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Lipopolysaccharide Interaction with Cell Surface Toll-like Receptor 4-MD-2: Higher Affinity than That with MD-2 or CD14

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Abstract

Toll-like receptors (TLRs) are innate recognition molecules for microbial products, but their direct interactions with corresponding ligands remain unclarified. LPS, a membrane constituent of gram-negative bacteria, is the best-studied TLR ligand and is recognized by TLR4 and MD-2, a molecule associated with the extracellular domain of TLR4. Although TLR4-MD-2 recognizes LPS, little is known about the physical interaction between LPS and TLR4-MD-2. Here, we demonstrate cell surface LPS–TLR4-MD-2 complexes. CD14 greatly enhances the formation of LPS–TLR4-MD-2 complexes, but is not coprecipitated with LPS–TLR4-MD-2 complexes, suggesting a role for CD14 in LPS loading onto TLR4-MD-2 but not in the interaction itself between LPS and TLR4-MD-2. A tentative dissociation constant (K_d) for LPS–TLR4-MD-2 complexes was ~3 nM, which is ~10–20 times lower than the reported K_d for LPS–MD-2 or LPS–CD14. The presence of detergent disrupts LPS interaction with CD14 but not with TLR4-MD-2. E5531, a lipid A antagonist developed for therapeutic intervention of endotoxin shock, blocks LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that required for blocking LPS interaction with CD14. These results reveal direct LPS interaction with cell surface TLR4-MD-2 that is distinct from that with MD-2 or CD14.

Key words: innate immunity • cell surface molecule • activation • macrophage

Introduction

The innate immune response is the first line of defense against microbial pathogens, and plays an important role in activating acquired immunity. Recently, the toll-like receptor (TLR) family has been discovered as specific pathogen recognition molecules in the innate immune system (1). Although TLRs specifically recognize microbial products (2), detection of direct interaction between TLRs and their ligands has been unsuccessful thus far.

LPS is a principal component of gram-negative bacteria that activates the innate immune system, and is one of the best-studied microbial products (3). Great progress has been made recently in the identification of LPS recognition molecules. Core components are CD14 (4), MD-2 (5), and toll-like receptor (TLR) 4 (6, 7). CD14 binds to LPS and augments LPS responses. TLR4-MD-2 is thought to work downstream of this initial binding. TLR4 is a type I membrane protein consisting of extracellular leucine-rich repeats and an intracellular signaling domain. MD-2 is associated with the extracellular leucine-rich repeats of TLR4. Both TLR4 and MD-2 are indispensable for LPS responses because mice lacking either TLR4 or MD-2 do not respond to LPS (8, 9). Despite these evidences for LPS recognition by

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Abbreviations used in this paper: PGN, peptidoglycan; TLR, toll-like receptor.

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TLR4-MD-2, little is known for LPS interaction with TLR4-MD-2.

Although soluble MD-2 was shown to bind to LPS (10), LPS–MD-2 needs to eventually form LPS–TLR4-MD-2 complexes on the cell surface to trigger a signal. However, little is known about LPS–TLR4-MD-2 complexes. Recent works suggested CD14-dependent LPS interaction with TLR4-MD-2 by using chemical cross-linking between LPS and TLR4-MD-2 (11–13). However, these papers showed merely the physical proximity between LPS and TLR4-MD-2, and do not necessarily demonstrate direct LPS interaction with TLR4-MD-2 in physiological condition. Direct interaction between LPS and TLR4-MD-2 still needs to be demonstrated and characterized in responding cells.

Here, we show, with a newly established monoclonal antibody to TLR4-MD-2, LPS–TLR4-MD-2 complexes on the cell surface, and we demonstrate direct LPS interaction with TLR4-MD-2 that is distinct from that with MD-2 or CD14.

Materials and Methods

Reagents. LPS from *Escherichia coli* 055:B5 and lipid A purified from *Salmonella minnesota* were purchased from Sigma-Aldrich. Synthetic lipid A and ^{3}H-labeled lipid A were described previously (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and *N*-octyl-β-D-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti–mouse TLR4 serum was established by immunized rabbits with TLR4 peptide (CSFNRIETSKGILQHFP), which is conjugated with keyhole limpet hemocyanin.

Expression Constructs and Stable Transfectants. IL-3–dependent Ba/F3 cells (15) were cultured in 10% FCS and RPMI 1640 supplemented with 100 μM 2-mercaptoethanol and IL-3. The mouse cDNAs encoding TLR4, MD-2, and CD14 were cloned into the retrovirus vector pMX-puromycin (16). MD-2 was tagged with the flag epitope at the COOH terminus. Ba/F3 cells expressing TLR4 and MD-2flag with or without CD14 were established by retroviral transduction. Ba/F3 cells expressing CD14 alone were established by electroporation of expression vector pcDNA3 (Invitrogen) encoding mouse CD14. We have described previously Ba/F3 cells expressing either MD-2 or TLR4, both of which were tagged with the flag epitope at the COOH terminus (indicated as MD-2f and TLR4f, respectively; reference 17). These cells were further transfected with pcDNA3 encoding CD14 (indicated as CD14/MD-2f and CD14/TLR4f, respectively).

Establishment of mAbs to Mouse TLR4-MD-2 and CD14. A rat was immunized on the foot pads with normal rat kidney cells expressing CD14 and TLR4-MD-2 that had been stimulated with 1 μg/ml lipid A. 1 wk later, cells from draining lymph nodes were fused with SP2/0 myeloma cells. Sa15-21 and Sa2-8 mAb (rat IgG2a/κ) were selected for further analyses because they specifically reacted with Ba/F3 cells expressing TLR4-MD-2 or CD14, respectively. The mAbs were purified from ascites obtained from SCID mice.

