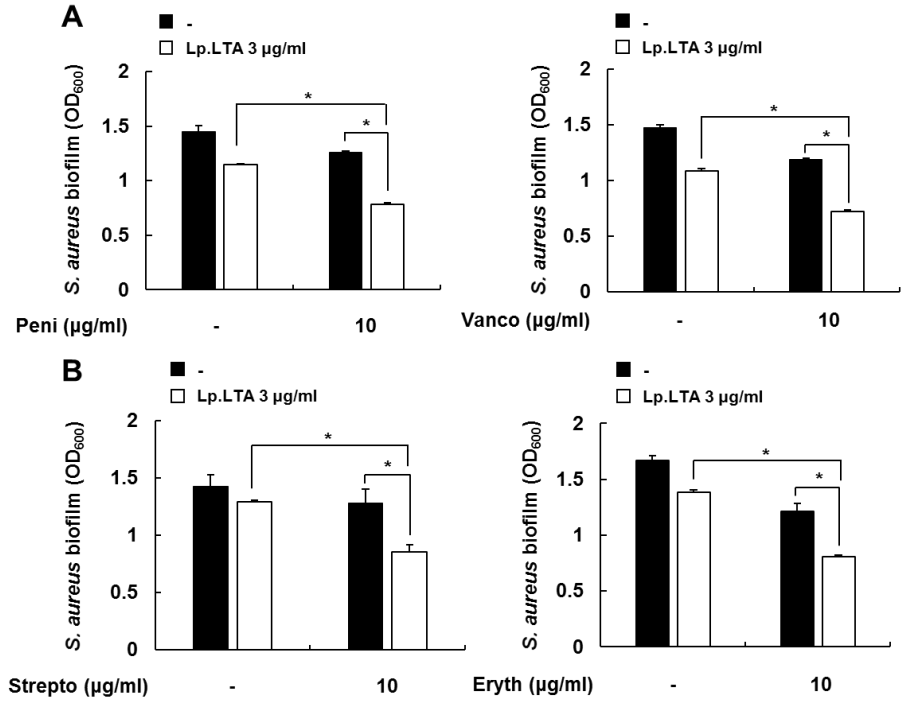


**Figure 29. *L. plantarum* LTA enhances the ability of antibiotics to inhibit biofilm of *S. aureus*.** (A) *S. aureus* ($5 \times 10^7$ CFU/ml) was grown on polystyrene plates with 3 μg/ml of Lp.LTA at 37°C for 24 h and further incubated for 6 h in the presence or absence of 10 μg/ml of antibiotics inhibiting the cell wall synthesis (penicillin and vancomycin) or (B) protein synthesis (streptomycin and erythromycin). The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with Lp.LTA- or antibiotics-treatment group.

### 2.2.8. *L. plantarum* LTA inhibits biofilm formation of *S. aureus* clinical isolates

In order to determine if Lp.LTA inhibits not only laboratory strain but also clinical strain, clinical isolates such as *S. aureus* USA300, ATCC29213, NCCP14780, and MRSA NCCP14769 were treated with Lp.LTA in a dose-dependent manner. As shown in Figure 30, USA300 and ATCC29213 strains, but not NCCP14780 and NCCP14769 were not significantly inhibited by 3 μg/ml of Lp.LTA. However, all clinical isolates of *S. aureus* was efficiently inhibited by 10 or 30 μg/ml of Lp.LTA in a dose-dependent manner.

