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Recombinant Soluble Forms of Extracellular TLR4 Domain and MD-2 Inhibit Lipopolysaccharide Binding on Cell Surface and Dampen Lipopolysaccharide-Induced Pulmonary Inflammation in Mice

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In this study, we sought the possibility of a new therapeutic strategy for dampening endotoxin-induced inflammation using soluble form of extracellular rTLR4 domain (sTLR4) and soluble form of rMD-2 (sMD-2). Addition of sTLR4 plus sMD-2 was significantly effective in inhibiting LPS-elicited IL-8 release from U937 cells and NF-κB activation in the cells transfected with TLR4 and MD-2 when compared with a single treatment with sTLR4 or sMD-2. Thus, we investigated the role of the extracellular TLR4 domain in interaction of lipid A with MD-2. Biotinylated sTLR4 failed to coprecipitate [3H]lipid A when it was sedimented with streptavidin-agarose, demonstrating that the extracellular TLR4 domain does not directly bind lipid A by itself. The amounts of lipid A coprecipitated with sMD-2 significantly increased when coincubated with sTLR4, and sTLR4 increased the affinity of lipid A for the binding to sMD-2. Soluble CD14 is required for the sTLR4-stimulated increase of lipid A binding to sMD-2. We also found that addition of sTLR4 plus sMD-2 inhibited the binding of Alexa-conjugated LPS to the cells expressing TLR4 and MD-2. Murine lungs that had received sTLR4 plus sMD-2 with LPS did not show any findings indicative of interstitial edema, neutrophil flux, and hemorrhage. Coinstillation of sTLR4 plus sMD-2, but not sTLR4 or sMD-2 alone, significantly decreased neutrophil infiltration and TNF-α levels in bronchoalveolar lavage fluids from LPS-treated mice. This study provides novel usage of sTLR4 and sMD-2 as an antagonist against endotoxin-induced pulmonary inflammation. The Journal of Immunology, 2006, 177: 8133–8139.

The innate immune system plays central roles in the first line of defense against microbial pathogens and in stimulating the clonal responses of adaptive immunity (1). LPS is a principal component of the outer membrane of Gram-negative bacteria that activates the innate immune system and elicits excessive release of proinflammatory cytokines from immune cells. TLRs have been implicated in recognition and signaling of pathogen-associated molecular patterns (2).

TLR4 has been shown to play a critical role in recognition and signaling of LPS (3). TLR4 is a type I membrane protein consisting of the extracellular domain that possesses a characteristic leucine-rich motif structure and an intracellular signaling domain. MD-2 (4) and CD14 (5) are pivotal components in LPS-induced TLR4 signaling. TLR4 does not respond efficiently to LPS without MD-2 (4). Secreted MD-2 confers LPS sensitivity to TLR4 (6). Soluble MD-2 has been demonstrated to bind bacterial LPS (7). CD14 and LPS-binding protein also bind LPS and play important roles in LPS-induced cellular activation (8). CD14 augments LPS responsiveness, which is thought to be an event occurring upstream of TLR4-MD-2 interaction.

We have previously generated a soluble form of rTLR (sTLR)3 lacking the putative intracellular and transmembrane domains and a soluble form of rMD-2 (sMD-2) (9). sTLR4 directly binds sMD-2, and LPS-conjugated beads precipitate sMD-2, but do not sediment sTLR4. However, LPS beads coprecipitate sTLR4 and MD-2 when both proteins are coincubated, suggesting that the extracellular TLR4 domain-MD-2 complex, but not TLR4 alone, is capable of binding LPS. Addition of sTLR4 and sMD-2 attenuates LPS-elicited NF-κB activation and IL-8 release in TLR4-expressing cells (9). One study has reported a 20-kDa naturally occurring soluble form of mouse TLR4 that is expressed by alternatively spliced TLR4 mRNA (10). The alternative spliced cDNA encodes 86 aa of the extracellular TLR4 domain and an additional 36 aa. Overexpression of this mutant partially inhibits TNF-α production and NF-κB activation in mouse macrophage cell line, suggesting critical roles of the extracellular TLR4 domain in interaction with LPS and in regulation of LPS responsiveness. Natural sTLR2 have been shown to exist in human milk and plasma and to modulate TLR2 signaling (11). However, the mechanisms by which these natural soluble forms of TLRs alter microbe-induced cell responses remain unknown.

