Regulatory Roles for MD-2 and TLR4 in Ligand-Induced Receptor Clustering

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J Immunol 2006; 176:6211-6218; doi: 10.4049/jimmunol.176.10.6211
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Regulatory Roles for MD-2 and TLR4 in Ligand-Induced Receptor Clustering


LPS, a principal membrane component in Gram-negative bacteria, is recognized by a receptor complex consisting of TLR4 and MD-2. MD-2 is an extracellular molecule that is associated with the extracellular domain of TLR4 and has a critical role in LPS recognition. MD-2 directly interacts with LPS, and the region from Phe\textsuperscript{119} to Lys\textsuperscript{132} (Arg\textsuperscript{132} in mice) has been shown to be important for interaction between LPS and TLR4/MD-2. With mouse MD-2 mutants, we show in this study that Gly\textsuperscript{129} was found to be a novel critical amino acid for LPS binding outside the region 119–132. LPS signaling is thought to be triggered by ligand-induced TLR4 clustering, which is also regulated by MD-2. Little is known, however, about a region or an amino acid in the MD-2 molecule that regulates ligand-induced receptor clustering. MD-2 mutants substituting alanine for Phe\textsuperscript{126} or Gly\textsuperscript{129} impaired LPS-induced TLR4 clustering, but not LPS binding to TLR4/MD-2, demonstrating that ligand-induced receptor clustering is differentially regulated by MD-2 from ligand binding. We further show that dissociation of ligand-induced receptor clustering and of ligand-receptor interaction occurs in a manner dependent on TLR4 signaling and requires endosomal acidification. These results support a principal role for MD-2 in LPS recognition. The Journal of Immunology, 2006, 176: 6211–6218.
to the cystic fibrosis airway. Human, but not murine, TLR4/MD-2 recognizes this adaptation and transmits robust proinflammatory signals in response to hexa-acetylated, but not penta-acetylated LPS from *P. aeruginosa*, suggesting that *P. aeruginosa* control host responses by changing their fine structure of LPS (23). It seems to be important to understand molecular mechanism by which TLR4/MD-2 discriminate the fine structure of LPS for understanding host-pathogen relationship during infection. Considering discrimination between lipid A and lipid A antagonist, a link between ligand binding and receptor clustering should be a critical step for recognition of the fine structures of LPS (16, 24). It is important to understand how receptor clustering is initiated and terminated. Whereas mouse TLR4 clustering upon lipid A stimulation was triggered by both mouse and human MD-2, that upon lipid IVa stimulation was triggered by mouse MD-2, but not by human MD-2 (16). MD-2 seems to have an important role in triggering receptor clustering after ligand binding.

In contrast to a number of structure-function studies on the interaction between LPS and MD-2, as mentioned above, little is known about the structure-function relationship for MD-2 in a link between ligand binding and receptor clustering. In the present study, we addressed this issue by identifying amino acid residues that are specifically required for receptor clustering, but not for ligand binding. We also show a role for TLR4 in terminating ligand-induced receptor clustering.

### Materials and Methods

#### Reagents

Lipid A purified from *Salmonella minnesota* (Re-595) and LPS from *E. coli* O55:B5 were purchased from Sigma-Aldrich. Both Lipid A and LPS did not contain TLR2 ligands, because both did not stimulate TLR2-deficient mice, which were able to respond to TLR2 ligands (data not shown). H-labeled lipid A was described previously (25). Anti-Flag Abs and anti-Flag agarose were purchased from Sigma-Aldrich. Anti-GFP Ab for immunoprecipitation and immunoprobing were purchased from Molecular Probes and Medical & Biological Laboratories, respectively. Rabbit IgG was purchased from Rockland. Anti-LPS mouse mAb (WN) (222-5) were purchased from HyCult Biotechnology. Mouse anti-GAPDH mAb was purchased from Chemicon International. Purified GAPDH from rabbit muscle was purchased from WAKO. Detergents such as Triton X-100, Brij98, and N-octyl-β-D-glucoside were purchased from Sigma-Aldrich and WAKO, respectively. Rat anti-mouse TLR4/MD-2 mAb (MTS510 or Sa15-21) and rat anti-mouse CD14 (Sa2-8) were established in our laboratory and described previously (24). Rat anti-mouse TLR4 mAb (MT439) was established in our laboratory and can be used for immunoprobing TLR4 in Western blotting.