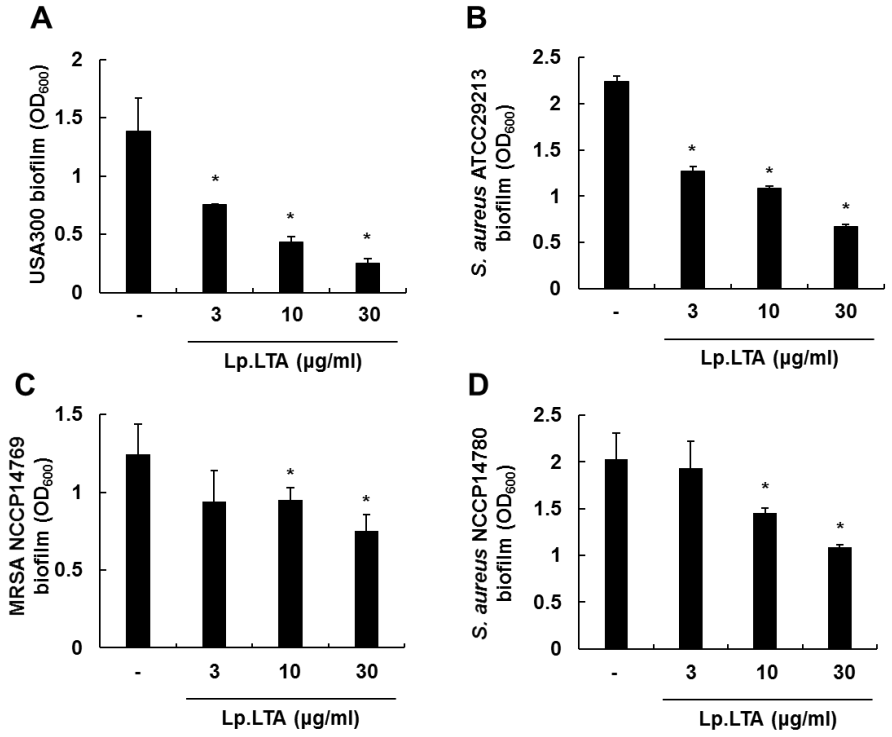


**Figure 30. *L. plantarum* LTA inhibits the biofilm formation of *S. aureus* clinical isolates.** (A) *S. aureus* USA300, (B) *S. aureus* ATCC29213, (C) methicillin-resistant *S. aureus* NCCP14769, or (D) *S. aureus* NCCP14780, were treated with 3, 10, or 30 μg/ml of Lp.LTA for 24 h. The biofilm formation was determined by a crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Asterisks indicate significant induction at $P < 0.05$ compared with non-treatment group.

# Chapter IV. Discussion

## 1. Lipoprotein in the cell wall of *S. aureus* is a major inducer of NO production in macrophages

Microbial infection induces various inflammatory mediators such as TNF-α, IL-6, PGE-2, or NO that participate in inflammatory responses and in the pathogenesis of inflammatory diseases. There is accumulating evidence that *S. aureus* substantially induces NO production, which is responsible for inflammation and bacterial sepsis in the host [134]. Nevertheless, which cell wall virulence factors are responsible for NO production has been unclear. In this study, it was demonstrated that lipoproteins of *S. aureus* play a crucial role in the induction of NO production in macrophages through activation of NF-κB and STAT-1 triggered by TLR2 (Figure 31).

These results are concordant with the previous report showing that Δ*lgt* induces significantly lower levels of inflammatory cytokine production in peritoneal macrophages than wild-type *S. aureus* [32]. Δ*lgt* also induces lower production of IL-6, IL-8, or MCP-1 than wild-type *S. aureus* in human pulmonary epithelial cells and umbilical vein endothelial cells [135]. Consistent with these findings, isolated lipoproteins from *S. aureus* induce NF-κB activation through TLR2 recognition [136] and induce IL-6 production in human corneal epithelial cells [127]. Moreover, previous study also demonstrated that wild-type, but not Δ*lgt*, *S. aureus* and synthetic lipopeptides mimicking bacterial lipoproteins induce inflammatory bone destruction [30].

Lipoprotein-deficient mutant strains of other bacteria such as *Mycobacterium tuberculosis* and *Yersinia pestis* exhibit attenuated virulence of tuberculosis and primary pneumonic plague, respectively [26, 137]. Furthermore, isolated lipoproteins from *M. tuberculosis* induce TNF-α, IL-10, and IL-12 production and regulate maturation of dendritic cells [138], and lipoproteins derived from *Y. pestis*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *Neisseria meningitides* are significantly involved in their pathogenesis [9, 137]. These observations indicate that lipoproteins are one of the most important cell wall components of bacteria for evoking the host immune responses.

It was observed that the Δ*lgt* strain of *S. aureus* did not induce NO production as a result of decreased iNOS mRNA and protein expression. The failure of Δ*lgt* to induce iNOS expression seems to be closely associated with decreased stimulation of TLR2. Lipoproteins are a representative TLR2 ligand [136]. Accumulating evidence suggests that TLR2 triggers MyD88-dependent signaling pathways leading to NF-κB activation [139]. Moreover, TLR2 activation also stimulates IFN-β expression and subsequent JAK/STAT-1 activation [140]. Concordant with the loss of iNOS inducibility, Δ*lgt* could not stimulate TLR2, NF-κB activation, or IFN-β/STAT-1 activation in this study. In light of the fact that *S. aureus*-induced inflammatory responses are known to be primarily mediated through TLR2 [6], loss of lipoproteins from *S. aureus* might seriously affect TLR2 stimulation and subsequent signaling pathways, especially those involved in inflammatory responses including NO production.

