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Pharmacological Inhibition of Endotoxin Responses Is Achieved by Targeting the TLR4 Coreceptor, MD-2[1]

Alberto Visintin,[2] Kristen A. Halmen, Eicke Latz, Brian G. Monks, and Douglas T. Golenbock

The detection of Gram-negative LPS depends upon the proper function of the TLR4-MD-2 receptor complex in immune cells. TLR4 is the signal transduction component of the LPS receptor, whereas MD-2 is the endotoxin-binding unit. MD-2 appears to activate TLR4 when bound to TLR4 and ligated by LPS. Only the monomeric form of MD-2 was found to bind LPS and only monomeric MD-2 interacts with TLR4. Monomeric MD-2 binds TLR4 with an apparent K_d of 12 nM; this binding avidity was unaltered in the presence of endotoxin. E5564, an LPS antagonist, appears to inhibit cellular activation by competitively preventing the binding of LPS to MD-2. Depletion of endogenous soluble MD-2 from human serum, with an immobilized TLR4 fusion protein, abrogated TLR4-mediated LPS responses. By determining the concentration of added-back MD-2 that restored normal LPS responsiveness, the concentration of MD-2 was estimated to be ~50 nM. Similarly, purified TLR4-Fc fusion protein, when added to the supernatants of TLR4-expressing cells in culture, inhibited the interaction of MD-2 with TLR4, thus preventing LPS stimulation. The ability to inhibit the effects of LPS as a result of the binding of TLR4-Fc or E5564 to MD-2 highlights MD-2 as the logical target for drug therapies designed to pharmacologically intervene against endotoxin-induced disease. ***The Journal of Immunology,*** **2005, 175: 6465–6472.**

The receptor complex composed of TLR4 and MD-2 constitutes a molecular antenna that recognizes and signals mammalian cells for the presence of LPS. TLR4 is a type I transmembrane glycoprotein characterized by the presence of 22 leucine-rich repeats on the extracellular domain (1). Initiation of the signal elicited by LPS depends on the dimerization of the cytoplasmic Toll-IL-1 resistance (TIR)[3] domain of TLR4 (2, 3). The activation signal is then propagated by the recruitment of a dedicated array of intracellular signaling protein adaptors followed by the activation of a complex serine/threonine kinase cascade, which eventually leads to the transcription of immunologically relevant genes (4). Recognition and signaling of LPS is absolutely dependent on the presence of MD-2 (5–7), a 160 amino acid secreted glycoprotein that coprecipitates with TLR4 (5). Viriyakosol et al. (8) reported that free MD-2 binds LPS with an apparent K_d of 65 nM.

In contrast to the known direct interactions of MD-2 with LPS, whether there is actually physical contact between TLR4 and LPS is an unresolved and contentious issue. We reported that TLR4 can be captured by a biotinylated form of LPS only when MD-2 is provided as a soluble molecule, or when cotransfected with TLR4 (9). These results suggest that the minimal cell surface LPS transmembrane signal transducer consists of MD-2 and TLR4 (10–12). Abs exist that appear to recognize the MD-2-TLR4 complex in the LPS-loaded or unloaded state (13), supporting the idea that LPS and MD-2-TLR4 form a stable complex on the cell surface.

Myeloid differentiation Ag, MD-2, is an Ig domain folded protein belonging to the MD-2-related lipid recognition family of lipid-binding receptors (14). Computational modeling suggests that MD-2 is capable of forming a barrel-like structure with a hydrophobic cavity large enough to accommodate the fatty acid moieties of lipid A (15, 16). We reported that a highly positively charged region of MD-2 that flanks this hypothetical hydrophobic cavity is required for stable binding to LPS. Mutations in the lysine residues of this region correlated with the loss of LPS binding and as a result, the loss of activity. Additional structural details necessary for MD-2 function have also been defined. For example, Cys^{95} is a critical residue for MD-2 activity (7). Cys^{95} is predicted to be located on the surface of the hypothetical barrel, as are all six of the other Cys residues save one, consistent with the idea that MD-2 is capable of forming covalently bound oligomers (10, 17, 18), though not precluding the existence of a monomeric form. The importance of this observation is that monomeric MD-2 has been reported to preferentially bind to a recombinant soluble TLR4 ectodomain (19).

In addition to existing as an integral component of the LPS receptor on the cell surface, it has also been demonstrated that LPS bound MD-2 behaves as an activating ligand for TLR4, in a way that is reminiscent of the interaction between *Drosophila* Toll and its soluble ligand spatzle. The binding of MD-2 by lipid A is greatly enhanced by serum components that have long been known to enhance LPS responses, i.e., soluble CD14 and LPS-binding protein (LBP) (8, 10, 20). MD-2 is unstable at 37°C, but the binding of LPS to MD-2 has been reported to dramatically stabilize its activity (21).

Taken together, the evidence supports a model in which LPS interacts with the MD-2/TLR4 surface heterodimer. The interaction of LPS with the receptor complex is reasonably high affinity, with a K_d that is estimated to be 3–10 nM (3, 13). The binding of LPS to MD-2 is then responsible for the aggregation of TLR4 and the recruitment/activation of the intracellular signal transducers.

Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01655

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[2] Address correspondence and reprint requests to Dr. Alberto Visintin, University of Massachusetts Medical School, Lazare Research Building, Room 270L, 364 Plantation Street, Worcester, MA 01605. E-mail address: alberto.visintin@umassmed.edu

[3] Abbreviations used in this paper: TIR, Toll-IL-1 resistance; LBP, LPS-binding protein; PAS, protein A-Sepharose; YFP, yellow fluorescent protein.

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However, these studies raised the questions: 1) what is the physiological role of the soluble form of MD-2 in blood and tissues, 2) does a soluble form of MD-2 exist in normal serum, and 3) can MD-2 can be pharmacologically targeted as a therapy for endotoxin-related conditions, especially sepsis?

In this report, we sought to address these fundamental questions. We explored in detail the minimal composition of the LPS receptor unit, and found that successful binding of LPS to its signaling receptor does not require other factors of cellular origin, except for MD-2, which can be provided and exists in blood as a soluble molecule. In the soluble phase, the ectodomain of TLR4, MD-2, and LPS form a stable complex, with an apparent K_d for TLR4-MD-2 interactions of 12 nM. Accordingly, TLR4-positive human cells could be efficiently triggered under protein-free conditions by supplementing the serum with <1 nM MD-2; activation levels were proportional to the concentration of soluble MD-2. The relative importance for CD14 and LBP and the absolute importance for MD-2 in LPS responses are in accordance with the data previously reported for the CD14, LBP, and MD-2 knockout mice (6, 22–24).

The importance of LPS binding to MD-2 is highlighted by the studies of LPS-inhibitory lipid A analogs, such as the synthetic compound E5564, which appear to function by preventing LPS-MD-2 interactions. We provide data supporting the hypothesis that monomeric MD-2 is the only physiologically relevant species of the molecule (19), having observed that only monomeric MD-2 interacts with LPS or TLR4 on the cell surface. By using soluble TLR4-Fc fusion proteins as a probe, we found that normal "healthy" human serum contains ~50 nM of functional monomeric soluble MD-2.