#### Expression constructs and transfectants

IL-3-dependent Ba/F3 cells were cultured in 10% FCS/RPMI 1640 supplemented with penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), 100 μM 2-ME, and murine rIL-3 (70 U/ml) at 37°C in a humidified atmosphere of 5% CO₂. The cDNAs encoding TLR4 (including mutants) and CD14 were cloned into the retrovirus vector pMx-puro/myc. MD-2 was tagged with the Flag epitope at the C terminus. The C terminus of TLR4 was tagged with the Flag epitope or the Flag epitope (TLR4-Flag) or GFP (TLR4-GFP). We used two TLR4 mutants, TLR4cy and TLR4HeJ. The cytoplasmic domain (from Cys662 to the C terminus) was deleted in TLR4cy, and the mic domain (from Cys662 to the C terminus) was deleted in TLR4HeJ. The cytoplasmic domain of mouse TLR4 was encoded in Western blotting. The cDNAs encoding TLR4 (including mutants) and CD14 were expressed in HEK293 cells and stably transfected into the cells with LipofectAMINE 2000 (Roche).

### ELISA

HEK293 stably expressing TLR4-GFP was plated on 6-well tissue culture plates 1 × 10⁶ cells/well a day before transfection. Expression vectors encoding mouse MD-2 mutants and mouse CD14 were transiently transfected into the cells with LipofectAMINE 2000. Transfected cells were collected 3 days later and replated onto 96-well plates. These cells were stimulated with various concentrations of lipid A for 5 h. Culture supernatants were then collected and stored at ~80°C until determination of IL-8 concentration with ELISA kit (BD Pharmingen). All samples were assayed in triplicates.

#### Bone marrow-derived dendritic cell (DC) preparation and gene transduction to bone marrow-derived DCs

Bone marrow cells from MD-2 knockout mice (13) were plated at 1 × 10⁶/well in the 24-well plates with 10% FCS in RPMI 1640 supplemented with 10 ng/ml murine rGM-CSF (R&D Systems). Following days 1 and 2, retrovirus encoding mouse MD-2 and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate transfection reagent (Roche) was added to each well. At days 6–8, loosely adherent cells were harvested and plated 5–10 × 10⁶/well in the 96-well plates. Transduced cells were detected by GFP-positive cells, because the retroviral vector contains internal ribosomal entry site-GFP. Transduction efficiencies were analyzed by the flow cytometer. TNF-α production in DCs expressing mutant MD-2 was adjusted to TNF-α production in those expressing wild-type MD-2 by comparing transduction efficiency.

#### Cell staining and flow cytometry

Single-cell suspensions were incubated at 4°C for 30 min with the primary Abs (biotin-conjugated MTS510, Sa15-21, or Sa2-8) diluted in staining buffer (PBS containing 1% BSA and 0.01% NaN₃). Cells were washed with staining buffer, and then incubated with PE-conjugated streptavidin (BD Pharmingen) for 20 min. Flow cytometry analysis was performed on FACSCalibur system (BD Biosciences).

#### Cell stimulation, immunoblotting, and immunoprecipitation

A total of 10⁵ cells/sample was stimulated with lipid A or LPS. After washing with HBSS three times, cells were lysed on ice for 30 min in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2 mM MgCl₂, 2 mM CaCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1% Brij, and 0.5% n-octyl-β-D-glucoside. Lysates were separated from debris by centrifuging 13,000 rpm at 4°C for 15 min and incubated with Ab-conjugated beads at 4°C for 2 h. Beads were washed three times and boiled at 99°C for 5 min in the sample buffer for SDS-PAGE. Bound proteins were subjected to SDS-PAGE and Western blotting. Reagents used for immunoprecipitation were anti-GFP and anti-Flag (M2) Abs. Abs used for detection in immunoblots were goat anti-mouse IgG-alkaline phosphatase conjugate (American Qualex) and goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad).

