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Reducing the Bioactivity of *Tannerella forsythia* Lipopolysaccharide by *Porphyromonas gingivalis*

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Tannerella forsythia **is considered a pathogen of periodontitis and forms a biofilm with multi-species bacteria in oral cavity. Lipopolysaccharide is a powerful immunostimulator and induces inflammation and shock. The purpose of this study was to investigate the characteristics of *T. forsythia* LPS in its co-cultivation with *Fusobacterium nucleatum* or *Porphyromonas gingivalis*. *T. forsythia* was co-cultured in the presence and absence of *F. nucleatum* and *P. gingivalis* and then *T. forsythia* LPS was extracted. The extracts were analyzed by SDS-PAGE and NF-κB reporter CHO cell lines. THP-1 cells were treated with the LPS and evaluated induction of cytokine expression by real-time RT-PCR and ELISA. For analysis of the bioactivity of *T. forsythia* LPS, the binding assay on LPS-binding protein (LBP) and CD14 was processed. The extracts did not contaminate other molecules except LPS and showed TLR4 agonists. Co-cultured *T. forsythia* LPS with *P. gingivalis* exhibited a lower level of induction of TNF-α, IL-1β, and IL-6 expression than single- or co-cultured *T. forsythia* LPS with *F. nucleatum* in the conditions of human serum. However, the three *T. forsythia* LPS did not show difference of cytokine induction in the serum free conditions. Co-cultured *T. forsythia* LPS with *P. gingivalis* exhibited a lower affinity to LBP and CD14 as binding site of O-antigen and attached at a lower level to THP-1 cells compared to single- or co-cultured *T. forsythia* LPS with *F. nucleatum*. The virulence of *T. forsythia* LPS was decreased by co-culturing with *P. gingivalis* and their affinity to LBP and CD14 was reduced, which may due to modification of O-antigen chain by *P. gingivalis*.**

Keywords: lipopolysaccharide, *Tannerella forsythia*, *Porphyromonas gingivalis*

Introduction

Periodontitis has been reported to be relate with three bacteria according to epidemiological experiments, and the three bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia* (previous names *Bacteroides forsythus*) and *Treponema denticola* were termed red complex (Socransky *et al.*, 1998; Park *et al.*, 2011; Murray *et al.*, 2014). Furthermore, they can co-adhere with each other, by which they are co-existed in a biofilm form *in vivo* (Jenkinson and Lamont, 2005; Socransky and Haffajee, 2005). In oral cavity, these bacteria form a biofilm with the commensal bacteria like oral streptococci through *Fusobacterium nucleatum* (Socransky and Haffajee, 1992). *P. gingivalis*, *T. forsythia*, and *T. denticola* are Gram-negative and obligated anaerobic bacteria in common and have glycolipid like lipopolysaccharide.

Lipopolysaccharide (LPS), which is an endotoxin, is a powerful immune stimulator and is found in the membrane component of Gram-negative bacteria (Seo *et al.*, 2012; Lee and Baek, 2013). LPS is composed of three domains as lipid A, core-oligosaccharide, and O-antigen chain (Raetz and Whitfield, 2002). O-antigen chain binds to LPS-binding protein (LBP) and is transported to CD14 (Schroder *et al.*, 2004). Lipid A part in LPS-CD14 complex binds to Toll-like receptor 2 (TLR2) or TLR4 and initiates an inflammatory signaling pathway (Chow *et al.*, 1999; Moreno *et al.*, 2004). The virulence of LPS differs by structure of lipid A (Hajjar *et al.*, 2002). Also, LPS binds TLR2 or TLR4 according to the structure of the acyl chain in lipid A (Lee, *et al.*, 2010).

Tannerella forsythia LPS is an inflammatory stimulator and is associated with chronic periodontitis (Lee *et al.*, 2010; Lappin *et al.*, 2011). *Tannerella forsythia* LPS showed rough-form (Posch *et al.*, 2013). Most studies about *T. forsythia* LPS were processed after cultivating *T. forsythia* alone. However, *T. forsythia* co-exists with various species of bacteria such as *F. nucleatum* in and *P. gingivalis* and forms biofilm in oral cavity. Also, they communicate using autoinducers or metabolites (Hojo *et al.*, 2009). Therefore, we investigated the characteristics of *T. forsythia* LPS in co-cultivation with periodontitis-related bacteria.

