

Targeting myeloid differentiation 2 for treatment of sepsis

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1. ABSTRACT

Sepsis continues to be a leading cause of intensive care unit (ICU) death. Gram-negative bacteria are among the most important pathogens of sepsis and their LPS content is regarded to be an important stimulator that elicits the systemic inflammatory reaction. MD-2 is a small secreted glycoprotein that can bind to both the hydrophobic portion of LPS and to the extracellular domain of TLR4. The interaction between MD-2 and LPS bridges the two TLR4 molecules and induces the dimerization of LPS-MD-2-TLR4, which forms the structural basis for biological functions of TLR4/MD-2 complex. Due to its essential role in mediating the interaction between LPS and TLR4, MD-2 has been extensively explored as a therapeutic target for treatment of inflammatory disorders such as sepsis. Eritoran is a synthetic tetraacylated lipid A that binds directly to MD-2 and antagonizes LPS binding to the same site. Although eritoran showed positive results in phase I and phase II clinical trials of severe sepsis, a phase III clinical study for severe sepsis has failed. More effective therapeutic strategies are in need to treat this devastating clinical disorder.

2. INTRODUCTION

Sepsis continues to be a leading cause of intensive care unit (ICU) death. Gram-negative bacteria are among the most important pathogens of sepsis and their lipopolysaccharide (LPS) content is regarded to be an important stimulator of the mammalian innate immune system that elicits the systemic inflammatory reaction. Toll like receptor 4 (TLR4), one of the pattern recognition receptors (PRRs) of the innate immune system, is the most important receptor that mediates the signal transduction of LPS. Recent studies demonstrated that an adaptor protein, myeloid differentiation-2 (MD-2), is requisite for LPS signaling of TLR4. MD-2 is physically associated with TLR4 on the cell surface and confers responsiveness to LPS (Figure 1). MD-2 is thus a link between TLR4 and LPS signaling. Researchers have gained deeper insights into the structure and function of MD-2, and its role in the pathogenesis of sepsis. Due to its essential role in mediating the interaction between LPS and TLR4, MD-2 has been extensively explored as a therapeutic target for treatment of sepsis. In this review, we outline the recent advances in the structure and biology of MD2, interaction

