Human MD-2 discrimination of meningococcal lipid A structures and activation of TLR4

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MD-2, a eukaryotic accessory protein, is an essential component for the molecular pattern recognition of bacterial endotoxins. MD-2 interacts with lipid A of endotoxins [lipopolysaccharide (LPS) or lipooligosaccharide (LOS)] to activate human toll-like receptor (TLR) 4. The structure of lipid A influences the subsequent activation of human TLR4 and the immune response, but the basis for the discrimination of lipid A structures is unclear. A recombinant human MD-2 (rMD-2) protein was produced in the Pichia pastoris yeast expression system. Human embryonic kidney (HEK293) cells were transfected with human TLR4 and were stimulated with highly purified LOS (0.56 pmol) from Neisseria meningitidis or LPS from other structurally defined bacterial endotoxins in the presence or absence of human rMD-2. Human rMD-2 restored, in a dose-dependent manner, interleukin (IL)-8 responsiveness to LOS or LPS in TLR4-transfected HEK293 cells. The interaction of endotoxin with human rMD-2 was then assessed by enzyme-linked immunosorbent assays. Wild-type meningococcal LOS (Wt m LOS) bound human rMD-2, and binding was inhibited by an anti-MD-2 antibody to MD-2 dose-dependently (P < 0.005). Wt m LOS or meningococcal KDO2-lipid A had the highest binding affinity for human rMD-2; unglycosylated meningococcal lipid A produced by meningococci with defects in the 3-deoxy-o-manno-2-octulosonic acid (KDO) biosynthesis pathway did not appear to bind human rMD-2 (P < 0.005). The affinity of meningococcal LOS with a penta-acylated lipid A for human rMD-2 was significantly less than that for hexa-acylated LOS (P < 0.05). The hierarchy in the binding affinity of different lipid A structures for human rMD-2 was directly correlated with differences in TLR4 pathway activation and cytokine production by human macrophages.

Key words: endotoxin/lipid A/MD-2/toll-like receptor 4

Introduction

The human toll-like receptor (TLR)-4 is a critical component of the inflammatory response to bacterial endotoxins (Medzhitov et al. 1997; Chow et al. 1999) that requires association with the accessory protein MD-2, an N-glycosylated, 19–27 kDa protein (Ohnishi et al. 2001) expressed in both a soluble and a membrane-bound form (Shimazu et al. 1999). Binding of endotoxin (LPS or LOS) to MD-2 in association with TLR4 leads to dimerization or oligomerization of two or more TLR4 receptors and activation of MyD88-dependent and MyD88-independent cellular pathways (Ozinsky et al. 2000; Visintin et al. 2001).

Endotoxin structures of different Gram-negative bacteria influence the potency of these molecules in TLR4-mediated activation of human macrophages for pro-inflammatory cytokine release. Conformational structural variations in lipid A, degree of lipid A phosphorylation, number and length of acyl chains, net charge of the molecule, and variations in hydrophobicity have been proposed as important for maximal TLR4 activation (Netea et al. 2002). For example, the analysis of enteric lipid A has shown that the pattern of phosphate and phosphoethanolamine (PEA) substitution influences the endotoxic activity of lipid A (Roth et al. 1992; Rietschel et al. 1994; Seydel et al. 2003). The structure of some lipid A may influence the ability of the lipid-binding pocket (LBP) to bind and deliver LPS to CD14 (Cunningham et al. 1996), and thus to initiate the inflammatory cascade. The potency of meningococcal lipid A for activation of human macrophages and dendritic cells depends on the structure of lipid A, particularly the number of acyl chains and lipid A glycosylation (Zughaier et al. 2006).