Interestingly, Δ*ltaS* and Δ*dltA* were more potent inducers of NO and iNOS

production than the wild-type strain. These observations are consistent with a previous report showing that UV-inactivated Δ*dltA* induces a greater increase in TNF-α production than wild-type *S. aureus* [141]. Although both lipoproteins and LTA are TLR2 ligands, lipoproteins are more potent than LTA in the stimulation of TLR2 [136] and the induction of proinflammatory mediators [142]. Indeed, Pam2CSK4, which mimics Gram-positive bacterial lipoproteins, is 100-fold more potent than LTA in the stimulation of immune responses [36]. Lipopeptide is 2 - 100 times more potent than LTA in the induction of IL-6 and IL-8 production in human odontoblasts [143]. Therefore, it is maybe not surprising that the Δ*ltaS* strain that expresses lipoproteins (a strong TLR2 ligand) but not LTA (a weak TLR2 ligand) is more potent than the wild-type because TLR2 molecules on macrophages might have a greater chance of encountering lipoproteins on the Δ*ltaS* strain compared with the wild-type. However, further investigation is required to determine whether the level of lipoproteins are indeed higher in Δ*ltaS* than in the wild-type.

In conclusion, the results of the current study suggest that lipoproteins are crucial for *S. aureus*-induced iNOS expression and NO production through TLR2. Notably, *S. aureus* is one of the most notorious of the Gram-positive bacteria that cause sepsis but no effective vaccines or therapeutics against *S. aureus* are currently available. Information provided by this study could be useful for the development of preventive vaccines and therapeutics against infectious diseases caused by *S. aureus*.

## 2. *S. aureus* LTA and MDP cooperatively induce COX-2 expression and PGE2 generation in macrophages

Although LTA has been known as TLR2 ligand of *S. aureus* to induce inflammatory responses of host, the ability of Δ*ltaS* strain to induce NO production was not decreased compared with wild-type strain in this study. Indeed, it has been reported that LTA is a lot less potent in inducing inflammatory responses than lipoproteins, but strongly induces inflammatory mediator expression by synergy with PGN. However, earlier LTA preparations were contaminated or structurally damaged by improper purification processes and molecular mechanisms have not been identified for the synergistic effect of LTA and PGN. Therefore, the synergistic effect of highly pure *S. aureus* LTA and PGN and action mechanism to induce inflammatory mediator production were investigated. In this study, it was demonstrated that MDP potentiates Sa.LTA-induced COX-2 expression and PGE2 production, where NOD2 and TLR2 are necessary (Figure 31). The synergy of MDP and Sa.LTA in inducing COX-2 expression is intriguing since Gram-positive bacteria have LTA and mostly thick PGN in their cell walls.

Synergism between LTA and MDP has been also reported in the induction of other inflammatory mediators in various cells or tissues. For example, co-treatment with Sa.LTA and MDP resulted in the enhanced recruitment of neutrophils to mammary glands by chemokine overproduction [144]. In rat microglia cells, Sa.LTA induced NO production through TLR2 that was

synergistically enhanced by MDP [145]. In addition, Sa.LTA and MDP synergistically induced IL-8 production in human monocytic cells [146]. It was reported that Sa.LTA and MDP synergistically induced the maturation and activation of human dendritic cells leading to the production of inflammatory cytokines [74]. Therefore, cooperative action between LTA and PGN or its derivatives might be important in inducing inflammation of host innate immunity upon Gram-positive bacterial infection.

Synergistic induction of COX-2 by MDP was observed in the treatment with Sa.LTA but not with Bs.LTA. The differential immuno-stimulating activity between Sa.LTA and Bs.LTA may be due to their structural differences. Indeed, the LTA structure of Gram-positive bacteria contains the different number of repeating units and the amount of D-alanine [147]. Sa.LTA contains longer repeating units with higher amount of D-alanine in the repeating unit than Bs.LTA [148]. In addition, loss of D-alanine resulted in the decrease of inflammatory cytokine production [149], suggesting that D-alanine content is critical for the immuno-stimulatory potential of LTA.

Both TLR2 and NOD2 are necessary for the maximal induction of COX-2 in Sa.LTA/MDP-stimulated macrophages. This potentiation effect was not observed in TLR2- or NOD2-deficient macrophages. Several mechanisms could cause the potentiation effect of MDP on Sa.LTA-induced COX-2 expression. First, MDP and Sa.LTA increased the expression of TLR2 and/or NOD2. MDP increases the expression of TLR2 on microglial cells resulting in the enhanced expression of MAPKs and NF-κB activation upon subsequent

stimulus [150]. MDP and TLR2 agonists upregulate the expression of NOD2 in human periodontal ligament cells [151]. TLR2 ligands including Sa.LTA and $Pam_2CSK_4$ upregulate NOD2 expression in human odontoblast-like cells [152]. Second, MDP cooperatively activated signaling pathways triggered by Sa.LTA. Indeed, the TLR2 and NOD2 signaling pathways seem to be closely related and mutually affected as shown in the cytokine expression in Crohn's disease [153].

