Novel Roles in Human MD-2 of Phenylalanines 121 and 126 and Tyrosine 131 in Activation of Toll-like Receptor 4 by Endotoxin*

The ability of mammalian cells to respond to minute (pm) concentrations of endotoxins, unique and abundant surface glycolipids of Gram-negative bacteria, is needed for optimal host defense against many invading Gram-negative bacteria. This remarkable sensitivity depends on sequential protein-endotoxin and protein-protein interactions involving lipopolysaccharide-binding protein, CD14, MD-2, and Toll-like receptor 4 (TLR4). TLR4 activation requires simultaneous binding of MD-2 to endotoxin (E) and the ectodomain of TLR4. We now describe mutants of recombinant human MD-2 that bind TLR4 and react with E-CD14 but do not support cellular responsiveness to endotoxin. The mutants F121A/K122A MD-2 and Y131A/K132A MD-2 react with E-CD14 only when co-expressed with TLR4. Single mutants K122A and K132A each react with E-CD14 ∓ TLR4 and promote TLR4-dependent cell activation by endotoxin suggesting that Phe₁²¹ and Tyr₁³¹ are needed for TLR4-independent transfer of endotoxin from CD14 to MD-2 and also needed for TLR4 activation by bound E-CD2. The mutant F₁₂₆A MD-2 reacts as well as wild-type MD-2 with E-CD14 ± TLR4. E-MD-2F₁₂₆A binds TLR4 with high affinity (K_d ∼ 200 pM) but does not activate TLR4 and instead acts as a potent TLR4 antagonist, inhibiting activation of HEK/TLR4 cells by wild-type E-MD-2. These findings reveal roles of Phe₁²¹ and Tyr₁³¹ in TLR4-independent interactions of human MD-2 with E-CD14 and, together with Phe₁₂₆, in activation of TLR4 by bound E-MD-2. These findings strongly suggest that the structural properties of E-MD-2, not E alone, determine agonist or antagonist effects on TLR4.

This remarkable sensitivity depends on sequential protein-endotoxin and protein-protein interactions involving at least four extracellular and cell surface host proteins: lipopolysaccharide-binding protein (LBP),² soluble (s) and GPI-linked membrane-associated forms of CD14, secreted and Toll-like receptor (TLR) 4-associated forms of MD-2 and TLR4 (1–4). LBP promotes extraction and delivery of individual molecules of endotoxin from endotoxin-rich interfaces (e.g. the Gram-negative bacterial outer membrane or aggregates of purified endotoxin) to CD14, yielding monomeric complexes of endotoxin (E)-CD14 that are the preferred substrate for MD-2 (2, 5–7). Transfer of endotoxin from CD14 to MD-2 coupled to binding of MD-2 to TLR4 triggers TLR4-dependent cell activation (2, 7). Thus, MD-2 has a pivotal role in endotoxin-induced TLR4 activation, bridging endotoxin recognition initiated by LBP and CD14 to receptor activation.

The requirement for simultaneous engagement by MD-2 of endotoxin and TLR4 for receptor activation strongly suggests that MD-2 contains structurally and topologically distinct binding sites for endotoxin and TLR4. Studies of the effects of mutagenesis of MD-2 have provided experimental support for this concept, showing that discrete mutations of MD-2 could markedly impair cellular endotoxin responsiveness without apparently affecting the ability of MD-2 to engage TLR4 (8–14). A subset of these MD-2 mutants also apparently retains normal endotoxin binding properties but fails to efficiently induce TLR4 oligomerization and receptor activation (15). These findings suggest that, in these select MD-2 mutants, the simultaneous engagement of endotoxin and TLR4 is somehow insufficient to trigger subsequent molecular events within the endotoxin:MD-2:TLR4 complex needed for receptor and cell activation.

The studies carried out to date seeking to characterize the structural requirements in MD-2 for endotoxin binding and TLR4 activation have presented endotoxin as aggregates ± serum (8–20). Under these conditions, it is likely that the added

---

*This work was supported by United States Public Health Service Grants R21 AI 54665 (to F.R.), P0144642 and AI59372 (to J.P.W.), and a Veterans’ Administration Merit Review grant (to T.L.G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

² The abbreviations used are: LBP, lipopolysaccharide-binding protein; CM, conditioned medium; E, endotoxin; HEK, human embryonic kidney; HSA, human serum albumin; LOS, lipooligosaccharide; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; sCD14, soluble CD14; TLR4_CD, Toll-like receptor 4 ectodomain; wt, wild type; DMEM, Dulbecco’s modified Eagle’s medium; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
endotoxin is present in a variety of physical and biochemical states, including as aggregates of endotoxin ± LBP ± sCD14 and as complexes with lipoproteins (21, 22) with little or no endotoxin present as a monomeric endotoxin–CD14 complex. This may explain the relatively high and variable apparent $K_d$ values (~3–65 nm) that were estimated for endotoxin–MD-2 interactions (15–20), in contrast to the pm reactivities (apparent $K_d$ ~ 100–200 ps) we have observed for transfer of endotoxin from CD14 to MD-2 (23). With wild-type recombinant human MD-2, efficient transfer of endotoxin monomers from CD14 to MD-2 occurs in solution with or without co-expression of TLR4, thus making it possible to assess separately direct CD14 to MD-2 occurs in solution with or without co-expression of TLR4, thus making it possible to assess separately direct CD14 to MD-2, and a potent agonist for the ectodomain of TLR4 (wild-type and variant) that are formed should provide a more direct and complete appraisal of the structural requirements for MD-2 function.

