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Lipoteichoic Acid Isolated from *Lactobacillus plantarum* Inhibits Lipopolysaccharide-Induced TNF-α Production in THP-1 Cells and Endotoxin Shock in Mice

Han Geun Kim,*† Na-Ra Kim,* Min Geun Gim,* Jung Min Lee,*† Seung Yeon Lee,* Mi Yeon Ko,* Joo Yun Kim,§ Seung Hyun Han,§ and Dae Kyun Chung2*†

In this study, the effect of *Lactobacillus plantarum* lipoteichoic acid (pLTA) on LPS-induced MAPK activation, NF-κB activation, and the expression of TNF-α and IL-1R-associated kinase M (IRAK-M) was examined. The expression of the pattern recognition receptor and the survival rate of mice were also examined. pLTA pretreatment inhibited the phosphorylation of ERK, JNK, and p38 kinase. It also inhibited the degradation of IkBα and IkBβ, as well as the activation of the LPS-induced TNF-α factor in response to subsequent LPS stimulation. These changes were accompanied by the suppression of the LPS-induced expression of TLR4, NOD1, and NOD2, and the induction of IRAK-M, with a concurrent reduction of TNF-α secretion. Furthermore, the overexpression of pattern recognition receptors such as TLR4, NOD1, and NOD2 and the degradation of IRAK-M by transient transfection were found to reinstate the production of TNF-α after LPS restimulation. In addition, the i.p. injection of pLTA suppressed fatality, and decreased the level of TNF-α in the blood, in LPS-induced endotoxin shock mice. In conclusion, these data extend our understanding of the pLTA tolerance mechanism, which is related to the inhibition of LPS-induced endotoxin shock, and suggest that pLTA may have promise as a new therapeutic agent for LPS-induced septic shock. *The Journal of Immunology*, 2008, 180: 2553–2561.

The lipoteichoic acid (LTA) of Gram-positive bacteria is considered to be analogous to the LPS of Gram-negative bacteria because LTA shares many of its biochemical and physiological properties (1). Like LPS, LTA is an amphiphile that is formed by linking a hydrophilic polyphosphate polymer to a glycolipid (2). Several previous investigations have shown that LTA is a ligand against TLR2 and TLR4. Recent studies, however, have clearly shown that repurified LTA from *Staphylococcus aureus* induces TNF-α secretion through the TLR2-signaling pathway (3, 4). These findings support the conclusion that the LTA preparations used in earlier studies were contaminated by endotoxin. Another problem associated with earlier LTA preparations is a loss of biological activity in purified LTA. The purification method included a phenol extraction step that contributed to the decomposition of LTA as a result of the loss of its alanine substituents (5). Recently, a novel purification technique using butanol extraction provided a highly purified biologically active LTA preparation.

The number of reported cases of sepsis and septic shock caused by Gram-negative and -positive bacteria, fungi, viruses, and parasites continues to grow every year (6). According to some reports, the proportion of sepsis due to Gram-negative bacteria varied between 30 and 80%, and that of sepsis due to Gram-positive bacteria varied between 6 and 24%. However, the contribution of Gram-positive bacteria to sepsis has increased in recent years. The mortality rates in patients with septic shock vary from 20 to 80% (7, 8). The production of cytokines, such as TNF-α and IL-1, initiated by bacterial components such as LPS, LTA, and peptidoglycan (PGN), can lead to the development of systemic inflammatory response syndrome and multiorgan system dysfunction. Therefore, many studies have attempted to elucidate the molecular pathways leading to an inflammatory response to abrogate them during clinical sepsis. Recent reports have described a general pathway involving the interaction between LPS and TLR: following release into the bloodstream, LPS from bacteria combines with the LPS-binding protein, and the resulting complex binds to CD14. CD14 recruitment, in turn, results in the release of inflammatory cytokines associated with sepsis (9, 10).

Recently, pathogen-induced tolerances using LPS, LTA, and PGN have been introduced to protect against the overproduction of proinflammatory cytokines associated with sepsis. However, the application of pathogen-induced tolerance to human disease entails some risks because the formulation of pathogenic fractions can induce septic shock. In this study, therefore, we isolated highly purified LTA from *Lactobacillus plantarum* (pLTA), known as a human health-promoting effector, and show that pLTA is more effective at inhibiting LPS-induced...
TNF-α production than pathogen-induced tolerance. We also identify a variation of signal transduction and demonstrate the regulation of pattern recognition receptors (PRRs), which occurred in our study through pLTA tolerance.