In this study, we sought the possibility of a new therapeutic strategy for dampening endotoxin-induced inflammation using sTLR4 and sMD-2. We have found that the extracellular TLR4 domain does not directly bind LPS by itself, but that it augments lipid A binding to sMD-2 and increases the affinity of lipid A for
the binding. Coadministration of sTLR4 and sMD-2 inhibits the binding of LPS to the TLR4- and MD-2-expressing cells, and dampens pulmonary inflammation caused by LPS in mice.

Materials and Methods

Cells and reagents
Macrophase-like cell line U937 (JCRB9021; obtained from Health Science Research Resource Bank, Osaka, Japan) and RAW264.7 (TIB-71; obtained from American Type Culture Collection) were maintained in RPMI 1640 (Sigma-Aldrich) containing 10% FCS. Human embryonic kidney (HEK) 293 cells (CRL-1573; obtained from American Type Culture Collection) were maintained in DMEM (Invitrogen Life Technologies) containing 10% FCS. LPS from Salmonella minnesota Re595 was purchased from Sigma-Aldrich. [3H]Lipid A was emulsified as described previously (12, 13).

Recombinant proteins
sTLR4 consisting of the putative extracellular domain (Met1-Lys631) and a 6×His tag at the C-terminal end, sTLR2 consisting of the putative extracellular domain (Met1-Arg682) and a 6×His tag at the C-terminal end, and sMD-2 containing V5 tag and 6×His tag were generated by the baculovirus-insect cell expression system, and the recombinant proteins were purified, as described previously (9, 14). sTLR4 and sTLR2 start at Glu21 and Glu21, respectively.

Antibodies
For preparation of anti-sTLR4 polyclonal Ab, 100 µg of sTLR4 protein was emulsified in TiterMax Gold (CytRx) and given intradermally to rabbits. After 3 wk, a booster with 80 µg of sTLR4 in the same adjuvant was injected. The blood samples were collected 2 wk later, and IgG was isolated from the sera using a protein G-Sepharose 4FF column (Amersham Biosciences). Anti-V5 mAb-conjugated agarose was obtained from Sigma-Aldrich. Anti-V5 polyclonal Ab was purchased from Medical & Biological Laboratories.

Binding of biotinylated sTLR4 to MD-2
sTLR4 was biotinylated using sulfo-N-hydroxysuccinimide-biotin (Pierce), according to the manufacturer’s instructions. Biotinylated sTLR4 (1.5 µg) was mixed with or without sMD-2 (1 µg) in PBS containing 10% FCS and incubated at 37°C for 2 h. Streptavidin-agarose beads were then added into the mixture, and the suspension (500 µl) was further incubated for 6 h. After the incubation, the agarose beads were washed with PBS containing 0.1% Triton X-100. The final pellets were subjected to SDS-PAGE, and the proteins on gel were transferred onto polyvinylidene difluoride membrane. Western blot analysis was performed to detect sMD-2 and sTLR4 by using anti-V5 polyclonal Ab and anti-sTLR4 polyclonal Ab, respectively.

Binding of biotinylated sTLR4 to [3H]Lipid A
Biotinylated sTLR4 (750 ng) or BSA (750 ng) was incubated with the indicated amounts of [3H]Lipid A in the presence or the absence of sMD-2 (500 ng) in PBS containing 10% FCS for 2 h at 37°C. Streptavidin-agarose beads were then added into the mixture, and the suspension (500 µl) was further incubated for 6 h. After the incubation, the agarose beads were washed with PBS containing 0.1% Triton X-100. The radioactivities of the final pellets were determined by a liquid scintillation counter.

Binding of sMD-2 to sTLR4
V5 tag-sMD-2 (100 ng) was mixed with or without sTLR4 (2.5 µg) in PBS containing 10% FCS and incubated at 37°C for 2 h. Anti-V5 mAb-agarose (10 µl, 50% suspension in PBS) was then added into the suspension, and total volume of the suspension was adjusted to 500 µl by the addition of PBS containing 3% BSA. The suspension was incubated at 4°C for 6 h with gentle rocking. After the incubation, the agarose beads were washed with PBS containing 0.1% Triton X-100. The final pellets were subjected to SDS-PAGE, and the proteins on gel were transferred onto polyvinylidene difluoride membrane. Western blot analysis was performed to detect sMD-2 and sTLR4 by using anti-V5 polyclonal Ab and anti-sTLR4 polyclonal Ab, respectively.

