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Interaction of Soluble Form of Recombinant Extracellular TLR4 Domain with MD-2 Enables Lipopolysaccharide Binding and Attenuates TLR4-Mediated Signaling

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TLRs have been implicated in recognition of pathogen-associated molecular patterns. TLR4 is a signaling receptor for LPS, but requires MD-2 to respond efficiently to LPS. The purposes of this study were to examine the interactions of the extracellular TLR4 domain with MD-2 and LPS. We generated soluble forms of rTLR4 (sTLR4) and TLR2 (sTLR2) lacking the putative intracellular and transmembrane domains. sTLR4 consisted of Glu24-Lys631. MD-2 bound to sTLR4, but not to sTLR2 or soluble CD14. BiACore analysis demonstrated the direct binding of sTLR4 to MD-2 with a dissociation constant of \( K_D = 6.29 \times 10^{-8} \) M. LPS-conjugated beads precipitated MD-2, but not sTLR4. However, LPS beads coprecipitated sTLR4 and MD-2 when both proteins were coincubated. The addition of sTLR4 to the medium containing the MD-2 protein significantly attenuated LPS-induced NF-κB activation and IL-8 secretion in wild-type TLR4-expressing cells. These results indicate that the extracellular TLR4 domain-MD-2 complex is capable of binding LPS, and that the extracellular TLR4 domain consisting of Glu24-Lys631 enables MD-2 binding and LPS recognition to TLR4. In addition, the use of sTLR4 may lead to a new therapeutic strategy for dampening endotoxin-induced inflammation. The Journal of Immunology, 2004, 173: 6949–6954.

The innate immune system protects against invasion of microorganisms as the first line of defense and stimulates the clonal responses of adaptive immunity (1). TLRs have been implicated in recognition and signaling of pathogen-associated molecular patterns (2). TLR2 and TLR4 have been shown to play critical roles in recognition and signaling of LPS, peptidoglycan, lipoteichoic acid, microbial lipoprotein, mycobacterial lipoprotein and zymosan (3–11). The characteristics of TLR4-deficient mice provide compelling evidence that TLR4 is a signaling receptor for LPS (12, 13). C3H/HeJ mice with a Pro712→His mutation in Tlr4 gene exhibit a defective responsiveness to LPS (14). TLR4 requires an accessory protein, MD-2, to respond efficiently to LPS (15). Secreted MD-2 confers LPS sensitivity to TLR4 (16). Immunoprecipitation analysis has revealed that MD-2 can be associated with TLR4 (17). The studies with site-directed mutagenesis of MD-2 indicate the importance of disulfide bonds and N-linked glycosylation in TLR4 signaling (18, 19). MD-2 has been demonstrated to bind bacterial LPS (20), but the region of MD-2 required for the association with TLR4 appears to be different from that for LPS responsiveness (21). The mechanisms of ligand recognition by TLRs have not been completely understood. The extracellular TLR2 domain directly binds to peptidoglycan derived from Staphylococcus aureus (22) and zymosan (23), and the extracellular TLR2 region containing Ser40-Ille64, but not Cys30-Ser39 is critical for peptidoglycan recognition (24), indicating the importance of the extracellular TLR2 domain in ligand recognition. LPS is cross-linked with TLR4 and MD-2 only when coexpressed with CD14 (25), suggesting that LPS is in close proximity to the receptor complex.

TLR4 is homologous to other TLRs and CD14 in point of having leucine-rich repeats. TLR4 consists of the amino-terminal region Met1-Phe44, the region Ser45-Phe567 containing leucine-rich repeats, the region Pro568-Lys631, and the region Thr632-Ile639 containing the putative transmembrane and cytoplasmic domains. The purposes of this study were to examine the direct interactions of TLR4 with MD-2 and LPS. In this study, we generated a soluble form of rTLR4 (sTLR4)1 lacking the putative intracellular and transmembrane domains, and analyzed the direct interaction of sTLR4 protein with MD-2 protein and LPS. This study demonstrates that the extracellular TLR4 domain alone cannot bind to LPS, but that the TLR4-MD-2 complex is capable of binding LPS, and that the TLR4 region containing Glu24-Lys631 enables MD-2 binding and LPS recognition. In addition, this study suggests that sTLR4 may lead to a new therapeutic strategy for dampening endotoxin-induced inflammation.

