이학석사 학위논문

Inhibitory effect of *Lactobacillus plantarum* lipoteichoic acid on Pam2CSK4-induced IL-8 production in Caco-2 cells

Caco-2 세포에서 Pam2CSK4에 의해 유도된 IL-8 발현에 대한 *Lactobacillus plantarum* lipoteichoic acid의 억제효과

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치의과학과 면역 및 분자미생물치의학 전공

노 수 영

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ABSTRACT

Inhibitory effect of *Lactobacillus plantarum* lipoteichoic acid on Pam2CSK4-induced IL-8 production in Caco-2 cells

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The genus *Lactobacillus* is considered as predominant bacteria in the gastrointestinal tract of humans and animals and has been associated with beneficial effects on the host. Although it has been well known that *Lactobacillus* alleviates intestinal inflammation, the precise mechanisms by which bacterial components of *Lactobacillus* attenuate the inflammatory responses in the intestine have not been clearly understood. In this study, we investigated the inhibitory effect

of a major bacterial component, lipoteichoic acid of *L. plantarum* on the production of interleukin (IL)-8 in a human intestinal epithelial cell line, Caco-2 cells. *Staphylococcus aureus* significantly induced mRNA expression of IL-8, but lipoprotein deficient *S. aureus* did not induce the expression of IL-8 in Caco-2 cells. Among microbial components tested in this study, Pam2CSK4, which is known to mimic Gram-positive bacterial lipoproteins, significantly induced IL-8 expression in a dose-dependent manner in Caco-2 cells. Although heat-inactivated *L. plantarum* slightly inhibited Pam2CSK4-induced IL-8 mRNA expression, *L. plantarum* LTA (Lp.LTA), but not its peptidoglycan, significantly inhibited Pam2CSK4-induced IL-8 expression. De-acylated or de-alanylated Lp.LTA failed to inhibit Pam2CSK4-induced IL-8 production, suggesting that lipid and D-alanine moieties are critical for the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 expression. Pam2CSK4-induced Toll-like receptor 2 (TLR2) activation was significantly reduced by Lp.LTA in CHO/CD14/TLR2 cells. Furthermore, Lp.LTA remarkably diminished Pam2CSK4-induced the phosphorylation of p38 kinase and JNK. In addition, IκBα was degraded in the presence of Pam2CSK4, but the degradation of IκBα was not observed in the presence of Lp.LTA. Collectively, these results suggest that Lp.LTA inhibits Pam2CSK4-induced IL-8 production through the decrease of phosphorylation of MAP kinases such as p38 kinase and JNK and the blocking of IκBα degradation via the decreased TLR2 activation in human intestinal epithelial cells.

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Keywords: *Lactobacillus plantarum*, Lipoteichoic acid, Pam2CSK4, Interleukin-8, Intestinal epithelial cells

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CONTENTS

I. INTRODUCTION

Gastrointestinal tract is a habitat of tremendous and complex population of bacteria [1]. An adult individual contains 10^{14} bacteria in the gastrointestinal tract that are ten times more than the number of human body cells [2]. In the gastrointestinal tract, over 400 different bacterial species exist as indigenous bacteria which constantly interact with the intestinal epithelium [3]. It has been reported that intestinal commensal bacteria play beneficial roles in nutrition and immune system of the host [4-7]. For example, carbohydrates are decomposed into short chain fatty acids including acetic acids, propionic acids, and butyric acids which give physiologic effects on the host [4, 5]. In addition, these commensal bacteria hydrolyze lipids, break down proteins, and produce vitamins. They also induce the production of IgA, leading to neutralization of exogenous pathogenic bacteria and/or their toxins in the gut lumen. Moreover, intestinal commensal bacteria contribute to the development of structure and function of epithelial cells [7]. Among the intestinal commensal bacteria, *Lactobacillus* and *Bifidobacterium* have been well known as beneficial bacteria to the host [8].

The genus *Lactobacillus* is a Gram-positive rod-shaped bacterium and can be characterized to have facultative anaerobic properties [9]. It mainly resides in the gastrointestinal and genital tracts without causing diseases [10, 11] and can be divided into over 100 species including *L. plantarum*, *L. sakei*, *L. delbruekii*, and *L. rhamnosus* GG [12]. It has been reported that *Lactobacillus* exerts a number of beneficial effects on the human health. For instance, soluble polys accharides from *L. acidophilus* have effective anti-cancer activities

[13]. In addition, consumption of *L. casei* and *L. bulgaricus* prevents antibiotic- and *Clostridium difficile*-associated diarrhea [14]. Enteropathogenic *Escherichia coli* (EPEC) O127:H6 adheres to intestinal epithelial cells and then disrupts intestinal epithelial barrier integrity to invade into the cells. However, *L. plantarum* DSM 2648 effectively inhibits EPEC-induced intestinal barrier malfunction [15]. Furthermore, *L. acidophilus* down-regulates gene expression of Niemann-Pick C1-like 1, which is related with intestinal absorption of cholesterol, and inhibited uptake of micellar cholesterol, reducing serum cholesterol level in human intestinal epithelial cells [16].

Intestinal inflammation is developed by several factors including genetic factors, environmental factors, and bacterial agents [7]. Pathogenic bacteria associated with the intestinal inflammation possess various pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins and peptidoglycan (PGN) in the cell wall, which are involved in bacterial adhesion, invasion, tissue destruction and the initiation of inflammatory responses [17]. Several intestinal pathogenic bacteria such as *Salmonella* and *Shigella* induce pro-inflammatory responses in the intestinal epithelial cells [18-20]. Excessive production of the pro-inflammatory mediators such as interleukin (IL)-8 results in severe intestinal inflammation including inflammatory bowel disease (IBD) [21, 22]. A number of reports have showed that *Lactobacillus* attenuates the intestinal inflammatory responses. For example, *L. reuteri* attenuated colitis and enhanced epithelial barrier function by reducing adherence and translocation of colonic mucosal aerobic bacteria [23]. *L. salivarius* decreased the colonic inflammation and production of pro-inflammatory cytokines in Peyer's patches and splenocytes [24]. More recently, *L. salivarius* suppressed IL-1β and CXCL2 production but increased IL-10

production through nucleotide-binding oligomerization domain (NOD)-containing protein 2 signaling pathway. In addition, PGN of *L. salivarius* alleviated 2,4,6-trinitrobenzene sulfonic acid-induced colitis by developing CD103-positive dendritic cells and regulatory T cells [25].

A member of the CXCL chemokine family, IL-8 is an important pro-inflammatory mediator in the early stage of infection. A major role of IL-8 is the recruitment of neutrophils to the site of infection and activation of neutrophils [26, 27]. In addition to neutrophils, other immune cells such as lymphocytes, monocytes [28], and eosinophils are also recruited to the infection site by IL-8. Despite the beneficial role of IL-8 to host immune system, excessive production of IL-8 could lead to over-infiltration and activation of neutrophils and consequently, exacerbating the inflammatory responses [29, 30]. For example, it has been reported that colonic mucosa in IBD patients contained higher amount of IL-8 than in healthy people [31]. Furthermore, overexpression of IL-8 results in the disruption of epithelial barrier, suggesting that excessive production of IL-8 may contribute to development of intestinal inflammation [32].

It has been reported that *Staphylococcus aureus* in the intestinal carriage was increased in hospitalized patients and infants [33, 34]. In addition, *S. aureus* in the intestine is closely related with necrotizing enterocolitis, resulting in systemic inflammation [35, 36]. Recently, it has been suggested that a cell wall molecule of *Lactobacillus*, lipoteichoic acid (LTA), exerts the immunomodulatory effects on pro-inflammatory responses under the inflamed condition in the presence of tumor necrosis factor-α and interferon-γ [37, 38]. Although several reports suggested that probiotic *Lactobacillus* ameliorates

enterocolitis such as *S. aureus*-mediated enterocolitis that might be mediated by bacterial components of *Lactobacillus*, it is not clear whether *Lactobacillus* LTA is able to reduce intestinal inflammatory responses. Therefore, in this study, we investigated the potential of *Lactobacillus* LTA to attenuate pro-inflammatory responses in human intestinal epithelial cells by examining IL-8 induction.