Cell Staining and Flow Cytometry. Single cell suspensions were incubated at 2×10^5 cells/100 μl on ice for 15 min with the primary antibodies diluted in staining buffer (PBS containing 2.5% FCS and 0.01% NaN_3). Cells were washed in staining buffer, and incubated with R-PE–conjugated goat anti–rat IgG Ab (Southern Biotechnology Associates Inc.), FITC-conjugated goat anti–mouse IgG (BD Biosciences), or R-PE–conjugated streptavidin (BD Biosciences) for 15 min. The mAbs used were as follows: rat anti–mouse TLR4-MD-2 (MTS510 and Sa15-21); rat anti–mouse CD14, 4C1 (18); or anti-LPS (Hycult Biotechnology). Flow cytometry analysis was performed using a FACSCalibur™ system (Becton Dickinson).

Cell Surface Biotinylation, Immunoprecipitation, and Immunoprobing. Cell surface proteins were biotinylated with 6-((6-((biotinoyl)amino)hexanoyl)amino) hexanoic acid, succinimidyl ester (biotin-XX; Molecular Probes) dissolved in saline containing 10 mM Hepes, pH 7.5, for 30 min at room temperature. Cells were washed and lysed in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, and 5 mM EDTA. After 30 min of incubation on ice, nuclei were removed by centrifugation. mAb-coupled beads were added to cell lysate and rotated for 2 h at 4°C. Beads were washed in 5× diluted lysis buffer, and bound proteins were subjected to SDS-PAGE and Western blotting with streptavidin–alkaline phosphatase conjugate (Calbiochem). The primary reagents for immunoprobing were as follows: Sa2-8 for CD14; anti-LPS (Hycult Biotechnology) for LPS; anti–IkB-α (BD Biosciences); and anti-actin (Sigma-Aldrich). The second reagents were alkaline phosphatase–conjugated as follows: goat anti–rabbit IgG (Bio-Rad Laboratories); goat anti–rat IgG; and goat anti–mouse IgG (American Qualex).

^{3}H-Lipid A Binding Assay. ^{3}H-lipid A was synthesized as described previously (14). The specific radioactivity was ∼10,000 cpm/ng. Ba/F3 stable transfectants (10^8 cells/sample) were stimulated with various concentrations of ^{3}H-lipid A for 30 min at 37°C. After washing, cells were subjected to immunoprecipitation as described in the preceding paragraph. Precipitated radioactivity was counted by a liquid scintillation counter (Aloka). To exclude nonspecific binding to TLR4-MD-2 in Fig. 6 (a and b), cpm associated with MTS510 was subtracted from that with Sa15-21.

Results

No Detectable LPS Binding to Cells Expressing TLR4-MD-2 but Not CD14. To investigate direct interaction between LPS and cell surface TLR4-MD-2, we used flow cytometry staining with an anti-LPS mAb. Ba/F3 stable transfectants separately expressing TLR4 alone, CD14 alone, TLR4-MD-2, or CD14 and TLR4-MD-2 were established, and cell surface expression of TLR4-MD-2 and CD14 was tested for in each cell line (Fig. 1, left and middle columns). These lines were stimulated with LPS, and cell surface LPS was detected with the anti-LPS mAb. Cell surface LPS was detected only on cells expressing CD14 (right column). LPS binding was largely abolished when anti-CD14 mAb was included during LPS stimulation (unpublished data). In sharp contrast, we could not detect any cell surface LPS on cells expressing TLR4-MD-2 (Fig. 1), revealing a clear difference from soluble MD-2 that showed CD14-independent binding to LPS with an affinity similar to that for CD14 (10).

Anti–TLR4-MD-2 mAb MTS510 Staining Is Down-regulated upon Lipid A Stimulation. Next, we studied a LPS-dependent change in TLR4-MD-2. We showed previously

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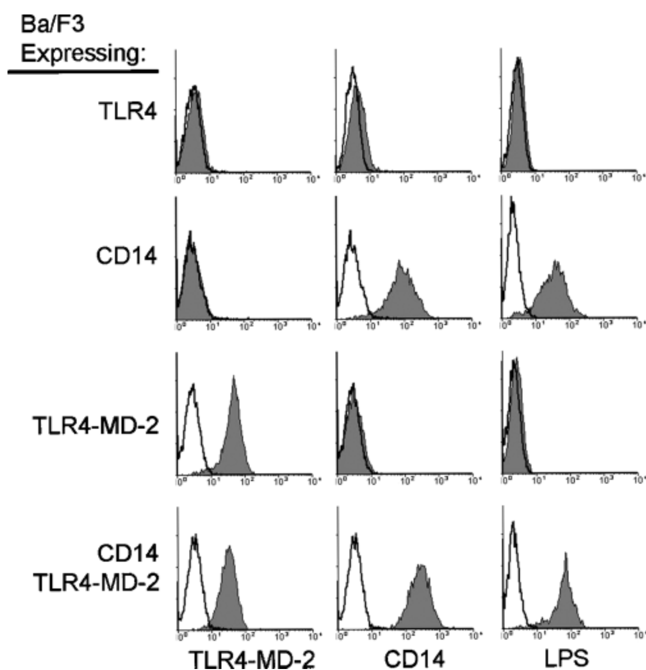

Figure 1. No detectable LPS binding to cells expressing TLR4-MD-2 without CD14. Ba/F3 stable transfectants were stained with anti–TLR4-MD-2 mAb (MTS510, left) or anti-CD14 mAb (4C1, middle), followed by goat anti–rat IgG-PE. (right) Ba/F3 transfectants were stimulated with 1 μg/ml LPS at 37°C for 30 min, washed, and stained with anti-LPS mAb, followed by goat anti–mouse IgG-FITC. All the open histograms depict staining with the second reagent alone.