Although much is known about the MD-2 protein and its hypothesized binding site for endotoxin (Viriyakosol et al. 2001; Re and Strominger 2003; Visintin et al. 2003; Nishitani et al. 2005), the structural requirements of endotoxin for MD-2 association and TLR4 activation are not fully understood. MD-2 has a conserved domain, the MD-2-related lipid (ML) recognition domain consisting of approximately 150 amino acids with N-terminal cysteine residues that are believed to be related to lipid recognition and trafficking (Inohara and Nunez 2002). MD-2 is predicted to interact directly with the lipid A component of endotoxin (Gruber et al. 2004). However, neither the molecular mechanisms by which endotoxin interacts with MD-2 to activate TLR4 nor the structural requirements of endotoxin for this interaction are defined. The ability of MD-2 to confer species-specific recognition of TLR4 activation points to MD-2 as a mediator of sensitivity between different endotoxin structures and TLR4 activation. For example, the response to lipid IVa, a lipid
A analog, is antagonistic to the human TLR4/MD-2, but acts as an agonist in the murine system (Akashi et al. 2001). Akashi et al. demonstrated that this effect is mediated by MD-2. Paclitaxel (PTX), a plant-derived anti-tumor agent, is recognized by mouse TLR4/MD-2, but not by human TLR4/MD-2. As demonstrated by chimeric experiments in which human TLR4 is expressed with murine MD-2, specific recognition of PTX requires the mouse MD-2 protein and is independent of the TLR4 species (Kawasaki et al. 2000; Kawasaki, Akashi S, et al. 2001; Kawasaki, Gomi, et al. 2001). Salmonella LPS is also recognized species specifically, with murine MD-2 as the discriminating member of the ligand–receptor complex (Muroi et al. 2002). These examples of species-specific recognition of TLR4 ligands by MD-2 and the evidence that MD-2 directly binds to lipid A suggest an important structure–function relationship between the endotoxin and the MD-2 protein. Using genetically and structurally defined meningococcal LOS preparations and recombinant human MD-2, we define the importance of lipid A structure and the TLR4/MD-2 receptor complex by studying the interactions between recombinant human MD-2 and hexa- and penta-acylated LOS structures.

Results

Recombinant human MD-2 produced in the Pichia pastoris expression system (human rMD-2)

Human MD-2 with a terminal 6×His tag was produced in a P. pastoris expression system (human rMD-2) and purified through Ni-NTA slurry on a chelating resin column. Expression of the recombinant protein was assessed using Coomassie blue staining of a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel (Figure 1). The expression of rMD-2 was confirmed by anti-MD-2 antibody and anti-6×His antibodies (Figure 1) and by functional studies (Figure 2). Cell lysates from P. pastoris expressing the empty pPICZB vector served as the negative control. Human MD-2 exists in multimeric and monomeric forms (Re and Strominger 2002).

Cell responsiveness to endotoxin provided by human rMD-2

Soluble MD-2 is known to confer responsiveness to bacterial endotoxins in cells expressing only TLR4 (Viriyakosol et al. 2001). To confirm and define the biological activity of P. pastoris-expressed human rMD-2, different concentrations of rMD-2 were added to human embryonic kidney (HEK293) cells alone or to TLR4-transfected HEK293 cells (Medvedev and Vogel 2003) (0.5 × 10⁶ cells/well) (Figure 2) and wild-type meningococcal LOS (Wt m LOS; 0.56 pmol). HEK293 cells alone or HEK293 cells transfected with TLR4 without the addition of human rMD-2 were unresponsive to meningococcal LOS. The addition of human rMD-2 conferred responsiveness to endotoxin by HEK293 cells transfected dose-dependently with TLR4 as measured by interleukin (IL-8) production (Figure 2). The concentration of MD-2 (approximately 1 μg/mL of eluted protein) that maximally stimulated TLR4-transfected HEK293 cells was defined as one unit of rMD-2, and was used as a standard for the remaining experiments. HEK293 cells exposed to human