Materials and Methods

Bacterial strain and cultivation

Fusobacterium nucleatum ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, and *Tannerella forsythia* ATCC 43037 were used for this study. *F. nucleatum* and *P. gingivalis* were cultivated in Brain Heart Infusion broth (BHI; BD bioscience, USA) supplemented with hemin (10 μg/ml) and vitamin K (0.2 μg/ml) at 37°C in an anaerobic atmosphere (5% H_2, 10% CO_2, and 85% N_2). *T. forsythia* was cultured in modified new oral spirochete (NOS) broth accord-

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ing to the method described by Lee *et al.* (2010) at 37°C anaerobically.

Co-culture of periodontopathogens

F. nucleatum or *P. gingivalis* was co-cultivated with *T. forsythia* using a Millicell cell culture insert (Millipore, USA). After mixing BHI and modified NOS broth at the same volume, the mixture was transferred to three new tubes. *F. nucleatum*, *P. gingivalis*, and *T. forsythia* were then inoculated into each tube. Millicell cell culture insert were hung into each well of 6-well plate and the suspension of *F. nucleatum* or *P. gingivalis* and suspension of *T. forsythia* were dispensed in the basolateral side and in the apical side of Millicell insert using the manufacturer's recommended volume, respectively. The plates were incubated for 36 h at 37°C in an anaerobic atmosphere. Contamination of each bacterium in the separating chamber was investigated by observation using a phase contrast microscope. Single cultivation of *T. forsythia* as control was carried out by inoculating the bacteria into the apical side and putting fresh the mixed media in the basolateral side.

Isolation of lipopolysaccharide (LPS)

LPS was isolated from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* according to the method described by Lee and Baek (2013). The *T. forsythia* were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C and then washed with cold phosphate buffered saline (PBS; pH 7.2). The bacteria were mixed with lysis buffer and vortexed until the bacterial clump disappeared, and then chloroform was added. The mixture was centrifuged 12,000 × *g* for 15 min at 4°C after votexing for 10 sec and the upper phase was transferred to a 1.5 ml new tube. The preparation was incubated with endonuclease (100 μg/ml) for 1 h at 37°C and then with proteinase K (250 μg/ml) for 1 h for 55°C. Lysis buffer was re-added to the preparation, which was then subjected to the protocol as described above and incubated with purification buffer for 10 min at -20°C. After centrifugation at 12,000 × *g* for 15 min at 4°C, the supernatant was removed. The pellet was washed with 1 ml of 70% ethyl alcohol, air-dried and dissolved with endotoxin-free water. After lyophilization, the dry weight of LPS was measured. The LPS was dissolved with endotoxin-free water at a concentration of 1 mg/ml. To analyze the pattern of O-antigen and lipid A, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) was carried out. The LPS was stained with silver nitrate.

Cell culture and treatment with LPS of *T. forsythia*

CHO/CD14/TLR2 and CHO/CD14/TLR4 which are NF-κB reporter cell lines were used to evaluate LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis,* 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, USA) including 10% FBS, 1 mg/ml of G418 (Calbiochem, USA) and 400 U/ml of hygromycin B (Calbiochem) at 37°C in a CO_2 incubator. When the cells were 80% confluent, *Escherichia coli* O111:B4 LPS (100 ng/ml; Invivogen, USA), *Staphylococcus aureus* lipoteichoic acid (1 μg/ml; Invivogen) and the LPS (10 μg/ml) from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* 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. The cells were then washed twice with cold PBS and incubated with fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD25 (BD Bioscience, USA) at 4°C for 30 min. After washing twice with cold PBS, CD25 expression was analyzed by flow cytometry (FACS Calibur; BD Biosciences).

THP-1 cells as a monocytic cell line were purchased from ATCC and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate). The cells were washed and resuspended with serum-free PRMI-1640. The cells (1×10^6 cells/ml) were plated in 6-well plates and treated with *E. coli* LPS (10 ng/ml) or the LPS (500 ng/ml) from *T. forsythia* single- or co-cultured with *F. nucleatum* or *P. gingivalis* at various concentrations for 12 h in the presence or the absence of 1% human serum (Sigma, USA) at 37°C in a CO_2 incubator. The cells treated with the LPS were used to analyze expression of inflammatory cytokines by real-time reverse transcription-polymerase chain reaction (RT-PCR). Also the conditioned media were collected to measure cytokine production using enzyme-linked immunosorbent assay (ELISA).