Materials and Methods

Cell culture

THP-1 and U-937, human monocyte-like cells, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. THP-1 cells were seeded onto 96- or 24-well plates. After incubation for 24 h, the THP-1 cells were used for stimulation with pLTA and/or LPS (Escherichia coli 055:B5; Sigma-Aldrich). Bone marrow-derived macrophages (BMM) were isolated from wild-type and TLR2-deficient BALB/c mice, which were provided by S. Akira (Osaka University, Osaka, Japan). Briefly, femurs and tibiae were taken out, and surrounding muscle was removed. The marrow was flushed out with medium using a syringe with a 0.45-mm (26 G) diameter needle, and then cells were washed with PBS. RBC were disrupted using 1 ml of RBC lysis buffer for 10 min and washed with PBS two times. To differentiate BMM, they were plated in 24-well plates at 1 × 10⁶ cells/ml in DMEM complete medium (1% penicillin/streptomycin, 10% FBS) adding 10% L-929-conditioned medium. After 3 days, medium was exchanged without L-929-conditioned medium in DMEM for 12 h and then stimulated with LPS and/or LTA.

Preparation and modification of LTA

Highly purified LTA was isolated from L. plantarum K8 (KCTC10887BP) by n-butanol extraction, as previously described (11). The purity of the purified LTA was determined by measuring the protein and endotoxin content with the conventional silver staining after PAGE and through the Limulus amebocyte lysate assay (BioWhittaker), respectively. DNA or RNA contamination was assessed by measuring UV absorption at 260 and 280 nm (12). The modification of LTA from L. plantarum was performed as previously described with minor modifications (5, 13). The dealanylation of LTA was achieved in increasing the pH of the water phase after butanol extraction, and stirring at pH 9–10.5 with a Tri buffer at room temperature (21°C) for 24 h. The desacylation 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 tri-alanine content of LTA was obtained through the indirect ELISA method, using a rabbit polyclonal anti-n-alanine Ab (Abcam).

Real-time PCR

To quantify the mRNA expression, real-time PCR amplification was conducted using the ABI Prism 7000 Sequence Detection System (Applied Biosystems), and the PCR products were detected with SYBR Green. The following sequences of the forward and reverse primer pairs were used: 5'-GGACGCTGACTGACTCTA-3' and 5'-GGCGACC TTCTCCCTTAATGT-3' for β-actin; 5'-AATTTGCTGCCATATTAGA ACTC-3' and 5'-GCTGTCCTGTCAGCTTAA-3' for TLR1; 5'-ACC CTAAAGGACACATCCT-3' and 5'-ACGCTGTAAGPTCATGC GACAT-3' for TLR2; 5'-TGAGAAGATCTCGATTGACAT-3' and 5'- AATGATCCACACTCAGGAG-3' for TLR4; 5'-GAACCTGTCTGA CAATCTCTG-3' and 5'-GACAGATTGAGGAGTTCG-3' for CD14; 5'-ATCCGAGATCTGACGAC-3' and 5'-AACCAGATAATCCCG TCTTC-3' for NOD1; 5'-CTAGAACACTCTCAGG-3' and 5'-AGTTGATCGTATTGACC-3' for NOD2; 5'-CACCT TCATCATGAGCTC-3' and 5'-CAACAAGCTGTCCTGCT-3' for LPS-induced TNF-α factor (LITAF). The expression of mRNA was normalized with β-actin.

ELISA

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

Transient DNA transfection and reporter assay

TLR or NOD expression vectors (Inoviogen) were transiently transfected into THP-1 cells using WelFec-Ex (JBI), according to the manufacturer’s manual with small modifications. Briefly, THP-1 cells were plated into 24-well plates at 1 × 10⁶ cells/well in RPMI 1640 (1% antibiotics and 10% FBS), grown overnight, and transfected for 6 h with the diverse concentrations of receptor expression and pUNO-empty vector by using 3 µg/ml WelFec-Ex transfection reagent. After 24 h, transfectants were used for the examination of TNF-α production by LPS and/or pLTA treatment. U937 or HEK293 cells were cotransfected with the pNF-κB-Luc vector and the pRL-SV40 vector as described above. The transfected cell lines were pre-stimulated with pLTA for 18 h followed by restimulation with LPS. After incubation for an additional 18 h, cells were harvested, lysed, and the cell lysates were assayed for firefly and Renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega) on an el 800 (Bio-Tek Instruments). The firefly luciferase activity of individual transfections was normalized against the Renilla luciferase activity.