![Fig. 1. Recombinant human MD-2 produced in the Pichia pastoris expression system (rMD-2). Protein markers are shown in lane 1. Coomassie blue staining demonstrates the purity of the rMD-2 protein after passage through the Ni-NTA column. Western blot of 12% SDS–PAGE-separated and transferred glycosylated human rMD-2 containing an N-terminal 6×His tag recognized by an anti-human MD-2 antibody and the same band recognized by an anti-6×His antibody in both the human rMD-2-expressing yeast cell lysates (lane 3) and the nickel resin-purified sample (lane 4). Pichia containing the empty pPICZB vector alone did not show this band (lane 2).](image-url)
rMD-2 without endotoxin were unresponsive. HEK293 cells co-transfected with both MD-2 and TLR4 were maximally responsive to meningococcal LOS. The addition of human rMD-2 to TLR4/MD-2 co-transfected cells, even at concentrations greater than the amount of human rMD-2 that resulted in maximal responsiveness in cells expressing TLR4 alone, did not significantly enhance IL-8 production. HEK293 cells alone or HEK293 cells transfected with TLR4 did not respond to the addition of rMD-2 alone.

Endotoxin binding to human rMD-2

The activation of TLR4 by endotoxin requires the binding of endotoxin to MD-2 (Hyakushima et al. 2004) presumably through the ability of MD-2 to interact with lipid A (Gruber et al. 2004). Wild-type Neisseria meningitidis LOS (0.3 μg/well, approximately 0.17 nmol based on lipid A content) blocked the recognition of increasing concentrations of human rMD-2 (0–100 units/well) coated on enzyme-linked immunosorbent assay (ELISA) plates by anti-6×His antibody (Figure 3A). Wt m LOS significantly inhibited human rMD-2 recognition by the above antibody at all concentrations of MD-2. In further support of these observations, human rMD-2 but not a bovine serum albumin (BSA) control recognized immobilized Wt m LOS (Figure 3B). Further, increasing concentrations of the anti-human MD-2 antibody (0–2 μg/well) inhibited the interaction of human rMD-2 with meningococcal LOS dose-dependently. An isotype control antibody had no effect on human rMD-2 recognition of LOS and optical density (OD) readings were stable between 0.5 and 0.45 nm (data not shown).

To examine the role of lipid A in endotoxin/MD-2 interactions further, meningococcal LOS with and without polymyxin B and human rMD-2 was assessed (data not shown). Polymyxin B (0–20 μg/mL), a cationic peptide that binds to lipid A (Thomas et al. 2001), interfered dose-dependently with the recognition of meningococcal LOS by
human rMD-2. Similarly, the incubation of polymyxin B (20 μg/mL) with meningococcal endotoxin inhibited LOS binding to all concentrations of human rMD-2 (10, 20, 50, and 100 U/mL).

**Relationship of MD-2 binding, biological activity, and lipid A structure**

The interaction between endotoxin and human rMD-2 and subsequent biological activity were dependent on lipid A structure or confirmation. Compared with the wild-type, fully glycosylated hexa-acylated meningococcal LOS [N. meningitidis serogroup B wild type LOS (NMB)] (Figure 4) and the KDO2–hexa-acylated meningococcal lipid A (gmhB) (Figure 4, dotted box), the corresponding penta-acylated meningococcal LOS (NMB-291) and penta-acylated KDO2–lipid A (gmhB-291) (Figure 4, solid box) in the same molar amounts had significantly lower activity (P < 0.05) in inducing MyD88-dependent tumor necrosis factor α (TNFα) release from THP-1 macrophages (Figure 5). Unglycosylated meningococcal lipid A (kdtA mutant) was inactive.

The interaction of the different lipid A structures with recombinant human rMD-2 correlated with the biological differences. LOS with hexa-acylated lipid A (wild type or KDO2–lipid A) had the greatest binding to human rMD-2, significantly more than LOSs with penta-acylated lipid A. Unglycosylated meningococcal lipid A did not bind to human rMD-2 (Figure 6). These differences were seen in experiments with LOS binding and increasing concentrations of human rMD-2 (0–100 U/mL) (Figure 6A), and when human rMD-2 bound immobilized LOS (Figure 6B).

