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Lipoteichoic Acid from *Lactobacillus plantarum* Inhibits the Expression of Platelet-Activating Factor Receptor Induced by *Staphylococcus aureus* Lipoteichoic Acid or *Escherichia coli* Lipopolysaccharide in Human Monocyte-Like Cells

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Platelet-activating factor receptor (PAFR) plays an important role in bacterial infection and inflammation. We examined the effect of the bacterial cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA) from *Lactobacillus plantarum* (pLTA) and *Staphylococcus aureus* (aLTA) on PAFR expression in THP-1, a monocyte-like cell line. LPS and aLTA, but not pLTA, significantly increased PAFR expression, whereas priming with pLTA inhibited LPS-mediated or aLTA-mediated PAFR expression. Expression of Toll-like receptor (TLR) 2 and 4, and CD14 increased with LPS and aLTA treatments, but was inhibited by pLTA pretreatment. Neutralizing antibodies against TLR2, TLR4, and CD14 showed that these receptors were important in LPS-mediated or aLTA-mediated PAFR expression. PAFR expression is mainly regulated by the nuclear factor kappa B signaling pathway. Blocking PAF binding to PAFR using a PAFR inhibitor indicated that LPS-mediated or aLTA-mediated PAF expression affected TNF-α production. In the mouse small intestine, pLTA inhibited PAFR, TLR2, and TLR4 expression that was induced by heat-labile toxin. Our data suggested that pLTA has an anti-inflammatory effect by inhibiting the expression of PAFR that was induced by pathogenic ligands.

Keywords: *Lactobacillus plantarum*, *Staphylococcus aureus*, lipoteichoic acid, platelet-activating factor, Toll-like receptor, monocyte-like cells

Introduction

Platelet-activating factor (PAF) is an endogenous phospholipid that mediates leucocyte functions, platelet aggregation, and inflammatory processes [24]. PAF receptor (PAFR) is a G-protein-coupled receptor that binds PAF. PAFR is expressed in various cells, including monocytes [16]. Increasing evidence indicates that PAFR is a key bacterial adhesion receptor. *In vitro* experiments and animal studies show that blockade of PAFR attenuates bacterial infection, and overexpression of PAFR increases vulnerability to infection [12, 15, 17]. In addition, PAFR is essential for cerebral malaria pathogenesis [26] and graft-versus-host disease in bone marrow transplant patients [3]. Mice lacking PAFR have markedly reduced production of certain pro-inflammatory factors that mediate pulmonary inflammation triggered by *Plasmodium berghei* ANKA infection [25]. Thus, blocking PAFR could be a novel therapeutic approach to treating acute and chronic airway infections.

Lactobacillus plantarum has been used in clinical trials on regulation of the immune system and treatment of gastrointestinal diseases with probiotics [9]. The beneficial and protective properties of probiotic gram-positive bacteria might be due to lipoteichoic acid (LTA) in the cell

wall. LTA is a major pathogen-associated molecular pattern (PAMP) molecule of gram-positive bacteria. LTA mediates innate immune and inflammatory responses [30] similar to the recognition of lipopolysaccharide (LPS) in gram-negative bacterial sepsis [10]. Like LPS, LTA is an amphiphile of a hydrophilic polyphosphate polymer linked to a glycolipid [7]. Toll-like receptor (TLR) 2 is a cognate pattern recognition receptor for LTA ligands in inflammatory responses to gram-positive bacteria [29, 32]. The key modulator in monocyte stimulation is an LTA anchor with two fatty acids and a glycerophosphate backbone with D-alanine substituents [5]. LTAs from different gram-positive species differ in the chemical composition of the repeating units in their polymeric backbone [1, 6, 11]. Recent conflicting reports on the immunological response to LTAs from different pathogenic gram-positive species might be explained by differences in LTA chemical structure [2, 14, 29]. In this study, we examined the effect of bacteria cell wall components on PAFR expression, especially the inhibition of *L. plantarum* LTA (pLTA) on LPS-mediated or *Staphylococcus aureus* LTA (aLTA)-mediated PAFR expression.

Materials and Methods

Materials and Reagents

LTAs were prepared from *L. plantarum* K8 (KCTC10887BP) and *S. aureus* (KCTC1621) as previously described [20]. The LTA purity was determined by measuring protein and endotoxin contents using polyacrylamide gel electrophoresis (PAGE) and conventional silver staining and *Limulus amebocyte* lysate assay (<0.01 EU/ml) (BioWhittaker). DNA and RNA contamination was assessed by measuring UV absorption at 260 and 280 nm. Biochemical inhibitors SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), PD98059 (ERK1/2 inhibitor), IKK inhibitor, NF-κB inhibitor, Akt inhibitor, and wortmannin (PI3K inhibitor) were from Calbiochem (USA). Anti-human TLR2 (clone TL2.1), anti-human TLR4 (clone HAT125), and anti-human CD14 (clone 134620) were from R & D Systems (USA). Antibodies against phospho-IκBα, phospho-p65, β-actin, and PAFR (C-20) were from Santa Cruz Biotechnology (USA). LPS (*Escherichia coli* 055:B5) was from Sigma-Aldrich (USA).