### H-labeled Lipid A-binding assay

Ba/F3 stable transfectants (10⁴ cells/sample) were stimulated with various concentrations of H-labeled lipid A for 30 min at 37°C. The specific radioactivity of H-labeled lipid A was ~10,000 cpm/ng. After three washings with HBSS, cells were lysed on ice for 30 min in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM PMSF, and 1% Triton X-100. Lysates were separated from debris by centrifuging 13,000 rpm at 4°C for 15 min and incubated with a mAb to TLR4 Sa15-21-conjugated beads at 4°C for 2 h. Beads were washed three times, and bound radioactivities were counted with a liquid scintillation counter (Aloka). To exclude nonspecific binding to Ig-coupled beads, cpm associated with rat anti-mouse CD14 (Sa2-8) beads were subtracted from that with Sa15-21. As described previously, anti-CD14 mAb did not precipitate lipid A (24).

#### Results

To reveal a role for MD-2 in receptor clustering, we used alanine-substituted MD-2 mutants, which Kawasaki et al. (27) previously studied with regard to cell surface expression of TLR4/MD-2 and their LPS responsiveness. We chose mutants that were normally stable expressing mouse TLR4 with the GFP tag (HEK293/mTLR4-GFP) were established by transfection of these constructs with LipofectAMINE 2000 (Roche).

**Abbreviation used in this paper: DC, dendritic cell.**
associated with TLR4 and expressed on the cell surface, but defective in LPS response, including Y34A, Y36A, G59A, V82A, F126A, P127A, K128A, G129A, Y131A, I153A, I154A, and H155A. We have shown previously that a unique mAb MTS510 reacts with cell surface TLR4/MD-2 before LPS stimulation, but not after LPS stimulation (24). The loss of the reactivity of MTS510 with TLR4/MD-2 may coincide with the receptor clustering, because the reactivity of MTS510 was hardly changed by lipid A antagonists, which bound to TLR4/MD-2, but did not trigger the receptor clustering (16, 24). We therefore used MTS510 staining as a first screening of MD-2 mutants. Ba/F3 transfectants expressing CD14, TLR4, and MD-2 mutants were established and stained with MTS510 after lipid A stimulation. Among 12 mutants examined, 3 mutants were distinct from wild-type MD-2 in that the reactivity of MTS510 was less changed after lipid A stimulation when compared with the other 9 mutants (Fig. 1a). Time course analyses revealed that the 3 mutants were delayed in ligand-dependent shift of the staining with MTS510, suggesting that LPS response with these 3 mutants were not completely defective, but partially impaired (Fig. 1b). Staining with the other mAb to TLR4, Sa15–21 will be described below. We decided to focus on these mutants: G59A, F126A, and G129A. These residues are highly conserved beyond species, including humans (Fig. 1c).

We first studied LPS responsiveness by using HEK293 cells stably expressing mouse TLR4, into which the expression vectors encoding mutant MD-2 were transiently transfected. In keeping with the previous study (27), TLR4 associated with MD-2 mutants did not respond to lipid A, as judged by IL-8 production (Fig. 2a). To further see the defects of these MD-2 mutants in normal immune cells, DCs from MD-2-deficient mice were transduced with

