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Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization

Shin-ichiroh Saitoh[1], Sachiko Akashi[1], Takenao Yamada[2], Natsuko Tanimura[2], Makiko Kobayashi[1], Kazunori Konno[1], Fumi Matsumoto[2], Koichi Fukase[3], Shoichi Kusumoto[3], Yoshinori Nagai[1], Yutaka Kusumoto[1], Atsushi Kosugi[2,4] and **Kensuke Miyake[1,4]**

[1]Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[2]Department of Medical Technology and Science, Graduate School of Medicine and [3]Department of Chemistry, Graduate School of Science, Osaka University, Osaka, Japan
[4]CREST, Japan Science and Technology Corporation, Tokyo, Japan

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Abstract

Toll-like receptor 4 (TLR4) and MD-2 recognizes lipid A, the active moiety of microbial lipopolysaccharide (LPS). Little is known about mechanisms for LPS recognition by TLR4–MD-2. Here we show ligand-induced TLR4 oligomerization, homotypic interaction of TLR4, which directly leads to TLR4 signaling. Since TLR4 oligomerization normally occurred in the absence of the cytoplasmic portion of TLR4, TLR4 oligomerization works upstream of TLR4 signaling. Lipid IVa, a lipid A precursor, is agonistic on mouse TLR4–MD-2 but turns antagonistic on chimeric mouse TLR4–human MD-2, demonstrating that the antagonistic activity of lipid IVa is determined by human MD-2. Binding studies with radioactive lipid A and lipid IVa revealed that lipid IVa is similar to lipid A in dose-dependent and saturable binding to mouse TLR4–human MD-2. Lipid IVa, however, did not induce TLR4 oligomerization, and inhibited lipid A-dependent oligomerization of mouse TLR4–human MD-2. Thus, lipid IVa binds mouse TLR4–human MD-2 but does not trigger TLR4 oligomerization. Binding study further revealed that the antagonistic activity of lipid IVa correlates with augmented maximal binding to mouse TLR4–human MD-2, which was ~2-fold higher than lipid A. Taken together, lipid A antagonist lipid IVa is distinct from lipid A in binding to TLR4–MD-2 and in subsequent triggering of TLR4 oligomerization. Given that the antagonistic activity of lipid IVa is determined by MD-2, MD-2 has an important role in a link between ligand interaction and TLR4 oligomerization.

Introduction

Innate immunity is the first line of defense against bacterial infection (1). The Toll family of receptors plays an essential role in innate recognition of microbial products (2). However, molecular mechanisms underlying microbial recognition by Toll-like receptors (TLRs) remain enigmatic. TLR4 recognizes lipopolysaccharide (LPS), one of the most immunostimulatory glycolipids constituting the outer membrane of the Gram-negative bacteria (3–7). LPS consists of lipid A, core oligosaccharide and an *O* side-chain (8). Lipid A is responsible for immunostimulating activity of LPS. Because of its potent activity, LPS has been implicated in a variety of diseases, such as septic shock (9). Lipid A antagonists are being developed for antagonizing LPS in septic shock (10,11), although their antagonistic mechanism has not been completely understood. It is arguably important to clarify the action mechanism of lipid A antagonist not only for therapeutic intervention of septic shock but also for understanding molecular mechanisms underlying LPS recognition.

LPS in the membrane poorly activates the immune system. LPS is transferred to CD14 by a lipid transferase LPS-binding

Correspondence to: K. Miyake; Email: kmiyake@ims.u-tokyo.ac.jp

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protein (LBP) (12,13). LPS–CD14 stimulates TLR4–MD-2 on the cell surface (14). TLR4 is a type I transmembrane protein that contains a large, leucine-rich repeat in the extracellular region and a Toll/IL-1 receptor homology (TIR) domain in the cytoplasmic region (3). MD-2 is an extracellular molecule that is associated with the extracellular domain of TLR4, and is indispensable for cell surface expression of, and LPS recognition by, TLR4 (14–17). LPS recognition begins with LPS interaction with TLR4–MD-2 and ends with an activation signal from TLR4. Recently, the direct interaction between LPS and TLR4–MD-2 was demonstrated (18). On the other hand, TLR4 is suggested to be activated by the oligomerization of the cytoplasmic domain, since chimeras consisting of the self-interacting extracellular domains fused with the TLR4 cytoplasmic domain were constitutively active (19). Interestingly, a study using scanning immunoelectron microscopy revealed that LPS stimulation results in the rapid formation of TLR4 aggregates on the cell surface (20). This study, however, has not directly demonstrated physical, homotypic interaction between TLR4. Moreover, little is known about a link between LPS interaction with TLR4–MD-2 and TLR4 aggregation. In the present study, we addressed these issues by comparing lipid A and its antagonist lipid IVa.

Methods

Reagents

LPS from *Escherichia coli* 055:B5 and lipid A purified from *Salmonella minnesota* were purchased from Sigma (St Louis, MO). Synthetic lipid A and ^{3}H-labeled lipid A were described previously (21). The tetraacyl lipid A precursor known as lipid IVa (compound 406) was synthesized as described previously (21). E5531 was obtained from Eisai Research Institute (Tsukuba, Japan). A mAb to LPS were purchased from Hycult biotechnology (Uden, the Netherlands). Anti-Flag antibody (M2) and anti-Flag (M2)–agarose were purchased from Sigma. Anti-GFP antibodies for immunoprecipitation and immunoprobing were purchased from Molecular Probes (Eugene, OR) and Medical & Biological Laboratories Co. (Nagoya, Japan), respectively. Anti-IRAK1 (interleukin-1 receptor associated kinase 1) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Detergents such as Brij98 and *n*-octyl-β-D-glucoside were purchased from Sigma and Nakalai (Tokyo, Japan), respectively.