Materials and Methods

Material

Human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 10% FCS. Re595 LPS was purchased from Sigma-Aldrich (St. Louis, MO). mAb to sTLR4, 4D9, was generated using the hybridoma technique based on the method described previously (26). Anti-FLAG Ab and anti-FLAG Ab-conjugated agarose beads were obtained from Sigma-Aldrich. Anti-V5 mAb was purchased from Invitrogen Life Technologies (Carlsbad, CA).

1 Abbreviations used in this paper: sTLR, soluble rTLR; HEK, human embryonic kidney; PVDF, polyvinylidene difluoride; sCD14, soluble CD14; wt, wild type.

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Expression vectors

The cDNAs for human TLR4 and human MD-2 were obtained, as described previously (15). TLR4-FLAG-His that contains the C-terminal fusion FLAG tag and 6 His tag was subcloned into pcDNA3.1(+)(Invitrogen Life Technologies). MD-2-V5-His that contains the C-terminal fusion V5 tag and 6 His tag was generated by using PCR and subcloned into pcDNA3.1/D/V5-His-TOPO (Invitrogen Life Technologies). The mutant proteins used in this study are schematically presented in Fig. 1.

A soluble form of extracellular sTLR4 domain and rMD-2

A soluble form of extracellular sTLR4 domain (sTLR4) consists of the putative extracellular domain (Met1-Lys631) and a 6 His tag at the C-terminal end (Fig. 1). sTLR4 cDNA was constructed by using PCR. The sense primers and antisense primers used were 5'-TTGCCAGGATGTCTGCGCC-3' and 5'-CTGGATCCATGATGAGGCACGATCTGAGCAGGATCTGAGCAG-3', respectively. Constructed sTLR4 cDNA was confirmed by DNA sequencing. The sTLR4 cDNA was subcloned into pVL1392 plasmid vector using NotI and BamHI sites. The MD-2 cDNA in pcDNA3.1(+) vector was digested with Pmel and subcloned into Smal site of pVL1392 vector. The proteins sTLR4 and MD-2 were expressed by a baculovirus-insect cell expression system using the method described by O'Reilly et al. (27). Viral titers were amplified to ~4 × 10^9 PFU/mL. The recombinant viruses were used to infect insect cell monolayers of Tri cells in serum-free medium at a multiplicity of 1 to 5. After 4-day incubation, the sTLR4 protein and the MD-2 protein were purified from the medium using a column of nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA, USA) by the method described previously (22, 28). sTLR4 produced in insect cells or soluble CD14 (sCD14) protein were subjected to SDS-PAGE (10% polyacrylamide gel) under reducing conditions. The proteins sTLR4 and MD-2 were expressed by a baculovirus-insect cell expression system using the method described by O'Reilly et al. (27). Viral titers were amplified to ~4 × 10^9 PFU/mL. The recombinant viruses were used to infect insect cell monolayers of Tri cells in serum-free medium at a multiplicity of 1 to 5. After 4-day incubation, the sTLR4 protein and the MD-2 protein were purified from the medium using a column of nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA, USA) by the method described previously (22, 28).

NF-kB reporter assay

Activation of NF-kB was measured, as previously described (22, 24). HEK293 cells were plated at 1 × 10^5 cells/well in 24-well plates on plastic before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland) with 30 ng of an NF-kB reporter construct (pNF-kB-Luc; Stratagene, La Jolla, CA) and 10 ng of a construct directing expression of Renilla luciferase (pRL-TK; Promega, Madison, WI) together with the indicated amount of cDNA for TLR2. Twenty-four hours after transfection, the cells were stimulated with the indicated concentrations of LPS for 6 or 12 h, and luciferase activity was measured by the dual luciferase reporter assay system (Promega), according to the manufacturer’s instructions.

Biotinylation of sTLR4

sTLR4 was biotinylated with EZ-Link sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Binding assay of sTLR4 to MD-2 with microtiter wells

sTLR4, MD-2, or sCD14, or BSA (2 μg/ml, 50 μg/well) was coated onto microtiter wells (Immulon 1B; Thermo Labsystems, Franklin, MA). After the wells were blocked with PBS containing 3% (w/v) BSA (blocking buffer), the indicated concentrations of MD-2 or biotinylated sTLR4 (50 μg/well) in the blocking buffer were added and the suspension was incubated at 37°C for 2 h. The wells were then washed with PBS containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100. MD-2 binding to the solid-phase protein was detected by anti-V5 mAb, followed by incubation with HRP-labeled anti-mouse IgG. MD-2 was detected with HRP-conjugated anti-V5 mAb. The proteins that reacted with the Abs were visualized by using a chemiluminescence reagent (SuperSignal; Pierce), according to the manufacturer’s instructions.

