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Inhibitory Effects of *Lactobacillus plantarum* Lipoteichoic Acid (LTA) on *Staphylococcus aureus* LTA-Induced Tumor Necrosis Factor-Alpha Production

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***Staphylococcus aureus* is a common etiologic agent for Gram-positive sepsis, and its lipoteichoic acid (LTA) may be important in causing Gram-positive bacterial septic shock. Here, we demonstrate that highly purified LTA (pLTA) isolated from *Lactobacillus plantarum* inhibited *S. aureus* LTA (aLTA)-induced TNF-α production in THP-1 cells. Whereas pLTA scarcely induced TNF-α production, aLTA induced excessive TNF-α production. Interestingly, aLTA-induced TNF-α production was inhibited by pLTA pretreatment. Compared with pLTA, aLTA induced a strong signal transduction through the MyD88, NF-κB, and MAP kinases. This signaling, however, was reduced by a pLTA pretreatment, and resulted in the inhibition of aLTA-induced TNF-α production. Whereas dealanylated LTAs, as well as native LTAs, contributed to TNF-α induction or TNF-α reduction, deacylated LTAs did not, indicating that the acyl chain of LTA played an important role in the LTA-mediated immune regulation. These results suggest that pLTA may act as an antagonist for aLTA, and that an antagonistic pLTA may be a useful agent for suppressing the septic shock caused by Gram-positive bacteria.**

Keywords: *Lactobacillus plantarum*, lipoteichoic acid, tolerance, Gram-positive sepsis

Staphylococcus aureus, recognized worldwide as an important foodborne pathogen, is a major cause of many serious infectious diseases. It has a wide range of virulence factors including enterotoxin genes, which can cause significant morbidity and mortality in renal patients [1, 4, 15]. Recent studies using highly purified LTA prepared from *S. aureus* have shown that staphylococcal LTA can efficiently stimulate monocytes through TLR2 to produce proinflammatory cytokines [7, 18]. Even though inflammation induced by proinflammatory cytokines is helpful in activating the host immune system against invading microbes, excessive inflammation causes severe inflammatory diseases such as septic shock, atherosclerosis, and cancer [22]. Along with *Streptococcus pneumoniae*, *S. aureus* is a common etiologic agent for Gram-positive sepsis, and its LTA is a potent inducer of acute inflammation and may be important in causing septic shock by stimulating TLR2 [6].

To treat septic shock without risks such as antibiotics resistance [10, 17], a tolerance study using bacterial cell wall components has been developed. Several studies have shown that pre-exposure to LPS or LTA induces a transient state of cellular hyporesponsiveness to subsequent LPS stimulation with a reduced production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-8, and enhances protection against endotoxic lethality [2, 12, 16, 19]. The application of those materials for clinical purposes, however, is limited, since the formulation of pathogenic fractions can induce synergistic septic shock [6].

LAB is known as probiotics with immune regulation and the control of pathogenic bacteria in the colon. In particular, *Lb. plantarum* reduces the total blood cholesterol, triglyceride, LDL-cholesterol, and free-cholesterol values, and inhibits the growth and adhesive activity of *H. pylori* [8, 14]. Cell wall components isolated from *Lb. plantarum* induce TNF-α and IL-6 production [11], and its cytoplasm fraction has antitumor activity [13]. Those molecules, however, were mixed with various materials such as LTA, lipoproteins, DNA, and others, so it is difficult to determine the precise signaling mechanism. In the present study, to determine the role of cell wall component on the immune regulation, we prepared highly purified LTA (pLTA) from *Lb. plantarum*, and examined its inhibitory effect against

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the *S. aureus* LTA-induced TNF-α production and signaling mechanism in Gram-positive sepsis.

Materials and Methods

Cell Culture

THP-1, U937, and HEK293 cells were maintained in RPMI 1640 or MEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. THP-1 cells were seeded onto 96-well or 24-well plates. After incubation for 24 h, THP-1 cells were used for stimulation with pLTA and/or aLTA.