Real-time RT-PCR

The LPS treated THP-1 cells were collected by centrifugation at 1,500 × *g* for 5 min at 4°C and washed twice with cold PBS. Total RNA from the THP-1 cells was isolated with a TRIzol® RNA isolation kit (Invitrogen Life Tech., USA) according to the manufarurer's protocol. Complementary DNA (cDNA) was synthesized by Maxime™ RT Premix (iNtRON, Korea) according to the manufarurer's instruction. cDNA were mixed with 10 μl of SYBR Premix EX Taq (TaKaRa Co., Japan), 0.4 μM of each specific primers and ROX dye in 20 μl final volume, and performed 40 PCR cycles (95°C for 15 sec, 60°C for 15 sec, and 72°C for 33 sec) using ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA). PCR productions were analyzed for each specific amplification product using a dissociation curve of amplification. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference to normalize expression levels and quantify changes of inflammatory cytokine. The 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′ for the TNF-α gene; 5′-AGC TGT ACC CAG AGA GTC C-3′ and 5′-ACC AAA TGT GGC CGT GGT TT-3′ for the IL-1β 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.

ELISA

The conditioned media of the cells treated with various LPSs were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C. The supernatants were analyzed for IL-1β, IL-6, and TNF-α levels using an ELISA kit (BD Biosciences) according to the manufacturer's protocol.

Labelling LPS with biotin

The LPS was labeled with biotin or fluorescence using EZ-Link Hydrazide-Biotin (Thermo Sci., USA). The LPS was incubated with 20 mM cold sodium metaperiodate at 4°C for 1 h and then dialyzed overnight in 0.1 M sodium acetate (pH 5.5). The preparation was transferred to a new tube and hydrazide-biotin solution was added to a final concentration of 5 mM. After agitation with rotator for 2 h, the mixture was dialyzed three times in triple 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.

Binding assay of the LPS for LBP and CD14

Anti-human LBP polyclonal Ab (50 ng/well in 50 μ; R&D Systems, USA) was coated on EIA plates (Corning Inc., USA) at 4°C overnight. The plates were washed three times with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. The plates were also washed three times with PBS including 0.1% Tween 20 (TPBS). Recombinant human LBP (rhLBP, 50 ng; R&D Systems) and biotin-labelled LPS (1 μg) were mixed and incubated for 1 h and then dispensed into the LBP-coated well. The plate was incubated at room temperature for 2 h and washed five times with TPBS. After adding horseradish peroxidase (HRP)-conjugated streptavidin (50 μl; 1 μg/ml) in TPBS including 1% BSA, the plate was incubated for 1 h and washed five times with TPBS. Solution of 3,3′,5,5′-tetramethylbenzidine (TMB) was added into the wells and incubated for 30 min. The enzyme reaction was halted with 1 N sulfuric acid, and the absorbance was measured at 450 nm wavelength with an ELISA reader. For CD14 binding, recombinant human CD14 (rhCD14, 100 ng; R&D Systems) was coated on an EIA plate (Coring Inc.) at 4°C overnight. The plates were washed three times with PBS and blocked with 1% BSA in PBS for 1 h. The plates were washed three times with TPBS, and biotin-labelled LPS (1 μg) was added. After washing five times with TPBS, the plate was incubated for 1 h and washed five times with TPBS. TMB solution was added into the wells and incubated for 30 min. The enzyme reaction was halted with 1 N sulfuric acid, and the absorbance was measured at 450 nm wavelength with an ELISA reader.

LPS biding assay to the cells

THP-l cells were treated with biotin-labelled LPS for 2 h in the presence of 1% human serum and washed three times with cold PBS. The cells were resuspended in PBS and incubated with FITC-conjugated streptavidin (1 μg) for 3 min. After washing three times with cold PBS, the cells were analyzed by flow cytometer (FACS Calibur; BD Biosciences).

Statistical analysis

Statistically significant differences were analyzed by Mann-Witney *U*-test using SPSS ver. 10 (SPSS Inc., USA). *p*-values less than 0.05 were considered statistically significant.

Results

SDS-PAGE of *T. forsythia* LPS in the various conditions

In order to evaluate the pattern of LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis*, 10% SDS-PAGE was carried out. The migration patterns of the LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* exhibited little different it (Fig. 1). Lipid A part showed a similar size compared with the molecular size marker. However, in the case of upper bands corresponding to the O-antigen chains, the bands of LPS from co-cultured *T. forsythia* with *P. gingivalis* displayed smaller size compared with those from single- and co-cultured *T. forsythia* with *F. nucleatum*. No visible band of nucleic acids and proteins were detected in agarose gel electrophoresis with ethidium bromide staining and SDS-PAGE with coomasie brilliant blue staining, respectively (data not shown).