**FIGURE 1.** The MD-2 mutants are not impaired in association with TLR4 and expression on the cell surface. a, Ba/F3 cells expressing mouse TLR4, mouse CD14, and mouse MD-2 with indicated mutation were stimulated with lipid A (1 μg/ml) for 15 min at 37°C, stained with anti-mouse TLR4/MD-2 Ab MTS510. MTS510 barely stained Ba/F3 cells stably expressing mouse TLR4 and mouse CD14, but not MD-2 (none). Open histograms depict staining cells without stimulation. Those in a thin line depict staining with the second reagent streptavidin-PE alone, whereas those in a thick line depict staining with MTS510. Gray histograms depict MTS510 staining of cells stimulated with lipid A. Representative data from five independent experiments are shown. b, Ba/F3 cells expressing mouse TLR4, mouse CD14, and mouse MD-2 with indicated mutation were stimulated with lipid A (1 μg/ml) for indicated periods of time, and stained with the two mAb to TLR4, MTS510 or Sa15–21, as indicated. Open histograms depict staining with the second reagent alone. c, The part of the MD-2 sequences (aa 51–63 and 119–133) from indicated species is shown. The residues investigated in the present study (G59, F126, and G129) are shown in boldface.
The delay in ligand-dependent shift of the MTS510 reactivity with TLR4/mutant MD-2 could be due to impaired ligand binding to TLR4/Mutant MD-2 complexes due to their impaired affinity to lipid A, altered conformation of TLR4/Mutant MD-2 after ligand interaction, or impaired TLR4 clustering. We addressed these possibilities. We first investigated lipid A binding to TLR4/Mutant MD-2. Ba/F3 cells expressing wild-type MD-2 and significantly responded to LPS when TLR4 was transfected (28). However, the amount of endogenous MD-2 was not large enough to influence relatively insensitive biochemical assays for lipid A binding to TLR4/Mutant MD-2 and subsequent TLR4 clustering, which were detectable only when MD-2 was cotransfected with TLR4 (16, 24). Ba/F3 cells were suitable for these biochemical assays, because a large number of cells was easily obtained. We therefore used Ba/F3 cells for further analyses. Ba/F3 cells expressing CD14/TLR4/Mutant MD-2 were stimulated with aH-labeled lipid A and TLR4/Mutant MD-2 was precipitated, and coprecipitated aH-labeled lipid A was counted. In this condition, CD14 was not coprecipitated with TLR4/Mutant MD-2, and unlabeled lipid A was able to inhibit the interaction between aH-labeled lipid A and TLR4/Mutant MD-2 (16, 24). Cell surface expression of TLR4/Mutant MD-2 (Fig. 1a) and CD14 (Fig. 3b) was similar among the transfectants used. The G129A mutants might be slightly impaired in lipid binding. The F126A mutant was not impaired at all when compared with wild-type MD-2, whereas G59A was very low in lipid A binding (Fig. 3a). The G59A mutation impaired lipid A binding.

The F126A and G129A mutations impaired ligand-induced TLR4 clustering

The F126A and G129A mutants were not impaired or only slightly impaired in interaction with lipid A, respectively (Fig. 3a). It was possible that these mutants had an influence on subsequent receptor clustering. We investigated ligand-induced receptor clustering with Ba/F3 transfectants expressing CD14, TLR4-Flag, TLR4-GFP, and MD-2 mutant. Cells were stimulated with lipid A, TLR4-GFP was precipitated, and coprecipitation of TLR4-Flag was detected (Fig. 4). TLR4-GFP was not precipitated by rabbit IgG, the retrovirus vector encoding wild-type or mutant MD-2. Transduced DCs were stimulated with lipid A or a TLR9 ligand CpG, and TNF-α production was determined by ELISA (Fig. 2b). TNF-α production was partially, but significantly impaired in DCs expressing mutant MD-2 when compared with those expressing wild-type DCs. These results demonstrated that these MD-2 mutations impaired LPS responses.

G59A mutant is impaired in interaction with lipid A

The delay in ligand-dependent shift of the MTS510 reactivity with TLR4/MD-2 complexes due to their impaired affinity to lipid A, altered conformation of TLR4/MD-2 after ligand interaction, or impaired TLR4 clustering. We addressed these possibilities. We first investigated lipid A binding to TLR4/MD-2. Ba/F3 cells expressed a small amount of endogenous MD-2 and significantly responded to LPS when TLR4 alone was transfected (28). However, the amount of endogenous MD-2 was not large enough to influence relatively insensitive biochemical assays for lipid A.
demonstrating the specificity of the anti-GFP Ab (Fig. 4a). Ba/F3 cells expressing F126A or G129A mutant, but not those expressing G59 mutant, were impaired in LPS-induced TLR4 clustering (Fig. 4b). The F126A mutant was specifically impaired in mediating TLR4 clustering, but not in ligand binding.