Expression constructs and stable transfectants

Interleukin-3 (IL-3)-dependent Ba/F3 cells were cultured in 10% FCS RPMI1640 supplemented with 100 μM 2-mercaptoethanol and recombinant murine IL-3 (~70 U/ml). The source of recombinant murine IL-3 was medium conditioned by Chinese hamster ovary (CHO) cells that had been genetically engineered to produce murine IL-3 up to ~70 000 U/ml (22). The cDNAs encoding muTLR4, muMD-2 and muCD14 and huMD-2 were cloned into the retrovirus vector pMX-puromycin. The cDNAs encoding huTLR4, huMD-2 and huCD14 were also cloned into the retrovirus vector pMX-puromycin. MD-2 was tagged with the flag epitope at the C-terminus. The C-terminus of TLR4 was tagged with either the flag epitope (TLR4F) or with GFP (TLR4G). The TLR4 mutant lacking the cytoplasmic domain (from Cys^{662} to the C-terminus) was also tagged either with the flag epitope (TLR4cyF) or with GFP (TLR4cyG). Ba/F3 cells expressing a variety of combination of TLR4F, TLR4G, muMD-2F, huMD-2F, muCD14 and huCD14 were established by retroviral transduction. Plat-E was used

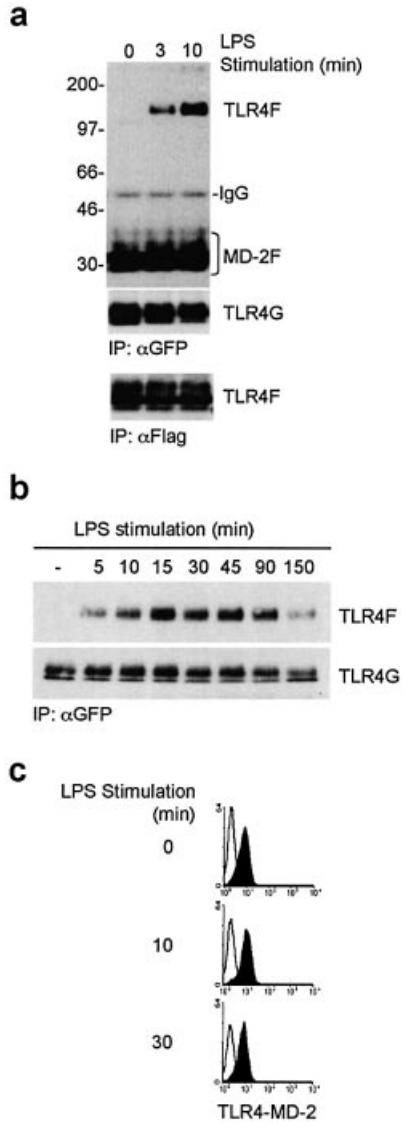

Fig. 1. LPS-dependent oligomerization of TLR4. (a) Ba/F3 cells expressing muTLR4F, muTLR4G, muMD-2F and muCD14 were subjected to LPS stimulation (1 μg/ml) for 0, 3, 10 min, detergent lysis, immunoprecipitation with anti-GFP antibody (upper and middle panels) or anti-flag antibody (lower panel), SDS–PAGE under reduced condition, and immunoprobing with anti-flag antibody (upper and lower panels) or anti-GFP antibody (middle panel). Non-specific IgG signal is indicated in the figure. (b) Ba/F3 cells expressing muTLR4F/muTLR4G/muMD-2F/muCD14 were subjected to LPS stimulation (1 μg/ml) for the indicated periods of time, detergent lysis, immunoprecipitation with anti-GFP, SDS–PAGE, immunoprobing with anti-flag (upper) or anti-GFP (lower) antibodies. Representative data from eight or five independent experiments are shown. (c) Ba/F3 cells expressing muTLR4F/muTLR4G/muMD-2F/muCD14 were subjected to LPS stimulation (1 μg/ml) for the indicated periods of time, and staining with a mAb to muTLR4–MD-2, Sa 15-21. Open histograms depict staining with the second reagent, streptavidin–PE, alone.

Table 1. Representative results in binding assay for interaction between TLR4–MD-2 and lipid A or lipid IVa

	Sa2-8	Sa15–21		
Lipid A				
Input c.p.m.	5,979,700	596,970	1,790,100	5,969,700
Mouse TLR4–MD-2	55	403	463	550
Mouse TLR4–human MD-2	50	456	531	627
Lipid IVa				
Input c.p.m.	3,227,600	322,760	968,280	3,227,600
Mouse TLR4–MD-2	83	200	311	363
Mouse TLR4–human MD-2	68	516	593	733

Ba/F3 stable transfectants expressing CD14 and mouse TLR4–MD-2 or mouse TLR4–human MD-2 were stimulated with 1, 3 and 10 μCi [^{3}H]lipid A or [^{3}H]lipid IVa for 30 min at 37°C. After washing, cells were subjected to immunoprecipitation with anti-TLR4–MD-2 mAb (Sa15-21) and anti-CD14 (Sa2-8) for non-specific association. Precipitated radioactivity was counted by a liquid scintillation counter. The counts associated with Sa2-8 did not change with input c.p.m. Three independent experiments were conducted with similar results.

as packaging cell line (23). Retrovirus vectors were transfected into the Plat-E cells using FuGene (Roche). Stable clones were established by limiting dilution method followed by selection with flow cytometry analysis.

