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Lipoteichoic acid of *Staphylococcus aureus* enhances IL-6 expression in activated human basophils

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ABSTRACT

At allergic inflammation, cross-linking of FcεRI with multivalent antigen-bound IgE triggers the signaling pathways via activation of protein kinase C (PKC) and mobilization of intracellular Ca²⁺ leading to the production of various mediators such as interleukin-6 (IL-6). Accumulating reports demonstrated that interaction of Toll-like receptor 2 (TLR2) expressed on basophils with a TLR2 ligand, lipoteichoic acid (LTA) of *Staphylococcus aureus*, exacerbated allergic inflammation. Here, we showed that staphylococcal LTA (Sa.LTA) substantially enhanced IL-6 expression at both the protein and the mRNA levels in the human basophil line, KU812, in the presence of a PKC activator (phorbol 12-myristate 13-acetate; PMA), and a calcium ionophore (A23187), whereas Sa.LTA alone could not induce IL-6 expression. PMA/A23187 augmented the expression of CD14 and TLR2 on the surface of KU812 cells and concomitantly increased the binding of fluorochrome-labeled Sa.LTA to the cells. Sa.LTA enhanced the phosphorylation of mitogen-activated protein (MAP) kinases in PMA/A23187-stimulated KU812 cells. Notably, Sa.LTA could not enhance PMA/A23187-induced IL-6 expression in the presence of inhibitors of MAP kinases, reactive oxygen species, or protein kinase C. Furthermore, Sa.LTA enhanced the PMA/A23187-increased DNA-binding activities of the transcription factors NF-κB and AP-1. Experiments using human peripheral blood mononuclear cells demonstrated that not only PMA/A23187 but also Sa.LTA increased the intracellular IL-6 expression in the basophil population and Sa.LTA plus PMA/A23187 further enhanced the IL-6 expression. Collectively, these results suggest that Sa.LTA exacerbates allergic inflammation by potentiating IL-6 production from activated basophils.

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1. Introduction

Basophils, which make up a small percentage of circulating leukocytes, are an important effector cell in allergic

inflammation and parasite-associated immune responses [1]. Basophils express large amounts of FcεRI, which is a high-affinity receptor for IgE [2]. Crosslinking of FcεRI with multivalent antigen-bound IgE triggers signaling pathways via activation of protein kinase C (PKC) and mobilization of intracellular Ca²⁺, leading to subsequent secretion of various preformed and newly formed mediators such as proteases, histamines, lipid mediators,

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and proinflammatory cytokines [3]. It is notable that not only antigen-bound IgE, but also infectious materials exacerbate allergic inflammation [4,5]. Furthermore, recent studies have demonstrated that basophils function as antigen-presenting cells during the development of the Th2 immune response [6] and express pattern-recognition receptors such as Toll-like receptor 2 (TLR2) and TLR4 for sensing and responding to pathogen-associated molecular patterns (PAMPs) [7].

One of the best-studied PAMPs is lipopolysaccharide (LPS), which is the major virulence factor of Gram-negative bacteria [8]. LPS has an amphiphilic structure and is recognized by TLR4/MD2, thereby stimulating both MyD88-dependent and MyD88-independent signaling pathways [9]. While Gram-negative bacteria express LPS, Gram-positive bacteria express lipoteichoic acid (LTA), which shares some common structural and functional features with LPS [10]. However, LTA is distinguishable from LPS in that (i) LTA is recognized by TLR2, therefore only activating MyD88-dependent signaling pathways [11], (ii) LTA is necessary but not sufficient to induce sepsis, whereas LPS alone is sufficient [12], and (iii) LTA is secreted at very high levels during bacterial division, but LPS is not [13].

Interleukin (IL)-6 is a pleiotropic cytokine that is involved in inflammatory responses, hematopoiesis, differentiation, activation of lymphocytes, and production of immunoglobulins [14]. IL-6 is able to facilitate IL-4-dependent IgE production [15] and is required for immunological memory response [16]. Enhanced secretion of IL-6 is also observed in inflammatory and autoimmune diseases including sepsis, rheumatoid arthritis, systemic juvenile idiopathic arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriasis, and Crohn's disease [17].

IL-6 is produced by various cell types including activated monocytes, macrophages, B cells, fibroblasts, endothelial cells, and glial cells in response to PAMPs or pro-inflammatory cytokines [18]. Although both transcriptional and post-transcriptional regulation of IL-6 gene expression has been reported [19], expression of the IL-6 gene is mediated mainly through activation of mitogen-activated protein (MAP) kinases such as ERK, JNK, and p38 kinase, and leads to the activation of transcription factors such as NF- κ B and AP-1 [20]. In addition, PKC, reactive oxygen species (ROS), and phosphoinositide 3-kinase (PI3K) signaling cascades are known to be involved in IL-6 gene expression in certain cell types [21,22].

Interestingly, among the various TLR subtypes, human basophils express TLR2 and TLR4 at high levels [7] and TLR2 ligands selectively activate human basophils in both IgE-dependent and IgE-independent manners [23]. Moreover, LTA from *Staphylococcus aureus* was shown to enhance allergen-specific IgE production [24] and allergen-induced dermatitis [25] in an experimental animal model. Thus, we hypothesized that staphylococcal LTA (Sa.LTA), as a major PAMP of bacteria, may exacerbate allergic inflammation by potentiating IL-6 production. To test this hypothesis, we investigated the effect of Sa.LTA on the IL-6 expression in a human basophil cell line, KU812, stimulated with a PKC activator (phorbol 12-myristate 13-acetate; PMA) and calcium ionophore (A23187).

2. Materials and methods

2.1. Reagents and chemicals

PMA, A23187, pyrrolidine dithiocarbamate (PDTC), glutathione (GSH), and catalase were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Inhibitors for MAP kinases, PKC, and PI3K were purchased from Calbiochem (Darmstadt, Germany). All other reagents were obtained from Sigma–Aldrich Chemical Co. unless otherwise indicated.

2.2. Purification of LTAs

LTAs were purified from *S. aureus* ATCC 29213 (American Type Culture Collection, Manassas, VA, USA) and *Lactobacillus plantarum* KCTC 10887BP (Korean Collection for Type Culture, Daejeon, Korea) as described previously [26]. The purified LTAs were not contaminated with biologically active impurities such as endotoxins, nucleic acids, or proteins [26]. The structural intactness of LTAs was confirmed using nuclear magnetic resonance and matrix-assisted laser desorption/ionization-time of flight mass spectrometry [27].

2.3. Cell culture

KU812 cells were purchased from the American Type Culture Collection and maintained in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin at 37°C in a humidified incubator with 5% CO₂. To stimulate KU812 cells, 40 nM of PMA and 1 μ M of A23187 were used in all experiments because these concentrations stimulated the cells maximally (*data not shown*).