II. MATERIALS AND METHODS

1) Bacteria

Lactobacillus plantarum KCTC 10887BP and *Streptococcus mutans* KCTC 3065 were obtained from Korean Collection for Type Culture (Daejeon, Korea). *Enterococcus faecalis* ATCC 29212 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *S. aureus* RN4220, T013, T065, M0674/pM101, and M0674/pM101-ltaS, and PGN of *L. plantarum* with or without wall teichoic acid (WTA) were kindly provided by Dr. Bok Luel Lee (Pusan National University, Busan, Korea). To prepare heat-inactivated *L. plantarum*, *L. plantarum* was grown in lactobacilli De Man, Rogosa and Sharpe (MRS) broth (Acumedia, Lansing, MI, USA) at 37°C for 16 h. After the bacterial pellets were obtained by centrifugation, the pellets were washed three times with phosphate-buffered saline (PBS) and *L. plantarum* was inactivated by heating at 80°C for 2 h. Optical density (OD) value of *L. plantarum* suspension was determined using spectrophotometer at 600 nm. To confirm the heat-inactivated *L. plantarum* was completely inactivated, the bacteria were plated in MRS agar plate at 37°C for 24 h. To prepare ethanol-inactivated *S. aureus* strains, *S. aureus* RN4220 [39] and *S. aureus* T013 [40], which is a mutant that lipoprotein diacylglyceryl transferase was deleted (RN4220 *lgt*::pM*lgt*), and *S. aureus* T065 [40], which is a Δ*lgt* strain containing a plasmid with *lgt* gene (pS*lgt*) were grown in Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA, USA), LB containing 10 μg/ml of erythromycin, or LB containing 10 μg/ml of erythromycin and 12.5 μg/ml of chloramphenicol at 37°C with shaking at 180 rpm for 18 h,

respectively. *S. aureus* M0674/pM101 [41], which is a mutant of which LTA was removed (RN4220 *ltaS::phleo*/pM101), and *S. aureus* M0674/pM101-ltaS [41], which is a Δ*ltaS* strain containing a plasmid with *ltaS* gene (Δp*ltaS::phleo*), were cultured in LB broth containing 50 μg/ml of kanamycin at 30°C with shaking at 180 rpm for 18 h. Next, bacterial suspensions were centrifuged and the bacterial pellets were obtained and washed three times with PBS. The bacteria were inactivated using 70% ethanol in PBS at room temperature with vigorous shaking for 2 h. To ensure that *S. aureus* was completely inactivated, the bacteria were plated in LB agar plate at 37°C for 24 h. LTAs from *L. sakei*, *L. delbruekii*, and *L. rhamnosus* GG were kindly provided by Dr. Dae Kyun Chung (Kyung Hee University, Suwon, Korea).

2) Reagents

Pam2CSK4 was purchased from EMC microcollections (Tuebingen, Germany). *Escherichia coli* LPS, Poly I:C, and muramyl dipeptide (MDP) were purchased from InvivoGen (San Diego, CA, USA). L-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP) was obtained from Anaspec (Freemont, CA, USA). Antibodies specific for p38 kinase, phospho-p38 kinase, JNK, phospho-JNK, ERK, phospho-ERK, and IκBα and HRP-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). FITC-conjugated anti-TLR2 monoclonal antibody, FITC-conjugated anti-CD25 monoclonal antibody, mouse IgG1, κ and mouse IgG2, κ were purchased from BioLegend (San Diego, CA, USA).

3) Purification of LTA

LTAs from *L. plantarum, S. aureus, S. mutans, and E. faecalis* were prepared as previously described [42]. The purity of LTAs was examined such as the contamination with endotoxins, nucleic acids, or proteins as previously described [42]. Structural intactness of LTA was determined using nuclear magnetic resonance spectrometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry [43]. De-alanylated Lp.LTA, which does not contain D-alanine contents in the LTA structure, and de-acylated Lp.LTA, which is the acyl chain-deficient structure of LTA, were prepared by incubating intact Lp.LTA in 0.1 M Tris-HCl for 24 h and in 0.5 N NaOH for 2 h, respectively. LTA was dialyzed against non-pyrogenic water for 2 days and lyophilized for 1 day. After phosphate assay to quantify the LTA [44], thin-layer chromatography was conducted to confirm that alanine and lipid were completely disappeared as described previously [45].

4) Cell culture

A human intestinal epithelial cell line, Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in complete Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, Utah, USA) containing 10% fetal bovine serum (GIBCO, Burlington, ON, Canada), 100 U/ml of penicillin and 100 μg/ml of streptomycin (HyClone) at 37°C in a 5% CO_2-humidified incubator. In order to polarize cells, Caco-2 cells were cultured on polycarbonate Transwell-permeable filters (0.4 μm pore size; Costar, Corning, NY, USA) for 16 days. Cell polarization was confirmed by

measuring trans-epithelial electrical resistance (>450 Ω/cm^2) with EVOM^2 (World Precision Instruments, Sarasota, FL, USA). Chinese hamster ovary (CHO) cells with overexpressing human CD14 and TLR2 (CHO/CD14/TLR2 cells) [46], were maintained in complete Ham's F-12 nutrient mixture (HyClone) containing 1 mg/ml of Geneticin® (GIBCO, Burlington, ON, Canada) and 0.4 mg/ml of hygromycin B (Invivogen) at 37°C in a 5% CO_2-humidified incubator.

5) Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by mixing total RNA (1 μg) with random hexamers (Promega, Madison, WI, USA) and a reverse transcriptase (Promega). Amplification of cDNA was performed by PCR in a total volume of 20 μl containing rTaq DNA polymerase (0.5 unit) and 10 picomole of primers for IL-8, A20, TOLLIP, IRAK-M, SOCS1, and β-actin (Table 1). PCR amplification was conducted as follows: 32 cycles at 94°C for 5 min, 94°C for 40 sec, 60°C for 40 sec, 72°C for 40 sec, and 72°C for 7 min for IL-8; 30 cycles at 94°C for 5 min, 94°C for 40 sec, 60°C for 40 sec, 72°C for 40 sec, and 72°C for 7 min for A20, SOCS1, and TOLLIP; 40 cycles at 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 72°C for 10 min for IRAK-M; 25 cycles at 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min for β-actin. PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Table 1. IL-8, A20, TOLLIP, IRAK-M, SOCS1, and β-actin primers

Primer	Forward primer	Reverse primer
IL-8	5'-TCT GCA GCT CTG TGT GAA GG-3'	5'-TGA ATT CTC AGC CCT CTT CAA-3'
A20	5'-CGC TCA AGG AAA CAG ACA CA-3'	5'-CTT CAG GGT CAC CAA GGG TA-3'
TOLLIP	5'-CAA GAA TCC CCG CTG GAA TA-3'	5'-ATG GCT TTC AGG TCC TCC TC-3'
IRAK-M	5'-TTT GAA TGC TGC CAG TCT GA-3'	5'-GCA TTG CTT ATG GAG CCA AT-3'
SOCS1	5'-CTC CTT CCC CTT CCA GAT TT-3'	5'-GGT GAC TGA ATG AGT TCA TTA ATG-3'
β-actin	5'-GTG GGG CGC CCC AGG CAC CA-3'	5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

6) Determination of IL-8 production using enzyme-linked immunosorbent assay (ELISA)

In order to determine IL-8 production, Caco-2 cells (4×10^5 cells/ml) were plated on a 96-well culture plate until the cells were fully confluent. The cells were starved with serum-free DMEM for 18 h and treated with the indicated stimuli for 24 h. After treatment, the cell culture supernatants were collected and the production of IL-8 was determined using a commercial IL-8 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In a separate experiment, polarized Caco-2 cells were apically treated with the Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 24 h and IL-8 production was measured as stated above.

7) Identification of TLR2 expression

Caco-2 cells (4×10^5 cells/ml) were cultured until the cells were fully confluent and further incubated in serum-free DMEM for 18 h. Subsequently, the cells were detached using 2 mM EDTA and centrifuged with PBS. The cells were washed with PBS containing 2% FBS for detection of surface expressing TLR2. To determine intracellular TLR2 expression, the cells were detached using 2 mM EDTA and washed with PBS containing 0.1% saponin. The cells were stained with FITC-conjugated anti-TLR2 monoclonal antibody or mouse IgG2, κ (BioLegend), as an isotype control for 30 min at 4°C. TLR2 expression was determined using FACSCalibur with CellQuest software (BD Biosciences).

8) Measurement of TLR2 activation

CHO/CD14/TLR2 cells (4×10^5 cells/ml) were incubated with Lp.LTA (30 μg/ml) in the absence or presence of Pam2CSK4 (0.1 μg/ml) for 24 h. The cells were washed with PBS and detached with 2 mM EDTA. After the cells were resuspended with PBS containing 2% FBS, the cells were centrifuged and stained with FITC-conjugated anti-CD25 monoclonal antibody or mouse IgG1, κ (BioLegend), as an isotype control, at 4°C for 30 min. Subsequently, the cells were washed with PBS containing 2% FBS and fixed with 1.5% paraformaldehyde. TLR2 activation was determined using a FACSCalibur with CellQuest software (BD Biosciences).

9) Competition binding assay for TLR2

In order to examine that Lp.LTA inhibits Pam2CSK4 binding to TLR2, CHO/CD14/TLR2 cells (4×10^5 cells/ml) were incubated with Lp.LTA (30 μg/ml) in the presence or absence of Pam2CSK4-rhodamine (Invivogen) for 1 h at 4°C. After the cells were washed with cold PBS, the cells were detached using 2 mM EDTA and fixed with 1.5% paraformaldehyde. Binding capacity of Pam2CSK4-rhodamine in the presence or absence of Lp.LTA was determined using FACSCalibur with CellQuest software (BD Biosciences).

