Mutational Analysis of Membrane and Soluble Forms of Human MD-2*

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Toll-like receptor 4 and MD-2 form a receptor for lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria. MD-2 is a 20–25-kDa extracellular glycoprotein that binds to Toll-like receptor 4 (TLR4) and LPS and is a critical part of the LPS receptor. Here we have shown that the level of MD-2 expression regulates TLR4 activation by LPS. Using site-directed mutagenesis, we have found that glycosylation has no effect on MD-2 function as a membrane receptor for LPS. We used alanine-scanning mutagenesis to identify regions of human MD-2 that are important for TLR4 and LPS binding. We found that mutation in the N-terminal 46 amino acids of MD-2 did not substantially diminish LPS activation of Chinese hamster ovary (CHO) cells co-transfected with TLR4 and mutant MD-2. The residues 46–50 were important for LPS activation but not LPS binding. The residues 79–83, 121–124, and 125–129 are identified as important in LPS activation but not surface expression of membrane MD-2. The function of soluble MD-2 is somewhat more sensitive to mutation than membrane MD-2. Our results suggest that the 46–50 and 127–131 regions of soluble MD-2 bind to TLR4. The region 79–120 is not involved in LPS binding but affects monomerization of soluble MD-2 as well as TLR4 binding. We define the LPS binding region of monomeric soluble MD-2 as a cluster of basic residues 125–131. Studies on both membrane and soluble MD-2 suggest that domains of MD-2 for TLR4 and LPS binding are separate as well as overlapping. By mapping these regions on a three-dimensional model, we show the likely binding regions of MD-2 to TLR4 and LPS.

Innate immunity is the first line of defense against pathogens. A key component of the mammalian innate immune system is a family of Toll-like receptors (TLRs)1, 2. Lipopolysaccharide (LPS), a major component of Gram-negative bacteria, activates a variety of cells to produce inflammatory cytokines that can lead to septic shock in humans. The innate immune mechanism that recognizes LPS involves a transfer of LPS to a pattern recognition molecule CD14 (3) by lipopolysaccharide-binding protein (LBP) (4). CD14 has no transmembrane domain and is not capable of signaling. TLR4 is a type 1 transmembrane protein that has extracellular leucine-rich repeats and an intracellular signaling domain that is responsible for LPS signaling (5, 6). TLR4 forms a complex with MD-2, a 22–25-kDa glycoprotein, on the cell surface (7).

A cascade of events leading to maximal cellular activation is likely to involve transferring of LPS by LBP to CD14 and then to TLR4-MD-2. Although CD14 and LBP enhance cellular activation, activation of TLR4 by LPS absolutely requires MD-2 (8).

MD-2 can be found on the cell surface in association with TLR4 or as a secreted protein. It shares a sequence homology to MD-1, a protein that binds to another TLR family member, R105 (9), which constitutes an LPS signaling complex on B-cells (10). MD-2 contributes to the ligand recognition of TLR4. It binds LPS with high affinity (11) and is responsible for the different responses of human and mouse to lipid IVA and taxol (12). Interaction of the cell surface TLR4-MD-2 complex with LPS induces clustering of TLR4 and may be the mechanism that triggers cellular activation (13).

Although proper glycosylation and trafficking of TLR4 to the cell surface requires intracellular association with MD-2 (14, 15), functional TLR4 can be presented on the cell surface without MD-2 in both transfected cells and human airway epithelial cells (11, 16). These cells can respond to LPS in the presence of soluble MD-2. Whereas soluble MD-2 is essential for LPS-induced activation of cells expressing only TLR4, high levels of soluble MD-2 can inhibit cellular responses (11). Soluble MD-2 exists as a heterogeneous collection of monomer and oligomers through inter- and intrachain disulfide bonds (17), and one group has presented data that argues that monomeric MD-2 preferentially binds to TLR4 and functions as a co-receptor with TLR4 (18).