Biological activity of LPS from single- or co-cultured *T. forsythia*

T. forsythia has both agonists for TLR4 and TLR2 such as

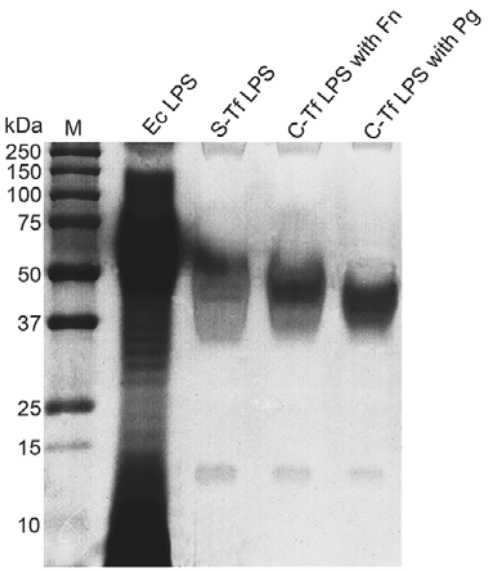

Fig. 1. SDS-PAGE of *T. forsythia* in the various conditions. After cultivating *T. forsythia* in the presence or absence of *F. nucleatum* or *P. gingivalis*, LPS was isolated from *T. forsythia* of each condition using phenol/water extraction methods. The LPS were performed SDS-PAGE (10%, acrylamine), and the gel then was stained with silver nitrate. Lanes: M, marker; Ec LPS, *E. coli* LPS; S-Tf LPS, LPS from single-cultured *T. forsythia*; C-Tf LPS with Fn, LPS from co-cultured *T. forsythia* with *F. nucleatum*; and C-Tf LPS with Pg, LPS from co-cultured *T. forsythia* with *P. gingivalis*.

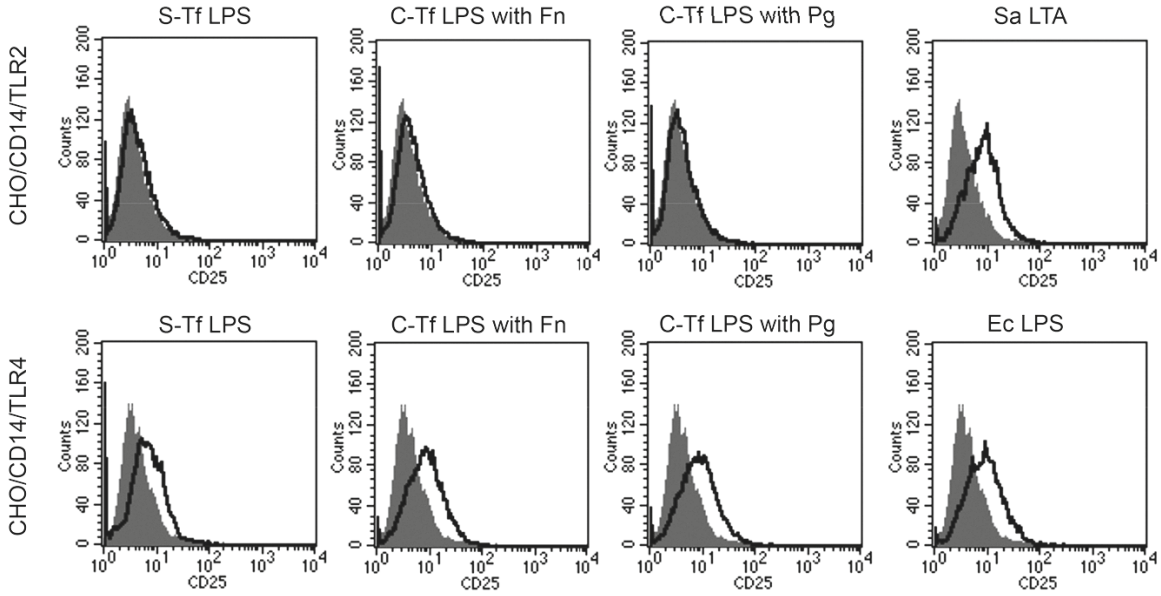

Fig. 2. TLR activation in NF-κB reporter CHO cells by LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis*. CHO/CD14/TLR2 or CHO/CD14/TLR4 was treated with LPS (10 μg/ml) from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis*, *E. coli* LPS (100 ng/ml) or *S. aureus* LTA (1 μg/ml) for 18 h. CD25 expression was analyzed by flow cytometer and expressed histogram using Cell Quest software. S-Tf LPS, LPS from single-cultured *T. forsythia*; C-Tf LPS with Fn, LPS from co-cultured *T. forsythia* with *F. nucleatum*; C-Tf LPS with Pg, LPS from co-cultured *T. forsythia* with *P. gingivalis*; Sa LTA, *S. aureus* LTA; Ec LPS, *E. coli* LPS.