PGN possesses several structural motives that can be recognized by NOD1 and NOD2 [81, 82]. Although NOD2 ligands modulate LTA-induced COX-2 expression, the NOD1 ligand that is exclusively found in the PGN of Gram-negative bacteria did not produce this effect in this study. Of note, no synergistic effect between NOD1 and TLR2 has been reported until now. Instead, NOD1 ligands appear to be synergistic with LPS, a major virulence factor of Gram-negative bacteria in the view of the fact that a NOD1 ligand, gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP), enhances LPS-induced IL-6 expression in macrophages [81]. Therefore, Gram-negative bacterial virulence factors seem to favorably cooperate with other virulence factors of Gram-negative bacteria but not with Gram-positive bacterial virulence factors.

Although Δ*lgt* has both LTA and PGN, it did not induce NO production in mice macrophages in this study. Lipid moieties are important part when LTA is recognized by TLR2 of the host cells [154]. Thus, it is expected that soluble LTA, such as released or purified LTA, will be suitable for binding to TLR2. However, the EKSA Δ*lgt* used in this study is in the state of physiologically inactivated whole cell and LTA is likely to be present as membrane anchored

LTA in which the lipid part is not exposed to the outside. For this reason, EKSA Δ*lgt* may not have induced NO production even though it has LTA and PGN, and the EKSA may be a model that is not suitable for measuring the effect of LTA in induction of immune responses.

Unlike LPS embedded in the cell wall of Gram-negative bacteria, LTA is secreted from Gram-positive bacteria [52]. Moreover, natural PGN fragments released from *S. aureus* stimulate NOD2 and are potent co-stimulators of the innate immune system together with TLR2 ligand [155]. Therefore, this study demonstrating that Sa.LTA and MDP cooperatively induce COX-2 expression and PGE2 generation suggest that these two virulence factors might contribute to the establishment of inflammation at sites of Gram-positive bacterial infection.

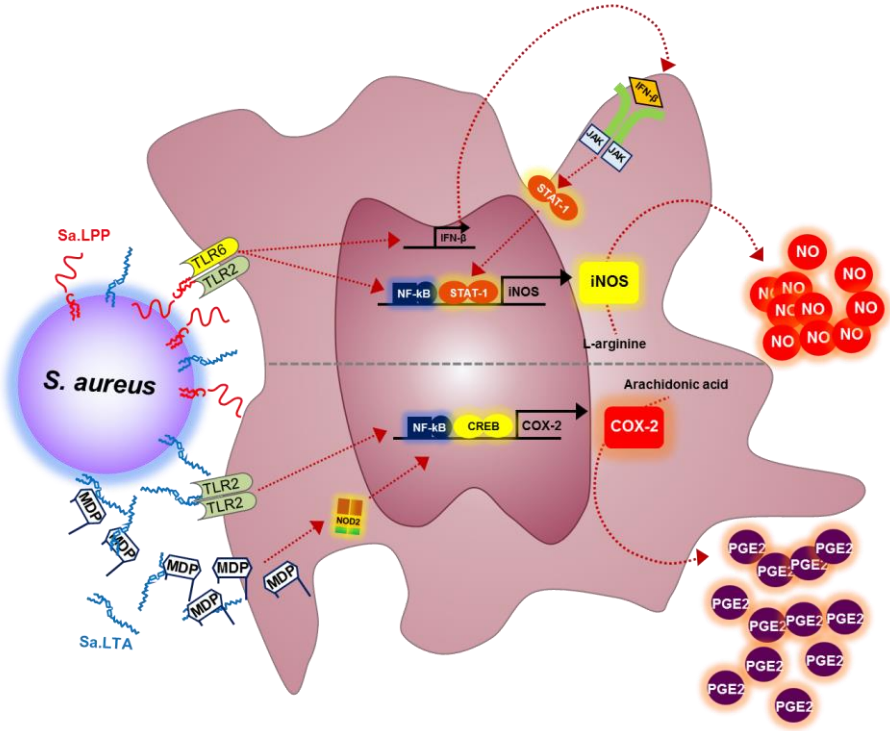


**Figure 31. Schematic illustration of the proposed action mechanism of cell wall-associated virulence factors of *S. aureus* in the induction of inflammatory responses.** Lipoproteins act as a major cell wall-associated virulence factor of *S. aureus* to induce iNOS expression through activation of NF-κB and STAT-1 triggered by TLR2, resulting in NO production. *S. aureus* LTA and MDP, minimal structural unit of PGN cooperatively induce COX-2 expression and PGE2 generation in macrophages through activation of NF-κB and CRE triggered by TLR2 and NOD2.