Western blot analysis

Cellular extracts were prepared as described with minor modification (14). Twenty micrograms of total protein were added in 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 TBST (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk, membranes were washed three times in TBST and probed for 2 h with anti-CD14-MAPK Ab (Santa Cruz Biotechnology) or anti-IκBα or anti-IκBβ (Santa Cruz Biotechnology) in TBS-Ti/0.5% nonfat milk. After being washed three times in TBST, the membranes were incubated with secondary HRP-conjugated donkey anti-rabbit Ig or sheep anti-mouse Ig (GE Healthcare Biosciences) for 2 h and washed five times in TBST; bands were detected using ECL reagents (GE Healthcare Biosciences), according to the manufacturer’s description.

In vivo study

Male BALB/c mice (4 wk old, Samtako) were used. Marine endotoxin shock was induced through the i.p. injection of 200 µl of aqueous solution containing LPS (25–45 mg/kg). The survival rate of pLTA and LPS of endotoxin shock mice was estimated by the i.p. injection of pLTA (10–300 mg/kg) or 15 mg/kg LPS before the i.p. reinjection of 45 mg/kg LPS. The survival rate was monitored over the next 4 days. The TNF-α level from serum was assayed by ELISA, as described above. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, and triacylglycerol in the mice were measured using Vital Scientific Selctra XL (Diamond Diagnostics). These studies have been approved by the Institutional Animal Care and Use Committee of Kyung Hee University.

Statistical analysis

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

Results

Highly purified pLTA inhibited LPS-induced TNF-α production

The LPS-induced TNF-α production in pLTA-pretreated THP-1 cells was reduced in a dose-dependent manner from 1 to 200 µg/ml (Fig. 1A). The inhibitory effects of pLTA were enhanced in proportion to pretreatment time (Fig. 1B). The inhibition of LPS-induced TNF-α production, however, was not exhibited in the simultaneous treatment of pLTA and LPS or pLTA posttreatment following LPS. These results suggest that pLTA-induced tolerance is different from LPS- or S. aureus LTA (aLTA)-induced tolerance which results in bilateral hyporesponsiveness to subsequent stimulation with LPS or aLTA (15).

Although pLTA significantly inhibited LPS-induced TNF-α production, other cell components, such as crude extract (CX), the cell wall (CW), PGN, teichoic acid (TA), genomic DNA, and the culture supernatant (sup), did not inhibit the induction of LPS-induced TNF-α production (Fig. 1C). In the other experiments, pLTA-induced tolerance against LPS was more effective than other ligand-induced tolerance (Fig. 1D). Although pLTA had a high inhibitory effect on LPS-induced TNF-α production, pLTA itself showed less immune activation than LPS in THP-1 cells.
The regulation of PRRs in pLTA- or LPS-tolerized THP-1 cells

4h. THP-1 cells were pretreated with 100 µg/ml LPS for 4 h. B. Cells were pretreated with 100 µg/ml pLTA for the indicated period of time before restimulation using 0.5 µg/ml LPS for 4 h. C. THP-1 cells were pretreated with subfractions isolated from L. plantarum, such as 100 µg/ml of the CW, CX, PGN, LTA, TA, and genomic DNA (DNA) and 10 µg/ml of culture sup for 18 h. Each batch of cells was then restimulated with 0.5 µg/ml LPS for 4 h. D. THP-1 cells were pretreated with 100 µg/ml pLTA, aLTA isolated from S. aureus, ILTA isolated from Lactobacillus lactis, pPGN isolated from L. plantarum, pFGN isolated from Shigella flexneri, and aPGN isolated from S. aureus. In the next experiments, therefore, we examined the role of PRRs on TNF-α reduction. The confocal microscope data showed that the expression of TLR4, NOD1, and NOD2 was reduced, while the expression of TLR2 and CD14 was up-regulated in the THP-1 cells pretreated with pLTA followed by LPS restimulation (data not shown). Given that TLR4, NOD1, and NOD2 contribute to septic shock, the down-regulation of those receptors by pLTA pretreatment may be related to the inhibition of LPS-induced septic shock. In the next experiments, therefore, we examined the role of PRRs on TNF-α reduction.

TLR2 down-regulated the production of sepsis-related receptors

LPS induces septic shock through PRRs, especially TLR4 (10). Therefore, we examined the role of pLTA pretreatment on the expression of PRRs. The mRNAs of TLR4, NOD1, and NOD2 were down-regulated on the THP-1 cells which were pretreated with 100 µg/ml pLTA for 18 h followed by restimulation with 0.5 µg/ml LPS for 2 h (Table I). TLR4, NOD1, and NOD2 mRNAs were reduced by 63.5, 60.6, and 51.3%, respectively, in the pLTA-pretreated group as compared with LPS alone. In contrast, the mRNA of other TLRs, including CD14, increased in the pLTA-pretreated group. When compared with only LPS, the mRNA production of TLR2 and CD14 was increased to 4.3- and 10.5-fold, respectively, in the pLTA pretreatment. The confocal microscope data showed that the expression of TLR4, NOD1, and NOD2 was reduced, while the expression of TLR2 and CD14 was up-regulated in the THP-1 cells pretreated with pLTA followed by LPS restimulation (data not shown). Given that TLR4, NOD1, and NOD2 contribute to septic shock, the down-regulation of those receptors by pLTA pretreatment may be related to the inhibition of LPS-induced septic shock. In the next experiments, therefore, we examined the role of PRRs on TNF-α reduction.