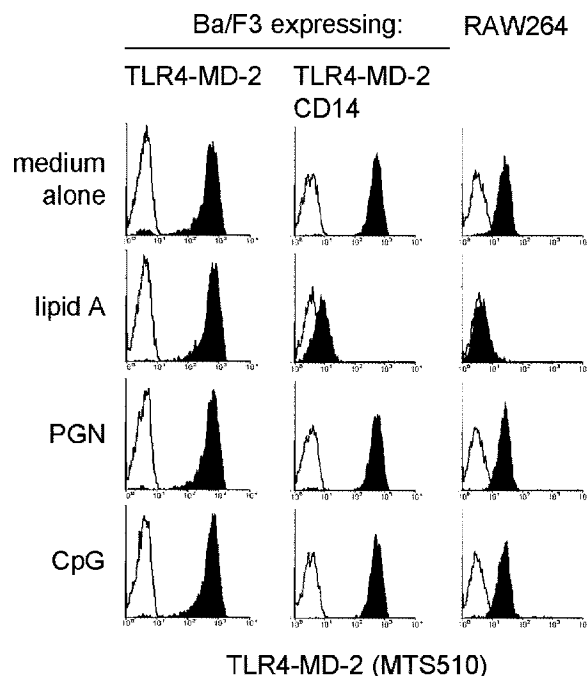

Figure 2. LPS down-regulates MTS510 binding to cell surface TLR4-MD-2. Ba/F3 cells expressing TLR4-MD-2 (left) or CD14$^+$TLR4-MD-2 (middle), or a macrophage line RAW264 (right) were stimulated with medium alone, 1 μg/ml lipid A, 10 μg/ml peptidoglycan (PGN), or 100 μM CpG DNA as indicated. After washing, cells were stained with biotinylated MTS510 followed by streptavidin-PE.

that cell surface expression of TLR4-MD-2 on peritoneal macrophages decreases upon LPS stimulation, as detected by a mAb to TLR4-MD-2, MTS510 (19). We were able to see a similar phenomenon in Ba/F3 transfectants and macrophage line RAW264. The decrease in MTS510 staining is observed with lipid A or LPS stimulation but not with other stimuli, such as peptidoglycan (PGN) or CpG oligodeoxynucleotide (Fig. 2 and not depicted). The down-regulation became apparent with as little as 20 ng/ml in RAW264 and 200 ng/ml in the transfectant, and as early as 10 min after LPS stimulation (unpublished data). Interestingly, lipid A–induced decrease in MTS510 staining was not clear in the absence of membrane CD14 (Fig. 2, left column). In keeping with this, anti-CD14 mAb was able to inhibit LPS-triggered decrease in MTS510 staining (unpublished data).

Newly Established mAb to TLR4-MD-2 Reveals the LPS-induced Change of TLR4-MD-2. We asked the cause of the lipid A–dependent decrease in MTS510 staining. We considered three possibilities. First, TLR4-MD-2 may be internalized upon LPS stimulation as described previously (20). Second, MD-2 may be dissociated from TLR4; MTS510 does not bind to TLR4 alone (17). Finally, TLR4-MD-2 remains on the cell surface but LPS stimulation may alter TLR4-MD-2 to a conformation, which MTS510 cannot bind. Biochemical analyses revealed that MTS510 reacted with TLR4-MD-2 before LPS stimulation but not after LPS stimulation. For further study, we established a novel mAb to TLR4-MD-2, Sa15-21, which was able to react with LPS-stimulated TLR4-MD-2, to which MTS510 did not bind. Sa15-21 was similar to MTS510 in that it specifically immunoprecipitated TLR4-MD-2 but not TLR4 alone (Fig. 3 a). However, Sa15-21 recognized an epitope distinct from the MTS510 epitope, because these two mAbs did not crossblock with each other (unpublished data). Sa15-21 remained reactive with LPS- and lipid A–stimulated cells expressing CD14 and TLR4-MD-2 as revealed by flow cytometry staining (Fig. 3 b). Similar results were obtained with RAW264 cells (unpublished data). Biochemical analyses were conducted with Sa15-21 mAb (Fig. 3 c). Ba/F3 cells expressing TLR4-MD-2 and CD14 or RAW264 cells were subjected to LPS stimulation, cell surface biotinylation, and immunoprecipitation with Sa15-21 or MTS510. MTS510 precipitated cell surface TLR4-MD-2 from cells treated with medium alone or a lipid A antagonist, E5531, but not from cells stimulated with LPS. In sharp contrast, Sa15-21 was able to precipitate cell surface TLR4-MD-2 from LPS-stimulated cells. Together, LPS-stimulated TLR4-MD-2 remains on the cell surface, but is likely to undergo the LPS-dependent conformation change, which is not recognized by MTS510 mAb.