Preparation and Modification of LTA

Highly purified LTA was isolated from *Lb. plantarum* (KCTC10887BP) or *S. aureus* by n-butanol extraction, as previously described [9]. Briefly, bacteria were cultured in MRS or BHI broth for 16 h at 37°C. The cells were harvested, suspended in 0.1 M sodium citrate buffer (pH 4.7), and disintegrated by ultrasonication. The cells were then mixed with an equal volume of n-butanol by stirring them for 30 min at room temperature. After centrifugation at 13,000 ×*g* for 20 min, the aqueous phase was evaporated, dialyzed against pyrogen-free water, and equilibrated with 0.1 M sodium acetate buffer containing 15% n-propanol (pH 4.7). The LTA was first purified by hydrophobic interaction chromatography on an octyl-Sepharose CL-4B (Sigma) column (2.5 by 20 cm). The column was eluted with a stepwise n-propanol gradient (100 ml of 20% n-propanol, 200 ml of 35% n-propanol, and 100 ml of 45% n-propanol). Then, the column fractions containing LTA were pooled after an inorganic phosphate assay, and the pool was dialyzed against water. The fractions containing LTA were further subjected to DEAE-Sepharose ion-exchange chromatography (2.5 by 9.5 cm) (Sigma), and equilibrated in the 0.1 M sodium acetate buffer (pH 4.7) containing 30% n-propanol. The column was eluted with 300 ml of a linear salt gradient (0 to 1 M NaCl in the equilibration buffer), and the eluate was collected in 10-ml aliquots. The purity of the purified LTA was determined by measuring the protein and endotoxin contents through the conventional silver staining after polyacrylamide gel electrophoresis and being through the *Limulus* amebocyte lysate (LAL) assay (pLTA<0.028 EU/ml; aLTA<0.042 EU/ml) (BioWhittaker, U.S.A.), respectively. DNA or RNA contamination was assessed by measuring UV absorption at 260 and 280 nm. The modification of LTA from *Lb. plantarum* was performed as previously described with minor modifications [9, 20]. Dealanylation of LTA was achieved by increasing the pH of the water phase after butanol extraction, and by stirring at pH≈8.5 with Tris buffer at RT (21°C) for 24 h. Deacylation of LTA was performed at 37°C for 24 h with 0.1 M NaOH. Both modified LTA fractions were dialyzed against water, and the D-alanine content of LTA was examined through the indirect ELISA method, using the rabbit polyclonal anti-D-alanine antibody (ABcam, U.K.). The completely deacylated LTA was confirmed by MALDI-TOF mass spectrometry according to a previous report [9].

ELISA Assay

After THP-1 cells were stimulated with pLTA and/or aLTA, cell supernatants were collected and assayed for cytokine production by standard sandwich ELISA. The TNF-α production was determined using the monoclonal anti-mouse IgG1 (clone #28401) and the biotinylated human TNF-α specific polyclonal anti-goat IgG for detection (R & D Systems, U.S.A.), according to the manufacturer's instructions.

Real-Time PCR

To quantify the mRNA expression, real-time PCR amplification was carried out using the ABI prism 7000 sequence detection system (Applied BioSystems, U.S.A.), and the PCR products were detected with SYBR Green. The sequences of the forward and reverse primer pairs used were the following: 5'-AGGAGGCATTGCTGATGATC-3' and 5'-AGTGAGGGTCTCTCTCTTCC-3' for GAPDH; 5'-AATGGTGCCATTATGAACTC-3' and 5'-GCTTGCTCTGTCAGC-TTAAT-3' for TLR1; 5'-ACCCTAGGGGAAACATCTCT-3' and 5'-AGCTCTGTAGATCTGAAGCATC-3' for TLR2; 5'-AGACCTGTC-TGACAATCCTG-3' and 5'-GACAGATTGAGGGAGTTCAG-3' for CD14; 5'-TCGACTGAAGTTGTGTGTGT-3' and 5'-AATCATCAG-AGACAACCACC-3' for MyD88; 5'-TCAAGTCAAAGAAGCTG-TCA-3' and 5'-GCATGGGTTTTCTGTTTCTA-3' for TIRAP; 5'-AAGAGAACACCCAGTCACAC-3' and 5'-GTCTTGTCTTACAA-GGCGAC-3' for TRAF6; 5'-ATCTACAAGAAGCACCTGGA-3' and 5'-TCTCTAGCCTCTCGTACACC-3' for IRAK1; and 5'-AATGAT-GCTGATTCCACTTC-3' and 5'-GCTGGTGAACCTTCTTAATG-3' for IRAK4. The expression of the mRNA was normalized with GAPDH.

Transient DNA Transfection and Reporter Assay

To determine the receptor-mediated NF-κB luciferase activity, HEK293 cells were transiently cotransfected with pNF-κB-Luc, pRL-SV40 and pCMV-Tag2A, pCMV/TLR2, or pCMV/CD14 using WelFec-EX (JBI, Korea), according to the manufacturer's manual. To determine the NF-κB luciferase activity induced by the treatment of pLTA and/or aLTA, U937 cells were cotransfected with pNF-κB-Luc and pRL-SV40. After 24 h, transfectants were used for the examination of NF-κB luciferase activity by pLTA and/or aLTA treatment. Cells were harvested, lysed, and the cell lysates were assayed for firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega, U.S.A.) on an el 800 (Bio-Tek instruments, Inc. U.S.A.). The firefly luciferase activity of the individual transfectants was normalized against the *Renilla* luciferase activity.