Cell staining and flow cytometry

Single cell suspensions were incubated at 5×10^5 cells/sample on ice for 25 min with the primary antibodies diluted in staining buffer (PBS containing 1% BSA and 0.01% NaN_3). Cells were washed with staining buffer twice, and incubated with biotin-conjugated rat anti-mouse TLR4–MD-2 mAb (MTS510 or Sa15-21) and rat anti-mouse CD14 mAb (Sa2-8) for 25 min. Cells were then incubated with R-phycoerythrin (PE)-conjugated streptavidin (BD Pharmingen) for 20 min. Flow cytometry analysis was performed on a FACSCalibur System (Becton Dickinson & Co., Mountain View, CA).

Cell stimulation, immunoblotting and immunoprecipitation

Cells ($3–10 \times 10^7$) were stimulated for the indicated time with LPS, lipid A or lipid IVa (1 μg/ml), and washed with cold RPMI1640. Prior to immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.6), 4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, with or without complete inhibitor cocktail tablets (Roche), 1% Brij, 0.5% *n*-octyl-β-D-glucoside, 0.5% Triton X-100 for 30 min on ice. The lysis buffer used in Fig. 1 did not include Triton X-100. Antibodies used for immunoprecipitation were bound to protein A before immunoprecipitation. After incubation of lysates with antibodies-protein A or anti-Flag mAb (M2)–agarose, these samples were washed with washing buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.6, 0.1% Brij, 0.1% *n*-octyl-β-D-glucoside, 0.1% Triton X-100) three times. Reagents used for immunoprobing were anti-GFP, anti-flag (M2) and anti-IRAK-1 antibodies. Antibodies used for detection in immunoblots were protein A–horseradish peroxidase (HRP) and goat anti-mouse IgG–HRP (Amersham and Chemicon).

[^{3}H]lipid A binding assay

The specific radioactivity of [^{3}H]lipid A or [^{3}H]lipid IVa was ~10 000 or 5 000 c.p.m./ng, respectively. Ba/F3 stable transfectants (1×10^8 cells/sample) were stimulated with various concentrations of [^{3}H]lipid A or [^{3}H]lipid IVa for 30 min at 37°C. For competitive inhibition of [^{3}H]lipid A binding to TLR4/MD-2 with nonradioactive lipid A or lipid IVa, cells were stimulated with 0.3 μCi/ml [^{3}H]lipid A with graded concentration of lipid A or lipid IVa simultaneously for 30 min at 37°C. After washing, cells were subjected to immunoprecipitation with anti-TLR4–MD-2 mAb (Sa15-21) as above. Precipitated radioactivity was counted by a liquid scintillation counter (Aloka, Tokyo, Japan). To exclude nonspecific binding to Ig-coupled beads, c.p.m. associated with beads coupled with rat anti-mouse CD14 (Sa2-8) was subtracted from that with Sa15-21. As described previously, anti-CD14 mAb did not precipitate lipid A (18). Representative results with [^{3}H]lipid A or [^{3}H]lipid IVa are shown in Table 1.

Results

LPS induces TLR4 oligomerization

To investigate homotypic interaction of TLR4, mouse TLR4 was tagged with two distinct epitopes, TLR4-Flag (TLR4F) and TLR4-GFP (TLR4G), and stably expressed in Ba/F3 cells together with mouse MD-2-Flag (MD-2F) and CD14 (see Methods). To detect TLR4 oligomerization, TLR4G was immunoprecipitated, and coprecipitation of TLR4F was immunoprobed with anti-flag antibody. Despite appreciable expression of TLR4–MD-2, physical interaction between TLR4F and TLR4G was not observed in the absence of ligand stimulation (Fig. 1a, left lane). Association between TLR4F and TLR4G was observed only after LPS stimulation (Fig. 1a). TLR4 oligomerization was detectable as early as 3 min after LPS stimulation, peaked between 15 to 45 min, and mostly disappeared within 150 min (Fig. 1b). The amount of precipitated TLR4G protein gradually decreased within 150 min after LPS stimulation, probably due to degradation (Fig. 1b, lower panel). Rapid induction of TLR4 oligomerization correlates very well with formation of the cell surface LPS/TLR4–MD-2 complexes (18). Cell surface staining with a mAb to TLR4–MD-2 revealed that TLR4–MD-2 remained on the cell surface 30 min after LPS addition (Fig. 1c), when TLR4 oligomerization has already peaked to the maximum (Fig. 1b). These results suggest that TLR4 oligomerization occurs right after LPS interaction with TLR4–MD-2 on the cell surface.

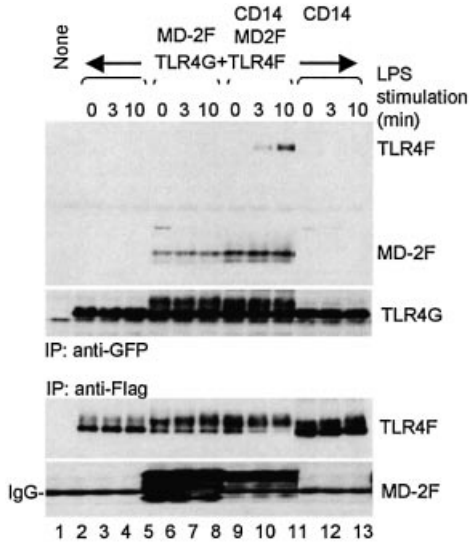

Fig. 2. Molecular requirement for TLR4 oligomerization. Ba/F3 cells expressing indicated molecules were stimulated with LPS (1 μg/ml) for the indicated periods of time. Plain Ba/F3 cells without stimulation was also used as a negative control and shown in lane 1. Cells were then subjected to immunoprecipitation with anti-GFP (upper two panels) or anti-flag antibodies (lower two panels), followed by immunoprobing with anti-flag mAb (upper, and lower two panels) or with anti-GFP antibody for TLR4G. It should be noted that the non-specific signals due to IgG light chains were detected in MD-2F (lower panel).

