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# Antagonistic effect of peptidoglycan of *Streptococcus sanguinis* on lipopolysaccharide of major periodontal pathogens

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***Streptococcus sanguinis* is often found in subgingival biofilm including periodontopathogens, and is correlated with a delay in colonization by periodontopathogens. However, the effect of *S. sanguinis* on inflammation induced by periodontopathogens is poorly understood. Thus, this study investigated the effect of *S. sanguinis* peptidoglycan (PGN) on induction of TNF-α, IL-6, and IL-8 expression by lipopolysaccharide (LPS) of periodontal pathogens. LPS was extracted from *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, and *Tannerella forsythia*, and PGN was isolated from *S. sanguinis*. THP-1 cells, a monocytic cell-line, were co-treated with LPS of the periodontal pathogens and *S. sanguinis* PGN, and then the expression of inflammatory cytokines was analyzed by real-time RT-PCR. To analyze the underlying mechanism, the binding assay of the LPS to CD14 or LPS-binding protein (LBP) was performed in the presence or absence of the PGN after coating recombinant human CD14 and LBP on EIA plate. The PGN inhibited the binding of LPS to CD14 and LBP in a dose-dependent manner. Also, THP-1 cells were co-treated with the LPS in the presence of *N*-acetylmuramic acid and *N*-acetylglucosamine, as components of PGN, and the competition binding assay to CD14 and LBP was performed. *N*-acetylmuramic acid inhibited the induction of inflammatory cytokine expression by LPS and the binding of LPS to CD14 or LBP whereas *N*-acetylglucosamine did not show such effect. Collectively, the results suggest that *S. sanguinis* PGN inhibited the cytokine expression induced by the LPS of periodontopathogens due to the inhibition of LPS binding to LBP and CD14. *N*-acetylmuramic acid of PGN may play a role in inhibition of the LPS binding of periodontopathogens to CD14 and LBP.**

***Keywords*:** antagonism, peptidoglycan, lipopolysaccharide, inflammation

## Introduction

Microbes of many species co-exist in oral cavity. Furthermore, normal flora are maintained by a balance between various Gram-positive and Gram-negative bacteria in healthy person (Socransky and Haffajee, 1992; Socransky and Haffajee, 2005). When the homeostasis of microbial ecosystem is broken down due to the change of oral condition, the number of periodontal pathogens increases compared to normal flora, and inflammatory diseases such as gingivitis and periodontitis may occur (Marsh, 2006). Periodontopathogens are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* and have lipopolysaccharide (LPS) as a potent stimulator of inflammatory responses (Schytte Blix *et al.*, 1999; Lee and Baek, 2013; Kim and Lee, 2014). Oral streptococci as a member of normal flora have peptidoglycan (PGN) and lipoteichoic acid (LTA) which are known to be weak immune stimulators compared to LPS (Hong *et al.*, 2014). Among oral streptococci, *S. sanguinis* is considered as a beneficial bacterium in the prevention of dental caries (Kreth *et al.*, 2005). Also, they are frequently found in subgingival biofilm including periodontopathogens of patients with periodontitis and their existence is correlated with a delay in colonization by periodontopathogens (Stingu *et al.*, 2008). Although both *S. sanguinis* and periodontal pathogens coexist and have pathogen-associated molecular patterns (PAMPs), the studies on the inflammatory response have focused on LPS of periodontal pathogens but rarely on PGN.

LPS is an endotoxin and a component in outer membrane of Gram-negative bacteria (Kim and Lee, 2014). LPS is composed of three domains including lipid A, core-oligosaccharide, and O-antigen. O-antigen has dideoxyhexoses of various lengths branched by phosphate group, and is hydrophilic (Lerouge and Vanderleyden, 2002). Structure of lipid A is 12 to 16 carbon atoms-composed pentamer or hexamer fatty acid linked to a phosphorylated β-1,6-linked D-glucosamine disaccharide and is hydrophobic (Lee and Baek, 2013). Therefore, most LPS in aqua can form micelles. Toll-like receptor (TLR) signaling is initiated by binding of lipid A to extracellular domain of TLR (Akira and Takeda, 2004). Micelles formed by LPS is disrupted by LPS-binding protein (LBP) and CD14, and lipid A is exposed by them (Lee *et al.*, 2006). Consequently, LPS is transferred to TLR by binding to LBP and CD14. LPS binds TLR2 or TLR4 according to the structure of acyl chain in lipid A (Lee *et al.*, 2010), and induces inflammation via the intracellular signaling molecules of TLR (Akira and Takeda, 2004). PGN is composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) cross-linked by short peptides. It is a major cell wall component of bacteria (Kurata, 2014), and has a slight difference in the molecular structure by bacterial species (Mychajlonka *et al.*, 1980; Moynihan *et al.*, 2014). MurNAc linked short peptide binds and stimulates nucleotide-bind-

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ing oligomerization domain (NOD) 1 and 2 (Dziarski and Gupta, 2005; Leung *et al*., 2009). PGN of *Staphylococcus aureus* and *Bacillus subtilis* stimulates immune response via NOD whereas PGN of *Lactobacillus* species did not show such effect (Volz *et al*., 2010; Wu *et al*., 2015).

