The Journal of Immunology

RESEARCH ARTICLE | MARCH 10 2023

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J Immunol (2023) 210 (9): 1386–1395.
https://doi.org/10.4049/jimmunol.2200872

Lipoteichoic Acid Inhibits Lipopolysaccharide-Induced TLR4 Signaling by Forming an Inactive TLR4/MD-2 Complex Dimer

Sachiko Watanabe, Kosuke Zenke, and Masashi Muroi

LPS interacts with TLR4, which play important roles in host-against-pathogen immune responses, by binding to MD-2 and inducing an inflammatory response. In this study, to our knowledge, we found a novel function of lipoteichoic acid (LTA), a TLR2 ligand, that involves suppression of TLR4-mediated signaling independently of TLR2 under serum-free conditions. LTA inhibited NF-κB activation induced by LPS or a synthetic lipid A in a noncompetitive manner in human embryonic kidney 293 cells expressing CD14, TLR4, and MD-2. This inhibition was abrogated by addition of serum or albumin. LTAs from different bacterial sources also inhibited NF-κB activation, although LTA from *Enterococcus hirae* had essentially no TLR2-mediated NF-κB activation. The TLR2 ligands tripalmitoyl-Cys-Ser-Lys-Lys-Lys-Lys (Pam_3CSK_4) and macrophage-activating lipopeptide-2 (MALP-2) did not affect the TLR4-mediated NF-κB activation. In bone marrow–derived macrophages from $TLR2^{-/-}$ mice, LTA inhibited LPS-induced IκB-α phosphorylation and production of TNF, CXCL1/KC, RANTES, and IFN-β without affecting cell surface expression of TLR4. LTA did not suppress IL-1β–induced NF-κB activation mediated through signaling pathways shared with TLRs. LTAs including *E. hirae* LTA, but not LPS, induced association of TLR4/MD-2 complexes, which was suppressed by serum. LTA also increased association of MD-2, but not TLR4 molecules. These results demonstrate that, under serum-free conditions, LTA induces association of MD-2 molecules to promote formation of an inactive TLR4/MD-2 complex dimer that in turn prevents TLR4-mediated signaling. The presence of LTA that poorly induces TLR2-mediated activation but inhibits TLR4 signaling provides insight into the role of Gram-positive bacteria in suppressing inflammation induced by Gram-negative bacteria in organs such as the intestines where serum is absent. ***The Journal of Immunology*****, 2023, 210: 1386–1395.**

Toll-like receptors are pattern recognition receptors that play important roles in host-against-pathogen immune responses. Excessive TLR activation can lead to overwhelming systemic inflammation and multiple organ injury, characterized as sepsis. Notably, LPS or endotoxin, derived from Gram-negative bacteria, activates TLR4 signaling and induces severe inflammatory responses (1). LPS is sensed by TLR4 in cooperation with LPS-binding protein (LBP), CD14, and MD-2. LBP attracts LPS and facilitates CD14-dependent transfer of LPS to TLR4 via the accessory protein MD-2 to promote dimerization of the TLR4/MD-2 complex. This TLR4/MD-2 complex dimer mediates translocation of NF-κB, and it ultimately results in production of proinflammatory cytokines. Therefore, uncontrolled LPS-induced inflammatory TLR4 signaling can cause acute sepsis (2–4).

Lipoteichoic acid (LTA) has been identified as a major pathogen-associated molecular pattern produced by Gram-positive bacteria. Although results of a previous study suggested that TLR4, but not TLR2, is required for the recognition of LTA as well as LPS (5), LTA is now known to be recognized by TLR2. In addition, LTA in the circulation was reported to activate TLR2 signaling with the aid of LBP and CD14 (6–9). Because LTA induces TLR2-mediated activation of NF-κB (10, 11), LTA is recognized as a major virulence factor that causes inflammatory responses (12). In contrast, LTA itself reportedly does not induce inflammatory responses and inhibits inflammatory cytokine release triggered by TLR3 stimulation in keratinocytes (13). LTA is also demonstrated to attenuate TLR-dependent dendritic cell activation and inflammatory responses in a TLR2-dependent manner (14, 15). Despite its importance as a pathogen-related factor that regulates host immune responses, the exact role of LTA during infection remains unclear (12).

In this study, we show that, under serum-free conditions, bacterial LTA suppresses TLR4-mediated signaling independently of TLR2. We found that LTA induces association of MD-2 molecules, which leads to formation of an atypical TLR4/MD-2 complex dimer that differs from that formed in response to LPS. Serum dependency of the formation of this atypical TLR4/MD-2 complex dimer strongly suggests that this atypical dimer is responsible for suppressing the initiation of TLR4 signaling.

Materials and Methods

Cells and mice

Human embryonic kidney 293 (HEK293) cell lines (obtained from the Human Science Research Resources Bank, Tokyo, Japan) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated FBS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). $TLR2^{-/-}$ mice were provided by Yusuke Murakami (Department of Pharmacotherapy, Musashino University, Tokyo, Japan). Bone marrow–derived macrophages (BMDMs) were generated from mouse bone marrow cells and grown in the culture medium described above supplemented with 20% conditioned media derived from LADMAC cells as a source of G-CSF. These cells were seeded

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Received for publication November 28, 2022. Accepted for publication February 19, 2023.

This work was supported in part by grants from Musashino University (Gakuin Tokubetsu Kenkyu and Joint Research Unit Promotion).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; HEK293, human embryonic kidney 293; LBP, LPS-binding protein; LgB, N-terminal large fragment derived from NanoLuc luciferase; LTA, lipoteichoic acid; MALP-2, macrophage-activating lipopeptide-2; Pam_3CSK_4, tripalmitoyl-Cys-Ser-Lys-Lys-Lys-Lys; SmB, C-terminal small fragment derived from NanoLuc luciferase; TIR, Toll/IL-1R; TLR4N, extracellular domain of TLR4.

on culture plates and cultured at 37°C and 5% CO_2 for 7 d prior to use. Adherent cells were considered to be murine BMDMs. When cells were stimulated with LPS in serum-free medium, 100 ng/ml LBP was included. Animal studies were conducted under protocols approved by the Institutional Animal Experiments Committee of Musashino University.

Reagents

Polymyxin B and LTAs from *Bacillus subtilis* or *Enterococcus hirae* were obtained from Sigma-Aldrich (St. Louis, MO). LTAs were further purified using EndoZero spin columns (Zymo Research, Irvine, CA) to remove any contaminating endotoxins. Purified LTA from *Staphylococcus aureus*, ultrapure LPS from *Escherichia coli* O111:B4, macrophage-activating lipopeptide-2 (MALP-2), and anti-HA Ab were obtained from InvivoGen (San Diego, CA). Tripalmitoyl-Cys-Ser-Lys-Lys-Lys-Lys (Pam_3CSK_4) was obtained from Bachem (Bubendorf, Switzerland) and suspended in 25 mM octyl glucoside. Polyclonal antiserum against the EIAV-tag epitope (amino acid sequence ADRRIPGTAEE) was a gift from Dr. Nancy Rice (National Cancer Institute–Frederick Cancer Research and Development Center). Abs against FLAG epitope, human serum globulin, and human serum albumin solution were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Anti–β-actin Ab (AC-15) from Sigma-Aldrich (St. Louis, MO), synthetic lipid A (compound 506) from Peptide Institute (Osaka, Japan), pooled human serum from Innovative Research (Novi, MI), IL-1β from PeproTech (Rocky Hill, NJ), an Ab against phosphorylated IκB-α (no. 9246) from Cell Signaling Technology (Danvers, MA), and an Ab against IκB-α (C-21) from Santa Cruz Biotechnology (Dallas, TX) were used. Recombinant LBP was prepared using the *Pichia* expression system (Invitrogen) as described earlier (16).