Measurement of molecular interaction by the BIAcore method

The interaction between sTLR4 and MD-2 was also analyzed with BIAcore 3000 (BIAcore AB, Uppsala, Sweden). MD-2 (30 μg/ml) in 10 mM sodium acetate (pH 5.0) was immobilized on a CM5 sensor chip using the amine-coupling method. sTLR4 in 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl at a concentration of 250 nM to 1.5 μM was passed over the sensor chip at a flow rate of 30 μl/min. The interaction was monitored as the changes of surface plasmon resonance response at 25°C. After 2 min of monitoring, the same buffer was introduced onto the sensor chip in place of the sTLR4 solution to start the dissociation. The sensor surface was regenerated with 10 mM HCl at the end of each experiment. Both the association rate constant (K_a) and the dissociation rate constant (K_d) were calculated according to the BIAevaluation software (version 3.1; BIAcore AB) using a program named 1:1 (Langmuir) binding model. The dissociation constant (K_d) was determined by K_d/K_a.

Binding of sTLR4 and MD-2 to LPS-Sepharose beads

LPS from Escherichia coli 026:B6 (2 mg; Sigma-Aldrich) was cross-linked to epoxy-activated Sepharose 6B (3 ml of gel volume; Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s instructions. A total of 2 μg each of sTLR4, MD-2, or sTLR4 plus MD-2 was added into 100 μl of the blocking buffer and preincubated at 37°C for 30 min. The mixture was precleared for 1 h at 4°C by incubation with 40 μl of protein G-Sepharose (50% suspension in PBS) and was incubated with 1 μg of anti-V5 mAb or 2 μg of anti-sTLR4 mAb 4D9 for 1 h at 4°C. Immune complexes were allowed to bind to 40 μl of protein G-Sepharose for 2 h at 4°C. The Sepharose beads were washed three times with PBS containing 0.1% (v/v) Triton X-100, and were finally resuspended in 20 μl of SDS sample buffer and boiled for 3 min under reducing conditions. The sample was subjected to SDS-PAGE (29), and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was immunoprobed with anti-sTLR4 mAb 4D9, followed by incubation with HRP-conjugated anti-mouse IgG. MD-2 was detected with HRP-conjugated anti-V5 mAb. The proteins that reacted with the Abs were visualized by using a chemiluminescence reagent (SuperSignal; Pierce), according to the manufacturer’s instructions.

Electrophoretic analysis of recombinant proteins. Recombinant proteins of MD-2, sTLR4, and sTLR2 produced by insect cells and of sCD14 produced by Chinese hamster ovary cells were subjected to SDS-PAGE (10% polyacrylamide gel) under reducing conditions. The proteins were visualized by Coomassie brilliant blue staining. A total of 10 μg/lane of MD-2 and 5 μg/lane of sTLR2, sTLR4, and sCD14 was loaded.
The extracellular TLR4 domain binds to MD-2. A. The binding of MD-2 to sTLR4. The indicated concentrations of MD-2 were incubated at 37°C for 2 h with sTLR4 (○), sTLR2 (△), CD14 (□), or BSA (■) that had been coated onto microtiter wells (2 μg/ml, 50 μl). The MD-2 protein binding to the solid-phase protein was detected with anti-V5 mAb, followed by the incubation with HRP-conjugated anti-mouse IgG, as described in Materials and Methods. The data shown are the means ± SE of three experiments. B. The binding of sTLR4 to MD-2. The indicated concentrations of biotinylated sTLR4 were incubated at 37°C for 2 h with MD-2 (○) or BSA (■) that had been coated onto microtiter wells (2 μg/ml, 50 μl). The sTLR4 protein binding to the solid-phase protein was detected using HRP-conjugated streptavidin, as described in Materials and Methods. The data shown are the means ± SE of three experiments. C. The binding of sTLR4 to MD-2 in solution. A total of 2 μg each of sTLR4, MD-2, or sTLR4 plus MD-2 was mixed in 100 μl of PBS containing 3% (v/v) BSA and incubated at 37°C for 30 min. After the incubation, total volume was adjusted to 500 μl by the addition of PBS containing 3% (v/v) BSA. The mixture was then precleared with protein G-Sepharose, and was incubated with anti-V5 mAb or with anti-sTLR4 mAb 4D9, followed by incubation with protein G-Sepharose beads. The immune complex that had been sedimented was subjected to SDS-PAGE under reducing conditions. The proteins on the gel were transferred to PVDF membrane. The membrane was immunoprobed with anti-sTLR4 mAb or anti-V5 mAb for the detection of sTLR4 and MD-2, respectively, as described in Materials and Methods.
sTLR4 can attenuate LPS-induced NF-κB activation and IL-8 secretion in TLR4-expressing cells