Human MD-2 contains 160 amino acid residues, including the N-terminal 17 amino acid signal sequence, with 7 cysteine residues and 2 N-glycosylation sites. Regions of functional importance on human and mouse MD-2 have been identified using peptide fragments (19), mutation analysis (13, 20–24), and structural modeling (25). In this study, we have investigated the structure/function relationships of membrane and soluble MD-2 by alanine-scanning mutagenesis.

MATERIALS AND METHODS

Reagents—Salmonella minnesota Re 595 LPS (Re LPS) was prepared as previously described (26). Recombinant soluble CD14 with C-terminal His tags were prepared as described previously (27). Anti-His tag and anti-HA tag were from Qiagen and Roche Diagnostics, respectively. Control mouse IgG1 and rabbit IgG were obtained from Caltag. All protein biotinylation were done using the EZ-Link Sulfo-NHS-LC biotinylation kit (Pierce). All reagents were tested for LPS contamination with Limulus Ameobocyte Lysate (BioWhittaker). All reagents had...
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<0.02 pg of endotoxin/μg of protein. When necessary, LPS was removed from the reagents using END-X (Associates of Cape Cod, Inc.).

Mutagenesis of MD-2—The human MD-2 gene with the gp64 signal peptide sequence as described in Ref. 11 was subcloned into the EcoRI and AgeI site of the plasmid pcdNA4/V5His (Invitrogen) for expression of secreted C-terminal His-tagged protein in mammalian cells. The MD-2 amino acids were changed using the QuikChange site-directed mutagenesis kit (Stratagene). The mutant MD-2 constructs were transfected into a CHO cell line stably transfected with TLR4 containing an N-terminal HA tag and a CD25 reporter plasmid as described previously (11). Stably transfected cell lines were generated by selection with Zeocin followed by immunomagnetic sorting using anti-His mAb (Qiagen).

Cell Culture and Transfection—Cell lines were maintained in the laboratory as previously described (26). EL1, a CHO cell line stably transfected with inducible membrane CD25 under the transcriptional control of a human E-selectin promoter containing NFκB binding sites, was a gift from Dr. D. Golenbock (28). Plasmid DNA was prepared using an EndoFree kit (Qiagen). Stably transfected cell lines were generated using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells expressing TLR proteins were sorted by immunomagnetic beads (Dynal) using anti-HA mAb (Roche Diagnostics). The stably transfected lines were generated by selection with G418 (Invitrogen). Transfected cells were assayed for surface expression of the HA-epitope by FACS analysis using anti-HA mAb followed by a F(ab′)2 fragment of goat anti-mouse Ig fluorescein isothiocyanate (Caltag).

Analysis of NFκB Activity by Flow Cytometry—CHO cells carrying NFkB reporter plasmids to express surface CD25 were plated in a 24-well plate 1 day prior to activation. The cells were stimulated overnight, harvested, and stained with phycoerythrin-CD25 mAb (BD Biosciences) and analyzed by FACS as previously described (11).

Expression of Soluble MD-2 and MD-1—Wild-type (WT) and mutant MD-2 (11) and MD-1 genes (Inivviron) were subcloned into pBlueBac4.5/V5-His (Invitrogen) or pBac11 (Novagen) and recombinant virus generated by the manufacturers’ protocols. Recombinant virus stock was verified to contain the correct mutation by sequencing PCR-amplified inserts. Expressed protein in the insect cell supernatant was purified by nickel-nitrioltriacetic acid affinity chromatography as described previously (11). The purity of all proteins was determined by Coomassie Blue staining of protein electrophoresed on a Nu-polyacrylamide gel (Invitrogen). The protein concentrations were determined by BCA assay (Pierce), ELISA using biotinylated anti-His tag, and Western blotting of the protein with anti-His tag. The control His tag protein was produced using control recombinant virus supplied by Novagen.

LPS Binding Assays—The assay for MD-2 binding using immobilized LPS was done in a similar manner to the method described previously (11).

Activation of U373 Cells—The cells were cultured in a 96-well plate and activated with various reagents in minimal essential medium with 10% fetal calf serum. The supernatant was harvested after 16 h of activation and assayed for IL-6 by ELISA as described previously (11).