Although, *S. sanguinis* plays a role in delay in colonization by periodontopathogens, the characteristics of immune response in co-existence of PGN of *S. sanguinis* and LPS of periodontopathogens have been unknown. Thus, this study investigated the effect of *S. sanguinis* PGN on the induction of cytokine expression by LPS of major periodontopathogens.

## Materials and Methods

### Bacterial species and cultivation

*A. actinomycetemcomitans* ATCC 43718, *P. gingivalis* ATCC 33277, and *T. forsythia* ATCC 43037 were used to extract lipopolysaccharide. *S. sanguinis* ATCC 10556 (formerly *S. sanguis*) and *S. aureus* ATCC 25923 were used to extract peptidoglycan. *A. actinomycetemcomitans*, *S. aureus*, and *S. sanguinis* were cultivated with brain heart infusion (BHI; BD Bioscience) broth, and *P. gingivalis* was cultured with BHI supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) in anaerobic condition at 37°C. *T. forsythia* was cultivated in the modified new oral spirochete (NOS) broth according to the methods as previously described (Kim and Lee, 2014) at 37°C in an anaerobic atmosphere (5% $H_2$, 10% $CO_2$, and 85% $N_2$).

### LPS extraction

LPS was extracted from *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* according to the method as described previously (Lee and Baek, 2013). The bacteria were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and washed three times with cold phosphate buffered saline (PBS; pH 7.2). The bacteria were mixed with lysis buffer, vortexed until the bacteria clump disappeared, and chloroform was added. The mixture was centrifuged 13,000 × *g* for 15 min at 4°C after votexing for 15 sec. The supernatant was transferred to a new 1.5 ml tube and incubated with endonuclease (100 μg/ml) for 1 h at 37°C followed by treatment with proteinase K (250 μg/ml) at 55°C for 1 h. The preparation was re-added lysis buffer to remove protein residues and performed the protocol as described above until before phase of the enzyme treatment. The suspension was incubated with purification buffer for 10 min at -20°C. After centrifugation at 13,000 × *g* for 15 min at 4°C, the supernatant was removed. The pellet was washed with 70% ethyl alcohol, air-dried and dissolved in endotoxin-free water. After lyophilization, the dry weight of LPS was measured. The LPS was dissolved in endotoxin-free water at a concentration of 1 mg/ml. After, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) was carried out, the gels were stained with coomassie brilliant blue G-250 to check protein contamination and with sliver nitrate to visualize the LPS. Also, agarose gel electrophoresis was performed and stained with ethidium bromide to confirm protein contamination.

### Peptidoglycan extraction

The cultured-*S. sanguinis* and -*S. aureus* were harvested by centrifugation. The pellet was washed three times with PBS and boiled for 40 min in distilled water including 5% SDS. The suspension was cooled in refrigerator, and centrifuged at 8,000 × *g* for 10 min. the pellet was washed distilled water until removing SDS and subsequently performed sonication (50 × 10 sec bursts at 200 W with 10 sec cooling period; Sonics & Materials, Inc.). After washing them, the pellet was suspended and incubated to remove nucleotides with PBS including DNase I (2 μg/ml) and RNase (2 μg/ml) at 37°C for 3 h. Trypsin (0.1 mg/ml) and $CaCl_2$ (10 mM) were added and subsequently incubated at 37°C for 12 h. The suspension was centrifuged at 10,000 × *g* for 10 min, and the pellet was resuspended and incubated with PBS including proteinase K (250 μg/ml) at 55°C for 2 h to eliminate bacterial proteins and used enzymes. After centrifugation at 8,000 × *g* for 10 min, the pellet was resuspended with 10% trichloroacetic acid solution at 4°C for 24 h to remove LTA. Finally, water-insoluble molecules were washed with cold endotoxin-free water until pH 7.

### Cell cultivation and treatment with LPS and PGN

CHO/CD14/TLR2 or CHO/CD14/TLR4 which is a NF-κB reporter cell lines was used to evaluate the TLR2- or TLR4-stimulating potential of LPS from *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia*, as described previously (Lee *et al*., 2010). The CHO cells were kindly provided by Douglas Golenbock (Medical School, University of Massachusetts). The cells have co-expressed CD14 and TLR2 or TLR4 and express a gene encoding a membrane CD25 driven by the human E-selectin promoter, which includes NF-κB binding sites. The cells were grown in Ham's F-12 medium (Hyclone) including 10% fetal bovine serum (FBS; Hyclone), 1 mg/ml of G418 (Calbiochem) and 400 U/ml of hygromycin B (Calbiochem) at 37°C in a $CO_2$ incubator. When the cells were 90% confluent, *Escherichia coli* O111:B4 LPS (100 ng/ml; Invivogen), Pam3CSK4 (100 ng/ml; Invivogen) and the LPS (500 ng/ml) from *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* were treated for 18 h in the presence of 5% FBS. The cells were washed twice with PBS and detached with 2 mM ethylenediamine tetra-acetic acid (EDTA) in PBS to analyze the expression of CD25.

