HHS Public Access

Author manuscript

Adv Carbohydr Chem Biochem. Author manuscript; available in PMC 2015 June 14.

Published in final edited form as:

Adv Carbohydr Chem Biochem. 2014 ; 71: 339–389. doi:10.1016/B978-0-12-800128-8.00005-4.

MODULATING LPS SIGNAL TRANSDUCTION AT THE LPS RECEPTOR COMPLEX WITH SYNTHETIC LIPID A ANALOGUES

Aileen F. B. White[a,1] and **Alexei V. Demchenko**[b,1]

[a]Dextra Laboratories Ltd., Science and Technology Centre, Earley Gate, Reading, United Kingdom

[b]Department of Chemistry and Biochemistry, University of Missouri–St. Louis, One University Boulevard, St. Louis, Missouri, USA

Abstract

Sepsis, defined as a clinical syndrome brought about by an amplified and dysregulated inflammatory response to infections, is one of the leading causes of death worldwide. Despite persistent attempts to develop treatment strategies to manage sepsis in the clinical setting, the basic elements of treatment have not changed since the 1960s. As such, the development of effective therapies for reducing inflammatory reactions and end-organ dysfunction in critically ill patients with sepsis remains a global priority. Advances in understanding of the immune response to sepsis provide the opportunity to develop more effective pharmaceuticals. This article details current information on the modulation of the lipopolysaccharide (LPS) receptor complex with synthetic Lipid A mimetics. As the initial and most critical event in sepsis pathophysiology, the LPS receptor provides an attractive target for antisepsis agents. One of the well-studied approaches to sepsis therapy involves the use of derivatives of Lipid A, the membrane-anchor portion of an LPS, which is largely responsible for its endotoxic activity. This article describes the structural and conformational requirements influencing the ability of Lipid A analogues to compete with LPS for binding to the LPS receptor complex and to inhibit the induction of the signal transduction pathway by impairing LPS-initiated receptor dimerization.

Keywords

Endotoxin; Immune response; Inflammatory reactions; Lipid A; Lipo-polysaccharide; LPS receptor complex; Mimetics; Sepsis; Toll-like receptor

I. Introduction

Interactions between the mammalian and microbial worlds are intimate, complex, and vital to the good health of both.[1] Over the course of almost a billion years, the mutual accommodation between microorganisms and multicellular hosts has enabled us both to survive and adapt to a changing environment. The evolution of multicellular organisms would not have been possible without intracellular microbial parasites, that is, the basic

[1]Corresponding authors: aileen.white@dextrauk.com; demchenkoa@msx.umsl.edu.

processes of cellular respiration in eukaryotes are possible only because of the presence of a microbial parasite in the cell called the mitochondrion.[2] However, this intricate, symbiotic relationship between humans and microbes has its darker side. From parasitic and acute infections to chronic illnesses such as peptic ulcer disease, cancer, and coronary heart disease, microorganisms have triggered a plethora of other human diseases.[3] In response, the innate immunity in humans has evolved into a complex system that enables it to respond to a microbial threat and achieve a survival advantage. The latter is often accomplished by exploiting features unique to the threat, just as the microbes have used the same defensive and subversive strategies to circumvent the human immune system.[1]

Accurate recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) is the cornerstone of the innate immune response.[4] A systemic activation of PRRs usually implies loss of control of the host's immune-response mechanisms and a rapid and vast nonspecific response known as the systemic inflammatory response syndrome (SIRS) may result. SIRS is a clinical condition defined by at least two of the four following criteria: (1) temperature below 36 or above 38 °C, (2) heart rate above 90 beats/min, (3) respiratory rate above 20 breaths/min or pCO_2 above 4.3 kPa, (4) white blood-cell count below 4×10^9/L or above 12×10^9/L, or more than 10% immature neutrophils.[5] When SIRS occurs in response to an infection, it is defined as sepsis. The disruption of homeostatic balance as a consequence of sepsis leads to massive production of proinflammatory mediators and dysregulation of the anti-inflammatory mechanisms. Sepsis, therefore, can be described as a pro- and anti-inflammatory disequilibrium syndrome.[6] The high mortality from sepsis is mostly due to an evolving multiorgan dysfunction resulting from local changes in blood flow, namely, sepsis-induced hypotension, diffuse intravascular coagulation, and cytokine-induced abnormalities to microcirculation.[7] A study by Angus *et al.* linking hospital discharge records from seven large states in the USA identified 192,980 cases of severe sepsis out of the 6,621,559 patients admitted.[8] The average cost per case was $22,100 and it adds to annual total costs of $16.7 billion in the USA alone. Despite many attempts to develop treatment strategies to manage sepsis in the clinical setting, the basic elements of treatment have not changed since the 1960s. As such, development of effective therapies for reducing inflammatory reactions and end-organ dysfunction in critically ill patients with sepsis remains a global priority.

Understanding the mechanisms underlying the recognition of invading pathogens through PRRs is of great interest when considering potential treatments. In the past four decades, our insight into how the immune system senses and identifies infective microorganisms to trigger the innate immune response, along with the link between innate and adaptive immunity, has improved dramatically. This understanding began with Hoffmann's pioneering discovery of the role of the Toll receptor in the defense against infections of the fruit fly *Drosophila melanogaster*.[9] Fruit flies lack an adaptive immune system, and its host defense against infections relies solely on the innate immune response. Using flies having mutations in genes of the Toll signaling pathway, Hoffmann and coworkers were able to demonstrate that Toll controlled the expression of antimicrobial peptide genes, and that deficient Toll signaling dramatically reduced survival after fungal infection. Subsequently, Toll was also found to be involved in the antibacterial defense of *Drosophila*.[10]

These results were extended to mammals after Beutler's discovery of homologous receptors, named Toll-like receptors (TLRs), in humans and in mice.[11] While searching for the receptor of the Gram-negative bacterial product involved in endotoxic shock (endotoxins, also known as lipopolysaccharides or LPSs), Beutler and coworkers observed that mice which displayed resistance to the LPS challenge carried constitutive mutations in a gene similar to the *Drosophila* Toll gene. This gene coded for a receptor now known as TLR4, and the latter was found subsequently to be a key player in the LPS receptor complex.

II. Immune Response to LPSs

In the 1890s, Pfeiffer and Centanni independently described a heat-stable pyrogenic toxin intrinsic to *Vibrio cholerae* and *Salmonella typhi*.[12] Pfeiffer initially called it "endotoxin," and in the 1930s, Boivin was able to extract and purify it using trichloroacetic acid.[13] Endotoxin purified through Boivin's method, however, was essentially a crude fraction containing many cell-wall proteins. This slowed the progress towards understanding the role of endotoxins and their biological impact remained inconclusive. In 1946, Westphal and Lüderitz were finally able to develop a method for obtaining pure active fractions of "endotoxin," which was shown to be LPS.[14]

LPSs are composed of three genetically, structurally, and antigenically distinct regions namely: (1) a hydrophobic membrane anchor called Lipid A; (2) a short chain of sugar residues with multiple phosphoryl substituents referred to as the core oligosaccharide; and (3) a structurally diverse, serospecific polymer composed of oligosaccharide repeat-units called the O-antigen (Fig. 1). While the LPSs of mucosal pathogens, for example, of *Haemophilus* and *Neisseria* species, lack the typical long-chain repeating O-antigen, the basic tripartite LPS framework stands for all characterized Gram-negative bacteria.