We now describe, using this approach, the identification and characterization of two different classes of MD-2 mutants that bind TLR4 and react with ECD14 but do not support TLR4-dependent cell activation by endotoxin. One class, exemplified by the mutants F121A/K122A and Y131A/K132A can react with form an E with wild-type human MD-2, unlike either endotoxin alone or a monomeric complex of hexa-acylated endotoxin E formed, a monomeric complex of hexa-acylated endotoxin E (25,000 cpm/pmol) from an acetate auxotroph of Neisseria meningitidis serogroup B was metabolically chased from GE Healthcare (Piscataway, NJ).

**Materials**—LBP and sCD14 were gifts from Xoma (Berkeley, CA) and Amgen Corp. (Thousand Oaks, CA), respectively. Insect-derived soluble MD-2 containing a hexapolyhistidine tag on the C-terminal end was prepared as previously described (7). Human serum albumin (HSA) was obtained as an E-free, 25% stock solution (Baxter Health Care, Glendale, CA). [3H]Lipopoligosaccharide (LOS; 25,000 cpm/pmol) from an acetate auxotroph of Neisseria meningitidis serogroup B was metabolically labeled and isolated as described (26). Chromatography matrices (Sephacryl HR S200 and S300, Ni FF Sepharose) were purchased from GE Healthcare (Piscataway, NJ).

**Production of Recombinant Proteins**—Human embryonic kidney (HEK) 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells (~80% confluence in 6-well plates or T75 flask for preparative amounts of material) were transfected with 4 μg (6-well plate) or 20 μg (T75 flask) of DNA using PolyFect reagent (Qiagen). After 12–16 h, plates were rinsed in PBS and 1 ml per well for 6-well plates or 10 ml for T-75 flask of serum-free medium (293 SFM, Invitrogen) + 0.4% HSA ± [3H]LOS±sCD14 (1 nM; see below) were added. Media containing expressed proteins were collected 24–48 h later. Conditioned medium containing secreted TLR4EC (wild type [wt]) or indicated mutants maintained activity to react with [3H]LOS±MD-2 or [3H]LOS±sCD14 for at least 6 months when stored at 4°C. Expression vectors containing DNA of interest for production of FLAG-TLR4EC, amino acids 24–634, (pFLAG-CMV-TLR4) and MD-2–FLAG-His (pEF-BOS) as well as MD-2 containing the indicated mutations have been previously described and characterized (27) or have been generated according to that protocol.

Preparative amounts of wt and F126A MD-2 were generated from infections of High Five insect cells with baculovirus containing the cDNA for either wt human MD-2–His6 or human MD-2F126A inserted into pBAC11 as described previously (7).

**Preparation of [3H]LOS–Protein Complexes—**[3H]LOS aggregates, [3H]LOS±sCD14, and [3H]LOS±MD-2 complexes were prepared as previously described (2, 7, 28). Briefly, [3H]LOS aggregates ($M_r > 20 \times 10^6$; 25,000 cpm/pmol) were obtained after hot phenol extraction of [3H]LOS, followed by ethanol precipitation of [3H]LOS and ultracentrifugation. Monomeric [3H]LOS–sCD14 complexes ($M_r \sim 60,000$) were prepared by incubating [3H]LOS aggregates for 30 min at 37°C with subsstoichiometric amounts of LBP (molar ratio 100:1 LOS:LBP and 1–1.5 x molar excess of sCD14 to LOS followed by gel exclusion chromatography (Sephacryl S200, 1.6 cm × 70 cm column) in PBS, pH 7.4, 0.03% HSA to isolate monomeric [3H]LOS–sCD14 complex. [3H]LOS±MD-2–His6 ($M_r \sim 25,000$) was generated by treatment of [3H]LOS±sCD14 (10 μg preparative or 10 ng analytical) by incubation for 30 min at 37°C with High Five insect cell medium containing MD-2–His6 (25 ml preparative or 0.025 ml analytical). Preparative samples were concentrated to 2 ml before application to 1.6 cm × 70 cm Sephacryl S200 column for isolation of [3H]LOS–MD-2.

Radiochemical purity of [3H]LOS aggregates, was confirmed by Sephacryl S500 (29) and that of [3H]LOS±sCD14, and [3H]LOS±MD-2 by Sephacryl S200 chromatography (7, 26).