Materials and Methods

Cells, constructs, and reagents

Unless otherwise stated, all other reagents were from Sigma-Aldrich. The $TLR4^{YFP}$ cell line with yellow fluorescent protein (YFP) fused in frame to the C terminus of TLR4 used in this study was previously described (12). Cell lines were maintained in "complete medium" (5% FCS in DMEM plus 10 g/ml ciprofloxacin) in a humidified 5% CO_2 atmosphere. Cells stably secreting the TLR-Fc chimeric constructs were generated by retroviral transduction of HEK293 cells (CRL-1573; American Type Culture Collection as a gift of J. Chow, Eisai Research Institute, Andover, MA) and were maintained in protein-free medium (293PF; Invitrogen Life Technologies). The TLR fusion proteins consisted of the entire extracellular domain of either human TLR2 (aa 1–587) or human TLR4 (aa 1–632) fused in frame with the C-terminal 233 aa Fc portion of mouse IgG2a, modified by the addition of the linker sequence GAAGGG. The cDNA for human MD-2 was PCR cloned from the original pEF-Bos-MD-$2^{FLAG/6\times His}$ provided by Dr. K. Miyake (University of Tokyo, Tokyo, Japan), and subcloned into the retroviral vector pCLCX4 (25). The resulting construct encodes for an N-terminal FLAG-tagged and a C-terminal 6× His-tagged human MD-2. Packaging of the virus and transduction of HEK293 cells was performed as described (25). Abs used in this study included: rabbit polyclonal anti-GFP Ab from Molecular Probes; HRP-conjugated anti-GFP rabbit antiserum from Abcam; mouse monoclonal anti-GFP from BD Clontech; mouse monoclonal anti-TLR4, clone HTA125 from Dr. K. Miyake; anti-6× His mAb from Novagen-EMD Biosciences; rabbit polyclonal HRP-conjugated anti-biotin from New England Biolabs. The lipid A antagonist E5564 (26) and the agonist ER112022 (27) previously referred to as B2038 (26) were provided by the Eisai Research Institute. The baculovirus encoding for a C-terminal 6× His-tagged human MD-2 was provided by Dr. S. Viriyakosol (University of California, San Diego, CA), and expanded in Sf9 insect cells. MD-2 was then purified as described (8). Soluble recombinant CD14 and LBP were gifts from Amgen.

Immunoprecipitation and Western blot

The protocol used for cell lysis and immunoprecipitation is described in detail (10). Cells were grown in an adhesive monolayer in 10-cm dishes (~7–8 × 10^6 cells) and lysed by adding 1 ml of ice-cold lysis buffer (20 mM Tris (pH 8), 137 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10% glycerol, and freshly added protease inhibitors (1 nM PMSF, 10 μg/ml leupeptin and aprotinin)) to the cell monolayer. Lysates were subjected to centrifugation at 10,000 × *g* for 5 min and the supernatants were incubated with 20 μl of packed protein A-Sepharose (PAS; Amersham Biosciences) and 2 μg of the indicated Ab for either 1 h or overnight at 4°C. Captured immunocomplexes were extensively washed in lysis buffer without the protease inhibitors, and subjected to SDS-PAGE under reducing (0.1 M DTT in the loading buffer) or nonreducing conditions, as indicated. When biotin-LPS was used to capture LPS-interacting proteins, 20 μl of packed streptavidin beads were used instead of PAS, and biotin-LPS was used at a final concentration of either 1 or 0.5 μg/ml as indicated in the figures. When the Fc fusion constructs were precipitated, 20 μl of packed PAS beads were used without additional Abs. Precast 4–16% polyacrylamide gels were purchased from VWR (Gradipore). The resolved proteins were electroblotted onto nitrocellulose membranes (Hybond C; Amersham Biosciences) that were blocked in nonfat powdered milk solubilized in PBS plus 0.1% Tween 20 (PBST). The membranes were probed with 1 μg of the indicated HRP-conjugated Ab per ml in PBST for 30 min at room temperature. When a secondary HRP-conjugated Ab was required for detection, Bio-Rad anti-mouse or anti-rabbit antisera were used at a 1/5000 dilution in PBST for an additional 15 min. After each step, membranes were washed in PBST for 10 min, and finally subjected to ECL per the manufacturer's instructions (Amersham). To detect surface proteins, cells were surface biotinylated using 10 μg/ml of the membrane impermeable compound NHS-biotin per the manufacturer's instructions (Pierce). Biotinylated proteins were detected in Western blot by using an HRP-conjugated polyclonal anti-biotin antiserum (New England Biolabs) diluted 1/1000 in PBST. To quantify the 6× His-tagged monomeric MD-2, we performed a comparative western blot analysis of purified MD-2 vs titrated amounts of a 30 kDa 6× His-tagged protein standard (Qiagen). The concentration of our MD-2 stocks was ~1 μM (data not shown). Because the preparation
of baculoviral MD-2 consisted of ~60% monomeric, 30% dimeric, and 10% multimeric MD-2, the concentration of monomeric MD-2 was ~0.6 μM.

LP-binding assay

Protein binding to LPS was studied as described (10). Briefly, the polysaccharide of *Escherichia coli* LPS (O111:B4) was labeled using hydrazide-biotin as per the manufacturer instructions (Pierce). Biotinylated LPS was gel-filtered in HBSS to remove free biotin, tested for activity and stored at 4°C. The assay is designed to detect the interaction of epitope-tagged recombinant proteins with LPS. To detect LPS binding to soluble proteins, biotin-LPS (0.5 or 1 μg/ml) and streptavidin beads (20 μl packed resin/point) were mixed with culture supernatants from transfected cells that secrete the candidate proteins for 1 h at 37°C or overnight at 4°C. To detect LPS binding to proteins that are expressed on the surface of cells, biotin-LPS was added to 5 ml of medium covering monolayers of growing cells at a final concentration of 1 μg/ml, and treated for 1 h in a 37°C incubator in a 5% CO_2 humidified atmosphere. Cells were lysed as described (10), and lysates were subjected to centrifugation at 10,000 × *g* for 5 min to remove insoluble material. LPS-interacting proteins were captured using 20 μl of packed streptavidin beads per sample directly added to the postnuclear whole cell lysates. Beads were then extensively washed in lysis buffer and proteins were eluted from the beads by boiling in SDS sample buffer. LPS-precipitated proteins were resolved by SDS-PAGE and Western blotted using Abs to their epitope tags (e.g., anti-GFP to detect $TLR4^{YFP}$, anti-mouse to detect TLR4-Fc, and anti-FLAG to detect MD-2^{FLAG} or $TLR4^{FLAG}$).

Cell stimulation

Cellular activation via TLR4 was monitored by measuring luciferase production driven by an NF-κB luciferase reporter vector consisting of five repeats of the NF-KB consensus sequence (28). Approximately 5 × 10^6 cells were suspended in 10 ml of complete medium. Fifteen microliters of GeneJuice (Novagen) were added to 250 μl of DMEM and allowed to stand at room temperature for 5 min; 2 μg of the reporter plasmid were added, and allowed to stand an additional 15 min. Finally, this mixture was added to the cell suspension. Cells were immediately seeded into 96-well plates and allowed to adhere for 4–24 h. The transfection medium was then replaced with fresh medium containing the stimulants as indicated in the figures. Treatments ranged from 4 to 20 h, after which luciferase activity was assessed using commercial reagents (Promega) per the manufacturer's instructions. Luminescence was assessed from duplicate wells using a Wallac Victor 2 luminometer.

When stimulations were performed under protein-free conditions, cells were transfected in complete medium on day 1 and plated on 10-cm dishes. The following morning, the cells were trypsinized and the pellet washed

twice in 50 ml of PBS before plating on poly-L-coated multiwell plates in protein-free medium (293PF; Invitrogen Life Technologies). Cells were allowed to rest until the following day. This washing procedure ensures the dilution of possible serum contaminants by a factor of $\sim 1 \times 10^8$ and allows the cells to recover and adapt to the protein-free conditions. No obvious cell death was observed. Stimulations were then performed as already described.