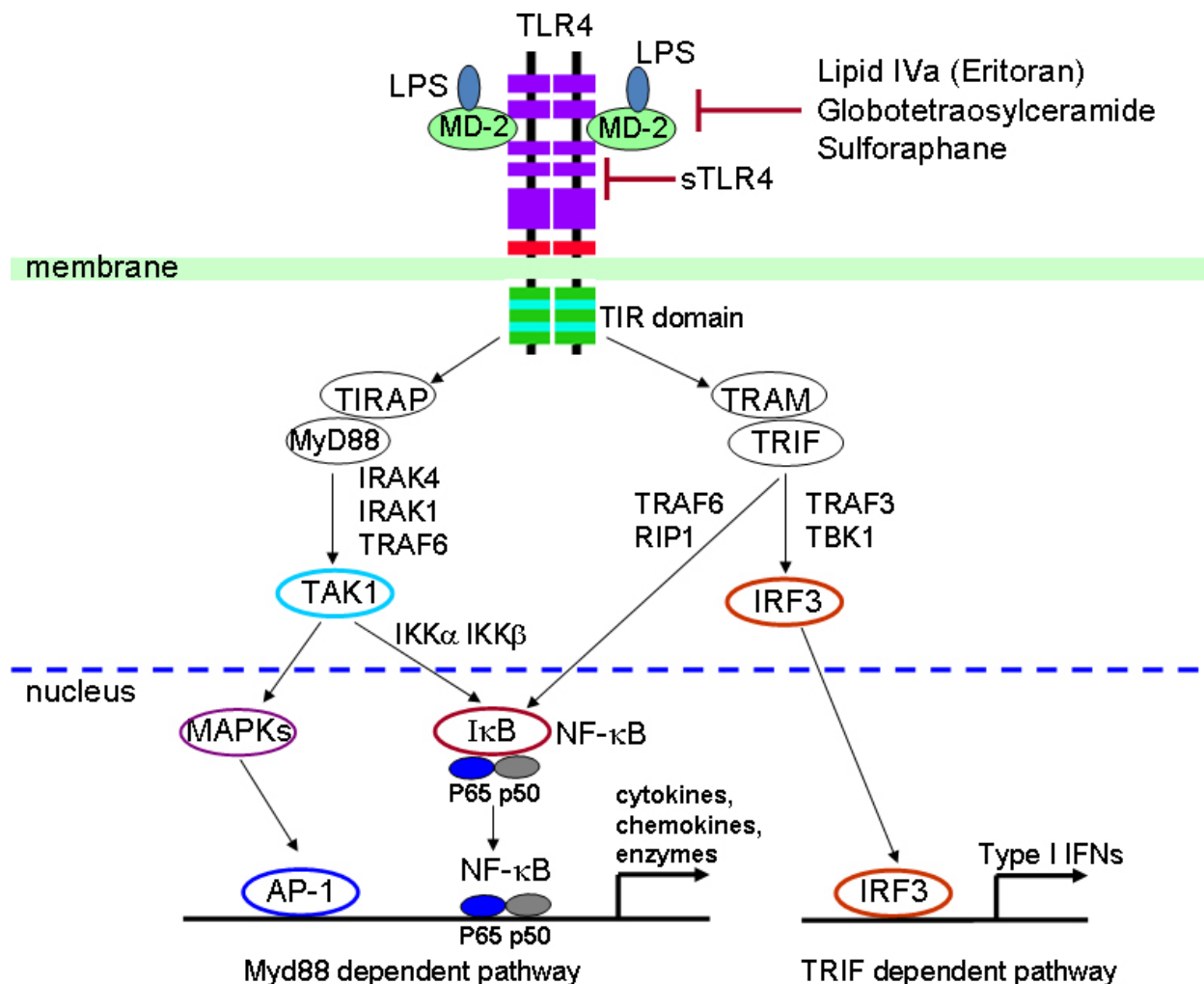


Figure 1. Schematic diagram of MD-2 and TLR4-mediated LPS signaling in the mammalian cells. Bounding of LPS to the TLR4/MD-2 complex leads to the formation of a receptor multimer of (LPS-MD-2-TLR4)₂, which is an activation hallmark of TLR4-mediated inflammatory cascade. TLR4 signaling is diverged into MyD88-dependent and TRIF-dependent pathways. Intracellular adaptor molecules include TIRAP (TIR domain-containing adaptor protein), MyD88, TRAM (TRIF-related adaptor molecule), and TRIF respectively. MyD88-dependent pathway activates the transcription factors of NF-κB and AP-1 for the induction of inflammatory cytokines, chemokines and inflammatory enzymes through multiple signaling molecules such as IRAK4 (IL-1 receptor-associated kinase 4), IRAK1, TRAF6 (TNF receptor-activated factor 6), TAK1 (transforming growth factor-β-activated kinase 1), IKKs (inhibitory κB kinase), and MAPKs (mitogen-activated protein kinases). TRIF-dependent pathway activates the transcription factors of NF-κB and IRF3 for the up-regulation of inflammatory genes, IFN-β and IFN-inducible genes through TRAF6, RIP1 (receptor-interacting protein1), TRAF3 and TBK1 (TRAF family member-associated NF-κB activator-binding kinase 1).

between LPS and TLR4/MD-2 complex, and highlight the MD-2-directed LPS antagonists, including natural and synthetic chemicals, to treat inflammatory disorders such as sepsis.

3. SEPSIS

Sepsis is a potentially fatal whole-body inflammation caused by the immune system's response to a serious infection, most commonly bacteria, but also fungi, viruses, and parasites in the blood, urinary tract, lungs, skin, or other tissues (1-3). Common symptoms of sepsis

include those related to a specific infection, but usually accompanied by high fevers, hot, flushed skin, elevated heart rate, hyperventilation, altered mental status, swelling, and low blood pressure (1).

Sepsis causes millions of deaths globally each year (4). It represents the second-leading cause of death in non-coronary ICU patients, and the tenth-most-common cause of death overall. Children under 12 months and elderly have the highest incidence of severe sepsis. Severe sepsis contributes to more than 200,000 deaths per year (5). Clinical trials in sepsis research have gone from failure to

failure (1). Novel treatment strategies to combat this epidemic are in need.

Pathophysiology of sepsis varies as it moves from an initiating early hyperinflammatory phase to a late hypoinflammatory and immunosuppressive phase (6). The early phase of sepsis is characterized by a systemic inflammatory response syndrome (SIRS), which is the predominantly cytokine-mediated, proinflammatory host response on invading pathogens and is considered the hallmark sign of sepsis (7). Molecular components of this response can be divided into cytokines, plasma cascades and acute phase proteins whereas the cellular components are the leukocytes and the endothelium. The compensatory anti-inflammatory response syndrome (CARS) is characterized by the induction of several anti-inflammatory mechanisms (8). SIRS is associated with early potentially cytokine storm inflicted deaths. As sepsis progresses, CARS becomes predominant, and it is during this later phase that secondary infections and viral reactivation occur associated with later deaths (9).