**Reaction of Secreted MD-2, TLR4EC, and MD-2–TLR4EC with [3H]LOS–Protein Complexes—**Conditioned serum-free medium of transfected HEK293T cells was harvested after 24 h in culture and used for subsequent incubation with [3H]LOS±sCD14. Alternatively, the culture medium was “spiked” with [3H]LOS±sCD14 (1 nM) at the time of addition of the medium to the transfected cells to permit reaction of MD-2 with [3H]LOS±sCD14 upon secretion. Media harvested without [3H]LOS±sCD14 are denoted as HEK/(secreted recombinant protein)EC, whereas media spiked with [3H]LOS±sCD14 during cell culture are represented as (HEK/recombinant protein)EC secreted + [3H]LOS±sCD14EC. Media harvested without [3H]LOS±sCD14 were incubated with 1 nM [3H]LOS±sCD14 for 30 min (or 24 h) at 37°C and then analyzed by gel sieving chromatography. Media spiked with [3H]LOS±sCD14 during cell culture were analyzed directly after harvesting the medium by gel sieving chromatography. Harvested media could be stored...
at 4 °C without any detectable change in either reactivity with freshly added [3H]-LOS-sCD14 or chromatographic profile of [3H]-labeled compounds in “spiked” media. Reaction products were analyzed by Sephacryl HR S200 or S300 (1.6 × 70 cm) chromatography in PBS, pH 7.4, 0.03% HSA as indicated (23). Fractions (1.0 or 0.5 ml) were collected at a flow rate 0.5 or 0.3 ml/min at room temperature using AKTA Purifier or Explorer 100 FPLC (GE Healthcare). Radioactivity in collected fractions was analyzed by liquid scintillation spectroscopy (Beckman LS liquid scintillation counter). Recoveries of [3H]LOS were ≥ 70% in all cases. All solutions used were pyrogen-free and sterile-filtered. After chromatography, selected fractions were sterile-filtered (0.22 μm) and kept at 4 °C for 3–6 months without any detectable changes in chromatographic or functional properties.

To measure the apparent Ki of [3H]LOS-MD-2-F126A interactions with TLR4-ECID, [3H]LOS-MD-2-F126A was incubated with concentrated (8–10×) conditioned medium containing TLR4-ECID, in a final volume of 0.5 or 1 ml in PBS, pH 7.4, for 30 min at 37°C. The same conditioned medium (containing secreted TLR4-ECID) was used with all concentrations of [3H]LOS-MD-2-F126A tested for Scatchard analysis. Scatchard analysis was done using GraphPad Prism 4.

**Immunoblotting**—Polyhistidine-labeled wt and mutant MD-2 were detected by SDS-PAGE/immunoblot, using an anti-polylhistidine antibody (Tetra-His antibody, Qiagen, Valencia, CA) as previously described (7). Samples were electrophoresed (Bio-Rad minigel system) through a 4–15% gradient acrylamide gel (Tris/HEPES/SDS buffer) and transferred to nitrocellulose. The nitrocellulose was washed with Tris-buffered saline (TBS), pH 7.5, containing 0.05% Tween-20 and 0.2% Triton X-100 (TBSTT), blocked to reduce nonspecific background with 3% bovine serum albumin in TBSTT for 1 hour at 25 °C, and incubated with the anti-His6 antibody in TBSTT overnight. After washing with TBSTT, the blot was incubated with donkey anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) for 1 hour at 25 °C in TBS containing 3% goat serum and washed with TBSTT exhaustively. Blots were developed using the Pierce SuperSignal substrate system.

**HEK293 Cell Activation Assay**—HEK293/TLR4 cell lines have been extensively characterized and were cultured as has been previously described (30) or according to recommendations of Invivogen, Inc. For cell activation assays by LOS-sCD14, cells were grown to confluency in a 6-well plate and then transfected with vector (4 μg of DNA) containing wt or mutant MD-2 as described above. After transfection, serum-free medium was added for 24 h at 37 °C in 5% CO2, and 95% humidity. The supernatants were removed; cells were dislodged and seeded in a 96-well plate (1 × 10^4 cells/well) for 24 h in DMEM, 0.1% HSA. The short incubation time (3 h) was used to preclude significant secretion of MD-2 that could compete with cell surface MD-2/TLR4 for reaction with LOS-sCD14. Supernatants were removed and evaluated for extracellular accumulation of IL-8 by ELISA.

For cell activation by LOS-MD-2 containing either wt or F126A MD-2, HEK293 cells ± TLR4 were seeded in a 96-well plate in triplicate (1 × 10^5 cells/well) and stimulated with increasing concentrations of LOS-MD-2 complex in DMEM, 0.1% HSA for 20 h. Activation of HEK293 cells was assessed by measuring accumulation of extracellular IL-8 by ELISA (BD Clontech, Inc., Palo Alto, CA).

**Assay of Binding of [3H]LOS from [3H]LOS-sCD14 to HEK293 Cells Expressing TLR4 ± wt or Mutant MD-2**—Parental HEK293T cells and cells transiently transfected with pFLAG-CMV-TLR4 ± pEFBOS-MD-2-FLAG-His, wt or mutant, (6 μg of each plasmid DNA) were grown for 24 h in DMEM/10% fetal bovine serum in T75 flasks followed by 24 h in serum-free medium. Cells were dislodged with PBS, sedimented at 1000 rpm for 5 min, washed once with 3 ml of PBS/0.1% HSA, and then aliquoted so that each sample contained 4 × 10^6 cells in 1 ml. The cells were again sedimented and resuspended in PBS/0.1% HSA containing 2 nM [3H]LOS-sCD14 (50,000 cpm). Samples were incubated for 30 min at 37 °C, with rotation. After the incubation, cells were washed twice with PBS/0.1% HSA and then transferred with 0.5 ml PBS/0.1% HSA into scintillation vials to measure cell-associated (bound) [3H]LOS by liquid scintillation spectroscopy. The percent of added [3H]LOS bound to cells was calculated as [(cpm in washed cell pellet)/(cpm in supernatant + washes + pellet)] × 100. Total recovery of added [3H]LOS was >90%.