Binding of sMD-2 to [3H]Lipid A
sMD-2 (100 ng) or BSA (100 ng) was incubated at 37°C for 2 h with the indicated amounts of [3H]Lipid A in the presence or the absence of sMD-2 in PBS containing 10% FCS. Anti-V5 mAb-conjugated agarose (10 µl, 50% suspension in PBS) was then added into the suspension, and total volume of the suspension was adjusted to 500 µl by the addition of PBS containing 3% BSA. The suspension was incubated at 4°C for 6 h with gentle rocking. After the incubation, the agarose beads were washed with PBS containing 0.1% Triton X-100. The radioactivities of the final pellets containing agarose beads were measured by a liquid scintillation counter.

Effects of sTLR4 and sMD-2 on LPS-induced IL-8 secretion from U937 cells
U937 cells (1 × 10⁶) were differentiated by incubating the cells with 10 nM PMA in RPMI 1640 containing 10% FCS for 24 h. The cells were further incubated in the absence of PMA for 24 h. The cells were then incubated for 6 h with the medium containing sTLR4 (5 µg/ml) and/or sMD-2 (0.25 µg/ml), which had been preincubated for 1 h with the indicated concentrations of LPS. After the LPS stimulation, the concentrations of IL-8 secreted from U937 cells were determined by ELISA using an OptEIA human IL-8 set (BD Pharmingen) according to the manufacturer’s instructions.

Effects of sTLR4 and sMD-2 on LPS-induced NF-κB activation in HEK293 cells cotransfected with TLR4 and MD-2
Activation of NF-κB was measured, as previously described (14, 15). HEK293 cells were plated at 0.15 × 10⁶ cells/well in 96-well plates on the day before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Molecular Biochemicals) with 7.5 ng of an NF-κB reporter construct (pNF-κB-Luc: Stratagene) and 3 ng of a constitutively expressing expression of Renilla luciferase (pRL-TK; Promega), together with 10 ng of each cDNA for wild-type (wt) TLR4 and wt MD-2. Twenty-four hours after transfection, the cells were stimulated for 6 h with 4 ng/ml LPS that had been preincubated with sMD-2 (0.25 µg/ml) and/or sTLR4 (5 µg/ml) for 1 h, and luciferase activity was measured by the dual luciferase reporter assay system (Promega), according to the manufacturer’s instructions.

Cell surface binding of Alexa-conjugated LPS
RAW264.7 cell and HEK293 cell line expressing TLR4, MD-2, and CD14 (InvivoGen) (1 × 10⁶ cells/ml) were incubated at 4°C for 45 min with Alexa Fluor 488-conjugated LPS (0.5 µg/ml, LPS from Escherichia coli 055:B5; Molecular Probes) that had been preincubated with sTLR4 (10 µg/ml) and sMD-2 (10 µg/ml) at 37°C for 30 min in RPMI 1640 or DMEM containing 10% FCS. After the incubation, the cells were washed with PBS containing 0.5% BSA. The binding of LPS to cell surface was analyzed using FACScalibur and CellQuest software (BD Biosciences).

Expression of cell surface TLR4 in HEK293 cells used in the experiments was confirmed by using PE-conjugated anti-TLR4 Ab (eBioscience).

Murine model of LPS-induced pulmonary inflammation
Female BALB/c mice from 7 to 8 wk of age were anesthetized by i.p. injection of 90 mg/kg ketamine HCl and 20 µg/kg xylazine hydrochloride. LPS (50 µg/kg in 20 µl of PBS) was instilled intratracheally with sTLR2 (150 µg/kg), sTLR4 (150 µg/kg), sMD-2 (125 µg/kg), or sTLR4 plus sMD-2, after tracheostomy. Sixteen hours after LPS instillation, mice were euthanized and a stainless 18G tube was installed as a tracheostomy cannula. The lungs were lavaged via the tracheal tube with Hanks’ balanced solution (1 × 1 ml; Sigma-Aldrich). The volume of collected bronchoalveolar lavage fluids was measured in each sample, and the number of leukocytes was counted. Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations (Autosmear, Sakura) after May–Giemsa staining. The concentrations of TNF-α in the lavage fluids were determined by ELISA using an OptEIA mouse TNF-α set (BD Pharmingen), according to the manufacturer’s instructions. To assess histological change of LPS-induced lung injury, lungs were inflated through the trachea with 1 ml of 10% formalin and fixed in 10% formalin by immersion for 24 h. The lung lobes were then washed for 8 h with tap water to remove formaldehyde. For light microscopy, the lung tissue was dehydrated with graded ethanol, put into xylene for 24 h, and then embedded in paraffin at 60°C. A series of 4.0-µm sections were cut and stained with H&E.