PRRs are the central components of the innate immune system that recognize invading pathogens and initiate the immune response (10, 11). PRRs can be categorized on the basis of their cellular localization. TLRs can be expressed on the cell surface (e.g. TLR1, TLR2, and TLR4) or intracellularly (e.g. TLR3, TLR7 and TLR8) where they guard the extracellular and endosomal compartments, respectively. TLR4 is a major receptor for the Gram-negative bacterial virulence factor LPS or endotoxin (11). Administration of very low dose of LPS resulted in signs and symptoms of clinical sepsis (12). Elevated LPS levels have been associated with poor outcome in septic patients (12-14). Recently, an adaptor protein, MD-2 was found to be required for LPS signaling of TLR4 (15, 16). MD-2 is physically associated with TLR4 on the cell surface and confers responsiveness to LPS.

4. MD-2

MD-2 was first described by Shimazu *et al.* (15). They did a query of the human transcript database by using the amino acid sequence of myeloid differentiation-1 (MD-1), which is a molecule involved in the regulation of LPS responses (17, 18). A highly homologous protein was identified and was named MD-2. The cDNA of MD-2 encodes for a 160 amino acids glycoprotein with a molecular weight of about 25–30 kDa (15). The first 16 hydrophobic amino acids correspond to the signal peptide, which directs MD-2 to the secretory pathway, thus MD-2 can be directly secreted into the cell outer membrane as a soluble protein. Human MD-2 is mainly expressed on B cell, dendritic cell and macrophage cell lineages. Mouse MD-2 was similar to a human homologue in the size of mRNA. The transcript of mouse MD-2 was ubiquitously distributed in the brain, thymus, liver, spleen, heart and kidney, among which spleen and kidney show pronounced expression (15). MD-2 is mainly synthesized in the endoplasmic reticulum/Golgi apparatus, where it physically associates with TLR4 and then forms the TLR4/MD-2

complex on the cell surface (19). The interaction of LPS-bound MD-2 with TLR4 is required for TLR4 mediated inflammatory responses (16, 20).

4.1. Structure of MD-2

Kim *et al.* analyzed structures of several molecules associated with MD-2, and proposed the crystal structure of MD-2 (21). MD-2 adopts a cup fold with two antiparallel β sheets that contain three and six β strands, respectively. In the cup fold, the two sheets become separated on one side of the protein and the hydrophobic internal core is open for ligand binding. The conserved disulfide bridge that connects the two β sheets in the immunoglobulin fold is absent from the β cup so that the two sheets can be separated, further permitting the formation of a large internal pocket. The MD-2 pocket is narrow and deep with a total surface area of 1000 Å². The overall shape and chemical behavior of the pocket appear to be suitable for binding large flat molecules such as LPS that contain multiple hydrophobic acyl chains and negatively charged phosphate groups. The generous internal surface of the pocket is completely lined with hydrophobic residues, and the opening region of the pocket contains positively charged residues that facilitate the binding of LPS. The disulfide bridge connecting Cys25 and Cys51, together with hydrogen bonds between Tyr34, Tyr36, and the backbone atoms of the bC strand, close the bottom of the pocket and stabilize the cup-like structure. The remaining two disulfide bridges buttress structures of the loops connecting the strands.

4.2. Interaction between TLR4 and MD-2

The surface of TLR4 that interacts with MD-2 has a long and narrow shape with dimensions 40 x 20 Å (21). It can be divided into two chemically and evolutionarily distinct areas, the A and B patches. The A patch is negatively charged and evolutionarily conserved, whereas the B patch is positively charged and located in a less conserved area, although the residues directly interacting with MD-2 are strictly conserved. The A and B patches of TLR4 are composed of the residues in the concave surface derived from the “LxLxxN” part of the LRR modules in the N-terminal domain and of the central domain, respectively. The interaction between TLR4 and MD-2 is mediated by an extensive net network of charge-enhanced hydrogen bonds. The negatively charged residues in the A patch interact with the positively charged Arg68 and Lys109 residues in MD-2. The positively charged B patch interacts with negatively charged residues in the loop between the Bf strand and the a helix of MD-2. The parts of MD-2 interacting with the A and B patches of TLR4 are named the A' and B' patches, respectively. The large interaction area and the complementary charge distribution over the interaction surface are consistent with the nanomolar binding affinity of TLR4-MD-2 (21, 22). Notably, Nishtani *et al.* showed that mutations of TLR4 residues Cys29 and Cys40, and a synthetic peptide including the residues from Glu24 to Lys47 of TLR4, block TLR4 binding to MD-2 (23, 24). This region is not only important for the structural integrity of the LRRNT module but also contains Asp41, a crucial component of the A patch of TLR4. Mutations of MD-2 residues Cys95,

Cys105, Asp99, Asp100, and Asp101 in the B0 patch have also been shown to disrupt TLR4 binding (25).