We next examined whether sTLR4 affected LPS-induced NF-κB activation in HEK293 cells that had been transfected with wild-type (wt) TLR4. When 100 ng/ml MD-2 was coexposed into the medium, LPS-induced NF-κB activation was significantly attenuated (Fig. 7). Addition of 4 μg/ml sTLR4 significantly attenuated NF-κB activity elicited with 10 and 100 ng/ml LPS. These results demonstrate that sTLR4 can neutralize LPS-induced NF-κB activation in cells transfected with wt TLR4.

We also examined the effect of sTLR4 on LPS-induced IL-8 secretion in cells endogenously expressing TLR4. The addition of sTLR4 into the culture medium containing MD-2 significantly attenuated IL-8 secretion from differentiated U937 cells (Fig. 8). The results confirm the data obtained from wt TLR4-transfected cells. Taken together, these data suggest that sTLR4 can neutralize LPS-induced inflammation in TLR4-expressing cells.

Discussion

This study demonstrates the direct binding of the extracellular TLR4 domain to MD-2 by microtiter well binding, immunoprecipitation, and BIACore analysis. The study further indicates that the extracellular TLR4 domain-MD-2 complex, but not TLR4 alone, can bind LPS. Because sTLR4 starts at Glu24, the study reveals that the TLR4 region of Glu24-Lys631 enables MD-2 binding and LPS recognition. This study demonstrates that sTLR4 can attenuate LPS-induced NF-κB activation in wt TLR4-transfected cells in the presence of MD-2.

TLR2, TLR4, and CD14 contain 19, 24, and 10 leucine-rich motifs, respectively. The proteins containing leucine-rich repeats appear to be involved in protein-protein interaction (31, 32). The structure of this motif adopts a curved shape resembling a horse-shoe forming with an α-helix lining its outer circumference and β-strands forming a parallel β-sheet along its inner circumference (31). TLR4 consists of the amino-terminal region Met1-Phe54, the region Ser20-Phe50 containing leucine-rich repeats, the region Pro568-Lys631, and the region Thr632-Ile636 containing the putative transmembrane and cytoplasmic domains. This study demonstrates that MD-2 binds to sTLR4, but not to sTLR2 or CD14 (see Fig. 4). Because sTLR4 consists of Glu24-Lys631, the data indicate that the extracellular TLR4 region of Glu24-Lys631, but not the transmembrane or intracellular domains, is the functional domain for the binding to LPS and MD-2. This is supported by the data showing that sTLR4 can attenuate LPS-induced signaling and IL-8 secretion in wt TLR4-expressing cells (see Figs. 7 and 8).

Previous studies (10, 24) have shown that cells transfected with TLR2 are able to induce NF-κB activation in response to peptidoglycan in the absence of CD14 or MD-2. Although cotransfection with CD14 enhances cell responsiveness, the studies indicate...
that an accessory protein is not required for the TLR2-mediated signaling. The previous studies (22, 23) from this laboratory consistently show that the extracellular TLR2 domain (sTLR2) directly binds to peptidoglycan and zymosan. The physiological relevance of the study is supported by the results showing that sTLR2 attenuates peptidoglycan-induced NF-κB activation and IL-8 secretion in TLR2-transfected HEK293 cells (22). In addition, anti-TLR2 mAb that blocks peptidoglycan-induced signaling also inhibits sTLR2 binding to peptidoglycan. In contrast, the present study has shown that sTLR4 fails to bind LPS, but that the MD-2-TLR4 complex is capable of binding LPS (see Fig. 6). This is consistent with the previous study (15) showing that TLR4 alone cannot transmit LPS signaling and that MD-2 is requisite for LPS signaling of TLR4. Although the extracellular domains of TLR2 and TLR4 are homologous in point of having leucine-rich repeats, these studies support the idea that the extracellular domain of each TLR exhibits different ligand specificities.

