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Functional Activity of MD-2 Polymorphic Variant Is Significantly Different in Soluble and TLR4-Bound Forms: Decreased Endotoxin Binding by G56R MD-2 and Its Rescue by TLR4 Ectodomain

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MD-2 is an essential component of endotoxin (LPS) sensing, binding LPS independently and when bound to the ectodomain of the membrane receptor TLR4. Natural variation of proteins involved in the LPS-recognition cascade such as the LPS-binding protein, CD14, and TLR4, as well as proteins involved in intracellular signaling downstream of LPS binding, affect the cellular response to endotoxin and host defense against bacterial infections. We now describe the functional properties of two nonsynonymous coding polymorphisms of MD-2, G56R and P157S, documented in HapMap. As predicted from the MD-2 structure, the P157S mutation had little or no effect on MD-2 function. In contrast, the G56R mutation, located close to the LPS-binding pocket, significantly decreased cellular responsiveness to LPS. Soluble G56R MD-2 showed markedly reduced LPS binding that was to a large degree rescued by TLR4 coexpression or presence of TLR4 ectodomain. Thus, cells that express TLR4 without MD-2 and whose response to LPS depends on ectopically produced MD-2 were most affected by expression of the G56R variant of MD-2. Coexpression of wild-type and G56R MD-2 yielded an intermediate phenotype with responses to LPS diminished to a greater extent than that resulting from expression of the D299G TLR4 polymorphic variant. The Journal of Immunology, 2008, 180: 6107–6115.

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ram-negative bacterial endotoxins (LPS, lipooligosaccharides (LOS)) are among the most potent inducers of innate immune responses (1). A maximally sensitive response to endotoxin requires sequential participation of several extracellular and cell-surface LPS-binding proteins, including the LPS-binding protein (LBP), CD14, MD-2, and TLR4 (2–5). LBP catalyzes extraction and delivery of endotoxin monomers from the Gram-negative bacterial outer membrane or from aggregates of endotoxin to membrane (GPI-linked) or extracellular soluble (s) CD14 (6). LPS is then transferred to MD-2 bound to the TLR4 ectodomain in host cell membranes or to secreted MD-2 that, as a monomeric LPS:MD-2 complex, can bind directly to and activate TLR4 (7–9). Crystal structure of the complex TLR4 ectodomain/MD-2/ligand shows that lipid A inserts its acyl tail into the hydrophobic pocket of MD-2, which in turn is anchored to the N-terminal domain of TLR4 (26). Together, the actions of LBP, CD14, MD-2, and TLR4 permit robust host responses to picomolar concentrations of many endotoxins, facilitating rapid mobilization of host defenses to small numbers of invading Gram-negative bacteria before the invading bacteria have time to further multiply, disseminate, and adapt to host conditions (10). This sensitivity, however, also raises the risk of endotoxin-induced immunopathology when Gram-negative bacterial infection and exposure to endotoxin are not adequately controlled.

Despite progress in identification and characterization of proteins participating in LPS sensing, the precise structural determinants of LPS recognition and LPS-induced TLR4 activation are still largely unknown. Natural variants of receptors, co-receptors, and adapters resulting from single nucleotide polymorphisms can provide novel insights concerning the structural requirements for ligand recognition, receptor activation, and signaling and the physiological consequences of such alterations. Mutant alleles of LPS-sensing elements that affect recognition and response to LPS have been reported (11, 12), including alleles of TLR4 that are associated with effects on susceptibility to infection and inflammation. Variations of in vitro and in vivo LPS responsiveness of different strains of mice and a subset of humans with the same TLR4 genotype indicate the role of additional genes in regulating host responsiveness to endotoxin (13).

TLR4 activation by endotoxin (E) requires its simultaneous binding either to MD-2 and subsequently the E:MD-2 complex to TLR4 or to the MD-2/TLR4 complex directly (4, 7, 9). Thus, MD-2 plays a pivotal role in LPS sensing, bridging recognition of endotoxin initiated by LBP and CD14 to activation of TLR4. Mutagenesis studies in mouse and human MD-2 have revealed that single amino acid substitutions can significantly alter MD-2 function either by affecting protein stability, reactivity with E:CD14,
reactivity with TLR4, and/or the structural and functional properties of E:MD-2 complexes (14–20). However, to date, polymorphisms of MD-2 have not been extensively investigated. A polymorphism in the MD-2 promoter region has been reported that causes increased mRNA production and correlates with the incidence of organ dysfunction and sepsis after major trauma (21), consistent with the potentially pathologic effects of excessive MD-2/TLR4 signaling. Only one natural coding variant of MD-2 has been reported. Expression of this variant decreased cellular LPS responsiveness, but the molecular basis of this functional defect has not been determined (22).