TLR4 and MD-2 binding assay

To determine the K_d for the interaction between TLR4 and MD-2, an indirect ELISA-like binding assay was developed. Fifty microliter of purified TLR4-Fc at 20 μg/ml (see Fig. 4*A*) or at the concentrations indicated were plated on protein A-coated, high-protein binding 96-well plates. The plates were then washed three times in PBS-Tween 20, blocked with 1% BSA, 5% sucrose, 0.1% Tween 20 in PBS for 1 h, and incubated with baculoviral-derived 6× His-tagged MD-2 in PBS at the indicated concentration or at 12 nM in a total volume of 50 μl at 37°C for 1 h. In some experiments, LPS was included at 100 ng/ml or as indicated in the MD-2 titrations (see Fig. 4). MD-2 bound to TLR4-Fc was detected using Ni-HRP (1/2000 in PBS-Tween 20; Sigma-Aldrich), and developed by incubation with its chromogenic substrate per the manufacturer's instructions (DakoCytomation). Each condition was measured in triplicate, the optical density of each sample was measured at 450 nm, and the results are presented as the average reading ± SD.

Depletion of serum with TLR-Fc chimeras

Human serum was collected from healthy volunteers using red-topped vacutainers (BD Biosciences) and allowed to clot for 30 min at 37°C. The blood clot was removed by centrifugation and serum-stored at −20°C in 1-ml aliquots until use. We sought to remove MD-2 from serum using immunoaffinity techniques using chimeric Ab-like proteins bound to PAS. Human Abs can potentially compete with the TLR chimeras for binding sites on protein A beads. Therefore, they were removed from serum by two preclearing steps, at 2 h each, with one-tenth the volume of packed PAS beads.

HEK293 cells that had been transduced with retrovirus encoding the TLR-Fc fusion proteins were grown in protein-free medium and served as the source of conditioned medium. TLR4-Fc and TLR2-Fc were captured from 50–100 ml of medium using 40 μl of packed PAS/ml for 1 h at 4°C. Coated beads were then added to sera that had been precleared with PAS alone for 1 h at 4°C. Human serum was diluted in DMEM to a final concentration of 20% v/v before use on reporter cells. Before each conjugation/treatment step, the beads were washed with 20% ethanol in PBS followed by equilibration in DMEM. Reconstitution with MD-2 was performed by adding the indicated amounts of recombinant purified baculoviral MD-2 to the TLR4-Fc-depleted stimulating media.

Results

Only monomeric MD-2 participates in TLR4 activation

Multimeric forms of MD-2 can be observed when the molecule is overexpressed by 293 cells (Fig. 1*A*), although it is unknown whether MD-2 multimerizes in vivo. We hypothesized previously that multimerization of MD-2 might be responsible for the aggregation of TLR4 because the triggering of TLR4 can be efficiently achieved by Ab cross-linking of either TLR4- (12) or TLR4-bound (9) MD-2.

Our initial impression of MD-2 was distorted by the fact that the anti-FLAG mAb (M2) recognizes recombinant polymeric MD-2 without difficulty, but poorly recognizes monomeric soluble MD-2. This lack of recognition was only a problem when MD-2 was engineered with a FLAG epitope immediately downstream from the 6× His tag at the C terminus (17, 18) and (data not shown). Reengineering the MD-2 molecule with the FLAG epitope at the N terminus allowed us to produce a protein with a monomeric form that was readily recognized by anti-FLAG Ab. When the newly engineered MD-2 was subjected to the biotin-LPS pull-down, the mature form of the monomeric MD-2 was precipitated almost exclusively (Fig. 1*A*, *lane 3*). We speculate that the minute amount of LPS-bound multimeric MD-2 still detectable in the overexposed portion of the gel (Fig. 1*A*, *lanes 2–4*) might be attributable to noncovalent binding of soluble monomeric MD-2 to its aggregated forms (18).

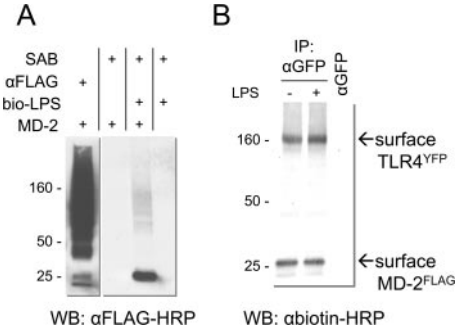

FIGURE 1. Monomeric MD-2 binds to LPS and TLR4 on the cell surface. *A*, Secreted N-terminal FLAG-tagged MD-2 (from the supernatants of stably transduced HEK293 cells) was immunoprecipitated with an anti-FLAG mAb (*lane 1*), or precipitated with biotin-LPS using streptavidin beads (SAB, *lanes 2* and *3*). The beads pellets were washed in lysis buffer, electrophoresed under nonreducing conditions, and Western blotted for the FLAG epitope. *Lanes 2–5* were developed with a longer exposure time, highlighting the fact that LPS almost exclusively binds to monomeric MD-2. *B*, Cells expressing a YFP-tagged TLR4 and MD-2 were left untreated or were treated with 1 μg LPS/ml for 1 h followed by surface labeling using a nonmembrane permeable biotinylation reagent (NHS-biotin). $TLR4^{YFP}$ was immunoprecipitated using a polyclonal anti-GFP Ab, separated by SDS-PAGE under nonreducing conditions, and detected by anti-biotin Western blotting. The 160-kDa band corresponds to surface TLR4, whereas the 25-kDa protein corresponds to the coprecipitated MD-2. LPS treatment neither affected binding of MD-2 to TLR4 nor altered the aggregation status of MD-2 on the cell surface (*lane 2*).

In addition to this important role of monomeric MD-2 in binding LPS, monomeric MD-2 was the only form capable of interacting with TLR4 on the cell surface. HEK/TLR4 cells were surfaced biotinylated and immunoprecipitated with anti-TLR4 mAb, followed by Western blot with an anti-biotin mAb (Fig. 1*B*). The addition of LPS before immunoprecipitation did not alter the aggregation status of TLR4-associated MD-2, ruling out the possibility that covalent multimerization of MD-2 is catalyzed by endotoxin or is in some way related to TLR4 aggregation (Fig. 1*B*, *lane 2*). As only monomeric MD-2 binds LPS, and only monomeric MD-2 binds TLR4, we conclude that only monomeric MD-2 participates in TLR4 activation by endotoxin.

Recognition of LPS by TLR4 and MD-2 does not require additional cellular components

An increasing amount of evidence is accumulating in the literature on the role of ancillary proteins in the LPS receptor complex, and in particular on the role of lipid rafts associated receptor in LPS signaling (29, 30). To establish whether the LPS recognition event by MD-2-TLR4 requires additional cellular cofactors, such as lipid rafts and the proteins associated with them, we performed the LPS-binding assay to TLR4 in the soluble phase with purified receptor components. Supernatants from cells stably expressing a recombinant soluble TLR4 extracellular domain (TLR4-Fc) and supernatants from MD-2 expressing cells were tested for the ability of binding biotin-LPS. As shown in Fig. 2, *lane 1*, biotin-LPS was unable to capture TLR4 in the absence of MD-2. However, the addition of MD-2 enabled LPS recognition, and both molecules were readily detected in the precipitate (Fig. 2, *lane 3*). PAS beads, which bind to the Fc portion of the IgG2a molecule, precipitated the chimeric construct and the associated MD-2 (Fig. 2, *lanes 4* and *5*). The addition of LPS did not alter the ability of TLR4 to bind MD-2 (Fig. 2, *lane 5*). Soluble MD-2 can therefore recognize