Analysis of Soluble MD-2 Binding to TLR4—TLR4-transfected CHO cells with or without the CD25 reporter gene were incubated with various amounts of soluble MD-2 in RPMI 1640 medium with 10% fetal calf serum for 15 min or 16 h at 20°C. After washing off the excess protein with medium, the cells were stained with mAb anti-His (Qiagen) to detect MD-2 or anti-HA to detect TLR4, followed by rabbit anti-mouse Ig-fluorescein isothiocyanate and analyzed by FACS. To quantitate the amount of MD-2 and TLR4 on the cell surface, the mean channel number (MCN) of fluorescence intensity was compared with the standard curve of Simply Cellular Microbeads (Bangs Laboratories) stained with the corresponding antibodies.

Antibody Sandwich ELISA for the Detection of Soluble MD-2—A 96-well microtiter plate (Immulon, Dynex) was coated with 1 μg/ml of three different monoclonal antibodies against soluble MD-2 developed in our laboratory and diluted in carbonate buffer, pH 9.6, overnight at 4°C. The plate was washed once with phosphate-buffered saline and 0.01% Tween (PBST) and blocked with 1% casein in phosphate-buffered saline for 1 h at room temperature. The culture supernatant was added to each well and the plate incubated for 1.5 h at 37°C. The wells were washed four times in PBST, and 2.5 μg/ml biotinylated polyacylamide rabbit anti-soluble MD-2 was added to each well. After incubation for 1 h at 37°C, the wells were washed five times, and 80 ng/ml Streptavidin-horseradish peroxidase conjugate (Zymed Laboratories Inc.) was added for 45 min at 37°C. The wells were washed five times, and the substrate (1% tetramethylbenzidine) was added. After 15 min, the reaction was stopped by adding 1.2 M H2SO4 to each well, and the absorbance was measured at 450 nm. The concentration of soluble MD-2 in the culture supernates was derived from a standard curve using purified wild-type soluble MD-2 expressed by baculovirus.

Immunoprecipitation and Western Blotting—CHO cells stably transfected with TLR4 were incubated with 1 μg of soluble MD-2 for 15 min at 25°C. The cells were washed three times in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) with 10% fetal calf serum and lysed in 50 mM Heps, 0.1% Nonidet P-40, 1 mM EDTA, and HALT protease inhibitor mixture (Pierce). The cell lysate was incubated with anti-HA-agarose (Profound HA, Pierce) overnight at 4°C and the precipitated protein eluted according to the manufacturer’s protocol. The eluted protein was electrophoresed on a reduced 4–12% Nu-polyacrylamide gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. The soluble MD-2 and TLR4 were detected using the biotinylated anti-His or anti-HA antibody, respectively. Streptavidin-horseradish peroxidase (Zymed Laboratories Inc.) and the enhanced chemiluminescence system (ECL, Amersham Biosciences) were used to detect the bound antibodies.
RESULTS

MD-2 Influences LPS Activation—To investigate the role of MD-2 in LPS receptor function, we varied the amount of soluble MD-2 and examined the response of TLR4-transfected EL1 cells (a CHO cell line stably transfected with a NF\(\kappa\)B/CD25 reporter plasmid) (28) to LPS. The amount of soluble MD-2 that bound to TLR4-transfected cells as well as the TLR4 expression level were assayed by FACS analysis using antibody to His and HA tags, respectively. The cells were also activated with 100 ng/ml of LPS, and NF\(\kappa\)B activation was assayed by FACS analysis of surface CD25 expression. These data plotted versus the concentration of soluble MD-2 are shown in Fig. 1. As the soluble MD-2 concentration increased, more MD-2 could be detected on the cell surface, whereas the TLR4 receptor number remained constant. At a concentration of 0.5 nM soluble MD-2, the calculated ratio of surface MD-2 per TLR4 was 0.5, yet a small amount of LPS-induced NF\(\kappa\)B activation occurred. The level of activation increased with more MD-2 on the cell surface and reached a maximum when the calculated ratio of MD-2 per TLR4 was \(-5\). This suggests that optimal receptor function requires multiple molecules of MD-2 bound to each TLR4.