4.3. Interaction between LPS, MD-2 and TLR4

The crystal structure of the TLR4-MD-2-LPS complex has recently been resolved (26, 27). LPS is bound to the hydrophobic pocket in MD-2. LPS binding has a minimal effect on the global structures of TLR4 and MD-2. The structural changes in MD-2 are limited to the flexible loops near the opening of its LPS-binding pocket, and there are only negligible changes in the radius of the TLR4 horseshoe-like structure and in the conformations of the side chains directly interacting with MD-2 and LPS. LPS binding induces the formation of a symmetrical complex of two TLR4-MD-2 heterodimers. The lipid chains and the phosphate groups of lipid A play direct roles in TLR4-MD-2 dimerization. The six lipid chains of the lipid A part of LPS are inserted into the hydrophobic pocket of MD-2. All the lipid chains except the R2 chain are completely buried inside the pocket, whereas the R2 chain is partially exposed and interacts directly with TLR4. A small hydrophobic patch formed by F440, F463, and L444 in the C-terminal domain of TLR4 binds to this exposed lipid chain and hydrophobic MD-2 residues. Surrounding hydrophilic interactions between TLR4 and MD-2 residues support this core hydrophobic part of the dimerization interface. Mutations of the interface residues F440 and F463 disrupt TLR4 signaling and dimerization (28). Gangloff *et al.* also established a modeling system and proposed that acyl chains of lipid A are buried within the hydrophobic core of MD-2 and this induces cross-linking of the two TLR4-MD-2 complexes, an event that is required to trigger signal transduction. Moreover, the mechanism of signal transduction is likely to involve concerted protein conformational changes (29). Taken together, the major driving force for TLR4-MD-2 dimerization is via the bridging of the two TLR4 molecules by the MD-2-LPS complex. Recognition of LPS by the TLR4-MD-2 heterodimer is facilitated *in vivo* by two accessory proteins, CD14 and LPS binding protein (30).

4.4. Soluble MD-2 and MD-2B

Indirect approaches have been employed to demonstrate the presence of soluble MD-2 in serum. Secreted MD-2 is found to be a large polymeric protein that efficiently confers LPS sensitivity to TLR4 (19). Depletion of endogenous soluble MD-2 from human serum with an immobilized TLR4 fusion protein abrogated TLR4-mediated LPS responses. The concentration of MD-2 was approximately 50 nM in healthy donors (31).

The mRNA for an alternatively spliced form of MD-2, MD-2B, has also been identified. The mRNA of MD-2B lacks the first 54 bases of exon 3. When overexpressed with MD-2, MD-2B competitively suppressed NF- κ B activity induced by LPS. MD-2B bound to TLR4 as efficiently as MD-2 and inhibited TLR4 from being expressed on the cell surface. MD-2B may compete with MD-2 for binding to TLR4 and decrease the number of TLR4-MD-2 complexes on the cell surface, resulting in the inhibition of LPS signaling (32). In a rat acute cholangitis model, MD-2B was shown to play a negative

regulatory role in the process of TLR4/MD-2-mediated inflammatory response to liver injury (33).

4.5. The role of MD-2 in the distribution of TLR4

Formation of the TLR4/MD-2 complex intracellularly is essential for the extracellular distribution of TLR4. For example, in MD-2 (-/-) embryonic fibroblasts, TLR4 was not able to reach the plasma membrane and predominantly resided in the Golgi apparatus, whereas TLR4 was distributed at the leading edge surface of cells in wild-type embryonic fibroblasts (16). There are two forms of TLR4 with different molecular masses (approximately 110 and 130 kDa) when TLR4 is expressed together with MD-2 (34). Only the 130-kDa form of TLR4 is expressed on the cell surface. N-glycosidase treatment of cells induced the convergence of two forms of TLR4 into a single band whose size was smaller than the 110-kDa form of TLR4. Mutation of TLR4 at Asn (526) or Asn (575) resulted in the disappearance of the 130-kDa form and prevented TLR4 from being expressed on the cell surface without affecting the ability of TLR4 to associate with MD-2. These results indicate that TLR4 is able to undergo multiple glycosylations without MD-2 but that the specific glycosylation essential for cell surface expression requires the presence of MD-2 (34). Interestingly, the 110-kDa form of TLR4 was found to exhibit the protein with high mannose type N-glycans and the 130-kDa protein with complex type N-glycans (35). Moreover, only the 130-kDa TLR4 with complex type N-glycans was expressed on the cell surface. The cells transfected with a mutant TLR4 (C88A) alone expressed only the 110-kDa TLR4 with a high mannose type N-glycan, which did not appear on the cell surface. However, TLR4 (C88A) acquired complex type N-glycans was expressed on the cell surface when MD-2 was co-transfected. Thus, MD-2 is critical for cell surface expression of TLR4 (35). By using monoclonal antibodies (mAbs) that are specific for complexed TLR4/MD-2 but unreactive with free TLR4 and MD-2, Tsukamoto *et al.* showed that cell surface TLR4 and extrinsically secreted MD-2 are capable of forming the functional complex extracellularly. The mAbs bound the extracellularly formed TLR4/MD-2 complex indistinguishably from the intracellularly formed complex in titration studies. The results indicate an additional or alternative pathway for the complex formation (36). It is notable that MD-2 is not the only factor that can influence the distribution of TLR4. For example, protein associated with TLR4 (PRAT4A), was reported to regulate cell surface expression of TLR4. PRAT4A is associated with the immature form of TLR4 but not with MD-2 or TLR2. PRAT4A knockdown abolished LPS responsiveness in HEK293 cells expressing TLR4/MD-2 probably through reducing cell surface expression of TLR4. PRAT4A knockdown down-regulated cell surface TLR4/MD-2 on dendritic cells. These results demonstrate an alternative mechanism regulating TLR4/MD-2 expression on the cell surface (37).