Results

sTLR4 plus sMD-2 is effective as an LPS antagonist
A previous study (9, 16) from this laboratory has revealed that the exogenously added TLR4 protein down-regulates LPS-induced signaling and cytokine release in TLR4-expressing cells. Thus, we...
first examined the efficiency of sTLR4 and/or sMD-2 in attenuating LPS-induced cell responses. Addition of sTLR4 alone into the culture medium did not affect LPS-induced IL-8 production (Fig. 1A). sMD-2 tended to decrease IL-8 secretion from U937 cells slightly. When both sTLR4 and sMD-2 were added, IL-8 secretion was significantly inhibited at all concentrations of LPS tested.

We next examined the effects of sTLR4 and/or sMD-2 on LPS-induced NF-κB activation in HEK293 cells cotransfected with wt TLR4 and wt MD-2. HEK293 cells (0.15 × 10⁶) were transfected with TLR4 cDNA and MD-2 cDNA together with an NF-κB reporter plasmid (pNF-κB-Luc) and Renilla luciferase control reporter plasmid (pRL-TK). Twenty-four hours after transfection, cells were incubated for 6 h with or without sMD-2 (0.25 μg/ml) and/or sTLR4 (5 μg/ml) that had been preincubated with LPS (4 ng/ml) at 37°C for 1 h. NF-κB activities were then determined. The data shown are the means ± SE from three separate experiments. *p < 0.05 when compared with the experiments with LPS alone.

**The role of the extracellular TLR4 domain in interaction of lipid A with MD-2**

We next pursued the role of the extracellular TLR4 domain in interaction of lipid A with MD-2. sTLR4 and sMD-2 were expressed by baculovirus-insect cell system, and recombinant proteins were purified from the medium by a column of nickel-nitritotriacetic acid beads. The purified proteins of sTLR4 and sMD-2 exhibited bands with apparent molecular masses of 80 and 23–30 kDa, respectively, under reducing conditions when analyzed by electrophoresis, as described previously (9). We first examined whether the extracellular TLR4 domain directly binds lipid A using biotinylated sTLR4 and streptavidin-agarose. Streptavidin-agarose coprecipitated the biotinylated sTLR4 protein (Fig. 2A). When biotinylated sTLR4 was incubated with increasing amounts of [³H]lipid A and was coprecipitated with streptavidin-agarose, almost no lipid A was associated with the precipitates of sTLR4 and the beads (Fig. 2B). The results clearly demonstrate that the extracellular TLR4 domain does not directly bind lipid A by itself. In contrast, a complex of sTLR4 and sMD-2, which was coprecipitated with the streptavidin-agarose (Fig. 2A), precipitated significant amounts of [³H]lipid A in a manner dependent upon lipid A concentration (Fig. 2B), indicating that the sTLR4-sMD-2 complex can bind lipid A.

We next examined the binding of sMD-2 to lipid A using anti-V5 mAb-conjugated agarose beads. When sMD-2 and sTLR4 were coincubated and sMD-2 possessing V5 tag was immunoprecipitated, sTLR4 was coprecipitated with sMD-2 (Fig. 3A). The result confirms the direct binding of the extracellular TLR4 domain to MD-2, and indicates the formation of TLR4-MD-2 complex.