**RESULTS**

**Presence of Monomeric EsCD14 during Secretion of MD-2 from HEK293T Cells Results in High Yields of Bioactive EMD-2**—We have previously demonstrated that bioactive monomeric endotoxin (meningococcal lipopoligosaccharide, LOS; or Escherichia coli lipopolysaccharide, LPS)-MD-2 can be efficiently generated by incubation of monomeric endotoxin-sCD14 with conditioned medium harvested from insect cells expressing and secreting recombinant human MD-2 (7). However, initial efforts to reproduce these findings with harvested conditioned medium from HEK293T cells transiently expressing human MD-2 were unsuccessful (Fig. 1A), despite secretion and extracellular accumulation of MD-2 (Fig. 1C). Kennedy et al. (17) had previously shown that recombinant human MD-2 secreted into serum-free culture medium lost activity (i.e. ability to support serum/LPS-dependent activation of HEK/TLR4 cells) in a time (24 h) and temperature (37 °C)-dependent manner unless serum (LBP and sCD14) and LPS were also present in the medium. These findings are consistent with the much greater stability at 37 °C of monomeric LPS-MD-2 (as compared with MD-2 alone) (2, 7) that could be formed rapidly by serum (LBP and sCD14)-dependent conversion of LPS aggregates to LPS-sCD14 and reaction of LPS-sCD14 with secreted MD-2. In an attempt to produce monomeric endotoxin-MD-2 from MD-2 secreted by transiently transfected HEK293T cells, the medium was supplemented (“spiked”) with [3H]LOS-sCD14 (1 nm). This change in experimental design resulted in virtually quantitative conversion of [3H]LOS-sCD14 to monomeric [3H]LOS-MD-2 in medium from cells expressing and secreting MD-2 (Fig. 1B) but not in medium of control cells (Fig. 1B). Note that in the absence of MD-2, some of the added [3H]LOS-sCD14 aggregates during incubation overnight and elutes in the void volume (V0) (Fig. 1B). The recovered [3H]LOS-MD-2 produced dose-dependent activation of HEK/
Human MD-2 Structure and Function

![Bioactive monomeric LOS/MD-2 is generated from MD-2 secreted from transfected HEK293T cells by “spiking” the culture medium with monomeric LOS/sCD14. A and B, HEK293T cells were transfected with expression vector (pE-F-BOS) encoding MD-2-FLAG-His6, or empty vector. After 12 h, medium was replaced with serum-free medium, 0.4% HSA without (A) or supplemented with ("spiked") 1 nM [3H]LOS/sCD14 (B). After an additional 24 h of incubation, medium was collected. A, medium was incubated for 30 min (or overnight; data not shown) at 37 °C with 1 nM [3H]LOS/sCD14, and 0.5 ml medium was applied directly to the Sephacryl S200 column equilibrated in PBS, pH 7.4. B, 0.5 ml was applied directly to the Sephacryl S200 column equilibrated in PBS, pH 7.4. As a control, [3H]LOS/sCD14 was incubated alone (–CM) and analyzed under the same chromatographic conditions (A). Reactants and products resolved by gel filtration chromatography were measured by liquid scintillation spectroscopy of eluted fractions. Profiles shown are representative of ≥3 experiments; overall recoveries were >80%. C, SDS-PAGE/immunoblots of media harvested at 24 h from HEK293T cells transfected with expression vector (pE-F-BOS) encoding MD-2-FLAG-His6, and cultured in serum-free medium/0.4% HSA with (lane 2) or without (lane 3) [3H]LOS/sCD14. MD-2 was detected using anti-(His) antibody. Lane 1 represents molecular weight markers (Perfect Protein, Novagen). D, HEK/TLR4 cells were incubated in DMEM, 0.1% albumin with increasing amounts of [3H]LOS/MD-2 complex isolated by gel filtration chromatography (Sephacryl S200) either from transfection of HEK293T cells “spiked” with [3H]LOS/sCD14 (●) or prepared by incubation of conditioned insect medium containing MD-2-His6 with [3H]LOS/sCD14 (○). After overnight incubation, extracellular accumulation of IL-8 was measured. Results are from one experiment in triplicate, representative of two similar experiments.

TLR4 cells (Fig. 1D) but not of parental cells lacking TLR4 (data not shown). The potency of [3H]LOS/MD-2 derived from recombinant MD-2 produced by HEK293T cells or by insect (High Five) cells was essentially the same (Fig. 1D), confirming the stability of mammalian cell-derived recombinant MD-2 when complexed, in monomeric form, with endotoxin.