Molecular requirement for TLR4 oligomerization

To address molecular requirement for TLR4 oligomerization, we examined Ba/F3 transfectants lacking MD-2F and/or CD14 (Fig. 2). Equivalent expression of CD14 and TLR4–MD-2 among these cell lines was confirmed by flow cytometry and immunoprecipitation, respectively (Fig. 2 and data not shown). As was described previously (18,24), TLR4F or TLR4G alone migrated faster than those associated with MD-2 due to hypoglycosylation (Fig. 2, middle two panels, lanes 2–4 and lanes 11–13). After stimulation with 1 μg/ml LPS, TLR4F coprecipitation was detected only in the presence of both MD-2 and membrane CD14, being precisely consistent with LPS interaction with TLR4–MD-2, which are also impaired in the absence of MD-2 or membrane CD14 (18).

Correlation between LPS interaction and TLR4 oligomerization

We further addressed the relationship between LPS interaction and TLR4 oligomerization. The absence of MD-2 resulted in neither LPS interaction nor TLR4 oligomerization (Fig. 2). This may be due to the absence of TLR4–MD-2 on the cell surface; TLR4 alone is not expressed on the cell surface (15). We therefore used Ba/F3 cells expressing mutant MD-2, in which two *N*-glycosylation sites (Asn^{26} and Asn^{114}) were replaced with alanine. Human TLR4–MD-2 with these mutations came out on the cell surface but was poorly crosslinked to LPS with a crosslinking reagent, suggesting impaired interaction with LPS (25,26). Mouse MD-2 lacking these two *N*-glycosylation sites (N26A and/or N114A) was transfected and expressed in Ba/F3 cells with CD14, TLR4F and TLR4G. In keeping with human MD-2 (25,26), cell surface expression of mouse TLR4–MD-2 was not impaired in the cell lines lacking MD-2 glycosylation (Fig. 3a, left column). LPS interaction and TLR4 oligomerization were impaired by these MD-2 mutations. LPS interaction and TLR4 oligomerization showed precise correlation with each other in that both were impaired only in Ba/F3 cells expressing mutant MD-2 that lacked the two *N*-glycosylation sites, but not in those lacking one of them (Fig. 3b). These strong correlations indicate that LPS interaction with TLR4–MD-2 precedes TLR4 oligomerization detected in the present study.

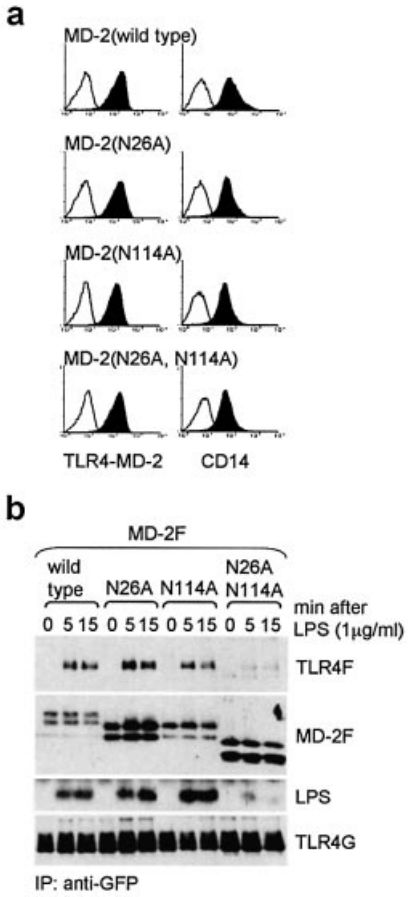

Fig. 3. *N*-glycosylation of MD-2 is important for LPS interaction and TLR4 oligomerization. (a) Ba/F3 cells expressing muTLR4F, muTLR4G, muCD14 and muMD-2 were stained with a mAb to TLR4–MD-2, MTS510, or to CD14, Sa2-8. Open histograms were staining with the second reagent, streptavidin–PE, alone. (b) Ba/F3 cells used in (a) were subjected to LPS stimulation (1 μg/ml) for the indicated periods of time, immunoprecipitation with anti-GFP antibody, immunoprobing with anti-flag mAb (upper two panels), anti-LPS mAb (middle lower panel) or anti-GFP antibody (lower panel). Representative data from three or four independent experiments are shown.