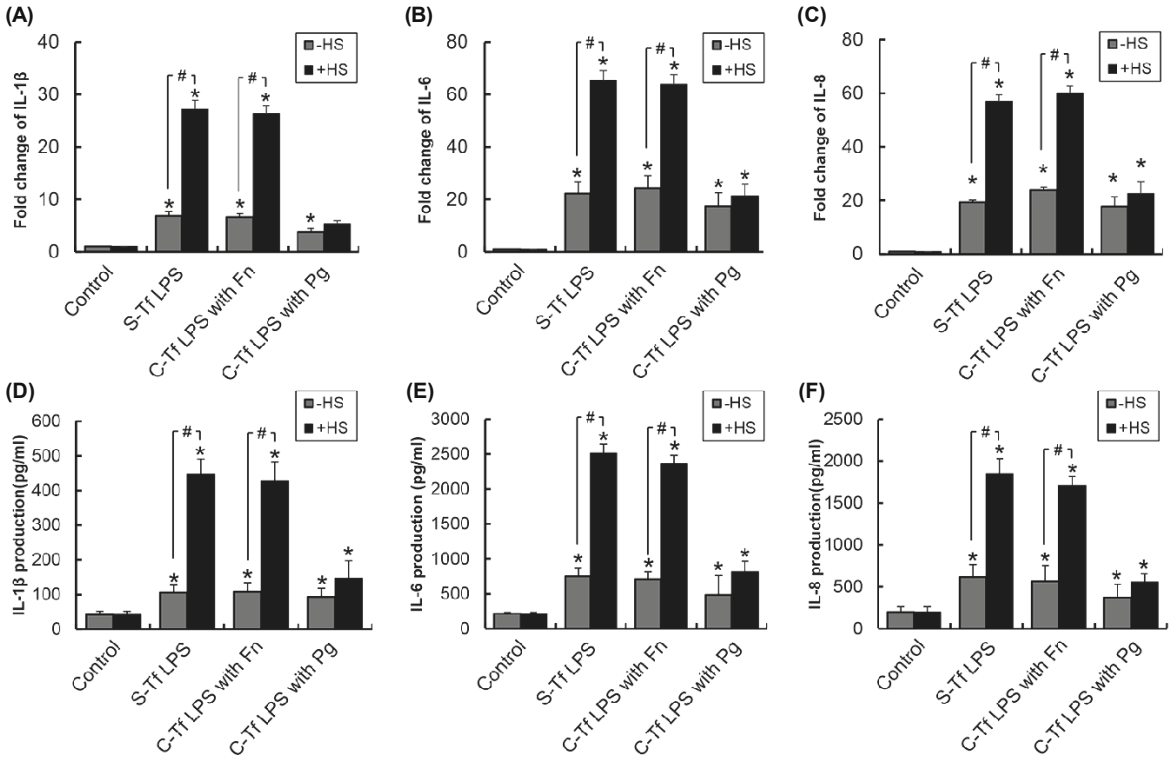

Fig. 3. Comparison of cytokine production by LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis*. THP-1 cells were treated with the LPS from *T. forsythia* (100 ng/ml) in the presence or the absence of human serum. After separating the cell and the conditioned media, the induction of cytokine expression was analyzed from the cells by real-time RT-PCR (A-C). The conditioned media were measured level of cytokines such as TNF-α, IL-1β, and IL-6 by ELISA (D-F). The experiments were carried out three times in duplicate and the average data are expressed. An asterisk (*) indicates significant difference compared to untreated (control) cells, and the sharp (#) represents a significant difference between the conditions of 1% human serum and the serum free conditions. Tf LPS, LPS from single-cultured *T. forsythia*; Tf-Fn LPS, LPS from co-cultured *T. forsythia* with *F. nucleatum*; Tf-Pg LPS, LPS from co-cultured *T. forsythia* with *P. gingivalis*.