2.4. Enzyme-linked immunosorbent assays (ELISA)

KU812 cells were plated at 1×10^6 cells/mL in 96-well plates and cultured for 3 h in the aforementioned media. Then, the cells were stimulated with 40 nM of PMA and/or 1 μ M of A23187 in the presence or the absence of the indicated concentrations of LTA for 24 h. For inhibition studies, the cells were incubated with the indicated inhibitors for 1 h followed by stimulation with PMA/A23187 plus LTA (10 μ g/mL) for an additional 24 h. At the end of the incubation period, the culture media were collected and subjected to cytokine analysis using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Real-time RT-PCR analysis

KU812 cells were plated at 1×10^6 cells/mL in 96-well plates and cultured for 3 h in the aforementioned media. Then, the cells were stimulated with Sa.LTA (0, 1, 3, 10, or 30 μ g/mL) in the presence or the absence of 40 nM PMA plus 1 μ M A23187 for 24 h. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's

instructions. Complementary DNA (cDNA) was synthesized using 5 µg of total RNA with random hexamers and reverse transcriptase (Promega Corporation, Madison, WI, USA). Semi-quantitative real-time PCR was conducted using the ABI Prism® Sequence Detection System 7500 (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex-Taq (Takara Bio Inc., Shiga, Japan). PCR conditions and primer pairs were as follows: denaturation at 95 °C for 1 min and amplification by cycling 40 times at 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 33 s. To determine IL-6 mRNA levels, the IL-6 copy number was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta C_t}$ method, and then the values were compared to that of the control group. The nucleotide sequences for the primers were as follows: IL-6 forward primer, 5'-AACCTGTCCACTGGGCACA-3' and IL-6 reverse primer, 5'-TCTGGCTCTGAACAAAGGA-3'; GAPDH forward primer, 5'-GTGGTGGACCTGACCTGC-3' and GAPDH reverse primer: 5'-TGAGCTTGACAAAGTGGTCG-3'.

2.6. Analysis of TLR2 and CD14 expression using flow cytometry

KU812 cells were seeded at 1×10^6 cells/mL in 6-well plates, cultured for 3 h, and then stimulated with PMA (40 nM) plus A23187 (1 µM) for various time periods. At the end of the incubation periods, the cells were stained with FITC-conjugated monoclonal anti-TLR2 antibodies (Biolegend, San Diego, CA, USA) plus APC-conjugated monoclonal anti-CD14 antibodies (Biolegend) or isotype-matched antibodies (FITC-mouse IgG1κ or APC-mouse IgG2a,κ) (BD Biosciences, San Diego, CA, USA), followed by flow cytometry using FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The data were analyzed further using Flow Jo software (Tree Star, San Carlos, CA, USA).

2.7. Preparation of fluorescent Sa.LTA and analysis of Sa.LTA binding to KU812 cells

Sa.LTA was conjugated with the fluorescent dye, Alexa Fluor 546 (Invitrogen, San Diego, CA, USA), according to the manufacturer's protocol. Briefly, Sa.LTA (1 mg) was incubated with Alexa Fluor 546 reactive dye (30 µg) in 0.1 M sodium bicarbonate (pH 8.3) for 1 h at room temperature by continuous and gentle mixing. Then, the mixture was subjected to size exclusion chromatography using a disposable PD-10 desalting column (GE healthcare, Piscataway, NJ, USA) to separate the conjugated Sa.LTA from free Alexa Fluor 546 dye. The concentration of Alexa Fluor 546-conjugated Sa.LTA was determined as described previously [28]. For the binding assay, KU812 cells were seeded at 1×10^6 cells/mL in 6-well plates, cultured for 3 h, and then stimulated with PMA (40 nM) plus A23187 (1 µM) for 24 h. At the end of the stimulation period, cells were incubated with Alexa Fluor 546-conjugated Sa.LTA for 1 h at 4 °C. After washing with PBS three times, binding of the fluorescent Sa.LTA to KU812 cells was analyzed by flow cytometry as described above.

2.8. Western blot analysis

KU812 cells were stimulated with Sa.LTA (10 µg/mL), PMA/A23187, or Sa.LTA plus PMA/A23187 for the indicated time periods. The cells were then washed twice with ice-cold PBS and lysed with PRO-PREP protein extraction solution (iNtRON Biotechnology Inc., Sungnam, Korea) containing 1 µg/mL each of leupeptin and aprotinin, 2 mM PMSF, and phosphatase inhibitors (2 mM Na_3VO_4 and 50 mM NaF). Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Thirty micrograms of total protein per lane was electrophoresed on 10% SDS-PAGE gels. Then, the gels were electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk for 1 h and then probed with specific antibodies for phosphorylated or non-phosphorylated forms of MAP kinases including ERK, JNK, and p38 kinase, or for β-actin. ECL reagents (Neuronex Co., Pohang, Korea) were used to visualize immunoreactive bands.

2.9. Electrophoretic mobility shift assays (EMSA)

KU812 cells were stimulated with Sa.LTA (10 µg/mL), PMA/A23187, or Sa.LTA plus PMA/A23187 for the indicated time periods. Nuclear extracts were prepared from KU812 cells as described previously [29]. Double-stranded oligonucleotide sequences used for the analysis of NF-κB and AP-1 bindings were as follows (binding sites indicated in italics): NF-κB; 5'-GATCTCAGAGGGGACTTCCGAGAGA-3', AP-1; 5'-GATCTGCATGAGTCAGACACA-3'. To confirm specific binding, each unlabeled oligonucleotide was used at 100-fold excess amount as a 'cold competitor'.

2.10. Analysis of human primary basophils

The experiments were performed under the approval of the Institutional Review Board of the Seoul National University. Peripheral mononuclear cells (PBMC) were isolated from fresh peripheral blood by density-gradient centrifugation using Ficoll-paque (GE healthcare, Uppsala, Sweden). For the analysis of TLR2 expression on the basophils, the PBMC were stained with PE-conjugated anti-CD123 antibodies (Biolegend), APC-conjugated anti-CD203 antibodies (Biolegend), and FITC-conjugated anti-TLR2 antibodies (Biolegend). For the analysis of CD14 expression on the basophils, the cells were stained with PE-conjugated anti-CD123 (Biolegend), FITC-conjugated anti-FcεRI antibodies (eBioscience, San Diego, CA, USA), and APC-conjugated CD14 (Biolegend) antibodies. Flow cytometric analyses were performed as mentioned above. For the analysis of intracellular IL-6 levels in the basophils, the PBMC were plated at 1×10^6 cells/mL in the RPMI 1640 media on a 48-well plate, cultured for 3 h, and then stimulated with Sa.LTA (30 µg/mL) with or without PMA (40 nM) plus A23187 (1 µM) for 24 h. Brefeldin A (5 µg/mL) was treated for the last 6 h of culture. At the end of the incubation period, the cells were harvested and stained with PE-conjugated anti-CD123 antibodies (Biolegend) and FITC-conjugated anti-FcεRI antibodies (eBioscience) to detect basophils. Then, the cells were fixed with PBS