Lipid A, the covalently linked lipid component of LPSs, is composed of six or more fatty acid residues linked to two phosphorylated glucosamine residues.[15] Four of the fatty acids have an (*R*)-3-hydroxyl group and the other two are devoid of it. Each Gram-negative bacterial species has a unique Lipid A composition, and the structural features that differentiate each molecule are as follows. First, the acylation pattern on each glucosamine residue can have either a symmetric (3+3) or an asymmetric (4+2) arrangement. Second, three or four different fatty acids can be present in the molecule, with a chain length that can be anywhere between 10 and 16 carbon atoms. Finally, a 4-amino-deoxy-L-arabinose and/or phosphonoethanolamine can be linked to the C-1 axial or C-4′ phosphate groups on the glucosamine residues.

The core region is a short polysaccharide chain showing moderate interbacterial variability. The inner core consists of two or more 3-deoxy-D-*manno*-octulosonic acid (Kdo) residues linked to C-6′ of the two glucosamine residues (Lipid A) on one side and two or three L-*glycero*-D-*manno*-heptose residues on the other.[16] In should be noted that both Kdo and L-*glycero*-D-*manno*-heptose are unique to bacterial species.[17] Under natural conditions, the smallest LPS produced by Gram-negative bacteria is Re-LPS—it consists of Lipid A with one or two Kdo residues—but longer LPSs are more common.[18] The Rd1- and Rd2-LPS serotypes contain a complete inner core and an inner core without two heptose residues,

respectively.[19] The outer core, on the other hand, consists of common sugars and is more variable than the inner core. It is normally two to three residues long and has one or more covalently bound polysaccharides as side chains.[20] LPSs consisting of the Lipid A and the complete inner and outer core are denoted Ra-LPS, whereas the Rb- and Rc-LPS serotypes only contain a part of the outer core.

The O-antigenic outer-core portion is the most variable part and consists of repeating units of oligosaccharides. Attached to the terminal sugar of the inner core, this portion extends from the bacterial surface and is highly immunogenic.[21] The chemical composition and structure of the O-antigen can be strain-specific (interstrain LPS heterogeneity), or it can vary within one bacterial strain (intra-strain LPS heterogeneity).[22] The inter- and intra-strain heterogeneity is characterized by variations at different levels. The first variation can occur through nonstoichiometric modification of the O-antigens with sugar moieties, that is, with glucosyl and fucosyl residues. The second variation occurs via addition of noncarbohydrate substituents—such as acetyl or methyl groups—to the O-antigen. This may arise with regularity, although, in most cases, these modifications are also nonstoichiometric. The length of the O-antigen may vary from 0 to as many as 40 repeating units, but it generally consists of 20 to 40 repeating units.

LPSs participate in physiological functions of the membrane and are therefore essential for bacterial growth and viability.[23] They contribute to low membrane permeability and enhance the resistance toward hydrophobic agents. LPSs are not toxic while they remain incorporated in the bacterial outer membrane. When released from the bacterial surface—either following cell division or death, as a consequence of antibacterial action of the immune system, or interaction with antibacterial agents—LPSs may form aggregates and interact with the cells of the immune system. Following this interaction, LPSs elicit multiple acute pathophysiological effects, such as fever, toxicity, Schwartzman reactivity, macrophage, and B-lymphocyte activation, among others.[24] In 1954, it was proposed that the Lipid A portion alone is responsible for the endotoxic properties of LPSs, and that the polysaccharide portion is dispensable.[25] After Shiba and coworkers completed the first targeted synthesis of the Lipid A of *Escherichia coli*,[26] comparative experiments between the natural LPS and the synthetic Lipid A confirmed that it is Lipid A that constitutes the source of toxicity of LPSs.[27]

Under physiological conditions, the immune cells are continuously exposed to low levels of LPSs derived from gastrointestinal bacteria. These LPSs are taken up by macrophages and may be essential to maintain a basal level of attentiveness of the immune system. It was originally believed that LPSs activated the immune cells through a nonspecific mechanism that involved intercalation of Lipid A into the mammalian lipid bilayer.[28] In the early 1980s, reports emerged suggesting that the biological actions of LPSs were facilitated by their binding to endogenous proteins. Indeed, the response of a host cell to LPSs is highly dependent on whether it encounters the latter in free or bound form.[29]

Over the past 20 years, one of the major aims in LPS research has been to elucidate the exact sequence of events from when the LPS binds to the cell to when it elicits a response from it. As mentioned earlier, distinct plasma membrane proteins mediate the initial interaction

between LPSs (Lipid A) and phagocytes (monocytes, macrophages, polymorphonuclear leukocytes (PMNs)). Some of these interactions may be solely involved in the removal and eventual degradation of LPSs, whereas others may play a critical role in transmembrane signaling. The receptors involved in transmembrane signaling are shown in Fig. 2 and are discussed in the succeeding sections.

III. The LPS Receptor Complex

1. LPS-Binding Protein

While studying binding of LPSs to high-density lipoproteins (HDLs) in normal and acute-phase rabbit serum, Tobias and coworkers observed that LPS was mainly complexed to a protein in the acute-phase serum.[31] Isolation of this protein from the rabbit serum led to the discovery of the LPS-binding protein (LBP). LBP was recovered as a glycoprotein having molecular weight of 58 and 60.5 kDa, wherein the difference in molecular mass reflects different degrees of glycosylation.[31,32] It is synthesized primarily by hepatocytes and released into the bloodstream after glycosylation.[33] Other sources of LBP include epithelial cells of the skin, the lung, the intestine, and human gingival tissues, as well as the small-muscle cells of the lung arteries, heart muscle cells, and renal cells.[34]

Human LBP consists of 452 amino acids and has the characteristic 25-amino acid signal sequence of secreted proteins.[33] Its amino acid sequence revealed a sequence homology to bactericidal/permeability-increasing protein (BPI), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP) of 45%, 23%, and 25%, respectively. It has also suggested a similarity in the tertiary structure of these proteins. After the three-dimensional structure of BPI was determined by X-ray crystallography, it provided a useful framework for modeling the three-dimensional structure of the LT/LBP family.[35] BPI appears as a boomerang-shaped molecule and consists of two symmetrical barrel domains connected by a proline-rich linker region. Each domain is composed of an antiparallel β-stranded layer twisted around a long α-helix and forms a hydrophobic pocket that can incorporate one phosphatidyl choline molecule. From these observations, Beamer *et al.* proposed a simulated three-dimensional model for LBP that is very similar to the structurally and functionally related BPI.[36] Analogously with BPI, the LPS-binding domain of LBP is located at the N-terminal region. Indeed, the three-dimensional LBP model showed that the cationic cluster of the LPS-binding site is fully exposed at the N-terminal tip.[36]

To verify the veracity of the claim that the LPS-binding site of LBP is at the N-terminus, Lamping *et al.* performed mutagenesis experiments.[37] LBP mutants with amino acid exchanges within the N-terminal region were expressed and tested in five different functional assays—binding to immobilized LPSs, facilitation of binding of LPS aggregates to monocytes, transfer of LPS monomers from aggregates to other LPS receptors, transfer of LPS monomers to HDL, and enhancement of LPS-induced cell activation. The double mutant glutamic acid 94/95 was completely lacking LPS binding, transfer, and cell-stimulatory activity, indicating that the integrity of amino acids 94 and 95 is required for LBP function.[37] While mutations of the amino acids Arginine-94 or Lysine-95 into alanine diminished the LPS-binding activity of LBP dramatically, the ability to facilitate binding of LPS aggregates to membrane-bound cluster of differentiation 14 (mCD14) at the cell surface

was retained. These findings emphasize the distinction between binding of LPS aggregates to cells and the binding of LPS monomers to CD14—the former is not associated with cell stimulation and the latter leads to cell stimulation.