Polymerase Chain Reaction

Total RNA from controls and stimulated THP-1 cells was extracted using the guanidium thiocyanate-acid phenol-chloroform extraction method. cDNA synthesis used the Improm-II Reverse Transcription System (Promega, USA). RT-PCR was in 20 μl containing 5 U Taq polymerase, 1× PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mixture, 10 μM each primer, and 1 μl cDNA. Amplification was 95°C for 10 min, followed by 25 to 35 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The PCR products were separated by 0.8% agarose gel electrophoresis. Forward and reverse primers were 5′-GTCTTC ACCACCATGGAGAA-3′ and 5′-AGTGAGGGTCTCTCTCTTCC-3′ for hGAPDH, and 5′-TCAAGACTGCTCAGGCCAAC-3′ and 5′-GAATTGCCAGGGATCTGGTT-3′ for hPAFR.

To quantify mRNA, real-time PCR was carried out using an ABI Prism 7500 sequence detection system (Applied BioSystems, USA); The PCR products were detected with SYBR. Forward and reverse primers for human mRNA were 5′-AGGAGGCATTGC TGATGATC-3′ and 5′-AGTGAGGGTCTCTCTCTTCC-3′ for hGAPDH; 5′-ACCCTAGGGGAAACATCTCT-3′ and 5′-AGCTCT GTAGATCTGAAGCATC-3′ for hTLR2; 5′-TGAAGAATTCCG ATTAGCAT-3′ and 5′-AATAGTCACACTCACCAGGG-3′ for hTLR4; 5′-AGACCTGTCTGACAATCCTG-3′ and 5′-GACAGA TTGAGGGAGTTCAG-3′ for hCD14; and 5′-TCACCAAGAAGTTCC GCAAG-3′ and 5′-GAATTGCCAGGGATCTGGTT-3′ for hPAFR. Primers for mouse samples were 5′-CTCCCACTCTTCCACCTTCG-3′ and 5′-TAGGGCCTCTCTTGCTCAGT-3′ for mGAPDH; 5′-AACTTCGTACGGAGCGAGTG-3′ and 5′-GGCTTTCCTCTCAAT GGGCT-3′ for mTLR2; 5′-TGGCTGGTTTACACGTCCAT-3′ and 5′-TGCAGAAACATTCGCCAAGC-3′ for mTLR4; and 5′-GGTGAC TTGGCAGTGCTTTG-3′ and 5′-GAAGGGTCACCTGGTCATGG-3′ for mPAFR.

Western Blotting

Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with appropriate primary antibodies. Positive signals were detected using a peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence with Super Signal West Detection Kits (Thermo Chemical Company, USA). Cellular β-actin was used as a protein loading control.

ELISA

After stimulation, cell supernatants were collected and assayed for TNF-α production by a standard sandwich ELISA method. TNF-α ELISA used monoclonal mouse IgG1 (clone 28401) for capture and biotinylated human TNF-α specific polyclonal goat IgG (R & D Systems, USA), followed by streptavidin-HRP for detection. ELISA was developed using *o*-phenylenediamine as a substrate, and the optical density was determined at the wavelength of 450 nm using a 550 nm reference wavelength.

Immunofluorescence

To examine the effect of pLTA on intestinal disease, 300 mg/kg pLTA or PBS was intraperitoneally injected into BALB/c mice (n = 10, 6 weeks old). After 24 h, small intestines were extracted, and heat-labile toxin (LT; Sigma-Aldrich, USA) was injected into the small intestines of mice treated with pLTA or into untreated controls. Tissues extracted from small intestines were embedded in CRYO-OCT compound (Sakura Finetek Europe B.V., The Netherlands) and sectioned to a 3–5 μm thickness. Tissue sections were fixed with cold acetone for 20 min at −20°C and washed with PBS. Sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for

10 min and cooled at room temperature. To inhibit endogenous peroxidase activity, samples were incubated with methanol containing 1% (v/v) hydrogen peroxide for 10 min, and incubated with 10% (v/v) normal goat serum (Dako, Glostrup, Denmark) for 1 h. Immunofluorescence staining used polyclonal antibodies against TLR2, TLR4, PAFR (1:50 dilution), and corresponding secondary antibody labeled with AlexaFluor 488 (Invitrogen, CA, USA). Tissues were examined under a confocal laser scanning microscope using the 488 nm line of the Argon laser. Tissues were digitized at ×40 magnification and images were captured.