N-linked Glycosylation of Membrane MD-2 Has Little Effect on Cellular Activation—There have been several reports in the literature that the glycosylation state of MD-2 was important for receptor function (23, 24). To test this hypothesis, we mutated the two N-glycosylation sites by replacing them with glutamines. The mutated MD-2 gene in pcDNA/V5His was transfected into the TLR4/CHO reporter cell line, and a stably transfected cell line was made. This cell line (TLR4-MD-2 NQ) expressed unglycosylated MD-2 protein, as shown by Western blotting of the cellular extract with antibody to His tag (Fig. 2A). The TLR4-MD-2 NQ expressed the MD-2 protein as a value 17 Kd protein, whereas the wild type expressed glycoforms of \(\sim20–27\) Kd. Because the level of MD-2 expression on the cell surface influences LPS activation, it was important that we compare the function of this mutant to the wild type expressed at a similar level. To achieve equal expression of mutant and WT MD-2, we sorted the TLR4-MD-2 NQ cell line several times using immunomagnetic beads to select cells expressing high levels of MD-2 NQ. Fig. 2B shows FACS analysis of the surface expression of TLR4 and MD-2 on the cell lines. The TLR4-MD-2 NQ and WT cell lines were activated with LPS, and NF\(\kappa\)B activation was assayed by CD25 surface expression. Fig. 3A shows that 100 ng/ml LPS activated the non-glycosylated mutant cells to express surface CD25 at the same level as the WT cells. We compared the level of CD25 expression after both cell lines were activated with concentrations of LPS stimulation from 1 ng/ml to 1 \(\mu\)g/ml and found similar levels of LPS activation, as shown in Fig. 3B. These data show that glycosylation is not important for the LPS receptor function of membrane MD-2.

Critical Regions of Human Membrane MD-2 for TLR4 Binding and Cellular Activation by LPS—We analyzed regions of functional importance of human MD-2 by site-directed mutagenesis. The protein sequence of human MD-2 was analyzed for Kyte-Dolittle hydrophilicity with McVector Software. Mutagenesis was designed by replacing blocks of 4–5 amino acids with alanine, as shown in Fig. 4. The expression
constructs with C-terminal His tags were stably transfected into TLR4/EL1 cells. We analyzed cell surface TLR4 and MD-2 by FACS using anti-HA and anti-His tags, respectively. TLR4 surface expression levels were relatively constant (data not shown), whereas MD-2 levels varied widely. (Fig. 5). Alanine replacement mutagenesis in the N-terminal 46 amino acids of MD-2 produced mutants that expressed as well or better than the wild type. Mutation in the rest of the molecule yielded protein that was expressed poorly on the cell surface, except for three regions: amino acids 79–83, 106–110, and 121–129. This suggests that most of the MD-2 sequence after amino acid 61 is important for cell surface expression. Fig. 5, left column, shows that all mutants were synthesized and secreted as soluble protein into the medium. The levels of secreted MD-2 varied among the mutants and were not correlated with the cell surface expression levels. These data rule out the possibility that MD-2 was not found on the cell surface, because the engineered mutants were not synthesized.

The stably transfected cells expressing TLR4 and MD-2 mutants were activated with 100 ng/ml LPS and NFκB activation assayed by CD25 expression. Each mutant was assayed in triplicate at least twice. Fig. 5 shows the LPS activation levels of different mutants as compared with the surface expression level. All mutants in the N-terminal 61 amino acids, which expressed on the cell surface, were responsive to LPS. Among this group, mutant 19–23 is more responsive to LPS than one would predict from the surface expression level. Mutants in the region 42–61 were expressed at a higher level than WT but responded to LPS less than half as well as WT. The three mutants at the C-terminal region (amino acids 79–83, 121–125, and 125–129) were expressed well but were not responsive to LPS. Mutants in the regions 66–79, 87–102, 116–122, and 127–150 were not expressed well on the cell surface.