Studies show that LBP has a dual role in interactions with LPSs. At low LPS concentrations, LBP enhances LPS signaling by extracting it from the bacterial membranes (LPS monomerization) and transferring the LPS monomers to CD14.[38] At high concentrations, it inhibits the LPS signaling by shuttling the LPS to the serum lipoproteins and by forming aggregates with LPS.[39] Indeed, the increased secretion of LBP as a result of LPS stimulation serves as an inhibitor of excessive response to LPS in the serum of septic patients.

The two domains of LBP have different functions. As with LBP and its homologue BPI, the amino-terminal domain has a high affinity for LPSs.[40] The C-terminal domain, on the other hand, is required for the interaction with CD14 or the cell membrane.[41] Adding LBP to a serum-free cell system enhances the LPS-mediated stimulation of CD14-positive cells by 100- to 1000-fold.[33,42] In addition, LBP also transfers LPS to soluble CD14 (sCD14), resulting in the activation of mCD14-negative cells (endothelial and epithelial cells). Thus, the ability of LBP to transfer disaggregated LPS to both mCD14 and sCD14 supports the view that LBP has a central role in mediating LPS responses. It was proposed that a single LBP molecule is able to transport hundreds of LPS molecules to sCD14, and that LBP is not consumed by this reaction.[43] Consequently, Yu and Wright demonstrated first-order kinetics for this enzymatic transfer and were further able to define catalytic constants for this reaction.[38b,43] To explain the catalytic reaction mechanism for the transfer of LPS to sCD14, two models were proposed. The “binary complex” model proposes that the initial step in the transfer involves a bimolar reaction between LBP and an LPS micelle. Following dissociation from the micelle with one molecule of LPS bound, LBP then binds to sCD14.[43] The “ternary complex” model, on the other hand, suggests a simultaneous interaction among LBP, LPS micelles, and sCD14.[44]

2. Cluster of Differentiation 14

While it was clear that the CD18 complex interacts with LPS by bridging bacteria to the surface of phagocytes,[45] it was not clear whether this interaction actually triggers cellular responses. To elucidate the exact role that CD18 plays in the cellular activation by LPS, Wright *et al.* performed experiments on mononuclear cells from CD18-deficient patients.[46] From these experiments, it became clear that CD18-deficient cells can bind LPS and that the binding event can result in cellular activation. Clearly, additional receptors must be present on the surface of macrophages and PMNs. Subsequently, Wright and coworkers identified this unknown receptor to be CD14—a differentiation antigen of monocytes.[47] Based on their report, CD14 binds complexes of LPS and LBP, and the blockade of CD14 with anti-CD14 antibodies prevents further binding of LPS-coated erythrocytes to macrophages. With the absence of a binding event, macrophages are unable to produce an LPS-induced inflammatory response. Golenbock and coworkers corroborated these findings by demonstrating that LPS-induced responsiveness can be transferred to a heterologous nonresponder cell type by expression of a single leukocyte-specific gene product.[48] Thus, transfection of human CD14 into Chinese hamster ovary (CHO) fibroblasts and treatment of

CD14-bearing CHO cells with LPS led to a macrophage-like responsiveness in otherwise LPS-unresponsive cells. Similarly, Lee and coworkers showed that CD14-bearing 70Z/3 cells bind LPS, and when LPS is complexed with LBP, the binding activity is even higher.[49] Consequently, Kirkland and coworkers determined the binding affinity of the LPS–LBP complex to CD14-transfected CHO cells and THP-1 cells and found K_d values of 2.7×10^{-8} to 4.8×10^{-8} M.[50]

CD14, a serum/cell surface glycoprotein and the first PRR to be described, is usually found in two forms: membrane bound (mCD14) and soluble (sCD14).[47,51] Since sCD14 lacks the glycosyl phosphatidylinositol (GPI) anchor, mCD14 and sCD14 have molecular masses of 53 and 48 kDa, respectively.[52] To determine the amino acid composition of CD14, Ferrero and Goyert cloned the CD14 gene and revealed a transcript encoding a 356-amino acid protein.[53] It was also found to have high leucine content (15.5%) and four putative N-glycosylation sites.[53,54] The site involved with LPS binding, as well as the sites involved in the interaction of human CD14 with supposed accessory receptors, has been identified in the N-terminal part of CD14.[55] This was determined by generating and transfecting 23 mutants in the N-terminal 152 amino acids of human CD14.[56] In each mutant, a block of 5 amino acids was substituted with Ala. Thus, Stelter and coworkers found that the region between amino acids 39 and 44 forms an essential part of the LPS-binding site of human CD14.[56] Moreover, for human sCD14, two other regions were found to be essential for eliciting LPS-induced responses from endothelial and smooth muscle cells: aa 9–13 and aa 91–101.[57]

CD14 is expressed by various cells such as cells of the myeloid lineage (monocytes, macrophages, PMNs), B cells, parenchymal cells of the liver, gingival fibroblasts, and microglial cells.[58] Each source expresses CD14 differentially: peritoneal and pleural macrophages exhibit a high level of constitutive CD14 expression, while (murine) Kupffer cells, alveolar macrophages, monocytes, and PMNs have a low level of constitutive CD14 expression.[58a,59] In addition, LPS and tumor necrosis factor-α (TNFα) induce the release of sCD14 by mononuclear cells and PMNs in a dose-dependent manner, whereas interferon-γ (IFN-γ) and interleukin 4 (IL-4) inhibit the release of sCD14.[60] In the steady state, human serum contains 2–6 μg/mL of sCD14.[61] This level increases in response to the presence of LPS, and consequently, Landmann *et al.* suggested the use of sCD14 levels as a diagnostic marker in patients with severe infections.[62] The level of sCD14 in human milk also explains why newborn infants are innately immune to bacteria with their hitherto sterile intestines. Thus, Labeta and coworkers found that the concentration of sCD14 is 10-fold higher in human milk than that in serum.[63]

Binding of LPS to a cell does not result in immediate response—a time lapse of 15–30 min is usually observed between LPS binding and LPS-induced cellular responses. Detmers and coworkers, along with Lichtman and coworkers, suggested that monomeric LPS is internalized in vesicles, and uptake may be required for signaling.[64] Indeed, several studies have revealed that blocking the internalization or endosome fusion also blocks LPS-induced signaling.[64,65] Although the precise mechanisms of this blocking event are not completely understood, it has been shown that monomeric LPS is transported into the cell to the Golgi complex and activates the cell from there on.[66] To determine if mCD14 directs the movement of LPS to the Golgi apparatus, Vasselon and coworkers used an mCD14 chimera

containing enhanced green fluorescent protein (mCD14-EGFP) to follow trafficking of mCD14 in stable transfectants.[67] It was found that monomeric LPS is transferred out of mCD14 at the plasma membrane and traffics within the cell independently of mCD14 involvement. In contrast, particulate (bacterium) and aggregated (micelles) LPSs were internalized to the lysosomes via a CD14-dependent pathway called macropinocytosis—a process resembling that of phagocytosis.[65b] After internalization, LPS induces mononuclear phagocytes (MPs) to produce three groups of powerful mediators: the reactive oxygen intermediates (O_2, H_2O_2, OH, and singlet oxygen), the proinflammatory cytokines, and a number of arachidonic acid metabolites, including prostaglandins and leukotrienes.