**MD-2 Mutants That Bind TLR4 but Do Not Support TLR4-dependent Cell Activation by Endotoxin:** Comparison of Reactivity with [3H]LOS/sCD14—We made use of the same experimental design to re-examine the functional properties of three previously described human MD-2 double mutants: F121A/K122A, K125A/F126A, and Y131A/K132A (11). These mutants had been previously shown to interact with TLR4, as judged by co-precipitation and/or FACS-based analyses, but did not support robust cellular endotoxin responsiveness (11). These results suggest either a defect in LPS binding (i.e. reactivity with endotoxin/sCD14) and/or activation of TLR4 by bound endotoxin/MD-2. Gel filtration chromatography analysis of medium from cells “spiked” with [3H]LOS/sCD14 and producing the mutant MD-2s indicated that only the K125A/F126A MD-2 reacted with [3H]LOS/sCD14 to produce monomeric [3H]LOS/MD-2 (Fig. 2A). Even though MD-2/K121A/K122A and MD-2/Y131A/K132A were secreted in amounts comparable to MD-2/K125A/F126A, neither medium containing MD-2/F121A/K122A nor MD-2/Y131A/K132A was detected in the same chromatographic fractions (A). Reactants and products resolved by gel filtration chromatography were measured by liquid scintillation spectroscopy of eluted fractions. Profiles shown are representative of ≥3 experiments; overall recoveries were >80%. C, SDS-PAGE/immunoblots of media harvested at 24 h from HEK293T cells transfected with expression vector (pE-F-BOS) encoding MD-2-FLAG-His6, and cultured in serum-free medium/0.4% HSA with (lane 2) or without (lane 3) [3H]LOS/sCD14. MD-2 was detected using anti-(His) antibody. Lane 1 represents molecular weight markers (Perfect Protein, Novagen). D, HEK/TLR4 cells were incubated in DMEM, 0.1% albumin with increasing amounts of [3H]LOS/MD-2 complex isolated by gel filtration chromatography (Sephacryl S200) either from transfection of HEK293T cells “spiked” with [3H]LOS/sCD14 (●) or prepared by incubation of conditioned insect medium containing MD-2-His6 with [3H]LOS/sCD14 (○). After overnight incubation, extracellular accumulation of IL-8 was measured. Results are from one experiment in triplicate, representative of two similar experiments.

MD-2 Mutants That Bind TLR4 but Do Not Support TLR4-dependent Cell Activation by Endotoxin: Comparison of Reactivity with [3H]LOS/sCD14—We made use of the same experimental design to re-examine the functional properties of three previously described human MD-2 double mutants: F121A/K122A, K125A/F126A, and Y131A/K132A (11). These mutants had been previously shown to interact with TLR4, as judged by co-precipitation and/or FACS-based analyses, but did not support robust cellular endotoxin responsiveness (11). These mutants of MD-2 that did not react with [3H]LOS/sCD14 (i.e. MD-2/F121A/K122A and MD-2/Y131A/K132A) and, for comparison, wild-type MD-2, were co-expressed and secreted with the TLR4 ectodomain into a culture medium “spiked” with [3H]LOS/sCD14. After 24 h, the culture medium was harvested and analyzed by gel filtration chromatography. A Sephacryl S200 gel filtration system that resolves [3H]LOS/sCD14 (M₉ ~ 60,000) from the two possible radiolabeled products formed, [3H]LOS/MD-2 (M₉ ~ 25,000), and ([3H]LOS/MD-2/TLR4ECD₂) (M₉ ~ 190,000) (23) was used to differentiate the products of transfer of [3H]LOS from [3H]LOS/sCD14 to MD-2 and/or MD-2/TLR4ECD₂, respectively. TLR4ECD expressed without MD-2 does not react with [3H]LOS/sCD14 (Fig. 3A) (23). Co-expression of wild-type MD-2 and TLR4ECD yielded both [3H]LOS/MD-2 and ([3H]LOS/MD-2/TLR4ECD₂) (Fig. 3A)
human MD-2 Structure and Function

Co-expression of TLR4EC and either MD-2K122A or MD-2K132A resulted in a marked increase in the amount of LPS/MD-2. The ability of MD-2K122A and MD-2K132A to bind LPS was also measured using a gel filtration assay. The results showed that MD-2K122A and MD-2K132A were able to bind LPS, and that this binding was inhibited by TLR4ECD (Fig. 3). These findings suggest that MD-2K122A and MD-2K132A are able to bind LPS in a TLR4-dependent manner.

To further investigate the role of MD-2 in TLR4 activation, we tested the ability of MD-2K122A and MD-2K132A to bind LPS in the presence of TLR4ECD. The results showed that the ability of MD-2K122A and MD-2K132A to bind LPS was inhibited by TLR4ECD. These findings suggest that MD-2K122A and MD-2K132A are able to bind LPS in a TLR4-dependent manner.
the absence of TLR4 (Fig. 2A) and to bind TLR4 without supporting robust TLR4-dependent cell activation by endotoxin (11) suggested that the LOS-MD-2 K125A/F126A mutant complex would act as a TLR4 antagonist rather than a TLR4 agonist. To test this hypothesis, we examined the dose-dependent effects of the purified LOS-MD-2 K125A/F126A on HEK/TLR4 cells, both alone and in the presence of wild-type LOS-MD-2. Fig. 5A shows that, in comparison to wild-type LOS-MD-2, LOS-MD-2 K125A/F126A produced much more limited activation of HEK/TLR4 cells and, in molar excess, reduced cell activation triggered by wild-type LOS-MD-2 to levels closely similar to that produced by the mutant complex alone (Fig. 5B). These findings strongly suggest that engagement of TLR4 by LOS-MD-2 K125A/F126A triggers only limited receptor activation and, in excess, inhibits activation of TLR4 by the wild-type complex.

To further define the importance of lysine 125 and/or phenylalanine 126 in MD-2 function, single site mutants of human MD-2 were produced and tested. As expected, both MD-2 K125A and MD-2 F126A reacted readily with [3H]LOS-CD4 to form...
monomeric $[^3H]$LOS-MD-2 (data not shown). LOS-MD-2 $^{K125A}$ was as potent as wild-type LOS-MD-2 in activation of HEK/TLR4 cells but LOS-MD-2 $^{F126A}$ produced essentially no activation of HEK/TLR4 cells (Fig. 6A). Instead, in molar excess, LOS-MD-2 $^{F126A}$ produced virtually complete dose-dependent inhibition of cell activation by wild-type LOS-MD-2 (Fig. 6B). Thus, the single amino acid alteration, F126A, in human MD-2 was sufficient to convert a potent TLR4 agonist (wild type LOS-MD-2) to a potent TLR4 antagonist.