In the present study we describe the effects of two missense mutations (G56R, P157S) of MD-2, reported in the HapMap database, on MD-2 function when MD-2 is expressed either in the presence or absence of TLR4. We show that the expression of the polymorphic variant G56R MD-2 reduces transfer of endotoxin from CD14 to MD-2 and, in parallel, TLR4-dependent cell activation. This effect was most pronounced when the MD-2 variant was expressed by cells in the absence of TLR4. The functional impact of this MD-2 variant may thus be particularly important in settings where there are TLR4-expressing cells (e.g., airway epithelial cells) that depend on ectopically expressed MD-2 for endotoxin responsiveness.

Materials and Methods

Cell culture and reagents

The human embryonic kidney (HEK) 293 cells were provided by Dr. D. Chow (Eisai Research Institute, Andover, MA). The HEK293 cells stably transfected with TLR4 (HEK293/TLR4 no. BF1) were provided by Dr. Douglas Golenbock (University of Massachusetts Medical Center, Worcester, MA) and Dr. Andrea Schromm (Research Center Borstel, Borstel, Germany). HEK293 T cells were used for the analysis of complex formation between LOS and MD-2 using gel filtration chromatography. Smooth LPS (S-LPS) (from Salmonella abortus equi HL83) was prepared by a phenol extraction procedure and was kindly provided by Dr. Brandenburg (Research Center Borstel, Borstel, Germany). Escherichia coli-type lipid A (compound 506) was obtained from the Peptide Institute (Osaka, Japan). [3H]LOS (25,000 cpn/pmol) from an acetate autoinjector of Neisseria meningitidis serogroup B was metabolically labeled and isolated as described (23). Chromatography matrices were purchased from GE Healthcare. Human serum albumin (HSA) was obtained as an endotoxin-free, 25% stock solution (Baxter Healthcare). Anti-FLAG Abs were obtained from Sigma-Aldrich, DyeMer 488/615 goat anti-rabbit IgG were from Molecular Probes, and anti-Tetra-His Abs and goat anti-mouse HRP-conjugated secondary Abs were from Qiagen and Jackson ImmunoResearch Laboratories, respectively.

Preparation of [3H]LOS aggregate ([3H]LOSagg) and [3H]LOS:sCD14 complex

[3H]LOSagg and [3H]LOS:sCD14 complex were prepared as previously described (4, 7, 24). Briefly, [3H]LOSagg (M Δ 20,000) were obtained after hot phenol extraction of [3H]LOS followed by ethanol precipitation of [3H]LOSagg and ultracentrifugation. Monomeric [3H]LOS:sCD14 complexes (M Δ 60,000) were prepared by treatment of [3H]LOSagg for 30 min at 37°C with substoichiometric LBP (molar ratio 100:1 LOS/LBP) and 1–1.5 × 106 excess sCD14 followed by gel exclusion chromatography (GE Healthcare Sephacryl S-200, 1.6 × 70 cm column) in PBS (pH 7.4), 0.03% HSA to isolate monomeric [3H]LOS:sCD14 complex. Radiochemical purity of [3H]LOS (H) and [3H]LOS:sCD14 was confirmed by gel filtration chromatography (GE Healthcare Sephacryl S-500 [LOSagg] or S-200 [([3H]LOS):sCD14] chromatography (7, 23).