LPS and TLR4-MD-2 Forms Stable Complexes on the Cell Surface. Next, we hypothesized that the change of TLR4-MD-2 may accompany the direct interaction between LPS and TLR4-MD-2. To address this possibility, we conducted immunoprecipitation of lipid A–stimulated TLR4-MD-2 with Sa15-21, and coprecipitation of lipid A was

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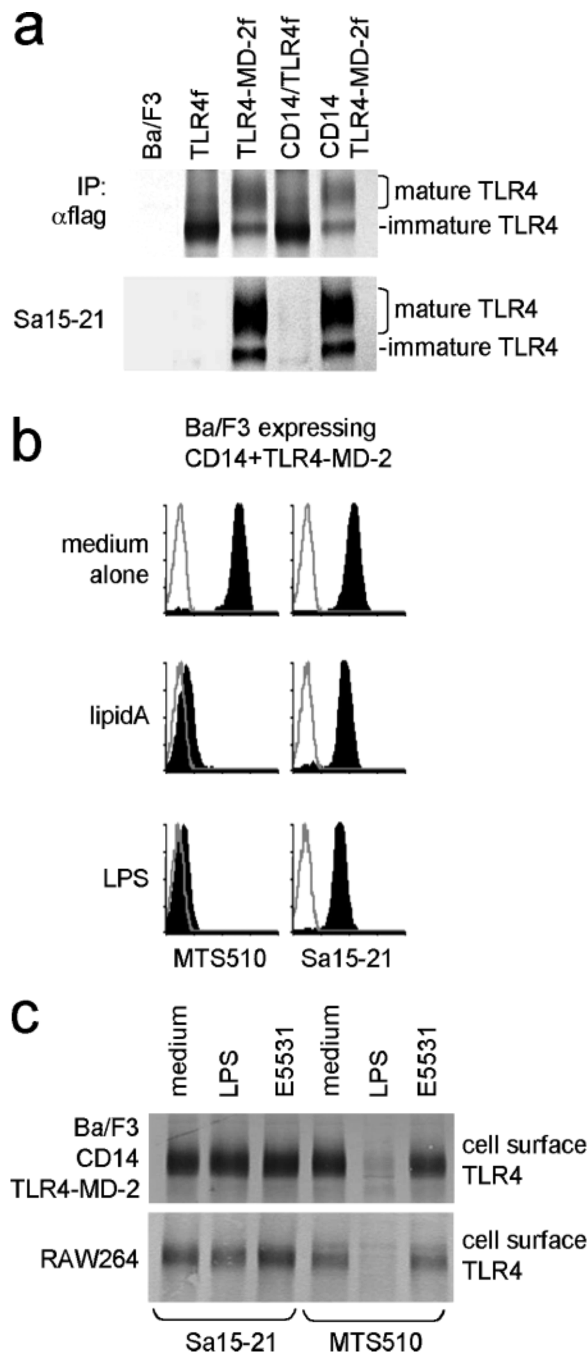

Figure 3. A novel mAb to TLR4-MD-2 reveals the LPS-triggered change of cell surface TLR4-MD-2. (a) Immunoprecipitation with anti-flag (top) or Sa15-21 (bottom) was conducted with Ba/F3 transfectants expressing the indicated molecules (Materials and Methods). The precipitates were probed with rabbit anti–mouse TLR4 sera followed by goat anti–rabbit alkaline phosphatase. Only immature, smaller TLR4 is detected in cells expressing TLR4 alone (top, TLR4f and CD14/TLR4f), because TLR4 without MD-2 cannot reach the cell surface. (b) Ba/F3 transfectants expressing CD14 and TLR4-MD-2 were stimulated with medium alone, 1 μg/ml lipid A, or 1 μg/ml LPS at 37°C for 30 min. Cells were stained with biotinylated MTS510 mAb or Sa15-21 as indicated, followed by streptavidin-PE. Open histograms depict staining with streptavidin-PE alone. (c) Ba/F3 cells expressing TLR4-MD-2 and CD14 (top) or RAW264 (bottom) were stimulated with medium, 2 μg/ml LPS, or 2 μg/ml lipid A antagonist E5531 as indicated at 37°C for 30 min. After washing, cells were subjected to cell surface biotinylation, detergent lysis, immunoprecipitation with MTS510 mAb (right three lanes) or Sa15-21 mAb (left three lanes), SDS-PAGE (7.5% polyacrylamide under nonreducing conditions), and electroblotting. Precipitated cell surface TLR4 was probed with streptavidin–alkaline phosphatase conjugate.

probed with anti–lipid A antibody (Fig. 4 a, bottom). We were able to detect lipid A coprecipitation with TLR4-MD-2 by Sa15-21 mAb but not by MTS510 mAb. Interestingly, MTS510 was able to precipitate TLR4-MD-2 from lipid A–stimulated cells as detected by anti-TLR4 polyclonal mAb (Fig. 4 a, top). The amount of precipitated TLR4 seems to be smaller than that from cells without stimulation. Precipitated TLR4-MD-2 is likely to be located inside the cells, because we could not detect the precipitation by MTS510 of LPS-stimulated, cell surface TLR4-MD-2 (Fig. 3 c). Lipid A was not present in these MTS510 precipitates containing intracellular TLR4-MD-2 (Fig. 4 a, bottom). We also examined CD14 coprecipitation with lipid A–TLR4-MD-2 complexes to address the possibility that association of lipid A with TLR4-MD-2 is mediated by CD14. We could not detect CD14 in lipid A–TLR4-MD-2 complexes precipitated by Sa15-21, demonstrating the direct interaction between lipid A and TLR4-MD-2 (Fig. 4 a, middle).

Similar results were obtained with LPS and anti-LPS antibody (unpublished data). We also detected LPS–TLR4-MD-2 complexes with native cells such as a macrophage line RAW264 (unpublished data). Thus, the LPS- or lipid A–dependent change of TLR4-MD-2 accompanies the formation of LPS–TLR4-MD-2 complexes on the cell surface.