THP-1 cell, a monocytic cell-line, was purchased from ATCC and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate). The cells ($1 \times 10^6$ cells/ml) were plated in 6-well plate and treated with the extracted LPS (500 ng/ml or 1 μg/ml) from periodontopathogens in the presence or absence of the extracted PGN from *S. sanguinis* or *S. aureus* for 8 h including 1% human serum at 37°C in $CO_2$ incubator for 12 h. Also, THP-1 cells were co-treated with the LPS extracted from various periodontal pathogens and MurNAc (1 μg/ml) or GlcNAc (1 μg/ml) at 37°C in $CO_2$ incubator for 12 h. The cells were used to analyze expression of inflammatory cytokines by real-time RT-PCR.

### Flow cytometry

To analyze CD25 expression of CHO cells, The cells were then washed twice with cold PBS and incubated with fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD25 (BD Bioscience) at 4°C for 30 min. After washing twice with cold PBS, CD25 expression was analyzed by flow cytometry (FACS Calibur™; BD Biosciences). The data were obtained by counting 15,000 cells, and the expression of the target molecules was analyzed using CellQuest program (BD Biosciences).

### Real-time RT-PCR

The LPS-treated THP-1 cells were collected by centrifugation at 1,200 × *g* for 5 min at 4°C and washed with cold PBS. Total RNA from the THP-1 cells was isolated with a TRIzol® RNA isolation kit (Invitrogen Life Tech.) according to the manufacturer's protocol. cDNA was synthesized by Maxime™ RT Premix (iNtRON). cDNA was mixed with 10 μl of SYBR® Premix EX Taq II (TaKaRa Co.), 0.4 μM of each specific primers and ROX II dye in 20 μl final volume, and performed 40 PCR cycles (95°C for 15 sec, 60°C for 10 sec, and 72°C for 33 sec) using ABI PRISM 7500 real-time PCR system (Applied Biosystems). PCR products were analyzed using a dissociation curve of amplification to examine non-specific amplificaiton. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference to normalize expression levels and quantify changes of inflammatory cytokine. Critical threshold cycle (Ct) was defined as the cycle at which fluorescence became detectable as against the background and was inversely proportional to the logarithm of the initial number of the template molecules. The sequences of primers for real-time RT-PCR were as follows: 5′-CAG GGA CCT CTC TCT AAT CA-3′ and 5′-AGC TGG TTA TCT CTC AGC TC-3′ the TNF-α gene; 5′-AAC CTG TCC ACT GGG CAC A-3′ and 5′-TCT GGC TCT GAA ACA AAG GAT-3′ for the IL-6 gene; 5′-GTG GTG GAC CTG ACC TGC-3′ and 5′-TGA GCT TGA CAA AGT GGT CG-3′ for the GAPDH gene.

### Labelling LPS with biotin

The LPS was labeled with biotin using EZ-Link Hydrazide-Biotin (Thermo Sci.). The LPS was incubated with cold 20 mM sodium metaperiodate at 4°C for 1 h and then dialyzed in 0.1 M sodium acetate (pH 5.5) for 12 h. The suspension was transferred into a new tube and hydrazide-biotin solution was added to a final concentration of 5 mM. After agitation with rotator for 3 h at room temperature, the mixture was dialyzed three times in distilled water for 24 h. After lyophilization, the dry weight of LPS was measured. The LPS was dissolved with endotoxin-free water at a concentration of 1 mg/ml.

### Competitive binding assay to LBP and CD14

Anti-human LBP polyclonal Ab (50 ng/well in 50 μl; R&D Systems) or anti-human CD14 polyclonal Ab (50 ng/well in 50 μl; R&D Systems) were coated on EIA plate (Corning Inc.) at 4°C for 12 h. The plates were washed four times with PBS containing 1% Tween 20 (PBST) and blocked with 1% bovine serum albumin (BSA) in PBS for 2 h. Recombinant human LBP (rhLBP) or human CD14 (rhCD14) were then added into each Ab-coated plate and incubated at room