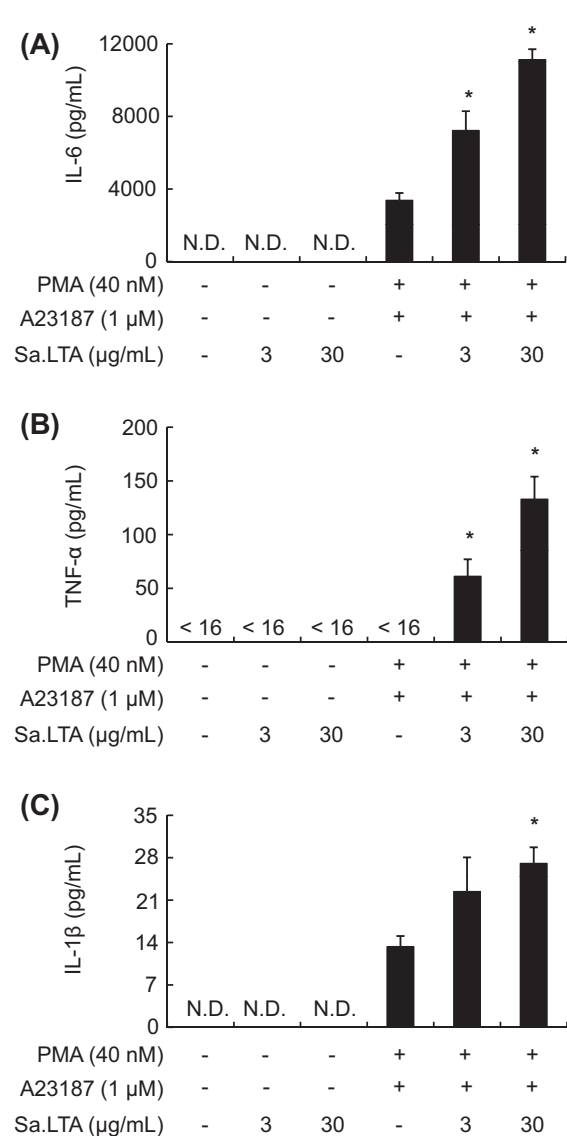


Fig. 1. Staphylococcal LTA (Sa.LTA) potentiates the PMA/A23187-induced secretion of proinflammatory cytokines. KU812 cells were stimulated with 3 or 30 μg/mL of Sa.LTA in the presence or the absence of 40 nM PMA and 1 μM A23187 for 24 h. The levels of IL-6 (A), TNF-α (B), and IL-1β (C) in the culture supernatants were measured using ELISA. Values are expressed as means ± standard deviations of triplicate samples. An asterisk (*) was given to the experimental groups significantly different (at $P < 0.05$) from the control group treated with PMA/A23187 in the absence of Sa.LTA. N.D. means 'not detected'. One of three similar results is shown.

containing 4% of paraformaldehyde for 15 min followed by permeabilization with PBS containing 0.1% of saponin for 15 min on ice. APC-conjugated anti-IL-6 antibodies (Biolegend) were used to stain intracellular IL-6. Flow cytometric analyses were performed as mentioned above.

2.11. Statistical analysis

All experiments were performed at least three times. Data are expressed as mean values ± standard deviations

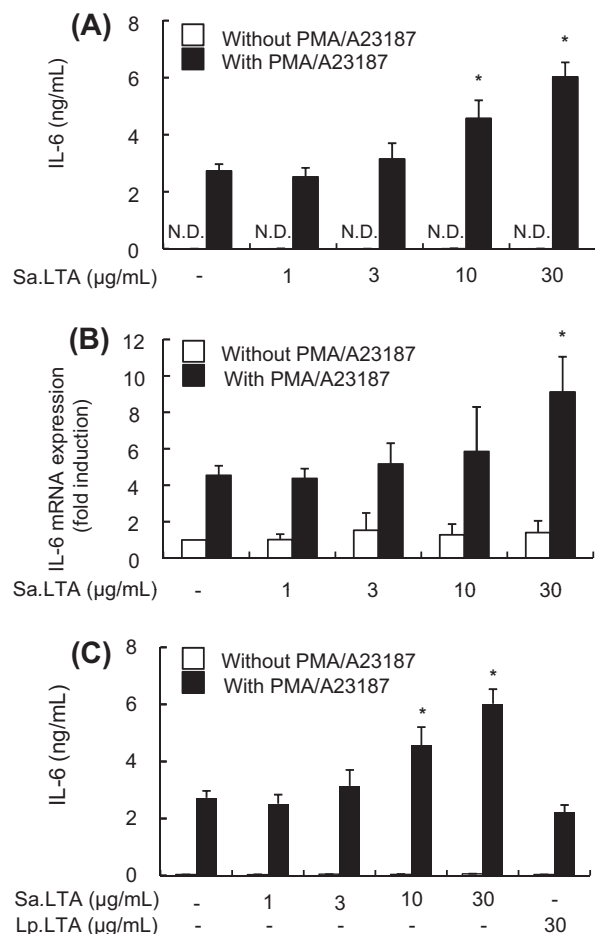


Fig. 2. Sa.LTA augments PMA/A23187-induced IL-6 expression at both the protein and the mRNA levels. KU812 cells were stimulated with Sa.LTA (0, 1, 3, 10, or 30 μg/mL) or *L. plantarum* LTA (Lp.LTA, 30 μg/mL) in the presence or the absence of PMA (40 nM) and A23187 (1 μM) for 24 h. (A, C) The level of IL-6 in the culture supernatants was determined by ELISA. (B) Total RNA was prepared with TRIzol reagent and IL-6 mRNA expression was determined by real-time RT-PCR. The IL-6 copy number was normalized to that of GAPDH, and relative expression was calculated by comparing the ratio of samples to that of the naïve control. Each data point represents the mean ± standard deviation of triplicate samples. An asterisk (*) was given to the experimental groups significantly different (at $P < 0.05$) from the control group treated with PMA/A23187 in the absence of LTA. N.D. means 'not detected'. One of three similar results is shown.

determined from triplicate samples. Statistical analysis was performed using two-tailed *t*-test. Asterisk (*) indicates significant difference by comparing experimental group with the appropriate control group ($P < 0.05$).

3. Results

3.1. Sa.LTA potentiates PMA/A23187-induced production of proinflammatory cytokines

To examine the effect of LTA on the production of proinflammatory cytokines such as IL-6, IL-1β, and