TLR4 oligomerization works upstream of TLR4 signaling

We next examined a requirement for the cytoplasmic portion of TLR4 in ligand-dependent TLR4 oligomerization. Ba/F3 cells were established that expressed CD14, MD-2F and TLR4 mutants (TLR4cyF and TLR4cyG) that lacked the cytoplasmic portion of TLR4. TLR4 oligomerization was observed with

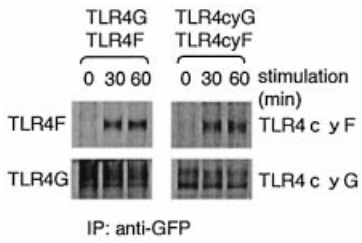

Fig. 4. The cytoplasmic portion of TLR4 is not required for TLR4 oligomerization. Ba/F3 cells expressing muCD14 and muMD-2 with wild-type muTLR4 (TLR4G and TLR4F), or with mutant muTLR4 (TLR4cyG and TLR4cyF) in which the TLR4 cytoplasmic portion is deleted, were stimulated with LPS (1 μg/ml). Cells were then subjected to immunoprecipitation with anti-GFP antibody and immunoprobed with anti-flag (upper) or anti-GFP (lower) antibody. Note that TLR4cyF migrates faster than TLR4F because of deletion in the TLR4 cytoplasmic portion. Representative data from three independent experiments are shown.

Ba/F3 cells expressing these TLR4 mutants (Fig. 4), demonstrating that ligand-dependent TLR4 oligomerization is not dependent on TLR4 signaling.

It is important to see the correlation between TLR4 oligomerization and TLR4 signaling. To address this issue, we used lipid A antagonist, lipid IVa. Lipid IVa is agonistic on muTLR4–muMD-2 but turns antagonistic on huTLR4–huMD-2 and chimeric muTLR4–huMD-2 (27). If TLR4 oligomerization directly triggers TLR4 signaling, TLR4 oligomerization has to correlate with the agonistic activity of lipid IVa, but not with the antagonistic activity of lipid IVa. We used Ba/F3 transfectants expressing huCD14, huTLR4G, huTLR4F and huMD-2. These transfectants were stimulated with lipid A, lipid IVa or both, and TLR4 oligomerization was examined. Human TLR4 oligomerization was induced by lipid A stimulation but not by lipid IVa stimulation, which clearly inhibited lipid A-induced human TLR4 oligomerization (Fig. 5a). We next used two Ba/F3 transfectants expressing muCD14, muTLR4F and muTLR4G with muMD-2 or with huMD-2. TLR4 signaling was concomitantly studied by the degradation of IL-1 receptor associated kinase-1 (IRAK-1). Significant TLR4 oligomerization was observed by lipid IVa stimulation on mouse TLR4–MD-2, albeit weaker than with lipid A (Fig. 5b, lane 4). In contrast, TLR4 oligomerization was not triggered at all when mouse MD-2 was replaced with human MD-2 (lane 8). Moreover, lipid IVa clearly inhibited lipid A-induced oligomerization of mouse TLR4–human MD-2 (lane 7). TLR4 oligomerization showed precise correlation with TLR4 signaling that was revealed by IRAK1 degradation (Fig. 5b, lower panel). Finally, we used another lipid A antagonist E5531 (10), which alone did not trigger TLR4 oligomerization and completely inhibited lipid A-triggered TLR4 oligomerization (Fig. 5c). Thus, TLR4 oligomerization correlated precisely with agonistic activity but not with antagonistic activity. Taken together with the result that TLR4 oligomerization is not dependent on TLR4 signaling (Fig. 4), TLR4 oligomerization works upstream of TLR4 signaling.

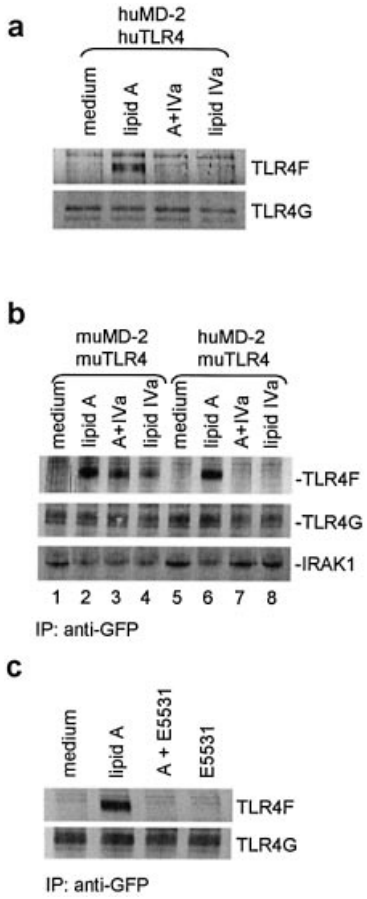

Fig. 5. MD-2 regulates lipid IVa-triggered TLR4 oligomerization. (a) Ba/F3 cells expressing huTLR4F, huTLR4G, huMD-2F and huCD14 were stimulated with medium, lipid A (1 μg/ml), lipid A (1 μg/ml) + lipid IVa (2 μg/ml), and lipid IVa (1 μg/ml), as indicated in the figure. Cells were then subjected to immunoprecipitation with anti-GFP antibody, immunoprobing with anti-flag mAb (upper) or anti-GFP antibody (lower). (b) Ba/F3 cells expressing TLR4F, TLR4G and CD14 with muMD-2F (lanes 1–4) or huMD-2F (lanes 5–8) were stimulated with medium, lipid A (1 μg/ml), lipid A (1 μg/ml) + lipid IVa (2 μg/ml) and lipid IVa (1μg/ml), as indicated in the figure. Cells were then subjected to immunoprecipitation with anti-GFP antibody, immunoprobing with anti-flag mAb (upper) or anti-GFP antibody (lower). In the lower panel, whole cell lysate was subjected to SDS–PAGE and probed by anti-IRAK1 antibody. (c) Ba/F3 cells expressing TLR4F, TLR4G, muMD-2 and CD14 were stimulated with medium, lipid A (1 μg/ml), lipid A (1 μg/ml) + E5531 (1 μg/ml) and E5531 (1 μg/ml), as indicated in the figure. Cells were then subjected to immunoprecipitation with anti-GFP antibody, immunoprobing with anti-flag mAb (upper panel) or anti-GFP antibody (lower panel). Representative data from two or four independent experiments are shown.

