Lipoteichoic Acid Isolated from *Weissella cibaria* Increases Cytokine Production in Human Monocyte-Like THP-1 Cells and Mouse Splenocytes

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Introduction

Lipoteichoic acid (LTA) is a cell wall component of gram-positive bacteria. The structure of LTA is different in every species [29]. However, most LTA is composed of long phosphate chains and glycolipids. The function of LTA is similar to that of lipopolysaccharide (LPS), which is a cell wall component of gram-negative bacteria. LTA and LPS increase secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [19, 25, 26], which cause inflammatory diseases such as arthritis, meningeal inflammation, and septic shock [6]. However, LTAs from different bacterial species and strains have different immune regulatory effects. For example, LTA from *Bacillus subtilis*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Staphylococcus aureus* increase the production of TNF-α in RAW 264.7 cells or splenocytes [19, 24]. On the other hand, LTA from *Lactobacillus plantarum* (pLTA) does not induce TNF-α production. The outstanding function of LTA isolated from *L. plantarum* is anti-inflammatory effects. It has been reported that LTA from *L. plantarum* inhibits the inflammatory cytokine production induced by LPS and LTA from *S. aureus* (aLTA) [12, 13]. LTA is recognized by Toll-like receptor 2 (TLR2) [19, 30] and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 mitogen-activated protein kinases, and c-Jun N-terminal kinases in THP-1 cells. The secretion of TNF-α and IL-6 was also increased in the cLTA-treated mouse splenocytes. These results suggest that cLTA, but not *W. cibaria* whole cells, has immune-boosting potential and can be used to treat immunosuppression diseases.

Keywords: *Weissella cibaria*, cytokine, lipoteichoic acid, immune regulation

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microflora and thereby affect intestinal health. LAB also have beneficial effects for reducing alcohol-induced hepatic inflammation and other anti-inflammatory activity [17, 18, 28]. In the food industry, LAB are used to produce fermented foods like kimchi and yogurt. LAB in foods produce some substances that create different tastes or smells and antimicrobial substances like bacteriocins that inhibit the growth of harmful bacteria [23]. LAB such as L. plantarum, Streptococcus thermophiles, and Bifidobacterium breve are used in cosmetic ingredients because they affect skin hydration, are antioxidative, and can enhance and produce hyaluronic acid that affects skin moisturization [9]. Weissella cibaria is a gram-positive bacterium that belongs to the Leuconostocaceae family. Weissella species are one of the most common LAB found in fermented foods, including kimchi [15]. Since W. cibaria has an inhibitory effect against volatile sulfur compounds and cancer preventive potential, it is considered as a novel probiotic [10, 14]. It is also known that W. cibaria inhibits the adherence of Fusobacterium nucleatum to the epithelium of the oral cavity by coaggregation with them, which results in the decrease of bad breath [11]. Previous studies have demonstrated that pLTA has anti-inflammatory effects [12, 13], and aLTA causes severe inflammation and septic shock [3, 27]. However, the immune-regulating effect of W. cibaria has not been demonstrated. In this study, we examined the effects of live or heat-killed W. cibaria and LTA isolated from W. cibaria (cLTA) on inflammatory cytokine production, using the human monocyte-like cell line THP-1 and mouse splenocytes.

Materials and Methods

Bacterial Strains

W. cibaria was isolated from Indian fermented food. Briefly, LAB have been isolated from Dosa, which is the most popular fermented product of South India. Dosa was spread on MRS agar plates after serial dilution, and 100 colonies were selected and cultured in MRS broth. The cultured LAB samples were numbered CDK1 to CDK100. The strain was identified using 16S rRNA sequencing, and the phylogenetic tree was drawn.

Purification of LTA

LTAs from W. cibaria, L. plantarum, and S. aureus were purified using the methods of Morath et al. [20]. Briefly, harvested cells were sonicated and then extracted with butanol. Octyl-Sepharose and DEAE Sepharose chromatography were used to purify LTAs. The purity of the LTAs was determined by measuring the protein content with silver staining, and endotoxin contents were identified with the LAL endotoxin kit (GenScript, NJ, USA).