## 3. *L. plantarum* LTA inhibits *S. aureus* biofilm formation

The biofilm has been implicated in more than 80% of human microbial infections and is responsible for various infectious diseases [100]. Although LTA and lipoproteins are major cell wall components of *S. aureus*, role of these molecules in *S. aureus* biofilm formation has not been clearly understood. In this study, LTA efficiently inhibits *S. aureus* biofilm formation by decreasing production of PNAG, which is an important component of staphylococcal biofilm, and increasing release of the AI-2.

These results are concordant with the previous reports showing that LTA inhibits bacterial colonization and bacterial adherence to host cells. For example, LTA purified from *Lactobacillus fermentum* and *Streptococcus mutans* are known to inhibit *S. mutans* colonization on glass surfaces through inhibition of glucosyltransferase activities [156] and *Streptococcus pyogenes* LTA is known to inhibit aggregation of streptococci [157]. Moreover, LTA purified from *E. faecalis* inhibits enterococcal binding to Caco-2 cells. These reports suggest that specific interaction of LTA with bacterial or host factors leads to inhibition of bacterial colonization or bacterial binding with host cells. Thus, it could be expected that LTA binds to specific receptor on *S. aureus* surfaces, leading to inhibition of biofilm-associated gene expression.

Interestingly, Lp.LTA increases the release of AI-2 from *S. aureus*, leading to the inhibition of *S. aureus* biofilm formation. AI-2 is a representative quorum sensing signaling molecule known to inhibit biofilms of *S. aureus* and *S.*

*epidermidis* via inhibition of the transcription of *icaA* [110, 111]. These reports support the data showing LTA-induced inhibition of biofilm through increase of AI-2. Although it has generally been known that the production of AI-2 is regulated by cell density, various external molecules can also regulate the production of AI-2. Fe(III), NaCl, glucose, dithiothreitol, and flavonoid increase AI-2 production, and amino acid starvation and IL-2 decrease AI-2 production in *Escherichia coli* [158]. Additionally, it was found that LTA act as a new regulator of AI-2 production in *S. aureus*, which is expected to affect various bacterial pathophysiology such as virulence, antibiotic resistance, motility, and biofilm formation.

D-alanine moieties play a critical role for inhibitory effects of Lp.LTA on *S. aureus* biofilm formation in the present study. Consistent with these findings, D-amino acids including D-leucine and D-tyrosine induce biofilm disassembly in *S. aureus, Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Bacillus subtilis* [159]. LTA contains D-alanine ester residues giving a positive charge to the negative charged LTA. Positive charge has been used for the prevention of biofilm formation. For example, along with the increase of *N*-deacetylation degrees in chitosan-streptomycin conjugates, anti-biofilm capacity is enhanced [160]. Moreover, positive charge is likely to be important for the inhibitory effect of unilamellar vesicle on biofilm formation by *S. aureus* and *P. aeruginosa* [161]. These reports support that positive charge of D-alanine is a determinant of the activity of LTA to inhibit biofilm formation of *S. aureus*. In addition, Lp.LTA significantly inhibited the expression of *icaA*, *icaB*, and

*icaC* whereas the Lp.LTA lacking D-alanine did not show such effect. It is speculated that D-alanine may be deeply involved in the binding of Lp.LTA with *S. aureus* surface receptors and activation of signaling pathway leading to the regulation of biofilm-associated gene expression. Further investigations are required to identify specific receptors of *S. aureus* for Lp.LTA.

There are five stages in the biofilm development, (i) attachment, (ii) irreversible attachment, (iii) early maturation, (i) late maturation, and (v) dispersion [101]. It was determined at which step Lp.LTA showed the inhibitory effect during the biofilm development. Interestingly, Lp.LTA not only inhibited the initial attachment but also biofilm at the maturation stage. Even Lp.LTA showed the destroying effect against preformed biofilm. These results are concordant with the previous report showing that rhamnolipid, biosurfactant, has similar structure with LTA which disrupts preformed biofilm of *P. aeruginosa* and *Yarrowia lipolytica* [162, 163]. In addition, production of rhamnolipid is governed by quorum sensing molecules in *P. aeruginosa* [164]. Since LTA increased AI-2 from *S. aureus*, it may also induce biosurfactant proteins that destroy preformed biofilm.

Recently, bacteria-derived molecules for inhibiting biofilm formation have been suggested as a natural biofilm-dispersing agent. For example, DspB, endogenous factor of *Actinobacillus actinomycetemcomitans*, induces detachment of *A. actinomycetemcomitans* biofilm cells [165] and *B. subtilis*-derived α-amylase inhibits various human pathogens biofilm formation [166].