Statistical Analysis

Results are expressed as the mean ± SD. Statistical analyses were performed using a two-tailed unpaired Student's t-tests or one-way ANOVA with GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant.

Results

PAFR Regulation by PAMP

To examine the effect of bacterial cell wall components on PAFR expression, THP-1 cells were treated with LPS, aLTA, or the pLTA and PAFR mRNA levels were measured by real-time PCR. PAFR mRNA increased about 7-fold with LPS treatment and about 2.5-fold with aLTA treatment compared with untreated cells. PAFR mRNA decreased moderately with pLTA treatment compared with untreated THP-1 cells (Fig. 1A). The increase in PAFR expression with LPS was dose-dependent (Fig. 1B) and peaked 3 h after LPS treatment before decreasing (Fig. 1C). PAFR mRNA showed a gradual increase with aLTA treatment up to 10 μg/ml, but 100 μg/ml aLTA resulted in less PAFR mRNA compared with cells treated with 10 μg/ml (Fig. 1D). PAFR mRNA mediated by aLTA treatment peaked at 3 h before decreasing (Fig. 1E). Priming with pLTA treatment significantly inhibited the induction of PAFR mRNA (Fig. 1F) and the protein level (Fig. 1G) in LPS- or aLTA-stimulated cells. Priming with pLTA also inhibited PAFR mRNA induction in TNF-α treated cells (Fig. 1H). These data suggested that pathogenic ligands induced PAFR expression, whereas the probiotic ligand pLTA inhibited the PAFR mRNA response to extracellular infection by pathogens and intracellular stimulation by cytokines.

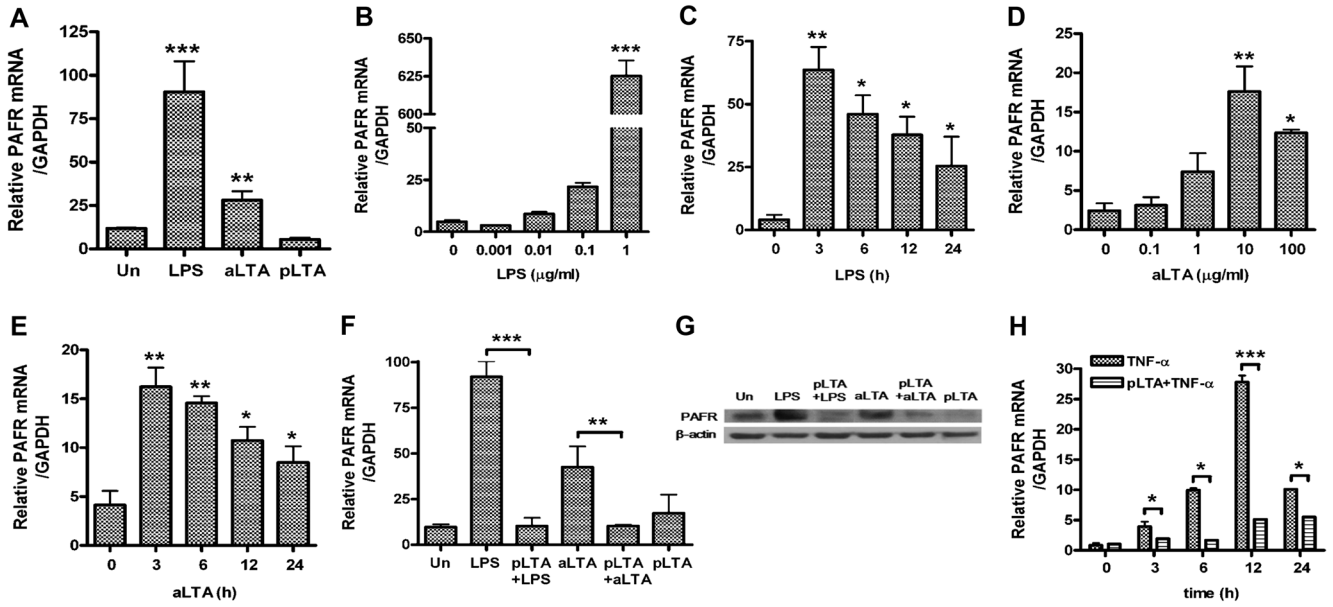

Fig. 1. PAFR regulation by PAMP.