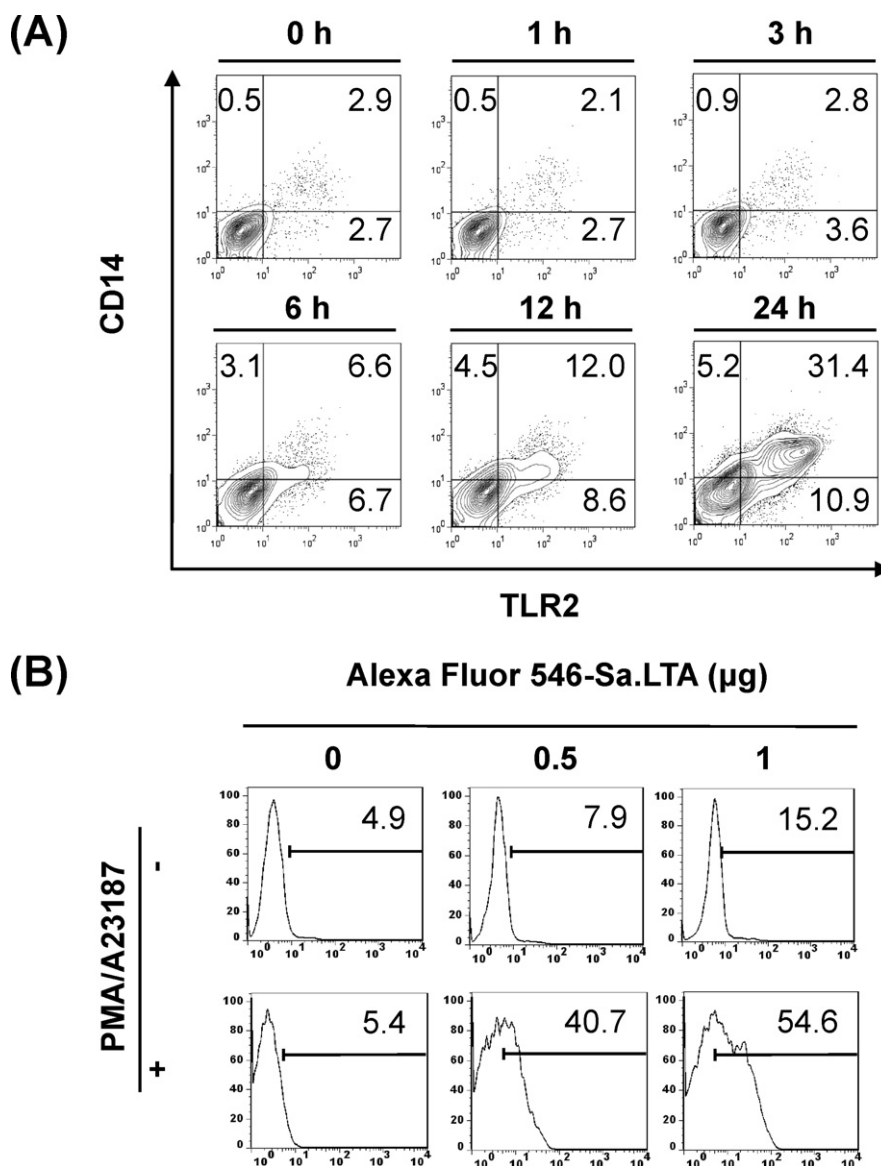
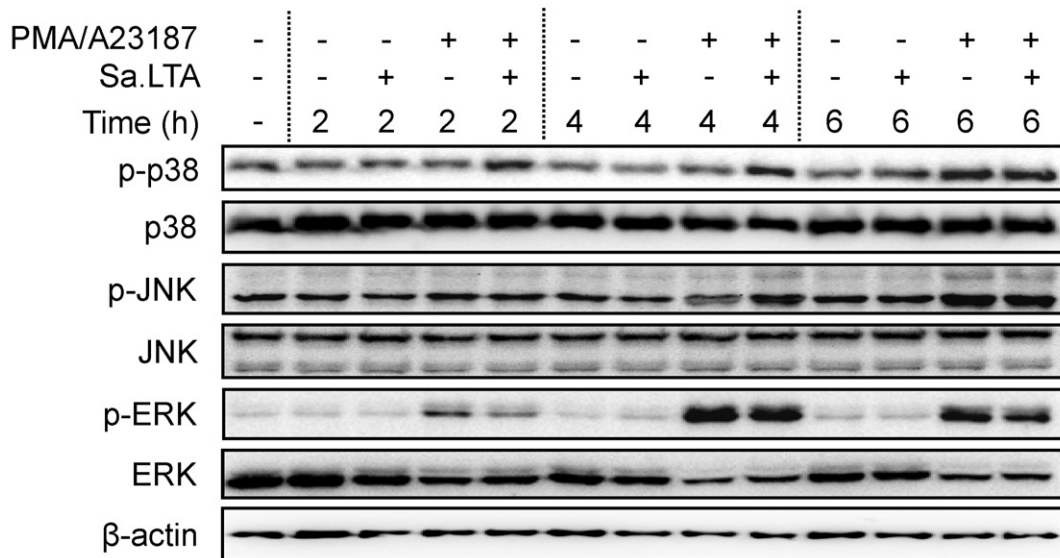
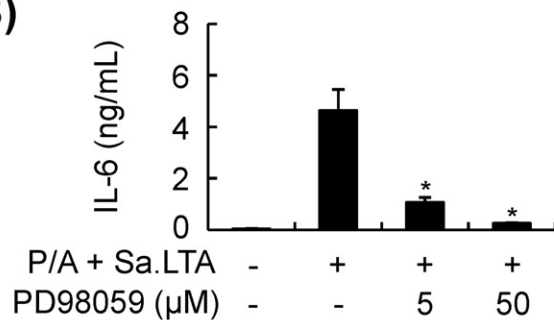
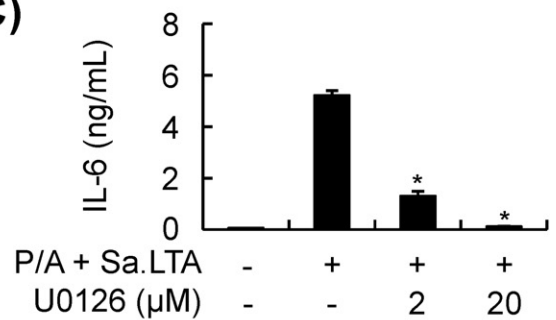
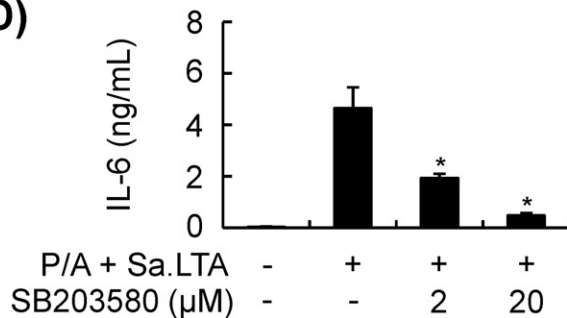
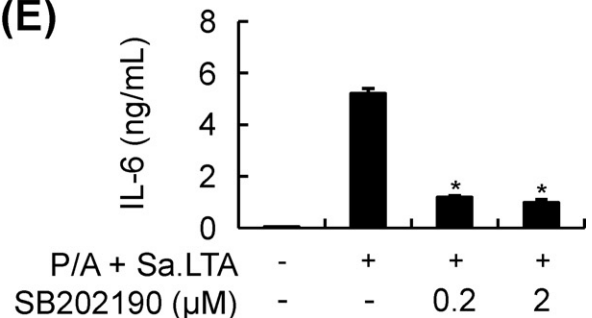
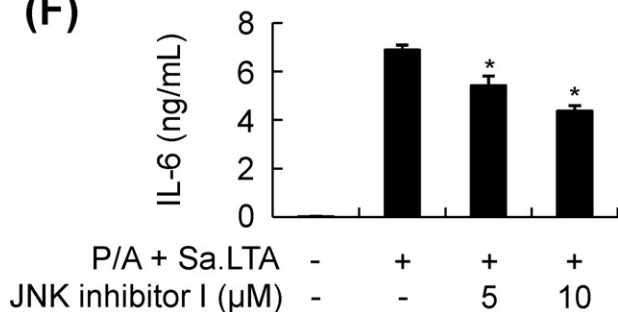
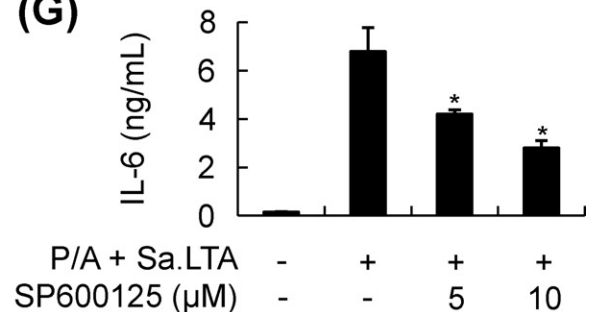