**Discussion**

The structural diversity of endotoxins of Gram-negative bacteria is remarkable as is the diversity of the biological activity of bacterial endotoxins. The interactions of lipid A structure and the mammalian TLR4 receptor complex are critical determinants of biological activity (e.g. strong agonist, weak agonist, and antagonist) (Seydel et al. 2003). Structural and biological conformation of lipid A (Seydel et al. 2005) is influenced by the number and length of acyl chains (Schromm et al. 2000; Seydel, Oikawa, et al. 2000), phosphorylation of lipid A (Seydel, Schromm, et al. 2000), and linkage of the KDO saccharides (Zughaier et al. 2004). While TLR4 is the cell-signaling molecule responding to endotoxin, the accessory protein MD-2 is essential for TLR4
**Fig. 5.** LOS-mediated differential TNFα induction by human macrophages. Hexa-acylated meningococcal LOS structures (NMB, gmhB), penta-acylated meningococcal LOS (NMB-291, gmhB-291), or meningococcal lipid A was used to stimulate THP-1 cells (10^6 cells/well). TNFα production was measured by ELISA. Unstimulated cells were used as a control. The error bars represent mean ± SD.

**Fig. 6.** Structure of meningococcal LOS and binding to human rMD-2. The binding of hexa-acylated Wt m LOS (NMB) and KDO2–lipid A (gmhB) (closed symbols) compared with the corresponding meningococcal LOS (NMB-291, gmhB-291) (open symbols) for human rMD-2 was assessed by ELISA. (A) Meningococcal LOS (0.17 nmol) was added to increase the concentration of immobilized human rMD-2 (0–100 U/mL). BSA (dash) and meningococcal lipid A (kdtA) (closed circle) were used as controls. (B) Binding of immobilized meningococcal LOS to human rMD-2 in the presence of increasing concentrations of anti-MD-2 antibody (0–2 μg/mL). The error bars represent mean ± SD.
activation (Shimazu et al. 1999; Nagai et al. 2002). Direct binding of lipid A to MD-2 but not to TLR4 has been shown in Viriyakosol et al. (2001). By site-directed mutagenesis, lipid A binds in a region of MD-2 (amino acids 119–132) with homology to the ML recognition domain, a segment distinct from the TLR4-binding site of MD-2 (Mancek et al. 2002; Re and Strominger 2003).

The data presented here provide further evidence for the role of human MD-2 in the discrimination of lipid A structures. Lipid A structure and conformation appear to determine the interaction with rMD-2. The species-specific recognition by MD-2 of other TLR4 ligands also supports this model. PTX is a potent activator of the mouse TLR4 receptor complex through species-specific interactions with mouse MD-2 but not with human MD-2 (Kawasaki, Akashi, et al. 2001; Kawasaki, Gomi, et al. 2001). Potent species-specific activation of the mouse TLR4 receptor complex by *Salmonella* lipid A is described in Muroi et al. (2002), and is dependent upon MD-2 rather than TLR4 or CD14. In addition, rMD-2 produced in *Escherichia coli* expressing LPS with a penta-acylated lipid A (BL21 lpxM−) does not co-purify with the LPS, whereas MD-2 produced in wild-type *E. coli* (Origami B strain) binds to hexa-acylated LPS (Tsuneyoshi et al. 2005). Further, Teghanemt et al. (2005), in a recent study of penta-acylated and chemically deacylated meningococcal endotoxins, demonstrated decreased biological activity of aggregates of underacylated endotoxin and MD-2 compared with hexa-acylated endotoxin. Thus, endotoxin binds MD-2 and not TLR4, and MD-2 discriminates between endotoxin structures.

The hierarchy in the binding affinity of human rMD-2 for lipid A structures was directly correlated with activation of TLR4. Meningococcal KDO₂–lipid A is the minimal structure required for maximal activation of the TLR4/MD-2 receptor complex (Zughaier et al. 2004) and Wt m LOS and meningococcal KDO₂–lipid A had similar affinity for human rMD-2. Unglycosylated meningococcal lipid A did not bind human rMD-2 and was not a TLR4 agonist. Unglycosylated meningococcal lipid A has characteristics of other biologically less active or inactive endotoxins: a dramatically reduced angle of inclination of the diglucosamine backbone of lipid A, increased rigidity of the acyl chains, and formation of unila-mellar aggregates (D.S. Stephens et al., unpublished data). Our data suggest that these structural and conformational changes to lipid A produced by loss of KDO glycosylation result in inefficient binding to human MD-2.