However, some reports have insisted that dispersing agents-mediated dispersed bacteria are highly virulent compared with biofilm or planktonic bacteria and involved in increasing disease severity. Thus, biofilm dispersal treatment should be combined with antimicrobial treatment which could remove the dispersed bacteria. It was observed that LTA enhances the antimicrobial effect of antibiotics against *S. aureus* biofilm formation, implying that this combination treatment will attenuate biofilm-associated inflammation and inhibit systemic dissemination of dispersed bacteria in the host.

There is possibility that LTA acts as a self-produced biofilm disassembly factor in Gram-positive bacteria because we found that released Sa.LTA in culture supernatants autocrinely inhibits biofilm of *S. aureus*. However, when we compared biofilm formation of *S. aureus* wild-type and Δ*ltaS* strains, Δ*ltaS* - weakly formed biofilm compared to wild-type, unexpectedly. This results is contrary to the inhibitory effect of LTA on the biofilm formation. There are two form of LTA, membraned anchored LTA and released LTA. Although the inhibitory effect of released LTA on the biofilm formation was firstly elucidated in this study, the effect of membrane anchored LTA on the biofilm formation has already been reported. LTA play an important role in biofilm formation of *E. faecalis* [167] and D-alanine acts as a key functional residue in LTA for colonization of *S. aureus* [84]. These suggest that the manner how membrane anchored LTA and released LTA are involved in biofilm formation may be different.

In conclusion, the results of this study suggest that LTA is a major inhibitory

molecule against *S. aureus* biofilm. Notably, biofilm is a major cause of various infectious diseases but there are no currently-available effective therapeutics. Information provided by this study could be useful for development of effective therapeutic agents to treat infectious diseases caused by *S. aureus* biofilms.

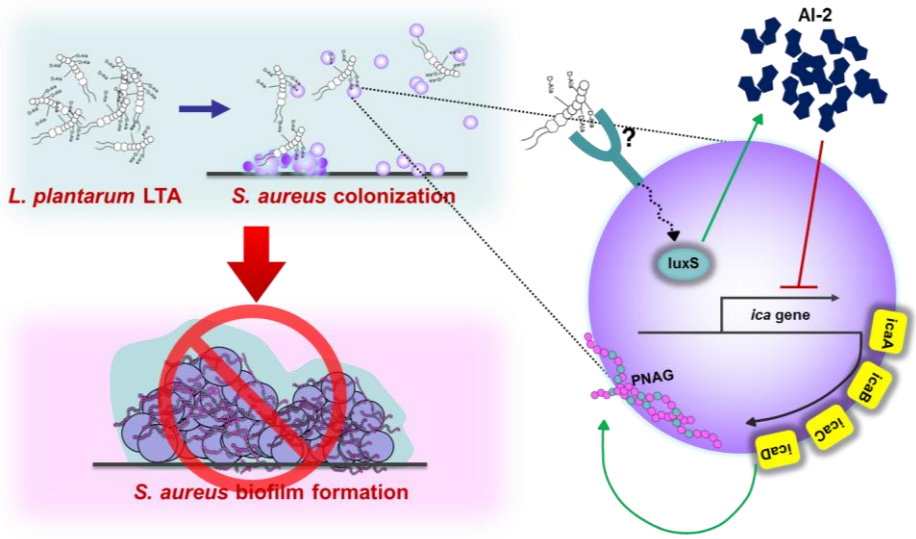


**Figure 32. Schematic illustration of the proposed action mechanism of inhibitory effect of LTA on the *S. aureus* biofilm formation.** Lp.LTA inhibits *S. aureus* biofilm formation though inhibition of *ica* gene expression and PNAG production. Moreover, LTA increases release of AI-2 from *S. aureus* that partly contributes to inhibition of *S. aureus* biofilm. D-alanine residues act as a crucial functional moieties of LTA for inhibitory effect against *S. aureus* biofilm formation.

# 4. Conclusion

In the present study, the roles of cell wall-associated virulence factors of *S. aureus* in the induction of inflammatory responses and biofilm formation were demonstrated. In *S. aureus*-mediated inflammatory responses, lipoprotein plays a crucial role in the induction of NO production in macrophages through activation of NF-κB and STAT1 triggered by TLR2 and LTA and MDP synergistically induced inflammatory response by overproducing COX-2 through NOD2 and TLR2. In *S. aureus* biofilm formation, released LTA, but not lipoprotein, inhibited *S. aureus* biofilm by inhibiting the production of PNAG, which is an important component of biofilm formed by *S. aureus*. In particular, LTA from a beneficial bacterium *L. plantarum* substantially inhibited *S. aureus* biofilm formation. Collectively, lipoprotein in the cell wall of *S. aureus* plays a major role in the induction of inflammatory responses through activation of TLR2 and cooperative action between LTA and MDP might contribute to the establishment of inflammation. LTA acts as a negative regulator against *S. aureus* biofilm and Lp.LTA could be an anti-biofilm agent for prevention or treatment of chronic inflammatory diseases caused by *S. aureus*.