Production and reaction of secreted MD-2 and MD-2/TLR4 ectodomain (TLR4-ECD) with [3H]LOS:sCD14 complex

HEK293 T cells were plated in 6-well plates with 10% FBS in DMEM. On the following day, cells were transfected with either expression plasmid encoding MD-2 alone or cotransfected with expression plasmids encoding MD-2 and TLR4ECD using PolyFect reagent (Qiagen). After 12–16 h, 1.5 ml of serum-free medium (293 SFM II; Invitrogen) + 0.1% HSA with or without [3H]LOS:sCD14 (5 ng/ml) was added. In most experiments, the serum-free medium was spiked with [3H]LOS:sCD14 at the time of addition of medium to the transfected cells to permit reaction of MD-2 with or without TLR4ECD, with [3H]LOS:sCD14 upon secretion. However, in selected experiments, conditioned medium harvested after 24 h of culture of transfected HEK293 T cells in serum-free medium was used for incubation with [3H]LOS:sCD14 (30 min at 37°C). Media harvested without [3H]LOS:sCD14 are shown as (recombinant protein) cm (where cm indicates conditioned medium), whereas media spiked with [3H]LOS:sCD14 during cell culture are represented as HEK/recombinant protein(s) secreted + spiked [3H]LOS:sCD14. Reaction products were analyzed by Sephacryl HR S-200 (1.6 × 70 cm) chromatography in PBS. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min at room temperature using AKTA Purifier or Explorer 100 fast protein liquid chromatography (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.

HEK293 cell activation assay

HEK293 cells were seeded into 96-well Costar plates (Corning) at 5 × 104 cells/well while HEK293 no. BF1 hTLR4 cells were seeded at 7 × 104 and incubated overnight in a humidified atmosphere (5% CO2) at 37°C. The next morning, cells were cotransfected for 4 h with Flag-CMV1-TLR4 and pEFPBOS-huMD-2-FLAG-His together with NF-κB, IP-10, or IFN-β-dependent luciferase and constitutive Renilla reporter plasmids using Lipofectamine 2000 (Invitrogen). After 4 h, medium was changed with DMEM + 10% FBS. The following day cells were stimulated with TLR4 agonists for 16 h. Cells were lysed in 1× reporter assay lysis buffer (Promega) and analyzed for reporter gene activities using a dual-luciferase reporter assay system on a Mithras LB940 luminometer. Relative luciferase activity...
(RLA) was calculated by normalizing each sample’s luciferase activity for constitutive Renilla activity measured within the same sample.

Site-directed mutagenesis

All mutations were introduced into pEFBOS-huMD-2-FLAG-His plasmid using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Several clones that resulted from the mutational procedure were sequenced to confirm the mutation. Primer sequences are available upon request.

Detection of MD-2 protein bound on cell surface-expressed TLR4

HEK293 no. BF1 hTLR4 cells were seeded in a 6-well plate (1.5 × 10^6 cells/well). After transfection, the cells were incubated for 24 h, harvested, and washed twice with FACS buffer. Cells (5 × 10^5) were resuspended with 100 μl FACS buffer containing 8 μg/ml anti-FLAG (Sigma-Aldrich), vortexed, and incubated on ice for 20 min. The cells were then washed twice with FACS buffer and incubated for 20 min in the dark with 2 μg/ml DyeMer 488/615 goat anti-rabbit IgG. The cells were then washed twice more with FACS buffer and resuspended in 300 μl FBS. Flow cytometry analysis was performed on EPICS ALTRA flow cytometer (Beckman Coulter). In each sample, 10,000 cells were analyzed. FACS data were analyzed by using WinMDI flow cytometry application.

Immunoblotting

To detect polyhistidine labeled wild-type (wt) and mutant MD-2s, an anti-polyhistidine Ab (Tetra-His Ab, Qiagen) was used. HEK293 T cells were transiently transfected with wt or mutant MD-2 using PolyFect as a transfection reagent. Twelve hours post-transfection the medium was changed. After 24 h, the supernatant was collected and equal volumes of supernatant from each sample were electrophoresed (Bio-Rad mini-gel system) through a 4–15% gradient acrylamide gel (Tris/HEPES/SDS buffer) and transferred to nitrocellulose membrane. The nitrocellulose membrane was washed with TBS (pH 7.5) containing 0.05% Tween 20 and 0.2% Triton X-100 (TBSTT), blocked to reduce nonspecific background with 3% BSA in TBSTT for 1 h at 25°C, and incubated with the anti-Tetra-His Abs in TBSTT.
overnight. After washing with TBSTT, the blot was incubated with goat anti-mouse IgG conjugated to HRP (Bio-Rad) for 1 h at 25°C in TBS containing 3% goat serum and washed with TBSTT exhaustively. Blots were developed using Pierce SuperSignal substrate system.