Since CD14 is a glycophosphatidylinositol-linked receptor that lacks a transmembrane domain, it was anticipated that it requires an accessory molecule for signal transduction.[68] This hypothesis was confirmed using different anti-CD14 antibodies that either blocked LPS binding to CD14 or did not block LPS binding while preventing LPS-induced cell activation.[44,69] This accessory receptor has been identified as a member of the TLR family.

3. Toll-Like Receptor 4

Nearly a decade after the importance of LBP and CD14 was initially delineated in seminal discoveries, the next main advance in understanding the mechanism of innate immunity emerged—the identification of the putative transmembrane protein that acted with CD14 to generate a transmembrane signal for LPS-induced cell activation. Two highly original and influential discoveries gave the impetus for this advance. First, it was found that TLRs play an important role in the innate immune response of *Drosophila* flies.[9,70] Second, a TLR homologue was identified as the gene responsible for LPS responses in two natural mouse mutants.[11,71] These results formed the basis for understanding how the innate immune system regulates responses to infection and how plasma membrane receptors control adaptive immune responses.[30]

It was well known that, despite the lack of an adaptive immune system, *Drosophila* flies are very resistant to microbial infections. The only rationale for this attribute, at the time, had been its demonstrated ability to synthesize potent antimicrobial peptides. Then, in the early 1980s, Anderson and coworkers conducted a mutagenesis screen for genes involved in dorsoventral patterning of the *Drosophila* embryo.[72] Their studies revealed a mutant gene that had an unusual appearance. Consequently, the authors named this gene “Toll,” meaning weird. The Toll gene, which encodes a single-pass transmembrane receptor, became highly important after it was found that it activates the signaling pathways that induce the synthesis of drosomycin, an antifungal peptide in *Drosophila* flies.[9,72]

Twelve years after Anderson’s discovery, Williams *et al.* showed that 18-wheeler, another TLR gene found in *Drosophila*, could induce the release of attacin,[70] one of the potent antibacterial peptides synthesized by *Drosophila.* As a result, it was established that the activation of a proteolytic cascade that produces peptidic ligands for the TLRs leads to the induction of these antimicrobial responses. It remains unanswered whether this mechanism is unique to *Drosophila*, or whether it is conserved in mammalian cells. What was remarkably inferred from these results, however, was that *Drosophila* TLRs were capable of discriminating between fungi and bacteria and, consequently, of inducing an appropriate and

distinct antimicrobial response. Subsequently, Imler and coworkers showed that the activation of TLR-induced pathways in *Drosophila* initiates an intracellular kinase cascade that ultimately produces a translocation of transcription factors, Dif and Relish, from cytoplasm to nucleus.[73] Dif and Relish are homologous to nuclear factor-kappa B (NF-κB), a transcription factor known to activate inflammatory mediators in humans, thereby linking *Drosophila* TLRs to the study of LPS biology.[72]

The apparent importance of TLRs, as well as the observation that the Toll gene shares a certain homology with the human IL-1 receptor, provided impetus for the field of Toll biology to move beyond flies.[74] Thus, in the mid-1990s, Janeway and coworkers began a search for dToll-related proteins in mammalian gene sequences. As a result of their efforts, the first human homologue of *Drosophila* Toll, initially termed human Toll and subsequently termed TLR4, was identified.[75] Human TLR is an 841-amino acid protein with a molecular mass of 92 kDa.[75] After cloning and characterization, human Toll was found to be a type I transmembrane protein, the cytoplasmic domain of which bears a structural homology to the human IL-1 receptor. Janeway and coworkers also determined that similarly with *Drosophila* Toll, human Toll could induce activation of NF-κB and subsequently induce the expression of NF-κB-controlled genes for the inflammatory cytokines. Finally, their observation that TLR4 could induce members of the B7 family—molecules that are required for the activation of naive T cells by antigen-presenting cells—provided a potentially important link between pathogen detection and induction of the adaptive immune response.

Other compelling evidence on the importance of TLRs in LPS-induced responses came when TLRs addressed the issue of why some strains of mice were unresponsive to LPS. For years, LPS has been known to be a very active mediator of inflammation in most mammalian system.[76] It was found, however, that LPS is relatively ineffective at inducing responses in the C3H/HeJ or C57BL/10ScCr strains of mice.[77] Then in 1998, Beutler and coworkers showed via positional cloning techniques that mutations of a gene termed the "LPS gene" selectively reduced the ability of C3H/HeJ and C57BL/10ScCr mice to sense LPS. The codominant Lps^d allele of the C3H/HeJ strain was a result of a mis-sense mutation in the third exon of TLR4, a mutation that was predicted to result in a Pro712→His substitution.[11,71a] When this mutation was introduced into wild-type TLR4, the receptor was converted into a dominant-negative mutant that inhibited LPS-dependent responses in a transfected macrophage cell line.[78] Similarly, Hoshino *et al.* demonstrated that C3H/HeJ mice have a single-point mutation of the amino acid that is conserved among the IL-1/Toll receptor family.[71b] Using genetically modified mice in which the TLR4 gene was deleted, the latter showed that TLR4 was essential for sensing LPS and mutations in this gene explained the lack of responsiveness in C3H/HeJ mice. Together, these seminal publications provided the first direct connection between TLRs and the physiological responses to LPS. Heine and coworkers provided further proof of this connection by showing that Chinese hamsters respond normally to LPS even though they carry a null allele for TLR2.[79] Their results implied that expression of TLR2 is sufficient, but not essential, for mammalian responses to endotoxin. Finally, it was shown that a dominant-negative mutant of TLR2 did not cause LPS responsiveness in transfected macrophages.[78]

4. Myeloid Differentiation Antigen 2

Despite the fact that several groups have already shown evidence of LPS-induced signal transduction through TLRs, direct binding of LPS to the latter is yet to be demonstrated.[80] Moreover, it was found that *in vitro* transfection of TLR4 cDNA did not confer LPS responsiveness on two LPS-unresponsive cell lines: human embryonic kidney-derived and a mouse IL-3-dependent pro-B cell-line Ba/F3.[81] Then in 1999, Shimazu and coworkers reported and characterized a novel LBP called myeloid differentiation antigen-2 (MD-2).[81b] In a series of experiments, they showed that MD-2 physically associated with TLR4 on the cell surface and confers responsiveness to LPS. In a similar fashion, Da Silva Correia determined that LPS binds directly to each of the members of a tripartite LPS receptor complex.[80b] Using modified and radio-iodinated LPS, they showed that LPS is cross-linked specifically to TLR4 and MD-2 when coexpressed with CD14. Thus, maximal cellular activation by LPS must be a cascade of events that likely involves transferring of LPS by LBP to CD14 and then to TLR4-MD-2. Moreover, although CD14 and LBP enhance cellular activation, activation of TLR4 by LPS was found to absolutely require MD-2.[82]