Fig. 3. PMA/A23187 augments the expression of TLR2 and CD14 and Sa.LTA binding to KU812 cells. (A) KU812 cells were stimulated with PMA (40 nM) and A23187 (1 μ M) for the indicated time periods. Then, the cells were stained with FITC-conjugated anti-TLR2 and APC-conjugated anti-CD14 antibodies. The expression of TLR2 and CD14 was analyzed by flow cytometry. (B) KU812 cells were stimulated with PMA (40 nM) and A23187 (1 μ M) for 24 h. At the end of the stimulation period, the cells were incubated with Alexa Fluor 546-conjugated Sa.LTA for 1 h at 4 °C. Binding of Sa.LTA to KU812 cells was analyzed by flow cytometry. One representative result from three similar results is shown.

TNF- α , KU812 cells were stimulated with the indicated concentrations of Sa.LTA (0, 3, or 30 μ g/mL) in the presence or the absence of 40 nM PMA plus 1 μ M A23187. As shown in Fig. 1, Sa.LTA alone did not significantly induce IL-6, IL-1 β , or TNF- α expression. However, Sa.LTA potentiated the PMA/A23187-induced production of all of these cytokines (Fig. 1A–C). Because the greatest increase in expression was observed for IL-6 among the cytokines examined, IL-6 was chosen to further investigate the mechanisms underlying the Sa.LTA-potential of PMA/A23187-induced basophil activation.

3.2. Sa.LTA augments PMA/A23187-induced expression of IL-6 at both the protein and the mRNA levels in a dose-dependent fashion

Because Sa.LTA promoted PMA/A23187-induced IL-6 production, we next examined whether Sa.LTA enhanced PMA/A23187-induced IL-6 production at both the protein and the mRNA levels in a dose-dependent manner. KU812 cells were stimulated with various concentrations of Sa.LTA (0, 1, 3, 10, or 30 μ g/mL) in the presence or the absence of PMA/A23187, and then IL-6 protein and mRNA expressions were measured using ELISA and real-time RT-PCR,

(A)**(B)****(C)****(D)****(E)****(F)****(G)**

respectively. While Sa.LTA alone barely induced IL-6 mRNA and protein expression, Sa.LTA significantly augmented the PMA/A23187-induction of IL-6 expression, at both the protein and the mRNA levels (Fig. 2A and B). These results suggest that Sa.LTA enhances PMA/A23187-induced IL-6 production at both the transcriptional and the translational levels. However, LTA of *L. plantarum* did not enhance IL-6 expression under the same condition (Fig. 2C) implying that LTAs from different Gram-positive bacteria may not show the same effect.

3.3. PMA/A23187 augments the expression of TLR2 and CD14 and Sa.LTA binding to KU812

To investigate how Sa.LTA enhances PMA/A23187-induced IL-6 gene expression, we tested the hypothesis that PMA/A23187 could modulate the expression of TLR2 and CD14, which have been known to be essential for Sa.LTA-mediated signaling pathways [30]. Flow cytometric analysis showed that KU812 cells in a resting state expressed low levels of TLR2 and CD14 on the surface (Fig. 3A). However, treatment with PMA/A23187 markedly increased TLR2 and CD14 expression on KU812 cells in a time-dependent manner (Fig. 3A). Next, we examined whether stimulation of cells with PMA/A23187 enhanced the binding of Sa.LTA to TLR2. When Alexa Fluor 546-labeled Sa.LTA was incubated with non-stimulated KU812 cells or PMA/A23187-stimulated cells, we observed a robust increase in the binding of Sa.LTA to the PMA/A23187-stimulated cells (Fig. 3B). These results suggest that stimulation of KU812 cells with PMA/A23187 enhances their responsiveness to Sa.LTA through an increase in the expression of TLR2 and CD14 on the cell surface.

3.4. Sa.LTA enhances the PMA/A23187-induced phosphorylation of p38 kinase and JNK, but not ERK

Because MAP kinase pathways have been reported to play an essential role in IL-6 expression [20], we investigated whether Sa.LTA could modulate the phosphorylation of MAP kinases in the presence or the absence of PMA/A23187. Sa.LTA alone barely influenced the phosphorylation of all three MAP kinases up to 6 h (Fig. 4). However, when the cells were stimulated with PMA/A23187, increased expression of phosphorylated MAP kinases was detected and in particular, levels of phosphorylated p38 kinases and JNK increased in the presence of Sa.LTA, particularly at 4 h (Fig. 4A). In contrast, levels of PMA/A23187-induced phosphorylated ERK barely changed in the presence or the absence of Sa.LTA (Fig. 4A).

3.5. MAP kinase pathways are essential for PMA/A23187 and Sa.LTA-induced IL-6 production

Next, we investigated the MAP kinase subtypes involved in Sa.LTA enhancement of PMA/A23187-induced IL-6 expression. When KU812 cells were pretreated with inhibitors against ERK (PD98059 and U0126), p38 kinases (SB203580 and SB202190), or JNK (JNK inhibitor I and SP600125), IL-6 production was attenuated in a dose-dependent manner (Fig. 4B–G). These results suggest that MAP kinase pathways are critical for the Sa.LTA enhancement of IL-6 expression induced by treatment of KU812 cells with PMA/A23187.

3.6. ROS and PKC pathways are also important in the Sa.LTA enhancement of PMA/A23187-induced IL-6 expression

To further analyze the mechanisms by which PMA/A23187 and Sa.LTA induce IL-6 production in KU812 cells, various intracellular signaling pathways were examined using specific inhibitors against ROS (PDTC, GSH, and catalase), PKC (calphostin C and staurosporine), and PI3K (LY294002 and wortmannin). All three ROS inhibitors markedly reduced Sa.LTA and PMA/A23187-induced IL-6 expression in a dose-dependent manner (Fig. 5A–C). The PKC inhibitors, calphostin C and staurosporine, marginally inhibited IL-6 production (Fig. 5D and E). In contrast, no significant changes were observed in cells treated with PI3K inhibitors (Fig. 5F and G). These results imply that signaling pathways involving ROS and PKC play a pivotal role in the Sa.LTA enhancement of PMA/A23187-induced IL-6 expression in KU812 cells.