When sMD-2 was incubated with [³H]lipid A in the absence of sTLR4 and [³H]lipid A binding to the immunoprecipitated sMD-2 was determined, sMD-2 coprecipitated significant amounts of [³H]lipid A (Fig. 3B), indicating direct binding of lipid A to MD-2. Addition of sTLR4 significantly increased the amounts of [³H]lipid A binding to the immunoprecipitated sMD-2, which was considered as a complex with sTLR4. The increase in the amounts of [³H]lipid A binding to the immunoprecipitated sMD-2 depended on the amount of sTLR4 added. When 2.5 μg of sTLR4 was incubated, the amount of lipid A associated with sMD-2 increased by a factor of 2.1 compared with that in the absence of sTLR4. These results clearly demonstrate that the extracellular TLR4 domain augments the binding of [³H]lipid A to a complex with sMD-2.

We next determined whether sTLR4 alters the properties of [³H]lipid A binding to sMD-2. Various concentrations of [³H]lipid A were incubated with sMD-2 in the presence or the absence of sTLR4, and [³H]lipid A binding to the immunoprecipitated sMD-2 was determined. When BSA was used instead of sMD-2, no [³H]lipid A was precipitated. Lipid A bound to sMD-2 in a concentration-dependent manner, and its binding reached saturation at ~250 ng of [³H]lipid A (Fig. 3C). Consistent with the results obtained (Fig. 2B), addition of sTLR4 into the reaction mixture containing sMD-2 significantly increased the binding of lipid A to the immunoprecipitated sMD-2 at all concentrations of lipid A tested.
are the means pellets were determined by a liquid scintillation counter. The data shown rocking, the agarose beads were washed and the radioactivities of the final mixture. After the suspension was incubated at 4°C for 6 h with gentle ti-V5 mAb-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4°C for 6 h with or without sTLR4 (2.5 g) at 37°C for 2 h. Anti-V5 mAb-conjugated agarose beads were then added into the reaction mixture. After the suspension was incubated at 4°C for 6 h with gentle rocking, the agarose beads were washed and the radioactivities of the final pellets were determined by a liquid scintillation counter. The data shown are the means ± SE from three separate experiments. *p < 0.05 when compared with the experiments in the absence of sTLR4. C. Concentration-dependent binding of lipid A to sMD-2. sMD-2 (100 ng) in the absence of LPS, sCD14 was not detected in the supernatant. D. Scatchard plot analysis of the data in C. The experiments shown above were performed in the presence of FCS. Thus, we examined whether serum factors including sCD14 and LBP are required for the sTLR4-mediated increase of lipid A binding to sMD-2. When sMD-2 was incubated with [3H]lipid A in the presence of sTLR4 in PBS containing 10% FCS or PBS containing 3% BSA, the enhanced binding of lipid A to the immunoprecipitated sMD-2 was not observed under serum-free condition (Fig. 4A). These results indicate that FCS is critical for sTLR4-mediated increase of lipid A to sMD-2.

Because FCS contains sCD14 and LBP, we examined whether addition of sCD14 or LBP restored the sTLR4-enhanced lipid A binding to sMD-2 in the absence of FCS. Addition of sCD14 significantly increased the binding of [3H]lipid A to sMD-2 in the presence of sTLR4 in a manner dependent upon sCD14 concentration, although sCD14 did not affect the lipid A binding to sMD-2 in the absence of sTLR4 (Fig. 4B). When the binding studies with various concentrations of lipid A were performed in the presence of 2.5 μg of sTLR4, the binding of lipid A to sMD-2 was concentration dependent and saturable regardless of the presence of sCD14 (Fig. 4C). However, the amount of lipid A binding to sMD-2 in the presence of sCD14 was significantly greater than that in the absence of sCD14 at all concentrations of lipid A tested. In contrast, LBP did not significantly alter the binding of [3H]lipid A to sMD-2 in the presence or the absence of sTLR4 (data not shown). Taken together, these results demonstrate that sCD14, but not LBP, is required for sTLR4-stimulated lipid A binding to sMD-2.

We next investigated whether sCD14 is coprecipitated with sMD-2 or a complex of sMD-2 and sTLR4. sMD-2 was incubated with or without sTLR4 and/or LPS, and was immunoprecipitated, and it was examined whether sTLR4 and sCD14 were coprecipitated (Fig. 4D). Although sTLR4 was coprecipitated with sMD-2 in the presence but not with sCD14.