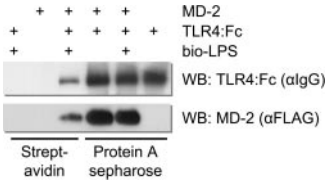

FIGURE 2. TLR4, MD-2, and LPS form a stable complex in the soluble phase. Conditioned media from MD-2 and TLR4-Fc-expressing cells were mixed in equal amounts (*lanes 3–5*) and captured with streptavidin beads in the presence (*lanes 1* and *3*) or absence (*lane 2*) of biotinylated LPS (1 μg/ml). Samples were then subjected to biotin-LPS precipitation (*lanes 1* and *3*) or protein A precipitation (*lanes 4–6*). After 1-h incubation at room temperature, beads were washed in lysis buffer, and the captured proteins were eluted from the beads by addition of reducing SDS sample buffer. The eluted proteins were separated on a 4–16% polyacrylamide gel, blotted on a nitrocellulose membrane, blocked, and probed with HRP-labeled anti-mouse polyclonal Ab for the TLR4-Fc chimera (*top lanes*) or an anti-FLAG mAb for MD-2^{FLAG} (*bottom lanes*). These results show that complexes of MD-2 and TLR4 bound to LPS without any other cell associated factors and that LPS did not interfere with the MD-2-TLR4 interaction.

TLR4 and LPS without the cooperation of additional proteins of cellular (i.e., membrane-bound) origin.

LPS does not alter the affinity of MD-2 for TLR4

Because MD-2 can be provided to TLR4 as a soluble molecule, we sought to determine the dissociation constant for the interaction of the monomer with TLR4. In Fig. 3*A*, saturating amounts of TLR4-Fc were adsorbed to protein A-coated 96-well plates, and purified soluble MD-2 was added in titrated amounts. The 6× His tag present at the C terminus of baculovirus-derived MD-2 was detected using a Ni-based HRP-labeled reagent, and the presence of MD-2 bound to TLR4 was detected using a chromogenic substrate. This experiment was repeated five times with four unrelated preparations of recombinant baculoviral MD-2. Note that in performing these experiments, the concentration of labeled MD-2 used was corrected to reflect only the concentration of monomeric, and not multimeric, MD-2. A concentration of 12 nM (range from 6 to 23 nM) of monomeric MD-2 corresponded to the half-saturating concentration of MD-2 (Fig. 3*A*). As MD-2 can bind to soluble LPS, and can act as an activating ligand for TLR4, it was conceivable that ligated MD-2 might have an altered affinity for TLR4. We therefore generated saturation curves in the presence of titrated amounts of LPS. In this experiment, the TLR4-Fc protein was adsorbed on protein A-coated plastic in titrated amounts. MD-2 was then added at its K_d concentration (12 nM), where changes in binding avidity could be most accurately measured, in the absence or in the presence of increasing amounts of LPS (0, 0.1, 1, and 10 ng/ml). As shown in Fig. 3*B*, the addition of LPS to MD-2 did not significantly change the dissociation rate constant of the binding, suggesting that MD-2 binding to TLR4 is independent from its interactions with LPS.

E5564 inhibits LPS binding to TLR4

E5564 is a synthetic LPS antagonist similar in structure to *Rhodobacter capsulatus* lipid A (26). Saitoh et al. (3) have previously reported that E5564 prevents LPS-induced TLR4 dimerization. Akashi et al. (13) reported that E5564 prevents the loss of immunoreactivity of the mAb MTS510 with LPS-bound mouse MD-2-TLR4 complex, suggesting that the inhibitor interacts with the complex without inducing the necessary conformational change that is required for TLR4 triggering. Because we hypothesize that MD-2 is the LPS-binding portion of the LPS receptor,

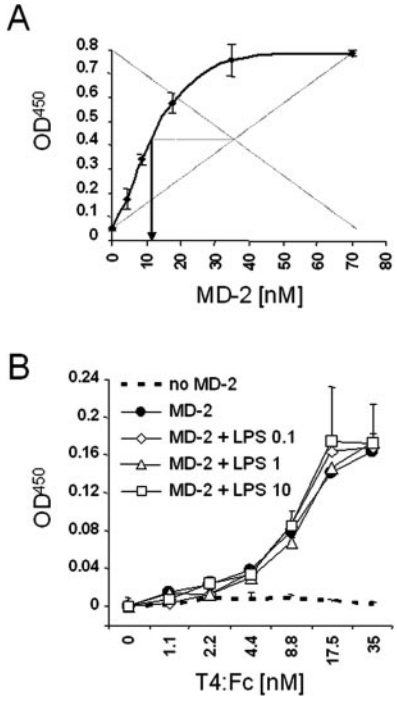

FIGURE 3. MD-2 binding to TLR4 has a K_d of ~12 nM and is unaffected by the presence of LPS. *A*, Protein A-coated 96-well plates were incubated for 1 h with TLR4-Fc, blocked and purified baculovirus derived MD-2$^{6\times\ His}$ was added at the indicated concentrations. After washing, MD-2 that was captured by protein A-bound TLR4-Fc was detected using Ni-HRP followed by incubation with a chromogenic substrate. MD-2 plated in the absence of TLR4-Fc did not give any signal over the background signal at the tested concentrations. Shown are the averages of triplicate optical density readings taken at 450 nm ± SD. The apparent of value of this interaction is a kDa of ~12 nM. *B*, TLR4-Fc was captured on protein A-coated plastic at the indicated concentrations and MD-2 added at fixed amounts (12 nM) without (●) or with (open symbols) LPS (0.1, 1, and 10 ng/ml). The background of the Ni-HRP reagent on the titrated TLR4-Fc, in the absence of added MD-2, is shown by the dashed line. Plates were washed and processed as in *A*.

we predicted that the inhibitory effects of E5564 on endotoxin-induced stimulation are due to competitive inhibition for a binding site on MD-2. As shown in Fig. 4*A*, we first determined the optimal concentration of E5564 necessary to achieve complete inhibition of LPS-induced responses. Using HEK293 cells expressing both human TLR4 and human MD-2, we observed that nearly complete inhibition of response was consistently achieved by using a 10-fold excess (w/v) of inhibitor over LPS (Fig. 4*A*).

We then tested titrated amounts of E5564 as competitors for biotin-LPS (0.5 μg/ml) binding to soluble MD-2 or cell-associated TLR4. As predicted by the functional titration, E5564 efficiently displaced biotin-LPS binding to soluble (HEK cell-derived) MD-2 when present in 10-fold (w/v) excess (Fig. 4*C*). Similarly, when LPS binding to MD-2 was assessed using cells that express TLR4-MD-2, the inhibitor displaced LPS in a dose-dependent manner, resulting in the inability to precipitate MD-2-bound TLR4 (Fig. 4*B*). As expected, unlabeled LPS also inhibited biotin-labeled LPS binding to MD-2. Previous studies have established that acyclic analogs of lipid A (i.e., analogs that do not contain the diglucosamine backbone), such as the synthetic compound known as ER112022, are pharmacologically similar to complete LPS. As one might have predicted based on the described experiments, ER112022 could displace biotin-LPS from MD-2 as well (Fig.