The ligand-induced clustering of TLR4/MD-2 is impaired in two TLR4 mutants

We next examined the consequence of the ligand/receptor complex after ligand-induced clustering. As we described previously, ligand-induced receptor clustering was transient and terminated by 120 min after ligand stimulation (16). To understand a relationship between TLR4 signaling and TLR4 clustering, we used two TLR4 mutants, TLR4cy and TLR4HeJ. The former does not have the cytoplasmic portion of TLR4, whereas the latter has the point mutation replacing Pro712 with histidine, which was reported in a spontaneous LPS-hyporesponsive mouse strain, C3H/HeJ (4–6). Both mutants are defective in signaling. Ba/F3 cells expressing these TLR4 mutants were used for analyzing roles for the cytoplasmic portion or the LPS signaling in ligand-induced TLR4-clustering. Both mutants were not impaired in ligand binding to TLR4/MD-2 and in triggering ligand-induced receptor clustering (Fig. 5, b and c), but receptor clustering did not terminate even at as late as 480 min after LPS stimulation in contrast, wild-type TLR4 was dissociated from each other at 240 min after stimulation (Fig. 5a). We simultaneously detected LPS bound to TLR4/MD-2 by immunoprecipitating with anti-LPS mAb, of which the specificity was shown by using an isotype control mAb (Fig. 5d). In keeping with TLR4 clustering, LPS was left bound to TLR4/MD-2 until 480 min after simulation of transfectants expressing TLR4 mutants (Fig. 5, b and c). These results suggest that dissociation of TLR4 clustering and of interaction between LPS and TLR4/MD-2 are both dependent on TLR4 signaling.

FIGURE 4. F126A and G129A mutants impaired ligand-induced TLR4 oligomerization. a, Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, MD-2, and CD14 were stimulated with lipid A (100 ng/ml) for 30 min at 37°C. Cells were then subjected to detergent lysis, lysate extraction, and immunoprecipitation with anti-GFP or rabbit IgG as an isotype control. Immunoprecipitated proteins were resolved on SDS-PAGE, and then immunoprobed with anti-GFP Ab or anti-Flag Ab. b, Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, CD14, and MD-2 with indicated mutation were stimulated with lipid A at the indicated concentrations for 30 min. Transfectants were then subjected to detergent lysis, lysate extraction, and immunoprecipitation with anti-GFP (upper and middle) or anti-Flag (lower). Precipitated TLR4 was subjected to SDS-PAGE and Western blotting. Precipitated TLR4-GFP and coprecipitated TLR4-Flag were detected by immunoprecipitation with anti-GFP Abs (middle) or anti-Flag Ab (upper), respectively. Expression of TLR4-Flag was shown by immunoprecipitation and immunoprecipitation with anti-Flag Ab (lower). Note that immature TLR4 was dominant in cells lacking MD-2 (middle and lower; the left three lanes), and only mature TLR4-Flag was coprecipitated with TLR4-GFP (upper). Similar results were obtained in four independent experiments.

FIGURE 5. Ligand-induced TLR4 oligomerization is not terminated in TLR4 mutants. Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, MD-2, and CD14 were stimulated with LPS (1 μg/ml) for indicated periods of time and lysed in the lysis buffer. TLR4-GFP was immunoprecipitated with anti-GFP, and precipitated TLR4-GFP and coprecipitated TLR4-Flag were probed with anti-GFP (middle panels in a–c) or anti-Flag Abs (upper panels in a, b, and c), respectively. Coprecipitated LPS was also probed with anti-LPS Abs (lower panels in a–c). Three Ba/F3 transfectants were used expressing wild-type TLR4 (a), TLR4Hej mutant in which P712 was substituted with histidine (b), or TLR4cy mutant of which the cytoplasmic portion was deleted (c). d, The indicated amount of LPS and GAPDH was subjected to SDS-PAGE and Western blotting. LPS was detected by immunoprecipitating with anti-LPS mAb (upper), but not with isotype control mouse mAb, anti-GAPDH (lower).
Termination of ligand-induced receptor clustering requires endosomal acidification