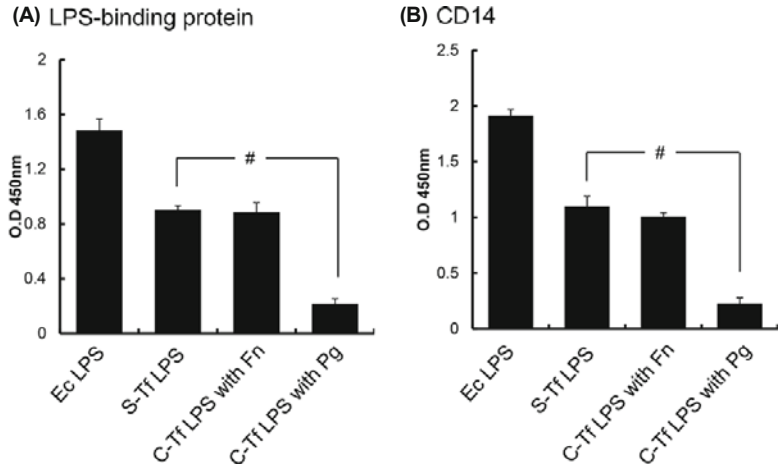

Fig. 4. LPS binding assay to LBP and CD14. Anti-human LBP Ab or rhCD14 was coated on EIA plates. The plates were treated and incubated with either the mixture of rhLBP and biotinylated *T. forsythia* LPS or biotinylated *T. forsythia* LPS for 2 h. The bound *T. forsythia* LPS was detected with HRP-conjugated streptavidin and TMB solution. The experiments were carried out three times and the average data was shown. Ec LPS, *E. coli* LPS; Tf LPS, LPS from single-cultured *T. forsythia*; Tf-Fn LPS, LPS from co-cultured *T. forsythia* with *F. nucleatum*; Tf-Pg LPS, LPS from co-cultured *T. forsythia* with *P. gingivalis*.

lipoprotein and LPS, respectively. Thus, the extracted LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* were analyzed by using CHO/ CD14/TLR2 cells and CHO/CD14/TLR4 cells. As shown in Fig. 2, the LPS from single- or co-cultured *T. forsythia* stimulated CHO/CD14/TLR4 cells. However, CHO/CD14/TLR2 cells were not affected by three LPS.

Induction of inflammatory cytokine expression by LPS from single- or co-cultured *T. forsythia*

Induction of cytokine expression on THP-1 cells by three LPS preparations was investigated. THP-1 cells were treated with the LPS (500 ng/ml) from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* in the presence or the absence of human serum. The three LPS significantly induced cytokine expression of THP-1 cells in the both conditions (Fig. 3). Interestingly, in case of 1% human serum condition, the LPS from co-cultured *T. forsythia* with *P. gingivalis* induced a significantly lower level of cytokine expression such as IL-1β, IL-6, and IL-8 than single- and co-cultured *T. forsythia* with *F. nucleatum* (Figs. 3A–3C). Also, LPS from co-cultured *T. forsythia* with *P. gingivalis* induced lower production of cytokines compared to single- and co-cultured *T. forsythia* with *F. nucleatum* (Figs. 3D–3F). However, three LPS did not show difference of cytokine induction in the serum free conditions.

LPS binding assay to LBP and CD14

LPS from co-cultured *T. forsythia* with *P. gingivalis* lower level of cytokine induction in 1% human serum condition. However, the three LPS did not exhibited difference of bioactivity in a serum free condition. LPS is amphiphilic and needs to bind to LBP and CD14 in order to attach itself to the cell (Lee *et al.*, 2006). Therefore, O-antigen chain as a binding site of LBP and CD14 was analyzed using a binding assay after biotinylation. As shown Fig. 3, the LPS from co-cultured *T. forsythia* with *P. gingivalis* exhibited less binding to LBP (Fig. 4A) and CD14 (Fig. 4B) compared to the LPS from single- and co-cultured *T. forsythia* with *F. nucleatum*. These results indicated that the LPS from co-