The role of TLR2 in pLTA-mediated tolerance

To determine the involvement of TLR2 in signaling on stimulation with pLTA, we compared TNF-α production by BMM from BALB/c mice lacking functional TLR2 and wild-type BALB/c mice. BMM from wild-type mice exhibited TNF-α release on pLTA stimulation with 10 µg/ml, whereas no TNF-α was released by TLR2-deficient BMM. The role of TLR2 in pLTA-mediated tolerance may be related to the inhibition of LPS-induced septic shock. In the next experiments, therefore, we examined the role of PRRs on TNF-α reduction.

Table I. The regulation of PRR in pLTA- or LPS-tolerized THP-1 cells

<table>
<thead>
<tr>
<th>Pretreatment/Challenge (µg/ml)*</th>
<th>TLR1**</th>
<th>TLR2***</th>
<th>TLR4****</th>
<th>NOD1*****</th>
<th>NOD2******</th>
<th>CD14*******</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLTA 0/LPS 0</td>
<td>508 ± 108</td>
<td>676 ± 38</td>
<td>905 ± 456</td>
<td>900 ± 6</td>
<td>275 ± 45</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>pLTA 100/LPS 0.5</td>
<td>1971 ± 431*</td>
<td>4757 ± 757*</td>
<td>989 ± 324**</td>
<td>1559 ± 356</td>
<td>2370 ± 684**</td>
<td>1643 ± 503**</td>
</tr>
<tr>
<td>LPS 0.1/LPS 0.5</td>
<td>2028 ± 520</td>
<td>3084 ± 359*</td>
<td>1175 ± 439*</td>
<td>1436 ± 888*</td>
<td>1491 ± 709*</td>
<td>2127 ± 545</td>
</tr>
<tr>
<td>pLTA 0/LPS 0.5</td>
<td>790 ± 306</td>
<td>1089 ± 342</td>
<td>1558 ± 181</td>
<td>3957 ± 1152</td>
<td>4870 ± 1113</td>
<td>156 ± 89</td>
</tr>
</tbody>
</table>

*Data from pLTA 100/LPS 0.5 and LPS 0.1/LPS 0.5 were significantly lower (*, p < 0.05; **, p < 0.01) than pLTA 0/LPS 0.5.
**TLR-1 cells were pretreated with 100 µg/ml pLTA or 0.1 µg/ml LPS for 18 h prior to challenge with 0.5 µg/ml LPS. Culture cells were harvested 4 h after challenge, and cDNAs were synthesized for real-time PCR. The amount of mRNA was normalized with β-actin.
***Data are represented as the mean ± SEM from three separate experiments.
from cells of TLR2-deficient mice (Fig. 2A). In contrast to stimulation with pLTA, BMM from TLR2-deficient mice were equally responsive as compared with wild-type mice in terms of LPS-induced TNF-α production (Fig. 2B). These results suggest that pLTA requires TLR2-dependent signal transduction to activate macrophages as aLTA does (19). Prior exposure to pLTA rendered cells hyporesponsive to subsequent stimulation with LPS. We tested whether prior exposure of cells to pLTA induced refractoriness to subsequent stimulation with LPS in TLR2-deficient mice. Release of TNF-α in response to pLTA was decreased in a dose-dependent fashion when wild-type BMM were pretreated with different concentrations of pLTA for 18 h before restimulation with 0.5 μg/ml LPS for 4 h (C). In all the experiments, murine TNF-α production was examined by ELISA from culture supernatants, and data are presented as means ± SD. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with pLTA-untreated cells.