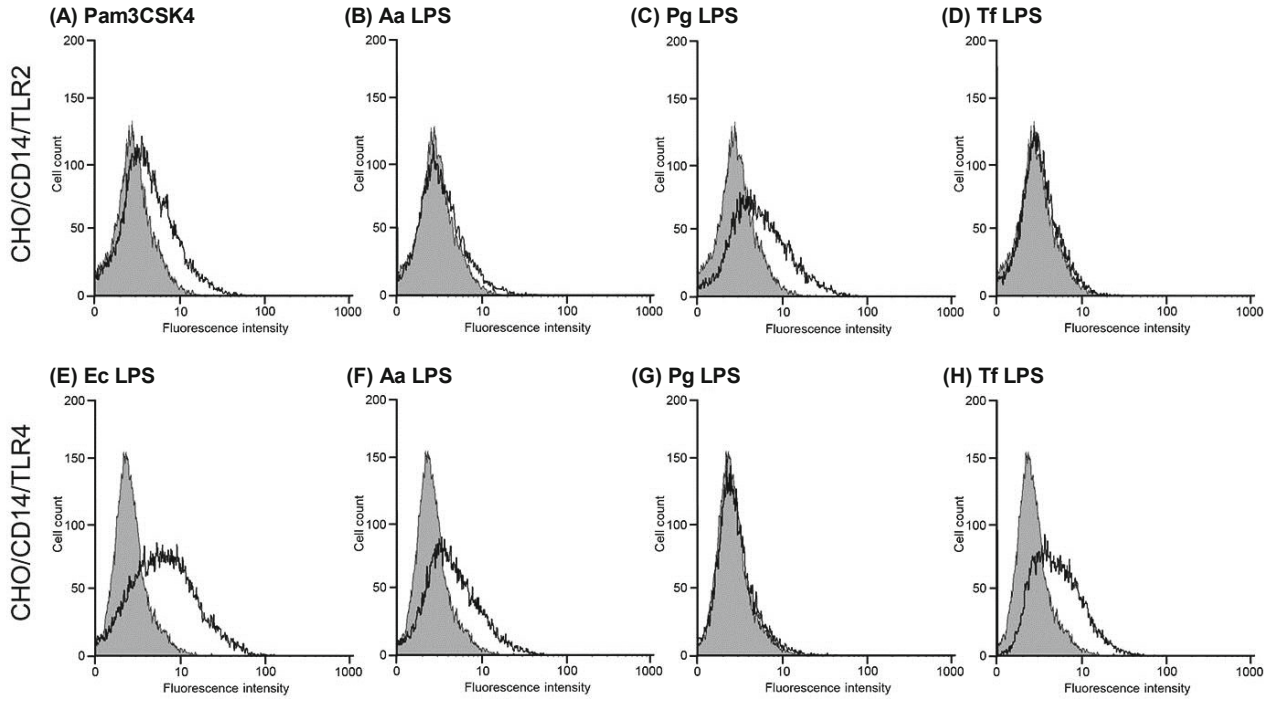


**Fig. 1. Verification of purity of the extracted LPS from major periodontal pathogens.** CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were treated with *A. actinomycetemcomitans* LPS (Aa LPS), *P. gingivalis* LPS (Pg LPS), and *T. forsythia* LPS (Tf LPS) for 18 h. CD25 expression on the cells was analyzed by flow cytometry. Pam3CSK4 and *E. coli* LPS (Ec LPS) were used as positive control for TLR2 and TLR4 activation, respectively.

temperature for 2 h. After washing the plate five times, the biotinylated LPS (500 ng/ml) and PGN, MurNAc (1 μg/ml) or GlcNAc (1 μg/ml) was added at various concentration and incubated at room temperature for 2 h. The plates were washed five times with PBST and exposed to 50 μl of horseradish peroxidase (HRP)-conjugated streptavidin (1 μg/ml) in PBS containing 2% BSA for 1 h to detect biotinylated LPS. After washing the plates with PBST, 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added. After 20 min, 50 μl of 1 N sulfuric acid was added to stop enzyme reaction. Optical density was measured at 450 nm wavelength by microplate reader (Molecular Devices, LLC.).

### Statistical analysis

Statistically significant differences between non-treated cells and LPS or co-treatment of LPS and PGN were analyzed by Kruskal-Wallis test and Mann-Whitney test using SPSS 10 (SPSS Inc.). *P*-values less than 0.05 were considered statistically significant.

## Results

### Biological activity of LPS extracted from the periodontal pathogens

To test the activity of the extracted LPS from periodontal pathogens, CHO/CD14/TLR2 and CHO/CD14/TLR4 were treated with the extracted LPS and CD25 expression was analyzed. LPS of *A. actinomycetemcomitans* and *T. forsythia* is known to as a TLR4 ligand, and LPS of *P. gingivalis* is known to be a of TLR2 ligand. In analyzing with CHO/CD14/TLR2 and CHO/CD14/TLR4, *P. gingivalis* LPS stimulated CHO/CD14/TLR2 cells (Fig. 1C), and LPS extract from *A. actinomycetemcomitans* and *T. forsythia* stimulated CHO/CD14/TLR4 cells (Figs. 1F and H). *S. aureus* PGN stimulated CHO/CD14/TLR2 cells, whereas *S. sanguinis* PGN stimulated neither CHO/CD14/TLR2 nor CHO/CD14/TLR4 (data not shown).