10) Western blot analysis

Caco-2 cells (4×10^5 cells/ml) were treated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 30 min. After treatment, the cells were lysed with a lysis buffer (PRO-PREP™, iNtRON biotechnology; Gyeonggi-Do, Korea) containing 2 mM PMSF, 2 mM Na_3VO_4, 2 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein concentration was determined using a BCA protein assay kit (Pierce; Rockford, IL. USA). Equal amounts of protein samples were electrophoresed on a 10% SDS-PAGE gel, and then, the gel was electro-transferred onto a PVDF membrane (Millipore; Bedford, MA, USA). Subsequently, the membrane was blocked with 5% skim milk for 1 h and then, probed with specific antibodies for the determination of phosphorylation of MAP kinases, including p38 kinase, ERK, or JNK. In a separate experiment, the membrane was incubated with IκBα antibody to examine IκBα degradation. The immunoreaction was visualized using a Luminescent

Image Analyzer (Fuji film, Japan).

11) Statistical analysis

All data are expressed as mean values ± standard deviations from at least three independent experiments unless otherwise stated. Treatment groups were compared with appropriate control and statistical analysis was performed using two-tailed *t*-test.

III. RESULTS

1) Lipoprotein is the crucial component of *S. aureus* for the induction of IL-8 in human intestinal epithelial cells.

S. aureus is well known as an opportunistic pathogen and causes wide variety of diseases including furuncles, foodborne illnesses, and sepsis [47-49]. Although, *S. aureus* is mainly found in nasal carriage [50], *S. aureus* is also found in intestinal carriage of hospitalized patients and aggravates intestinal inflammation [33, 51]. To elucidate whether *S. aureus* can induce pro-inflammatory responses in a human intestinal epithelial cells, Caco-2 cells were stimulated with 0 to 300 μg/ml of ethanol-inactivated *S. aureus* RN4220. Figs. 1A and 1B indicated that ethanol-inactivated *S. aureus* RN4220 induced IL-8 mRNA expression in a dose-dependent manner. Next, to determine which components of *S. aureus* are responsible for the expression of IL-8, Caco-2 cells were stimulated with 100 μg/ml of ethanol-inactivated *S. aureus* RN4220, T013, T065, M0674/pM101, and M0674/pM101-ltaS for 3 h marked as WT, Δ*lgt*, p*lgt*, Δ*ltaS*, and p*ltaS*, respectively. As shown in Figs. 1C and 1D, *S. aureus* T013 did not induce the expression of IL-8, suggesting that *S. aureus* lipoprotein is a crucial factor to induce pro-inflammatory response in human intestinal epithelial cells.

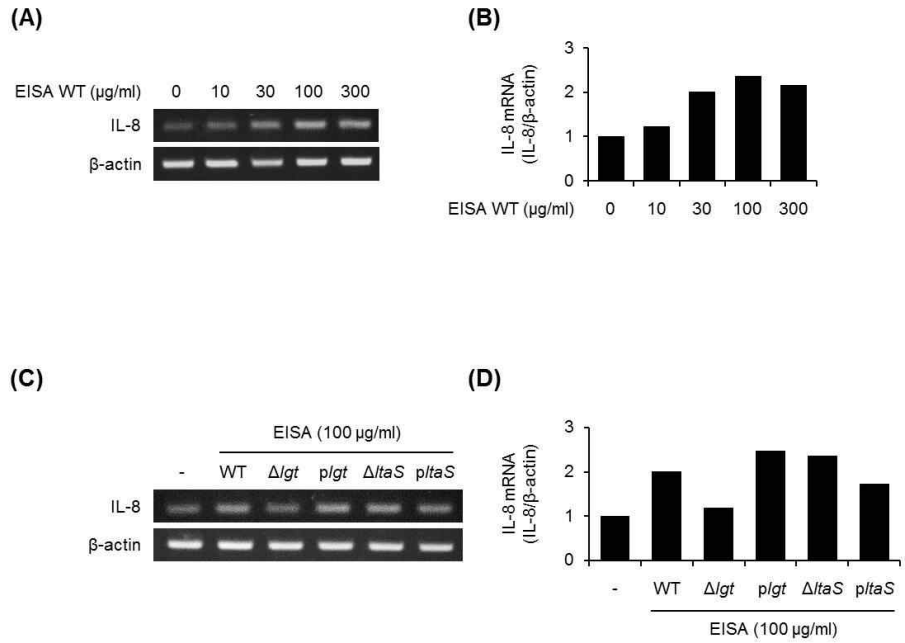

Figure 3. Expression of IL-8 after stimulation with ethanol-inactivated *S. aureus* RN4220, T013, T065, M0674/pM101, and M0674/pM101-ltaS in human intestinal epithelial cell line, Caco-2 cells. (A and B) Caco-2 cells were stimulated with ethanol-inactivated *S. aureus* (EISA) RN4220 marked as EISA WT for 3 h. (C and D) Caco-2 cells were stimulated with *S. aureus* RN4220, T013, T065, M0674/pM101, and M0674/pM101-ltaS marked as WT, Δ*lgt*, p*lgt*, Δ*ltaS*, and p*ltaS*, respectively, for 3 h. Then, total RNA was extracted and mRNA expression was determined using RT-PCR. Fig. B and D show the level of IL-8 mRNA through the density ratio between IL-8 and β-actin mRNA with normalization to control.

2) Pam2CSK4 induces IL-8 expression in Caco-2 cells.

As shown in Fig. 1, *S. aureus* T013, which is a lipoprotein-deficient strain, did not induce IL-8 expression in Caco-2 cells. To ensure the inhibitory effect of bacterial lipoprotein, we tested the effect of various PAMPs including Pam2CSK4, which is a diacylated lipoprotein model of Gram-positive bacteria. Caco-2 cells were stimulated with various PAMPs for 1 h and IL-8 expression was measured. As shown in Fig. 2A and 2B, only Pam2CSK4 potently induced IL-8 expression whereas other PAMPs such as LPS and LTA did not induce IL-8 expression in Caco-2 cells. To further examine whether PamCSK4 elicits IL-8 expression in a dose-dependent manner, the cells were stimulated with the various concentrations (0 to 1 μg/ml) of Pam2CSK4 for 1 h. IL-8 expression was significantly up-regulated at the treatment with 0.01 μg/ml of Pam2CSK4 and further increased up to the treatment with 1 μg/ml of Pam2CSK4 (Fig. 2C and 2D). For the time kinetics of IL-8 expression, the cells were stimulated with Pam2CSK4 (0.1 μg/ml) for 0 to 24 h. Fig. 2E and 2F indicated that IL-8 expression was peaked at 1 h and gradually decreased after 3 h. These results suggest that among the selective PAMPs, Pam2CSK4 strongly induced IL-8 expression in human intestinal epithelial cells. Collectively, the results of Fig. 1 and Fig. 2 suggest that Gram-positive bacterial lipoprotein and its mimicking model lipopeptide, Pam2CSK4, are major factors to induce IL-8 that might be important in the intestinal inflammation.