Overexpression of PRRs reinstated the production of TNF-α in pLTA-mediated tolerance

We tried to verify whether overexpression of PRRs would alter pLTA-mediated tolerance. The expression of pUNO-hTLR4 (InvivoGen) was confirmed by RT-PCR in THP-1 cells, and by confocal microscope in HEK293 cells (data not shown). TNF-α production was inhibited in the control cells transiently transfected with an empty vector after stimulation with 0.5 μg/ml
LPS following 100 μg/ml pLTA pretreatment, whereas TNF-α was reinstated in the cells transiently transfected with pUNO-hTLR4 in a dose-dependent manner (Fig. 3A). In the next experiments, we examined the role of NOD1 and NOD2 in pLTA-mediated tolerance. The expression of NOD1 and NOD2 was confirmed after transient transfection of pUNO-hNOD1 and pUNO-hNOD2. Specific down-regulation of IRAK-M reinstated the production of TNF-α in pLTA tolerant cells. A, THP-1 cells stimulated with 100 μg/ml pLTA or the medium for 18 h, and then the cells were restimulated with 0.5 μg/ml LPS for the indicated amounts of time (h) or (B) THP-1 cells were stimulated with the indicated concentration of pLTA for 18 h, and the total cell lysates were blotted with a specific Ab for IRAK-M. C, THP-1 cells were transfected with 0.1–8 μg of siRNAs for IRAK-M or control RNAs, and incubated for 24 h. The cell lysates were then blotted with a specific Ab for IRAK-M. D, THP-1 cells were transfected with graded concentrations of siRNAs for IRAK-M or control RNAs, and incubated for 24 h. Cells pretreated with 100 μg/ml pLTA for 18 h were restimulated with 0.5 μg/ml LPS for 4 h. The amount of TNF-α was measured by ELISA. The data represent the mean ± SD, and the data shown are representative of three independent experiments. The statistical significance (*, p < 0.05) was compared with the control siRNA (prestimulated with 100 μg/ml pLTA) and IRAK-M siRNA transfectants; these were compared with the control siRNA stimulated with 0.5 μg/ml LPS alone, and control siRNA stimulated with 0.5 μg/ml LPS following 100 μg/ml pLTA.

FIGURE 4. Specific down-regulation of IRAK-M reinstated the production of TNF-α in pLTA tolerant cells. A, THP-1 cells stimulated with 100 μg/ml pLTA or the medium for 18 h, and then the cells were restimulated with 0.5 μg/ml LPS for the indicated amounts of time (h) or (B) THP-1 cells were stimulated with the indicated concentration of pLTA for 18 h, and the total cell lysates were blotted with a specific Ab for IRAK-M. C, THP-1 cells were transfected with 0.1–8 μg of siRNAs for IRAK-M or control RNAs, and incubated for 24 h. The cell lysates were then blotted with a specific Ab for IRAK-M. D, THP-1 cells were transfected with graded concentrations of siRNAs for IRAK-M or control RNAs, and incubated for 24 h. Cells pretreated with 100 μg/ml pLTA for 18 h were restimulated with 0.5 μg/ml LPS for 4 h. The amount of TNF-α was measured by ELISA. The data represent the mean ± SD, and the data shown are representative of three independent experiments. The statistical significance (*, p < 0.05) was compared with the control siRNA (prestimulated with 100 μg/ml pLTA) and IRAK-M siRNA transfectants; these were compared with the control siRNA stimulated with 0.5 μg/ml LPS alone, and control siRNA stimulated with 0.5 μg/ml LPS following 100 μg/ml pLTA.

FIGURE 5. pLTA tolerance down-regulated the activated signaling pathways. A, THP-1 cells were pretreated with either the medium or 100 μg/ml pLTA for 18 h, and then restimulated with 0.5 μg/ml LPS for the indicated amounts of time (min). The cell lysates were blotted with phosphospecific Abs for ERK1/2, p38, JNK1/2, IκB-α, and IκBβ. To verify the amount of loaded protein, they were also probed with anti-ERK1/2. B, U937 cells were transiently cotransfected with the pNF-κB-luc and pRL-SV40 control vectors. They were preincubated with the medium or 100 μg/ml pLTA for 18 h, and then restimulated with LPS at the indicated concentrations for 20 h. Cells were lysed and firefly and Renilla luciferase activities were measured. Data are expressed as the mean ± SD of three experiments performed in triplicate and normalized for Renilla luciferase activity. C, THP-1 cells were pretreated with 100 μg/ml pLTA for 18 h, and then restimulated with 0.5 μg/ml LPS for 4 h. The total RNA was extracted, and cDNA synthesis was performed. The level of LITAF was examined by quantitative real-time PCR methods using specific primers for human LITAF. Data are expressed as the mean ± SD of three independent experiments. *, p < 0.05 vs untreated cells, indicated as C. D, THP-1 cells were pretreated with 100 μg/ml pLTA for 18 h, and then restimulated with 0.5 μg/ml LPS for 4 h. The cell lysates were blotted with a specific Ab for human LITAF (Upstate Biotechnology).
pUNO-hNOD2 into THP-1 cells for RT-PCR and HEK293 cells for confocal microscope (data not shown). Although TNF-α production was inhibited in the THP-1 cells transfected with the empty vector after 0.5 μg/ml LPS stimulation following 100 μg/ml pLTA pretreatment, LPS-induced TNF-α production was reinstated in NOD1 and NOD2 transfectants in a dose-dependent manner (Fig. 3B). Similarly, NF-κB activation was inhibited in U937 cells cotransfected with the empty vector and the pNF-κB-luc vector after stimulation with 0.5 μg/ml LPS following 100 μg/ml pLTA pretreatment, whereas it was increased in U937 cells cotransfected with PRR and the pNF-κB-luc vector in a PRR dose-dependent manner (Fig. 3C). These results may suggest that TLR4, NOD1, and NOD2 participated in LPS-induced TNF-α production, and that the down-regulation of those receptors by pLTA pretreatment resulted in the inhibition of TNF-α production.