Lipid A antagonist is distinct from lipid A in maximal binding to TLR4–MD-2

To act as an antagonist, lipid IVa has to interact with TLR4–MD-2 without TLR4 activation. Interaction with TLR4–MD-2 does not necessarily lead to TLR4 signaling. To gain insight into a link between ligand interaction and TLR4 oligomerization, we compared lipid A and lipid IVa in interaction with TLR4–MD-2. Ba/F3 transfectants were stimulated with ^{3}H-labeled lipid A or [^{3}H]lipid IVa, and TLR4–MD-2 was immunoprecipitated with a mAb to TLR4–MD-2, Sa15-21. The amount of coprecipitated lipid A or lipid IVa was counted with a scintillation counter. Importantly, antagonistic lipid IVa was similar to lipid A in dose-dependent and saturable binding

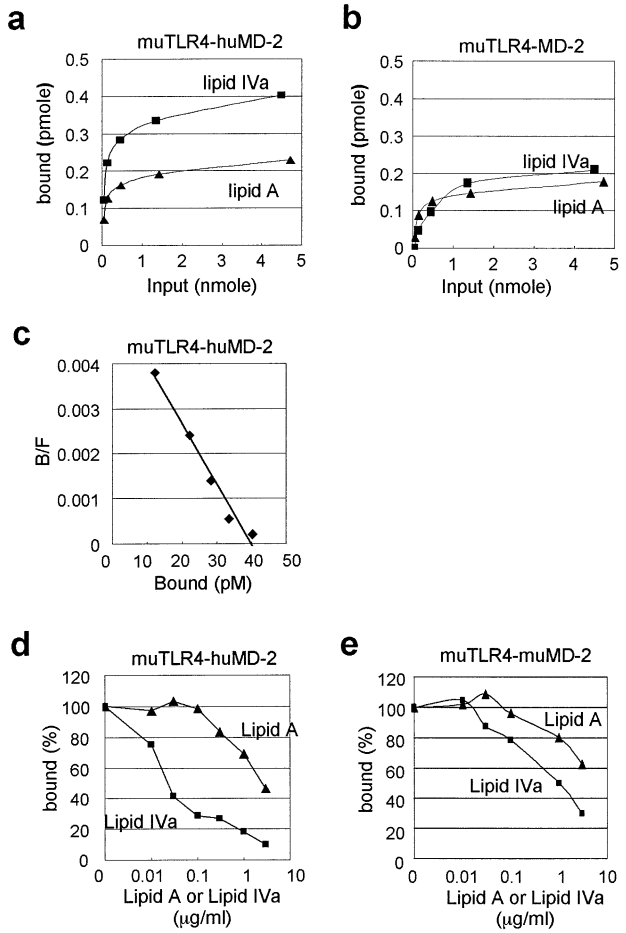

Fig. 6. Lipid IVa is distinct from lipid A in interaction with TLR4–MD-2. Ba/F3 cells (10^8 cells/10 ml medium) expressing TLR4F, TLR4G, CD14 with huMD-2F (a) or muMD-2F (b) were incubated with graded doses of [^{3}H]lipid A (triangles) or [^{3}H]lipid IVa (squares). TLR4–MD-2 was immunoprecipitated with Sa15-21 and coprecipitated lipid A or lipid IVa was counted by a scintillation counter. Specific binding was obtained by subtracting bound c.p.m. with a rat mAb Sa2-8 from that with Sa15-21. Bound lipid A or lipid IVa (pmole) were plotted against input lipid A or lipid IVa (nmole). Scatchard plot for lipid IVa on muTLR4–huMD-2 is shown in (c). Bound lipid IVa/free lipid IVa (B/F) was plotted against bound lipid IVa (pM). (d and e) Ba/F3 cells expressing TLR4F, TLR4G, CD14 and huMD-2 (d) or muMD-2 (e) were incubated with 0.261 μg/ml [^{3}H]lipid A and graded doses of cold lipid A (triangles) or lipid IVa (squares). Cells were immunoprecipitated, and coprecipitated [^{3}H]lipid A was detected by a scintillation counter. Percentages of bound [^{3}H]lipid A in the absence of cold inhibition were plotted against graded doses of cold inhibitors. Representative data from two or three independent experiments are shown.

to chimeric mouse TLR4–human MD-2 (Fig. 6a). The scatchard plot analyses revealed a single dissociation constant for antagonistic lipid IVa binding to mouse TLR4–human MD-2, suggesting a single, homogenous binding site (Fig. 6c). As lipid A agonist, lipid IVa also revealed dose-dependent and saturable binding to mouse TLR4–MD-2 with a single dissociation constant (Fig. 6b and data not shown).

We tentatively calculated dissociation constants for TLR4–MD-2 interaction with lipid A or lipid IVa. A dissociation constant for interaction between lipid A and mouse TLR4–MD-2 was ~10 nM, which was ~3-fold higher than the value (~3 nM) calculated previously (18). Currently we can not explain the difference between the present and the previous studies. Dissociation constant for lipid A interaction with chimeric mouse TLR4–human MD-2 was ~5 nM. Lipid IVa was slightly higher than lipid A in dissociation constants for interaction with TLR4–MD-2 (28 nM for mouse TLR4–MD-2 and 10 nM for chimeric mouse TLR4–human MD-2).