(**A**) THP-1 cells were stimulated with 0.5 μg/ml LPS, 100 μg/ml aLTA, or 100 μg/ml pLTA for 6 h. (**B**) THP-1 cells were treated with the indicated doses of LPS. (**C**) THP-1 cells were incubated with 0.5 μg/ml LPS for the indicated time points. (**D**) THP-1 cells were treated with the indicated doses of aLTA. (**E**) THP-1 cells were treated with 100 μg/ml aLTA for the indicated times. (**F**) THP-1 cells were pre-incubated with 100 μg/ml pLTA for 24 h, and then treated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 6 h. (**G**) THP-1 cells were pre-treated with 100 μg/ml pLTA, then re-treated with 0.5 μg/ml LPS for 24 h. PAFR protein was detected by western blotting. (**H**) THP-1 cells were pre-treated with or without 100 μg/ml pLTA for 24 h, and then treated with 50 ng/ml TNF-α for the indicated time points. PAFR mRNA was measured by real-time PCR and normalized to GAPDH. "Un" indicates untreated cells. *P* was determined using a two-tailed *t*-test: **p* < 0.05; ***p* < 0.01; ****p* < 0.001 compared with untreated cells or cells at the zero time point (**A** to **E**).

TLRs and PAFR Expression

Since LPS and LTA are recognized by TLRs, we examined TLR expression in stimulated THP-1 cells. LPS treatment increased the mRNA of TLR2, TLR4, and CD14 in a dose-dependent manner, although 0.1 μg/ml LPS treatment resulted in a moderate reduction of TLR2 mRNA compared with 0.01 μg/ml LPS treatment (Fig. 2A). TLR2 mRNA peaked at 12 h after LPS stimulation, TLR4 mRNA peaked at 3 h, and CD14 mRNA peaked at 24 h (Fig. 2B). A dose-dependent time course of TLR2, TLR4, and CD14 mRNA was also seen in aLTA-treated cells (Figs. 2C and 2D). Similar to PAFR inhibition by pLTA, TLR mRNA was significantly inhibited by pLTA priming (Fig. 2E). To analyze the effect of TLRs on PAFR expression, neutralizing antibodies against TLR2, TLR4, and CD14 were used to pretreated cells before stimulation with LPS or aLTA. CD14 antibodies strongly inhibited PAFR expression after LPS or aLTA treatment in THP-1 cells, and other neutralizing antibodies slightly inhibited PAFR expression (Fig. 2F, upper panel). Variation of PAFR mRNA expression was analyzed by densitometry scanning (Fig. 2F, lower panel). These results indicated the importance of CD14 in LPS-mediated and aLTA-mediated PAFR mRNA production.

NF-κB-Mediated PAFR Expression

Next, we examined signaling molecules related to PAFR expression after stimulation of THP-1 cells with LPS or aLTA. PAFR expression is regulated by NF-κB in the human monocytic cell line Mono-mac-1 [4]. LPS-mediated or aLTA-mediated PAFR expression was inhibited by NF-κB inhibitor in THP-1 cells (Fig. 3A). Inhibitors of signaling by p38, JNK, ERK, and PI3K did not have an inhibitory effect. Inhibition of PAFR mRNA in LPS-stimulated or aLTA-stimulated cells was dependent on NF-κB inhibitor concentration (Fig. 3B). The variation in NF-κB was examined. Degradation of IκBβ, an NF-κB inhibitor, increased in THP-1 cells stimulated with ligands, but degradation did not occur in pLTA-treated or untreated cells. No difference was seen in IκBβ degradation between cells treated by pLTA priming following re-treatment with LPS or aLTA, and cells treated with LPS or aLTA only. In cells treated with LPS or aLTA, p65 phosphorylation increased, but this