Results
Wild-type and variant MD-2 show similar secretion from HEK293 cells and surface expression with TLR4

Two coding mutations of MD-2, G56R and P157S, are described in the HapMap database. These MD-2 variants have been observed only in populations of European origin at the frequency of 2.5% for both the G56R and P157S alleles. According to the recently determined crystal structures of MD-2 (25) and MD-2/TLR4ECD (26), both mutations are located on the same side of MD-2, separated by ~16 Å (Fig. 1). As seen from the crystal structure of MD-2/TLR4ECD and mutagenesis studies (17, 19), this region of MD-2 does not overlap with sites of MD-2 that mediate TLR4 binding. Neither G56 nor P157 is in direct contact with the ligands lipid IVa (25) or eritoran (26) bound to MD-2 in crystal structures. However, G56 lies on the β-strand lining the LPS binding pocket, while P157 is located within the C-terminal extension with relatively little contact with the main protein domain. P157 is not completely conserved among MD-2 sequences from different species; serine is present at this position in bovine MD-2. In contrast, G56 is conserved among all reported mammalian MD-2 species but not in MD-1, a closely related protein that lacks the LPS binding properties of MD-2 (16). On the basis of these considerations, we predicted that the G56R variant could affect MD-2 function, whereas the P157S variant should not significantly affect MD-2 functional properties. To test this prediction, we expressed wt and the variant MD-2 species in HEK293 T (Fig. 2, A and B) and HEK293/TLR4 (Fig. 2C) cells. Without transfection with expression plasmids encoding MD-2, these cells do not express MD-2 (7, 27). Immunoblots of harvested culture media from transfected HEK293 T cells showed closely similar levels and profiles (i.e., presence of MD-2 monomers and multimers) of secreted wt and variant MD-2 (Fig. 2, A and B). Under nonreducing conditions (Fig. 2A), both wt and variant MD-2 migrated during SDS-PAGE mainly as dimers ($M_r$ of ~25,000) and higher order multimers with only a small fraction of the wt and variant MD-2 species migrating as monomers ($M_r$ of ~50,000). Under reducing conditions (Fig. 2B), each of these MD-2 species was converted to the monomeric form, migrating as a doublet because of differences in glycosylation (28). When coexpressed with TLR4 (Fig. 2C), MD-2 was expressed at the cell surface as determined by FACS.

![FIGURE 4. Effect of G56R MD2 variant on TIR domain-containing adapter inducing IFN-β-dependent pathway compared with wt MD-2. HEK293/TLR4 cells were transiently transfected with expression plasmids (pEF-BOS) encoding wt or mutant MD-2, and IP-10- or IFN-β-dependent luciferase reporter plasmid. After 24 h, cells were stimulated with different concentrations of (A and B) S-LPS, (C) lipid A/506, or (D) meningococcal LOS for 16 h in the presence of 10% FBS. The cells were lysed and lysates were analyzed for luciferase activity. Data are presented as described in the legend to Fig. 3 and represent the means ± SEM of three determinations. The symbols represent the p values as follows: *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$ (compared with wt MD-2 by Student’s t test).

FIGURE 5. Reduced transfer of [3H]LOS from [3H]LOS:sCD14 to G56R variant MD-2 vs wt or P157S MD-2. HEK293 T cells were transfected with plasmids encoding wt, G56R, or P157S MD-2. After ~24 h, the transfection medium was changed with serum-free medium and spiked with 1 nM [3H]LOS:sCD14. Medium was harvested after 24 h and analyzed by Sephacryl S-200 chromatography. Resolved reactants and products were monitored by liquid scintillation spectroscopy. Gray dashed line represents medium from cells that were not transfected, as control, spiked with 1 nM [3H]LOS:sCD14. Note that the reaction of monomeric [3H]LOS:sCD14 with sMD-2 yields monomeric [3H]LOS:MD-2, as shown. The results shown are representative of three or more closely similar experiments.
Reduced endotoxin responsiveness of HEK293/TLR4 cells expressing G56R MD-2