4.6. The role of MD-2 and TLR4 in regulation of LPS signaling

MD-2 plays a pivotal role in LPS recognition and TLR4 is mainly involved in the transmission of

inflammatory signaling across the plasma membrane (38, 39). The intracellular Toll/interleukin (IL)-1 receptor (TIR) domain of TLR4 within the activated (LPS-MD-2-TLR4)₂ multimer recruits an adaptor molecule TIR domain-containing adaptor protein (TIRAP), which functions to recruit MyD88 to TLR4. MyD88 in turn recruits IL-1R-associated kinase (IRAK) 4, IRAK1, and/or IRAK2 to the TLR/MyD88 signaling complex. IRAK1 or IRAK2 is phosphorylated by IRAK-4, leaves the receptor complex, and then associates with tumor necrosis factor- α receptor-associated factor (TRAF) 6. Binding of TRAF6 to IRAK1 or IRAK2 leads to the activation of transforming-growth-factor- β -activated kinase 1 (TAK1) that ultimately results in activation of signaling cascades leading to the activation of mitogen-associated protein kinases (MAPKs), and NF- κ B and subsequent expression of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α), IL-1 β and IL-6 (40-42). On the other hand, activated (LPS-MD-2-TLR4)₂ multimer is also able to recruit another adaptor molecule TRIF-related adaptor molecule (TRAM), which functions to recruit TIR domain-containing adaptor inducing IFN β (TRIF) to TLR4. TRIF is required for the TLR4-induced MyD88-independent pathway (43, 44). TRIF recruits receptor-interacting protein (RIP) 1 and two TRAF family members, TRAF6 and TRAF3. RIP1 and TRAF6 are required for the TRIF-dependent NF- κ B activation and pro-inflammatory cytokine production, whereas TRAF3 recruits TANK-binding kinase (TBK) 1 and IKK ϵ to activate IRF3, resulting in the production of IFN- β and chemokines (45-47).

5. MD-2 AND SEPSIS

The physiological and pathological importance of MD-2 in response to LPS has been investigated using MD-2 gene-targeted mice. MD-2-deficient mice do not respond to *E. coli* LPS and thus are resistant to the septic shock (16). MD-2-null mice are also insensitive to pulmonary inflammation following nasal instillation of LPS from *Neisseria meningitidis* (48). Pugin *et al.* reported the existence of soluble MD-2 in body fluids of septic patients, but not in healthy individuals (49). They found that plasma from patients with severe sepsis and septic shock but not normal plasma supports LPS activation of epithelial cells expressing TLR4. Recombinant soluble MD-2 complemented normal plasma and allowed LPS activation of epithelial cells to levels measured with "septic" plasma, whereas soluble MD-2-depleted plasma lost its effects. The same "MD-2 activity" was found in urine from a patient with septic shock and in lung edema fluids from patients with adult respiratory distress syndrome (ARDS). An anti-MD-2 monoclonal antibody blocked LPS activation of TLR4-expressing cells only in the presence of septic plasma or septic urine (49). These results suggest that septic plasma containing soluble MD-2 leaking into the extravascular space supports LPS activation of TLR4-expressing epithelial cells and soluble MD-2 is an important mediator of organ inflammation during sepsis.

6. TARGETING MD-2

Due to its essential adaptive role in mediating the interaction between LPS and TLR4, MD-2 represents an

attractive therapeutic target of inflammatory disorders such as sepsis.

The current efforts in exploration of MD-2-directed LPS antagonists to treat inflammation and sepsis, including those of natural and synthetic chemicals, were highlighted.