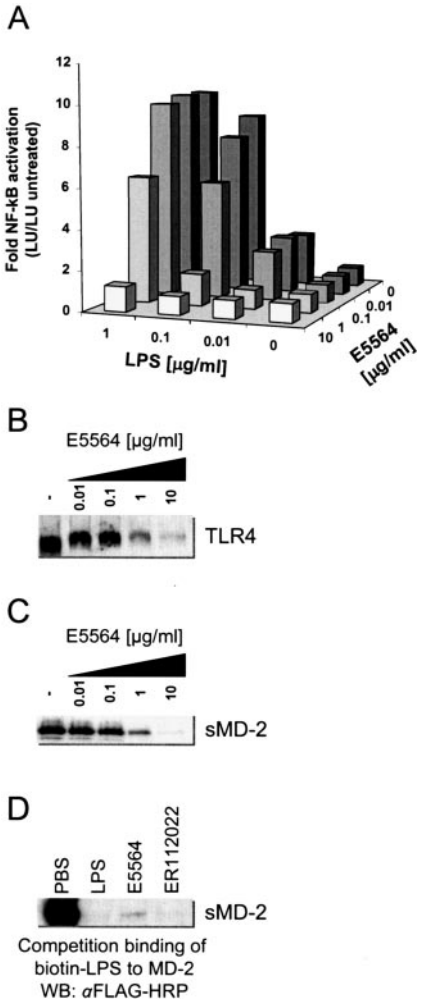

FIGURE 4. LPS antagonists and mimetics directly interact with MD-2. *A*, HEK293 cells stably expressing TLR4^{YFP} and MD-2^{FLAG} were transiently transfected with a NF-κB luciferase reporter plasmid and seeded on a 96-well plate at a density of ~50,000 cells/well. The cells were then stimulated with increasing amounts of LPS (*x*-axis, from right to left) in the presence of increasing amounts of the LPS antagonist E5564 (*y*-axis, dark to light bars). After an overnight incubation, luciferase activity was measured using a multiplate luminometer and shown as in Fig. 1*A*. E5564 consistently abrogated LPS signaling when used 10-fold in excess (w/v). *B*, The cells used in *A* were plated in 10-cm dishes and treated with biotin-LPS (0.5 μg/ml) for 1 h at 37°C in the absence (*lane 1*) or presence (*lanes 2–5*) of increasing amounts of the LPS antagonist E5564. Biotinylated LPS was then captured in the lysates using streptavidin beads, eluted and resolved in a reducing SDS-PAGE. TLR4^{YFP} was detected by Western blot analysis with an anti-GFP mAb. *C*, A similar experiment was performed by adding biotin-LPS plus variable amounts of E5564 to conditioned medium containing soluble MD-2 secreted by HEK293 cells (10 ml/lane); soluble MD-2 was precipitated with streptavidin beads and analyzed by Western blot with an anti-FLAG mAb as in Fig. 3*A*. *D*, Binding of biotin-LPS to soluble MD-2 (*lane 1*) could be abrogated using a 10-fold excess (w/v) of nonlabeled LPS (*lane 2*), E5564 (*lane 3*) or the synthetic TLR4 agonist, ER112022 (*lane 4*). Note that the conditioned medium in which these experiments were performed contained 5% FCS as a source of soluble CD14 and LBP.

4*D*). Deacylated LPS, in which the lipid A moiety has been subjected to base hydrolysis, is neither a TLR4 agonist nor an LPS antagonist (31, 32). Deacylated LPS bound to MD-2, but failed to displace fully acylated LPS (data not shown). Hence, the ability of a ligand to bind to MD-2 is a prerequisite for TLR4 activity, either stimulatory or inhibitory. The ability of the ligand to subsequently activate a signal transduction program is presumably the result of an alteration in the conformation of MD-2 that results in a change in aggregation status of TLR4, and the recruitment of TIR domain adapter molecules to the "receptosome" (9).

Soluble MD-2 in human normal serum

Human normal serum contains soluble MD-2 at a concentration of ~50 nM (1.7 μg/ml). MD-2 research has been plagued by the lack of reagents, particularly mAbs that could be used to detect native protein. Efforts to establish mAbs and polyclonal Abs able to recognize endogenous soluble MD-2 have, to date, only been marginally successful. For example, although Abs provided to us by Viriyakosol et al. (8) (clone 5H10 and a rabbit polyclonal antiserum) proved efficient in detecting baculoviral MD-2, we were unable to detect the endogenous protein in serum by immunoprecipitation or Western blot. MD-2 in the lysates of LPS-responsive immune cells or even transfected HEK293 was also undetectable (data not shown). Similarly, other available polyclonal Abs (Imgenex) or mAbs (eBioscience) failed to detect native MD-2. Therefore, we developed an alternative strategy to demonstrate the presence of soluble MD-2 in human serum.

We reasoned that TLR4 can efficiently bind to monomeric soluble MD-2 in whole blood or serum (this work and Ref. 19). We used TLR4-Fc chimeric protein immobilized on PAS beads to deplete MD-2 from the serum of healthy human donors from endogenous soluble MD-2. Depleted sera were then tested for the ability to confer LPS responses in TLR4-KB/Luc 293 reporter cells. Human serum, incubated with protein A beads only and used at a final concentration of 20% in DMEM, enabled LPS responsiveness to TLR4-expressing cells up to 3- to 4-fold when compared with unstimulated cells (Fig. 5*A*; representative of eight separate donors). Mock depletion of serum MD-2 with TLR2-Fc (Fig. 5*A*) did not alter the response. However, pretreatment of the serum with the TLR4-Fc chimera completely abrogated the serum-enhanced response, due to the elimination of soluble endogenous monomeric MD-2 (Fig. 5*A*). To confirm that loss of function was attributable to loss of endogenous soluble MD-2, we reconstituted the TLR4-Fc-treated serum by adding purified recombinant baculoviral-derived MD-2. Addition of exogenous soluble MD-2 restored (and even enhanced) LPS responsiveness (Fig. 5*B*), suggesting that TLR4-Fc depleted the serum of an enhancing capability that was identical in function to soluble MD-2.

To quantify soluble MD-2 in human serum, we reconstituted TLR4-Fc-treated serum with an increasing concentration of baculovirus-derived MD-2 and sought to determine the amount of purified recombinant monomeric MD-2 required to functionally match the physiological activation levels conferred by untreated human serum at the same nonsaturating LPS concentration. Although complicated, the assay was highly reproducible. TLR4-expressing 293 cells were stimulated with three fixed concentrations of LPS (250, 100, and 50 ng/ml) in 20% human serum that had been previously depleted of monomeric MD-2 with TLR4-Fc. The cells were stimulated without the addition of protein, as well as in the presence of carefully titrated amounts of purified baculoviral MD-2. We then determined the concentration of added monomeric MD-2 that repleted the ability of serum to enable LPS responses at each of the three concentrations of LPS. This determination of functional soluble human MD-2 was nearly identical in each of the three concentrations of LPS tested. Therefore, in Fig. 5*C* the concentration of MD-2 is represented as the average ± the range of the three determinations. We conclude that the concentration of soluble monomeric MD-2 in human normal serum is ~50 nM, i.e., 1.7 μg/ml. As all of the volunteers are thought to be