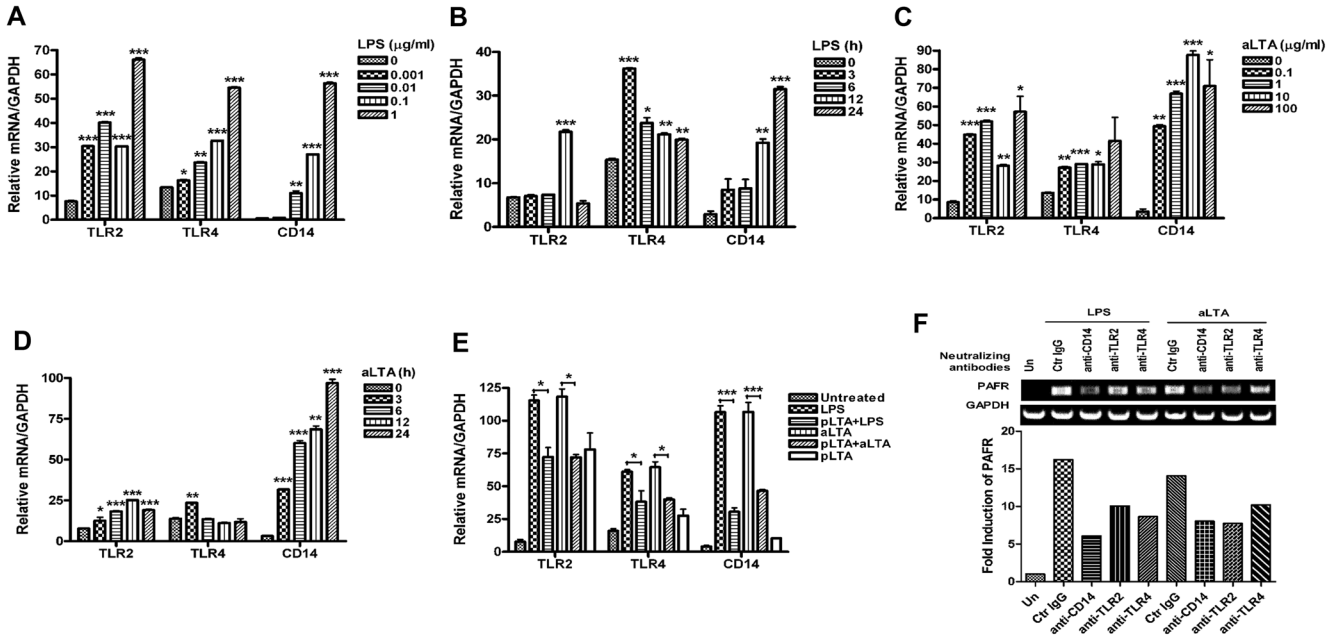

Fig. 2. TLR effects on PAFR expression.

(**A**) THP-1 cells were stimulated with LPS at indicated doses for 24 h. (**B**) THP-1 cells were treated with 0.5 μg/ml LPS for the indicated time points. (**C**) THP-1 cells were stimulated with the indicated doses of aLTA for 6 h. (**D**) THP-1 cells were treated with 100 μg/ml aLTA for the indicated time points. (**E**) THP-1 cells were pre-treated with or without 100 μg/ml pLTA for 24 h, and then treated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 6 h. TLR2, TLR4, and CD14 mRNAs were measured by real-time PCR and normalized to GAPDH (**A** to **E**). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. (**F**) THP-1 cells were pre-incubated with antibodies against CD14, TLR2, or TLR4 or control IgG for 30 min and then incubated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 6 h. PAFR and GAPDH mRNAs were measured by RT-PCR and products were visualized on 0.8% agarose gels (upper panel). Fold induction of PAFR mRNA intensity was analyzed by ImageJ software (lower panel).