**Discussion**

We have described in this study the application of novel experimental approaches to address the identification of specific structural determinants in MD-2 for activation of TLR4 by bound E:MD-2 and the role of TLR4 in the transfer of endotoxin from CD14 to MD-2.

These approaches have led to several new insights concerning the structure and function of human MD-2. First, our findings indicate that the structural requirements in human MD-2 for transfer of E from CD14 to MD-2 are more stringent when MD-2 is presented as a soluble extracellular protein in the absence of TLR4 than when pre-associated with the ectodomain of TLR4.

**Activation of HEK/TLR4**

FIGURE 5. Endotoxin-MD-2 $^{K125A/F126A}$ acts as a TLR4 antagonist. A, HEK/TLR4 cells were incubated overnight with increasing amounts of LOS-MD-2 complex containing either wt (○) or K125A/F126A (□) MD-2, and cell activation was measured by determining extracellular accumulation of IL-8. B, purified LOS-MD-2 $^{K125A/F126A}$ complex (20 µM) with increasing amounts of LOS-MD-2 $^{K125A/F126A}$ was incubated with HEK293/TLR4 cells in DMEM, 0.1% HSA overnight. Extracellular IL-8 was measured by ELISA. Results shown represent mean ± S.E. of three experiments, each in triplicate.

**Inhibition of activation of HEK/TLR4 by LOS-MD-2 $^{K125A/F126A}$**

FIGURE 6. Endotoxin-MD-2 $^{F126A}$ is a potent TLR4 antagonist. A, HEK/TLR4 cells were incubated overnight with increasing amounts of LOS-MD-2 complex containing either wt (○) or K125A/F126A (□) MD-2. Cell activation was measured by determining extracellular accumulation of IL-8 by ELISA. Results shown are from one experiment (triplicate samples) representative of three independent experiments. B, purified LOS-MD-2 $^{K125A/F126A}$ complex (20 µM) with increasing amounts of LOS-MD-2 $^{K125A/F126A}$ was incubated with HEK293/TLR4 cells in DMEM, 0.1% HSA overnight. Extracellular IL-8 was measured by ELISA. Results shown represent mean ± S.E. of three experiments, each in triplicate.
Human MD-2 Structure and Function

MD-2K132A co-expressed with full-length TLR4 supported potent cell activation by LOS-sCD14 (Fig. 4B), presumably reflecting reactivity of cell surface MD-2K132A/TLR4 with LOS-sCD14.

Second, we demonstrate that several aromatic residues situated either just inside the opening of the hydrophobic cavity in MD-2 (i.e. Phe121 and Tyr131; (32); Fig. 8) or extending outward from the rim of this cavity (Phe126; Fig. 8), are important for activation of TLR4 by bound E:MD-2. This role was demonstrated most clearly for Phe126 but also strongly suggested for Phe121 and Tyr131, where substitution with alanine nearly ablated cell activation by endotoxin (Fig. 4B) (11) despite maintenance of MD-2 binding to TLR4 (11) (data not shown) and significant reactivity of MD-2/TLR4ECOD with LOS-sCD14 (Figs. 3 and 4A). Our findings extend earlier observations by other investigators who showed a role for each of these three residues in cellular responsiveness to endotoxin (9) and, in the case of phenylalanine 126, a role in receptor clustering induced by endotoxin binding to MD-2/TLR4 (15). The conservation of Phe121, Phe126, and Tyr131 in almost all of the mammalian MD-2 species reported to date (15) is consistent with a crucial role for each of these three aromatic residues in cellular responsiveness to endotoxin.

Third, we have demonstrated that simply by substituting phenylalanine 126 with alanine, we could produce an E:MD-2 complex that was a potent TLR4 antagonist, despite the fact that the bound endotoxin was normally a potent activator of TLR4. Previous studies have demonstrated contrasting agonist versus antagonist effects of tetraacylated lipid A in mouse and human cells, respectively. These contrasting species-dependent effects are due, substantially, to discrete structural differences between murine and human MD-2 (10, 12, 18, 33–35), indicating that activation or blocking of activation of TLR4 can be determined not only by the structural properties of endotoxin/lipid A bound by MD-2 but also by the structure of MD-2 itself. The properties we have described for human MD-2K132A support this view and demonstrate for the first time that a single amino acid substitution in MD-2 is sufficient to convert a potent TLR4 agonist (hexa-acylated LOS:MD-2K132A) to a potent TLR4 antagonist (hexa-acylated LOS:MD-2F126A). This finding underscores the remarkably discrete structural variables that distinguish TLR4 agonists from TLR4 antagonists and demonstrates that what specifies TLR4 agonist properties is not the structural properties of endotoxin alone but rather that of the monomeric endotoxin:MD-2 complex.