**FIGURE 3.** sTLR4 enhances the binding of [3H]lipid A to sMD-2. A, sTLR4 is coprecipitated with sMD-2. sMD-2 (100 ng) possessing V5 tag was mixed with or without sTLR4 (2.5 μg) and incubated at 37°C for 2 h. Anti-V5 mAb-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4°C for 6 h with gentle rocking. After the incubation, the agarose beads were washed and the final pellets obtained were subjected to SDS-PAGE. Western blot (WB) analysis was performed to detect sMD-2 and sTLR4 by using anti-V5 Ab and anti-sTLR4 Ab, respectively. B, The binding of [3H]lipid A to sMD-2 in the presence or the absence of sTLR4. sMD-2 (100 ng) was incubated with 250 ng of [3H]lipid A in the absence or the presence of increasing amounts (0.31, 0.62, 1.25, 2.5, and 5 μg) of sTLR4 at 37°C for 2 h. Anti-V5 mAb-conjugated agarose beads were then added into the reaction mixture. After the suspension was incubated at 4°C for 6 h with gentle rocking, the agarose beads were washed and the radioactivities of the final pellets were determined by a liquid scintillation counter. The data shown are the means ± SE from three separate experiments. *p < 0.05 when compared with the experiments in the absence of sTLR4. C, Concentration-dependent binding of lipid A to sMD-2. sMD-2 (100 ng) or BSA (100 ng) was incubated with the indicated amounts of [3H]lipid A in the presence or the absence of sTLR4 (2.5 μg) at 37°C for 2 h. Anti-V5 mAb-conjugated agarose beads were then added, and the amounts of lipid A binding to the immunoprecipitated sMD-2 were determined, as described above. The data shown are the means ± SE from three separate experiments. *p < 0.05 when compared with the experiments with sMD-2 alone. D, sMD-2; ○, BSA; ●, sTLR4 plus sMD-2. D, Scatchard plot analysis of the data in C. ●, sTLR4 plus sMD-2; □, sMD-2. E and F, Electrophoretic analysis of sMD-2. E, Five micrograms of rsMD-2 was resolved by 13% SDS-PAGE under reducing (R) and nonreducing (NR) conditions, and the proteins were visualized by Coomassie brilliant blue staining. F, For native PAGE analysis, 15 μg of sMD-2 was subjected to a NativePAGE™ Novex 4–16% bis-Tris gel (Invitrogen Life Technologies). St, Molecular mass standards.
Addition of sTLR4 and sMD-2 inhibits LPS binding on cell surface

Because addition of sTLR4 plus sMD-2 was the most effective in inhibiting LPS-elicted signaling and cytokine release, as shown in Fig. 1, we next examined whether addition of sTLR4 plus sMD-2 affects cell surface binding of Alexa-conjugated LPS on RAW264.7 cell (Fig. 5A) and HEK293 cell line expressing TLR4, MD-2, and CD14 (Fig. 5B). When 0.5 μg/ml Alexa-conjugated LPS was incubated with the cells at 4°C, a significant labeling of LPS was observed on the surface of these cell lines (Fig. 5, solid line). Inclusion of sTLR4 and sMD-2 in LPS-containing medium inhibited cell surface labeling of Alexa-conjugated LPS in these cells (Fig. 5, dotted line). These results clearly demonstrate that the sTLR4-sMD-2 complex inhibits cell surface binding of LPS, and suggest that the exogenously added sTLR4-sMD-2 complex competes with membrane-bound receptor complex of TLR4 and MD-2 for LPS binding.
**Intratracheal instillation of sTLR4 and sMD-2 dampens LPS-induced pulmonary inflammation in vivo**

Intratracheal instillation of LPS causes pulmonary inflammation. Thus, we examined whether coadministration of sTLR4 and sMD-2 with LPS protects the lungs against LPS-induced pulmonary inflammation. LPS instillation induced interstitial edema, neutrophil influx, and hemorrhage in the lung tissue (Fig. 6B), whereas PBS-treated murine lungs did not show any findings indicative of pulmonary inflammation (Fig. 6A). When sTLR4 and sMD-2 were coadministered with LPS, the histological findings indicative of LPS-induced inflammation were not observed (Fig. 6C).