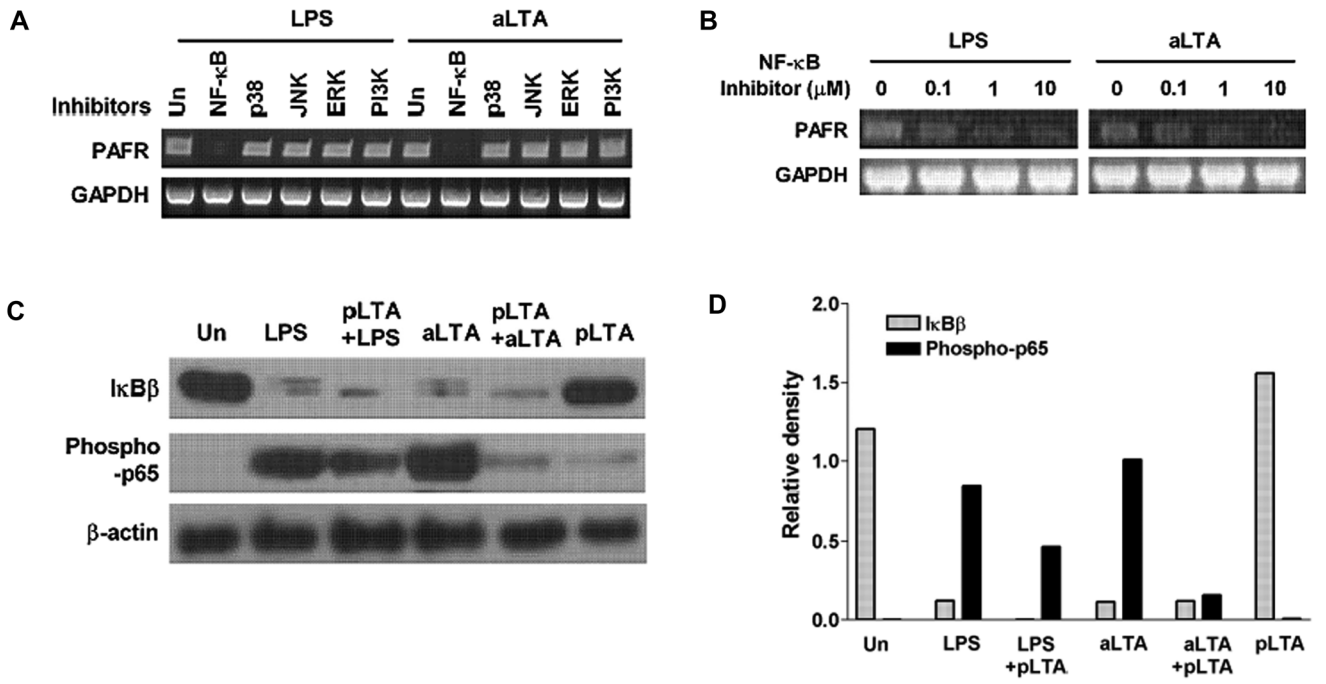

Fig. 3. NF-κB-mediated PAFR expression.
(**A**) THP-1 cells were pre-treated with or without 10 μM of the indicated inhibitor for 30 min, and then incubated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 6 h. (**B**) THP-1 cells were pre-treated with the indicated NF-κB inhibitor for 30 min, and then stimulated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 6 h. PAFR mRNA was measured by RT-PCR and products were visualized on 0.8% agarose gels (**A** and **B**). GAPDH was the internal control. (**C**) THP-1 cells were pre-treated with 100 μg/ml pLTA for 24 h, and then treated with 0.5 μg/ml LPS or 100 μg/ml aLTA for 30 min. Separately, cells were stimulated with 0.5 μg/ml LPS, 100 μg/ml pLTA, or 100 μg/ml aLTA for 30 min. Cells were lysed with 2× reducing sample buffer and used for western blotting. (**D**) Intensity of IκBβ and phosphor-p65 bands from (**C**), normalized to β-actin intensity.

phosphorylation decreased with pLTA priming (Fig. 3C). The variation in phosphorylated p65 was analyzed by densitometry scanning (Fig. 3D). These data suggested that pLTA inhibition of NF-κB signaling was mediated by specific inhibition of p65 phosphorylation, but not by inhibition of IκBβ degradation.

TNF-α Regulation by PAFR Inhibitor

THP-1 cells secrete TNF-α in response to pathogen-associated molecular patterns [21, 22]. As shown in Fig. 4 panel A, aLTA and LPS significantly increased TNF-α secretion from THP-1 cells, whereas pLTA showed mild expression of TNF-α. Previously, we have shown that aLTA- or LPS-mediated TNF-α secretion was significantly inhibited by pLTA [21, 22]. PAF induces TNF-α synthesis in peripheral blood, and PAF-mediated TNF-α production was inhibited by a specific PAF-receptor antagonist, WEB 2170 [8]. To determine the influence of PAF secreted by aLTA or LPS on TNF-α secretion, THP-1 cells were treated with PAFR inhibitor before stimulation with LPS or aLTA. LPS induction of TNF-α was inhibited by 1 or 10 μM PAFR inhibitor (Fig. 4B). PAFR inhibitor also dose-dependently inhibited aLTA-mediated TNF-α production (Fig. 4C). These data indicated that LPS and aLTA increased PAF production in THP-1 cells, and PAF bound to PAFR to induce TNF-α production. Together, these results suggest that PAFR down-regulation by pLTA priming treatment following LPS or aLTA may affect PAF-mediated cytokine production.

PAFR Regulation by pLTA in Mouse Small Intestine

Inhibition of PAFR expression by pLTA was also observed in the mouse intestine. PAFR expression was inhibited in the small intestine of LT-treated mice after pLTA injection, compared with PAFR expression in the small intestine of LT-treated mice injected with PBS. Furthermore, TLR2 and TLR4 expressions were reduced by pLTA treatment (Fig. 5A). The relative intensity of immunofluorescence is shown (Fig. 5B). TLR mRNA in the small intestines of LT-treated mice was lower in pLTA-injected mice compared