MD-2 mutants defective in endotoxin binding have been previously described (8, 12–14). These studies have relied on assays of recombinant MD-2 either expressed in and purified from bacteria or from conditioned culture medium of insect cells or present in impure form in harvested conditioned medium from mammalian cells (e.g. HEK293 cells) (8, 12–14). MD-2-endotoxin interactions have been measured by assays of recombinant MD-2 either expressed in and purified from bacteria or from conditioned culture medium of insect cells or present in impure form in harvested conditioned medium from mammalian cells (e.g. HEK293 cells) (8, 12–14). MD-2-endotoxin interactions have been measured by assays of recombinant MD-2 either expressed in and purified from bacteria or from conditioned culture medium of insect cells or present in impure form in harvested conditioned medium from mammalian cells (e.g. HEK293 cells) (8, 12–14).
three interactions require further study. The experimental approaches described in this study may be used to juxtapose the structural requirements in MD-2 and MD-2/TLR4ECD for reaction with endotoxin aggregates and monomeric ECD14.

The functional instability of secreted MD-2 in the absence of the TLR4 ectodomain adds an additional layer of complexity to interpretations of the effects of changes in MD-2 structure on MD-2 function. By spiking the cell culture medium with [3H]LOS scCD14 (25,000 cpm/pmol), we could measure transfer of [3H]LOS from [3H]LOS scCD14 to secreted MD-2 before inactivation of MD-2 and thus assay directly the reactivity of MD-2 with LOS scCD14 in the absence of TLR4. In the case of wild-type MD-2 and certain mutant MD-2 species (e.g. K122A, K125A, F126A), the ratio of the products ([3H]LOS-MD-2/[3H]LOS-MD-2/TLR4ECD)2 formed roughly corresponded to the ratio of expression and secretion of MD-2 and TLR4ECD, i.e. ratio of MD-2 to MD-2/TLR4ECD as determined by immunoblot (data not shown), suggesting that for these secreted MD-2 species the reactivity of MD-2 with LOS scCD14 is not significantly altered by prior engagement of MD-2 with TLR4ECD. In contrast, for MD-2 F126A, MD-2 Y131A, and MD-2 K132A, interaction with TLR4ECD was apparently crucial for reactivity of these MD-2 species with LOS scCD14. Whether or not binding of MD-2 to TLR4ECD in these MD-2 mutants is needed to induce a conformational change in MD-2 or simply to preserve longer the functionality active conformation of MD-2 is not known. MD-2, when secreted in molar excess of TLR4, can form an array of oligomers as well as persist as a monomer (27, 38). It is generally believed that the functionally reactive form of MD-2, both with respect to endotoxin and TLR4 binding, is monomeric MD-2 (20, 27) and that the state of the resting MD-2/TLR4 heterodimer (i.e. before binding of endotoxin) may also be monomeric (14, 18, 39, 40). Thus, it is possible that, in the case of MD-2 F126A, MD-2 Y131A, and MD-2 K132A, the functional half-life of monomeric MD-2, unless co-expressed with full length TLR4 or TLR4 ectodomain, is too short to have a chance to react with extracellular LOS scCD14.

The orientation of the side chains of Phe121 and Tyr131, as revealed between MD-2 and MD-2 or MD-2 and TLR4, that may be induced when MD-2 is simultaneously engaged with activating endotoxin species and TLR4 (7, 14, 18, 25, 39, 40). One possibility is that occupation of the hydrophobic cavity of MD-2 by the multiple acyl chains of lipid A of endotoxin species that are normally potent TLR4 agonists induces displacement of side chains within the hydrophobic cavity (e.g. Phe121 and Tyr131) and, secondarily, Phe126 leading to the downstream protein-protein interactions needed for signal/transduction. The fact that LOS MD-2wt/TLR4ECD and LOS MD-2 F126A/TLR4ECD are structurally indistinguishable by gel sieving (Figs. 3 and 7) and immunocapture (data not shown) analyses suggest more subtle conformational differences and/or a role of other (intramembrane, cytosolic) regions of TLR4 or other cellular proteins in determining the agonist versus antagonist action of wt and variant EMD-2 complexes. The remarkably stable and soluble properties of monomeric LOS MD-2wt and LOS MD-2 F126A, and of LOS MD-2wt or F126A/TLR4ECD, in contrast to MD-2 alone, should make these complexes valuable reagents for better defining the structural basis of TLR4 activation by bound EMD-2.

Acknowledgments—We thank Xoma Corp. (Berkeley, CA) for recombinant LBP and Amgen Corp. (Thousand Oaks, CA) for scCD14. We are also grateful to DeSheng Zhang for preparation, isolation, and characterization of radiolabeled LOS.