Cell Culture

THP-1 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 100 U/ml of penicillin and 100 µg/ml of streptomycin (P/S). They were cultured at 37°C in 5% CO₂. Male 5-week-old BALB/c mice were obtained from NaraBio (Korea). Mice were housed at 23°C in a 12 h light/dark cycle for 7 days. Mouse spleens were isolated and washed with DPBS. Single-cell suspensions were prepared by grinding the spleens into small pieces with a strainer and centrifuging at 800 × g for 5 min. The cell pellet was resuspended in RBC lysis buffer for 5 min on ice and centrifuged. After washing in RPMI 1640 medium, the cells were adjusted to a final concentration of 5 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS and P/S and cultured at 37°C in 5% CO₂.

Enzyme-Linked Immunosorbent (ELISA) Assay

THP-1 and splenocytes were seeded at 1 × 10⁶ cells in a 96-well round plate. To examine the induction of cytokines by live or heat-killed bacteria and LTA, cells were treated for time- and dose-dependent studies. To examine the inhibitory effect of LTA, cells were pretreated with 100 µg/ml LTA for 18 h and then with 500 ng/ml LPS for 6 h. The secretion of cytokines in the supernatants was analyzed by sandwich ELISA. hTNF-α, hIL-1β, hIL-8, mTNF-α, mIL-1β, mIL-6, and mIL-12 were measured using specific antibodies, purchased from R&D Systems (USA).

Western Blot Analysis

THP-1 cells at 1 × 10⁶ cells/ml were seeded in 6-well plates. After 24 h, cells were treated with cLTA, pLTA, or aLTA for 1 h and then washed twice with DPBS. Cells were lysed with Laemmli loading buffer and boiled at 100°C for 5 min. Proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes at 100 V for 1 h. The membranes were blocked with 5% skim milk and washed three times with TBST before being incubated with primary antibody overnight. After washing, secondary HRP-conjugated antibody was applied for 2 h. Protein bands were detected with ECL, and β-actin was used as a loading control.

Statistical Analysis

All the experiments were performed at least three times. The data shown are representative results of the mean ± SD of triplicated experiments. Differences were considered statistically significant when the p value was <0.05.

Results

W. cibaria Was Isolated from Dosa, a Traditional Indian Fermented Food

CDK18 was isolated from an Indian fermented food as described in Materials and Methods. A single colony of

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bacteria was sequenced for phylogenetic analysis. The 16S rRNA gene was sequenced, and sequence alignment was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov). A neighbor-joining tree based on 16S rRNA gene sequences showed the phylogenetic relationships between Weissella and CDK18, with a Bar 0.02 substitutions per nucleotide position (Fig. 1). Bootstrap analysis with 1,000 replicates was also conducted in order to obtain confidence levels for the branches. Most of the species in Weissella were included in the phylogenetic tree. CDK18 was identified as W. cibaria and renamed W. cibaria K401 for further studies.

cLTA Isolated from W. cibaria Induced Cytokine Production in THP-1 Cells

To examine the effects of W. cibaria on cytokine production, live and heat-killed W. cibaria were applied dose- and time-dependently. TNF-α secretion was increased to 1 × 10^8 CFU/ml, but the production level was less than that of S. aureus, which significantly increased TNF-α production after 24 h stimulation (Fig. 2A). TNF-α secretion was not altered in heat-killed W. cibaria-treated cells, whereas it was significantly increased by heat-killed S. aureus (Fig. 2B). Live and heat-killed S. aureus-treated cells significantly increased TNF-α after 3 h stimulation. Similarly, time-dependent induction of TNF-α was shown in live and heat-killed W. cibaria-treated cells, but it was not significant (Figs. 2C and 2D). These results indicate that live and heat-killed W. cibaria do not affect immune responses.

Next, the biological function of cLTA was examined. To examine the cytotoxicity of cLTA, the viability of cLTA-treated THP-1 cells was identified using the WST-1 assay. cLTA, pLTA, and aLTA did not induce cell death, which indicates that cLTA does not affect the viability of THP-1 cells (Fig. 3A). To investigate the effects of cLTA on cytokine production, culture supernatants from cLTA-treated THP-1 cells were collected and subjected to ELISA. As shown in

![Phylogenetic tree of CDK18](image-url)

**Fig. 1.** Phylogenetic tree of CDK18.
A single colony of CDK18, isolated from an Indian fermented food, was sequenced for its 16S rRNA. The data show that CDK18 is closely related to Weissella cibaria. Thus, we renamed CDK18 as W. cibaria K401.