Western Blot Analysis

Twenty μg of total cellular protein was added to a Laemmli buffer, boiled for 5 min, resolved by 12% SDS-PAGE in a Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and blotted onto nitrocellulose membranes (100 V, 1.5 h, 4°C). After blocking for 1 h in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk, the membranes were washed three times in TBS-T and probed for 2 h with anti-phospho-MAP kinase antibodies (R & D Systems, U.S.A.) in TBS-T/0.5% nonfat milk. Following washing three times in TBS-T, the membranes were incubated with secondary HRP-conjugated donkey anti-rabbit Ig or sheep anti-mouse Ig for 2 h and washed five times in TBS-T; bands were detected using enhanced chemiluminescence (ECL) reagents (GE Healthcare, U.K.), according to the manufacturer's description.

Immunofluorescence Staining

THP-1 cells were grown on cover slips. The cells were fixed with 4% paraformaldehyde following pLTA or aLTA treatment. The cells

were washed with PBS and incubated with monoclonal anti-LTA (clone BGN/1140/2G10; Biogenesis, U.K.) for 90 min, and then washed and incubated with FITC-conjugated anti-rabbit antibodies (Santa Cruz Biotechnology, U.S.A.) for 60 min. The cover slides were washed with PBS, mounted, and examined using a confocal microscope.

Statistical Analysis

All of the experiments were performed at least three times. The data shown are representative results of the means ± the standard deviation of triplicate experiments. Differences were judged to be statistically significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

pLTA Inhibited aLTA-Induced TNF-α Production

When THP-1 cells were treated with various concentrations of *S. aureus* LTA (aLTA) for 6 h, TNF-α production was displayed in a dose-response manner, which peaked in response to 50 μg/ml of aLTA. THP-1 cells incubated with pLTA, however, scarcely induced TNF-α production compared with aLTA-treated cells (Fig. 1A). Interestingly, THP-1 cells pretreated with 100 μg/ml of pLTA followed by restimulation with 50 μg/ml of aLTA significantly inhibited aLTA-induced TNF-α production (Fig. 1B). The inhibitory efficiency of pLTA gradually increased with increasing pLTA pretreatment times (Fig. 1C). In contrast, post-treatment with pLTA for 1 to 4 h following 50 μg/ml of aLTA did not reduce the aLTA-induced TNF-α production.

pLTA-Induced Tolerance Required TLR2 and CD14

To determine the expression level of pattern recognition receptors (PRRs) by pLTA or aLTA, THP-1 cells were treated with pLTA or aLTA, and the expression level was determined by a real-time PCR method. Cells treated with aLTA induced more TLR2 and CD14 expression than pLTA-treated cells ($p<0.05$), whereas pLTA showed a moderate induction of those receptors. The expression of those receptors, however, was highly induced by both pLTA- and aLTA-cotreatment compared with pLTA or aLTA treatment only (TLR2, $p<0.05$; CD14, $p<0.01$) (Fig. 2A). These results indicate that LTA isolated from pathogens is more inducible on the immune cell activation than that isolated from lactic acid bacteria, and PRRs such as TLR2 and CD14 may be important in LTA-mediated signaling. To examine the role of TLR2 and CD14, we tested the NF-κB luciferase activity. The NF-κB luciferase activity was highly induced in response to aLTA after the cotransfection of both TLR2 and CD14 into HEK293 cells, whereas cells transfected with either TLR2 or CD14 moderately induced NF-κB luciferase activity in response to pLTA or aLTA (Fig. 2B). In addition, the inhibition of aLTA-induced NF-κB luciferase activity by pLTA pretreatment was only shown in cotransfected HEK293 cells with TLR2 and CD14, indicating that those receptors are essential to induce pLTA-mediated tolerance. In general, aLTA induces TNF-α production through the TLR2 and CD14 complex [9, 18], and our results show that pLTA also responds to the TLR2 and CD14 complex. Compared with pLTA, however, aLTA induced a strong signal transduction, suggesting that aLTA may have a higher affinity for TLR2 and CD14.