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# 국문초록

# 황색포도상구균 바이오필름 형성과 염증반응 유도에서 세포벽 병독성인자들의 역할

안기범

서울대학교 치의학대학원

치의과학과 면역 및 분자미생물 전공

## 목 적

황색포도상구균은 통성혐기성 그람양성세균으로 사람의 피부 또는 점막에 집락을 형성하고높은 보균율을 가지고 있으며 인체 내 가벼운 피부염증에서부터 패혈증, 폐렴, 심내막염 등과 같은 중증 질환까지 유발하는 치명적인 병원균이다. 특히 황색포도상구균은 세균 최 외곽에 존재하는 세포벽 병독성인자를 통해 강한 면역반응을 일으켜 비정상적인 염증반응을 유도하고 바이오필름 형성을 통해 감염 질환을 유발하는 것

으로 알려져 있다. 하지만 현재까지 이에 대한 핵심 세포벽 병독성인자 규명 및 작용기전에 대한 연구는 매우 미비한 상태이다. 황색포도상구균에 의한 감염과 감염질환을 예방하고 치료하기 위해서는 세균 감염기전에 대한 이해가 필요하며 이를 바탕으로 한 조절물질 개발 연구가 이루어져야 한다. 따라서 본 연구에서는 황색포도상구균에 의해 유도된 면역반응 및 바이오필름을 매개하는 주요 세포벽 병독성인자 발굴 및 작용기전을 규명하고 이를 조절할 수 있는 물질개발 및 조절 기전을 규명하고자 하였다.

## 실험방법

황색포도상구균 세포벽 병독성인자들의 선천성 면역활성 능력을 평가하기 위해 마우스 대식세포 (마우스 대식세포주인 RAW 264.7 세포, C57BL/6 정상마우스 또는 TLR2-, NOD2 결손마우스의 골수유래 대식세포) 또는 인체 대식세포 (인체 단핵구유래 대식세포)를 황색포도상구균 정상균주, 지질단백질 결여균주, 리포테이코익산 결여균주 또는 정제된 지질단백질, 리포테이코익산 및 펩티도글리칸, 펩티도글리칸 소단위체 (MDP, Tri-DAP, M-tri DAP) 물질로 자극한 뒤 생성된 염증매개인자들을 측정 후 비교 분석하였다. 황색포도상구균 세포벽 병독성인자의 염증매개인자 생성 유도능력을 평가하기 위해 마우스 대식세포에 황색포도상구균 정상균주, 지질단백질 결여균주, 리포테이

코익산 결여균주를 처리한 후 nitric oxide (NO)의 생성을 Griess시약을 이용한 아질산염 측정법울 통하여 확인하였고 inducible NO synthase (iNOS)의 생성을 RT-PCR과 Western blotting을 통하여 확인하였다. 리포테이코익산과 MDP의 염증매개인자 생성 유도능력을 평가하기 위해 마우스 대식세포와 인체 대식세포에 리포테이코익산과 MDP를 단독 또는 병용처리 후 프로스타글란딘 E2 (Prostaglandin E2, PGE2)의 생성을 효소면역측정법(Enzyme-linked immunosorbent assay, ELISA)을 통하여 확인하였고 cyclooxygenase-2 (COX-2)의 생성을 RT-PCR과 Western blotting을 통하여 확인하였다. 리포테이코익산과 MDP의 염증매개인자 발현관련 전사인자 활성 능력을 평가하기 위해 마우스 대식세포에 리포테이코익산과 MDP를 단독 또는 병용처리 후 NF-κB, AP-1, CRE의 활성을 루시퍼라아제(발광효소) 리포터 유전자 분석기법을 통해 확인하였다. 세포벽 병독성인자들에 의한 톨유사수용체(Toll-like receptor, TLR)의 신호전달 활성을 조사하기 위해 TLR2 또는 TLR4 의존적인 전사활성에 따라 세포막에 분화항원(Cluster of differentiation, CD) 25를 발현하도록 유전적으로 조작된 중국 햄스터 난소(Chinse hamster ovary cell, CHO)/CD14/TLR2 세포주와 CHO/CD14/TLR4 세포에 황색포도상구균 정상균주, 지질단백질 결여균주, 리포테이코익산 결여균주를 처리한 후 유세포 분석기를 이용하여 CD25 발현을 분석하였다. 황색포도상구균 바이오필름에 대