MD-2 is a 20- to 25-kDa extracellular glycoprotein that belongs to the MD-2-related lipid-recognition family of lipid-binding receptors.[83] Since MD-2 lacks a transmembrane domain that would anchor it to the cell membrane, several groups performed studies to verify the process by which MD-2 associates with TLR4—whether it is a soluble intracellular protein that binds to TLRs in the endoplasmic reticulum (ER) or it is first secreted into the medium and then binds to TLRs on the cell surface. To this end, Visintin *et al.* found that, in some cells, MD-2 is synthesized in large excess to TLR4 and it saturates all available TLR4 molecules in the ER. The excess MD-2 is then secreted into the medium.[84] Although proper glycosylation and trafficking of TLR4 to the cell surface require intracellular association with MD-2,[85] functional TLR4 can be presented on the cell surface without MD-2 in both transfected[86] and epithelial cells of the human airway.[87] For reporter cells that expressed TLR4, but not MD-2, secreted MD-2 (sMD2) was found to restore LPS responsiveness.[84] Thus, even at concentrations as low as 50 pM, Visintin and colleagues showed that MD-2 significantly enhances LPS reactivity and suggested that TLR4 has a functional affinity constant for MD-2 in the range of 50–500 pM.[84]

Human MD-2 contains 160 amino acid residues, prominent regions of which are the 17-amino acid sequence at the N-terminus, 7 cysteine residues, and 2 N-glycosylation sites.[88] To identify the regions of functional importance on human and mouse MD-2, common analytical methods—namely, analysis of peptide fragments,[89] mutation analysis,[90] and computational modeling[91]—have been utilized. Computer modeling suggests that MD-2 is capable of forming a barrel-like structure with a hydrophobic cavity sufficient to accommodate the fatty acid moieties of Lipid A.[91,92] In addition, Visintin and coworkers reported that a positively charged region flanking the hypothetical hydrophobic cavity of MD-2 is required for stable binding to LPS.[93] On the other hand, site-directed mutagenesis identified the regions of human MD-2 involved in TLR binding, and consequently, in conferring LPS responsiveness.[90d] Thus, Re and coworkers found that MD-2 binding to TLR4 took place via Cys95 and Cys105, probably through the formation of an intermolecular disulfide bond.[90d] Several studies predict that Cys95 is located on the surface

of the hypothetical barrel, along with the other Cys residues, except for Cys133.[84,94] This prediction is consistent with the idea that MD-2 is capable of forming covalently bound oligomers, but it does not preclude the existence of a monomeric form. Indeed, monomeric MD-2 has been reported to bind preferentially to a recombinant soluble TLR4 ectodomain.[95] Hydrophilic and charged residues surrounding this area, such as R90, K91, D100, and Y102, also contributed to the formation of the TLR4–MD-2 complex.[90d] Re and Strominger found, however, that a different region of MD-2 was responsible for conferring LPS responsiveness.90d This region is not involved in TLR4 binding and is rich in basic and aromatic residues, several of which contribute to LPS responsiveness and might represent an LPS-binding site. Consequently, mutations in the lysine residues of this region are correlated with the loss of LPS binding and, as a result, the loss of activity.

Finally, it was found that binding of MD-2 by Lipid A was greatly enhanced by serum components that had long been known to enhance LPS responses, namely, sCD14 and LBP.[86,94a,96] MD-2 is unstable at 37 °C, but the binding of LPS to MD-2 has been reported to dramatically stabilize its activity.[97] Overall, the evidence supports a model in which LPS interacts with the MD-2/TLR4 surface heterodimer. The interaction of LPS with the receptor complex occurs with high affinity, and the K_d is estimated to be 3–10 nM.[98] The binding of LPS to MD-2 is then responsible for the aggregation of TLR4 and the recruitment of intracellular signal transducers.

IV. Host-Derived Mediators and the Pathogenesis of Sepsis

Once TLR4 binds to its LPS ligand, two possible pathways of cellular activation can occur —either through the myeloid differentiation factor 88 (MyD88) or through the TLR-domain-containing adapter-inducing interferon-β (TRIF) pathway.[99] In each pathway, signaling events lead to the sequential activation of specific tyrosine and threonine/serine kinases. This signaling cascade ultimately results in phosphorylation, ubiquitination, and degradation of inhibitory kappa-B (IκB) and other transcriptional activators. IκB degradation leads to translocation of NF-κB into the nucleus. Once NF-κB is translocated into the nucleus, it binds to specific DNA sequences located in the promoter regions and participates in the activation of a large variety of genes including cytokines, chemokines, stress-response proteins, and antimicrobial and antiapoptotic peptides.[100] The outpouring of inflammatory cytokines and other inflammatory mediators after LPS exposure contributes to generalized inflammation, procoagulant activity, tissue injury, and septic shock.[101]

In macrophages, Lipid A activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation and activates the production of costimulatory molecules required for the adaptive immune response.[4] Once activated, macrophages are the fundamental secretory cells of the immune system.[102] To date, more than 100 macrophage products have been identified—with molecular weights ranging from 32 (superoxide anion) to 440,000 Da (fibronectin).[103] Among these, inflammatory cytokines such as TNFα, IL-1β, and IL-6 are the most studied.

1. The Cytokine Networks

The cytokine TNFα, an endogenous monocyte/macrophage-derived protein, is one of the most important soluble mediators of inflammation. It is mainly synthesized by activated monocytes/macrophages and is responsible for a wide range of signaling events within cells. In response to an LPS challenge, TNFα is synthesized very quickly and the production peaks in a matter of 1.5 h.[104] Secretion of this molecule triggers a proinflammatory response in neutrophils and endothelial cells and leads to cell damage.[105] TNFα exerts most of its effects by binding, as a trimer, to either a 55-kDa cell membrane receptor called TNFR-1 or the 75 kDa cell membrane receptor TNFR-2; both are members of the TNF receptor superfamily.[106] In animal studies, the administration of TNFα has been shown to have lethal consequences.[107] In human volunteers, dramatic hemodynamic, metabolic, and hematologic changes are observed after administration of TNFα. Perhaps the most dramatic demonstration of the pathophysiologic significance of systemic cytokine release was observed recently in a phase I study of an experimental anti-CD28 monoclonal antibody.[101a] The antibody was well tolerated in animal studies, but was found to be markedly toxic to humans. Within a few hours of receiving the antibody, all six healthy human volunteers developed shock, disseminated intravascular coagulation (DIC), and multiorgan failure. The “cytokine storm” that often accompanies septic shock was clearly demonstrated by the striking elevations in IL-1, TNF, IL-8, IFN-γ, and other cytokines and chemokines that were released almost immediately into the patient’s bloodstream.