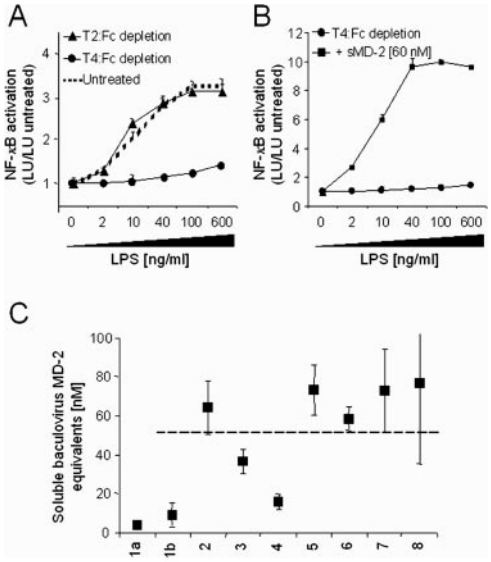

FIGURE 5. Human serum contains ~50 nM (1.7 μg/ml) of soluble monomeric MD-2. HEK293 cells stably expressing TLR4^{YFP} were transiently transfected with an NF-κB luciferase reporter plasmid, plated in a 96-well plate and treated overnight with increasing amounts of LPS in 20% human serum that had been previously treated as indicated in the figures. *A*, Human serum was pretreated with PAS (dashed line), TLR2-Fc loaded PAS (▲), or TLR4-Fc loaded PAS (●). *B*, MD-2-depleted serum (●, same as in *A*) was reconstituted with 60 nM MD-2 and used in the stimulation assay (■). Results are shown as average of duplicate luciferase readings divided by the untreated point (0, no LPS) ± SD. Note that the experiment shown is representative of one of 20 experiments performed with sera from eight different human volunteers (*A* and *B*). *C*, The concentration of functional monomeric soluble MD-2 in human healthy serum is ~50 nM. Human serum was depleted of MD-2 using TLR4-Fc and reconstituted with increasing amounts of soluble purified recombinant baculoviral MD-2 at three different concentrations of LPS (250, 100, 50 ng/ml; see *Materials and Methods* for MD-2 quantitation procedure). Shown are the levels of MD-2 in sera from eight unrelated healthy individuals normalized for the activity (and mass) of baculoviral MD-2. Note that donor 1, who was assayed on two occasions separated by several months, appears to have consistently low levels of functional soluble MD-2 (1a and 1b). The dashed line represents the average soluble active MD-2 concentration of donor 1b to donor 8.

healthy, the functional consequences of low MD-2 (e.g., subjects 1 and 4) are unknown.

Purified TLR4 ectodomain inhibits the effects of LPS by neutralizing MD-2

To date, there has been no direct evidence that LPS binds directly to TLR4, although previous molecular genetic studies with pharmacological antagonists of LPS suggested such an interaction (33, 34). Instead, most of the evidence suggests that MD-2 is the binding portion of the TLR4-MD-2 signal transduction complex. Thus, any therapeutic strategy of inhibiting the effects of LPS during clinical disease by infusing large amounts of TLR4 ectodomain, in the hopes of binding and neutralizing LPS, seem unrealistic. In contrast, if the access of MD-2 to TLR4 were a rate-limiting step in the initiation of LPS signaling, we reasoned that excess amounts of the TLR4 ectodomain would inhibit endotoxin responses by preventing the interaction of MD-2-LPS with surface TLR4.

Thus, we purified the TLR4-Fc fusion construct to near homogeneity. We first tested to see whether this fusion protein, denoted as TLR4-Fc, could inhibit the effects of LPS in TLR4-transfected HEK293 cells to which recombinant MD-2 was added as a tissue culture supernatant from MD-2-expressing cells. Cells were transfected with an NF-κB reporter construct, and the following day were stimulated with increasing amounts of LPS. As can be seen from Fig. 6*A*, LPS responses in these cells were inhibited when the concentration of TLR4 was 4 μg/ml and nearly completely inhibited at a concentration only 10-fold higher.

Similarly, we tested the Fc fusion protein with human PBMC under serum free conditions, in which the only MD-2 was cell bound on the surface of monocytes. Under these conditions, TLR4-Fc was again capable of inhibiting the effects of LPS, although LPS-binding studies of TLR4-Fc under identical serum-free conditions failed to show any direct interaction of the TLR4 ectodomain with endotoxin (Fig. 6*C*). When 60% autologous serum was added to the PBMC, TLR4 was still capable of inhibiting the effects of LPS, albeit to a somewhat attenuated degree due to the presence of LBP, soluble CD14 and, of course, soluble MD-2 (Fig. 6*D*).

To determine whether the effects of TLR4-Fc were, in fact, due to its interactions with MD-2 (rather than LPS), and as a result preventing the formation of a functional LPS receptor, we plated HEK-TLR4^{YFP} cells overnight. The cells were washed the next day in protein-free medium, and fresh supernatants from MD-2-transduced HEK293 cells were added as a source of soluble MD-2 in the absence or presence of TLR4-Fc. Biotinylated LPS was then added, and we determined whether we could use the binding of

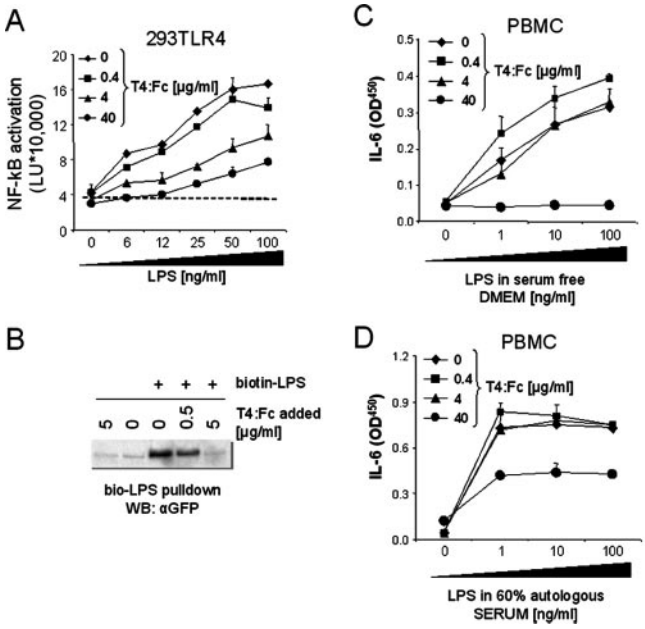

FIGURE 6. Soluble TLR4-Fc prevents LPS-induced cellular stimulation by interfering with MD-2/TLR4 binding. *A*, Cells expressing TLR4^{YFP} and an NF-κB luciferase reporter plasmid were seeded in a 96-well plate and stimulated for 4 h with increasing concentrations of LPS in the absence (◆) or in the presence of TLR4-Fc at the indicated concentrations. MD-2 was provided as conditioned medium from cells secreting soluble MD-2. Cells were analyzed as in Fig. 5. Shown is the average of duplicate luciferase reading ± SD. *B*, The same cells used in *A* were plated in 10-cm dishes and treated for 1 h with 1 μg of biotin-LPS/ml in the absence (*lanes 2* and *3*) or in the presence of TLR4-Fc (*lanes 3–5*). Cells were washed, lysed, and subjected to streptavidin precipitation as in Fig. 4*B*. As a control, the maximum amount of TLR4-Fc was added to the cells in the absence of biotin-LPS. Note that the presence of TLR4-Fc prevented the interaction of biotinylated LPS with MD-2. Adherent human PBMC were treated as in *A* in the absence (*C*) or in the presence (*D*) of 60% autologous human serum for 4 h; the release of IL-6 was measured by ELISA. Shown are the averages of optical density units ± SD.

LPS to MD-2 as a means to precipitate full-length YFP-tagged TLR4. These monolayers were washed, lysed, and subjected to precipitation with streptavidin beads. The precipitants were then analyzed by immunoblotting against YFP (Fig. 6*B*). Notably, streptavidin failed to pull down the full-length $TLR4^{YFP}$ when TLR4-Fc was present (Fig. 6*B*, *lane 5*), indicating that the TLR4-Fc fusion protein inhibited the ability of MD-2 to bind TLR4.