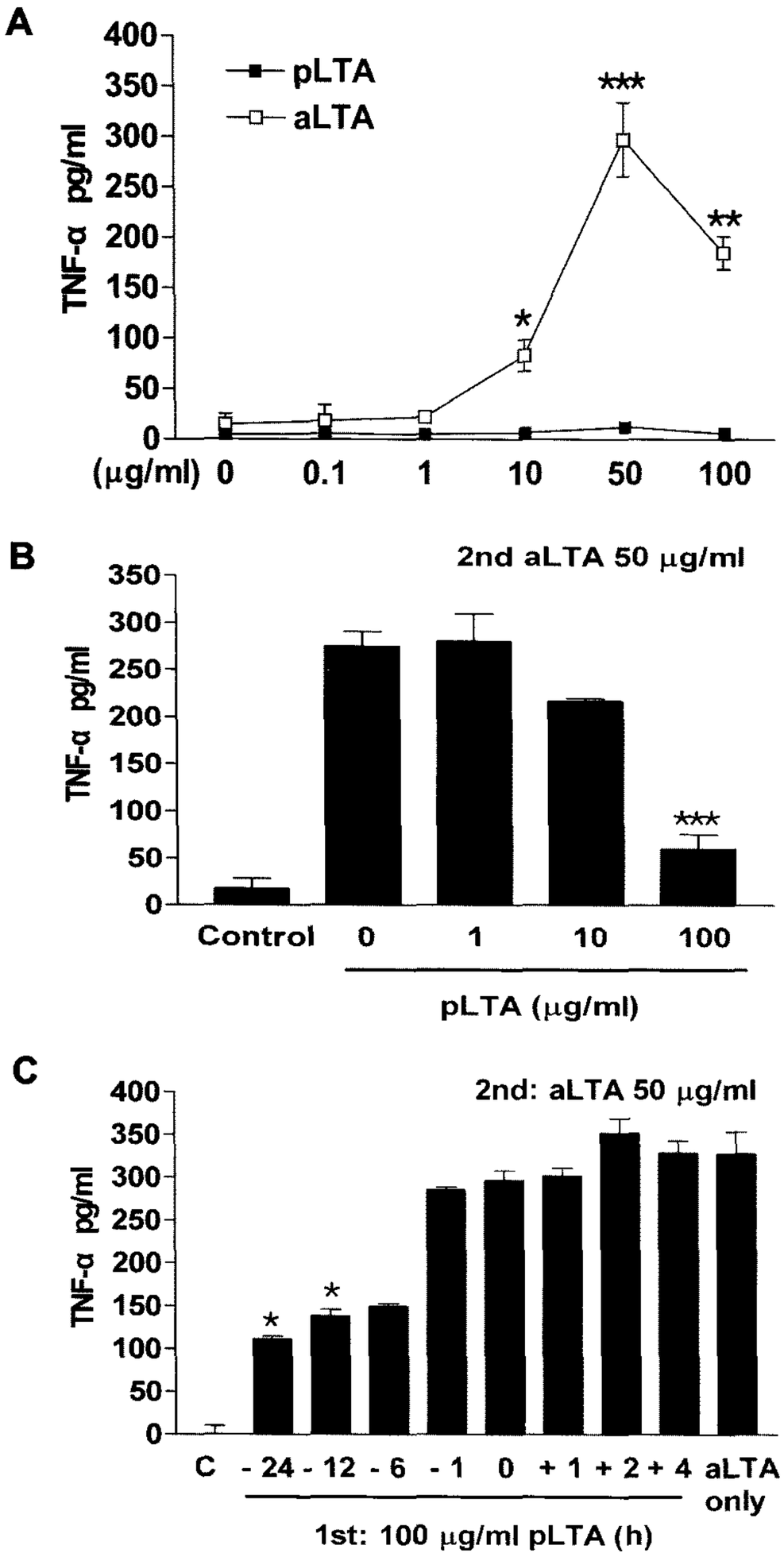

Fig. 1. The effect of pLTA and aLTA on THP-1 cells.

A. THP-1 cells were treated with pLTA or aLTA at the indicated concentrations for 6 h. **B**. THP-1 cells were pretreated with pLTA at the indicated concentrations for 20 h followed by restimulation with 50 μg/ml of aLTA for 6 h. **C**. THP-1 cells were pretreated with 100 μg/ml of pLTA for the indicated times, and then restimulated with 50 μg/ml of aLTA for 6 h. In all of the experiments, the amount of TNF-α was estimated by ELISA from the culture supernatants, and the data are presented as means±standard deviation. *$p<0.05$; **$p<0.01$; ***$p<0.001$ as compared with the pLTA-treated samples (**A**) or aLTA treatment only (**B**).