6.1. Lipid IVa

Tetraacylated lipid IVa, a synthetic lipid A precursor with four fatty acid chains, acts as a weak agonist to mouse TLR4/MD-2, but as an antagonist to human TLR4/MD-2 or chimeric mouse TLR4/human MD-2 (50-52). Meng *et al.* used site-directed mutagenesis to identify the MD-2 residues that determine the differential roles of lipid IVa (53). A single mutation of murine MD-2 at the hydrophobic pocket entrance, E122K, substantially reduced the response to lipid IVa. Combining the murine MD-2 E122K with the murine TLR4 K367E/S386K/R434Q mutations completely abolished the response to lipid IVa, effectively converting the murine cellular response to a human-like response. In human cells, however, simultaneous mutations of K122E, K125L, Y41F, and R69G on human MD-2 were required to promote a response to lipid IVa. Combining the human MD-2 quadruple mutations with the human TLR4 E369K/Q436R mutations completely converted the human MD-2/human TLR4 receptor to a murine-like receptor. Because MD-2 residues 122 and 125 reside at the dimerization interface near the pocket entrance, surface charge differences here directly affect receptor dimerization. In comparison, residues 42 and 69 reside at the TLR4/MD-2 interaction surface opposite the dimerization interface. Surface charge differences there likely affect the binding angle and/or rigidity between MD-2 and TLR4, exerting an indirect influence on receptor dimerization and activation (53). Therefore, surface charge differences at the two TLR4/MD-2 interfaces determine the agonistic or antagonistic effect of lipid IVa.

Visintin *et al.* showed that lipid IVa is similar to lipid A in saturable binding to mouse TLR4-human MD-2 in a dose-dependent manner. However, Lipid IVa did not induce TLR4 oligomerization, but inhibited lipid A-dependent oligomerization of mouse TLR4-human MD-2. Moreover, binding study further revealed that the antagonistic activity of lipid IVa correlates with augmented maximal binding to mouse TLR4-human MD-2, which was approximately 2-fold higher than lipid A (31). The results suggested that MD-2 has an important role in a link between ligand interaction and TLR4 oligomerization.

Ohno *et al.* further provided structural evidence that LPS and lipid IVa show agonistic or antagonistic activities in a species-specific manner (54). They resolved the crystal structures of mouse TLR4/MD-2/LPS and TLR4/MD-2/lipid IVa complexes at 2.5 and 2.7 Å resolutions, respectively. Mouse TLR4/MD-2/LPS exhibited an agonistic "m"-shaped 2:2:2 complex similar to the human TLR4/MD-2/LPS complex. Mouse TLR4/MD-2/lipid IVa complex also showed an agonistic structural feature, exhibiting architecture similar to the 2:2:2

complex. Remarkably, lipid IVa in the mouse TLR4/MD-2 complex occupied nearly the same space as LPS, although lipid IVa lacked the two acyl chains. Human MD-2 binds lipid IVa in an antagonistic manner completely differently from the way mouse MD-2 does (54). These results deepened understanding of the ligand binding and dimerization mechanism by the structurally diverse LPS variants.

Recently, Artner *et al.* reported on design, synthesis, and biological activities of a series of conformationally confined Lipid A mimetics based on β , α -trehalose-type scaffold (55). Replacement of the flexible three-bond β (1 \rightarrow 6) linkage in diglucosamine backbone of Lipid A by a two-bond β , α (1 \leftrightarrow 1) glycosidic linkage afforded novel potent TLR4 antagonists. Synthetic tetraacylated bisphosphorylated Lipid A mimetics based on a β -GlcN (1 \leftrightarrow 1) α -GlcN scaffold selectively block the LPS binding site on both human and mouse MD-2 and completely abolish LPS-induced pro-inflammatory signaling, thereby serving as antisepsis drug candidates (55). The results indicate that besides the chemical structure, the three-dimensional arrangement of the diglucosamine backbone of MD-2-bound Lipid A also determines endotoxic effects on TLR4.