### Antagonistic effect of PGN of *S. sanguinis* on the expression of inflammatory cytokines by LPS

Since *S. sanguinis* is frequently found in subgingival biofilm including periodontopathogens, PGN of *S. sanguinis* was investigated for the effect on the inflammatory cytokine expression induced by LPS of periodontopathogens. LPS of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* significantly induced the expression of inflammatory cytokines such as TNF-α, IL-6, and IL-8 in the presence or absence of human serum though the level of cytokine expression was different in both conditions (Fig. 2). *S. sanguinis* PGN significantly inhibited the induction of cytokine expression by the LPS of periodontal pathogens in the presence

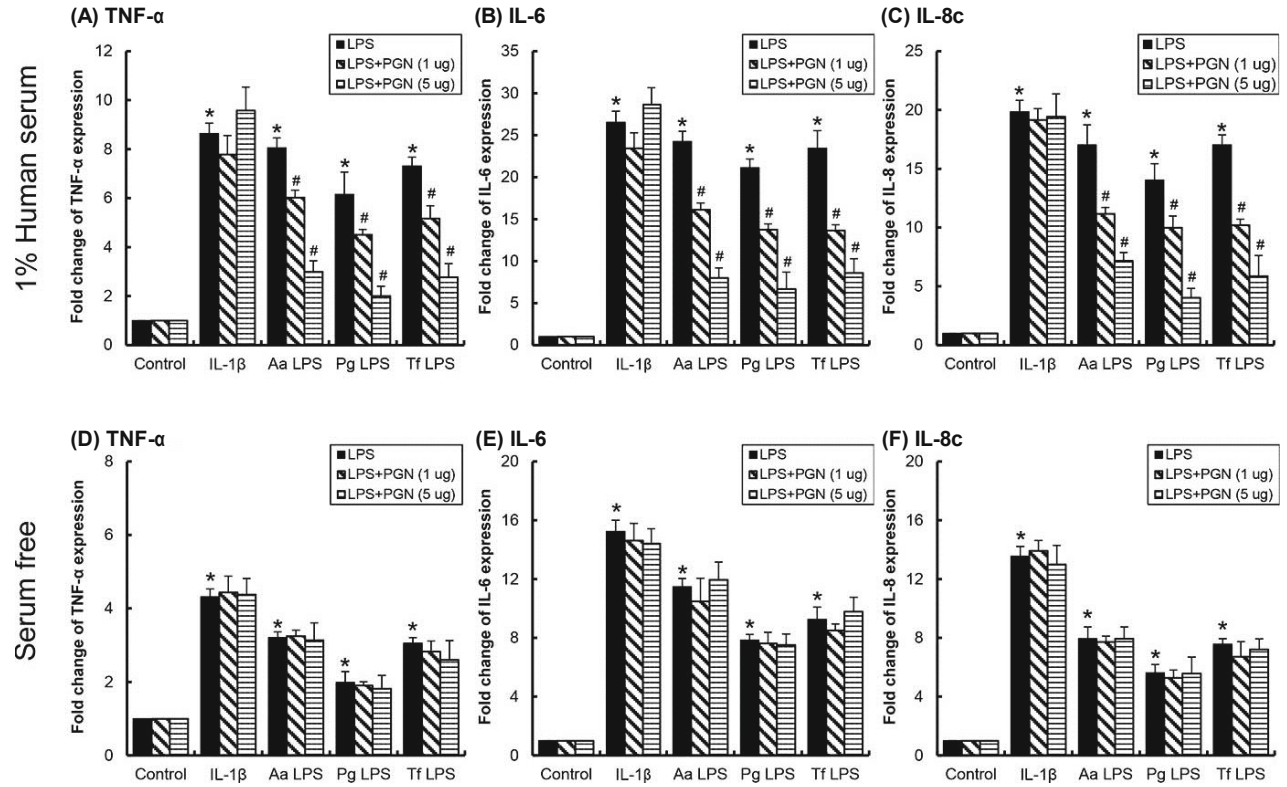


**Fig. 2. Inhibitory effect of *S. sanguinis* PGN on the cytokine expression induced by LPS of major periodontopathogens.** THP-1 cells were co-treated with *A. actinomycetemcomitans* LPS (Aa LPS), *P. gingivalis* (Pg LPS), *T. forsythia* LPS (Tf LPS) or IL-1β together with the PGN in the presence or the absence of 1% human serum. The induction of cytokine expression was analyzed by real-time RT-PCR. The experiments were performed three times in duplicate and representative data for mean value and standard deviation are shown. * indicates a significant difference compared to the non-treatment control group ($P<0.05$) and # indicates a significant difference compared to the LPS treatment group ($P<0.05$).