Conformation of Soluble MD-2 Mutants—Selected MD-2 mutants with different levels of expression on the cell surface, mutants that expressed well but were poorly activated by LPS, as well as the unglycosylated mutant were chosen for expression as soluble protein. The mutant genes were subcloned into baculoviral plasmids and soluble proteins expressed in insect cells. All recombinant viruses used were purified and DNA sequenced to confirm the correct mutation. The protein was purified by nickel-nitriilotriacetic acid affinity chromatography. The purified protein was analyzed on non-reducing PAGE. Fig. 6 shows isoforms of different soluble MD-2 mutants on non-reduced PAGE. Soluble mutants 38–42, 46–50, and 125–129 are most similar to the WT MD-2 in the amount of monomer. These mutants also expressed well on the cell surface. Mutants 75–79, 79–83, 106–110, and 116–120 did not express well on the cell surface. The soluble forms of these mutants contained very little monomer. These data suggest that monomer formation may correlate positively with membrane MD-2 expression.

Analysis of Soluble MD-2 Mutant Function—We tested for soluble MD-2 binding to LPS by using an LPS-coated plate assay (11). Fig. 7A shows that soluble mutants 38–42, 46–50, and 79–83 bound LPS similarly to the wild type. Mutant 75–79 bound LPS better than the wild type. The unglycosylated mutant (NQ) bound LPS as well as the wild type. Two mutants, 125–129 and 127–131, bound LPS but less well than the wild type. Our control His tag protein and MD-1 showed no binding to LPS.
Previously we have shown that an excess of soluble MD-2 inhibited soluble CD14-dependent LPS activation in the human epithelial cell line U373 (11). We tested soluble MD-2 mutants for the ability to inhibit LPS activation in this assay. Fig. 7B shows that WT-soluble MD-2 at 50 nM efficiently inhibits IL-6 secretion from U373 cells activated with 20 nM soluble CD14 and 100 ng/ml LPS. MD-2 mutants 106–110, 116–120, and 127–131 and MD-1 fail to inhibit LPS-induced activation in this assay. The rest of the mutants inhibited IL-6 secretion to various degrees. Although the direct LPS binding assays showed that most of these mutants bind LPS similarly to the wild type, none was as efficient as the WT in the U373 inhibition assay.

To determine the ability of soluble MD-2 mutants to bind to TLR4, we added the purified proteins to TLR4-transfected EL1 cells, washed off the excess protein after 15 min or overnight incubation, stained them with an anti-His tag antibody, and analyzed them by FACS for MD-2. We also studied binding of soluble MD-2 to TLR4-transfected EL1 cells by co-immunoprecipitation with TLR4 with anti-HA tag to precipitate TLR4 from the cell lysate (Fig. 8B). After 15 min of incubation of the sMD-2 with the cells, sMD-2 wild-type, 125–129, and 127–131 were found to co-immunoprecipitate with TLR4. The difference between these results and the FACS analysis of binding in Fig. 8A suggests that co-immunoprecipitation is a more stringent measure of binding and may less successfully reflect what is bound to TLR4 on the cell surface.

We analyzed LPS responsiveness by adding soluble MD-2 mutants with 100 ng/ml LPS to TLR4-transfected EL1 cells and assaying for NFκB-dependent CD25 expression by FACS (Fig. 9). We found that mutant 38–42 and the unglycosylated mutant (NQ) proteins were analyzed on SDS-polyacrylamide gel under non-reducing conditions and Western blotted with anti-His tag antibody. Molecular mass (in kDa) are indicated to the left of the panels.