We also analyzed inflammatory indicators obtained from bronchoalveolar lavage fluids after LPS challenge. Intratracheal LPS instillation induced neutrophil infiltration and increased TNF-α in the lavage fluids (Fig. 6, D and E). When sTLR2, sTLR4, or sMD-2 was separately administered intratracheally, no significant reduction of neutrophil infiltration or TNF-α level was observed. In contrast, coadministration of sTLR4 and sMD-2 resulted in significant decreases of neutrophil infiltration and TNF-α concentration in the lavage fluids. These results indicate that a complex of sTLR4 and sMD-2 modulates TLR4-mediated signaling to minimize acute lung inflammation caused by LPS challenge to murine lung.

Taken together, the results indicate that intratracheal instillation of sTLR4 with sMD-2 dampens LPS-induced pulmonary inflammation in vivo, and suggest that the exogenous administration of sTLR4 and sMD-2 can be used as a new therapeutic strategy.

**Discussion**

In this study, we pursued the possibility that our recombinant soluble form of the extracellular TLR4 domain can modulate LPS-induced inflammation in cooperation with sMD-2. We have shown that the complex of sTLR4 and sMD-2 is the most potent inhibitor for LPS-induced IL-8 release and NF-κB activation in U937 cells and HEK293 cells cotransfected with TLR4 and MD-2 when compared with sTLR4 or sMD-2 alone (see Fig. 1). A recent study (16) has shown that TLR4-Fc fusion protein down-regulates LPS-induced cellular responses in TLR4-expressing cells. Streptavidin fails to pull down wt TLR4 in the presence of TLR4-Fc when the binding of biotinylated LPS to MD-2 is used as a means to precipitate wt TLR4, suggesting that the TLR4-Fc fusion protein inhibits the ability of MD-2 to bind TLR4. In this study, we have shown that the presence of sTLR4 plus sMD-2 inhibits LPS binding to the cells expressing TLR4 and MD-2 (see Fig. 5). Thus, the mechanism of down-regulation by sTLR4 plus sMD-2 is suggested to be competition for LPS binding between sTLR4-sMD-2 complex and membrane-bound receptor complex of wt TLR4 and MD-2. In addition, the in vivo experiments demonstrate that intratracheal instillation of sTLR4 with sMD-2 dampens LPS-induced pulmonary inflammation (see Fig. 6). Although sMD-2 alone can bind LPS by itself, it does not exhibit strong inhibition of LPS-induced inflammation in vitro and in vivo. Because the affinity of lipid A for sMD-2 binding and the amount of lipid A binding to MD-2 in the absence of sTLR4 are less than those in the presence of sTLR4 (see Fig. 3), sMD-2 by itself may not be enough to compete with the receptor complex of wt TLR4 and wt MD-2 for LPS recognition. Addition of both sTLR4 and sMD-2 results in strong inhibition of LPS-induced inflammation, presumably because sTLR4 dramatically increases the affinity and the amount of LPS binding, which becomes sufficient to compete with wt TLR4-MD-2 complex for LPS binding.

This study clearly demonstrates that the extracellular TLR4 domain does not directly bind lipid A by itself. This is consistent with previous studies showing that TLR4 cannot transmit LPS signaling without MD-2 (4) and that the extracellular TLR4 domain alone is not coprecipitated with LPS-conjugated beads (9). However, this is in stark contrast to TLR2. Transfection of TLR2 cDNA into HEK293 cells confers responsiveness to peptidoglycan and zymosan (18, 19), and the extracellular TLR2 domain directly binds to these ligands (14, 20). Although it is now well recognized that interaction of TLR4 with MD-2 is critical for recognition and signaling of LPS, little is known about the role of the extracellular TLR4 domain. Thus, in this study, we focused on the extracellular TLR4 domain in interaction with MD-2 and lipid A. We have demonstrated that the extracellular TLR4 domain increases lipid A binding to MD-2 in terms of the affinity and the amount of binding, implicating the extracellular TLR4 domain in LPS-MD-2 interaction. One recent study (21) has shown that lipid A interacts with cell surface TLR4-MD-2 with higher affinity than MD-2 or CD14 expressed on the cell surface. The binding of MD-2 to TLR4 is dependent upon Cys106 and Cys110 of MD-2 that form a interchain disulfide bond (22, 23). The MD-2 region of amino acid residues 119–132, which is rich in basic and aromatic residues, has been found to be responsible for LPS responsiveness (23, 24), indicating that separate functional domains of MD-2 mediate TLR4 binding and LPS responsiveness. Thus, the binding of TLR4 to MD-2 may cause a conformational change that brings greatly increased lipid A binding into the MD-2 molecule. In contrast, there is also a possibility that TLR4 acquires a lipid A-binding property after a complex of TLR4 and MD-2 is formed, although we have demonstrated that the extracellular TLR4 domain does not directly bind lipid A by itself, at least not in the absence of MD-2. The region of leucine-rich repeats in the extracellular TLR4 domain may still be available for ligand binding even after MD-2 binds to TLR4 because the N-terminal region of TLR4 containing no leucine-rich repeats has been shown to be responsible for MD-2 binding (25, 26).