Plasmids

Expression plasmids for TLR2, TLR4, CD14, and MD-2, as well as NF-κB–dependent luciferase reporter plasmid pELAM-L, were described previously (17, 18). Plasmids in which the CMV promoter was replaced with a Rous sarcoma virus promoter were also used. The coding sequence of IL-1R1 lacking the signal peptide region was amplified from a human spleen cDNA library (Clontech, Palo Alto, CA) and subcloned downstream of a mammalian expression vector having a preprotrypsin signal peptide sequence preceding an N-terminal epitope tag. The coding sequence of the N-terminal large (LgB) or C-terminal small (SmB) fragment derived from NanoLuc luciferase (Promega, Madison, WI) was linked N-terminally (downstream of the signal peptide sequence) or C-terminally to the coding sequence of MD-2 or the extracellular domain of TLR4 (TLR4N) with a spacer insert comprising two copies of the Gly^4-Ser–encoding sequence.

NF-κB reporter assay

The NF-κB–dependent luciferase reporter assay was performed as previously described (19, 20). Briefly, HEK293 cells ($2–5 \times 10^5$ cells) were plated in 12-well plates and transfected on the following day by calcium phosphate precipitation or using FuGENE HD transfection reagent (Promega, Madison, WI) with the indicated plasmids together with 0.1 μg of pELAM-L and 2.5 ng of phRL-TK (Promega, Madison, WI) for normalization. At 24–32 h after transfection, cells were stimulated with the indicated ligands for 6 h either in the culture medium or FreeStyle 293 serum-free medium (Life Technologies, Gaithersburg, MD). Following stimulation, cellular extracts were prepared by adding lysis buffer (10 mM HEPES-KOH [pH 7.9], 400 mM NaCl, 10 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM DTT, 0.5% Nonidet P-40, 30 mM NaF, 1 mM Na_3VO_4) containing a protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan). A portion of the cellular extract was used to measure reporter gene activity using a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega).

Western blotting

Cellular extracts were prepared and subjected to SDS-PAGE as described previously (19, 20). Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) for immunoblotting with the indicated Abs followed by visualization using an ECL system (Millipore).

Flow cytometry

BMDMs were stimulated with LTA for 15 min followed by LPS for 1 h in FreeStyle 293 serum-free medium (Life Technologies, Gaithersburg, MD). The cells were stained with PE-conjugated anti-mouse CD284 (TLR4) Ab (BioLegend, San Diego, CA) and examined by flow cytometry (FACSLyric; BD Biosciences, San Jose, CA). The analysis was performed using the FlowJo software program (version 10; Tree Star, San Carlos, CA). Isotype control Abs were used as negative controls to exclude nonspecific background staining.

Cytokine and chemokine measurement

BMDMs were stimulated with LTA for 15 min followed by LPS in FreeStyle 293 serum-free medium for 4 or 6 h. Following stimulation, culture supernatants were collected and TNF, CXCL1/KC, RANTES, and IFN-β concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN). After removing culture supernatants, cell viability was measured using a CellTiter 96 AQ_{ueous} One solution cell proliferation assay kit (Promega, Madison, WI). Cytokine and chemokine levels were normalized to these cell viabilities (OD) to compensate for differences in cell numbers and viability between wells (21).

NanoBiT protein–protein interaction assay

Culture medium from cells transfected with indicated plasmids was mixed with Nano-Glo live cell reagent according to the manufacturer's instructions (Promega), and the luciferase activity was measured using a luminometer (Centro LB 960 detection system; Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis

Data are presented as the mean ± SEM. The p values were calculated according to a two-tailed Student t test, and p values <0.05 were considered to be statistically significant.

Results

LTA inhibits LPS-induced activation of NF-κB under serum-free conditions

In this study, we examined the effect of LTA in HEK293 cells, which are known to be unresponsive to TLR2 ligands and thus avoid LTA-mediated TLR2 activation while allowing examination of the direct effect of LTA on TLR4-mediated signaling. We examined NF-κB–dependent luciferase reporter activity in HEK293 cells transiently transfected with expression plasmids for CD14, TLR4, and MD-2. As expected, LTA alone had almost no effect on activation of the NF-κB reporter (Fig. 1A). In contrast, LPS treatment strongly induced the reporter activity, which was not affected by up to 1 μg/ml LTA (Fig. 1A). Although a low level of reporter activation was observed at a higher concentration (10 μg/ml) of LTA alone (Fig. 1A), this activation was suppressed by polymyxin B, which binds the lipid A portion of LPS to block its biological effects (22). In contrast, polymyxin B did not inhibit LTA-induced NF-κB reporter activation in HEK293 cells expressing TLR2 (Supplemental Fig. 1). Thus, this slight activation induced by the higher concentration of LTA appears to be caused by the presence of small amounts of contaminating LPS in the LTA preparation.

The experiments described above (Fig. 1A) were performed in a culture medium containing 10% FBS. When a similar experiment was performed in serum-free medium, however, activation of the NF-κB reporter induced by LPS was unexpectedly and concentration-dependently reduced by LTA (Fig. 1B). The expression levels of CD14, TLR4, and MD-2 were not significantly affected by LTA (Supplemental Fig. 2A, 2B), indicating that the inhibitory effect of LTA was not due to suppressed expression of these proteins. Furthermore, this inhibitory effect of LTA was abrogated when an increasing amount of FBS was added to the serum-free medium (Fig. 1C). In contrast, FBS did not affect TLR2-mediated activation of the NF-κB reporter induced by LTA (Fig. 1D), indicating that FBS itself did not inactivate LTA activity. In these experiments, the expression levels of CD14, TLR4, MD-2, and TLR2 were not significantly affected by increasing amounts of FBS (Supplemental Fig. 2C, 2D). Similar to FBS, human serum also abrogated the inhibitory effect of LTA on LPS-induced activation of the NF-κB reporter in a concentration-dependent manner (Fig. 2A). Among serum proteins, albumin is the most abundant. Thus, we examined the effect of human albumin and