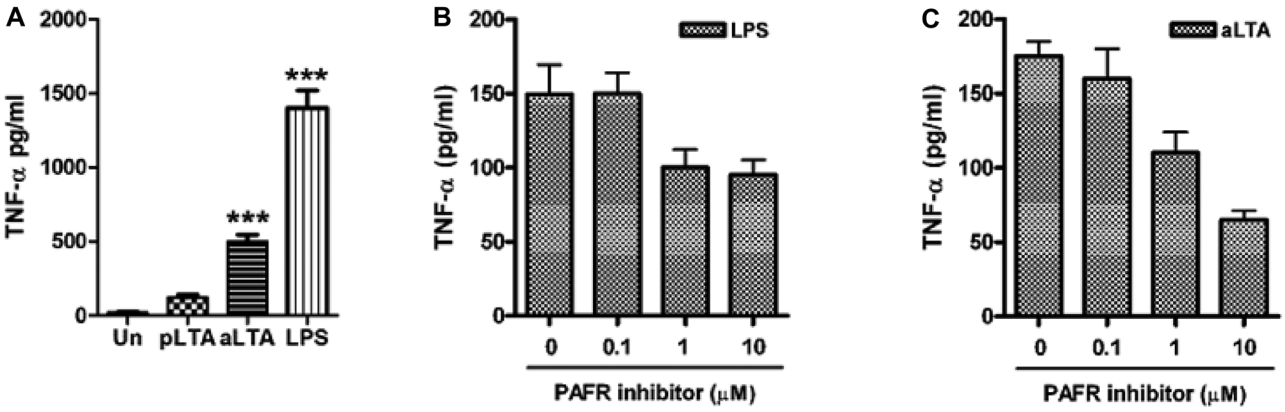

Fig. 4. TNF-α regulation by PAFR inhibitor.
THP-1 cells were stimulated by 100 μg/ml pLTA or aLTA, and 0.5 μg/ml LPS, and then TNF-α secretion was examined by ELISA with culture supernatants (**A**). THP-1 cells were pre-incubated with the indicated PAFR inhibitor for 30 min, and then stimulated with 0.5 μg/ml LPS (**B**) or 100 μg/ml aLTA (**C**) for 24 h. TNF-α in culture supernatants was examined by ELISA. "Un" indicates untreated cells. P was determined using a two-tailed *t*-test: ***$p < 0.001$ compared with untreated cells.