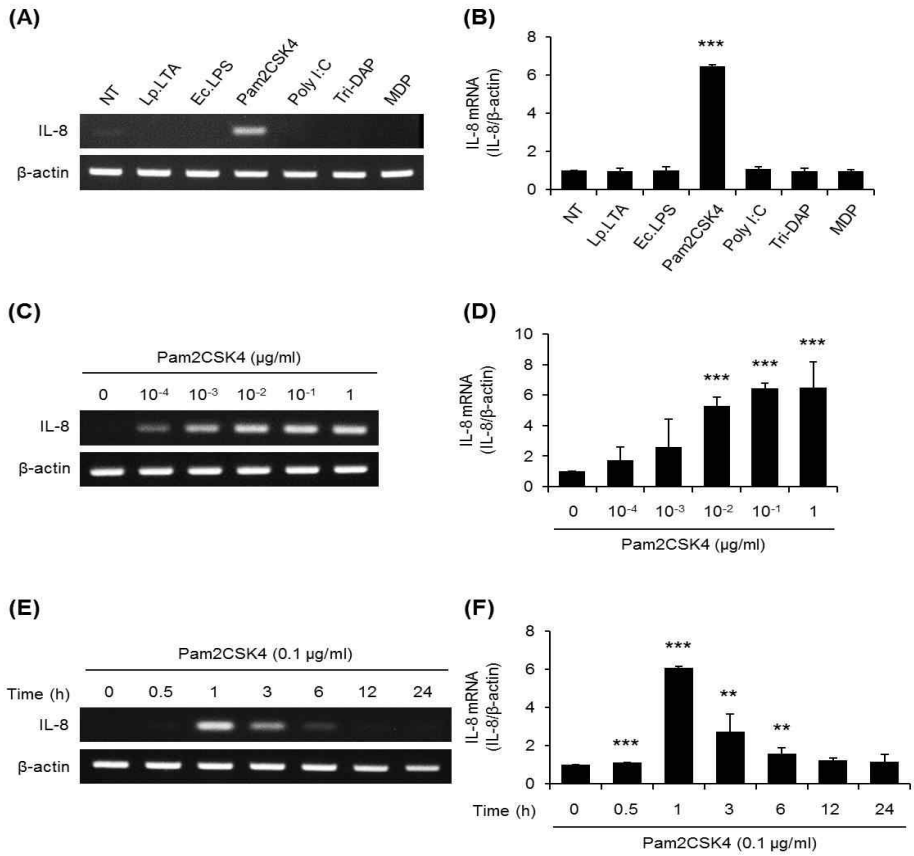

Figure 4. Induction of IL-8 expression by Pam2CSK4 in Caco-2 cells. (A and B) Caco-2 cells were stimulated with Lp.LTA (30 μg/ml), Ec.LPS (0.1 μg/ml), Pam2CSK4 (0.1 μg/ml), Poly I:C (10 μg/ml), Tri-DAP (1 μg/ml), and MDP (1 μg/ml) for 1 h. (C - F) Caco-2 cells were incubated with Pam2CSK4 in at various concentrations (0 to 1 μg/ml) and for various time periods (0 to 24 h). Then, total RNA was extracted and IL-8 mRNA expression was determined using RT-PCR. Relative mRNA expression of IL-8 to that of β-actin was obtained with densitometric analysis. All results were conducted at least three times. The asterisks (*) indicate that $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3) Lp.LTA specifically inhibits Pam2CSK4-induced IL-8 expression.

It has been reported that *Lactobacillus* possesses several beneficial effects on human health including the attenuation of intestinal inflammation [23]. Accordingly, we examined whether *L. plantarum* attenuates the Pam2CSK4-induced IL-8 expression. Heat-inactivated *L. plantarum* (HILP) by itself did not induce IL-8 expression but HILP slightly inhibited Pam2CSK4-induced IL-8 expression (Fig. 3A and 3B). To further elucidate whether the cell wall components of *Lactobacillus* attenuates Pam2CSK4-induced IL-8 expression, Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of LTA or PGN of various *Lactobacillus* strains. Figs. 3C and 3D indicated that LTA from *L. plantarum* significantly inhibited Pam2CSK4-induced IL-8 expression. However, LTAs from other lactobacilli such as *L. delbruekii* or *L. rhamnosus* GG slightly inhibited Pam2CSK4-induced IL-8 expression and moreover, LTA from *L. sakei* failed to inhibit Pam2CSK4-induced IL-8 expression. In contrast to LTA, either PGN with WTA or without WTA from *L. plantarum* did not suppress Pam2CSK4-induced IL-8 expression (Fig. 4A and 4B). To examine whether the inhibitory effect of other bacterial LTAs on Pam2CSK4-induced IL-8 expression, Caco-2 cells were stimulated with 30 μg/ml of LTAs from *S. aureus*, *E. faecalis*, *S. mutans*, and *L. plantarum* in the absence or presence of Pam2CSK4 (0.1 μg/ml) for 24 h. Then, the supernatants were collected and IL-8 production was determined using ELISA. Pam2CSK4-induced IL-8 production was also suppressed by LTAs of *S. aureus*, *E. faecalis*, *S. mutans*, and *L. plantarum*. However, Lp.LTA most potently inhibited Pam2CSK4-induced IL-8 production among the bacterial LTAs (Fig. 4C). Therefore, these results indicate that Lp.LTA preferentially and

potently inhibits Pam2CSK4-induced IL-8 expression in Caco-2 cells.

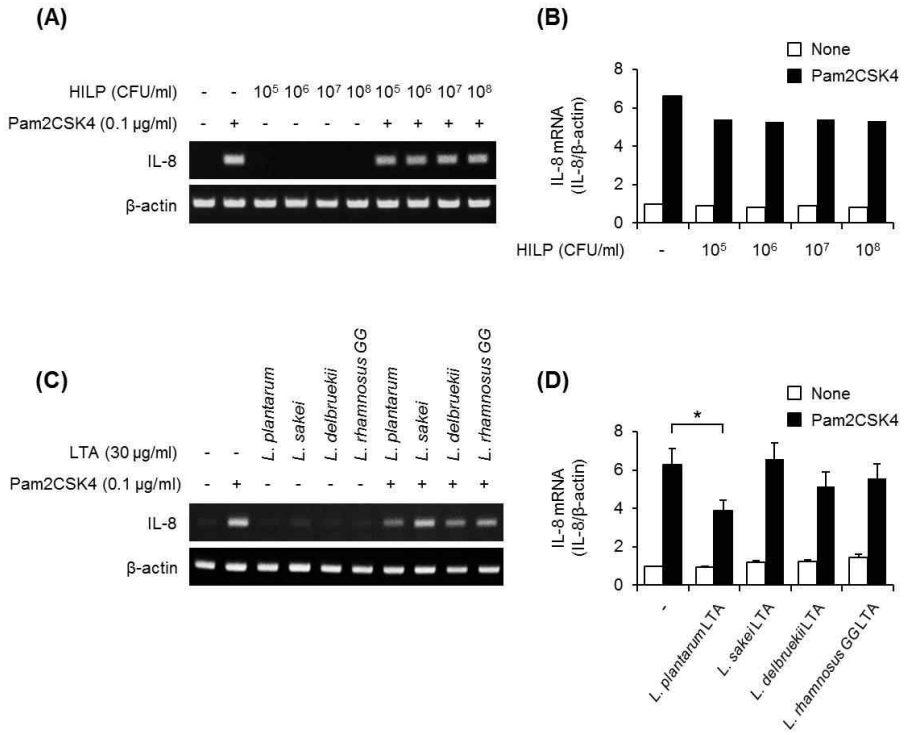

Figure 5. Effect of heat-inactivated *L. plantarum* and LTAs of various lactobacilli on Pam2CSK4-induced IL-8 expression. (A and B) Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of heat-inactivated *L. plantarum* marked as HILP (10^5 to 10^8 CFU/ml) for 1 h. (C and D) Caco-2 cells were incubated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of 30 μg/ml of Lp.LTA, *L. sakei* LTA, *L. delbruekii* LTA, or *L. rhamnosus* GG LTA for 1 h. Total RNA was extracted and IL-8 mRNA expression was determined using RT-PCR. Relative mRNA expression of IL-8 to that of β-actin was obtained with densitometric analysis. The asterisk (*) indicates that $*P < 0.05$. Fig. D is one of three-independent expreiments.

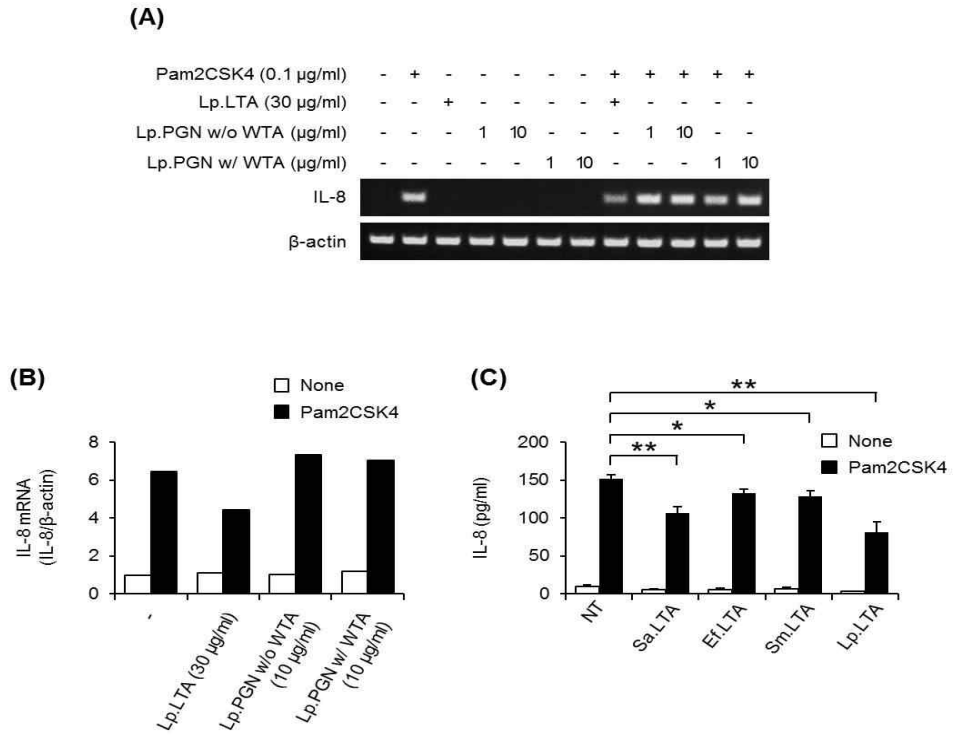

Figure 6. Effect of *L. plantarum* PGN and other bacterial LTAs on Pam2CSK4-induced IL-8 production. (A and B) Caco-2 cells were stimulated with Pam2CSK4 in the presence or absence of Lp.LTA, or *L. plantarum* PGN (Lp.PGN) covalently linked with or without WTA for 1 h. Total RNA was extracted and IL-8 mRNA expression was determined using RT-PCR. Fig. B shows the relative mRNA expression of IL-8 to that of β-actin calculated with densitometric analysis (C) Caco-2 cells were treated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of 30 μg/ml of LTAs from *S. aureus*, *E. faecalis*, *S. mutans*, and *L. plantarum* for 24 h. The culture supernatants were collected and IL-8 production was determined using ELISA. Fig C. was conducted in triplicated experiments. The asterisks (*) indicate that $*P < 0.05$ and $**P < 0.01$.