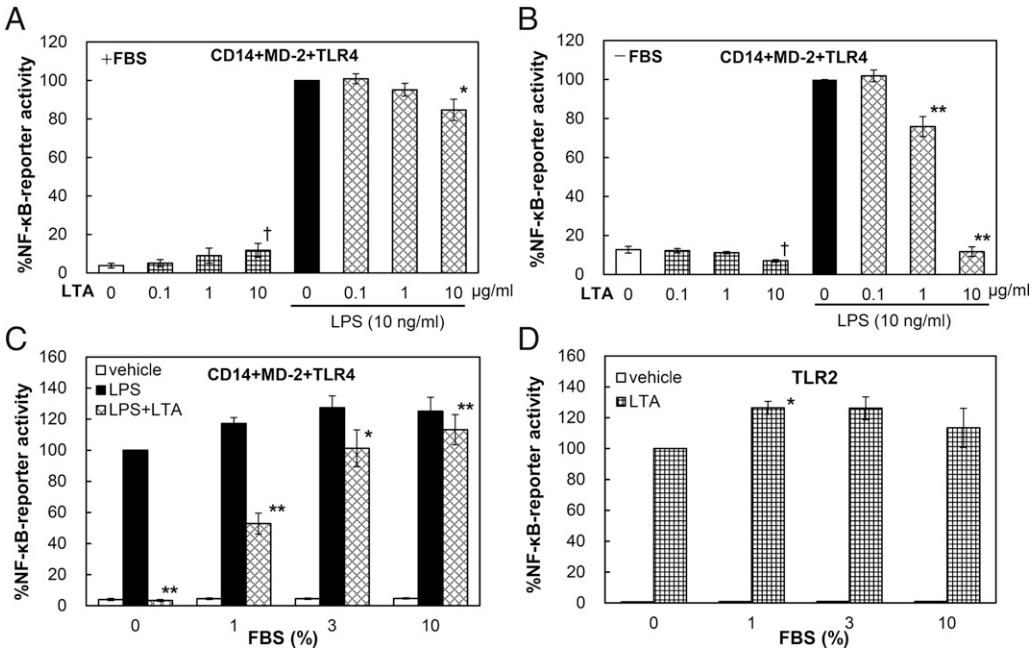

FIGURE 1. LTA inhibits LPS-induced activation of NF-κB in the absence of serum. (**A**–**D**) HEK293 cells were transiently transfected with expression plasmids for CD14, MD-2, and TLR4 (A–C) or TLR2 (D) together with an NF-κB–dependent luciferase reporter plasmid. After 24 h, cells were stimulated for 6 h with increasing concentrations of *S. aureus* LTA in the absence or presence of LPS (10 ng/ml) in culture medium containing 10% FBS (A) or serum-free medium (B), or cells were stimulated for 6 h with LPS (10 ng/ml) in the absence or presence of LTA (10 μg/ml) from *S. aureus* (C) or with LTA (10 μg/ml) from *S. aureus* (D) in serum-free medium containing increasing concentrations of FBS. Cellular extracts were then prepared and subjected to luciferase reporter activity measurements. The reporter activity in response to LPS alone (A and B), LPS alone in the absence of FBS (C), or LTA in the absence of FBS (D) is expressed as 100%. Values are means ± SEM from three to eight independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with cells in response to LPS alone (A–C) or LTA in the absence of FBS (D); $^{\dagger}p < 0.05$, compared with cells treated with vehicle.

found that 1 and 10 mg/ml albumin abrogated the inhibitory effect of LTA (Fig. 2B). Globulin is also known as a major serum protein; however, 10 mg/ml globulin as well as other serum proteins apolipoprotein C3 (0.1 mM) and neutrophil-activating protein 2 (1 μg/ml) did not affect the inhibitory effect of LTA (Supplemental Fig. 3).

To determine whether the inhibitory effect of LTA on LPS-induced NF-κB reporter activation was caused by the direct competition of LTA with LPS, we examined the effect of LTA on the concentration–response curve of LPS in HEK293 cells expressing CD14, TLR4, and MD-2. Increasing the concentration of LPS (1 and 10 μg/ml) could not overcome the inhibitory effect of 1 and 3 μg/ml LTA (Fig. 3), indicating that LTA noncompetitively inhibited LPS-induced activation of the NF-κB reporter.

LTA inhibits LPS-induced activation of TLR4 signaling independently of TLR2

Although LTA from most bacteria has a similar basic structure, LTAs prepared from different bacteria induce distinctive immunostimulatory activity, which may be due to subtle structural differences among LTAs (12). Thus, we examined the effect of LTAs from *S. aureus*, *B. subtilis*, and *E. hirae*. All LTAs dose-dependently suppressed NF-κB reporter activity induced by LPS in HEK293 cells expressing CD14, TLR4, and MD-2 (Fig. 4A). LTA also suppressed reporter activity induced by synthetic lipid A, the biologically active part of LPS (Fig. 4B). However, these LTAs inhibited TLR4-mediated activation of the NF-κB reporter at similar concentrations (Fig. 4A, 4B), whereas TLR2-mediated activation of the reporter differed among the LTAs. In particular, LTA derived from *E. hirae* induced little activation (Fig. 4C).

Other TLR2 ligands such as Pam_3CSK_4 or MALP-2 did not affect TLR4-mediated NF-κB activation induced by LPS (Fig. 5A), but they did strongly activate reporter activity in cells expressing TLR2 (Fig. 5B). These results strongly suggest that the inhibitory effect of LTA on LPS-induced activation of NF-κB is independent of TLR2.

Although IL-1R and TLRs possess structurally distinct extracellular domains, they share an intracellular Toll/IL-1R (TIR) homology domain that is essential for interactions with downstream signaling components and that activate NF-κB through the same intracellular signaling pathway (23–26). Thus, we examined the effect of LTA on IL-1β–induced NF-κB reporter activation in HEK293 cells expressing type I IL-1R. LTA did not inhibit the reporter activity, but it did inhibit LPS-induced activation of the NF-κB reporter in cells expressing CD14, TLR4, and MD-2 (Fig. 5C). Thus, LTA likely does not affect intracellular TLR4 signaling.

The above experiments were performed with HEK293 cells expressing CD14, TLR4, and MD-2. We next examined the inhibitory effect of LTA on BMDMs. Because LTA activates TLR2, we used primary BMDMs from $TLR2^{-/-}$ mice. As expected, under serum-free conditions, LTA suppressed LPS-induced phosphorylation and proteasome-dependent degradation of IκB-α, which are prerequisites for NF-κB activation (Fig. 6A, 6B). Cell surface TLR4 expression levels in BMDMs were not affected by LTA treatment, suggesting that the inhibitory effect of LTA was not due to changes in the TLR4 expression levels (Fig. 6C). We also examined the effect of LTA on LPS-induced cytokine or chemokine production by BMDMs from $TLR2^{-/-}$ mice. LTA concentration-dependently inhibited production of CXCL1/KC and TNF, which are both produced from the MyD88-dependent pathway, as well as RANTES and IFN-β, which are produced from the TRIF-dependent pathway (Fig. 6D–G). These results indicate that LTA inhibits LPS-induced TLR4 signaling through interactions at the cell surface under serum-free conditions.