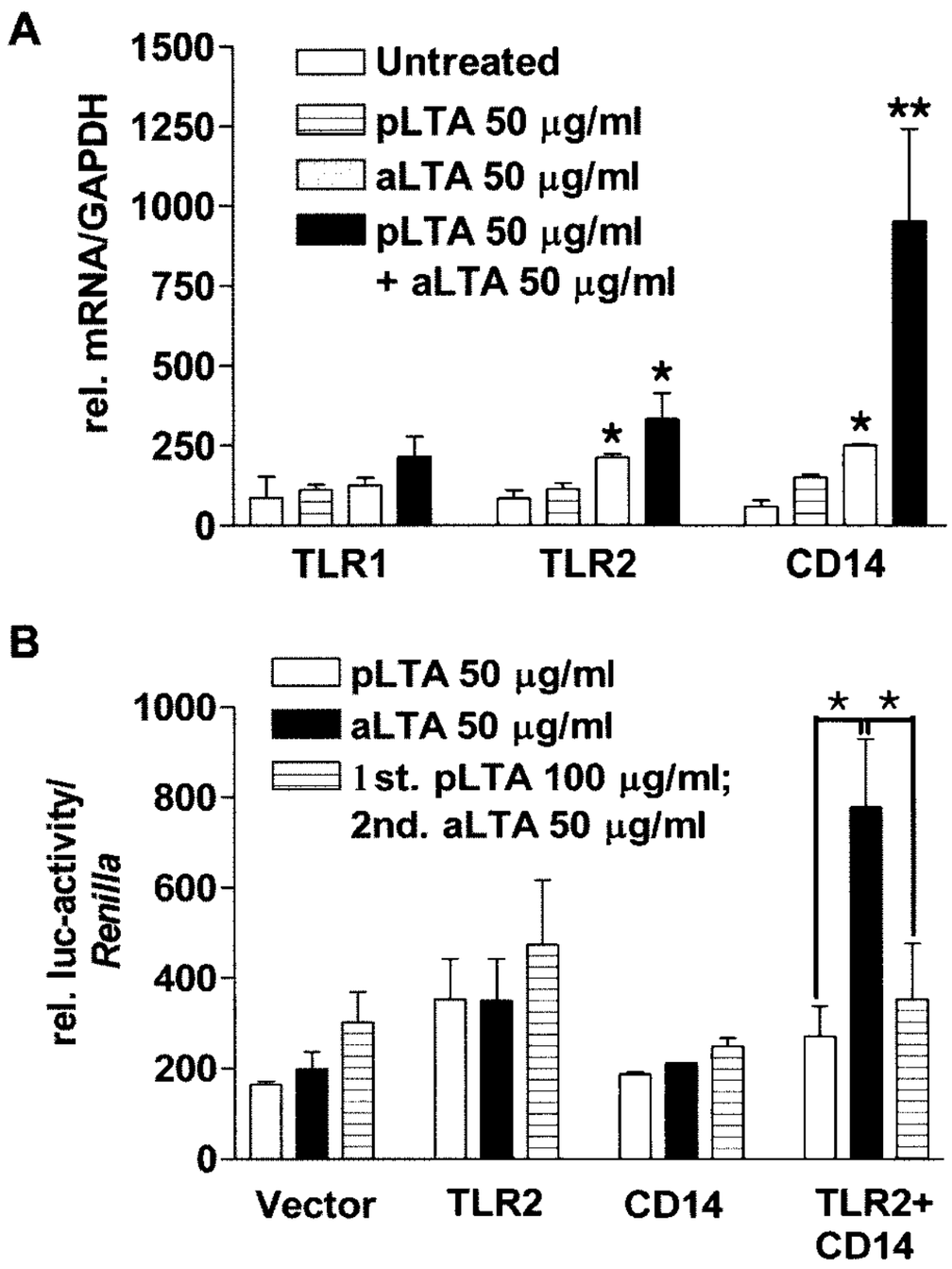

Fig. 2. LTA required TLR2 and CD14 for immune regulation.
A. THP-1 cells were treated with 50 μg/ml of pLTA and/or aLTA, and then the mRNA level of the PRRs was determined by real-time PCR. **B**. HEK293 cells were transiently cotransfected with pNF-κB-luc, pRL-SV40, pCMV-Tag2A, pCMV/TLR2, and/or pCMV/CD14. Transfectants were prestimulated with or without 100 μg/ml of pLTA for 20 h, and then restimulated with 50 μg/ml of pLTA or aLTA for 18 h. The cells were lysed, and the luciferase activity was measured. Data are expressed as the mean±standard deviation and normalized for GAPDH (**A**) or *Renilla* luciferase activity (**B**). *$p<0.05$; **$p<0.01$.

aLTA Induced a Strong Signal Transduction, and pLTA Downregulated the aLTA-Mediated Signal Transduction

To determine the difference between pLTA and aLTA in the signal transduction, we examined the expression level of signaling components including the TLR adaptor molecule MyD88. The expression of MyD88 was significantly induced by aLTA, but it was reduced by pLTA pretreatment followed by aLTA. Other signaling molecules, such as TIRAP, TRAF6, IRAK1, and IRAK4, were induced moderately (Fig. 3A). These results suggest that MyD88 played an important role in the pLTA-mediated tolerance as well as in the LTA-induced signal transduction.