4) Lipid and D-alanine moieties of Lp.LTA are crucial for the inhibition of Pam2CSK4-induced IL-8 production.

Next, we determined the decrease of IL-8 expression with the various concentration of Lp.LTA. Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence of various concentrations of Lp.LTA (0 to 30 μg/ml). Pam2CSK4-induced IL-8 mRNA expression was gradually decreased by Lp.LTA in a dose-dependent manner (Figs. 5A and 5B). In addition, the release of Pam2CSK4-induced IL-8 was significantly diminished in the presence of Lp.LTA (30 μg/ml) (Fig. 5C). It has been reported that Caco-2 cells are non-polarized cells but they have a capacity to polarize to enterocytes by culturing the cells for 14 days culture after the cells were fully confluent [52]. Caco-2 cells were polarized using the transwell cell culture system and apically stimulated with Lp.LTA (30 μg/ml) in the presence or absence of Pam2CSK4 (0.1 μg/ml) for 24 h. Pam2CSK4-induced IL-8 production was observed only in the supernatants of apical side of Caco-2 cells. In addition, Pam2CSK4-induced IL-8 production was significantly inhibited by Lp.LTA. However, IL-8 production was not detected in the supernatants of basolateral side of Caco-2 cells (Fig. 6A). In order to identify functional moieties of Lp.LTA structure responsible for the inhibitory effect, we prepared de-alanylated Lp.LTA and de-acylated Lp.LTA and then, determined the inhibitory effect of these LTAs on IL-8 expression. Caco-2 cells were stimulated with 30 μg/ml of Lp.LTA, de-alanylated Lp.LTA, and de-acylated Lp.LTA in the presence or absence of Pam2CSK4 (0.1 μg/ml). The culture supernatants were collected and IL-8 production was determined using ELISA. Fig. 6B indicated that Pam2CSK4-induced IL-8 production was

suppressed by Lp.LTA. However, the inhibitory effect of Lp.LTA on IL-8 production was not observed when the cells were stimulated with de-alanylated Lp.LTA or de-acylated Lp.LTA. Taken together, Pam2CSK4-induced IL-8 production was decreased by Lp.LTA in a dose-dependent manner, and the lipid moieties and D-alanine contents of Lp.LTA played a key role in the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 production.

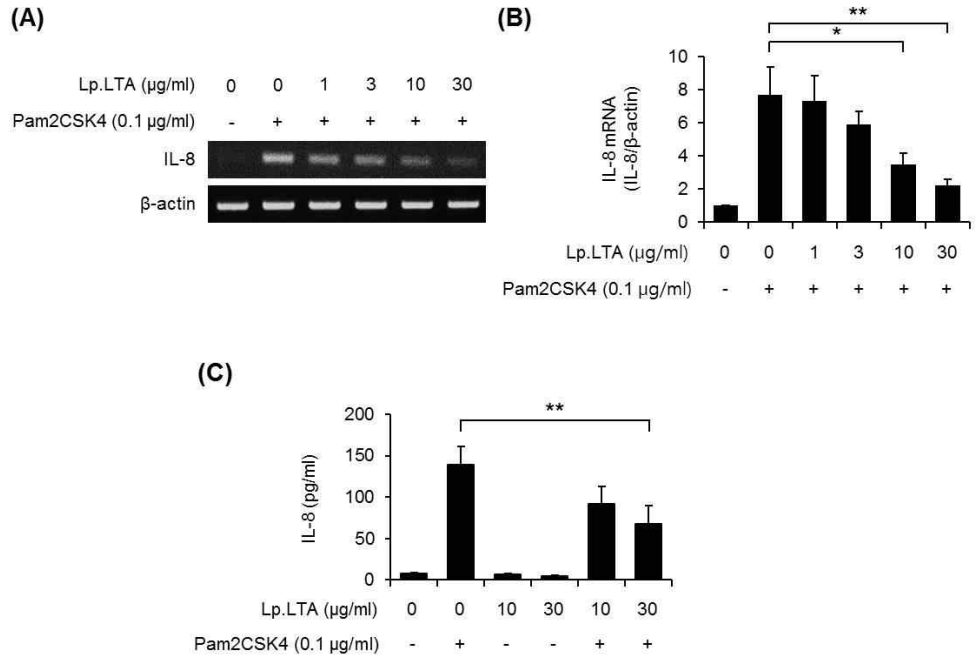

Figure 7. Effect of Lp.LTA on Pam2CSK4-induced IL-8 production. (A and B) Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (1 to 30 μg/ml) for 1 h. Total RNA was extracted and IL-8 expression was determined using RT-PCR. Fig. B shows the relative mRNA expression of IL-8 to that of β-actin was obtained with densitometric analysis. (C) Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (10 or 30 μg/ml) for 24 h. Culture supernatants were collected and IL-8 production was determined using ELISA. The asterisks (*) indicate that $*P < 0.05$ and $**P < 0.01$. All results were performed in triplicated experiments.

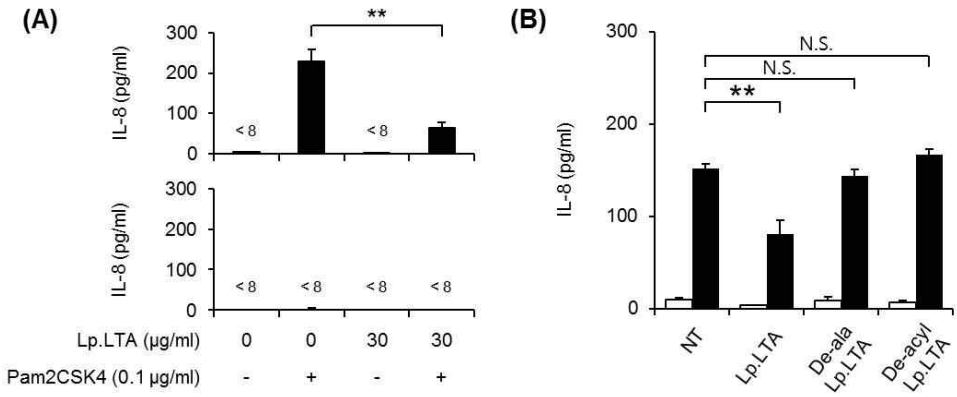

Figure 8. Effect of Lp.LTA and its structural variants on Pam2CSK4-induced IL-8 production. (A) Polarized Caco-2 cells were apically stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (10 or 30 μg/ml) for 24 h. Both apical and basolateral culture supernatants were collected and IL-8 production was determined using ELISA. Upper panel indicates apical IL-8 production and lower panel indicates basolateral IL-8 production. (B) Caco-2 cells were incubated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of 30 μg/ml of Lp.LTA, de-alanylated Lp.LTA, and de-acylated Lp.LTA marked as Lp.LTA, De-ala Lp.LTA, and De-acyl Lp.LTA, respectively, for 24 h. Caco-2 cell supernatants were collected and IL-8 production was detected using ELISA. N.S. means no significance. The asterisks (*) indicate that $*P < 0.05$ and $**P < 0.01$. All results were performed in triplicated experiments.

5) Intracellular negative regulators are not involved in the regulation of Pam2CSK4-induced IL-8 production.

It has been reported that intracellular negative regulators play an important role in the maintenance of intestinal homeostasis [53]. Furthermore, a number of studies have reported that *Lactobacillus* regulates the expression of negative regulators such as IRAK-M, A20, Bcl-3, and MKP-1 to attenuate intestinal inflammatory responses [54, 55]. To examine the expression of negative regulators, Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 0, 1, 3, 6, 12, or 24 h. The expression of negative regulators including IRAK-M, SOCS1, TOLLIP, and A20 were not affected by Lp.LTA, suggesting that intracellular negative regulators are not involved in the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 production (Fig. 7).