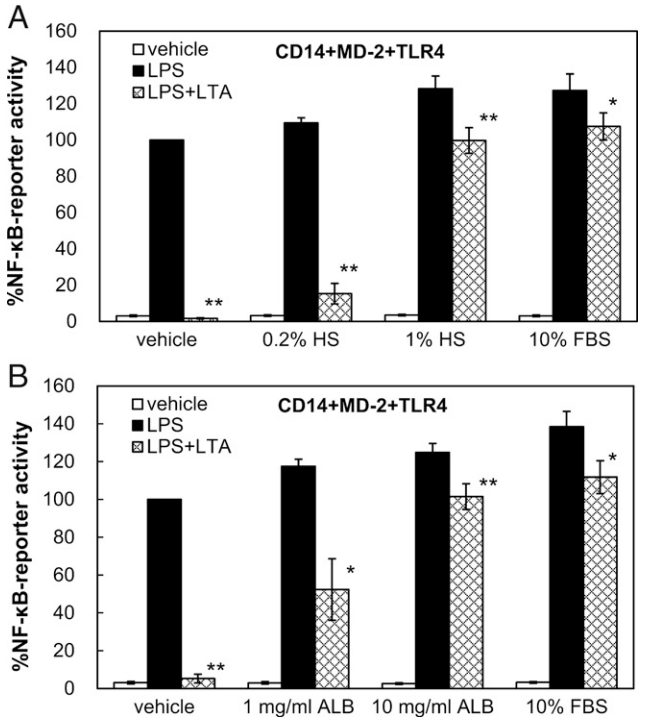

FIGURE 2. Inhibitory effect of LTA on LPS-induced activation of NF-κB is abrogated in the presence of human serum or albumin. HEK293 cells were transiently transfected with expression plasmids for CD14, MD-2, and TLR4 together with an NF-κB–dependent luciferase reporter plasmid. (**A** and **B**) After 24 h, cells were stimulated for 6 h with LPS (10 ng/ml) in the absence or presence of *S. aureus* LTA (10 μg/ml) in serum-free medium containing FBS, increasing concentrations of human serum (HS; A), or human serum albumin (ALB; B). Cellular extracts were then prepared and subjected to luciferase reporter activity measurements. The reporter activity in response to LPS in vehicle alone is expressed as 100%. Values are means ± SEM from four to six independent experiments. $*p < 0.05$, $**p < 0.01$, compared with cells in response to LPS alone.

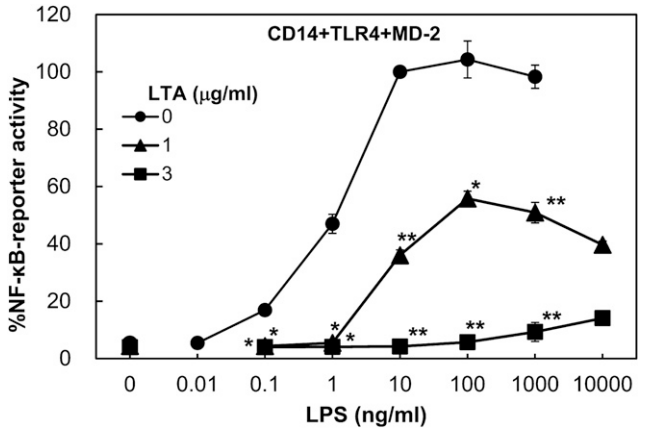

FIGURE 3. LTA noncompetitively inhibits LPS-induced NF-κB activation in the absence of serum. HEK293 cells were transiently transfected with expression plasmids for CD14, MD-2, and TLR4 together with an NF-κB–dependent luciferase reporter plasmid. After 24 h, cells were stimulated for 6 h with increasing concentrations of LPS in the absence or presence of *S. aureus* LTA (1 or 3 μg/ml) in serum-free medium. Cellular extracts were then prepared and subjected to luciferase reporter activity measurements. The reporter activity in response to 10 ng/ml LPS alone is expressed as 100%. Values are means ± SEM from three to four independent experiments. $*p < 0.05$, $**p < 0.01$, compared with cells in response to the corresponding concentration of LPS alone.

LTA induces formation of atypical TLR4/MD-2 complex dimers

The above results suggested that LTA interacts with cell surface components. Thus, we next examined the effect of LTA on the interaction between TLR4 and MD-2 in real time with a NanoBiT protein–protein interaction assay, which involves structural complementation of luciferase to assess protein–protein interactions (27, 28). We created expression plasmids for SmB fused N-terminally to the extracellular domain (aa 25–630) of the TLR4 (SmB-TLR4N) and LgB fused C-terminally to MD-2. HEK293 cells were transfected with these plasmids and cultured in serum-free medium for 48–72 h. Then, the culture supernatants were collected for use in the NanoBiT protein–protein interaction assay. Because TLR4 and MD-2 are known to interact, luciferase activity was detected when the NanoLuc luciferase substrate was added to the culture supernatant. When LTA was added to the culture supernatant, the luciferase activity unexpectedly increased, and this increase could be inhibited by the addition of FBS (Fig. 7A). The increase in the luciferase activity was observed for all LTAs derived from *S. aureus*, *B. subtilis*, and *E. hirae*, but not with LPS (Fig. 7B, 7C).

To examine whether LTA acts on MD-2 or TLR4N, we assessed the interaction of each protein separately. For TLR4N, no increase in luciferase activity was detected in response to LTA, regardless of whether SmB or LgB was fused either C-terminally or N-terminally (Fig. 8A). In contrast, for the combination of SmB or LgB fused C-terminally to MD-2 (MD-2-SmB+MD-2-LgB), a significant increase in the luciferase activity was observed upon addition of LTA (Fig. 8B). These results indicate that LTA induces association of MD-2 molecules, leading to an increase in the formation of TLR4N/MD-2 complex dimers.

From these results, we concluded that LTA induces formation of atypical TLR4/MD-2 complex dimers that differ from those formed by LPS. This atypical TLR4/MD-2 complex dimer cannot activate TLR4-mediated intracellular signaling pathways (Fig. 9).

Discussion

In this study, we showed a previously unrecognized function for LTA. In this study, LTA, which is known as a TLR2 ligand, functioned as an inhibitor of TLR4 signaling under serum-free conditions. A series of experiments to explore the mechanism behind this inhibitory effect revealed that LTA could inhibit initiation of LPS-induced TLR4 activation by promoting formation of an atypical TLR4/MD-2 complex dimer. The mode of this inhibitory effect for LTA differs from that of several other inhibitors of LPS-induced TLR4 activation that were reported in previous studies.

Our results indicated that LTA acted as a noncompetitive inhibitor toward LPS-induced NF-κB activation, as increasing concentrations of LPS could not overcome the inhibitory effect of LTA (Fig. 3). Several other studies reported the ability of synthetic or naturally occurring small molecules, particularly various kinds of glycolipids having a structure that resembles that of LPS, to inhibit LPS-induced TLR4 activation. Most of these molecules are competitive inhibitors of LPS binding to TLR4/MD-2 complexes (29, 30). The competition of LTA with LPS for CD14 or LBP binding has also been reported (31–33). CD14 and LBP are required for full activation of TLR4 by LPS and are also known to augment LTA recognition by TLR2 (8). The noncompetitive nature of the LTA results obtained in this study suggests that the inhibitory mechanism of LTA likely essentially differs from the abovementioned inhibitors, and also indicates that LTA does not inhibit LPS binding to the receptor by capturing LPS under serum-free conditions. In addition, our observation that LTA did not