To examine whether pLTA tolerance affects aLTA-induced signal transduction, we estimated the NF-κB luciferase activity in U937 cells after transiently cotransfecting them with pNF-κB-Luc and pRL-SV40 vectors followed by the treatment of pLTA and/or aLTA. The NF-κB luciferase activity was highly induced by the aLTA treatment, whereas

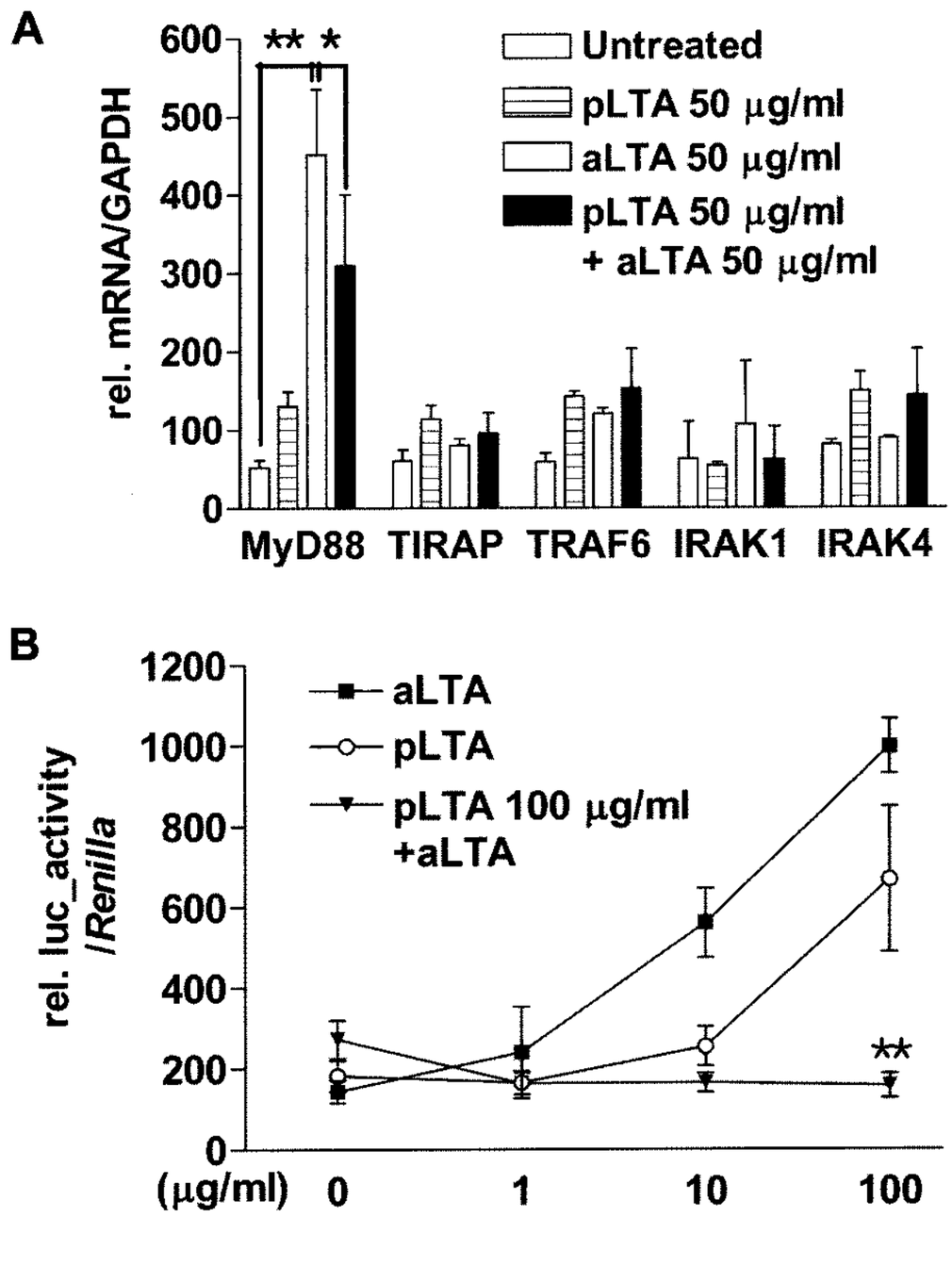

Fig. 3. aLTA induced the activation of signaling molecules that were inhibited by a pLTA pretreatment.
A. THP-1 cells were treated with 50 μg/ml of pLTA and/or aLTA, and then the mRNA level of TLR-mediated molecules was determined by real-time PCR. Data are normalized for GAPDH. *$p<0.05$; **$p<0.01$. **B**. U937 cells were transiently cotransfected with pNF-κB-luc and pRL-SV40. Transfectants were prestimulated with or without 100 μg/ml of pLTA for 20 h, and then restimulated with 50 μg/ml of pLTA or aLTA for 18 h. The cells were lysed, and the luciferase activity was measured. Data are normalized for *Renilla* activity. **$p<0.01$ compared with aLTA alone. **C**. THP-1 cells were pretreated with either the medium or 100 μg/ml of pLTA for 20 h, and then restimulated with 50 μg/ml of pLTA or aLTA for the indicated times (min). The cell lysates were blotted with phospho-specific antibodies for ERK1/2, p38, and JNK1/2. To verify the amount of loaded protein, they were also probed with β-actin. Data are expressed as the mean±standard deviation (**A** and **B**).

pLTA showed less induction of NF-κB luciferase activity. The aLTA-induced NF-κB luciferase activity, however, was significantly inhibited after pLTA pretreatment (Fig. 3B).

aLTA-mediated phosphorylation of ERK1/2, JNK1/2, and p38 was evident after 90 min, and the phosphorylation of ERK1/2 was induced most strongly. Whereas the pLTA treatment scarcely induced the phosphorylation of the three MAP kinases, that phosphorylation was significantly inhibited in pLTA-pretreated cells following a 60 min restimulation with 50 μg/ml of aLTA (Fig. 3C).