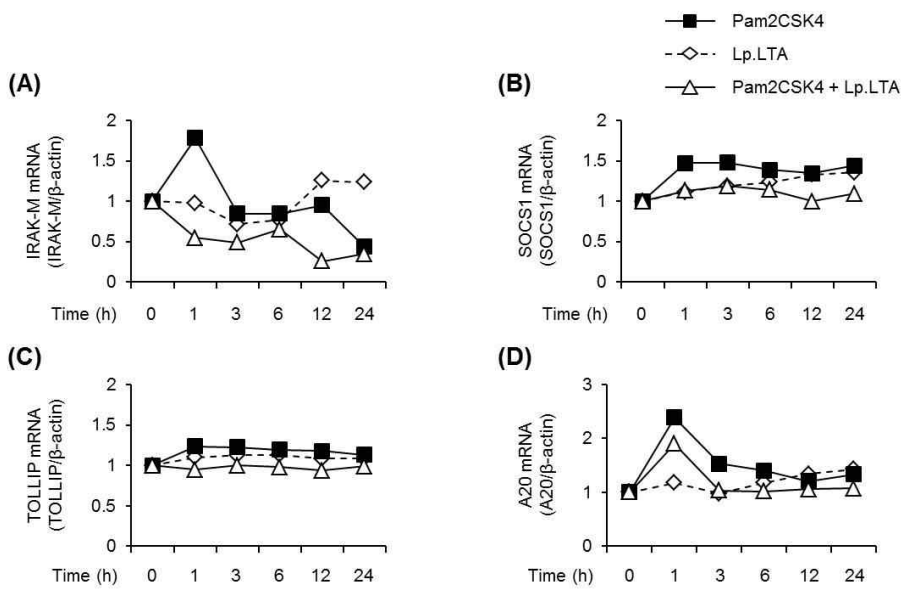

Figure 9. Effect of negative regulators on the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 production. (A - D) Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 0, 1, 3, 6, 12, or 24 h. Total RNA was extracted and mRNA expression of negative regulators was determined using RT-PCR. Relative mRNA expression of IRAK-M, SOCS1, TOLLIP, and A20 to that of β-actin was obtained with densitometric analysis.

6) Lp.LTA reduces Pam2CSK4-induced TLR2 activation in CHO/CD14/TLR2 cells.

It has been well known that both Pam2CSK4 and LTA are agonists of TLR2 [56, 57]. Therefore, we hypothesized that the Lp.LTA might inhibit the binding capacity of Pam2CSK4 to TLR2. Surface and intracellular TLR2 was stained with anti-TLR2 FITC monoclonal antibody and TLR2 expression was determined using flow cytometric analysis in and on Caco-2 cells. A549 cells and CHO/CD14/TLR2 cells were used as controls for TLR2 expression. As shown in Fig. 8, TLR2 expression on the cell surface was about three times higher than intracellular TLR2 expression in Caco-2 cells. Next, competition assay between Pam2CSK4 and Lp.LTA for TLR2 was performed. CHO/CD14/TLR2 cells were treated with Pam2CSK4-rhodamine (0.1 μg/ml) in the absence or presence of Lp.LTA (30 μg/ml) for 1 h at 4°C. Binding capacity of Pam2CSK4-rhodamine was determined using flow cytometric analysis. As shown in Fig. 9A, Pam2CSK4-rhodamine slightly bound to TLR2 compared with non-treatment group. Contrary to expectations, binding of Pam2CSK4-rhodamine was not interrupted in the presence of Lp.LTA. These results suggest that Lp.LTA does not interfere with the binding ability of Pam2CSK4 to TLR2. Next, to examine the inhibitory effect of Lp.LTA on the Pam2CSK4-induced TLR2 activation, CHO/CD14/TLR2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the absence or presence of Lp.LTA (30 μg/ml) for 24 h. CD25 expression on the cell membrane was analyzed using anti-CD25 FITC monoclonal antibody by flow cytometry. As shown in Fig. 9B, Pam2CSK4 potently induced TLR2 activation but Lp.LTA slightly induced TLR2 activation. However, Pam2CSK4-induced TLR2 activation was significantly decreased in the presence of Lp.LTA, suggesting that Pam2CSK4-induced TLR2

activation is decreased by Lp.LTA.

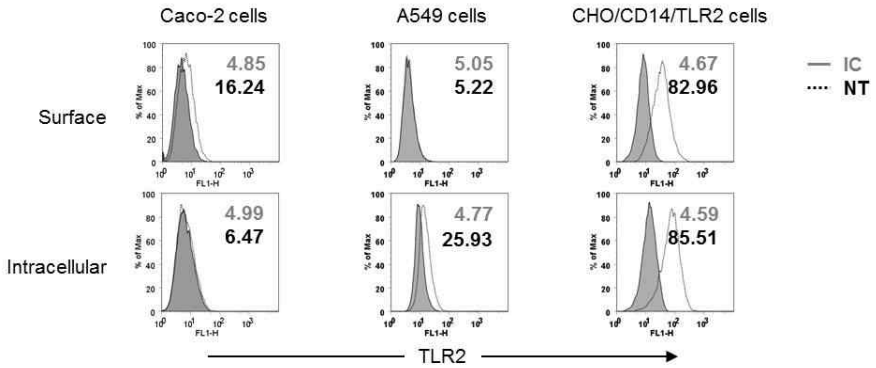

Figure 10. TLR2 expression in Caco-2, A549, and CHO/CD14/TLR2 cells. Caco-2, A549, and CHO/CD14/TLR2 cells were stained with FITC-conjugated anti-TLR2 monoclonal antibody and mouse IgG2, κ as an isotype control at 4°C for 30 min. The cells for intracellular staining were permeabilized with PBS containing 0.1% saponin for 10 min before staining. And other cells for surface staining were blocked and washed with PBS containing 2% FBS. TLR2 expression was determined using flow cytometric analysis.

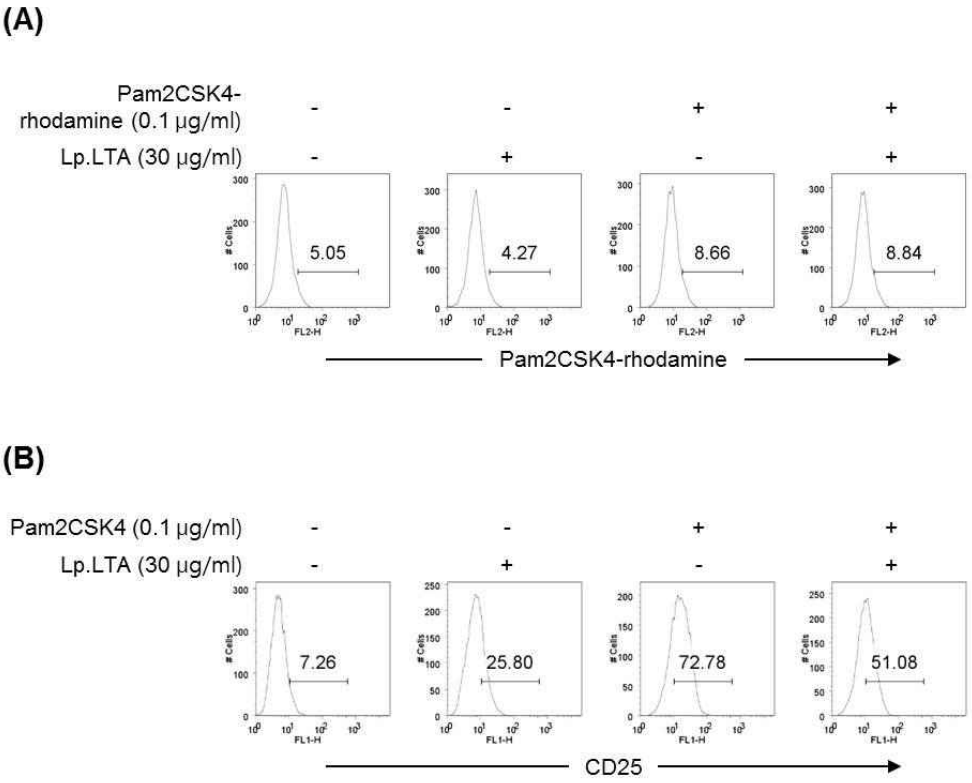

Figure 11. Effect of Lp.LTA on Pam2CSK4-induced TLR2 activation and binding of Pam2CSK4 to TLR2. (A) CHO/CD14/TLR2 cells were stimulated with Pam2CSK4-rhodamine (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) at 4°C for 1 h. Binding of Pam2CSK4-rhodamine was determined using flow cytometry. (B) CHO/CD14/TLR2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 24 h. The cells were stained with FITC-conjugated anti-CD25 monoclonal antibody and mouse IgG1, κ as an isotype control at 4°C for 30 min. CD25 expression was determined using fluorescence-activated cell sorting analysis. CD25 expression indicates TLR2 activation

7) Lp.LTA suppresses Pam2CSK4-induced MAP kinases phosphorylation and IκBα degradation.