Another important cytokine in host defense during sepsis is the IL-1 gene family.[108] This family consists of three members: IL-1α, IL-1β (both agonists with proinflammatory character), and the IL-1 receptor antagonist (IL-1ra, anti-inflammatory counterpart). While IL-1β is solely active in its processed and secreted form, IL-1α is active in its intracellular precursor, membrane-associated, or secreted form.[109] The activation of numerous cell types by IL-α and IL-β leads to diverse proinflammatory events.[110]

Both IL-1 and TNFα act synergistically in the initiation of the inflammatory cascade in sepsis, leading to the expression of further factors.[111] These factors include other proinflammatory cytokines (IL-12, IL-18),[71b,112] and chemokines (IL-8, monocyte chemoattractant protein-1/MCP-1).[113] The chemokines IL-8 and MCP-1 are key factors in chemotaxis—IL-8 is involved in neutrophil chemotaxis, while MCP-1 is involved in the chemotaxis of monocytes. IL-8 also causes neutrophils to degranulate and cause tissue damage.[114]

2. The Coagulation Cascade

Activation of the coagulation cascade has traditionally been synonymous with the need for hemostasis (stoppage of bleeding) at sites of injury. Over the past several decades, however, it has been increasingly recognized that initiation of coagulation is an integral and consistent element of the local and systemic response to inflammatory stimuli. The precise mechanism whereby coagulation contributes to the full expression of inflammation is an area of active study.

Tissue factor (TF) expression on the surface of endothelial cells and monocytes—induced by the presence of endotoxins or inflammatory cytokines—initiates the coagulation process.[115] Thus, TF on the cell surface activates factor VII, and the resulting complex of factor VIIa and TF converts factor X into factor Xa. In concert with factor Va, factor Xa converts prothrombin into thrombin, which in turn results in the cleavage of fibrinogen to fibrin. Deposition of fibrin plays a critical role in hemostasis and in the localization of microorganisms within an abscess cavity. This process, however, can impede delivery of oxygen to tissues and can induce further inflammatory injury indirectly through the response to hypoxia (lack of oxygen) and directly through signals delivered to the thrombin receptor. Engagement of the thrombin receptor activates the nuclear transcription factor NF-κB,[116] causing the transcription of a broad array of proinflammatory gene products and resulting in release of nitric oxide.[117] The thrombin receptor is not a unique mechanism through which an inflammatory response is amplified. Clustering of TF has also been shown to initiate gene expression for proinflammatory cytokines, including TNF.[118]

In general, the activation of the coagulation pathway induces anticoagulant mechanisms that function to limit progression of the coagulation cascade. During sepsis, however, an imbalance of the procoagulant and anticoagulant systems occurs, resulting in a sustained hypercoagulable state. The specific abnormalities of the coagulation system that occur following endotoxemia and cytokinemia have been documented in detailed studies involving human volunteers and septic patients. Thus, in human volunteers injected with small doses of TNFα or LPS, there is gradual activation of coagulation, as shown by increases in thrombin–antithrombin (TAT) complexes, prothrombin activation fragments, and fibrinopeptide A.[119] This process begins by the second hour, peaks at 4–5 h, and persists for 6–12 h. There is also an early increase in plasma fibrinolytic activity (1–2 h) due to the presence of plasminogen activators, which leads to plasmin generation. The anticoagulant effect of the latter, however, is rapidly neutralized by an increase in the amount of antifibrinolytic plasminogen activator inhibitor-1 protein (PAI-1) released into the bloodstream. Both ATIII and protein C are also rapidly consumed during the septic state.[120] Additionally, the downregulation of thrombomodulin due to both local and systemic release of such cytokines as TNF and IL-1 results in impaired activation of the anticoagulant APC–protein S complex.[121]

The increased procoagulant activity, decreased anticoagulant activity, and impaired fibrinolysis in septic patients lead to the development of the clinical syndrome called DIC. DIC is clinically defined as an overexuberant systemic clotting that depletes coagulation proteins and platelets from the blood and leads to bleeding complications.[122] It becomes increasingly common as patients advance from sepsis (SIRS) to septic shock.[123] The microvascular thrombosis that develops concomitantly results in organ injury, partly on an ischemic basis. As such, while DIC is considered in terms of bleeding complications, the clinical outcome is ultimately decided by the accompanying microvascular thrombosis and end-organ damage.

In summary, multiple and diverse pathways lead to activation of the cytokine networks and the coagulation cascade. To alleviate the fatal outcome of sepsis and septic shock, many therapeutic interventions have been targeted toward the later stages of endotoxin response:

(a) blocking cytokine synthesis/release by interfering with the transduction of cell surface signals,[124] (b) neutralizing released cytokines by passive immunization[125] and soluble receptors,[126] and (c) blocking cytokine cell surface receptors with a specific receptor antagonist.[127] The majority of these approaches have demonstrated efficacy in both *in vitro* and animal models, but none has proven to be effective at treating human sepsis. Due to the large diversity and quantity of cytokines released by activated cells, it is likely that the approach of blocking only a single cytokine may be inadequate. Since no single therapeutic agent has proven to be unequivocally beneficial for managing the abnormalities of sepsis, it has become increasingly clear that the therapeutic path to sepsis does not lie on the treatment of the downstream events. Under the assumption that end-organ damage ensues from an exuberant or hyperactivated immunological response that becomes unresponsive to supervening counter-regulatory mechanisms, the approach to treatment of sepsis lies in interrupting the cascading inflammatory response by blocking the initial signaling events. Current progress towards this goal is discussed next.

V. Modulation of the LPS Receptor Complex by Lipid A Analogues

It is understood, thus far, that endotoxin is essentially a signaling molecule that alerts the vertebrate host to the presence of a Gram-negative pathogen. While the endotoxin molecule itself is not intrinsically toxic, the exaggerated host response to endotoxin accounts for septic shock from Gram-negative bacterial organisms. To summarize from the foregoing, endotoxin mediates its injurious effects through systemic activation of host-derived inflammatory mediators.

While the evolution of organ dysfunction in septic shock is a complex, highly variable and multifactorial process involving many mediators, the past few decades have seen enormous advances toward understanding the cellular and molecular basis of the initial events in this process. The thorough characterization of these events has allowed researchers to design rational therapies directed at blocking the initial signaling events in LPS-induced sepsis.

A successful approach to downregulating LPS signaling involves the use of compounds that compete with LPS binding to MD-2 and inhibit the induction of the signal transduction pathway by impairing LPS-initiated receptor dimerization. To date, several Lipid A variants that specifically block the LPS-binding site on human (h) MD-2 have been identified: lipid IV_A (a biosynthetic precursor of the Lipid A of *E. coli*, **1**, Fig. 3)[128] and a nonpathogenic Lipid A from *Rhodopseudomonas sphaeroides* (**2**),[129] which served as the structural basis for the synthetic antisepsis drug candidate Eritoran (E5564, **3**).[130]

The central structure of Lipid A, as exemplified by the *E. coli* Lipid A structure (**4**), is a highly conserved glycosidically β-(1→6)-linked di-D-glucosamine backbone bisphosphorylated at the 1-O- and 4′-O-positions.[131] As mentioned earlier, LPS and Lipid A trigger innate immune responses through the TLR4/MD-2 complex, the activation of which leads to two distinct signaling pathways—the Myd88-dependent pathway and the TRIF-dependent pathway.[132] The Myd88-dependent pathway results in the production of proinflammatory cytokines such as TNFα, IL-1β, and IL-6. The TRIF-dependent pathway, on the other hand, results in interferon-β and nitric oxide production.