3.7. Sa.LTA enhances the PMA/A23187-induced DNA binding activities of the transcription factors, NF- κ B and AP-1

The transcription factors NF- κ B and AP-1, have been reported to play a crucial role in transcriptional activation of IL-6 [31]. We therefore examined whether Sa.LTA could potentiate the PMA/A23187-induced activation of NF- κ B and AP-1. When KU812 cells were stimulated with Sa.LTA, PMA/A23187, or both Sa.LTA and PMA/A23187, Sa.LTA enhanced the PMA/A23187-induced DNA-binding activities of NF- κ B and AP-1 at 4 h, with maximal binding observed at 6 h (Fig. 6).

3.8. Sa.LTA enhances PMA/A23187-induced IL-6 expression in human primary basophils

In order to confirm the results obtained from KU812 cells whether it is true for human primary basophils, freshly

Fig. 4. Sa.LTA enhances PMA/A23187-induced phosphorylation of MAP kinases and MAP kinase pathways are essential for PMA/A23187- and Sa.LTA-induced IL-6 production. (A) KU812 cells were stimulated with Sa.LTA (10 μ g/mL) in the presence or the absence of PMA (40 nM) and A23187 (1 μ M) for the indicated time periods. At the end of the stimulation periods, the cells were lysed and equal amounts of lysates were subjected to Western blot analysis using antibodies specific to the non-phosphorylated or phosphorylated forms of p38 kinases, JNK, and ERK. KU812 cells were pretreated with various concentrations of specific inhibitors to ERK (B and C), p38 kinases (D and E), or JNK (F and G) for 1 h, and then stimulated with Sa.LTA (10 μ g/mL) in the presence of PMA (40 nM) and A23187 (1 μ M) for an additional 24 h. After the incubation period, cell culture media were collected and IL-6 protein levels were determined by ELISA. Values are expressed as means $\pm$ standard deviations of triplicate samples. Experimental groups that were significantly different from the control group treated with PMA/A23187/Sa.LTA at $P < 0.05$ are indicated with asterisk (*). One of the three similar results is shown.

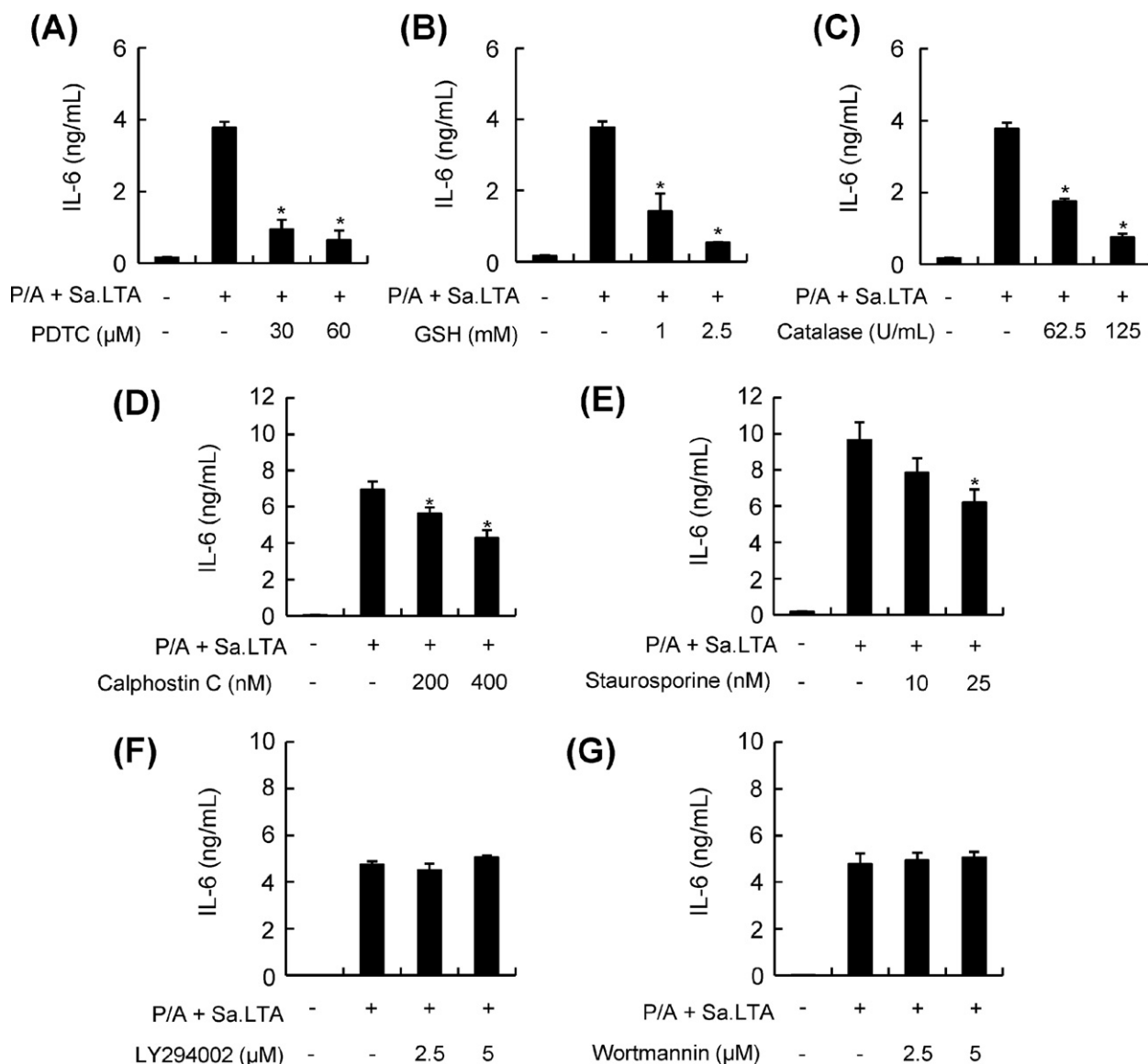


Fig. 5. ROS and PKC pathways are important for the Sa.LTA enhancement of IL-6 expression induced by PMA/A23187. KU812 cells were pretreated with various concentrations of ROS inhibitors, PDTCC (A), GSH (B), and catalase (C), PKC inhibitors, calphostin C (D) and staurosporine (E), and PI3K inhibitors, LY294002 (F) and wortmannin (G) for 1 h. Then, the cells were stimulated with 10 μg/mL of Sa.LTA in the presence of 40 nM of PMA and 1 μM of A23187 for an additional 24 h. After the indicated culture periods, the cell culture media were harvested and IL-6 production was measured using ELISA. Values are expressed as means ± standard deviations of triplicate samples. Experimental groups that were significantly different from the control group treated with PMA/A23187/Sa.LTA at $P < 0.05$ are indicated with asterisk (*). One of three similar results is shown.

isolated PBMC were treated with Sa.LTA in the presence or the absence of PMA/A23187 for 24 h followed by flow cytometric analysis. The cells which express both CD123 and FcεRI were gated and regarded as basophils and then intracellular level of IL-6 was analyzed. As shown in Fig. 7A, not only PMA/A23187 but also Sa.LTA increased the intracellular IL-6 expression in the basophils, and Sa.LTA plus PMA/A23187 further enhanced its expression. The Sa.LTA-induced IL-6 expression, which was not observed in KU812 cells, was associated with higher expression of both TLR2 and CD14 on the primary basophils (Fig. 7B) than on KU812 cells (Fig. 3A).