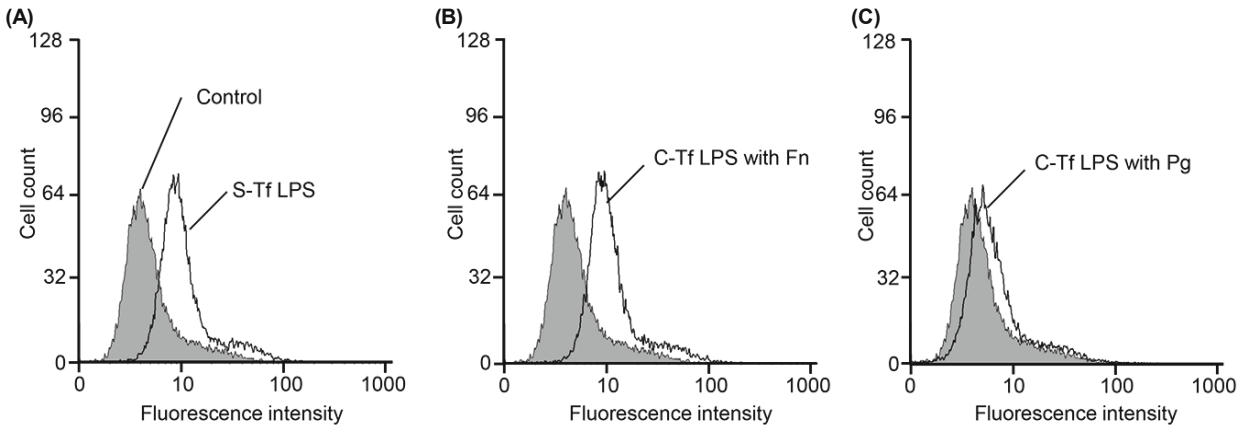

Fig. 5. The LPS binding to THP-1 cells. After labelling LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* with biotin, THP-1 cells were treated and incubated with the biotinylated LPS for 2 h. The cells were washed and treated FITC-labelled streptavidin. The bound LPS were analyzed by flow cytometer. Control, untreated control cells; S-Tf LPS, LPS from single-cultured *T. forsythia*; C-Tf LPS with Fn, LPS from co-cultured *T. forsythia* with *F. nucleatum*; C-Tf LPS with Pg, LPS from co-cultured *T. forsythia* with *P. gingivalis*.

cultured *T. forsythia* with *P. gingivalis* may adhere in smaller amounts to the cell than the other LPS.

LPS binding assay to the cell

The LPS from co-cultured *T. forsythia* with *P. gingivalis* showed less binding to LBP and CD14, and we inferred from these results that co-cultured *T. forsythia* LPS with *P. gingivalis* may attach in lesser quantities to the cells. To confirm this hypothesis, an experiment on LPS binding assay to THP-1 cells was carried out. When THP-1 cells were treated with biotinylated LPS in the presence of human serum, the three LPS showed different levels of bindings to the cells. The amount of LPS from co-cultured *T. forsythia* with *P. gingivalis* was significantly reduced compared to the LPS from single- and co-cultured *T. forsythia* with *F. nucleatum* (Fig. 5).

Discussion

T. forsythia, which is a Gram-negative bacteria and obligated anaerobe, is associated with chronic periodontitis (Socransky and Haffajee, 2005). Also, this bacterium is considered a contributor to systemic diseases like cardiovascular disease by increasing C-reactive protein level (Noack, *et al.*, 2001). The known virulence factors of *T. forsythia* include lipopolysaccharide, BspA (adhesins), sustenins (trypsin-like proteinase), and S-layer protein (immune stimulator) (Yoneda *et al.*, 2003; Holt and Ebersole, 2005; Lee *et al.*, 2010), which have been studied primarily by cultivating *T. forsythia* alone, although *T. forsythia* co-exists with *F. nucleatum*, *P. gingivalis*, and *T. denticola* and forms biofilm in subgingival tissue (Liljemark and Bloomquist, 1996). Therefore, when *T. forsythia* was co-cultured with other periodontopathogens, the bioactivity of its LPS was first investigated in this study.

LPS is a powerful stimulant to induction of inflammation and shock (Raetz and Whitfield, 2002) and is a contributor with the induction of local or systemic disease (Cooke *et al.*, 2002). *T. forsythia* LPS is also an inflammatory factor with regards to inducing cytokines such as IL-1β, IL-6, and IL-8 (Lee *et al.*, 2010). In this study, *T. forsythia* was co-cultured with *F. nucleatum* or *P. gingivalis*, and then the changes in characteristics of *T. forsythia* LPS were investigated. The extracted LPS from single- or co-cultured *T. forsythia* with *F. nucleatum* and *P. gingivalis* were evaluated contamination of other molecules such as lipoprotein, bacterial proteins and nucleic acids using SDS-PAGE and agarose gel electrophoresis. The extracts were detected in silver-stained gel. However, no visible bands were detected in coomasie blue-stained gel and ethidium bromide-stained agarose gel. These results indicated that the extracts were not contaminated by other molecules except glycolipid. Also, in the experiments using CHO/CD14/TLR2 and CHO/CD14/TLR4, the extracts showed TLR4 agonist. Thus, we were convinced that the extracts are LPS.