pLTA tolerance up-regulated the production of IL-1R-associated kinase M (IRAK-M), negative regulator of TLR

To determine the variation of TLR-negative regulators, we examined the mRNA levels of ST-2, IRAK-M, suppressor of cytokine signaling 1, MyD88s, and single immunoglobulin domain IL-1R-related from pLTA and LPS tolerance cells. THP-1 cells did not express ST-2, a negative regulator of TLR4: pLTA and LPS tolerant cells had a slight reduction in suppressor of cytokine signaling 1, MyD88s, and single immunoglobulin domain IL-1R-related production compared with LPS treatment alone (data not shown). Therefore, these molecules were not considered to be involved in pLTA tolerance. IRAK-M, which inhibits the release of the IRAK-TNFR-associated factor 6 complex from MyD88, was induced by pLTA pretreatment as compared with LPS treatment alone (Fig. 4A). The expression of IRAK-M might be affected by pLTA because its expression was dependent on the concentration of pLTA (Fig. 4B).

To examine whether the down-regulation of IRAK-M expression would alter pLTA-mediated tolerance, the IRAK-M expression was degraded in THP-1 cells transfected with a specific small interference RNA (siRNA) for IRAK-M (Fig. 4C). Although TNF-α production was inhibited in control siRNA-transfected cells, which were pretreated with 100 μg/ml pLTA followed by 0.5 μg/ml LPS restimulation, it was reinstated in THP-1 cells transfected with IRAK-M siRNAs in a dose-dependent manner (Fig. 4D). This result...

FIGURE 6. pLTA improved the survival rate of endotoxin shock mice induced by LPS. A, BALB/c mice (6 wk old, 8 mice) were injected i.p. with the indicated concentrations of LPS. B, Mice (16 mice) were injected i.p. with 45 mg/kg LPS. C, Mice (eight mice) were injected i.p. with the indicated concentrations of pLTA or PBS and, after 24 h, reinjected i.p. with 45 mg/kg LPS. *p < 0.05 compared with PBS preinjection. D, The effect of pLTA on plasma TNF-α level in endotoxin shock mice. The plasma was collected 24 h after i.p. injection of 45 mg/kg LPS following pre-i.p. injection of 15 mg/kg LPS or 300 mg/kg pLTA for 24 h. The TNF-α level from plasma was determined by ELISA. Each value represents the mean ± SD of two independent experiments in duplicate. **p < 0.01; ***p < 0.001 compared with PBS preinjection. E and F, The effect of pLTA on serum ALT (E) and triacylglycerol (F) levels in endotoxin shock mice 24 h after i.p. injection of 45 mg/kg LPS. Each value represents the mean ± SD of three experiments. *p < 0.05, compared with PBS.
indicates that IRAK-M, which was up-regulated by pLTA pretreatment, played an important role as a negative regulator for TLR4 signaling and inhibited LPS-induced TNF-α production.

**pLTA tolerance down-regulated the activated signaling pathways**

LPS-mediated phosphorylation of p38, ERK1/2, and JNK1/2 in medium-pretreated THP-1 cells was evident after 15 min, and optimal responses were reached by 30 min of LPS stimulation. MAPK phosphorylation, however, was significantly inhibited in pLTA-pretreated THP-1 cells following restimulation with 0.5 μg/ml LPS (Fig. 5A). Although LPS caused a degradation of IκBα and IκBβ after 60 min in the untolerized cells, pLTA-pretreated cells exhibited a markedly suppressed degradation of both IκBα and IκBβ in response to LPS restimulation (Fig. 5A). As shown in Fig. 5B, pLTA pretreatment inhibited the capacity of U937 cells to induce NF-κB-dependent transcription of the luciferase reporter gene in response to subsequent stimulation with LPS. THP-1 cells stimulated with LPS induced a high level of LITAF production as compared with untreated or pLTA-treated cells, whereas pLTA pretreatment reduced LPS-induced LITAF production (Fig. 5C). As expected, the protein level of LITAF was also inhibited by LPS stimulation. pLTA pretreatment, however, inhibited LPS-induced LITAF protein production (Fig. 5D). Taken together, these results suggest that the activation of MAPKs, NF-κB and especially LITAF, were involved in LPS-induced TNF-α production, and that pLTA tolerance inhibited TNF-α production via the down-regulation of signal transduction associated with the MAPKs, NF-κB and LITAF.