Previous reports have shown that the lipid A analogs E5531 and DY-9973 inhibited excessive TNF-α production induced by LPS in human and mice, respectively [3, 23]. However, it is not reported that those molecules can inhibit Gram-positive sepsis. Unlike lipid A analogs, pLTA inhibited aLTA-induced TNF-α production (Fig. 1B), suggesting that pLTA tolerance may have a different signaling mechanism with lipid A analogs. It is reported that the inhibition of Gram-negative sepsis by LPS-containing lipid A requires the downregulation of signal transduction such as NF-κB and MAP kinases [19]. In the present study, we showed that aLTA induced signal transduction through the MAP kinases, especially ERK1/2, as well as NF-κB activation, and might have resulted in the induction of TNF-α production. Additionally, our results suggest that pLTA tolerance against Gram-positive sepsis may be led by the inhibition of signal transduction caused by cell wall components of Gram-positive bacteria.

An Acyl Chain was Essential for the aLTA-Induced TNF-α Production and pLTA-Induced Tolerance

A previous report has shown that deacylated aLTA did not induce a signal transduction through TLR2 [9, 21], and another report showed that the D-alanine (D-ala) content of LTA plays an important role in the TNF-α production [20]. We tried to determine whether structural differences in LTA affect aLTA-induced TNF-α production and pLTA-induced tolerance. Both pLTA and aLTA were treated with Tris-HCl pH 8.5 buffer to delete D-ala and 0.1 M NaOH to delete the fatty acid chain from LTA according to the previous protocols [20]. Like native aLTA, dealanylated aLTA significantly induced TNF-α production, but deacylated aLTA failed to induce TNF-α (Fig. 4A). In addition, the phosphorylation of ERK1/2 was induced by not only native aLTA but also dealanylated aLTA, whereas it was not induced by deacylated aLTA (Fig. 4B). These results suggest that the acyl group, not the D-ala content, of aLTA was essential for inducing TNF-α production. Next, we tested whether modified pLTAs affect the inhibition of aLTA-induced TNF-α production. THP-1 cells were treated with native or modified pLTA for 20 h, and then restimulated with 50 μg/ml of aLTA. Whereas dealanylated pLTA was as effective as native pLTA in suppressing TNF-α production, deacylated pLTA was not suppressive (Fig. 4C). This suggested that the acyl chain of LTA was important for inducing tolerance.

Inflammation is the first response to infection and injury and is critical for body defenses. Basically, the inflammatory