DISCUSSION

MD-2 is absolutely required for LPS activation of cells via TLR4 (7). In this study, we have found that the level of MD-2 expressed on the sur-
face of TLR4-transfected cells influences the level of LPS-induced activation. Because MD-2 is present in relatively small amounts as compared with CD14 or LBP, small changes in MD-2 levels are likely to have large effects on cellular activation by LPS. This observation has implications for the analysis of MD-2 function. We showed in this study that unglycosylated MD-2 functions normally as a cell

FIGURE 7. Soluble MD-2 mutant interaction with LPS. An ELISA assay for soluble MD-2 binding to an LPS-coated plate was used. The plate was coated with LPS, and increasing concentrations of MD-2 WT, alanine replacement MD-2 mutants, unglycosylated mutant (NQ), soluble MD-1, or control His tag protein were added to the plate. The protein binding to the plate was detected using biotinylated anti-His antibody. A, effect of soluble MD-2 mutants on CD14-dependent LPS activation of U373 cells. U373 cells were activated with 10 ng/ml LPS and 1 μg/ml CD14. Inhibition of the response with 50 and 125 nM of MD-2, MD-2 mutants, or MD-1 were shown as the fold difference of IL-6 response from cells in the absence of the added MD-2 or MD-1; OD, optical density.
surface receptor when expressed at levels equal to the wild type. Our results differ from some previous studies (23, 24), which showed that lack of glycosylation impaired MD-2 function in assays of LPS activation in transiently transfected cell lines with a luciferase reporter assay. The previous analysis of MD-2 levels relied on immunoprecipitation and Western blotting, which is less quantitative than FACS. The unglycosylated mutant-soluble protein bound LPS as well as wild type, but bound TLR4 less well and inhibited LPS activation less well than the wild type. Therefore, although glycosylation is not important for membrane MD-2 function, it appears to play a role in optimum soluble MD-2 function. Our results are in agreement with the report by Gruber et al. (29) that MD-2 expressed in bacteria was active, but no comparison to the wild type was made in that study.

Analysis of the alanine replacement mutation of membrane MD-2 in stably transfected cell lines showed that the N-terminal 61 amino acids of human MD-2 are not required for cell surface expression. It is interesting that we found certain mutants that were overexpressed as well as
hyper-responsive to LPS, given their level of expression. Further studies of the properties of these mutants in soluble forms are in progress. Nearly all of the rest of MD-2 is involved in surface expression, except for three areas. The areas that affect surface expression include both hydrophilic and hydrophobic residues. They may be involved in direct binding of monomeric MD-2 to TLR4 or affect MD-2 oligomerization that could then interfere with its binding to TLR4. Variable amounts of sMD-2 were found in the culture supernatants of EL1 cells expressing

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**TABLE 1**

Summary of MD-2 mutants characteristics

The function of the same mutant in both membrane and soluble forms derived from the data in Figs. 5–9 were summarized. The “good” activity was defined when the activity of the mutants was greater than or equal to the wild type. The activity “intermediate” was defined when the function was less than the WT. The loss of function was defined as “poor.”

| Mutation | Surface expression | Activation | LPS binding | TLR4 binding | U373 inhibition | Activation |
|----------|-------------------|------------|-------------|--------------|----------------|------------|
| 38–42    | Good              | Good       | Good        | Intermediate | Poor           |
| 46–50    | Good              | Intermediate | Good        | Intermediate | Poor           |
| 75–79    | Poor              | Poor       | Good        | Poor         | Poor           |
| 79–83    | Intermediate      | Poor       | Good        | Poor         | Poor           |
| 106–110  | Poor              | Poor       | Good        | Poor         | Poor           |
| 116–120  | Poor              | Poor       | Poor        | Poor         | Poor           |
| 125–129  | Good              | Poor       | Poor        | Intermediate | Poor           |
| 127–131  | Poor              | Poor       | Poor        | Poor         | Poor           |

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**FIGURE 10.** Structural model of MD-2. The coordinates of Gruber et al. (25) were used together with Protein Explorer software to generate the stereo images shown. Color coding is: brown, mutation to A no effect on function; green, mutation to A causes poor association of MD-2 with TLR4; magenta, mutation to A causes either poor response to LPS or poor binding of LPS; orange, mutation to A causes poor association of MD-2 with TLR4 as well as poor response to LPS or poor binding of LPS. B shows our data together with published data from other laboratories (13, 20–22, 25).
wild-type and mutant sMD-2, indicating that all mutants were synthesized.