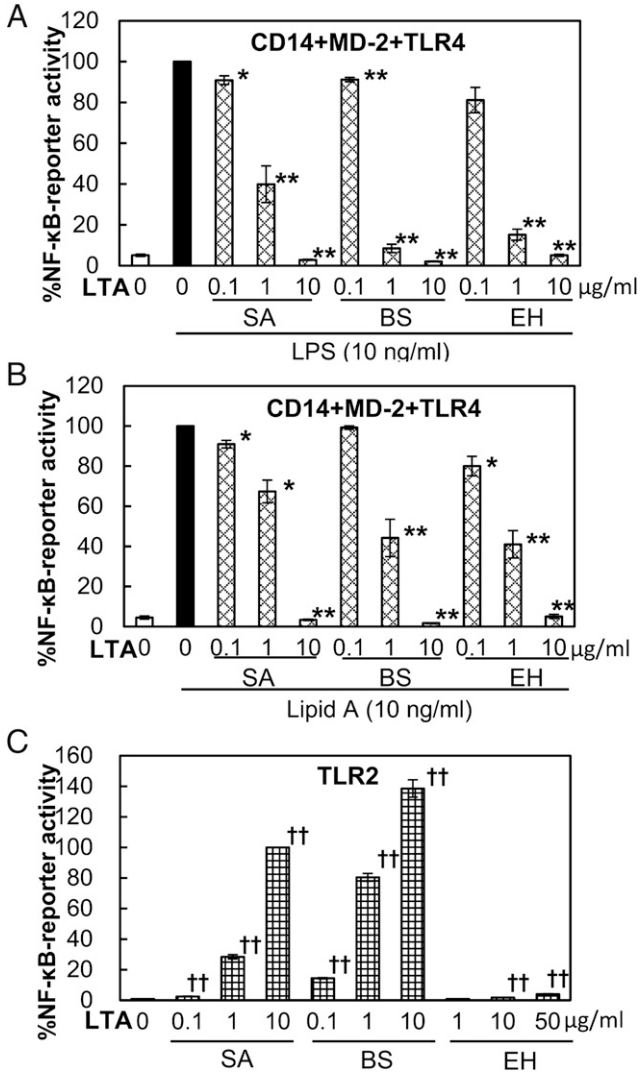

FIGURE 4. LPS-induced activation of NF-κB is inhibited by LTA from different bacteria in the absence of serum. (**A**–**C**) HEK293 cells were transiently transfected with expression plasmids for CD14, MD-2, and TLR4 (A and B) or TLR2 (C) together with an NF-κB–dependent luciferase reporter plasmid. After 24 h, cells were stimulated for 6 h with 10 ng/ml LPS (A) or synthetic lipid A (B) in the absence or presence of increasing concentrations of LTA from *S. aureus* (SA), *B. subtilis* (BS), or *E. hirae* (EH) in serum-free medium, or were stimulated for 6 h with these LTAs in culture medium (C). Cellular extracts were then prepared and subjected to luciferase reporter activity measurements. The reporter activity in response to LPS alone (A and B) or 10 μg/ml SA LTA (C) is expressed as 100%. Values are means ± SEM from three to eight independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with cells in response to LPS alone; $^{\dagger\dagger}p < 0.01$, compared with cells treated vehicle alone.

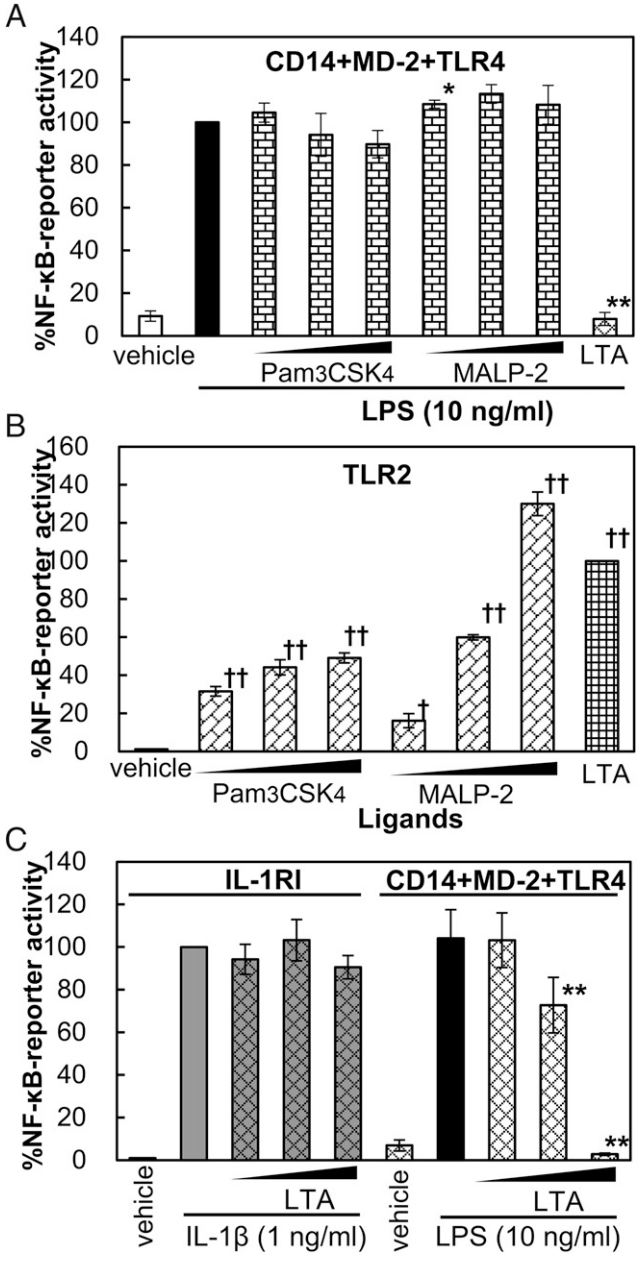

FIGURE 5. Inhibitory effect of LTA on LPS-induced activation of NF-κB is independent of TLR2 or intracellular signaling molecules. (**A**–**C**) HEK293 cells were transiently transfected with expression plasmids for CD14, MD-2, and TLR4 (A and C, right half), TLR2 (B), or type I IL-1R (C, left half) together with an NF-κB–dependent luciferase reporter plasmid. After 24 h, cells were stimulated for 6 h with increasing concentrations of Pam_3CSK_4, MALP-2, or 10 μg/ml LTA from *S. aureus* in the absence (A and B) or presence of 10 ng/ml of LPS (A), or were stimulated for 6 h with increasing concentrations (0.1, 1, and 10 μg/ml) of LTA from *S. aureus* in the absence or presence of 1 ng/ml IL-1β or 10 ng/ml LPS (C) in serum-free medium (A and C) or culture medium (B). Cellular extracts were then prepared and subjected to luciferase reporter activity measurements. The reporter activity in response to LPS alone (A), LTA (B), or IL-1β alone (C) is expressed as 100%. Values are means ± SEM from three to five independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with cells in response to LPS alone; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, compared with cells treated vehicle alone.

inhibit IL-1β–induced NF-κB activation (Fig. 5C) suggested that LTA does not target signal transduction downstream of MyD88 activation because both the IL-1β receptor and TLR4 involve MyD88 and the same subsequent downstream signaling proteins to induce NF-κB activation.

The inhibitory effect of LTA observed in our study was not related to TLR2 signaling, as the inhibitory effect was observed both in BMDMs derived from $TLR2^{-/-}$ mice (Fig. 6) and HEK293 cells that have low levels of TLR2, which was reflected by nonresponsiveness to LTA in terms of NF-κB activation (Fig. 1A, Supplemental Fig. 1). Our observation that LTA derived from *E. hirae*, which induced very little NF-κB activation through TLR2 but still efficiently inhibited LPS-induced NF-κB activation, also indicates that TLR2 activation is not involved in the inhibitory effect of LTA (Fig. 4). Several studies demonstrated that LTA (or LPS) can induce so-called "tolerance" to exposure to a second TLR ligand (34–36). In this tolerance effect, pretreatment with LTA (or LPS) induced desensitization to a second exposure to LTA or other TLR ligands (cross-tolerance), although the