The closely similar surface expression of wt and variant MD-2 in HEK293/TLR4 cells facilitated comparison of the ability of each of these MD-2 species to promote responsiveness of HEK293/TLR4 cells to added endotoxin. Sensitive responses to endotoxin required expression of MD-2 and addition of serum, with the latter providing LBP and sCD14 to convert the added endotoxin (LPS or lipid A) aggregates to monomeric E:sCD14 complexes that are highly reactive with MD-2/TLR4 (9). Expression of wt and P157S MD-2 yielded closely similar dose-dependent responses of transfected HEK293/TLR4 cells to S-LPS (Fig. 3A). In contrast, cells expressing G56R MD-2 showed statistically significant lower responses to S-LPS, meningococcal LOS (Fig. 3B), and lipid A (compound 506; Fig. 3C). Reduced responsiveness to endotoxin of cells expressing G56R MD-2, in comparison to cells expressing wt MD-2, was observed when induction of luciferase was under the control of either NF-κB (Fig. 3), IP-10 (Fig. 4, A, C, and D), or IFN-β (Fig. 4B) promoters. These findings indicate that expression of the G56R variant of MD-2 results in reproducibly lower responses to several different endotoxin species, including both MyD88-dependent (NF-κB) and TIR domain-containing adapter inducing IFN-β-dependent (IFN-β- and IP-10-based reporters) signaling pathways.

G56R MD-2 variant exhibits very low LPS binding

Activation of cells expressing MD-2/TLR4 requires transfer of endotoxin from CD14 to MD-2 in the MD-2/TLR4 heterodimer (9). Thus, the differences in the potency of cell activation by endotoxin toward cells expressing TLR4 and MD-2 (wt vs G56R) (Figs. 3 and 4) could reflect differences in the efficiency of transfer of endotoxin from CD14 to MD-2. To test this possibility, we made use of a recently developed assay to sensitively and quantitatively measure transfer of [3H]LOS from [3H]LOS:sCD14 to MD-2 in a culture medium of transfected HEK293 T cells expressing and secreting MD-2 (27). As shown in Fig. 5, in conditioned medium containing either wt or P157S MD-2, nearly all [3H]LOS:sCD14 added (spiked) to the culture medium was converted to [3H]LOS:MD-2. In contrast, there was very little conversion of [3H]LOS:sCD14 to [3H]LOS:MD-2 when G56R MD-2 was expressed. This suggests a marked effect of this single amino acid alteration on the ability of MD-2 to react with [3H]LOS:sCD14.

Ability of sMD-2 to support activation of HEK293/TLR4 cells by LPS is markedly reduced in G56R MD-2 variant

Cells can be activated by LPS either through binding of LPS to MD-2 that is already associated with the ectodomain of TLR4 or through binding of the MD-2:LPS complex to the ectodomain of TLR4. Thus, the marked defect in the ability of secreted G56R MD-2 to react with [3H]LOS:sCD14 suggested that this polymorphic variant would have much less ability to support activation by LPS (i.e., LPS:sCD14) of cells expressing TLR4 without MD-2. To test this hypothesis, we compared the ability of secreted wt and G56R MD-2 to confer activation of HEK293/TLR4 cells by LPS. Fig. 6A shows that the responsiveness of HEK293/TLR4 cells to LPS in the presence of medium containing G56R MD-2 was at least 10-fold lower in comparison to the addition of medium containing wt MD-2, roughly paralleling the differences seen in the reaction of wt versus G56R MD-2 with [3H]LOS:sCD14 (Fig. 5). It has been reported that MD-2 rapidly looses its biological activity at 37°C in serum-free medium (29). Presence of LPS significantly stabilized MD-2, and we therefore performed the same experiment by spiking the cell medium with LPS so that all secreted MD-2 could immediately bind LPS and increase its stability. However, biological activity of G56R MD-2 variant was reduced to the same extent in comparison to wild type as in the case when LPS was added subsequently. These findings clearly demonstrate that the diminution in host cell responsiveness to endotoxin when G56R MD-2 is expressed is much greater when TLR4 and MD-2 originate from different cells (Fig. 6) than when TLR4 and MD-2 are coexpressed by the same cell (Figs. 3 and 4).