6.2. Eritoran as a MD-2 antagonist

Eritoran is a synthetic tetraacylated lipid A that was designed from the nonpathogenic LPS of *R. sphaeroides*, and the clinical studies about it for the treatment of septic death have been completed (39, 56, 57). Eritoran competes with endotoxic LPS in binding to the same site of MD-2, but it does not directly interact with TLR4 (56). Eritoran impairs the formation of the LPS-activated receptor multimer and thus sequentially inhibits signal transmission across the plasma membrane (56). Eritoran has been highlighted as a drug candidate for treating endotoxemia in the phase I clinical studies. Single eritoran doses of 50–250 μ g ameliorate LPS-injected endotoxemia in healthy human volunteers with statistically significant reductions in the elevated body temperature, heart rate, C-reactive protein levels, white blood cell counts, and cytokine (TNF- α and IL-6) levels over the placebo group (57). A phase II trial of eritoran has been conducted in patients with severe sepsis. Eritoran at a high dose (105 mg) reduces the mortality rate to 33%, as compared with the 56% death in the placebo group of patients who are at a high risk of mortality according to the acute physiology and chronic health evaluation II (APACHE II) score, whereas it produces a trend towards increased mortality in the low risk population (58). More recently, a randomized, double-blind, placebo-controlled, multinational phase III trial of eritoran in 197 intensive care units was performed. Patients with severe sepsis ($n = 1961$) were randomized and treated within 12 hours of onset of first organ dysfunction in a 2:1 ratio with a 6-day course of either eritoran tetrasodium (105 mg total) or placebo, with $n = 1304$ and $n = 657$ patients, respectively. In the modified intent-to-treat analysis (randomized patients who received at least 1 dose) there was no significant difference in the primary end point of 28-day all-cause mortality with 28.1% (366/1304) in the eritoran group vs 26.9% (177/657) in the

placebo group ($P = .59$; hazard ratio, 1.05; 95% CI, 0.88–1.26; difference in mortality rate, -1.1; 95% CI, -5.3 to 3.1) or in the key secondary end point of 1-year all-cause mortality with 44.1% (290/657) in the eritoran group vs 43.3% (565/1304) in the placebo group, Kaplan-Meier analysis of time to death by 1 year, $P = .79$ (hazard ratio, 0.98; 0.85–1.13). No significant differences were observed in any of the prespecified subgroups. In conclusion, the study demonstrated that the use of eritoran did not result in reduced 28-day mortality among patients with severe sepsis as compared with placebo, which is very disappointing (59).

6.3. RP105

RP105 is a TLR-4 homologue. RP105 lacks a signaling domain, having the apparent structure of a TLR inhibitor. RP105 is mainly expressed on B cells, dendritic cells and macrophages. Surface expression of RP105 is dependent upon co-expression of MD-1. RP105/MD-1 interacts directly with TLR-4/MD-2, inhibiting the ability of this signaling complex to bind LPS (60). RP105 is as an endogenous inhibitor of TLR-4 and plays a negative regulatory role in the activation of mouse peritoneal macrophages by *Staphylococcus aureus* (60, 61). Using recombinant RP105 may represent a promising strategy to target TLR-4/MD-2 complex.

6.4. Globotetraosylceramide

A globo-series glycosphingolipid, globotetraosylceramide (Gb4) was found to attenuate the toxicity of lipopolysaccharides (LPSs) by binding to TLR4-MD-2. α 1, 4-galactosyltransferase (A4galt)-deficient mice lacking globo-series glycosphingolipids showed higher sensitivity to LPS than wild-type mice (62). Cultured endothelial cells lacking A4galt showed higher expression of LPS-inducible genes upon LPS treatment. In turn, introduction of A4galt cDNA resulted in the neo expression of Gb4, leading to the reduced expression of LPS-inducible genes. Exogenous Gb4 induced similar effects. Mechanistically, specific binding of Gb4 to the LPS receptor TLR4-MD-2 was demonstrated by coprecipitation of Gb4 with recombinant MD-2 and by native PAGE. Gb4 was colocalized with TLR4/MD-2 in lipid rafts after LPS stimulation. More importantly, administration of Gb4 significantly protected mice from LPS-elicited mortality (62). Therefore, Gb4 is an endogenous ligand for TLR4-MD-2 and is capable of attenuating LPS toxicity, indicating the possibility for its therapeutic application in endotoxin shock and sepsis.

6.5. Sulforaphane

Koo *et al.* showed that sulforaphane (SFN) interfered with the binding of LPS to MD2 as determined by *in vitro* binding assay and co-immunoprecipitation of MD2 and LPS in a cell system (63). The inhibitory effect of SFN on the interaction of LPS and MD2 was reversed by thiol supplementation with N-acetyl-L-cysteine or dithiothreitol, which indicated that the inhibitory effect of SFN was dependent on its thiol-modifying activity. Moreover, SFN preferentially formed adducts with Cys133 in the hydrophobic pocket of MD2. Molecular modeling showed that SFN bound to Cys133 blocks the engagement

of LPS and lipid IVa to hydrophobic pocket of MD2 (63). The data demonstrate that SFN interrupts LPS engagement to TLR4/MD2 complex by direct binding to Cys133 in MD2, thus SFN might be a novel target for the regulation of TLR4-mediated inflammatory and immune responses.