Despite considerable data on the activity of both isolated[133] and synthetic Lipid A derivatives,[134] there is no universal correlation between the chemical structure of Lipid A and its activity in the TLR4/MD-2 complex. An important approach to understanding how a receptor system functions is to define its pharmacology. Recent work on the crystal structure of the TLR4/MD-2 complex, bound to either agonistic LPS[135] or antagonistic Lipid IV_A[128] (**1**), provided a deeper understanding of the structural requirements of the LPS receptor complex. Thus, a large hydrophobic cavity is noted in MD-2, whereby all four lipid chains of compound **1** are contained, as opposed to five of the six lipid chains of LPS. The remaining chain of LPS is exposed to the surface of MD-2 and forms hydrophobic interactions with conserved phenylalanine residues in TLR4. Consequently, it is speculated that structural properties of MD-2 play a critical role in differentiating among varying Lipid A structures and potentiating a biological response.

Subtle differences in the length and distribution pattern of acyl chains, the phosphorylation status of the di-glucosamine backbone, and changes in the di-glucosamine backbone of Lipid A are known to profoundly affect its biological activity.[134b–d] The following sections summarize the effect of these variables on the agonistic or antagonistic activity of Lipid A derivatives.

1. Length and Distribution of Acyl Chains

The acyl chains of Lipid A, particularly its number, length, symmetry, and saturation, have been shown to be a major determinant of the potency of LPS in eliciting TLR4-dependent host responses.[136] The relationship between acyl-chain length and bioactivity has been investigated to some extent with tetraacyl disaccharide analogues[137] of lipid IV_A (**1**). Recently, having demonstrated that the toxic effects of Lipid A of *Salmonella minnesota* R595 (**5**, Fig. 4) could be ameliorated by selective hydrolysis of the 1-*O*-phosphono and (*R*)-3-hydroxytetradecanoyl groups (**6**),[138] Johnson and coworkers proceeded to clarify the importance of normal fatty acid chain length by preparing and evaluating chain-length homologues of **5** (also known as monophosphoryl Lipid A, MPL). Thus, derivatives **7a–f** were synthesized and evaluated against MPL **6** for induction of nitric oxide synthase (iNOS) in murine macrophages and production of cytokine in human peripheral monocytes.[139] It was found that the induction of both iNOS and proinflammatory cytokines exhibit a profound bimodal dependence on the length of the normal fatty acid chains, reaching a maximum when n=8 (**7d**) in each case. The iNOS response is more sensitive than cytokine induction to variations in chain length, showing a 100-fold difference in potency between **7d**, which possesses a greater macrophage-stimulating ability, and **7f**. However, both models show a similar threshold chain length for activity—iNOS and cytokine responses are abolished when n=4 (**7b**) and n=6 (**7c**), respectively, and for shorter chain derivatives.

In general, increasing or decreasing the number of acyl residues present in disaccharide Lipid A derivatives from the optimum of six diminishes endotoxic activity.[140] The known[141] monosaccharide GLA-47, corresponding to the (tetraacylated) non-reducing sugar portion of compound **7f**, is devoid of activity in both the iNOS and cytokine models.[142] These observations are consistent with the hypothesis that subtle modifications to the hydrophobic side chains of Lipid A derivatives induce conformations that dramatically

affect cellular activation and the expression of endotoxic activities.[143] Indeed, Lipid A having six lipid chains has optimal inflammatory activity, Lipid A molecules with five lipid chains are 100-fold less active, and those with four lipid chains, such as Eritoran, lack agonistic activity completely.[144]

The contribution of acyl chains on ligand specificity and receptor activation mechanism of the TLR4–MD-2–LPS complex is evident in the crystal structure.[135] Thus, Park and coworkers demonstrated that binding of LPS induced the formation of an m-shaped receptor multimer composed of two copies of the TLR4–MD-2–LPS complex arranged symmetrically. LPS interacts with a large hydrophobic pocket in MD-2, whereby five of the six lipid chains of LPS are buried deep inside the pocket, and the remaining chain is exposed to the surface of MD-2. The latter forms a hydrophobic interaction with the conserved phenylalanine residues of TLR4. The F126 loop of MD-2 undergoes localized structural change and supports this core hydrophobic interface by making hydrophilic interactions with TLR4. Comparison with the structures of Eritoran (**3**, Fig. 3) and Lipid IV_A (**1**) bound to MD-2 indicates that two lipid chains in LPS displace the phosphorylated glucosamine backbone by 5 Å towards the solvent area.[128,145] This structural shift allows phosphate groups of LPS to contribute to receptor multimerization by forming ionic interactions with a cluster of positively charged residues in TLR4 and MD-2. Thus, the bioactivity of Lipid A is mainly influenced by the length, number, and symmetry of acyl chains, as well as the number and distribution of negative charges.[146]

Lipid A from the LPS of *Rhizobium sin*-1 (**8**, Fig. 5), a nitrogen-fixing bacterial species, is structurally unusual and differs in almost every aspect from endotoxic Lipid A molecules.[147] *R. sin*-1 Lipid A is devoid of phosphates, and a 2-amino-2-deoxy-D-gluconolactone moiety is present at the reducing end. In particular, it contains a very long-chain fatty acid, 27-hydroxyoctacosanoic acid, which can be esterified by 2-hydroxybutanoate. Since Lipid A of *R. sin*-1 (**8)** prevents the induction of TNFα by Lipid A of *E. coli*, Boons and coworkers decided to investigate the contribution of the unusual acyl chain to the antagonistic activity of **8**, and they synthesized derivatives **9a** and **9b**.[148] Their data show that the hydroxyl moiety of the 27-hydroxyoctacosanoic acid moiety of *R. sin*-1 Lipid A is not important for antagonistic properties, whereas shortening the octacosanoic acid moiety (**9b**) decreases the inhibitory potential. Derivative **9a**, which contains the octacosanoic acid moiety without the hydroxyl group, exhibits the same LPS-antagonistic activity as Lipid A of *R. sin*-1 (**8)**. It should also be noted that while performing detailed biological evaluations on synthetic *R. sin*-1 Lipid A derivatives, Boons and coworkers[149] observed that the ester group at C-3 of the reducing-end residue in **9a** is prone to elimination and readily produces enone derivative **10**. Also obtained was compound **11**, wherein the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether-linked moiety. Interestingly, compound **11**, which has a much improved chemical stability in comparison to that of **9a**, was found to be as potent as **9a** in antagonizing LPS-induced cytokine production by a human monocytic cell line. Moreover, compound **11** was found to inhibit both MyD88- and TRIF-dependent cell-signaling events.

The chemical and biological properties of *Porphyromonas gingivalis* LPS and its Lipid A are different from those of enterobacterial LPSs and their Lipid A structures.[150] The Lipid A

moiety of the LPS of *P. gingivalis* also displays considerable heterogeneity. The elucidated structures of four Lipid A molecules from *P. gingivalis* (**12–15**, Fig. 6) differ in their fatty acid substitution pattern. A common structural feature of these derivatives is the presence of unusual branched fatty acids, such as (*R*)-3-hydroxy-13-methyltetradecanoic acid and (*R*)-3-hydroxy-15-methyl hexadecanoic acid. In a recent report, innate host responses to Lipid A species from *P. gingivalis* LPS were found to be unusual in that these Lipid A molecules were able to function as an agonist for TLR2 and also as an antagonist or agonist for TLR4.[151] In order to identify the *P. gingivalis* Lipid A capable of antagonizing *E. coli* Lipid A, compounds **12–15** were chemically synthesized.[152] Thus, Ogawa and coworkers[152a] demonstrated that compounds **12** and **13** were agonists for TLR4 but not TLR2, and that both compounds were antagonists for *E. coli* Lipid A. On the other hand, Boons *et al.* showed that while compound **14** was a potent LPS antagonist, compound **15** showed significantly diminished activity.[152b] This prompted their conclusion that the acylation pattern of **14** is critical for optimal antagonistic activity.