TLR4_eccd rescues LPS binding of G56R variant

The modest diminution in responsiveness to endotoxin of cells coexpressing TLR4 and G56R (vs wt) MD-2 suggested that preassociation of the G56R MD-2 with the TLR4 ectodomain might increase the reactivity of G56R MD-2 with E:CD14 as compared with G56R MD-2 expressed and secreted in the absence of TLR4.
To test this hypothesis, experiments were repeated with cells coexpressing MD-2 (wt or G56R) and TLR4ECD. Addition (spiking) of [3H]LOS:sCD14 to the culture medium of cells expressing wt MD-2 and TLR4ECD resulted in nearly complete conversion of [3H]LOS:sCD14 to earlier eluting (larger) and later eluting (smaller) [3H]LOS-containing complexes (Fig. 7A). Formation of the larger complex reflects transfer of [3H]LOS from sCD14 to MD-2/TLR4ECD yielding (3H]LOS:MD-2/TLR4ECD)2. The absence of [3H]LOS:MD-2 as a product in this way, a small (~20–30%) but reproducible difference in the generation of [3H]M1 ~ 190,000 complex (3H]LOS:MD-2/TLR4ECD)B, whereas reaction of [3H]LOS:sCD14 with sMD-2 yields [3H]LOS:MD-2. The absence of [3H]LOS:MD-2 as a product in C even when wt MD-2 was expressed indicates that added TLR4ECD is in sufficient excess to convert all monomeric sMD-2 to MD-2/TLR4 or [3H]LOS:MD-2 to ([3H]LOS:MD-2/TLR4ECD). The results shown are representative of two or more closely similar experiments.

FIGURE 7. TLR4ECD rescues the reactivity of G56R MD-2 with [3H]LOS:sCD14. A and B, HEK293T cells were cotransfected with plasmids encoding TLR4ECD and wt or G56R MD-2. After ~24 h, the transfection medium was changed with serum-free medium with (A, "spiked") or without (B) 1 nM [3H]LOS:sCD14. Medium was harvested at 24 h and analyzed directly by Sephacryl S-200 chromatography (A) or after 30-min incubation with 1 nM [3H]LOS:sCD14 (B). C: HEK293 T cells were transfected with plasmids encoding wt or G56R MD-2. After ~24 h, the transfection medium was changed with serum-free medium and spiked with 1 nM [3H]LOS:sCD14 and 200 μl of conditioned cell culture medium from HEK293T cells transfected with plasmid encoding TLR4ECD. Medium was harvested at 24 h and analyzed directly by Sephacryl S-200 chromatography.Resolved reactants and products were monitored by liquid scintillation spectroscopy. Gray dashed line represents medium from cells that were not transfected, as control, spiked with 1 nM [3H]LOS:sCD14. Note that the reactions of [3H]LOS:sCD14 with MD-2/TLR4 ECD yield a [3H]M1 ~ 190,000 complex ([3H]LOS:MD-2/TLR4ECD)B, whereas reaction of [3H]LOS:sCD14 with MD-2 yields [3H]LOS:MD-2. The absence of [3H]LOS:MD-2 as a product in C even when wt MD-2 was expressed indicates that added TLR4ECD is in sufficient excess to convert all monomeric sMD-2 to MD-2/TLR4 or [3H]LOS:MD-2 to ([3H]LOS:MD-2/TLR4ECD).

To determine whether coexpression of wt and G56R MD-2, as in the case of heterozygotes, confers reduced cellular responsiveness to endotoxin, we transfected HEK293/TLR4 (Fig. 8A) or HEK293 (Fig. 8B) cells with equal amounts of plasmids encoding wt and
G56R MD-2 and then measured cellular LPS responsiveness as described above. As shown in Figs. 8, A and B, coexpression of wt and G56R MD-2, either with (Fig. 8A) or without (Fig. 8B) TLR4, conferred an intermediate level of LPS responsiveness. As seen above, the effects of expression of the G56R MD-2 variant were more dramatic when MD-2 was expressed without TLR4 and was needed to render cells expressing TLR4 alone (i.e., no MD-2) responsive to endotoxin. These findings indicate that heterozygotes expressing both wt and G56R MD-2 will have a blunted TLR4-dependent response to endotoxin and suggest a gene dosage effect in which reduced expression of maximally active wt MD-2 results in diminished endotoxin responsiveness.