Fig. 3. cLTA significantly induced TNF-α, IL-1β, and IL-8 production compared with untreated cells (Figs. 3B, 3C, and 3D). Unlike whole-cell bacteria, cLTA seems to have a strong immune regulatory effect. Previous studies have shown that pLTA isolated from *L. plantarum* inhibited excessive inflammation caused by LPS [12]. To examine the inhibitory effects of cLTA, LPS-mediated TNF-α production was examined after pretreatment with cLTA. Unexpectedly, cLTA pretreatment did not inhibit LPS-mediated TNF-α production (Fig. 3E). LPS-mediated IL-1β and IL-8 production was also not changed by cLTA pretreatment (data not shown).

**cLTA Increased the Phosphorylation of NF-κB and MAPK in THP-1 Cells**

Previous studies have shown that activation of MAPK and NF-κB signals is important to produce inflammatory cytokines [4, 16, 32]. Thus, to identify the effect of cLTA on signaling activation, western blot assay was performed to measure the activation of ERK, JNK, p38, and NF-κB. As shown in Fig. 4, the level of p-ERK was not changed by LTA. However, the levels of p-JNK, p-p38, and NF-xB were significantly increased by cLTA. Similar results were shown in aLTA-treated cells, whereas pLTA did not alter signaling variation. These results indicate that cLTA induces the production of inflammatory cytokines by activating the JNK, p38, and NF-κB signaling pathways.

**cLTA Induces Inflammatory Cytokines in Mouse Splenocytes**

When mouse splenocytes were treated with cLTA, unlike with THP-1 cells, the secretion of TNF-α from splenocytes was increased by all LTAs used in this study (Fig. 5A), including cLTA. Interestingly, the results of production of TNF-α by aLTA at 100 µg/ml and 10 µg/ml were similar. We did not examine the toxicity of LTA on splenocytes, but it seems that splenocytes were killed by aLTA at 100 µg/ml, because aLTA is toxic to cells and causes shock and organ failure [3]. The secretion of IL-6, like that of TNF-α, was also increased by LTAs (Fig. 5B). The cLTA-mediated
production of IL-6 was higher than that of pLTA, but this was not significant compared with aLTA. IL-1β and IL-12 were not increased by LTA (data not shown).

Next, we examined the inhibitory effects of cLTA on LPS-treated splenocytes. Pretreatment with cLTA did not inhibit the production of TNF-α induced by LPS (Fig. 5C). Like THP-1 cells, it seems that cLTA is involved in the TNF-α pathway, but not in the inhibition pathway. IL-6...
and IL-1β were increased by LPS, but they were not inhibited by any LTA (data not shown).

**Discussion**

Most LAB are probiotics that have many beneficial effects on immune and intestinal regulation. LTA has effects on aging, skin whitening, and modulating inflammatory cytokines [12, 13]. Our study focused on the immune-regulating effect of *W. cibaria*. The effects of live and heat-killed *W. cibaria* on the production of inflammatory cytokines were very weak. However, cLTA showed immune-stimulating effects. Inflammatory cytokines, such as TNF-α, IL-1β, and IL-8, were increased by cLTA, which also activated the phosphorylation of NF-κB and MAPK. These inducing effects by cLTA were shown on THP-1 cells, so we performed these experiments with mouse splenocytes. The inducing effects of cLTA were also shown in mouse splenocytes, and cLTA increased TNF-α and IL-6 in a dose-dependent manner. However, the phosphorylation of ERK was decreased in cLTA-treated THP-1 cells. The activation of MAPK induces different cell signaling. For example, JNK plays a role in T cell differentiation and the cellular apoptosis pathway. The activation of this signaling pathway contributes to inflammatory responses in mammals [8]. P38 MAPK, which is activated by inflammatory cytokines, LPS, ultraviolet light (UV), and growth factors, is involved in

![Fig. 4](image-url). Activation of MAPK and NF-κB signaling by cLTA, pLTA, and aLTA in THP-1 cells. THP-1 cells were cultured with 100 µg/ml LTAs for 1 h. The levels of p-JNK, p-ERK, p-p38, and NF-κBp65 (Cell Signaling Technology, MA, USA) were measured using a western blot assay and normalized to β-actin.