The number of lipid A acyl chains was also critical in determining the affinity for MD-2 and the biological activity of lipid A. Tetra-acylated lipid A is an antagonist of the human TLR4/MD-2 receptor complex and acts as a weak agonist in mice (Lien et al. 2000). Tetra-acylated and penta-acylated LPS structures, such as that of *Porphyromonas gingivalis* or *Rhodobacter sphaeroides*, are antagonists or weak agonists (Golenbock et al. 1991; Coats et al. 2005), and penta-acylated lipid A meningococcal LOS structures are less potent activators of human TLR4 (Zughaier et al. 2004). Seydel, Oikawa, et al. (2000) have demonstrated that hexa-acylated lipid A structures assume a physico-chemical conformation (“cone-shaped”), including acyl chain fluidity, distinct from tetra-acylated lipid A and the penta-acylated lipid A. These data correlate the number of acyl chains with binding affinity for human rMD-2. Hexa-acylated lipid A appears optimal for binding to the hydrophobic-binding pocket of human MD-2 or for facilitating dimerization of TLR4 necessary for signal transduction. The lipid A decaying enzyme (acyloxyacyl hydrolase) exists as part of the human innate immune system and decreases the inflammatory response to LPS by the removal of the secondary acyloxyacyl chains from lipid A (Erwin and Munford 1991; Luchi and Munford 1993). This enzyme would be predicted to decrease the ability of lipid A to interact effectively with human MD-2 and downregulate the inflammatory response.

Acyl chain conformation and fluidity were also critical determinants of MD-2 affinity. KDO₂ glycosylation influences lipid A conformation, acyl chain fluidity, acyl chain inclination angle, biological activity (Akashi et al. 2003; Zughaier et al. 2004), and now MD-2 binding. In addition to the LBP, charged basic residues around the rim of the hydrophobic pocket of MD-2 are involved in the “fit” between MD-2 and the charged lipid A phosphate head groups (Gruber et al. 2004; Ichikawa et al. 2005). Specific variations in lipid A head group phosphorylation or PEA substitution are thus predicted to affect the interaction between MD-2 and LPS. The ability of the cationic peptide, polymyxin B, that binds to these headgroups (Tzeng et al. 2005), to inhibit the binding of human MD-2 to endotoxin supports the model of charged headgroups binding to the outer rim of the MD-2-binding pocket.

While our studies are limited by the inability to quantify exact molar ratios of protein and ligand interactions based on the mixture of monomeric and multimeric species present in recombinant MD-2 preparations, a problem encountered by many in the field, they do provide important information about the ability of MD-2 to discriminate structural differences between endotoxin molecules and understand the model of activation of human TLR4 by endotoxin. Site-directed mutagenesis has been used to summarize important structural components of the human MD-2 molecule (Viriyakosol et al. 2006). Further directions of study include molecular and computer-assisted modeling to understand the role of human MD-2 structure in endotoxin binding.

Our studies confirm the role of human MD-2 in binding to bacterial lipid A and conferring responsiveness to endotoxin on TLR4-expressing cells. Human MD-2 was produced in a eukaryotic expression system and the role of human MD-2 in the discrimination between lipid A in a fashion that parallels biological activity of these molecules was demonstrated. *Pichia* expression may offer an advantage over insect cell expression, allowing more complete glycosylation of the protein. These data emphasize the importance of lipid A structure and conformation and the interaction with MD-2 in determining downstream signaling. Activation of TLR4 is predicted to require aggregation of dimeric or multimeric TLR4 complexes in lipid rafts and conformational changes of the TIR domain of TLR4 to trigger recruitment of cytoplasmic accessory molecules in the signaling pathway. Rather than an “on or off” switch, the flexibility of the MD-2 LBP (suggested by the hinged “clamshell” structure) may permit differences in biological activity by different lipid A structures. The vast range of bacterial lipid A structures and discrimination of these structures by MD-2 may have clinical and biological importance in areas such as host homeostasis, adaptive immunity, allergy, and autoimmunity.
Materials and methods