LPS responsiveness of cells expressing minor polymorphic variants of TLR4 at residues 299 and 399 has been extensively studied both in vitro and in vivo (12, 30, 31). In HEK293 cells expressing MD-2 and TLR4, expression of G56R rather than wt MD-2 reduced cellular endotoxin responsiveness to a slightly greater degree than did expression of D299G rather than wt TLR4 (Fig. 9). Coexpression of the G56R MD-2 and D299G TLR4 alleles did not further blunt cellular responsiveness to LPS beyond that caused by the expression of G56R MD-2 alone.

**Discussion**

The response of human cells to bacterial infection has to be delicately balanced, because both exaggerated as well as deficient responses may result in detrimental effects to the host. This view has gained recent added support by the description and characterization of genotypic variants of innate immune signaling pathways that are associated with altered incidence of specific infectious and/or inflammatory diseases even in certain instances in which the described in vitro phenotype is relatively modest. These observations have included elements of the LPS signaling pathway such as CD14 and TLR4 (32, 33). However, despite its pivotal role in TLR4 activation by endotoxin, genetic variation of MD-2 has not been extensively studied. An apparently low-frequency polymorphism in human MD-2 at residue 35 has been published (22). This mutation confers decreased cellular responses to LPS, but the mechanistic basis of decreased responsiveness (e.g., reduced LPS binding, TLR4 binding, or a defect in the activation process) has not been investigated and remains unknown, with no helpful clues being provided by the recent crystal structure studies of MD-2 with and without TLR4ECD other than to indicate that this residue is remote from sites in MD-2 involved in LPS and TLR4 binding. In fact, alanine scanning of murine MD-2 did not produce a statistically significant decrease in LPS responsiveness or TLR4 binding in an equivalent S35A mutant of murine MD-2 (19).

Our studies of missense mutations of MD-2 that were documented in HapMap have shown that the G56R, but not the P157S mutation, results in significantly decreased cellular responsiveness.
to LPS. The effect of the G56R mutation reflects diminished function of the expressed protein and not reduced expression. In transfected HEK293 cells that we used as a model to study MD-2 expression and function, no differences were observed between wt and G56R MD-2 either in secretion of sMD-2 from cells that did not coexpress TLR4 (Fig. 2, A and B) or in surface expression of MD-2 (MD-2/TLR4) when MD-2 was coexpressed with TLR4 (Fig. 2C). Reduced cellular responsiveness to endotoxin when G56R MD-2 was expressed was associated with reduced transfer of endotoxin from E:CD14 to G56R (vs wt) MD-2. Remarkably, the extent to which expression of the G56R variant of MD-2 reduced cellular responsiveness depended on the cellular context of MD-2 expression. Thus, cells that coexpress MD-2 and TLR4 showed a relatively modest diminution of endotoxin responsiveness when expressing G56R MD-2 rather than wt MD-2 (Figs. 3, 4, and 8). In contrast, cells (e.g., HEK/TLR4) that depended on exogenous sMD-2 to respond to LPS (i.e., E:scD14) showed a much greater reduction in endotoxin responsiveness when G56R rather than wt MD-2 was added (Figs. 6 and 8). These differences in effects of G56R vs wt MD-2 expression on cell activation by endotoxin were paralleled by differences in the reactivity of G56R vs wt MD-2 with \(^{3}H\)LOS:scD14: reduced transfer of \(^{3}H\)LOS from CD14 to G56R (vs wt) MD-2 was much more pronounced when MD-2 was expressed and secreted from cells without (Fig. 5) or in excess of TLR4 (Fig. 7A). Taken together, these findings strongly suggest that diminished cellular endotoxin responsiveness when G56R rather than wt MD-2 is expressed is due, at least in significant part, to reduced endotoxin transfer from CD14 to MD-2.

To date, mutagenesis studies have strongly suggested that increased positive electrostatic potential in the region surrounding the endotoxin binding site of MD-2 increases TLR4-dependent cellular responsiveness to endotoxin (14, 15, 20, 34). Thus, the deleterious effect of the G56R mutation on MD-2 reactivity with LPS was somewhat surprising. However, a glycine residue at this site is conserved in all known MD-2 orthologs, suggesting an essential role for glycine (56). Glycine residues are often instrumental in supporting conformational flexibility, a property that would be lost by substitution of the bulkier side chain of arginine. Conformational changes accompanying opening of the lipid-binding pocket have been documented in crystal structures of the structurally related ML superfamily member GM2-activating protein produced in the absence and presence of lipid ligands of different size (35). This expansion requires adjustment of the \(\beta\)-sheets of the binding pocket and thus may explain the importance of the glycine residue at this site for LPS binding. In murine MD-2, even the more conservative substitution of glycine (56) with alanine had a deleterious effect of the G56R mutation on MD-2 reactivity with LPS when G56R rather than wt MD-2 is expressed is due, at least in significant part, to reduced endotoxin transfer from CD14 to MD-2.