A Role for Membrane CD14 in LPS Interaction with TLR4-MD-2. Membrane CD14 was required for the LPS-dependent conformation change of TLR4-MD-2 (Fig. 2), but CD14 was not present in lipid A–TLR4-MD-2 complexes (Fig. 4 a). Next, we addressed a role for membrane CD14 in LPS interaction with TLR4-MD-2 by using Ba/F3 transfectants separately expressing MD-2flag, TLR4flag, CD14, TLR4-MD-2flag, CD14 and TLR4flag, CD14 and MD-2flag, and CD14 and TLR4-MD2flag (Materials and Methods). We used ^{3}H-lipid A to stimulate the transfectants. Immunoprecipitation was conducted with MTS510, Sa15-21, anti-flag mAb, or anti-CD14 mAb. Precipitated radioactivity is shown (Fig. 4 b and not depicted). Appreciable counts were detected in the precipitate with Sa15-21 from Ba/F3 transfectant expressing CD14 and TLR4-MD-2, but not clearly from that expressing TLR4-MD-2. Lipid A coprecipitation was also observed with anti-flag mAb only from the transfectant expressing CD14 and TLR4-MD-2flag (Fig. 4 b). We could not detect lipid A coprecipitation with the anti-flag mAb from cells separately expressing MD-2flag, TLR4flag, TLR4-MD-2flag, CD14 and TLR4flag, or CD14 and MD-2flag (Fig. 4 b and not depicted). We had similar results with LPS, coprecipitation of which was detected by anti-LPS mAb (unpublished data). These results demonstrate an important role for membrane CD14 in facilitating the establishment of the interaction between lipid A and TLR4-MD-2. However, CD14 is not required for the interaction itself, because membrane

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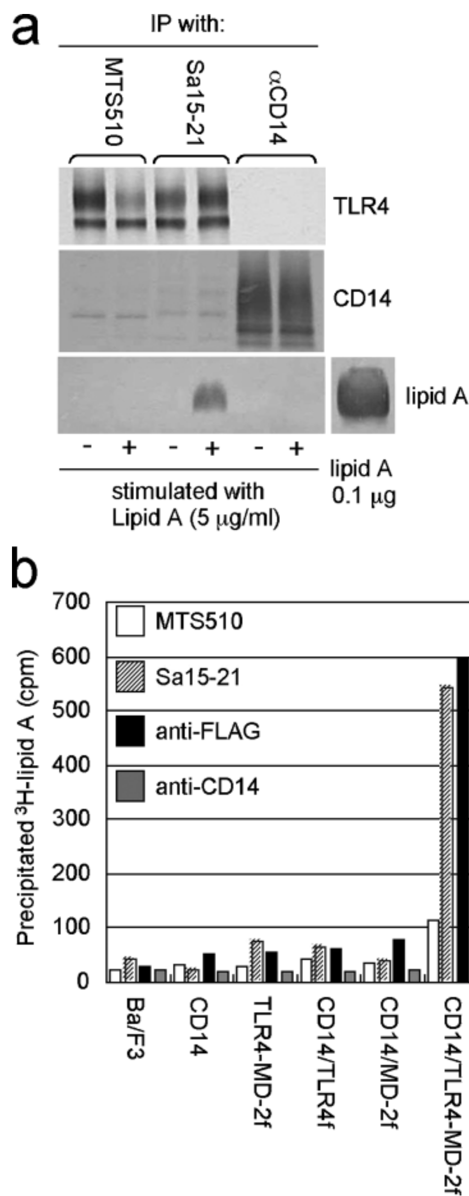

Figure 4. Lipid A–TLR4-MD-2 complexes. (a) Ba/F3 cells expressing TLR4-MD-2 and CD14 (10^8/10 ml sample) were incubated with 5 μg/ml lipid A at 37°C for 30 min. Cells were subjected to washing, detergent lysis, immunoprecipitation (with mAbs to TLR4-MD-2, MTS510, and Sa15-21, or an mAb to CD14, Sa2-8, and SDS-PAGE [17.5% for lipid A, 10% for CD14, and 7.5% for TLR4; under nonreducing conditions]), and electroblotting. Precipitates were probed with anti-TLR4 polyclonal Ab, anti-CD14 mAb (Sa2-8), or anti–lipid A mAb, followed by alkaline phosphatase–conjugated secondary antibodies. Nonspecific signals detecting IgG heavy chains were observed in the precipitates probed with anti-CD14 mAb (left four lanes). (b) A variety of Ba/F3 stable transfectants (2.5×10^7/sample) were stimulated with ^{3}H-lipid A (0.75 μCi/sample) at 37°C for 30 min. Cells were subjected to washing, detergent lysis, and immunoprecipitation with MTS510, Sa15-21, anti-flag, or anti-CD14 mAbs. Precipitated radioactivity was counted by scintillation counter. Similar results were obtained from three independent experiments using ^{3}H-lipid A.

CD14 is not present in lipid A–TLR4-MD-2 complexes (Fig. 4 a). It should be noted that only 1–2% of cell bound lipid A was coprecipitated with TLR4-MD-2 (Fig. 4 b).

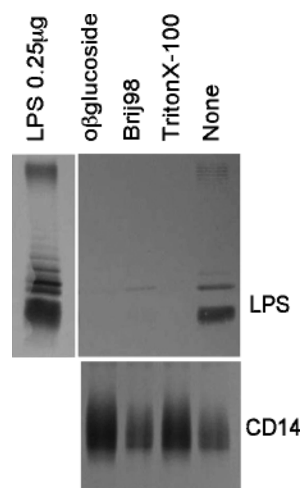

Figure 5. LPS–CD14 complexes are disrupted by detergents. Supernatant from Ba/F3 cells expressing 1 ml CD14 (cultured up to 4–5 × 10^6/ml) was incubated with 3 μg/ml LPS at 37°C for 30 min; sCD14 in the supernatant from Ba/F3 cells expressing CD14 was immunoprecipitated with anti-CD14 mAb (4C1). The indicated detergents (1% Triton X-100, 0.5% *N*-octyl-β-D-glucoside, and 1% Brij98) were included before immunoprecipitation. Precipitated LPS or CD14 was probed with anti-LPS mAb and anti-CD14 mAb (Sa2-8), followed by alkaline phosphatase–conjugated secondary antibodies.