Reagents

RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, sodium pyruvate, and nonessential amino acids were obtained from Cellgro Mediatech (Herdon, VA). Phorbol myristate acetate (PMA) was purchased from GibcoBRL (Grand Island, NY). IL-8 and TNFα ELISA kits were obtained from R&D systems (Minneapolis, MN), THP-1 and HEK293 cell lines were obtained from ATCC (Manassas, VA). Pichia pastoris strain GS115 and the yeast expression kit (EasySelect Pichia Expression Kit) including pPICZB expression vector were obtained from Invitrogen (Carlsbad, CA). Media used for the yeast culture were as follows: yeast extract–peptone–dextrose (YPD) medium: 1% yeast extract, 2% peptone, and 2% dextrose. Buffered glycerol-complex medium (BMGY): 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.4 mg/L biotin, and 1% glycerol. Buffered methanol-complex medium (BMMY) was prepared with 0.5% methanol instead of glycerol. Human MD-2 and TLR4 plasmids (pEFBOS MD-2 and pEFBOS TLR4) were kind gifts from K. Miyake (University of Tokyo). Nickel-NTA slurry and protein purification columns were obtained from Qiagen (Valencia, CA). Wash buffers and elution buffers were prepared following the manufacturer’s instructions (Qiagen). Mouse anti-human MD-2 antibody was obtained from Sigma (St. Louis, MO).

LOS purification and quantification

Endotoxin from the serogroup B N. meningitidis strain NMB (encapsulated, L2/L4 immunotype) (Rahman et al. 1998) and previously genetically defined mutants of this strain [NMB-penta: penta-acylated NMB (NMB-291)] (Y.L. Tzeng and D.S. Stephens, unpublished data); gmhB:KDO2–lipid A (Shih et al. 2001); gmhB-penta:KDO2–lipid A (gmhB-291)] (Y.L. Tzeng and D.S. Stephens, unpublished data); kdtA: bisphosphorylated lipid A (Tzeng et al. 2002)] (Figure 4) were initially extracted using phenol–water method (Rahman et al. 1998). In addition to genetic definition of the mutants, the precise chemical structure of the oligosaccharide and the expected LOS fatty acyl components of 3-OH C12:0, 3-OH C14:0, and C12:0, as well as the absence of membrane phospholipids were confirmed by mass spectroscopy (R. Carlson, University of Georgia). These preparations were further purified and quantified on the basis of lipid A content as described previously (Zughaier et al. 1998). The correct nucleotide sequence PCR product was confirmed by DNA sequencing (Emory University Biochemical facility), digested with EcoRI and XbaI, and inserted into the pPICZB expression vector that had been cut with the same enzymes. The cloned product (pPICZB–MD-2) in the expression vector flanked by the yeast AOXI gene and a 6 × His selection marker was confirmed by sequencing using the AOXI forward and reverse primers (Invitrogen).

P. pastoris strain GS115 was transformed by electroporation with 10 µg of linearized pPICZB–MD-2 as described previously by Nomura et al. (2003) for the production of recombinant CD14. Briefly, P. pastoris strain GS115 was grown in YPD medium to an OD600 of 1.5. The cell pellet was washed with ice-cold water and re-suspended in 1 mL of ice-cold 1 M sorbitol. Eighty microliters of cells in sorbitol were mixed with 10 µg of linearized DNA in a 0.2 cm cuvette and electroporated with Gene Pulser (Bio-Rad) under a charging voltage of 2500 V, a capacitance of 25 µF, and a resistance of 400 Ω. Zeocin-resistant transformants were incubated at 30 °C and selected on YPDS plates containing 100 µg/mL of Zeocin (Invitrogen). Zeocin-resistant colonies appeared after 4 days and the presence of the MD-2 construct was confirmed by colony PCR. Selected colonies were cultured in 25 mL of BMGY medium and allowed to grow overnight at 30 °C with shaking. Larger scale cultures were produced by inoculating 500 mL of buffered minimal media and histidine medium with 50 mL of overnight culture at 30 °C.