The ligand/receptor complex on the cell surface, LPS/TLR4/MD-2, was detected by a mAb to TLR4, Sa15-21, but not by the other mAb to TLR4, MTS510 (24). MTS510 did not react with cell surface TLR4/MD-2 even after stimulation with lipid A (Fig. 1b). Sa15-21 bound to cell surface TLR4/MD-2 even after stimulation with lipid A, but its reactivity gradually disappeared by 4 h after ligand stimulation (Fig. 1b). This time course correlated very well with the termination of TLR4 clustering and LPS dissociation from TLR4/MD-2 (Fig. 5a). We therefore stained cell surface TLR4cy/MD-2 or TLR4hej/MD-2 with Sa15–21 after ligand stimulation. TLR4cy/MD-2 or TLR4hej/MD-2 remained detectable on the cell surface even after ligand stimulation (Fig. 6). The disappearance of TLR4/MD-2 is probably due to internalization of TLR4/MD-2, because LPS stimulation was reported to cause TLR4 endocytosis and its profound redistribution around LPS-containing vesicles (29, 30). Considering that we could not see either the termination of receptor clustering or the disappearance of cell surface TLR4/MD-2 in cells expressing TLR4 mutants (Figs. 5 and 6), we hypothesized that dissociation of receptor clustering occurred after internalization of cell surface TLR4/MD-2. Acidification after internalization in endosome might be responsible for dissociation of receptor oligomerization and of ligand-receptor interaction. To address this issue, we used weak bases, NH₄Cl and chloroquine, which affect endosomal acidification. Ba/F3 cells were treated with 10 mM NH₄Cl or 1 μM chloroquine for 1 h and stimulated with LPS. NH₄Cl or chloroquine had no effect on initiation of ligand-induced receptor clustering and the time course of cell surface TLR4/MD-2 disappearance (Fig. 7). Dissociation of the TLR4/MD-2 cluster was observed in non-treated cells at 2 h after LPS stimulation, but not in cells treated with NH₄Cl or chloroquine even at as late as 4.5 h after LPS stimulation (Fig. 7b). Endosomal acidification is likely to contribute to dissociation of the TLR4/MD-2 cluster.

Discussion

Kawasaki et al. (27) previously demonstrated amino acids in mouse MD-2 that were important for LPS responsiveness, but not for cell surface expression of TLR4/MD-2, dissecting a role for MD-2 in LPS response from that for cell surface expression of
TLR4/MD-2. In this work, we extended this study and demonstrated amino acid residues important for LPS binding or subsequent ligand-induced TLR4 oligomerization. To dissect LPS binding from ligand-induced TLR4 oligomerization and to study roles for MD-2, it was important to have MD-2 mutants that were specifically impaired in LPS binding or in ligand-induced TLR4 oligomerization. G59A mutation did not influence cell surface expression of TLR4/MD-2, but impaired interaction with lipid A (Figs. 1 and 3), whereas the F126A mutant was impaired in receptor clustering, but not in cell surface expression or interaction with LPS (Figs. 1, 3, and 4). Such specific mutations were found to impair LPS responses only partially when expressed in DCs (Fig. 2b), demonstrating that these mutations were mild in phenotype. This is probably due to the cell surface expression of CD14 on DCs, because cotransfection of CD14 with mutant MD-2 compensated LPS-induced IL-8 production in HEK293 cells expressing TLR4 (data not shown). Membrane CD14 was shown to augment LPS binding to TLR4/MD-2 (24). Augmented LPS loading onto MD-2 was likely to compensate for specific, but mild mutation in the MD-2 protein.

Responsiveness to LPS is highly conserved between mouse and human TLR4/MD-2, and mouse TLR4 is able to use human MD-2 as a coreceptor (16), suggesting that critical amino acids for LPS recognition are conserved between humans and mice. In this regard, it is of note that all the 3 aa studied in this work were conserved between humans and mice (Fig. 1c). These amino acids are likely to have similar roles in human MD-2.

Mancek et al. (17) searched LPS-binding motif of MD-2 by comparing MD-2 with other LPS-binding proteins (31–33). They found high amphipathicity and high content of positively charged residues at the region from aa 119 to 132 in human MD-2. The importance of this region (aa 119–132) was underscored by the studies using MD-2 mutants (18–20). In the present study, we identified G59 as a critical amino acid for LPS binding, which was not in the region 119–132 and has never been reported before. It is of note that the difference between glycine and alanine is only a methyl residue. Such a small difference was sufficient for impairing lipid A binding to TLR4/MD-2 (Fig. 3a). Alternatively, the G59A mutant might have a problem in interaction with CD14 during LPS transfer from membrane CD14 to TLR4/MD-2. Cocrystallography of lipid A and MD-2 would reveal how the methyl residue contributes to LPS binding. G59A mutation was impaired in ligand binding, but we were still able to detect ligand-induced receptor clustering (Fig. 4). Diminished, but significant lipid A binding to the G59A MD-2 mutant might have been sufficient for inducing TLR4 clustering. Insufficient lipid A binding to TLR4/MD-2 was likely to impair TNF-α production in DC (Fig. 2b).