Secreted MD-2 forms a mixture of monomers, dimers, and higher order multimers (Ref. 36; Fig. 2A). The monomeric form of MD-2 is required both for transfer of endotoxin from CD14 to MD-2 and for binding to the ectodomain of TLR4 (7-9, 27). Therefore, it is possible that the G56R mutation reduces MD-2 function by promoting aggregation of MD-2. Immunoblots of secreted MD-2 do not show differences in the relative abundance of sMD-2 monomer and multimers in wt and G56R MD-2 (Fig. 2A).

However, the use of SDS-PAGE for these analyses may mask differences in the amount of wt vs G56R sMD-2 monomer present in the cell culture medium before SDS treatment. The protective effect of coexpression of TLR4 or TLR4\(_{ECD}\) on G56R MD-2 function could thus be due to interactions between MD-2 and the TLR4 ectodomain that capture functional MD-2 monomers before these monomers self-associate to form nonfunctional oligomers.

Our findings suggest that the effect of expression of the G56R polymorphic variant will be greatest in settings in which MD-2 is secreted without TLR4. The fact that spiking the cell culture medium with TLR4\(_{ECD}\) yielded G56R MD-2-2TLR4\(_{ECD}\) complexes reactive with \(^{3}H\)LOS:scD14 (Fig. 7C) suggests that G56R MD-2 function can be rescued not only by intracellular interactions with coexpressed TLR4 but also by extracellular interactions with neighboring cell surface TLR4, provided that sMD-2 can find TLR4 before it becomes inactive. In the absence of TLR4, the marked impairment in functional reactivity of G56R sMD-2 was observed in both serum-free medium (Fig. 5) and culture medium containing 10% serum (Fig. 6). Thus, the reduced function of G56R MD-2 seems likely to be manifested in biological fluids in vivo as well as in culture medium in vitro. The physiological function of sMD-2 in vivo is still largely unknown and potentially complex. In vitro studies have demonstrated several possible effects of sMD-2 on TLR4-dependent cell activation by endotoxin. This includes reaction of sMD-2 with cells expressing surface TLR4 without MD-2, thereby rendering these cells responsive to E:CD14. Secreted MD-2 can also react with E:scD14 to form E:MD-2 (Figs. 5 and 7A) that, depending on neighboring cell-surface levels of mCD14, MD-2:TLR4, and TLR4, can either promote (cells with TLR4 alone) or blunt (cells with MD-2:TLR4) cell activation by endotoxin. It is known that levels of soluble MD-2 rise in plasma in sepsis where it could promote activation of blood monocytes (37). In this circumstance, expression of G56R MD-2 could be protective by reducing endotoxin-driven systemic inflammatory responses by monocytes or it could be harmful by reducing sequestration of the circulating LPS from cells expressing surface MD-2:TLR4 with little or no mCD14 (e.g., endothelial cells). Similarly, along mucosal epithelia where low expression of MD-2 limits epithelial responses to endotoxin (38), MD-2 secreted from neighboring cells such as macrophages and dendritic cells could bind to TLR4 on the epithelial cell surface and amplify epithelial responsiveness to endotoxin or, if in molar excess to TLR4, sequester endotoxin and reduce TLR4-dependent proinflammatory responses. In experimental settings, the contrasting properties of wt and G56R MD-2 may help to decipher the relative roles of TLR4-associated vs sMD-2 cell activation. Even though the frequency of the G56R MD-2 allele is relatively low (2.5% among Caucasians of European origin), the demonstrable phenotype in cells with heterozygous expression of wt and G56R MD-2 (Fig. 8) should encourage analysis of the distribution of this polymorphism among populations of patients in which (endotoxin-driven) TLR4-dependent inflammatory responses may contribute significantly to the observed pathophysiology.

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Disclosures
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References