Discussion

The syndrome of Gram-negative sepsis has long been studied as a disease with a pathogenesis that is thought to be due to the toxic effects of LPS. Although formal proof of this association has never been established, the circumstantial evidence that LPS causes the initial toxicity associated with a deeply invasive Gram-negative infection is overwhelming. In part, the lack of formal proof is related to the essential nature of LPS, from the standpoint of the bacterium. Only a single mutant Gram-negative bacterium that is entirely lacking LPS has been engineered, and in an organism for which no good animal model exists (*Neisseria meningitidis*) (35). Nevertheless, there are numerous published reports relating endotoxin effects to sepsis, and Gram-negative organisms that express attenuated endotoxins are less proinflammatory (e.g., Ref. 36). Certainly, of all of the immune modulating molecules expressed by Gram-negative organisms, endotoxin is the most potent initiator of proinflammatory events.

Faced with this overwhelming circumstantial evidence, investigators and pharmaceutical companies have long desired to identify molecules that might be used therapeutically for sepsis, and perhaps for other diseases said to be due to endotoxin. Many such molecules have been identified, including LPS neutralizing proteins and peptides, although the value of such molecules to patients remains to be proven. One relatively newer category of anti-endotoxin agents is lipid A-based LPS inhibitors. These analogs of toxic lipid A have been thought since their discovery to be LPS receptor antagonists. The problem with these agents was that their mechanism of action could not be defined because until recently, the LPS receptor was only a hypothetical entity. With the identification of LBP and CD14, there was initial optimism that either molecule might be their target. This proved to be incorrect, because both LBP and CD14 are simply LPS-enhancing proteins (albeit potent ones) that work together with MD-2 on the surface of bacteria to bring the LPS present in the outer leaflet of the outer membrane to the TLR4 signal transducer. The essence of the difference between TLR4-MD-2 and LBP-CD14 is that the latter two molecules are not absolutely required for LPS responses.

In contrast, both TLR4 and MD-2 appear to be essential for cells to respond to LPS, at least with the respect to the induced production of the immune mediators that are associated with the sepsis syndrome. As the importance of these molecules becomes clear, it seemed only logical that endotoxin receptor inhibitors would function by binding to one or both of these receptor components. This appears to be the case, as we have described in this current report. Indeed, we have taken great pains to identify the drug target for the lipid A analogs as MD-2. This is somewhat a surprise for us, as our molecular genetic studies of pharmacological antagonists such as lipid IVa suggested that this class of drugs would primarily function by interacting with TLR4 (33). Although there may, in fact, be an interaction of compounds such as lipid IVa with TLR4, there is little doubt that compounds such as lipid IVa, including E5564, inhibit LPS signaling primarily by interfering with LPS binding to MD-2.

In addition to small molecules that interfere with LPS binding to MD-2, the results shown in this study demonstrate that one can also interrupt LPS signaling by blocking the binding of MD-2 to TLR4. The use of the TLR4-Fc fusion protein, of course, was the most straightforward way to achieve this goal. A recent similar report has demonstrated that the ectodomain of TLR4 can interfere with TLR4 signaling in transformed cell lines (37), and we have clearly extended this concept to primary human cells. However, there is little reason why small molecules or mAbs that block MD-2-TLR4 interactions would not have the same effects. Patients at the highest risk for developing sepsis, such as those with penetrating trauma to the abdomen or large bowel incarceration, would be ideal candidates for prophylaxis with agents that inhibit MD-2 function.

There are a variety of unresolved questions concerning MD-2 that remain to be answered before anti-endotoxin strategies can be most effectively moved into the clinical arena. First, although our data suggest an approximate concentration of MD-2 in human blood, these data are indirect, and are not entirely in agreement with the published works of others, who failed to detect MD-2 in blood from normal volunteers (38). Secondly, the role of MD-2 monomers vs polymers remains to be resolved. Indeed, although Hyakushima et al. (37) previously reported on the avidity of TLR4 with MD-2, using a different binding assay, they did not consider this issue, providing us with the incentive to more carefully define the binding of MD-2 to TLR4 accounting for the influence of MD-2 polymerization. If MD-2 is present in human blood primarily as a polymer, it remains possible that strategies to exhaust the supply of MD-2 to the immune system will be thwarted by a convertase of some sort that has not yet been identified in tissue culture. High affinity Abs that bind native MD-2 would be an important first step in identifying the biology of MD-2 in vivo.

Finally, and most importantly, how does LPS-bound MD-2 result in the activation of the TLR4 signal transducer? On the basis of our experimental evidence, we favor a model of cellular activation in which aggregation of TLR4 is not induced by multimerization of prebound MD-2 via disulfide exchange, as we had earlier hypothesized. Instead, we suggest that MD-2 undergoes an LPS-dependent conformational change that in turn induces the homotypic aggregation of TLR4-MD-2, followed by the recruitment of MyD88 and, presumably, the other adapter molecules except for TIR domain-containing adapter inducing IFN-β-related adapter molecule, which appears to be constitutively localized to the plasma membrane, due to its N-terminal myristoylation (data not shown).

If it is true that MD-2 undergoes a conformational change after binding LPS, and that this conformationally altered MD-2 is capable of activating TLR4, then MD-2 might be compared with spatzle, the endogenous peptide ligand in the fly that binds to Toll and hence activates antimicrobial peptide production in the *Drosophila* fat body (2, 39). Indeed, it is common parlance to refer to MD-2 (or at least "LPS-bound" MD-2) as the true ligand for TLR4 (20, 21), making the comparison to Toll/spatzle inevitable. However, it is not entirely a semantic argument to reject this comparison because the approach to finding a therapeutic agent that interferes with MD-2 function would be quite different if MD-2 were the mammalian equivalent of spatzle. In contrast, MD-2 directly binds to LPS and, although we and others have failed to demonstrate this conclusion, it remains possible that LPS directly binds to TLR4. That was the conclusion of previous studies designed to explain the curious pharmacology of lipid A precursors, such as lipid IVa, which had stimulatory "LPS-mimetic" activity when tested in rodent immune cells, but LPS inhibitory activity when tested with human cells (33). A rapid off time, for example, or a need for LPS to be cobound by MD-2 to interact with TLR4, might

explain why binding studies to date have failed to detect an interaction of LPS with TLR4.

We expect that any meaningful answer to the question of how LPS-bound MD-2 activates cells will require a structural physicochemical approach. Such efforts are clearly underway in a variety of labs, but the purification of monomeric MD-2, the only physiologically relevant form, has proved to be difficult. Regardless of the mechanism whereby MD-2-bound LPS activates the TLR4 signal transducer, it is clear that MD-2 is a critical component of the LPS receptor and is the best target for the development of novel therapeutics designed to interfere with LPS receptor function.

Disclosures

The authors have no financial conflict of interest.