Lipid IVa, as lipid A antagonist, bound to mouse TLR4–human MD-2 but did not trigger TLR4 oligomerization (Figs 5a and 6a). Lipid IVa is likely to inhibit lipid A binding to TLR4–MD-2 by occupying the binding site on TLR4–MD-2. We therefore examined competitive inhibition of ^{3}H-labeled lipid A binding to mouse TLR4–human MD-2. Whereas lipid IVa was slightly higher than lipid A in dissociation constants for interaction with chimeric mouse TLR4–human MD-2 (10 nM and 5 nM, respectively), lipid IVa was much more effective than lipid A in inhibiting ^{3}H-labeled lipid A binding (Fig. 6d). This difference between lipid A and lipid IVa seems to be, at least in part, due to the antagonistic activity of lipid IVa, because a difference between lipid A and lipid IVa appeared smaller in competitive inhibition of ^{3}H-labeled lipid A binding to mouse TLR4–MD-2 (Fig. 6e). Given that lipid IVa was slightly higher than lipid A in dissociation constants for interaction with TLR4–MD-2, these results suggest a difference, which is not explained by dissociation constant, between lipid A and lipid IVa in their interaction with mouse TLR4–human MD-2. Interestingly, antagonistic lipid IVa appeared to be ~2-fold higher than lipid A in its maximal binding to chimeric mouse TLR4–human MD-2 (Fig. 6a). This was not due to a difference between lipid A and lipid IVa, since these two radioactive reagents were similar to each other in maximal binding to mouse TLR4–MD-2 (Fig. 6b). The antagonistic activity of lipid IVa is likely to correlate with higher maximal binding to chimeric mouse TLR4–human MD-2. The maximal binding of a ligand is determined by the number of the ligand binding site on responding cells, and lipid IVa and lipid A share TLR4–MD-2 as a receptor. It might be possible that lipid IVa is distinct from lipid A in the stoichiometry of interaction with chimeric mouse TLR4–human MD-2.

Discussion

Using two epitope tags, we showed ligand-induced, homotypic interaction of TLR4. Homotypic interaction of TLR4 became detectable only after ligand stimulation (Fig. 1). Membrane CD14 and MD-2 are required for detecting both LPS interaction and TLR4 oligomerization (Fig. 2). Two *N*-glycosylation sites on MD-2 were both important for LPS interaction and TLR4 oligomerization (Fig. 3). TLR4 oligomerization shown in the present study seems to be dependent on, and preceded by, ligand interaction with TLR4–MD-2. TLR4 oligomerization does not result from TLR4 activation, since the cytoplasmic portion was dispensable for TLR4 oligomerization (Fig. 4). On the other hand, TLR4 signaling is dependent on TLR4 oligomerization, because the agonistic and antagonistic activity of lipid IVa correlated exactly with the presence and absence of TLR4 oligomerization, respectively (Fig. 5a). Taken

together, TLR4 oligomerization shown here works between ligand interaction and TLR4 signaling.

The experiments for TLR4 oligomerization were conducted in the presence of fetal bovine serum that contained soluble CD14. Our system did not show a positive role for soluble CD14 in LPS interaction with TLR4–MD-2 (18) and in TLR4 oligomerization (Fig. 2). Many types of cells normally respond to LPS in the presence of soluble CD14 but not of membrane CD14. We believe that LPS complexed with soluble CD14 interacts with only a small percentage of TLR4–MD-2 on the cell surface and triggers their oligomerization. This may be sufficient for TLR4 signaling, but not for biochemical detection of LPS interaction with TLR4–MD-2 and TLR4 oligomerization. Membrane CD14 probably helps LPS interact with much higher percentage of TLR4–MD-2 than soluble CD14. Further studies are required to understand a difference between membrane CD14 and soluble CD14 in LPS recognition.

Recently, Lee *et al.* (28) demonstrated constitutive, MD-2-independent TLR4 oligomerization, which we confirmed by using HEK293 cells transiently expressing TLR4F and TLR4G with or without MD-2F. Even in the absence of MD-2, TLR4F was coprecipitated with TLR4G without LPS stimulation (data not shown). The difference between the present results and the previous results by Lee *et al.* (28) could be due to either the cell line used or transfection procedure. Lee *et al.* employed transiently transfected HEK293 cells, whereas we used stable Ba/F3 transfectants. Very importantly, the transient overexpression of TLR4 highly activates HEK293 cells without LPS stimulation (28). This result is consistent with the present study, in that TLR4 oligomerization activates TLR4 signaling, but is distinct from our results in requirement for ligand and MD-2. It has to be stressed that constitutive, MD-2-independent TLR4 signaling remains to be clarified with regard to its role in LPS response *in vivo*. In sharp contrast, TLR4 oligomerization in the present study has similarities to LPS response in normal cells, in that it requires ligand stimulation and MD-2 (Figs 1 and 2). Moreover, we clearly demonstrated a direct link between TLR4 oligomerization and TLR4 signaling by using lipid A antagonist lipid IVa (Fig. 5a). Along this line, TLR2 was shown to be constitutively associated with TLR1 or TLR6 in transiently transfected HEK293 cells (29–31). Tapping and Tobias (30) suggested that a TLR2 ligand, lipoarabinomannan (LAM), altered the physical interaction between TLR1 and TLR2. TLR2 ligands may have a role in strengthening the physical interaction between TLR2 and TLR1, or TLR2 and TLR6.