4. Discussion

Basophils mediate allergic inflammation by producing various inflammatory molecules following cross-linking of allergen-bound IgE and its high affinity receptor FcεRI. Since *S. aureus* is a commensal bacterium which is widely present in human body and also is a notorious opportunistic pathogen, the microorganism and/or its components may be able to affect the severity of allergic inflammation. This hypothesis is further supported by the previous reports demonstrating that Sa.LTA induced Th2-prone dermatitis and enhanced allergen-specific IgE production in

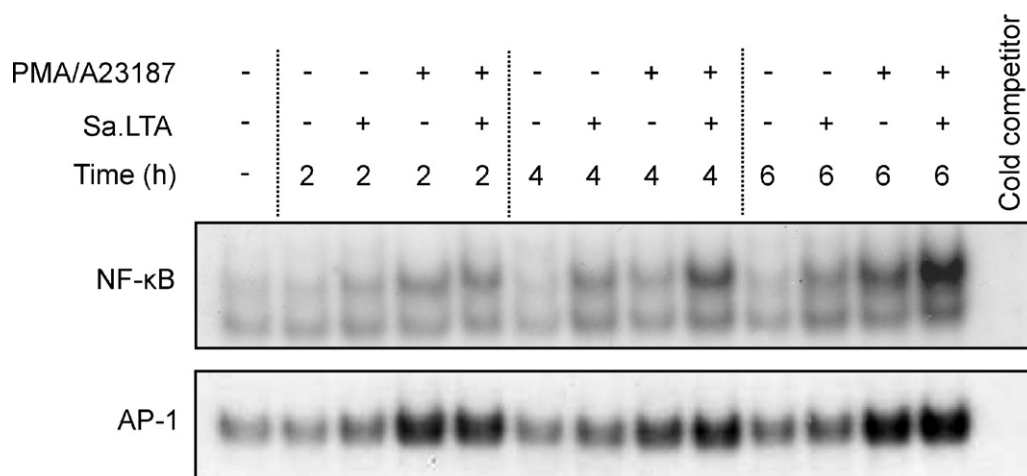


Fig. 6. Sa.LTA enhances the PMA/A23187-induced DNA binding activities of the transcription factors, NF- κ B and AP-1. KU812 cells were treated with 10 μ g/mL of Sa.LTA in the presence or the absence of 40 nM of PMA and 1 μ M of A23187 for the indicated time period. Nuclear extracts were isolated and incubated with ^{32} P-labeled consensus oligonucleotides recognizing NF- κ B and AP-1. To verify specific binding, competition assays were conducted using one picomole of unlabeled probe, marked as 'cold competitor' in the figure. After incubation, reaction products were electrophoresed and autoradiographed. One representative result from three similar results is shown.

mice sensitized with an allergen [24,25]. In agreement with those studies, we showed that Sa.LTA further increased the already considerable amount of IL-6 produced by KU812 cells stimulated with PMA plus A23187. In addition, we demonstrated that the induction of IL-6 is dependent on signaling pathways involving MAP kinases, ROS, and PKC. Furthermore, we found that Sa.LTA can potentiate the DNA-binding activities of the transcription factors, NF- κ B and AP-1, which activate IL-6 gene transcription. These results suggest that Sa.LTA is at least partially involved in the exacerbation of allergic inflammation in response to Gram-positive bacterial infections.

KU812, a human basophilic cell line, has been widely used for allergic experimental setting [32–34]. However, unlike primary basophils, KU812 cells are seldom reactive to allergen-IgE complexes because of low expression of high affinity receptor for IgE, Fc ϵ RI [35]. Thus, in light of the fact that activation of PKC and calcium signaling is known to be indispensable for IgE-mediated allergic inflammation [3], PMA and A23187 instead of allergen-IgE complex were used for the activation of KU812 cells in the current study. Indeed, PMA and A23187 were reported to induce the activation and the differentiation of KU812 cells to mature into basophils and even leading to the expression of IL-6 [32,36,37]. Nevertheless, results from KU812 cells seem to be coincident with those of primary basophils since similar phenomena were observed when the primary cells were used in the present study.

In our study, Sa.LTA alone could not induce the secretion of proinflammatory cytokines such as IL-6, TNF- α , and IL-1 β , but in the presence of PMA and A23187, Sa.LTA significantly enhanced IL-6 production. An increase in intracellular calcium levels and activation of PKC are likely responsible for this potentiation effect because the calcium ionophore A23187 and the PKC activator PMA markedly augmented the surface expression of TLR2 and CD14, both of which are involved in the sense of and response to Sa.LTA, on basophils. In previous studies, PMA plus ionomycin

markedly enhanced TLR2 and TLR4 gene expression in the S49.1 mouse T cell line [38] and in bovine $\gamma\delta$ T cells, PMA plus ionomycin significantly increased the expression of CD36, a scavenger receptor that recognizes Sa.LTA together with TLR2 and CD14, thereby contributing to the recognition of Sa.LTA [39]. Consistent with these findings, we found that stimulation of KU812 cells and primary basophils with PMA/A23187 increased the expression of TLR2 and CD14 on the cell surface, thereby augmenting the adherence of Sa.LTA to the cells and enhancing their responsiveness to Sa.LTA.

We demonstrated that MAP kinases signaling pathways involving ERK, p38 kinases, and JNK play an important role in the Sa.LTA enhancement of PMA/A23187-induced IL-6 production in KU812 cells. The importance of MAP kinases in IL-6 production has been noted in other studies. Consistent with our results, the activation of ERK, p38 kinases, and JNK was shown to be essential for thymic stromal lymphopoietin-induced IL-6 expression [40]. In contrast, TNF- α -induced IL-6 production was shown to be mediated by p38 kinases and ERK pathways in human primary mesangial and proximal tubular cells [41] while p38 kinases and NF- κ B were shown to cooperate to induce IL-6 expression in TNF- α -stimulated primary myocytes [42]. Meanwhile, ERK and JNK pathways are important in IL-1 β -induced IL-6 production in mouse skeletal muscle cells [43], while bacterial flagellin and LPS have been shown to up-regulate IL-6 gene expression through ERK-dependent NF- κ B and AP-1 activation [44]. Despite the crucial role of MAP kinases in IL-6 expression, the differences in the MAP kinase subtypes involved in IL-6 expression reported by the various studies may be due to difference in cell types and stimuli used in the various studies.