In contrast to G59, F126 and G129 had a minimal role in ligand binding (Fig. 3a). Especially, the F126A MD-2 mutant was not less than wild-type MD-2 in lipid A binding (Fig. 3a). Impaired LPS responses with these F126A and G129A mutants were explained by diminished ligand-induced TLR4 clustering, but not by a defect in lipid A binding (Figs. 3 and 4). Although MD-2 influenced both lipid A binding and TLR4 clustering, TLR4 clustering seemed to be differentially regulated from lipid A binding. It is of note that F126 and G129 are both in the region 119–132, which is important not only for ligand binding, but also for subsequent TLR4 clustering. This region could be a target for therapeutic intervention in endotoxin shock.

Ligand-induced receptor clustering and ligand/receptor interaction were transient and terminated within 4 h (Fig. 5a). A TLR4 mutant deleted in the cytoplasmic portion was apparently delayed in the termination of LPS-induced TLR4 clustering and of interaction between LPS and TLR4/MD-2 (Fig. 5c), suggesting a requirement for TLR4 signaling. In keeping with this, similar results were obtained with another TLR4 mutant with the substitution of Pro712 with histidine (Fig. 5b). A mechanism for terminating receptor clustering was suggested by disappearance of TLR4/MD-2 from the cell surface, which occurred with a time course similar to the termination of TLR4 clustering and was not observed in cells expressing TLR4 mutants (Figs. 5 and 6). LPS-induced disappearance of cell surface TLR4/MD-2 can be due to either shedding off from the cell surface or endocytosis. We could not detect soluble TLR4/MD-2 from the culture supernatant, although there remains a possibility that TLR4 and MD-2 were dissociated with each other and therefore not detected by a mAb specific for TLR4/MD-2. However, shedding off cannot explain the effect of the inhibitors for endosomal acidification on the termination of receptor clustering (Fig. 7). Moreover, LPS-induced TLR4 endocytosis was recently reported in Chinese hamster ovary cell line expressing TLR4/MD-2 and CD14 (29). TLR4/MD-2 was likely to be internalized after ligand stimulation and endosomal acidification would be responsible for disrupting the receptor oligomerization and ligand-receptor interaction. TLR4 mutants were impaired in both the disappearance of TLR4/MD-2 from the cell surface and the termination of TLR4 clustering (Figs. 5 and 6), suggesting that the LPS signal led to dissociation of TLR4 cluster and the interaction between LPS and TLR4/MD-2 by triggering TLR4/MD-2 internalization. It is important to determine a signaling pathway that leads to TLR/MD-2 internalization. TLR4 is able to activate two signaling pathways, which are MyD88 dependent and MyD88 independent. The TLR4 mutants used in the present study were defective in both pathways. It is informative to study DCs or macrophages from mice lacking MyD88.

Prolonged TLR4 clustering with NH4Cl treatment might lead to up-regulation of LPS responses. We have examined the effect of NH4Cl on LPS-induced TNF-α production in bone marrow-derived DC and a macrophage cell line RAW. We, however, could not see consistent effect of NH4Cl on these LPS responses. We believe that LPS signaling would be terminated by negative regulators such as IRAK-M before internalization (34).

What is a fate of TLR4/MD-2 after endocytosis: being degraded in phagolysosome or having another role? TLR4 was demonstrated recently to be degraded by ubiquitination, and LPS responses are inhibited by proteasome inhibitors (35, 36). It is possible that endocytosis of TLR4/MD-2 may not just be linked with degradation, but has a functional role. In this regard, it is of note that LPS stimulation facilitated lysosomal fusion of the phagosome containing bacteria, but not apoptotic cells (37). Such a difference between phagosomes containing bacteria and apoptotic cells is speculated to be due to TLRs residing in the phagosome with bacteria, but not with apoptotic cells. Signal-dependent internalization may be important for phagosome maturation.

In conclusion, we showed in this study regulatory roles for MD-2 and TLR4 in initiating and terminating ligand-induced TLR4 oligomerization.

Disclosures
The authors have no financial conflict of interest.

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