References

1. Bell, J. K., G. E. Mullen, C. A. Leifer, A. Mazzoni, D. R. Davies, and D. M. Segal. 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* 24: 528–533.
2. Weber, A. N., S. Tauszig-Delamasure, J. A. Hoffmann, E. Lelievre, H. Gascan, K. P. Ray, M. A. Morse, J. L. Imler, and N. J. Gay. 2003. Binding of the *Drosophila* cytokine spatzle to Toll is direct and establishes signaling. *Nat. Immunol.* 4: 794–800.
3. Saitoh, S., S. Akashi, T. Yamada, N. Tanimura, M. Kobayashi, K. Konno, F. Matsumoto, K. Fukase, S. Kusumoto, Y. Nagai, et al. 2004. 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. *Int. Immunol.* 16: 961–969.
4. Beutler, B. 2004. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 430: 257–263.
5. Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* 189: 1777–1782.
6. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol.* 3: 667–672.
7. Schromm, A. B., E. Lien, P. Henneke, J. C. Chow, A. Yoshimura, H. Heine, E. Latz, B. G. Monks, D. A. Schwartz, K. Miyake, and D. T. Golenbock. 2001. Molecular genetic analysis of an endotoxin nonresponder mutant cell line: a point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. *J. Exp. Med.* 194: 79–88.
8. Viriyakosol, S., P. S. Tobias, R. L. Kitchens, and T. N. Kirkland. 2001. MD-2 binds to bacterial lipopolysaccharide. *J. Biol. Chem.* 276: 38044–38051.
9. Visintin, A., E. Latz, B. G. Monks, T. Espevik, and D. T. Golenbock. 2003. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J. Biol. Chem.* 278: 48313–48320.
10. Latz, E., A. Visintin, E. Lien, K. A. Fitzgerald, T. Espevik, and D. T. Golenbock. 2003. The LPS receptor generates inflammatory signals from the cell surface. *J. Endotoxin Res.* 9: 375–380.
11. Latz, E., A. Visintin, E. Lien, K. A. Fitzgerald, B. G. Monks, E. A. Kurt-Jones, D. T. Golenbock, and T. Espevik. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the Toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J. Biol. Chem.* 277: 47834–47843.
12. Akashi, S., S. Saitoh, Y. Wakabayashi, T. Kikuchi, N. Takamura, Y. Nagai, Y. Kusumoto, K. Fukase, S. Kusumoto, Y. Adachi, et al. 2003. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J. Exp. Med.* 198: 1035–1042.
13. Inohara, N., and G. Nunez. 2002. ML: a conserved domain involved in innate immunity and lipid metabolism. *Trends Biochem. Sci.* 27: 219–221.
14. Gangloff, M., and N. J. Gay. 2004. MD-2: the Toll 'gatekeeper' in endotoxin signalling. *Trends Biochem. Sci.* 29: 294–300.
15. Gruber, A., M. Mancek, H. Wagner, C. J. Kirschning, and R. Jerala. 2004. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. *J. Biol. Chem.* 279: 28475–28482.
16. Mullen, G. E., M. N. Kennedy, A. Visintin, A. Mazzoni, C. A. Leifer, D. R. Davies, and D. M. Segal. 2003. The role of disulfide bonds in the assembly and function of MD-2. *Proc. Natl. Acad. Sci. USA* 100: 3919–3924.
17. Visintin, A., A. Mazzoni, J. A. Spitzer, and D. M. Segal. 2001. Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. *Proc. Natl. Acad. Sci. USA* 98: 12156–12161.
18. Re, F., and J. L. Strominger. 2002. Monomeric recombinant MD-2 binds TLR4 tightly and confers LPS responsiveness. *J. Biol. Chem.* 277: 23427–23432.
19. Gioannini, T. L., A. Teghanemt, D. Zhang, N. P. Coussens, W. Dockstader, S. Ramaswamy, and J. P. Weiss. 2004. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc. Natl. Acad. Sci. USA* 101: 4186–4191.
20. Kennedy, M. N., G. E. Mullen, C. A. Leifer, C. Lee, A. Mazzoni, K. N. Dileepan, and D. M. Segal. 2004. A complex of soluble MD-2 and lipopolysaccharide serves as an activating ligand for Toll-like receptor 4. *J. Biol. Chem.* 279: 34698–34704.
21. Jack, R. S., X. Fan, M. Bernheiden, G. Rune, M. Ehlers, A. Weber, G. Kirsch, R. Mentel, B. Furll, M. Freudenberg, et al. 1997. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. *Nature* 389: 742–745.
22. Wurfel, M. M., B. G. Monks, R. R. Ingalls, R. L. Dedrick, R. Delude, D. Zhou, N. Lamping, R. R. Schumann, R. Thieringer, M. J. Fenton, et al. 1997. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J. Exp. Med.* 186: 2051–2056.
23. Perera, P. Y., S. N. Vogel, G. R. Detore, A. Haziot, and S. M. Goyert. 1997. CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. *J. Immunol.* 158: 4422–4429.
24. Wang, R. F., X. Wang, S. L. Johnston, G. Zeng, P. F. Robbins, and S. A. Rosenberg. 1998. Development of a retrovirus-based complementary DNA expression system for the cloning of tumor antigens. *Cancer Res.* 58: 3519–3525.
25. Mullarkey, M., J. R. Rose, J. Bristol, T. Kawata, A. Kimura, S. Kobayashi, M. Przetak, J. Chow, F. Gusovsky, W. J. Christ, and D. P. Rossignol. 2003. Inhibition of endotoxin response by E5564, a novel Toll-like receptor 4-directed endotoxin antagonist. *J. Pharmacol. Exp. Ther.* 304: 1093–1102.
26. Lien, E., J. C. Chow, L. D. Hawkins, P. D. McGuinness, K. Miyake, T. Espevik, F. Gusovsky, and D. T. Golenbock. 2001. A novel synthetic acyclic lipid A-like agonist activates cells via the lipopolysaccharide/Toll-like receptor 4 signaling pathway. *J. Biol. Chem.* 276: 1873–1880.
27. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the Toll adapters TRAM and TRIF. *J. Exp. Med.* 198: 1043–1055.
28. Triantafilou, M., K. Brandenburg, S. Kusumoto, K. Fukase, A. Mackie, U. Seydel, and K. Triantafilou. 2004. Combinational clustering of receptors following stimulation by bacterial products determines lipopolysaccharide responses. *Biochem. J.* 381: 527–536.
29. Pfeiffer, A., A. Bottcher, E. Orso, M. Kapinsky, P. Nagy, A. Bodnar, I. Spreitzer, G. Liebisch, W. Drobnik, K. Gempel, et al. 2001. Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Eur. J. Immunol.* 31: 3153–3164.
30. Seid, R. C., Jr., and J. C. Sadoff. 1981. Preparation and characterization of detoxified lipopolysaccharide-protein conjugates. *J. Biol. Chem.* 256: 7305–7310.
31. von Eschen, K. B., and J. A. Rudbach. 1976. Antibody responses of mice to alkaline detoxified lipopolysaccharide. *J. Immunol.* 116: 8–11.
32. Lien, E., T. K. Means, H. Heine, A. Yoshimura, S. Kusumoto, K. Fukase, M. J. Fenton, M. Oikawa, N. Qureshi, B. Monks, R. W. Finberg, R. R. Ingalls, and D. T. Golenbock. 2000. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest.* 105: 497–504.
33. Poltorak, A., P. Ricciardi-Castagnoli, S. Citterio, and B. Beutler. 2000. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proc. Natl. Acad. Sci. USA* 97: 2163–2167.
34. Steeghs, L., R. den Hartog, A. den Boer, B. Zomer, P. Roholl, and P. van der Ley. 1998. Meningitis bacterium is viable without endotoxin. *Nature* 392: 449–450.
35. Somerville, J. E., Jr., L. Cassiano, B. Bainbridge, M. D. Cunningham, and R. P. Darveau. 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J. Clin. Invest.* 97: 359–365.
36. Hyakushima, N., H. Mitsuzawa, C. Nishitani, H. Sano, K. Kuronuma, M. Konishi, T. Himi, K. Miyake, and Y. Kuroki. 2004. Interaction of soluble form of recombinant extracellular TLR4 domain with MD-2 enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. *J. Immunol.* 173: 6949–6954.
37. Pugin, J., S. Stern-Voeffray, B. Daubeuf, M. A. Matthay, G. Elson, and I. Dunn-Siegrist. 2004. Soluble MD-2 activity in plasma from patients with severe sepsis and septic shock. *Blood* 104: 4071–4079.
38. Brennan, C. A., and K. V. Anderson. 2004. *Drosophila:* the genetics of innate immune recognition and response. *Annu. Rev. Immunol.* 22: 457–483.