LPS Interaction with TLR4-MD-2 Is More Stable than That with CD14. Unexpectedly, lipid A coprecipitation was not detected in the CD14 precipitates with anti-CD14 mAb (Fig. 4, a [right lane]). We prepared cell lysates for immunoprecipitation using lysis buffer containing 1% Triton X-100, which might disrupt LPS interaction with CD14. To address this possibility, soluble CD14–LPS complexes were immunoprecipitated with anti-CD14 in the absence of detergents (Fig. 5). LPS was clearly coprecipitated with soluble CD14. Moreover, LPS coprecipitation was abolished by the presence of detergents such as 1% Triton X-100, 0.5% *N*-octyl-β-D-glucoside, and 1% Brij98, indicating that these detergents disrupted LPS interaction with CD14. In view of the possibility that interaction between LPS and TLR4-MD-2 can only be observed in the presence of detergent, we conducted immunoprecipitation of sonicated cell membrane in the absence of detergent. The results using mechanically solubilized cell lysate clearly demonstrated physical association between LPS and TLR4-MD-2 (unpublished data). Thus, LPS interaction with TLR4-MD-2 seems to be distinct from and more stable than with CD14.

We could not detect lipid A–MD-2 complexes by immunoprecipitation of MD-2 from cells expressing MD-2flag or CD14+ MD-2flag (Fig. 4 b and not depicted). It is possible that lipid A–MD-2 interaction might be disrupted in the presence of detergents as lipid A–CD14 interaction.

Binding Properties of Lipid A Interaction with TLR4-MD-2. It was difficult to directly compare lipid A–TLR4-MD-2 with lipid A–MD-2 or with lipid A–CD14, because lipid A was not coprecipitated with CD14 or MD-2 (Fig. 4, a and b). Immunoprecipitation in our binding assay seems to be too rigorous for lipid A–CD14 or lipid A–MD-2 complexes. Despite such a rigorous binding assay, the binding of lipid A and TLR4-MD-2 was dose-dependent and reached saturation (Fig. 6 a). Therefore, we tentatively figured out an apparent dissociation constant (K_d) for lipid A–TLR4-MD-2 complexes (Fig. 6 b). We were able to obtain the tentative K_d of ~3 nM for lipid A–TLR4-MD-2, which was, even in a rigorous condition, ~10–20 times

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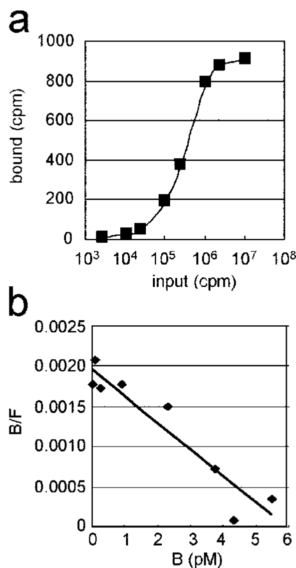

Figure 6. Binding properties of lipid A interaction with TLR4-MD-2. Ba/F3 stable transfectant expressing CD14 and TLR4-MD-2 (10^8 cells/10 ml medium) were stimulated with various concentrations of ^{3}H-lipid A (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μCi) for 30 min at 37°C. After washing, cells were subjected to immunoprecipitation with Sa15-21 or with MTS510. Precipitated radioactivity was counted by a liquid scintillation counter (Aloka). Specific binding was obtained by subtracting bound cpm with MTS510 from that with Sa15-21. Bound lipid A (cpm) was plotted against input lipid A (cpm) in panel a. Scatchard plot is shown in panel b. Two independent experiments were conducted and similar results were obtained.

lower than the reported dissociation constant for LPS–MD-2 or LPS–CD14 complexes (65 and 30–74 nM, respectively; references 10, 21).

Lipid A Antagonist E5531 Acts on TLR4-MD-2. To further characterize interaction between LPS and TLR4-MD-2, we used E5531, a potent LPS antagonist developed for therapeutic intervention of endotoxin shock (22). E5531 was shown to block LPS binding to macrophage cells, supposedly by antagonizing LPS binding to membrane CD14 (23). However, further analysis demonstrates that E5531 inhibits CD14-independent, TLR4-MD-2 agonists (24). We hypothesized that E5531 acts on TLR4-MD-2 rather than on CD14. Ba/F3 cells expressing CD14 and TLR4-MD-2 were pretreated with graded concentrations of E5531 for 30 min and stimulated with LPS at 3 μg/ml. We added E5531 up to 30 μg/ml, 10 times higher than the concentration of LPS. E5531 was able to block CD14-dependent LPS binding to cells at concentrations higher than 10 μg/ml (Fig. 7 a, right). For further confirmation of the antagonistic effect of E5531, we also conducted LPS coprecipitation with soluble CD14. The supernatant from Ba/F3 cells expressing CD14 was exposed to 3 μg/ml LPS with or without the indicated concentration of E5531. Soluble CD14 was precipitated with anti-CD14 mAb and LPS coprecipitation was probed with anti-LPS. LPS coprecipitation gradually decreased with increasing concentrations of E5531, leading to complete inhibition at 10 μg/ml of E5531 (Fig. 7 b).