Purification of recombinant human MD-2 from P. pastoris

Using pyrogen-free reagents, cell pellets were washed with breaking buffer [50 mM sodium phosphate, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride] in the presence of protease inhibitor cocktail with broad specificity for the inhibition of serine, cysteine, aspartic, and aminopeptidases, and thromolin-like activities (Sigma). P. pastoris were vortexed with one-fifth of the volume of 0.5 mm glass beads 7 times and incubated on ice. Lysed cells were then centrifuged at 12 000g for 10 min and the supernatant was harvested for purification. Five milliliters of cleared supernatant was mixed with 1 mL of Ni-NTA slurry (Qiagen) and gently shaken at 200 rpm on a rotary shaker at 4 °C for 2 h. Washing and elution of rMD-2 was done following the manufacturer’s instructions. Briefly, the slurry and supernatant were washed with wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 20 mM imidazole) twice and eluted 4 times with elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 250 mM imidazole).

SDS–PAGE and western blotting

The eluted MD-2 proteins were analyzed by 10% SDS–PAGE according to the Laemmli method (Cleveland et al. 1977) and Coomassie blue staining, and protein concentration was estimated using the Bradford assay (Bradford 1976) comparing the elutions of rMD-2 with known concentrations of BSA. One unit of rMD-2 (approximately 1 µg of eluted protein) was defined by the maximal activation of TLR4-transfected HEK293 cells stimulated with LOS and this concentration was used as a standard for all remaining experiments. For western blotting, resolved proteins in the gel were transferred to a polyvinylidene difluoride membrane using a semi-dry electroblot apparatus (Bio-Rad) at 15 V for 30 min in a transfer buffer.
buffer composed of 25 mM Tris–192 mM glycine. The membrane was then blocked with 3% BSA for 1 h at 20°C and incubated with mouse anti-hMD-2 antibody or anti-6×His antibody diluted at 1:1000 with tris-tween buffered saline (TTBS) for 1 h. The membrane was then washed 3 times with TTBS, and incubated with biotinylated goat anti-mouse immunoglobulin G (IgG) at room temperature for 1 h. The membrane was washed 3 times with TTBS, incubated with streptavidin-conjugated alkaline phosphatase for 1 h, and developed with alkaline phosphatase developer (20 mL Tris-buffered saline, 70 µL 5-Bromo-4-chloro-3-indolyl-phosphate p-toluidine-salt, 90 µL nitroblue tetrazolium).

**Cell cultures**

THP-1 human macrophage-like cell lines were grown in RPMI 1640 with l-glutamate supplemented with 10% FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. HEK293 cells were grown in DMEM supplemented as above. Culture flasks were incubated at 37°C with humidity under 5% CO2.

**Cytokine induction**

THP-1 human monocytes were differentiated into macrophage-like cells using PMA at a final concentration of 10 ng/mL and incubated at 37°C for 24 h. Differentiated macrophages were washed with phosphate-buffered saline, counted, adjusted to 10^6 cell/mL, and transferred into a 24-well tissue culture plate (1 mL/well). The cells were stimulated with 0.56 pmol per 10^6 cells of endotoxin overnight at 37°C with 5% CO2. Cell culture supernatants were harvested and saved at −20°C.

**Cytokine and chemokines quantification by ELISA**

Human TNFα and IL-8 Duoset kits (R&D Systems) were used for cytokine quantification according to the manufacturer’s instructions. Maxisorp ELISA plates were obtained from Nalge Nunc International (Rochester, NY). The cells were stimulated with 0.56 pmol of endotoxin for 24 h and cytokine production was measured by ELISA.