Recent studies (21, 33) elucidate the MD-2 structure critical for TLR4 binding and LPS responsiveness by site-directed mutagenesis of MD-2. MD-2 binding to TLR4 is dependent on Cys105 and Cys102 (21). Although the MD-2 mutant F121G-K122A-Y131G-K132A is able to bind TLR4, this mutant is totally unresponsive to LPS. The MD-2 mutants of positively charged amino acids at Lys128 and Lys132 have also been shown to fail to interact with LPS, although these mutants can bind to TLR4 (34). These studies suggest that MD-2 interaction with TLR4 is necessary, but not sufficient for cellular response to LPS. Collectively, the MD-2 domain involved in TLR4 binding is likely to be distinct from that involved in LPS recognition. Thus, it is reasonable that MD-2 that binds to TLR4 is still able to interact with LPS. In addition, LPS can be associated with TLR4 when the cells were transfected with cDNAs for both wt TLR4 and MD-2, but not with TLR4 cDNA alone (33). Taken together, these studies are consistent with the conclusion that the extracellular TLR4 domain-MD-2 complex can bind LPS.

The addition of sTLR4 to the culture medium containing the MD-2 protein reduces the LPS-induced NF-κB activation and IL-8 secretion in TLR4-expressing cells (see Figs. 7 and 8). Because the sTLR4-MD-2 complex binds LPS (see Fig. 6), it is likely that the inhibitory effect of sTLR4 on the wt TLR4-mediated LPS signaling is due to the sTLR4-MD-2 complex competing with wt TLR4-MD-2 receptor complex for LPS recognition. The previous study (23) from this laboratory has also shown that sTLR4 attenuates peptidoglycan-induced cell response. Taken together, these results raise the possibility that the extracellular TLR domain can function as a modulator of TLR-mediated inflammation. One recent study (34) supports this possibility. Blood monocytes have been shown to release natural sTLR2 constitutively. The natural soluble forms of TLR2 that exhibit the major polypeptide bands with 83, 70, and 66 kDa appear to be released from Mono Mac-6 cells and to originate from cell surface TLR2. This likely result from the posttranslational modification. This natural sTLR2 has been shown to inhibit IL-8 and TNF-α production by Mono Mac-6 cells in response to synthetic bacterial lipopeptide Pam3Cys. The use of the soluble forms of rTLR2 and rTLR4 may lead to new therapeutic strategies for dampening TLR-mediated inflammation in infectious diseases.

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References


FIGURE 7. sTLR4 protein can attenuate LPS-induced NF-κB activation. HEK293 cells were transfected with wt TLR4 cDNA (100 ng) together with an NF-κB reporter plasmid and control reporter plasmid, as described above. Total amount of plasmids was adjusted to 200 ng/well by means of pcDNA3.1 (+) vector. Twenty-four hours after transfection, the cells were incubated for 12 h with 10(10) or 100 ng/ml (■) LPS in medium that had been preincubated with or without 4 μg/ml sTLR4 and/or 0.1 μg/ml rMD-2 proteins. NF-κB activities were then determined, as described in Materials and Methods. The data shown are the means ± SE of three experiments. *, p < 0.05, and **, p < 0.02, when compared with the experiments in the presence of MD-2 plus LPS without sTLR4.

FIGURE 8. sTLR4 down-regulates LPS-induced IL-8 secretion from U937 cells. Differentiated U937 cells (1 × 106) were incubated for 6 h with the medium containing sTLR4 (10 μg/ml) and MD-2 (0.25 μg/ml) that had been preincubated with the indicated concentrations of LPS at 37°C for 1 h. After the LPS stimulation, concentrations of IL-8 secreted into the medium were determined by ELISA, as described in Materials and Methods. The incubation with medium containing LPS alone (■). The incubation with the medium containing LPS, sTLR4, and MD-2. The data shown are the means ± SE of three experiments. *, p < 0.01, when compared with the experiments in the absence of sTLR4 and MD-2.