Plasma from patients with severe sepsis and septic shock and even normal plasma appears to contain soluble MD-2 that elicits LPS activation in TLR4-transfected HEK293 cells and HUVECs (16, 27), whereas the patients’ plasma tends to somewhat decrease, although not significantly, LPS responsiveness in leukocytes and differentiated THP-1 cells expressing both TLR4 and MD-2 on cell surface. Previous studies (6, 28) have shown that soluble MD-2 interacts with the extracellular TLR4 domain and confers LPS responsiveness on TLR4-transfected cells. In contrast, this study shows that addition of soluble MD-2 significantly down-regulates LPS-elicited responses in vitro and in vivo when it is coadministered with sTLR4. Taken together, these studies indicate that the opposing effects of the activities of soluble MD-2 depend on the extracellular TLR4 domain.

Only a small amount of lipid A bound to sMD-2 and a complex of sMD-2 plus sTLR4 when 500-2000 ng of lipid A was added (see Figs. 2B and 3, B and C). Native PAGE analysis indicated that most of the sMD-2 existed as noncovalent aggregates and that there was a very small population of monomeric sMD-2 (see Fig. 3F). Because only the monomeric form of MD-2 can interact with LPS (16), the small amount of lipid A binding to sMD-2 may have been due to the fact that there was little monomer in our sMD-2 preparation.

sTLR4 (5 μg/ml) plus sMD-2 (0.25 μg/ml) significantly down-regulates IL-8 secretion from differentiated U937 cells stimulated with low concentrations (~25 ng/ml) of LPS (see Fig. 1A). When LPS concentrations were increased up to 0.1 and 1 μg/ml, sTLR4
plus sMD-2 failed to inhibit IL-8 secretion. Much higher concentrations of sTLR4 and sMD-2 may be required to inhibit the cytokine production elicited with high doses of LPS. If the proportion of sMD-2 monomer in our preparation increases, it may be possible to efficiently down-regulate the cytokine production induced by high doses of LPS, because our preparation contains only a small population of sMD-2 monomer (see Fig. 3F) and only the MD-2 monomer can interact with LPS (16). There is also a possibility that high concentrations of LPS transmit signaling through other mechanisms besides the TLR4-MD-2-mediated mechanism.

We also tested whether sTLR4 plus sMD-2 modulates serum TNF-α concentration after i.p. LPS injection. Peritoneal injection of LPS (250 μg/kg) into mice increased serum TNF-α concentration up to 68.7 ± 15.1 pg/ml (means ± SE; n = 11), resulting in systemic inflammation. When sTLR4 and sMD-2 were coadministered with LPS, serum TNF-α levels were decreased to 40.1 ± 16.3 pg/ml (n = 11), suggesting the possibility that sTLR4 plus sMD-2 could become a target for the development of a novel therapy against systemic inflammation caused by a TLR4-mediated mechanism. Further studies to establish appropriate doses and ratios of the recombinant proteins to be used and to determine the increase of sMD-2 monomer population in the recombinant protein preparation are required for efficient inhibition of systemic inflammation.

This study demonstrates that sTLR4 enhances the binding of lipid A to sMD-2 and increases the affinity of lipid A for the binding, although sTLR4 does not directly bind lipid A by itself. Co-administration of sTLR4 and sMD-2 inhibits the binding of LPS to the TLR4-expressing cells and down-regulates LPS-induced inflammation in vitro and in vivo, raising the possibility of a new therapeutic strategy for dampening endotoxin-induced pulmonary inflammation.

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Disclosures

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References


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