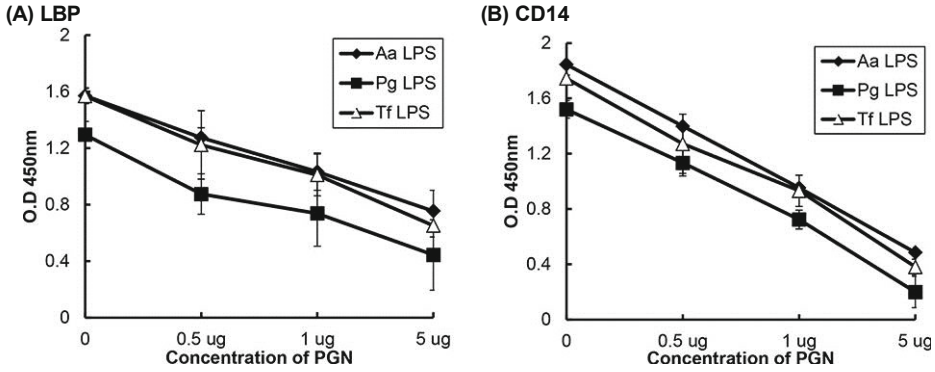


**Fig. 3. Inhibition of the binding of LPS to LBP and CD14 by *S. sanguinis* PGN.** EIA plates were coated with rhLBP and rhCD14 after pre-coating with anti-human LBP and anti-human CD14, respectively. The plates were incubated with the biotinylated LPS in the presence of the PGN at various concentrations. The bound LPS of periodontal pathogens was detected by HRP-conjugated streptavidin and TMB solution. Optical density was measured at a wavelength of 450 nm by spectrophotometer.

of human serum (Figs. 2A, B, and C). However *S. sanguinis* PGN did not affect the induction of them in serum-free condition (Figs. 2D, E, and F). Furthermore, IL-1β-induced cytokine expression was not affected by the PGN in those conditions (Fig. 2). Notably, IL-1 receptor has the same intracellular signaling domain with TLR2 and TLR4 (Lee *et al.*, 2006).

### Inhibitory effect of PGN on binding of LPS to LBP and CD14

The PGN of *S. sanguinis* exhibited inhibitory effect on the induction of cytokine expression by the LPS in 1% human serum condition, and did not affect it under serum-free condition or IL-1β-induced cytokine expression. Thus, the relationship between extracellular components of LPS signaling and inhibitory effect of the PGN was investigated. When the competition between PGN and the biotinylated-LPS for binding to LBP or CD14 was tested, binding of LPS from *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* to LBP and CD14 was significantly reduced by the PGN (Fig. 3). The binding of LPS to CD14 was more inhibited than that of LBP by PGN.

### Comparison between PGN of *S. sanguinis* and *S. aureus* on LPS-induced cytokine expression

In order to examine if the inhibitory effect of PGN on LPS-induced cytokine expression was observed only by *S. sanguinis* PGN, the induction of cytokine expression was investigated after co-treatment of the LPS of periodontopathogens with PGN of *S. aureus*. PGN of *S. sanguinis* significantly reduced LPS-induced cytokine expression of THP-1 cells whereas PGN of *S. aureus* did not produce such reduction but instead significantly enhanced it (Fig. 4).

### Inhibitory effect of *N*-acetylmuramic acid on LPS signaling

The inhibitory effects of PGN from *S. sanguinis* were investigated using partial molecules of PGN. In co-treating with MurNAc or GlcNAc and the LPS, MurNAc significantly decreased LPS-induced cytokine expression in THP-1 cells. However, GlcNAc did not affect it (Fig. 5). In investigating the inhibitory mechanism of MurNAc with same protocol of the PGN test, MurNAc significantly inhibited binding of LPS to LBP and CD14, which was similar trend with the inhibitory effect of the PGN (Fig. 6).

## Discussion

Hundreds of bacterial species co-exist in oral cavity. Furthermore, they are mixed with Gram-positive and Gram-negative bacteria and have maintained the balance in healthy person (Socransky and Haffajee, 1992, 2005). When the homeostasis of microbial ecosystem was broken down due to the change of oral condition by various factors, the number of periodontal pathogens increases compared to normal flora, and inflammatory disease such as gingivitis and periodon-

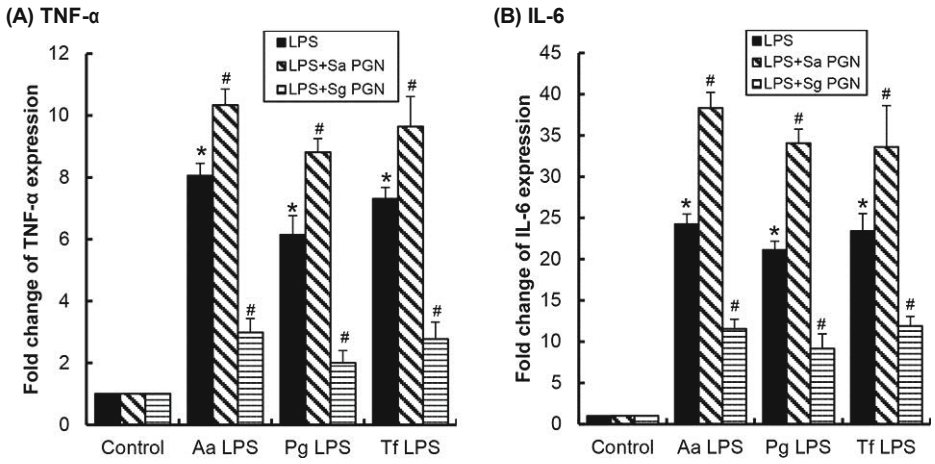


**Fig. 4. Effect of *S. sanguinis* PGN and *S. aureus* PGN on LPS-induced cytokine expression.** THP-1 cells were co-treated with *A. actinomycetemcomitans* LPS (Aa LPS), *P. gingivalis* (Pg LPS), *T. forsythia* LPS (Tf LPS) or IL-1β and *S. sanguinis* PGN or *S. aureus* PGN in the presence of 1% human serum. The induction of cytokine expression was analyzed by real-time RT-PCR. The experiments were performed three times in duplicate and representative data for mean value and standard deviation are shown. * indicates a significant difference compared to the non-treatment control group ($P<0.05$) and # indicates a significant difference compared to the LPS treatment group ($P<0.05$).