The present study clearly demonstrated that lipid A antagonists, lipid IVa and E5531, do not trigger TLR4 oligomerization (Fig. 5). Importantly, lipid IVa bound to chimeric mouse TLR4–human MD-2 (Fig. 6a), indicating that ligand interaction is not directly coupled with TLR4 oligomerization. We therefore asked about molecular mechanism(s) regulating a link between ligand interaction and TLR4 oligomerization. The antagonistic activity of lipid IVa is determined by the species origin of MD-2 (27,32). Lipid IVa acts agonistic on mouse TLR4–MD-2 but turns antagonistic when mouse MD-2 is replaced with human MD-2. We took this advantage to see the difference between agonist and antagonist. Lipid IVa appeared to be more effective than lipid A in inhibiting radioactive lipid A binding, particularly to chimeric mouse TLR4–human MD-2 (Fig. 6d). Such a difference between lipid A and lipid IVa was not explained by a difference in their dissociation constants. Lipid IVa was slightly higher than lipid A in dissociation constants for their interaction with mouse TLR4–human MD-2 (Fig. 6a). We found that lipid IVa was distinct from lipid A in maximal binding to mouse TLR4–human MD-2 (Fig. 6a), but not distinct from lipid A in maximal binding to mouse TLR4–MD-2 (Fig. 6b). The antagonistic activity of lipid IVa is likely to result in the difference between lipid A and lipid IVa in maximal binding to chimeric mouse TLR4–human MD-2. Maximal binding of a ligand is thought to be determined by the number of the binding sites. Lipid A and lipid IVa share the binding site on mouse TLR4–human MD-2, since lipid IVa inhibited radioactive lipid A binding to mouse TLR4–human MD-2 (Fig. 6d). These results might suggest an interesting possibility that lipid A antagonist is distinct from lipid A in the stoichiometry of ligand–receptor interaction, i.e. single ligand–single receptor for lipid IVa and single ligand–two receptor for lipid A. Further studies are under way to address this issue.

Given that MD-2 regulates the antagonistic activity of lipid IVa, MD-2 has an important role in a link between ligand interaction and TLR4 oligomerization. How does MD-2 regulate TLR4 oligomerization? We could not detect spontaneous TLR4 oligomerization in cells overexpressing TLR4 alone (Fig. 2a, lanes 1–3). TLR4 is not able to interact with itself. Toll, the prototype of TLR4, is similar to TLR4 in that Toll does not spontaneously interact with itself (33). Dimerization of Toll is triggered by the interaction with the homodimeric form of the ligand Spätzle. LPS, TLR4–MD-2 ligand, seems to be distinct from Spätzle in that LPS in an aggregated state has little or no effect on the stimulatory potency of LPS (34). CD14 binds only LPS monomers, not aggregates (35). Since LPS is transferred from CD14 to TLR4–MD-2 (18), LPS is likely to be presented as a monomer to TLR4–MD-2. MD-2 was reported to be secreted in a dimeric or multimeric form (36,37). However, only the monomeric form of MD-2 is associated with TLR4 (37,38). TLR4 association might inhibit the oligomerization of MD-2. Such inhibition could be removed by LPS stimulation. We prefer the possibility that MD-2 interaction with an agonist but not with an antagonist triggers self-interaction of MD-2, which, in turn, leads to TLR4 oligomerization. This possibility is currently addressed.

Previous studies with soluble MD-2 demonstrated that MD-2 binds to LPS (20,39,40). The present study clearly demonstrated that MD-2 also regulates TLR4 oligomerization. Lipid IVa bound to both muTLR4–MD-2 and chimeric muTLR4–huMD-2, but induced TLR4 oligomerization of muTLR4–MD-2 but not of chimeric muTLR4–huMD-2 (Fig. 5). The difference between human MD-2 and mouse MD-2 might locate the domain regulating TLR4 oligomerization. Recently, a domain important for MD-2 binding to TLR4 was suggested to locate at Cys^{95} and Cys^{105}, and surrounding hydrophilic and charged residues (39). In contrast, a domain required for MD-2 binding to LPS is suggested to be a highly basic region between Phe^{121} and Lys^{132}. MD-2 might have another functional domain regulating TLR4 oligomerization. Human MD-2 exhibited 66% amino acid sequence identity with mouse MD-2 (38). Taxol, an anti-cancer reagent, stimulates mouse TLR4–MD-2 but not human MD-2. Kawasaki *et al.* (41) determined Gln^{22} of mouse MD-2 as an amino acid reside regulating species-specific

responsiveness of TLR4–MD-2 to taxol. It is possible that the N-terminal region including Gln^{22} might regulate ligand-induced TLR4 oligomerization.

Despite aggressive management, many hospitalized patients die of endotoxin shock. Lipid A antagonists are being developed with an aim to neutralize endotoxin *in vivo*. We showed here that lipid A antagonists target TLR4–MD-2, which mediates the adverse effects of endotoxin (6,15). The present results revealed that lipid A antagonist interacts with TLR4–MD-2 but halts subsequent TLR4 oligomerization, and suggest that lipid A antagonist may be distinct from lipid A in the stoichiometry of ligand–receptor interaction. Further study would contribute to further improvement of lipid A antagonists and development of novel therapeutic interventions for endotoxin shock.

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Abbreviations

GFP	green fluorescent protein
TLR4F	TLR4–flag fusion protein
TLR4G	TLR4–GFP fusion protein

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