![Fig. 5](image-url). Effects of cLTA, pLTA, and aLTA on the production of cytokines in splenocytes. Pure splenocytes were seeded at 1 × 10⁶ cells in a 96-well round plate. After 1 day, cLTA, pLTA, and aLTA were treated at the indicated concentrations for 6 h. Culture supernatants were collected, and the levels of TNF-α (A) and IL-6 (B) were determined using ELISA. (C) Splenocytes were cultured with the indicated concentrations of LTAs for 18 h and stimulated with 500 ng/ml LPS for 6 h. The supernatants were collected, and TNF-α levels were measured using ELISA. Data are presented as the mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 compared with untreated or LPS-treated only cells.
cell differentiation, apoptosis, and autophagy [7]. ERK is activated by many stimuli, including cytokines, viruses, carcinogens, and growth factors, and is involved in the regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells [2]. Interestingly, αLTA and βLTA did not alter the ERK phosphorylation, whereas γLTA slightly decreased it, indicating that the ERK signaling pathway may not be altered by LTA-TLR2 stimulation. Although the phosphorylation of ERK by γLTA decreased in THP-1 cells, we do not expect that decreased ERK phosphorylation affects cell states, including cytokine production, since cells were activated by other signaling pathways. After stimulation by LTA, the activation of signaling molecules, including NF-κB and MAPKs, begins within 15 min and returns to the steady state in 2 h. The activated signaling molecules induce the transcription and translation of certain genes. The mRNA level of pro-inflammatory cytokines, for example, reaches a peak at 2 to 3 h and the protein level reaches a peak at 3 to 4 h. After reaching a peak, cytokine production is returned to the steady-state level. However, TNF-α production in the bacteria-treated THP-1 cells was maintained up to 24 h. It might be because whole-cell bacteria have plenty of ligands to stimulate immune cells on their surface. In this experiment, induction was not shown in live or heat-killed W. cibaria. Many studies have shown different results between live and heat-killed bacteria on stimulation of the immune response. For example, live L. reuteri has anti-inflammatory effects, but heat-killed and gamma-irradiated bacteria do not have these effects [18]. On the other hand, both live and heat-killed L. paracasei have effects on perennial allergic rhinitis [22]. It seems that the similar effects of live and heat-killed bacteria are due to the cell wall component.

In this study, live and heat-killed W. cibaria showed similar moderate cytokine production, indicating that cell wall components are involved in the immune regulation. In the study using LTA, one of the gram-positive bacteria cell wall components, cLTA, showed cytokine-inducing effects, unlike live and heat-killed W. cibaria. LTA of various bacteria have different immunostimulatory effects [24]. Previous studies have shown that different LTAs have different effects. For example, αLTA increases the production of TNF-α and induces organ failure [3]. LTA isolated from L. sakei (sLTA) induces TNF-α production, but γLTA can inhibit the production of TNF-α induced by LPS [31]. pLTA does not increase TNF-α production and inhibits TNF-α induced by αLTA and LPS [12, 13]. cLTA induced the inflammatory cytokine but did not inhibit the cytokines induced by LPS. This effect seems to be due to structural differences of LTA. For example, Streptococcus pneumoniae R6 and S. pneumoniae Fp23 are different strains of S. pneumoniae that have different structures of LTA and immunostimulatory potencies [5]. αLTA also stimulates an immune response, but has other side effects such as inducing sepsis and other diseases. However, W. cibaria is one LAB that is harmless and well known for its probiotic effects. Thus, it can be used in the treatment of immune-compromised patients. Inflammation is a double-edged sword. Cytokine induction is required for the activation of the immune system, but over-activation leads to the inflammatory diseases. Thus, the fine regulation of inflammation is important to maintain homeostasis. Any components from bacteria can be used for therapies if they are highly purified and do not induce any side effects. Commercially available monophosphoryl lipid A (MPLA), for example, is extracted from lipopolysaccharide (LPS or endotoxin) produced by Escherichia coli or Salmonella Minnesota [1]. MPLA is a TLR4 agonist that is currently used as a vaccine adjuvant in humans, although it is developed from pathogens. Cytokine-inducible LTAs, including cLTA and αLTA may be applied to induce inflammation in immunosuppressed patients, but they should not be applied to patients who have systemic inflammatory response syndrome including sepsis.

In conclusion, this study demonstrated the immune-boosting effects of cLTA. cLTA induces the production of inflammatory cytokines such as TNF-α by activating the phosphorylation of NF-κB and MAPK. These results suggest that cLTA has treatment potential in immune suppression. However, further studies are needed to verify the effect of cLTA on treatment of immunosuppressed patients.

Acknowledgments

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