ROS also appear to be crucial mediators of the Sa.LTA-enhanced expression of IL-6 in PMA/A23187-stimulated KU812 cells, based on our observation that ROS inhibitors such as PDTC, GSH, and catalase remarkably attenuated the Sa.LTA-enhanced expression of IL-6 in these cells. In

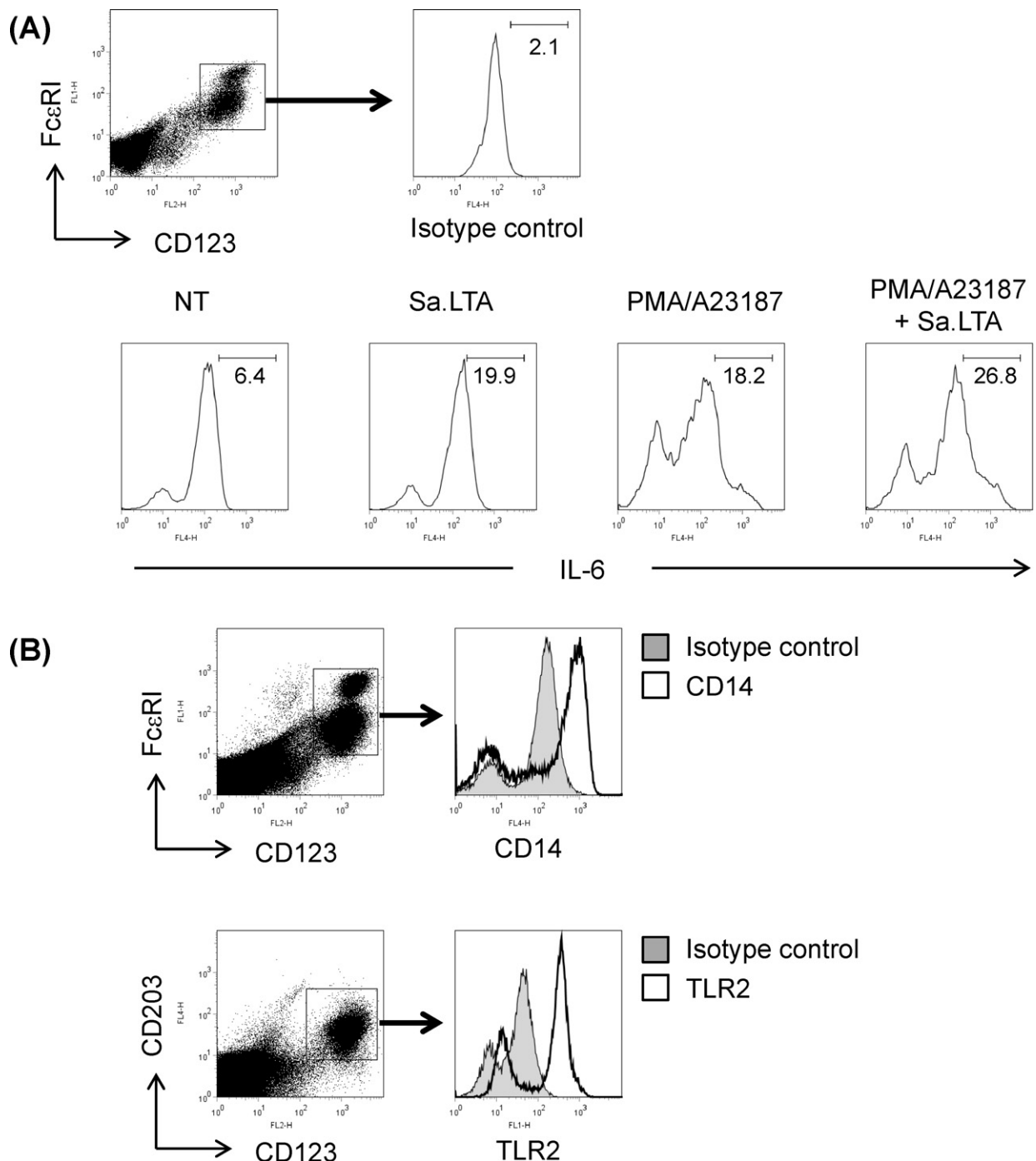


Fig. 7. Sa.LTA enhances PMA/A23187-induced IL-6 expression in human primary basophils. (A) PBMC were stimulated with 30 $\mu\text{g}/\text{mL}$ of Sa.LTA in the presence or the absence of 40 nM of PMA and 1 μM of A23187 for 24 h. The cells were stained with PE-conjugated anti-CD123 antibodies and FITC-conjugated anti-FcεRI antibodies to identify basophils. Then, the cells were fixed, permeabilized, stained with APC-conjugated anti-IL-6 antibodies, and subjected to flow cytometry to measure intracellular IL-6 expression. The result is a representative of three independent experiments. (B) PBMC were stained with PE-conjugated anti-CD123, FITC-conjugated anti-FcεRI antibodies, and APC-conjugated CD14 antibodies to analyze CD14 expression on the basophils (upper panel) or with PE-conjugated anti-CD123 antibodies, APC-conjugated anti-CD203 antibodies, and FITC-conjugated anti-TLR2 antibodies to analyze TLR2 expression on the basophils (lower panel). One of the three similar results is shown.

fact, a number of studies have demonstrated that ROS induced by various stimuli such as asbestos, IL-4, and PMA in human lung epithelial cells, endothelial cells, and alveolar macrophages, play a pivotal role in IL-6 expression

[45–47]. There are various kinds of ROS involved in intracellular signal transduction including hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and the hydroxyl radical ($\cdot\text{HO}$) [48]. Among these molecules, H_2O_2 may be the

major species involved in the Sa.LTA-enhanced expression of IL-6 in PMA/A23187-stimulated cells, because catalase markedly attenuated this induction. Indeed, it has been reported that H₂O₂ is involved in TGF- β 1-induced IL-6 expression since ROS inhibitors significantly attenuated the TGF- β 1-induced induction of IL-6 expression in human lung fibroblast cells [21]. Conversely, treatment with H₂O₂ was sufficient to up-regulate IL-6 expression and its promoter activity in human prostate and cervical cancer cells, leading to the activation of NF- κ B [49]. Thus, H₂O₂ might be an important mediator in intracellular signaling cascades activated in response to PMA/A23187 and Sa.LTA.

Although Sa.LTA could enhance the IL-6 expression in PMA/A23187-activated basophils, not all LTAs are likely to have the same function since *L. plantarum* LTA (Lp.LTA) did not show such effect. In fact, Lp.LTA seems to be less potent than Sa.LTA in the stimulation of innate immunity as demonstrated with macrophage models [26,50]. The distinct activity of two LTAs is due to subtle differences in their molecular structures. Our recent studies using NMR and MALDI-TOF mass spectrometry demonstrated that Lp.LTA has different molecular structures from Sa.LTA: Lp.LTA has three acyl chains in the glycolipid region whereas Sa.LTA has two, saturated acyl chains [27]. Indeed, di-acylated molecules and tri-acylated molecules are differentially recognized by the host [51] and molecules with saturated fatty acid would preferentially target the lipid rafts comprising highly saturated lipids such as sphingolipid and ganglioside which are essential for mounting effective immune responses [52].

Collectively, our results lead us to suggest that Sa.LTA potentiates IL-6 production by basophils through signaling pathways associated with MAP kinases, ROS, and PKC. In view of the fact that (i) human basophils predominantly express TLR2 and TLR4 [7], (ii) TLR2 ligands selectively activate human basophils in both IgE-dependent and IgE-independent manners [23], and (iii) LTA from *S. aureus* enhances allergen-specific IgE production [24] and allergen-induced dermatitis [25] in experimental animal models, the Sa.LTA could possibly exacerbate allergic inflammation, especially in response to Gram-positive bacterial infections.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

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