Our data agree with previous reports both in mouse and human MD-2 (13, 19, 20, 22) that the basic residues 121–129 are not important for surface expression but are critical for LPS responsiveness. The soluble MD-2 mutant 125–129 bound to TLR4 cells normally but bound poorly to LPS. It is possible that this property extends to membrane MD-2, which suggests that this region in membrane MD-2 is important for LPS binding. However, we have no data on LPS binding to cells. The critical regions on human membrane CD14 for LBP-dependent LPS activation were mapped to the N-terminal region with three acidic residue regions and one hydrophobic region (26). The difference in LPS binding regions of CD14 and MD-2 suggests that membrane MD-2 and CD14 may bind to different parts of LPS.

The mutant in hydrophobic residues 79–83 was expressed as a membrane protein but was LPS-unresponsive. The purified protein of this mutant contained little monomer and failed to bind TLR4-transfected cells. It is possible that oligomers may be less capable of binding to TLR4 than the monomer. This is in agreement with the work of Re and Strominger (18) who show that MD-2 bound to TLR4 is monomeric.

It was described previously that LPS interaction with cell surface TLR4-MD-2 is different from that with soluble MD-2 (30). A body of evidence points to structural differences of MD-2 bound to TLR4 and soluble MD-2. We have produced a number of mAbs that recognize soluble MD-2 but failed to recognize TLR4-MD-2 on the cell surface by FACS analysis (data to be published elsewhere).

A summary of our observations in the figures is in Table 1. All soluble mutants, except for the mutants in the basic residues 125–131, bound LPS quite well but inhibited U373 activation less well than the wild type. U373 cell activation by LPS requires soluble CD14. This result may indicate that these mutants of MD-2 are not able to compete effectively with CD14.

Gruber et al. (25) have recently modeled the three-dimensional structure of MD-2 based on structural analyses of several related proteins. Their model proposes a β sandwich-type fold composed of seven strands arranged in two layers of anti-parallel β-sheets. Additionally, they propose two loops between residues Cys35–Cys105 and Cys37–Cys51, respectively. In Fig. 10A, we have mapped several of our mutants, whose properties are summarized in Table 1, onto the structure proposed (25). In Fig. 10A, the functional consequences of the mutation to A of various regions are indicated by color coding. Brown indicates a region in which mutation to A has no functional consequences; green indicates a region in which mutation to A causes poor binding to TLR4; magenta indicates a region in which mutation to A causes poor LPS binding; and orange indicates a region in which mutation to A causes decreased binding to TLR4 and poor binding of LPS.

Mutant 38–42, in the Cys37–Cys51 loop, is actually expressed at higher levels and more functional than the wild-type structure. Also in this loop, but with quite a different functional result, is mutant 46–50, which is an intermediate LPS cell membrane receptor and a poor soluble receptor. Mutant 46–50 was a good LPS binder, and an intermediate TLR4 binder, which suggests that the defect in this mutant may be in the TLR4 binding site. In the Cys95–C105 loop, the inactive membrane mutant 98–102, containing negatively charged residues, did not express well on the cell surface. Soluble protein of this mutant is being studied. Gruber et al. (25) found that mutants in this loop can be co-immunoprecipitated with TLR4, despite poor or no surface expression. It is possible that this loop may play a role in surface translocation of the MD-2-TLR4 complex. The mutants 125–129 and 127–131 that replaced mostly positively charged residues had poor LPS binding capacity. Both were inactive LPS receptors, both as a membrane protein and a soluble molecule. Mutant 125–129 was a good TLR4 binder, whereas the overlapping mutant 127–131, despite containing a similar amount of monomer, was a poor TLR4 binder. This suggests that this TLR4 binding site is closely associated with the LPS binding site. These mutants, color coded in Fig. 10, begin to suggest the separate, as well as overlapping, surfaces involved in LPS and TLR4 binding, both of which are required for cellular activation. In Fig. 10B, we have added data from several other studies (13, 20–22, 25). The composite data suggest that a large portion of MD-2 is involved in binding to TLR4, whereas a much smaller area is involved in MD-2 binding to LPS. All of the data taken together further delineate likely binding surfaces for TLR4 and LPS.
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