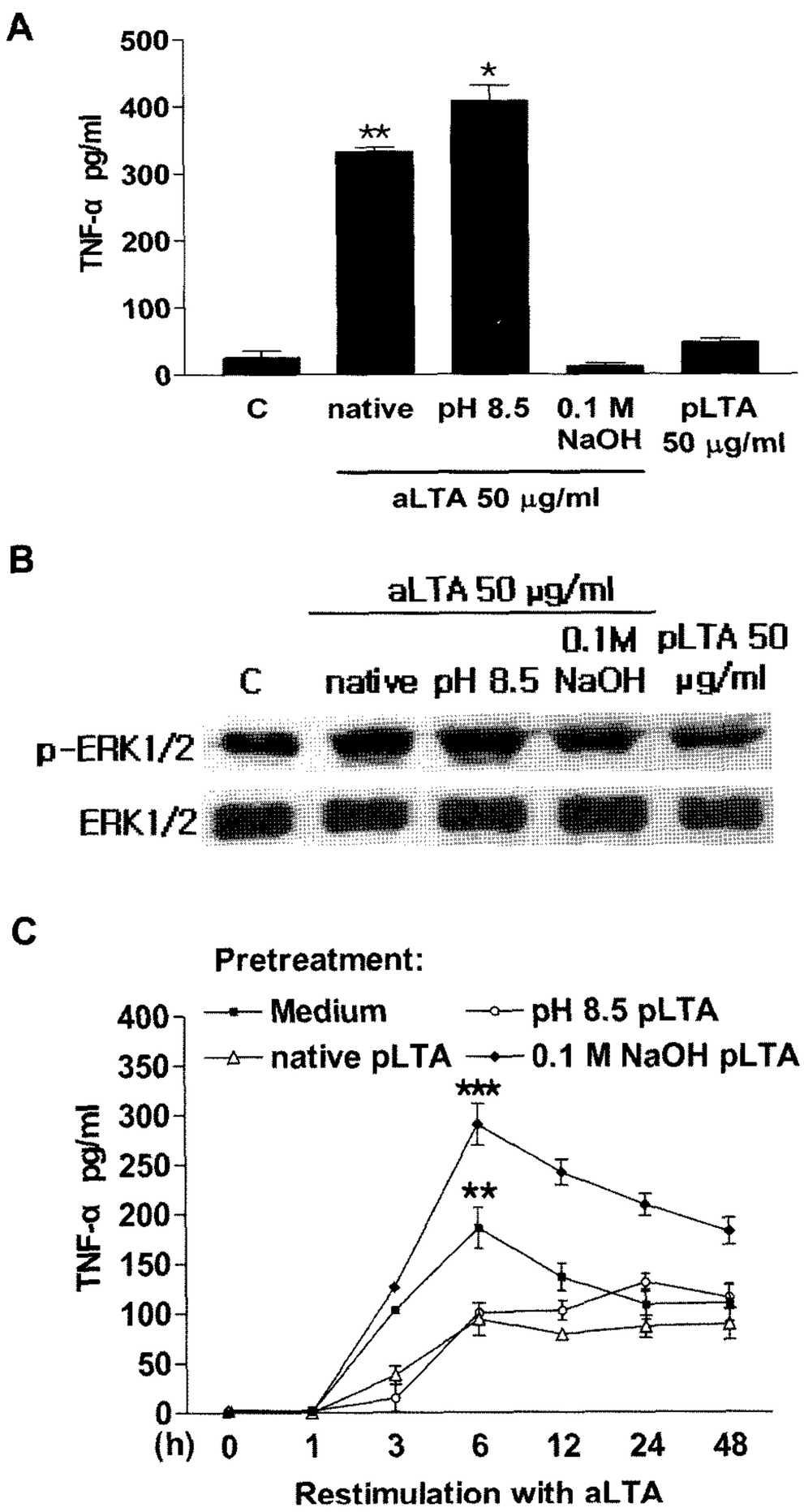

Fig. 4. The acyl group of LTA was critical in immune regulation. **A**. THP-1 cells were treated with native or modified aLTAs for 6 h. $*p<0.05$; $**p<0.01$ compared with C. **B**. THP-1 cells that were treated with native or modified aLTAs for 90 min. The cell lysates were blotted with phospho-specific antibodies for ERK1/2. To verify the amount of loaded protein, they were also probed with nonphosphorylated ERK1/2. Native pLTA was used for the control experiments in **A** and **B**. **C**. THP-1 cells were pretreated with native or modified pLTA for 20 h and then restimulated with 50 μg/ml of aLTA for the indicated times. The amount of TNF-α was determined by ELISA from culture supernatants. Data are expressed as the mean±standard deviation (**A** and **C**). $**p<0.01$; $***p<0.001$ compared with native pLTA at 6 h.

response is an attempt by the body to restore and maintain homeostasis after injury. Excessive inflammation can, however, lead to inflammatory diseases such as septic shock [5]. In the present study, we showed that pLTA did not induce an excessive immune activation, but it inhibited the excessive inflammation caused by a pathogenic infection.

pLTA tolerance might result from an impaired expression and/or functioning of the common signaling intermediates involved in LTA and TLR2 signaling. These data extend our understanding of the aLTA-signaling mechanisms that are inhibited in pLTA-treated THP-1 cells and suggest that pLTA may be a good candidate to treat Gram-positive septic shock.

Acknowledgments

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