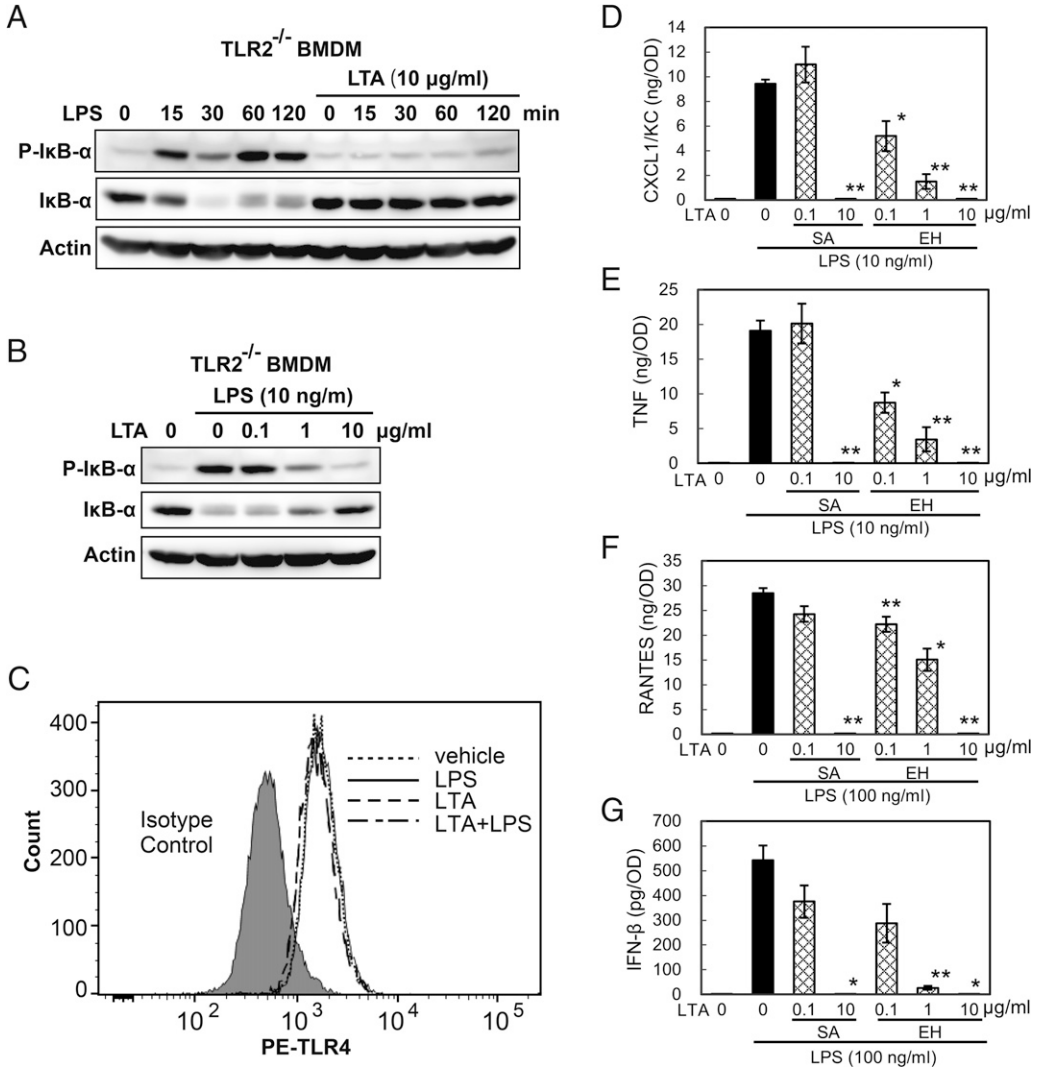

FIGURE 6. LTA inhibits LPS-induced phosphorylation of IκB-α and cytokine production in TLR2$^{-/-}$ mouse BMDMs. (**A** and **B**) BMDMs isolated from TLR2$^{-/-}$ mice were treated with or without LTA from *S. aureus* (A, 10 μg/ml; B, 0.1–10 μg/ml) for 15 min followed by LPS (10 ng/ml) for the indicated time period (A) or 30 min (B) in serum-free medium. (A and B) Cellular extracts were then used for immunoblotting to detect p-IκB-α, IκB-α, and β-actin. BMDMs were treated with or without LTA from *S. aureus* (10 μg/ml) for 15 min followed by vehicle or LPS (10 ng/ml) for 1 h. (**C**) Then, TLR4 cell surface expression was detected by flow cytometry. (**D**–**G**) BMDMs were treated with or without indicated concentrations of LTA from *S. aureus* (SA) or *E. hirae* (EH) for 15 min followed by vehicle or LPS (D and E, 10 ng/ml; F and G, 100 ng/ml) for 6 h (D, F, and G) or 4 h (E). The culture supernatants were then collected and used to measure amounts of CXCL1/KC (D), TNF (E), RANTES (F), and IFN-β (G). After removing the culture supernatant, cell viability was measured as described in *Materials and Methods*. Cytokine and chemokine levels were normalized to the cell viability (OD values) to compensate for differences in cell numbers and viability between wells. Data are representative of two to three independent experiments with similar results (A–C), and values are means ± SEM from three to four independent experiments (D–G). *$p < 0.05$, **$p < 0.01$, compared with cells in response to LPS alone.

establishment of tolerance was dependent on cell type and the bacterial species from which the LTA originated (35). Importantly, LTA-induced tolerance requires TLR2 signal activation (34, 36), which did not occur in BMDMs derived from TLR2$^{-/-}$ mice or the HEK293 cells used in our study. Therefore, the inhibitory effect of LTA we observed is likely not caused by induction of tolerance. This possibility is further supported by our observation that the inhibitory effect of LTA was not induced by other TLR2 ligands (e.g., MALP-2 or Pam_3CSK_4), even though tolerance toward LPS responsiveness has been reported to be induced by MALP-2 (37) and Pam_3CSK_4 (38).

As described above, LTA is suggested to interact with cell surface components. Thus, we examined the effect of LTA using a NanoBiT protein–protein interaction assay and found that LTA induced formation of atypical TLR4/MD-2 complex dimers. As shown schematically in Fig. 9, TLR4 adopts a horseshoe-like shape whereas MD-2 has a β-cup fold structure shaping the globular form (39). Prior to LPS binding, the contact interface of the TLR4/MD2 complex involves two distinct areas in the N-terminal domain and the central domain of TLR4. Upon LPS recognition, the side of MD-2 opposite of the primary TLR4/MD-2 dimerization interface forms a secondary dimerization interface in which LPS bridges to another TLR4 (3, 39, 40). The formation of the TLR4/MD-2 complex dimer then brings the TIR domain of each TLR4 molecule in close proximity (41) to initiate downstream signal transduction. In our NanoBiT protein–protein interaction assay to detect TLR4 and MD-2 interactions, the increase in luciferase activity, which is related to the formation of the TLR4/MD-2 complex dimer, was detected only in the presence of LTA and not LPS (Fig. 7). The result of this luciferase complementation assay indicated that LPS and LTA likely induce formation of a different TLR4/MD2 complex dimer. Namely, LTA induces formation of an atypical TLR4/MD2 complex dimer that