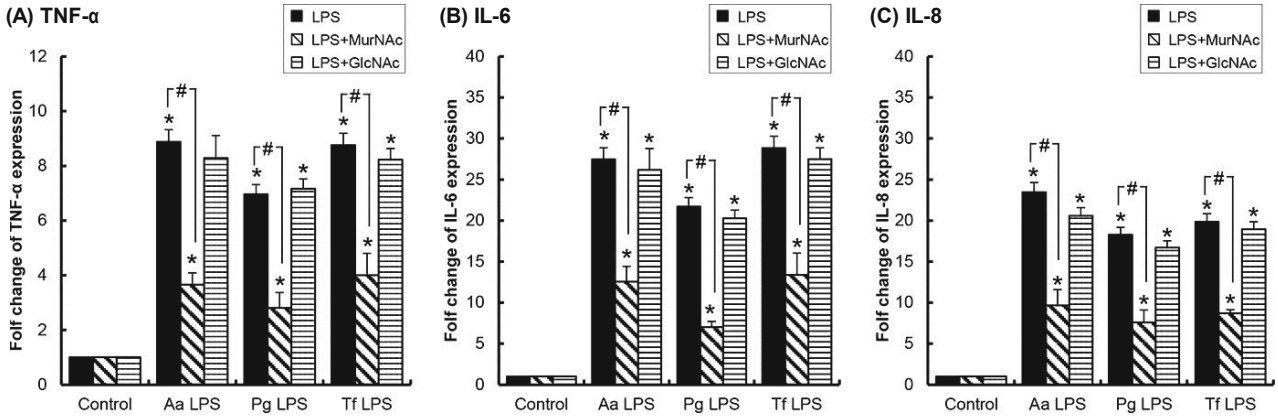


**Fig. 5. Effect of partial PGN on cytokine expression induced by the LPS.** THP-1 cells were co-treated with *A. actinomycetemcomitans* LPS (Aa LPS), *P. gingivalis* (Pg LPS) or *T. forsythia* LPS (Tf LPS) and MurNAc or GlcNAc in the presence of 1% human serum. The induction of cytokine expression was analyzed by real-time RT-PCR. The experiments were performed three times in duplicate and representative data for mean value and standard deviation are shown. * indicates a significant difference compared to the non-treatment control group ($P<0.05$) and # indicates a significant difference compared to the LPS treatment group ($P<0.05$).

titis may induce (Marsh, 2006). Although, both of Gram-positive and Gram-negative bacteria in oral cavity have PAMPs such as PGN and LPS, the expression of the inflammatory cytokines is induced in the higher proportion of Gram-negative bacteria compared Gram-positive bacteria in gingival pocket (Socransky and Haffajee, 2005). LPS is a potent stimulator of immune response and induces the expression of inflammatory cytokines (Schytte Blix, *et al*., 1999; Lee and Baek, 2013; Kim and Lee, 2014). Most Gram-positive bacteria as commensal bacteria in oral cavity are oral streptococci. *S. sanguinis* is an early colonizer in oral cavity and often detected with periodontal pathogens in subgingival biofilm (Stinson *et al*., 1991; Deng *et al*., 2004; Stingu *et al*., 2008). Since *S. sanguinis* co-exists with periodontopathogens, their PAMPs may co-exist. Also, the inflammation related study about PGN of *S. sanguinis* has been poorly understood. Thus, this study investigated the effect of the PGN on the LPS-induced cytokine expression.

PGN was extracted from S. san and LPS from *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia*. The purity of PGN and LPS was examined by SDS-PAGE followed by coomassie blue staining or silver nitrate staining to detect protein contamination. PGN and LPS were also subjected to agarose gel electrophoresis to detect nucleic acid contamination. The extracted PGN and LPS were not contaminated with bacterial proteins or nucleic acids. Also, the bioactivity of the extracted PGN and LPS was evaluated using CHO/CD14/TLR2 and CHO/CD14/TLR4. The extracted LPS from *P. gingivalis* stimulated CHO/CD14/TLR2, and the extracted LPS from *A. actinomycetemcomitans* and *T. forsythia* stimulated CHO-CD14/TLR4. The results indicate that LPS of *P. gingivalis* is a ligand for TLR2, and LPS of *A. actinomycetemcomitans* and *T. forsythia* is a TLR4 ligand. LPS of periodontal pathogens induced the expression of inflammatory cytokines such as TNF-α, IL-6, and IL-8 in THP-1 cells, whereas the extracted PGN from *S. sanguinis* did not stimulate THP-1 cells. Furthermore, *S. sanguinis* PGN inhibited the induction of cytokine expression by the LPS in the presence of 1% human serum. Interestingly, the PGN did not affect the LPS-induced cytokine expression in serum-free condition. These results indicate that the inhibitory effect of the PGN might be related with some serum components required for the LPS signaling. Also, the PGN did not affect IL-1β-induced cytokine expression in the presence or absence of human serum. The intracellular signaling domain of IL-1 receptor is equal to those of TLR2 and TLR4