To determine the effect of Lp.LTA on Pam2CSK4-induced intracellular signaling pathway, Caco-2 cells were stimulated with Lp.LTA (30 μg/ml) in the absence or presence of Pam2CSK4 (0.1 μg/ml) for 30 min. Then cell lysates were obtained and MAP kinases phosphorylation was examined using antibodies for phosphorylated or non-phosphorylated forms of p38 kinase, JNK, and ERK. Pam2CSK4 induced phosphorylation of p38 kinase and JNK but not ERK. In addition, Lp.LTA remarkably suppressed Pam2CSK4-induced p38 kinase and JNK phosphorylation (Fig. 10A, 10B, and 10C). Next, to see the effect of Lp.LTA on IκBα degradation, which is known as an NF-κB inhibitory cytoplasmic molecule and an essential component of the signaling pathway [58], Caco-2 cells were stimulated with Lp.LTA (30 μg/ml) in the presence or absence of Pam2CSK4 (0.1 μg/ml) for 30 min. IκBα was degraded in the presence of Pam2CSK4. However, Pam2CSK4 failed to degrade IκBα in the presence of Lp.LTA (Fig. 10D). These results suggest that Lp.LTA may suppress Pam2CSK4-induced IL-8 production through the inhibition of p38 kinase and JNK phosphorylation and prevention of IκBα degradation in Caco-2 cells.

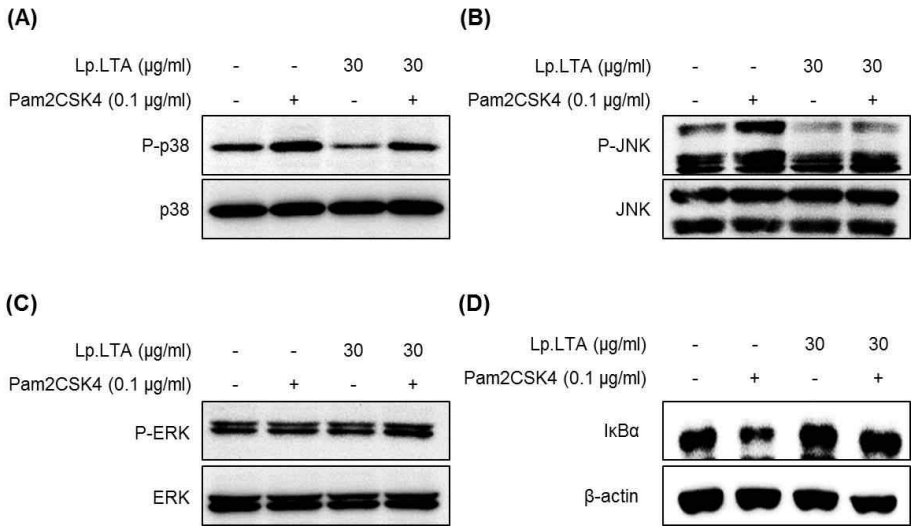

Figure 12. Effect of Lp.LTA on MAP kinases and IκBα expression. (A - C) Caco-2 cells were incubated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 30 min. Cell lysates were obtained from the cells and subjected to Western blot analysis to determine phosphorylation of p38 kinase, JNK, and ERK. (D) Caco-2 cells were stimulated with Pam2CSK4 (0.1 μg/ml) in the presence or absence of Lp.LTA (30 μg/ml) for 30 min. The cell lysates were subjected to Western blot analysis to detect IκBα expression.

IV. DISCUSSION

It has been reported that *Lactobacillus* plays an important role in the attenuation of acute and chronic inflammation in the gastrointestinal tract [25, 59]. Certain *Lactobacillus* strains suppress production of pathogen-induced pro-inflammatory mediators such as IL-8 [60]. However, it has not been clearly demonstrated that *Lactobacillus* LTA can inhibit IL-8 production in human intestinal epithelial cells. Even though IL-8 plays an important role in the recruitment of leukocytes such as neutrophils during inflammatory condition [61] and migration of fibroblasts to enhance wound healing [62], it also facilitates tissue damage by neutrophil accumulation at the inflammatory site, eventually resulting in chronic inflammation [63, 64]. Harada et al. [65] reported that LPS-induced dermatitis, LPS/IL-1-induced arthritis, and lung reperfusion injury model resulted in increased level of IL-8 production and a number of neutrophil infilteration. However, treatment with IL-8 neutralizing antibody in inflammatory diseases which are described in above diminished neutrophil-induced tissue damage. The current study demonstrated that bacterial lipoproteins are major cell wall components to induce IL-8 expression in human intestinal epithelial cells. Lp.LTA, but not the PGN, significantly inhibits Pam2CSK4-induced IL-8 expression. The lipid and D-alanine moieties of Lp.LTA are crucial for the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 expression in Caco-2 cells.

Previous reports have suggested that lipid and D-alanine moieties are critical structural components of LTA to modulate immune responses [66, 67]. Native *S. aureus* LTA significantly induced the production of TNF-α, while de-alanylated *S. aureus* LTA did not show such effect

[66]. Replacing D-alanine with L-alanine in the LTA structure makes LTA lost its ability to induce inflammatory responses [67]. Moreover, it has been reported that *S. aureus* LTA has 75% of D-alanine contents whereas those of *B. subtillis* and Group B streptococcus have 25% and 46%, respectively. The difference in D-alanine contents is considered to result in higher immuno-stimulatory potential of *S. aureus* LTA than those of *B. subtilis* and Group B streptococcus [68]. In addition, de-acylated LTAs of *S. aureus*, *E. faecalis*, and *Streptococcus pneumoniae* hardly induced the production of inflammatory mediators such as TNF-α and nitric oxide compared with native LTAs [44, 45, 67]. Moreover, de-acylated LTA did not bind to TLR2 and this failure of TLR2 binding abolished the immunomodulatory capacity of LTA, suggesting that the lipid moieties are crucial for LTA to bind to TLR2 leading to activation [67]. Our data also suggest that lipid and D-alanine moieties are crucial for the immunomodulatory properties of Lp.LTA since de-alanylated or de-acylated Lp.LTA does not attenuate Pam2CSK4-induced IL-8 production.

Pam2CSK4-induced IL-8 expression was more potently suppressed by Lp.LTA than LTAs from other *Lactobacillus* strains and several pathogenic bacteria tested in this study. Kang et al. [56] have reported that the lipid chains of LTA sit into the hydrophobic pocket in TLR2. This leads to the conformational changes of leucine-rich repeats of TLR2 and head groups of LTA resulting in the triggering of intracellular signaling transduction [56]. On the other hand, ester-bound lipid attached to the lipid pocket of TLR2. While *L. plantarum* LTA has three ester-bound lipid chains [43], *S. aureus* and *L. rhamnosus* GG have two ester-bound lipid chains [69, 70]. Therefore, since LTAs of various bacteria have various numbers of lipid moieties, LTAs of

differential bacteria may have differential potential to bind to TLR2, consequently resulting in the differential immunomodulatory activities. In addition, arrangement of hydrogen donor and acceptor atoms of TLR2 by head groups of LTA makes non-effective TLR2 and TLR6 heterodimer [56]. Because the composition of head group of each bacterial LTA is various [68], the immunomodulatory effects of each bacterial LTA may be various. Collectively, it can be assumed that the structural differences of LTA between various bacterial species result in differences in binding capacity to TLR2 and the ability to form the effective receptor complexes and, consequently, different immunological properties.

It has been well known that one of the mechanisms to maintain intestinal homeostasis is the regulation of TLR signaling pathways by up-regulation of intracellular negative regulators [53]. Previously, it has been reported that *Lactobacillus* regulates pro-inflammatory responses through the negative regulators such as IRAK-M, A20, Bcl-3, and MKP-1 [54, 55]. In this study, co-treatment with Pam2CSK4 and Lp.LTA did not upregulate the expression of negative regulators compared with treatment with Pam2CSK4 alone, suggesting that Lp.LTA does not inhibit Pam2CSK4-induced IL-8 production through regulating the expression of intracellular negative regulators. However, some research groups have suggested that the activation of peroxisome proliferator-activated receptor-γ, which is a member of nuclear hormone receptor, has an essential role in the inhibitory effect of LTA through the negative regulation of TLR signaling [71, 72]. Both Pam2CSK4 and LTA are well known for the agonists of TLR2 [56, 57]. According to our study, since Lp.LTA antagonized Pam2CSK4-induced inflammatory response, we examined whether Lp.LTA inhibits the effect of Pam2CSK4 on TLR2. While Lp.LTA

failed to inhibit binding capacity of Pam2CSK4-rhodamine to TLR2, Lp.LTA inhibited Pam2CSK4-induced TLR2 activation. These results suggest that Lp.LTA regulates pro-inflammatory responses by inhibiting Pam2CSK4-induced TLR2 activation. Moreover, in addition to the binding to TLR2, the bacterial LTA may be able to bind to and to interact with additional receptor(s), although the receptor(s) has not been decisively studied, contributing to the immunomodulation. Recently, it has been described that LTA interacts with paired Ig-like receptor-B and substantially, inhibits bacterial lipoproteins-induced inflammatory responses in macrophages [73], indicating that LTA may alleviate intestinal inflammation by transferring the inhibitory signals into the cells through other receptors. However, it needs to be further studied the mechanisms by which Lp.LTA attenuates intestinal inflammation through which mechanisms.