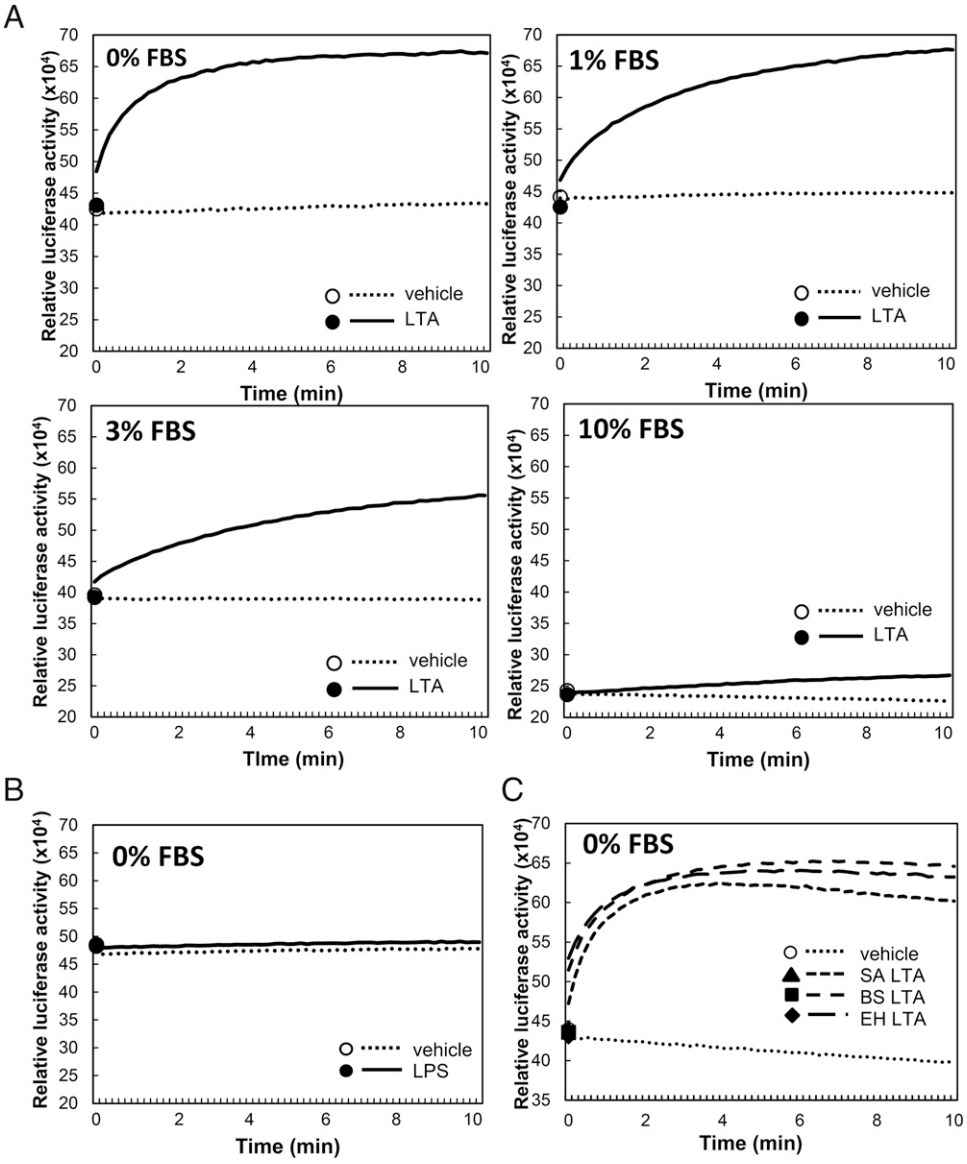

FIGURE 7. LTA induces association of TLR4/MD-2 complexes. HEK293 cells were transiently transfected with expression plasmids for SmB-TLR4N and MD-2-LgB. After 24 h, the culture medium was replaced with serum-free medium and cells were cultured for an additional 48–72 h. Culture supernatants were then collected and subjected to a NanoBiT protein–protein interaction assay in the presence of indicated concentrations of FBS. (**A** and **B**) The luciferase activity before the addition of vehicle or LTA (A), or of vehicle or 100 ng/ml LPS (B), is shown by open circles. The activity after addition of vehicle or 10 μg/ml LTA (A), or of vehicle or LPS (B), is indicated by lines. The luciferase activity in response to LTAs (10 μg/ml) from *S. aureus* (SA), *B. subtilis* (BS), or *E. hirae* (EH) is shown in (**C**). Data are representative of three to five independent experiments with similar results.

allowed the SmB (or LgB) domain of luciferase in one TLR4/MD2 complex to be in close proximity to LgB (or SmB) in another TLR4/MD2 complex. Although further study is needed to elucidate the precise structure of this atypical TLR4/MD-2 complex dimer, one possible assignment of an atypical TLR4/MD2 complex dimer compared with the typical LPS-induced complex is shown in Fig. 9. In the atypical TLR4/MD2 complex dimer, the TIR domain of each TLR4 is likely not close enough to elicit downstream signal transduction. Our observation that the interaction between MD-2 but not TLR4 molecules was induced by LTA (Fig. 8) suggests that the formation of the atypical TLR4/MD-2 complex dimer is mediated through the bridging of MD-2 molecules of each TLR4/MD-2 complex by LTA, whereas the typical TLR4/MD-2 complex dimer induced by LPS is formed by bridging MD-2 of one TLR4/MD-2 complex and TLR4 of another TLR4/MD-2 complex (39). LTAs usually consist of a poly(1,3-glycerophosphate) backbone covalently linked to a glycolipid or phosphatidyl glycolipid (12). Because lipid chains of LPS are known to bind to the hydrophobic pocket in MD-2 (33), it is expected that lipid chains of LTA may be involved in the binding to MD-2. However, other TLR2 ligands such as MAPL-2 and Pam_3CSK_4 also possess lipid chains. Thus, it is likely that both of the lipid chains and the poly(glycerophosphate) chain, which is unique to LTA among TLR2 ligands, may be involved in bridging MD-2 molecules, although further studies are required to know whether LTA directly binds to MD-2. Whether LPS could still bind to an LTA-induced atypical TLR4/MD-2 complex dimer is unclear, but presumably atypical TLR4/MD-2 complex dimer formation predominates relative to typical LPS-induced TLR4/MD-2 complex dimers. Beyond a recent study reporting that ferulic acids, anti-inflammatory molecules found in plants, could inhibit LPS-induced TLR4 activation by disrupting TL4/MD-2 complex formation (42), few other molecules that can disturb typical TLR4/MD-2 complex dimer formation via mechanisms that do not involve LPS competition have been reported.

Interestingly, in this study, we found that the inhibitory effect of LTA on LPS-induced NF-κB activation was abrogated by serum (Figs. 1, 2). Previous proteomic studies reported that several proteins in normal human serum such as apolipoprotein, neutrophil-activating protein, and albumin could bind LTA (43). Among these LTA-binding proteins in serum, albumin is the most abundant with a concentration typically between 35 and 50 mg/ml (44), but despite these high levels, it is reported that albumin did not affect the ability of LTA to induce production of proinflammatory cytokines (45). In our study, 1% human serum, which would contain ~0.5 mg/ml albumin,