**HEK293 transfection with TLR4 and MD-2**

HEK293 cells seeded in 12-well plates (5×10^5 cells/well) were transiently transfected with 0.5 µg/well of the pEFBOS-human TLR4, the pEFBOS-human MD-2, or both plasmids together and SuperFect transfection reagent (Qiagen, Inc.) for 3 h. An empty vector was used as a control. Transfection efficiency was confirmed by responsiveness of co-transfected cells to bacterial endotoxin. Fresh media were applied and the cells were allowed to recover for 12–18 h. The cells were stimulated with 0.56 pmol of endotoxin/5×10^5 cells for 24 h.

**LOS binding to MD-2**

In methods similar to those of Viriyakosol et al. (2001), 96-well Maxisorp microtiter plates were coated overnight with 100 µL of increasing concentrations of MD-2 (0–100 µg/mL) or BSA (10 µg/mL) in 0.2 M sodium acetate buffer, pH 5.0. Plates were then washed 3 times with wash buffer (1 mg/mL BSA, 50 mM HEPES, 0.15 M NaCl, pH 7.4) and blocked for 1 h at 37°C with blocking buffer (10 mg/mL BSA, 50 mM HEPES, 0.15 M NaCl, pH 7.4). Plates were again washed 3 times in wash buffer and then incubated with 100 µL of endotoxin (0.3 µg/mL) in wash buffer for 3 h at 37°C. Plates were again washed 3 times and incubated with 100 µL of mouse anti-human MD-2 antibody (eBioscience, San Diego, CA) diluted to 1:500 in wash buffer for 2 h at room temperature. After three washings, 100 µL of a secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (1:10 000 in wash buffer), was added for 1 h at room temperature. The ELISA was developed with p-nitrophenyl phosphate in 0.5 M diethanolamine buffer containing 0.5 mM MgCl2, pH 9.5, and the OD405 was read. In some experiments, polymyxin B (20 µg/mL) was incubated with the LPS before addition to the microtiter plates.

**MD-2 binding to LOS**

Maxisorp microtiter plates were coated for 3 h with LPS (0.3 µg/mL) or BSA (10 µg/mL) in 0.1 M Na2CO3, 20 mM EDTA, pH 9.6, at 37°C. Plates were washed with water, allowed to air dry, and then blocked for 3 h with blocking buffer. Plates were then washed 3 times with wash buffer and incubated for 3 h at 37°C with 100 µL/well of MD-2 (10 µg/mL). The MD-2 had been pre-incubated on ice without or with increasing concentrations of anti-human MD-2 antibody or an isotype control antibody (0–2 µg/well). Plates were then washed 3 times with wash buffer, incubated with biotinylated anti-6×His antibody (Sigma) (1:200) for 2 h at room temperature, again washed 3 times with wash buffer and incubated with 100 µL/well of 1:200 streptavidin-conjugated horse radish peroxidase (R&D Systems) for 30 min at room temperature. The ELISA was developed with substrate solution (R&D Systems), stopped with 50 µL/well of 2 N H2SO4 and the OD405 was read. In a variation to the above experiment, increasing concentrations of polymyxin B (0–100 µg/mL) were incubated with the MD-2 instead of the anti-human MD-2 antibody.

**Statistical analysis**

Mean values ± standard deviations and P-values (Student t-test) of at least three independent determinations were calculated with Microsoft Excel software.

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**Conflict of interest statement**

None declared.

**Abbreviations**

BMGY, buffered glycerol-complex medium; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEK, human embryonic kidney; IgG, immunoglobulin G; IL-8, interleukin-8; KDO, 3-deoxy-d-manno-2-octulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; ML, MD-2-related lipid; NMB, N. meningitidis serogroup B wild type; OD, optical density; PCR, polymerase

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chain reaction; PEA, phosphoethanolamine; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; PTX, paclitaxel; rMD-2, recombinant human MD-2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TTBS, tris-tween buffered saline; Wt m LOS, wild-type meningococcal LOS; YPD, yeast extract–peptone–dextrose.

References