**pLTA suppressed LPS-induced endotoxin shock in mice**

To examine the inhibitory effects of pLTA on endotoxin shock induced by LPS in BALB/c mice, we examined the survival rate of endotoxin shock mice. In the absence of pLTA or LPS, ~85% of the mice died within 2 days of an i.p. injection of the aqueous solution containing 40–45 mg/kg LPS (Fig. 6A). When 15 mg/kg LPS was injected i.p. 24 h before the i.p. injection of 45 mg/kg LPS, the rate of survival was improved by 58%. Interestingly, the i.p. preinjection of 300 mg/kg pLTA, followed 24 h later by i.p. injection of 45 mg/kg LPS, significantly increased the survival rate of endotoxin shock mice (Fig. 6B). When mice were preinjected i.p. with various concentrations of pLTA 24 h before i.p. injection of 45 mg/kg LPS, the survival rate increased in a dose-dependent manner; mice injected with over 100 mg/kg pLTA did not show mortality (Fig. 6C). In this study, mice preinjected i.p. with over 100 mg/kg pLTA recovered their health within 2 days, whereas mice preinjected with below 50 mg/kg pLTA recovered painfully after 4 days. Mice preinjected with 300 mg/kg pLTA recovered their health most rapidly among the experimental groups. LPS-tolerized mice, however, showed severe diarrhea and hardly recovered their health after LPS restimulation, as compared with the pLTA-tolerized mice. In addition, the plasma TNF-α levels 24 h after i.p. injection with 45 mg/kg LPS, following a preinjection of 300 mg/kg pLTA or 15 mg/kg LPS 24 h earlier, significantly decreased compared with the preinjection of PBS (Fig. 6D). In the PBS-preinjected mice, the serum levels of ALT and triacylglycerol were markedly augmented, but pLTA strikingly inhibited the increase in these levels. However, LPS preinjection did not alter the triacylglycerol level, while reducing the serum ALT level slightly, as compared with PBS preinjection (Fig. 6E, E and F). Serum AST and serum total cholesterol levels in the LPS and pLTA preinjection groups did not show significant difference from the PBS preinjection group, although these levels in the LPS and pLTA preinjection groups were higher than those in the control group (data not shown). These results suggest that pLTA and LPS significantly
suppress lethality in endotoxin shock mice induced by LPS, probably resulting from the reduction of hepatic inflammation.

The acyl group of pLTA played an important role in the inhibition of LPS-induced TNF-α production, but not D-alanine

In the previous report, the quality and level of D-alanine substitu-

The inhibition of LPS-induced TNF-α production occurred by both intact pLTA and dealanylated pLTA, but not by deacylated pLTA (Fig. 7B). The D-alanine contents of both modified pLTA were confirmed through indirect ELISA methods using the anti-D-alanine Ab, and Fig. 7C shows the reduction of D-alanine contents from both modified pLTAs as compared with the intact pLTA. In addition, the phos-

Discussion

Inflammation is the first response to infection and injury and is critical to body defense. Basically, the inflammatory response is an attempt by the body to restore and maintain homeostasis after injury. Excessive inflammation can, however, cause inflammatory diseases such as septic shock, Legionnaire’s disease, systemic lupus erythematosus, Crohn’s disease, atherosclerosis, and so on (21). To treat these inflammatory diseases, researchers have tried to induce tolerance against endotoxin, which induces excessive inflammation, and several reports have shown that bacterial cell wall components induced homologous tolerance (15, 16, 22, 23). However, most bacterial cell walls used in tolerance-induction tests were isolated from pathogenic bacteria, making them harmful for use in clinical applications. The reason for this is that it is difficult to decide the proper concentration to induce the tolerance, and the composition of these bacterial cell walls can cause synergistic inflammation or septic shock (17, 18). In our present experiments, the treatment of LPS or aLTA in THP-1 cells induced excessive proinflammatory cytokine production, and the mouse survival rate was reduced by 50% in the LPS-induced tolerance as compared with endotoxin shock mice, indicating the risk of pathogenic bacterial cell walls as a clinical material. Therefore, we selected an LTA isolated from L. plantarum as a material that would induce tolerance without the trouble of side effects. Unlike LPS or aLTA, pLTA did not induce the high level of proinflammatory cytokines and showed low cytotoxicity (data not shown). In contrast, pLTA efficiently inhibited LPS-induced TNF-α production, and results in a high survival rate among mice with endotoxin shock.