Next, we examined the effect of E5531 on TLR4-MD-2. E5531 completely antagonized LPS-dependent down-regulation of MTS510 staining at a concentration as low as 0.1 μg/ml (Fig. 7 a, middle). LPS association with TLR4-MD-2 was disrupted with concentrations of E5531 higher than 0.1 μg/ml (Fig. 7 c). Furthermore, E5531 was able to inhibit LPS-stimulated IkBα degradation at 0.1 μg/ml (Fig. 7 d). With regard to sensitivity to E5531 treatment, LPS signaling correlated precisely with LPS interaction with TLR4-MD-2 but not with CD14.

Discussion

A newly established mAb to TLR4-MD-2, Sa15-21, demonstrated lipid A–TLR4-MD-2 complexes on the cell surface by precipitating lipid A–TLR4-MD-2 complexes from cells expressing TLR4-MD-2 and CD14 (Fig. 4). Lipid A interaction with TLR4-MD-2 was dose-dependent and saturable (Fig. 6 a). Cell surface TLR4-MD-2 interacts with lipid A at a tentative K_d of ∼3 nM, which is ∼10–20 times lower than that of soluble MD-2 (65 nM) or CD14 (74 nM; reference 10). This binding assay uses immunoprecipitation and is different from a conventional ligand binding assay. We could not directly compare, with the binding assay, TLR4-MD-2 with CD14 or MD-2 with regard to lipid A interaction because lipid A was not coprecipitated with CD14 or MD-2, probably due to the presence of detergents (Figs. 4 and 5). However, even in such a rigorous condition where affinity decreased, our binding assay still yielded the K_d for lipid A–TLR4-MD-2 that is still lower than the reported K_d for lipid A–CD14 or lipid A–MD-2. In keeping with the lower K_d, LPS–TLR4-MD-2 complexes were disrupted by lipid A antagonist E5531 at ∼100 times lower concentration than LPS–CD14 complexes (Fig. 7). Together, the present results demonstrated LPS–TLR4-MD-2 complexes that interact with each other at higher affinity and are more stable than LPS–MD-2 or LPS–CD14 complexes. The higher affinity for TLR4-MD-2 than that of soluble MD-2 may be due to associated TLR4 that might directly bind to lipid A or strengthen interaction between MD-2 and lipid A.

Although LPS interaction with soluble MD-2 does not require CD14 (10), membrane CD14 was required for LPS interaction with TLR4-MD-2 (Fig. 4 b). Recent papers suggest that LPS triggers CD14 association with TLR4-MD-2 (11, 13). However, we could not detect CD14 association with LPS–TLR4-MD-2 complexes (Fig. 4 a). Even if CD14 associates itself with LPS–TLR4-MD-2 complexes, the association probably plays a minimal role in sustaining the direct interaction between LPS and TLR4-MD-2; LPS interaction with TLR4-MD-2 is higher in affinity and more stable than that with CD14. CD14 seems to have a role in loading LPS onto TLR4-MD-2, which by itself showed little binding to LPS (Fig. 1). It should be noted that CD14 is not essential for LPS response. CD14 KO mice still show significant LPS responses (25, 26). A CD14-independent mechanism for loading LPS onto TLR4-MD-2 must be present and is likely to be accelerated by membrane CD14. We believe that a small number of LPS–TLR4-MD-2 complexes are present on cells expressing TLR4-MD-2 without CD14. The number of complexes is probably too small to be detected in the present detection system. Interestingly, only 1–2% of cell-associated lipid A was coprecipitated with TLR4-MD-2, even with membrane CD14 (Fig. 4 b). The CD14-independent lipid A loading mechanism might restrict the amount of lipid A to be loaded onto TLR4-MD-2. Further study has to focus on molecular mechanisms underlying LPS loading onto TLR4-MD-2.

The unique mAb to TLR4-MD-2, MTS510, binds to cell surface TLR4-MD-2 before LPS stimulation, but not af-