In the condition of 1% human serum, the induction of cytokine expression between single-cultured *T. forsythia* LPS and co-cultured *T. forsythia* LPS with *F. nucleatum* did not show any difference. However, co-cultured *T. forsythia* LPS with *P. gingivalis* induced a lower level of cytokine expression compared to single-cultured *T. forsythia* LPS. Interestingly, in serum-free condition, the three LPS did not exhibited difference of biological activity. Furthermore, lipid A of single-cultured *T. forsythia* LPS and co-cultured *T. forsythia* LPS with *P. gingivalis* was analyzed using mass spectrometer because the bioactivity of LPS differs according to the structure of lipid A (Hajjar *et al.*, 2002; Raetz *et al.*, 2007). The peaks showed similar pattern in the range of *m/z* 1,500 to *m/z* 2,500 as detecting range of lipid A (data not shown). Hajjar *et al.* (2002) reported that LPS shows different bioactivity for human and murine cells by modifications of lipid A structure. This result indicates that structure of the three lipid A is same, and the subsequent cytokine-inducing capacity of the three LPS does not show any difference. However, the three LPS showed significantly different bioactivity in the experiments of cytokine induction in the condition of 1% human serum. We thought that the difference may be attributed to a certain factor(s) in human serum. Next, we analyzed O-antigen chains. Since LPS needs to bind to LBP or soluble CD14 for attachment on TLR2 or TLR4, and O-antigen chains of LPS are a binding site on LBP or soluble CD14 (Jack *et al.*, 1997; Moreno *et al.*, 2004), the three LPS were investigated in an affinity test using rhLBP and rhCD14. After biotinylation of the three LPS, the preparations were carried out by binding assay on rhLBP- or rhCD14-coated 96-well EIA plate. Co-cultured *T. forsythia* LPS with *P. gingivalis* was found to have a significantly low level of both rhLBP and rhCD14 compared to single-cultured *T. forsythia* LPS and co-cultured *T. forsythia* LPS with *F. nucleatum*. These results indirectly indicate that co-cultured *T. forsythia* LPS with *P. gingivalis* binds less amounts on TLR than other LPS even though the amount of the LPS is the same. So, we lastly investigated whether co-cultured *T. forsythia* LPS binds less to THP-1 cells than the others or not. For analysis of LPS binding to the cells, the LPS was labelled with biotin, treated on THP-1 cells and detected with FITC-conjugated HRP. The amount of co-cultured *T. forsythia* LPS on the cell surface exhibited a low level compared to single-cultured *T. forsythia* LPS and co-cultured *T. forsythia* LPS with *F. nucleatum.* Also, in the analysis of SDS-PAGE, the bands of co-cultured *T. forsythia* LPS with *P. gingivalis* located lower compared to the others. Ultimately, *P. gingivalis* may bring about some changes in O-antigen chains of *T. forsythia* LPS.

In a previous study, when *P. gingivalis* and *F. nucleatum* are co-cultivated, the virulence of *P. gingivalis* LPS was increased by up-regulation of LPS biosynthesis-related genes (Lee and Baek, 2013). However, the virulence of *T. forsythia* LPS was decreased by *P. gingivalis* in this study. Multi-species bacteria dwell in oral cavity and communicate with one another by metabolite and quorum-sensing molecules, by which the virulence and characteristics of oral bacteria are changed (Kolenbrander, 2000). Especially, the quorum-sensing system is related with bacterial virulence and adhesion (Morkunas *et al.*, 2012; Jang *et al.*, 2013). The change of *T. forsythia* LPS by quorum sensing in co-cultivation with periodontopathogens may be excluded because *F. nucleatum* did not affect the LPS. In a further study, we will investigate how the molecules of *P. gingivalis* related to O-antigen chains of *T. forsythia*.

In conclusion, the virulence of *T. forsythia* LPS was de-

creased by co-culturing it with *P. gingivalis* and their affinity to LBP and CD14 was reduced. This may be due to modification of O-antigen chains by *P. gingivalis*.

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