REFERENCES

1. Beutler, B. (2003) Annu. Rev. Pharmacol. Toxicol. 43, 609–628
2. Giovannini, T. L., Teghanemt, A., Zhang, D., Levis, E. N., and Weiss, J. P. (2005) J. Endotoxin Res. 11, 117–123
3. Miyake, K. (2003) Int. Immunopharmacol. 3, 119–128
4. Ulevitch, R. J., and Tobias, P. S. (1999) Curr. Opin. Immunol. 11, 19–22
5. Giovannini, T. L., Teghanemt, A., Zhang, D., Prohinar, P., Levis, E. N., Munford, R. S., and Weiss, J. P. (2007) J. Biol. Chem. 282, 7877–7884
6. Post, D. M., Zhang, D., Eastvold, J. S., Teghanemt, A., Gibson, B. W., and Weiss, J. P. (2005) J. Biol. Chem. 280, 38383–38394
7. Giovannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., and Weiss, J. P. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 4186–4191
8. Gruber, A., Mancek, M., Wagner, H., Kirschning, C. J., and Jerala, R. (2004) J. Biol. Chem. 279, 28475–28482
9. Kawaski, K., Nogawa, H., and Nishijima, M. (2003) J. Immunol. 170, 413–420
10. Muroi, M., and Tanamoto, K. (2006) J. Biol. Chem. 281, 5484–5491
11. Re, F., and Strominger, J. L. (2003) J. Immunol. 171, 5272–5276
12. Tsuneyoshi, N., Fukudome, K., Kohara, J., Tomimasu, R., Gauchat, J. F., Nakatake, H., and Kimoto, M. (2005) J. Immunol. 174, 340–344
13. Viriyakosol, S., Tobias, P. S., and Kirkland, T. N. (2006) J. Biol. Chem. 281, 11955–11964
14. Visintin, A., Latz, E., Monks, B. G., Espevik, T., and Golenbock, D. T. (2003) J. Biol. Chem. 278, 48313–48320
15. Kobayashi, M., Saito, K., Tanimura, N., Takahashi, K., Kawasaki, K., Nishijima, M., Fujimoto, Y., Fukase, K., Akashi-Takamura, S., and Miyake, K. (2006) J. Immunol. 176, 6211–6218
16. Hyakushima, N., Matsuzawa, H., Nishitani, C., Sano, H., Kuronuma, K., Konishi, M., Himi, T., Miyake, K., and Kuroki, Y. (2004) J. Immunol. 173, 6949–6954
17. Kennedy, M. N., Mullen, G. E., Leifer, C. A., Lee, C., Mazzoni, A., Dilipan, K. N., and Segal, D. M. (2004) J. Biol. Chem. 279, 34698–34704
18. Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Kobayashi, M., Konno, K., Matsumoto, F., Fukase, K., Kusumoto, S., Nagai, Y., Kusumoto, Y., Kosugi, A., and Miyake, K. (2004) Int. Immunol. 16, 961–969
19. Viriyakosol, S., Kirkland, T., Soldau, K., and Tobias, P. (2000) J. Endotoxin Res. 6, 489–491
20. Visintin, A., Halm, K. A., Latz, E., Monks, B. G., and Golenbock, D. T. (2005) J. Immunol. 175, 6465–6472
21. Kitchens, R. L., Thompson, P. A., Viriyakosol, S., O’Keefe, G. E., and Munford, R. S. (2001) J. Clin. Invest. 108, 485–493
22. Giovannini, T., and Weiss, J. P. (2007) Immunol. Res. 39, 249–268
23. Prohinar, P., Re, F., Widstrom, R., Zhang, D., Teghanemt, A., Weiss, J. P.

A. Teghanemt, R. Widstrom, T. L. Giovannini, and J. P. Weiss, unpublished data.
and Gioannini, T. L. (2007) J. Biol. Chem. 282, 1010–1017
24. Jia, H. P., Kline, J. N., Penisten, A., Apicella, M. A., Gioannini, T. L., Weiss, J., and McCray, P. B., Jr. (2004) Am. J. Physiol. Lung Cell Mol. Physiol. 287, 1428–1437
25. Teghanemt, A., Zhang, D., Levis, E. N., Weiss, J. P., and Gioannini, T. L. (2005) J. Immunol. 175, 4669–4676
26. Giardina, P. C., Gioannini, T., Buscher, B. A., Zaleski, A., Zheng, D. S., Stoll, L., Teghanemt, A., Apicella, M. A., and Weiss, J. (2001) J. Biol. Chem. 276, 5883–5891
27. Re, F., and Strominger, J. L. (2002) J. Biol. Chem. 277, 23427–23432
28. Gioannini, T. L., Zhang, D., Teghanemt, A., and Weiss, J. P. (2002) J. Biol. Chem. 277, 47818–47825
29. Gioannini, T. L., Teghanemt, A., Zarember, K. A., and Weiss, J. P. (2003) J. Endotoxin Res. 9, 401–408
30. Yang, H., Young, D. W., Gusovsky, F., and Chow, J. C. (2000) J. Biol. Chem. 275, 20861–20866
31. Gangloff, M., and Gay, N. J. (2004) Trends Biochem. Sci. 29, 294–300
32. Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007) Science 316, 1632–1634
33. Akashi, S., Nagai, Y., Ogata, H., Oikawa, M., Fukase, K., Kusumoto, S., Kawasaki, K., Nishijima, M., Hayashi, S., Kimoto, M., and Miyake, K. (2001) Int. Immunol. 13, 1595–1599
34. Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. (2002) Nat. Immunol. 3, 354–359
35. Kawasaki, K., Akashi, S., Shimazu, R., Yoshida, T., Miyake, K., and Nishijima, M. (2001) J. Endotoxin Res. 7, 232–236
36. Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., and Beutler, B. (2005) Nat. Immunol. 6, 565–570
37. Montminy, S. W., Khan, N., McGrath, S., Walkowicz, M. J., Sharp, F., Conlon, J. E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K., Akira, S., Cotter, R. J., Goguen, J. D., and Lien, E. (2006) Nat. Immunol. 7, 1066–1073
38. Mullen, G. E., Kennedy, M. N., Visintin, A., Mazzoni, A., Leifer, C. A., Davies, D. R., and Segal, D. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3919–3924
39. Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Matsumoto, F., Fukase, K., Kusumoto, S., Kosugi, A., and Miyake, K. (2004) J. Endotoxin Res. 10, 257–260
40. Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007) Cell 130, 906–917