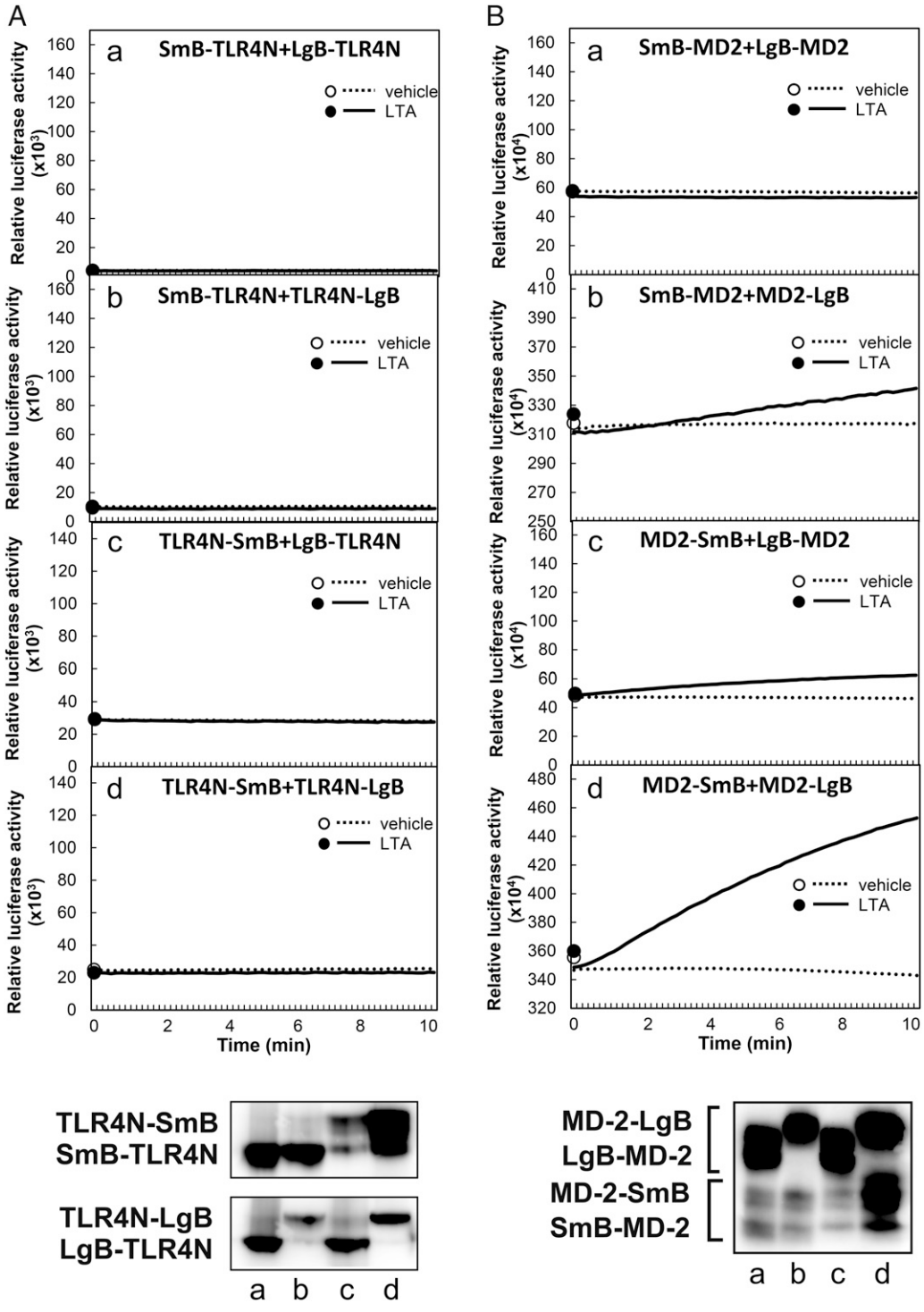

FIGURE 8. LTA induces association of MD-2. (**A** and **B**) HEK293 cells were transiently transfected with expression plasmids for TLR4N fused to SmB or LgB (A) or for MD-2 fused to SmB or LgB (B). After 24 h, the culture medium was replaced with serum-free medium and cells were cultured for an additional 48–72 h before the culture supernatant was collected and subjected to a NanoBiT protein–protein interaction assay. Luciferase activity before the addition of vehicle or LTA is shown by open circles, and the activity after the addition of vehicle or 10 μg/ml LTA is shown by lines. Data are representative of four independent experiments with similar results. SmB or LgB fused to N-terminal or C-terminal of TLR4N or MD-2 is shown as SmB (LgB)-TLR4N or SmB (LgB)-MD-2 and TLR4N-SmB (LgB) or MD-2-SmB (LgB), respectively. Expression levels of each protein detected by immunoblotting are shown at the bottom of each panel.

abrogated the inhibitory effect of LTA. However, alone 1–10 mg/ml albumin was required to abrogate the inhibitory activity of LTA, indicating the involvement of other serum components. We at least found that another major serum protein, globulin, as well as apolipoprotein C3 and neutrophil-activating protein 2 could not abrogate the inhibitory effect of LTA (Supplemental Fig. 3), suggesting that albumin could indeed be a major factor in abrogation of LTA activity. Interestingly, the compound FP13, a synthetic LPS inhibitor, was also suggested to be neutralized by serum or albumin (46). FP13 is an analog of LPS and functions as an LPS inhibitor by occupying the LPS-binding pocket formed by MD-2 in the TLR4/MD-2 complex.

Because LTA could not inhibit LPS-induced activation of NF-κB efficiently in the presence of serum, LTA may not inhibit LPS-induced activation in blood in vivo. This possibility is rational because, upon bacterial invasion of the bloodstream, induction of innate and adequate immune response requires activation of NF-κB, which should not be inhibited by possible coexisting LTA derived from Gram-positive bacteria. In contrast, an inhibitory effect of LTA might be beneficial under certain circumstances such as in gut microbiota. An important role for interactions between gut microbiota and host immune systems is becoming increasingly recognized (47), but the mechanism by which bacterial species of gut microbiota can evade

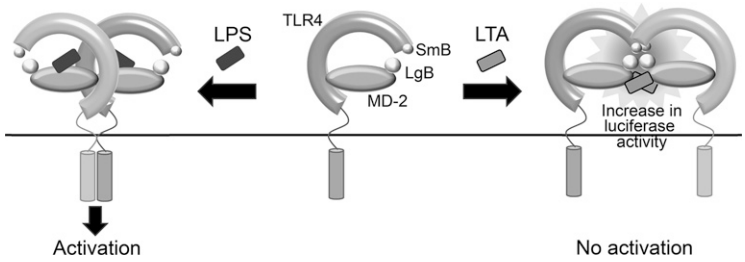

FIGURE 9. Model illustrating active and inactive TLR4/MD-2 complex dimer formation induced by LPS and LTA, respectively. LTA induces association of two MD-2 molecules in complex with TLR4. This association brings LgB fused to MD-2 of one TLR4/MD-2 complex close to SmB fused to TLR4 in another TLR4/MD-2 complex, leading to an increase in luciferase activity. This TLR4/MD-2 complex dimer is not close enough to allow interaction of the TIR domains, and thus intracellular signaling is not activated. Meanwhile, LPS induces formation of a TLR4/MD-2 complex dimer in which the TIR domains can recruit adaptor proteins that leads to activation of intracellular signaling. This dimer formation does not bring LgB fused to MD-2 of one TLR4/MD-2 complex close to SmB fused to TLR4 of another TLR4/MD-2 complex, and thus luciferase activity is not increased.

activation of host immune responses remains unclear. Because LTA derived from *E. hirae*, a common member of gut microbiota, poorly induced TLR2-mediated immune responses, our finding of the ability of LTA to suppress LPS-induced TLR4 activation might be an additional mechanism by which the gut microbiota evade host immunity.

Acknowledgments

We thank Yusuke Murakami (Department of Pharmacotherapy, Musashino University) for providing TLR2$^{-/-}$ mice. We also thank Shunya Abe, Hikaru Takano, Kaito Koizumi, and Misa Kataoka for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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