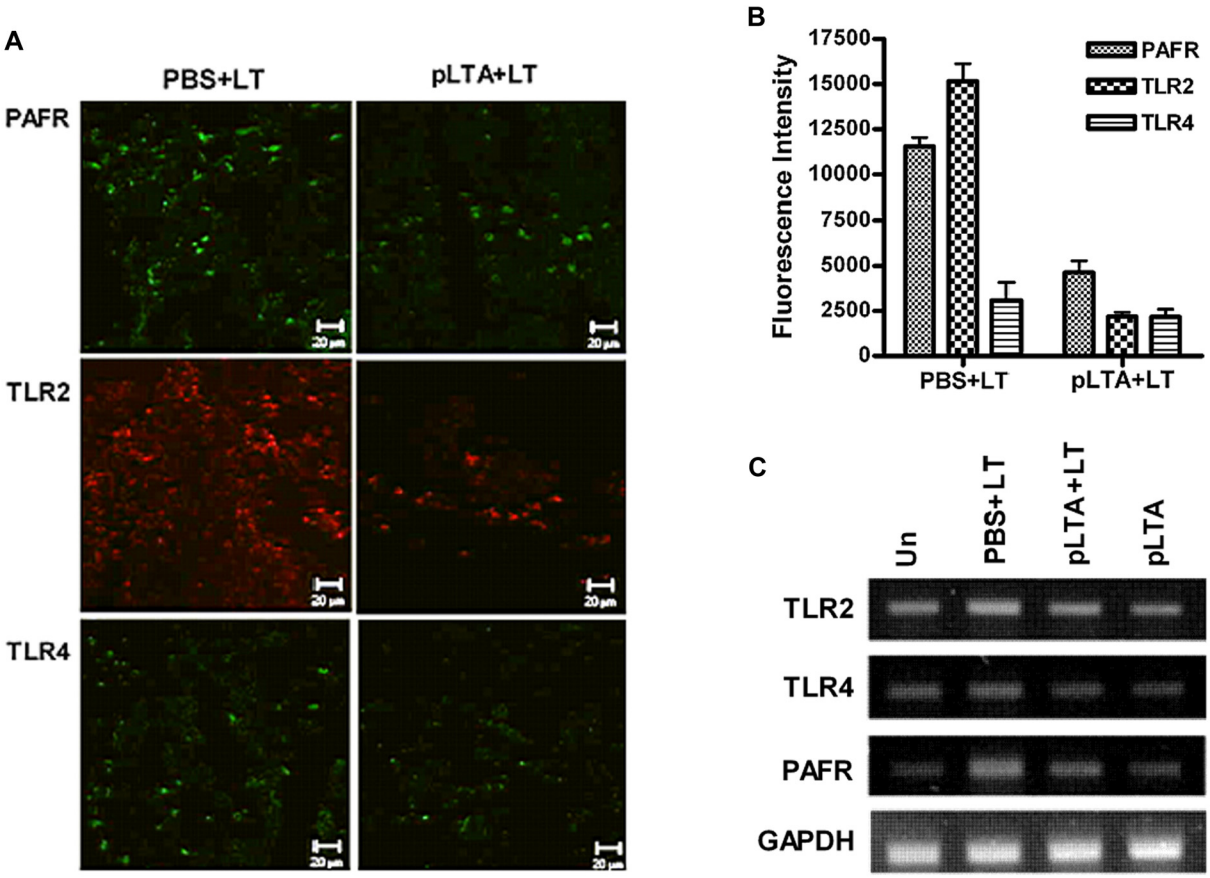

Fig. 5. *In vivo* PAFR regulation by pLTA.
(**A**) Tissue samples from the small intestine of mice injected with pLTA or PBS were treated with LT and stained with antibodies against PAFR, TLR2, or TLR4. (**B**) The fluorescence intensity of receptors was calculated with ImageJ software. (**C**) Small intestine samples were dissolved after TI treatment. After total RNA extraction, cDNA was synthesized and mRNAs for TLR2, TLR4, and PAFR were measured by RT-PCR.

with mice injected with PBS (Fig. 5C). These data suggested that pLTA inhibited inflammatory receptor expression, alleviating PAFR-mediated inflammation.

Discussion

LTA is a major outer cell wall component of gram-positive bacteria that is implicated in the inflammatory response to bacterial infection. pLTA and aLTA have different effects on immune regulation. For example, pLTA significantly induces IL-23p19 mRNA, whereas IL-10 production is moderate; aLTA significantly increases IL-10 production, but not IL-23p19 [20]. This physiological difference in LTA effects might be caused by structural differences in the LTAs [18], which might affect their interactions with TLR2 [19] and subsequent signaling pathways. aLTA increases PAFR expression, but pLTA does not affect the expression of PAFR in THP-1 cells. However, pLTA has shown its inhibitory effect against LPS- or aLTA-mediated PAFR expression. Previously, we have shown that pLTA induces tolerance in inflammatory responses against LPS or aLTA. Priming with pLTA inhibits the NF-κB and MAPK signaling pathways, alleviating mouse septic shock [21, 22]. pLTA also inhibits NF-κB signaling related to PAFR expression. The PAFR gene on chromosome 1 is reported to have distinct transcription initiation sites and promoters that transcribe two mRNAs. Transcript 1 is expressed ubiquitously, especially in a differentiated eosinophilic cell line (Eol-1) and in leukocytes. Transcript 2 expression is tissue specific and transcript 2 is not expressed in leukocytes or in the brain. Transcript 1 has three tandem repeats of NF-κB and SP-1 sites and responds to inflammatory reagents, including PAF, LPS, and phorbol ester [31]. The expression of the human PAFR gene is differentially regulated by estrogen and TGF-β1. In a human stomach cancer cell line, transcript 2 levels increase with estrogen, but decrease with TGF-β1 treatment [28]. Thus, pLTA seems to inhibit the activation of one of the transcription factors involved in PAFR regulation.

PAFR is associated with diverse physiological functions such as melanoma metastasis [27] and lung inflammation [25], and is a bacterial adhesion receptor [12]. These functions suggest that the inhibition of PAFR could alleviate bacterial infection or excessive inflammation caused by infection by a pathogen. LTA and PAFR appear to have a sophisticated relationship. For example, LTA-mediated NO production is inhibited by blocking PAFR. PAFR inhibition also blocks the phosphorylation of JAK2 and STAT1, which are involved in inducing NO synthase. These findings suggest that LTA induces NO production using a PAFR signaling pathway to activate STAT1 *via* Jak2 [13]. Knapp *et al*. [23] suggested that TLR2 is the most important receptor for signaling the presence of LTA in the lungs, and TLR4 and PAFR influence lung inflammation induced by LTA by sensing LTA directly or recognizing LTA and signaling using endogenous mediators induced by interactions between LTA and TLR2. In our study, treatment with LPS or aLTA significantly increased TLR2, TLR4, CD14, and PAFR production. The association or activation of these receptors might increase the inflammatory response *via* activation of the NF-κB and MAPK signaling pathways. Treatment of cells with neutralizing antibodies against TLR2, TLR4, and CD14 decreased aLTA-mediated or LPS-mediated PAFR expression. In contrast, neutralizing antibody against pLTA acted as an antagonist to the responses generated by aLTA and LPS treatment. Priming with pLTA inhibited aLTA-mediated or LPS-mediated receptor expression and PAFR production. PAFR expression was regulated by the NF-κB signaling pathway, and pLTA priming inhibited p65 phosphorylation. In conclusion, our results suggest that pLTA alleviates the inflammatory response by down-regulation of inflammatory receptors, including TLRs and the PAFR and NF-κB signaling pathways.

Acknowledgments

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