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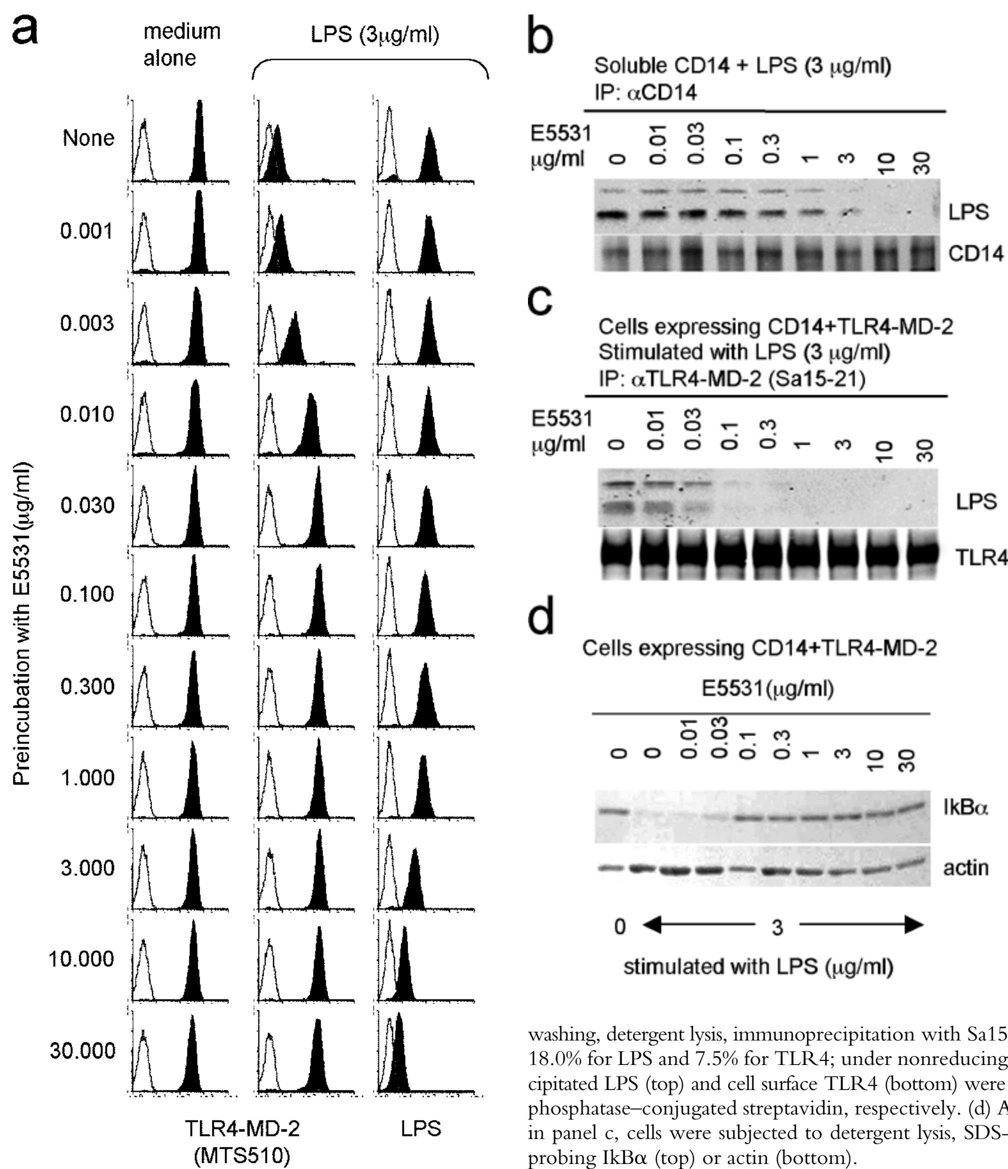

Figure 7. E5531 acts on LPS interaction with TLR4-MD-2 at a concentration that does not affect LPS binding to mCD14. (a) Ba/F3 cells expressing TLR4-MD-2 and CD14 were pretreated with or without E5531 (indicated concentration) at 37°C for 30 min. Cells were stimulated with medium alone or 3 μg/ml LPS at 37°C for 30 min. After washing, cells were stained with biotinylated MTS510 mAb followed by streptavidin-PE (left and middle columns), or with anti-LPS followed by goat anti–mouse IgG-FITC (right column). Open histograms depict staining with the secondary reagent alone. (b) 3 μg/ml LPS with indicated concentrations of E5531 was added to the supernatant from Ba/F3 cells expressing CD14. sCD14 in the supernatant was precipitated with anti-CD14 mAb, followed by immunoprobing with anti-LPS (top) or anti-CD14 (bottom). (c) After treatment with E5531 and LPS as in panel a, cells were subjected to cell surface biotinylation, washing, detergent lysis, immunoprecipitation with Sa15-21, SDS-PAGE (polyacrylamide gel: 18.0% for LPS and 7.5% for TLR4; under nonreducing conditions), and electroblotting. Precipitated LPS (top) and cell surface TLR4 (bottom) were probed with anti-LPS mAb or alkaline phosphatase–conjugated streptavidin, respectively. (d) After treatment with LPS and E5531 as in panel c, cells were subjected to detergent lysis, SDS-PAGE, electroblotting, and immunoprobing IkBα (top) or actin (bottom).

ter stimulation, demonstrating the ligand-dependent change of TLR4-MD-2 (Figs. 2 and 3). The LPS-triggered change of TLR4-MD-2 correlates perfectly with LPS–TLR4-MD-2 complex formation. It is possible that the LPS-dependent change of TLR4-MD-2 reflects the conformation change of TLR4-MD-2, which leads to dimerization of TLR4-MD-2 and delivering a signal. The LPS-dependent change of TLR4-MD-2 may reveal a link between LPS interaction and LPS signaling. Further studies on the antagonistic action of E5531 may be important to address this issue, because E5531 does not induce the LPS-dependent change and prevents LPS interaction with TLR4-MD-2 (Fig. 7 a).

It has been described previously that E5531 inhibits LPS binding to cells, suggesting competition with LPS in binding to CD14 as an antagonistic mechanism (23). Because CD14 binds other microbial products in addition to LPS (27), E5531 should be able to compete CD14 interaction with these microbial products. However, E5531 is antagonistic to LPS but not to other microbial products (e.g., PGN; reference 23). Moreover, E5531 inhibits CD14-independent TLR4-MD-2 ligands (24). These results suggest that E5531 directly acts on TLR4-MD-2. The present experimental system enabled us to address this question. E5531 inhibited LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that required for inhibiting LPS interaction with CD14 (Fig. 7). Given that E5531 acts on TLR4-MD-2, it is reasonable that E5531 antagonizes TLR4-MD-2 ligand other than LPS. Thus, the present paper has revealed a novel mechanism for the action of this lipid A antagonist.

The reported incidence of sepsis syndromes is increasing dramatically in hospitalized patients. Despite aggressive management, many patients die of endotoxin shock. E5531 was developed with an aim to neutralize endotoxin in vivo (22). Here, we showed that E5531 targets TLR4-MD-2, which mediates the adverse effects of endotoxin (8, 9). TLR4-MD-2 is arguably a target for therapeutic interven-

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tion of endotoxin shock. The present work provides a theoretical background and assay system for further improvement of lipid A antagonists and for development of other therapeutic interventions for endotoxin shock.

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