A previous study has shown that the impaired expression and functions of common signaling, which intermediates LPS and IL-1 signaling, induce LPS-mediated tolerance (14). In particular, the regulation of TLR4 expression, which plays an important role in the induction of septic shock, may depend on the material that induces tolerance. The expression of the TLR4-MD2 complex was reduced in LPS-tolerized mouse peritoneal macrophages (24), whereas macrophage-activating lipoprotein 2-induced tolerance did not reduce the expression of the TLR4-MD2 complex (25). Similar to LPS-induced tolerance, pLTA-induced tolerance reduced the expression of the TLR4-MD2 complex. In addition, the expression of NOD1 and NOD2, which induces the activation of NF-κB through LPS stimulation, and resulted in the up-regulation of inflammatory cytokines (26, 27), was also reduced through pLTA tolerance. The excessive expression of NOD1 and NOD2, as well as TLR4, causes inflammatory diseases. Therefore, the reduction of TLR4, NOD1, and NOD2 may inhibit excessive inflammation through the limited interaction between those receptors and LPS. Interestingly, the expression of CD14, which plays an important role in the recognition of LPS via TLR4, increased in the pLTA-tolerized cells. This phenomenon may be explained in that CD14 also participates in the interaction between TLR2 and LTA (28). A previous study has shown that LTA competes with LPS for binding to the CD14 of human monocytes, and LPS-CD14 binding is inhibited completely by Streptococcus sanguis LTA at a 100-fold higher concentration than LPS (23). These results suggest that the increased CD14 in pLTA tolerance may interact with pLTA, and result in the decreased interaction between CD14 and LPS.

The activation of MAPKs such as ERK1/2, JNK1/2, and p38 is important in mediating many macrophage functions, including the activation of various transcription factors and the production of pro- and anti-inflammatory cytokines (29, 30). Recent studies have reported that endotoxin- or bacterial lipoprotein-induced tolerance down-regulated the phosphorylation of ERK, JNK, and p38 in mouse macrophages and human THP-1 cells, respectively (14, 31). In addition, the LPS-induced activation of the MAPK pathways plays an important role in mediating NF-κB activation. NF-κB activation is induced by the dissociation of IκBα and IκBβ, which are phosphorylated by IκB kinases. Therefore, the amount of degradation of IκBα and IκBβ is commonly used as an indicator to confirm the activation of NF-κB. In the present study, pretreatment of pLTA on THP-1 cells inhibited the phosphorylation of MAPKs and the degradation of IκBα and IκBβ. These results suggest that pLTA tolerance and LPS tolerance may share similar intracellular signal transduction pathways. As a result of the inhibition of intracellular signaling pathways, the expression of LITAF was inhibited in the pLTA-tolerized cells. LITAF is known as the LPS-induced TNF-α factor, which mediates TNF-α transcription (32, 33). It has been reported that the inhibition of LITAF mRNA expression in THP-1 cells resulted in a reduction of TNF-α transcripts. These results suggest that LITAF, as the final factor in the signaling cascade, plays an important role in LPS-induced TNF-α production, and the inhibition of LITAF expression through pLTA tolerance mediates the inhibition of LPS-induced TNF-α production.

Unlike other bacterial cell walls, such as aLTA, LPS, and lipoproteins, pLTA was prepared from lactic acid bacteria, which contributes to the health of the host. pLTA had low cytotoxicity and did not induce excessive inflammation. In particular, pLTA significantly reduced the excessive TNF-α production caused by endotoxin and increased the survival rate of endotoxin shock mice. These inhibitory effects were induced by the complex mechanism of 1) the inhibition of intracellular signaling pathways such as MAPKs and NF-κB; 2) the reduction of sepsis-related PRRs such as TLR4, NOD1, and NOD2; and 3) the induction of IRAK-M. The alteration of these mechanisms reduced LITAF expression and resulted in the inhibition of excessive LPS-induced TNF-α production; thus, there was a high survival rate among endotoxin shock mice. Therefore, pLTA tolerance may be more effective in the prevention and treatment of endotoxin shock, because pLTA is
safer and more inducible in the induction of tolerance compared with other bacterial cell wall components.

Disclosures
The authors have no financial conflict of interest.

References