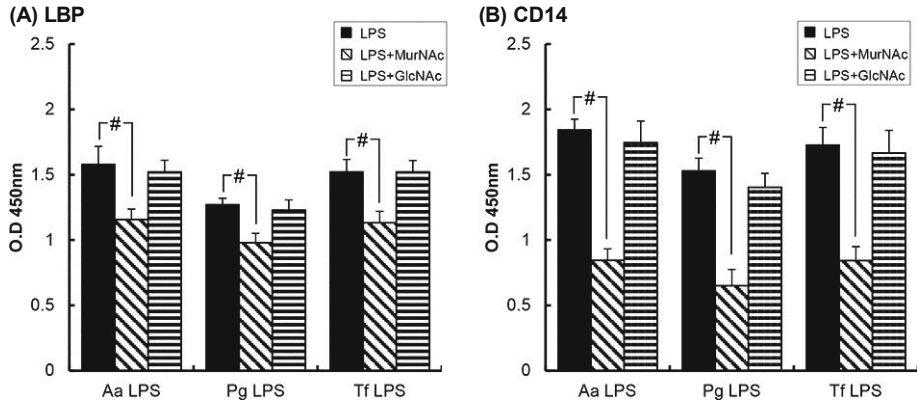


**Fig. 6. Inhibition of LPS binding to LBP and CD14 by MurNAc.** EIA plates were coated with rhLBP and rhCD14 after precoating with anti-human LBP and anti-human CD14, respectively. The plates were incubated with the biotinylated LPS in the presence of MurNAc or GlcNAc. The bound LPS of periodontal pathogens were detected by HRP-conjugated streptavidin and TMB solution. Optical density was measured at a wavelength of 450 nm by spectrophotometer.

(Lee *et al*., 2006). Taken together the results, *S. sanguinis* PGN might inhibit binding of LPS to the serum components such as LBP and CD14.

Next, a competitive binding assay was performed using anti-human LBP Ab, rhLBP, anti-human CD14 Ab, and rhCD14 after biotinylating the LPS. The PGN reduced the binding of the biotinylated LPS to LBP and CD14. PGN of some species is known to bind to LBP and CD14 (Rietschel *et al*., 1998; Muhvic *et al*., 2001; Weber *et al*., 2003). As a result, *S. sanguinis* PGN may inhibit cytokine expression induced by the LPS of periodontopathogens due to inhibition of LPS binding to LBP and CD14. On the other hand, even though the PGN of *S. sanguinis* inhibited the cytokine expression induced by the LPS of periodontal pathogens, the PGN of *S. aureus* enhanced the induction of cytokine expression. A possible explanation can be found in the difference in the molecular structures of two PGNs. In general, PGN consists of GlcNAc, MurNAc, and short peptides, and the short peptides crosslink with MurNAc (Dziarski and Gupta, 2005). Short peptides comprised of L-Ala-D-glu-meso-DAP-D-Ala (DAP-type PGN) or L-Ala-D-glu-L-Lys-D-Ala (Lys-type PGN) are found according to the difference between species. Moreover, DAP-type PGN is a NOD1 ligand, but Lys-type PGN is not (Chamaillard, *et al*., 2003; Davis and Weiser, 2011). MurNAc and GlcNAc are backbone of PGN, and exhibit a slight difference in the molecular structure by bacterial species (Davis and Weiser, 2011). Thus, the difference of PGN effect of *S. sanguinis* and *S. aureus* on LPS-induced cytokine expression may be due to the structural difference of PGN.

Finally, to search the binding site of PGN to LPB and CD14, MurNAc and GlcNAc as partial PGN components were investigated their inhibitory effect on cytokine expression induced by the LPS. Since we do not have the technique for purification of MurNAc from the PGN, commercial MurNAc was used in this study. MurNAc significantly inhibited the cytokine expression induced by the LPS of periodontal pathogens and the LPS binding to LBP and CD14. However, GlcNAc showed no such effect. These results suggest that MurNAc may be a binding site of CD14 and LBP. Although the binding site of PGN to CD14 has not been clearly identified, Weber *et al*. (2003) suggested that O-acetate at 3-carbon position of MurNAc is a binding site of LBP.

In summary, the PGN of *S. sanguinis* inhibited the cytokine expression induced by the LPS of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* by acting on interference of LPS signal-related extracellular molecules such as LBP and CD14. MurNAc of PGN may play a role in the inhibition of binding of the LPS of periodontopathogens to CD14 and LBP.

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