한 세포벽 병독성인자들의 영향을 조사하기 위해 황색포도상구균에 정제된 리포테이코익산, 지질단백질, 펩티도글리칸을 처리한 후 형성된 바이오필름을 crystal violet 염색법, 공초점현미경, 주사전자현미경을 통해 확인하였다. 황색포도상구균의 쿼럼센싱 물질 생성에 대한 리포테이코익산의 영향을 조사하기 위해 황색포도상구균에 리포테이코익산을 처리한 후 생성된 autoindcuer-2 (AI-2)을 비브리오 하베이 (*Vibrio harveyi*) BB170 균주를 이용한 루시퍼라아제 리포터 유전자 분석기법 통해 확인하였다.

## 결 과

황색포도상구균 지질단백질 결여균주는 대식세포에서 정상균주와 리포테이코익산 결여균주에 비해 NO와 NO 합성 효소인 iNOS의 생성을 유도하지 못하였다. 또한 정상균주와 리포테이코익산 결여균주는 iNOS 생성에 관여하는 전사인자인 NF-κB와 STAT-1의 활성을 유의적으로 증가시켰지만 지질단백질 결여균주는 이들의 활성화를 유도하지 못하였다. 특히 정상균주와 리포테이코익산 결여균주는 TLR2의 활성을 농도 의존적으로 증가시켰지만 지질단백질 결여균주의 자극은 TLR2 활성에 아무런 영향을 주지 못하였다. 따라서 지질단백질은 황색포도상구균에 의한 TLR2 활성과 이로 인한 iNOS 발현 및 NO 생성에 관여하는 핵심 세포벽 병독성인자임을 확인하였다. 정제된 황색

포도상구균 리포테이코익산과 펩티도글리칸은 대식세포에서 상승효과를 보이며 COX-2와 PGE2 생성을 유도하였다. 특히 펩티도글리칸의 소단위체 물질들 중 MDP가 리포테이코익산과 COX-2 발현에 상승효과를 보이는 것을 확인하였다. 하지만 리포테이코익산과 MDP의 COX-2 발현에 대한 상승효과는 TLR2와 NOD2 결손마우스 대식세포에서 실험 시 사라졌다. 리포테이코익산과 MDP는 COX-2 발현 관련 전사인자인 NF-κB와 CRE 활성에도 상승효과를 보였다. 따라서 리포테이코익산은 펩티도클리칸의 소단위체 물질인 MDP와 협력하여 황색포도상구균에 의한 염증반응에 관여하는 것을 확인하였다. 분비된 황색포도상구균 리포테이코익산을 포함하고 있는 정상세균의 배양액은 황색포도상구균의 바이오필름을 억제하였지만 리포테이코익산 결여균주의 배양액은 바이오필름을 억제하지 못하였다. 정제된 황색포도상구균 리포테이코익산 역시 바이오필름을 억제하였고 지질단백질은 아무런 영향을 주지 못하였다. 다양한 그람양성세균들에서 정제한 리포테이코익산 모두 황색포도상구균의 바이오필름을 억제하였고 이 중 특히 유산균 *Lactobacillus plantarum*에서 정제된 리포테이코익산이 매우 큰 억제효과를 보였다. *L. plantarum* 리포테이코익산은 황색포도상구균 바이오필름에 핵심 구성물질인 poly-*N*-acetylglucosamine의 생성을 억제하였고 바이오필름의 음성 조절물질로 알려진 AI-2의 생성을 증가시킴으로써 황색포도상구균 바이오필름을 억제하였다. 또

한 리포테이코익산의 D-alanine 잔기가 바이오필름 억제 효과에 필수적으로 작용하는 것을 확인하였다

## 결 론

이상의 연구결과들로부터 다음과 같은 결론을 얻을 수 있었다. 지질단백질은 황색포도상구균에 의한 염증반응에 관여하는 핵심 세포벽 병독성인자이며 리포테이코익산은 MDP와 협력하여 황색포도상구균에 의한 염증반응에 기여한다. 또한 리포테이코익산은 황색포도상구균 바이오필름에 대한 음성조절인자로 작용하며 특히 *L. plantarum* 리포테이코익산은 황색포도상구균에 의한 염증질환을 예방 및 치료 할 수 있는 항바이오필름 작용제로 활용될 수 있다.

**주요어:** 황색포도상구균, 지질단백질, 리포테이코익산, 염증반응, 바이오필름, 유산균

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