6.6. Soluble TLR4 as a therapeutic approach to target MD-2

Soluble decoy receptors provide important negative regulatory mechanisms for cytokines and chemokines, and their interaction with their membrane-bound receptor. In mice, Iwami *et al.* cloned a splice TLR4 mRNA that encodes a soluble 20-kDa protein (64). When expressed in Chinese ovary (CHO)-K1 cells, this protein is secreted in the culture medium. It inhibits LPS-mediated TNF α secretion and NF- κ B activation in a mouse macrophage cell line. Interestingly, LPS stimulation increased the sTLR4 mRNA expression, suggesting a negative feedback to inhibit excessive cytokine production (64). Thus targeting soluble TLR4 might be of potential interest in treating patients with sepsis.

Hyakushima *et al.* generated soluble forms of rTLR4 (sTLR4) consisting of Glu (24)-Lys (631) but lacking the putative intracellular and transmembrane domains (22). BIAcore assay demonstrated the direct binding of sTLR4 to MD-2 with a dissociation constant of $K(D) = 6.29 \times 10^{-8}$ M. Although LPS-conjugated beads did not precipitate sTLR4, LPS beads coprecipitated sTLR4 and MD-2 when both proteins were coincubated. Importantly, the addition of sTLR4 to the medium containing the MD-2 protein significantly attenuated LPS-induced NF- κ B activation and IL-8 secretion in wild-type TLR4-expressing cells. The use of sTLR4 may effectively dampen endotoxin-induced inflammation (22). Moreover, Mitsuzawa *et al.* showed that addition of sTLR4 plus sMD-2 was significantly effective in inhibiting LPS-elicited IL-8 release from U937 cells and NF- κ B activation in the cells transfected with TLR4 and MD-2 when compared with a single treatment with sTLR4 or sMD-2 (65). Mechanistic study showed that the extracellular TLR4 domain does not directly bind lipid A by itself. The amounts of lipid A coprecipitated with sMD-2 significantly increased when coincubated with sTLR4, and sTLR4 increased the affinity of lipid A for the binding to sMD-2. Addition of sTLR4 plus sMD-2 inhibited the binding of Alexa-conjugated LPS to the cells expressing TLR4 and MD-2. Murine lungs that had received sTLR4 plus sMD-2 with LPS did not show any findings indicative of interstitial edema, neutrophil flux, and hemorrhage. Administration of sTLR4 plus sMD-2, but not sTLR4 or sMD-2 alone, significantly decreased neutrophil infiltration and TNF- α levels in bronchoalveolar lavage fluids from LPS-treated mice. Thus, combination of sTLR4 and sMD-2 represents an attractive approach against endotoxin-induced pulmonary inflammation (65).

7. CONCLUDING REMARKS

MD-2 plays an essential role in physical association with TLR4 on the cell surface and conferring responsiveness to LPS, thus involves in the transmission of

inflammatory signaling across the plasma membrane. Binding of LPS to the hydrophobic pocket of MD-2 to form the LPS-MD-2 complex bridges the two TLR4 molecules and induces the dimerization of LPS-MD-2-TLR4, which is the structural basis for biological functions of TLR4/MD-2 complex. Among various medications targeting MD-2, a synthetic tetraacylated lipid A, eritoran, is the most advanced MD-2-directed LPS antagonist. Although eritoran displayed positive results in phase I trial and phase II trial of severe sepsis, the phase III study of eritoran for severe sepsis has been disappointing. There was no statistically significant difference in mortality rate between the eritoran treatment and the placebo group. We are waiting for the proof of MD-2 as a therapeutic target for sepsis and other inflammatory diseases such as myocardial or renal ischemia-reperfusion. Positive outcomes in these areas will provide novel insights into the drug discovery to treat these devastating diseases.

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Abbreviations: APACHE II: acute physiology and chronic health evaluation II; ARDS: adult respiratory distress syndrome; CARS: compensatory anti-inflammatory response syndrome; IRAK: IL-1R-associated kinase; ICU: intensive care unit; LPS: lipopolysaccharide; MAPKs: mitogen-associated protein kinases; mAbs: monoclonal antibodies; MD-1: myeloid differentiation-1; MD-2: myeloid differentiation-2; PRR: pattern recognition receptors; PRAT4A: protein associated with TLR4; RIP: receptor-interacting protein; SFN: sulforaphane; TAK1: transforming-growth-factor- β -activated kinase 1; TBK: TANK-binding kinase; TIRAP: TIR domain-containing adaptor protein; TRIF: TIR domain-containing adaptor inducing IFN β ; TIR: Toll/interleukin (IL)-1 receptor; TLR: Toll like receptor; TRAM: TRIF-related adaptor molecule; TNF- α : tumor necrosis factor α ; TRAF: tumor necrosis factor- α receptor-associated factor; SIRS: systemic inflammatory response syndrome

Key Words: Eritoran, lipopolysaccharide, myeloid differentiation 2, sepsis, Toll like receptor 4, Review

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