We observed Lp.LTA decreased Pam2CSK4-induced p38 kinase and JNK phosphorylation but not ERK phosphorylation. p38 kinase is activated and phosphorylated immediately in response to various stimuli including PAMPs and inflammatory cytokines [74, 75]. In addition, it has been reported that activation of p38 kinase pathway is closely related with inflammation. For example, activated p38 kinase pathway induced the production of inflammatory mediators such as IL-1β, TNF-α, IL-6, COX-2, and iNOS [76, 77]. JNK also related with inflammation. For instance, JNK mediated the expression of LPS-induced inflammatory mediators such as MCP-1 and IL-6 in microglia [78]. Therefore, decline of p38 kinase and JNK phosphorylation are related with decrease of Pam2CSK4-induced IL-8 expression by Lp.LTA. On the other hand, ERK1/2 is mainly related to cell survival and proliferation rather than inflammation [79]. In addition, our result showed that Lp.LTA did not regulate ERK1/2

phosphorylation. This result suggests that regulation of ERK1/2 phosphorylation is not involved in the inhibitory effect of Lp.LTA on the production of Pam2CSK4-induced IL-8. IκBα, which is a regulatory molecule that inhibits the activation of NF-κB should be degraded for the induction of IL-8 [58]. However, Lp.LTA suppressed Pam2CSK4-induced degradation of IκBα, suggesting that the inhibition of IκBα degradation by Lp.LTA results in inactivation of NF-κB and consequentially, the reduction of IL-8 production.

In this study, we demonstrated the inhibitory effect of Lp.LTA on Pam2CSK4-induced IL-8 production in human intestinal epithelial cells. Distinct structural composition of LTA such as the number of repeated units, the amount of D-alanine contents, and the number of acyl chains and unsaturated fatty acid was supposed to affect the inhibitory effect of LTA. More specifically, we showed that D-alanine contents and lipid moieties are essential for the inhibitory effect of Lp.LTA. Collectively, Lp.LTA strongly attenuates Pam2CSK4-induced IL-8 production via the inhibition of MAP kinases phosphorylation such as p38 kinase and JNK and the prevention of IκBα degradation through the suppression of TLR2 activation in human intestinal epithelial cells. These results may inform us that *Lactobacillus*, one of the intestinal commensal bacteria, defends the host against pathogenic microbes using its LTA in a normal state. In addition, our results suggest that Lp.LTA could be used to attenuate pathogen-induced intestinal inflammation as an alternative therapy.

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국문초록

Caco-2 세포에서 Pam2CSK4에 의해 유도된 IL-8 발현에 대한 *Lactobacillus plantarum* lipoteichoic acid의 억제 효과

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1. 연구목적

유산균은 인간과 동물의 소화기관에 존재하며 숙주에 유익한 영향을 주는 세균으로 알려져 있다. 유산균에 의해 장내 염증반응이 완화된다고 보고되고 있지만 정확히 유산균의 어떤 물질에 의해 장내 염증반응이 억제되는지에 대해서는 아직 잘 알려져 있지 않다. 따라서 본 연구는 *Lactobacillus plantarum*의 주요 구성물질 중 하나인 lipoteichoic acid (LTA)가 인간 대장 상피세포에서 IL-8과 같은 전염증성 사이토카인의 생산을 조절할 수 있는지에 대해 알아보았다.

2. 연구방법

인간의 대장상피세포주인 Caco-2 세포에 *S. aureus* RN4220과 그 돌연변이체들을 처리하여 IL-8의 발현양상을 역전사효소 중합연쇄반응(RT-PCR)로 알아보았다. 세균의 지질단백질은 염증 유발에 중요하다고 알려져 있는데, 이를 모방하여 합성한 Pam2CSK4를 Caco-2 세포에 다양한 농도 및 시간으로 처리하여 IL-8의 발현양상을 RT-PCR과 효소결합 면역분석법 (ELISA)을 이용하여 관찰하였다. 유산균 및 그 구성요소가 Pam2CSK4에 의해 유도된 IL-8 발현에 어떠한 영향을 주는지 알아보기 위해, Caco-2 세포에 열에 의해 불활성화된 *L. plantarum*을 다양한 농도로 처리하거나, *L. plantarum*의 펩티도글리칸과 여러 종류의 유산균 LTA를 Pam2CSK4와 동시에 처리하여 IL-8의 발현을 RT-PCR로 확인하였다. Caco-2 세포에 Pam2CSK4와 *L. plantarum* LTA (Lp.LTA)를 동시에 처리하여 IL-8의 생산을 ELISA를 통해 측정하였다. 또한, Lp.LTA의 구조적 차이에 따른 영향을 알아보기 위해 Caco-2 세포에 D-alanine이 제거된 Lp.LTA와 아실 사슬이 제거된 Lp.LTA를 Pam2CSK4와 동시에 처리하여 IL-8 생산을 ELISA를 통해 측정하였다. 톨유사수용체-2 (TLR2)의 활성을 알아보기 위해, CD14와 TLR2를 과발현하며 CD25 리포터 유전자를 가진 CHO/CD14/TLR2 세포에 Pam2CSK4와 Lp.LTA를 동시에 처리한 후 NF-κB 활성을 통한 CD25 발현을 유세포분석기로 측정하였다. 세포내부 신호전달을 알아보기 위해 Caco-2 세포에 Pam2CSK4와 Lp.LTA를 동시에 처리한 후 p38, ERK, JNK 등의 세포 내 신호전달 매개인자와 IκBα의 발현양상과 인산화 정도를 웨스턴 블롯법으로 관찰하였다.

3. 연구결과

Caco-2 세포에서 *S. aureus* RN4220에 의해 IL-8이 생산되었으나 지질단백질을 가지고 있지 않은 *S. aureus* 돌연변이체에 의해서는 IL-8이 생산되지 않았다. 또한, Pam2CSK4는 IL-8의 발현을 강하게 유도하는 것을 관찰하였다. 따라서 *S. aureus*의 지질단백질이 장에서 염증을 일으키는 중요한 인자라는 것을 알 수 있었다. 열에 의해 불활성화된 *L. plantarum*

은 IL-8의 발현을 유도하지 못했지만 Pam2CSK4 자극에 의한 IL-8의 발현을 조금 억제하였다. 유산균의 세포벽 구성물질 중 Lp.LTA의 농도가 증가할수록 Pam2CSK4에 의해 유도된 IL-8의 발현이 현저히 억제되었다. 하지만, *L. delbruekii, L. rhamnosus* GG의 LTA는 IL-8 발현을 조금밖에 억제하지 못했으며 *L. sakei* LTA는 전혀 억제하지 못했다. Lp.LTA와는 대조적으로 *L. plantarum*의 펩티도글리칸은 IL-8 발현을 전혀 억제하지 못했다. 또한, 아실 사슬이 결여된 Lp.LTA는 Pam2CSK4에 의해 유도된 IL-8 생산을 억제하지 못했다. 이것은 Lp.LTA에서 지질 성분이 Pam2CSK4에 의해 유도된 IL-8 생산을 억제하는데 중요하다는 것을 의미한다. CHO/CD14/TLR2 세포에 Pam2CSK4를 처리했을 때 TLR2의 활성이 매우 강하게 유도된 반면 Pam2CSK4에 의해 유도된 TLR2의 활성은 Lp.LTA에 의해 억제되었다. 또한, Pam2CSK4가 p38 kinase 과 JNK와 같은 마이토젠활성 단백키나아제의 인산화를 증가시켰지만, Lp.LTA가 이들의 인산화를 눈에 띄게 감소시켰다. IκBα는 Pam2CSK4에 의해 현저히 분해되었지만 Lp.LTA가 존재할 때는 Pam2CSK4에 의한 IκBα 분해가 일어나지 않았다. 결론적으로, Lp.LTA는 Pam2CSK4에 의한 TLR2 활성화를 억제함으로써 p38 kinase 와 JNK와 같은 세포 내 신호전달 매개인자의 인산화를 감소시키고 IκBα의 분해를 방해하여 Caco-2 세포에서 Pam2CSK4에 의해 유도되는 IL-8 생산을 억제한다는 것을 알 수 있었다.

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주요어 : *Lactobacillus plantarum*, Lipoteichoic acid, Pam2CSK4, Interleukin-8, 대장상피세포

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