2. Degree of Phosphorylation of the Di-Glucosamine Backbone

As mentioned earlier, the phosphorylation status of the di-glucosamine backbone is known to profoundly affects the biological activity of Lipid A.[134b–d] At the same time, it has been identified that Lipid A from *R. sin*-1, which lacks phosphate groups, does not stimulate production of TNFα by human monocytes[153] and prevents the induction of TNFα by *E. coli* LPS. The Lipid A of *R. sin*-1 is perhaps the most structurally unusual Lipid A reported to date; its structure (**8**, Fig. 5) differs in almost every aspect from those known to contribute to the toxicity of enteric Lipid A.[147] In particular, the disaccharide moiety of rhizobial Lipid A is devoid of phosphate groups and the glucosamine phosphate is replaced by 2-amino-2-deoxy-D-glucono-1,5-lactone. The microheterogeneity of rhizobial Lipid A limits the identification of specific structural features that make it an antagonist rather than an agonist. To study the contribution of the reducing-end phosphate group to the antagonistic activity of *R. sin-1* Lipid A, Boons and coworkers[153a] synthesized compounds **16** and **17** (Fig. 7) and compared their biological activity. Thus, they found that **17** was able to antagonize *E. coli* LPS, while **16** was devoid of this activity. These results suggest that the gluconolactone moiety is important for this property. Compound **17** is the first example of a synthetic Lipid A derivative that lacks phosphate groups and inhibits production of cytokine initiated by *E. coli* LPS.

On a similar note, Ribi and coworkers[154] found an LPS-mimetic compound that exhibits potent adjuvant activity, but is 100- to 10,000-fold less toxic than LPS.[154,155] This compound, MPL,[156] is a Lipid A derivative that lacks the phosphate moiety at the reducing-end glucosamine residue. To understand the molecular mechanism underlying the low toxicity of MPL, Nishijima and coworkers[157] examined the effects of *E. coli* Lipid A and MPL on the production of IL-1β and the activation of caspase-1 in mouse peritoneal macrophages. They found that MPL is defective in the induction of IL-1β secretion and is incapable of activating caspase-1. Since caspase-1 has been shown to be essential for the induction of endotoxin shock (via caspase-1-deficient mice),[158] these results suggest that the lack of caspase-1 activation in MPL-stimulated macrophages contributes to the low toxicity of MPL. Similarly, Mitchell and coworkers[159] reported that the low toxicity of MPL in mice

is associated with a bias toward TRIF signaling. To determine whether alteration of a single phosphate group can cause TRIF-biased signaling, Mitchell and coworkers[160] performed extensive comparisons of the signaling activities of synthetic MPL (sMPL) and diphosphate Lipid A (sDPL), in the context of an *E. coli*-type Lipid A structure. They found that sMPL largely retained their TRIF bias as compared to sDPL of *E. coli*, indicating that the loss of a single phosphoryl group is sufficient to bring about TRIF-biased activation of TLR4.

3. Changes in the β-(1→6)-Linked-Di-Glucosamine Backbone

Partial structures of Lipid A have been useful in investigating the mechanism of LPS binding and cell activation. These include LPS antagonists such as deacylated LPS, lipid IV_A, and *R. sphaeroides* Lipid A,[161] as well as several unnatural synthetic Lipid A-like structures.[162] An example for the latter are derivatives synthesized by Shiozaki and coworkers,[163] whereby they altered the β-(1→6)-linked di-glucosamine backbone common to natural Lipid A structures with a β-(1→6)-linked glucosamine-glucose disaccharide. They found that these novel derivatives, based on the structure of Eritoran (**3**, Fig. 3), had almost the same (or stronger) LPS-antagonistic activities toward both human blood cells and murine macrophages, as compared to classic Lipid A-type disaccharides having the glucosamine–glucosamine moiety. Following their initial success, Shiozaki's group decided to reverse their aforementioned backbone design and created Lipid A derivatives featuring β-(1→6)-linked glucose-glucosamine backbones (**18–23**, Fig. 8).[164] Thus, they found that, except for compound **21**, these synthetic derivatives exhibited LPS-antagonistic activity comparable to *R. sphaeroides* Lipid A. Compounds **20** and **23** were even more potent than E5564 (**3**) in inhibiting production of TNFα in LPS-challenged mice, while compound **19** was more potent than **3** in protecting mice from lethal LPS challenge.

Peri and coworkers[165] developed synthetic Lipid A derivatives that, on the other hand, largely depart from natural Lipid A structural motifs. Thus, compound **24** (Fig. 9) features a β-(1→6)-N(OMe)-linked di-glucose backbone, four linear C-14 hydrophobic alkyl chains, and no phosphate groups, while compound **25** is the β-O-linked analogue of **24**. Compounds **24** and **25** antagonized the inflammatory effect of *E. coli* Lipid A on MT2 macrophages and did not exhibit proinflammatory effects on the same cell lines. Their most significant result, however, is the observation that N- and O-linked disaccharides have very similar activities. It indicates that the chemical nature of the interglycosidic bridge does not influence LPS antagonist activity.

In another example of exploring structure–function relationships in the LPS receptor complex with nonnatural Lipid A derivatives, Zamyatina and coworkers[166] synthesized Lipid A mimetics featuring a β,α−(1→1′)-linked di-glucosamine scaffold (**26**–**32**, Fig. 9). With the restricted internal flexibility imposed on the di-glucosamine backbone, these trehalose-type derivatives aim to elucidate the parameters whereby the three-dimensional molecular shape of MD-2-bound Lipid A/Lipid IV_A determines endotoxicity. Thus, they found that the variably acylated Lipid A mimetics **26–32** lacked proinflammatory activities in hTLR4/MD-2-transfected HEK293 cells. Except for compound **26**, which is a conformationally constrained counterpart of Lipid IV_A, the compounds were shown to potently inhibit proinflammatory responses in TLR4-transfected HEK293 cells stimulated

with *E. coli* Lipid A (**4**, Fig. 3). The failure of **26** to compete with **4** for the binding site on MD-2 could be due to an increase in the hydrophobic volume of the lipid chains, which would prevent the ligand from entering the binding groove of MD-2. These Lipid A mimics were further examined for their capacity to inhibit *E. coli* O111 LPS-induced activation of the TLR4/MD-2 complex in TLR4-transfected HEK293 cells. Upon application of **27** and **29** at a concentration of 500 ng/mL, the inflammatory responses to *E. coli* LPS were entirely abrogated. The shorter chain Lipid A mimetics **31**, **29**, and **30** allowed for 50% inhibition at the submolar concentration of 5 ng/mL. Compound **32**, which contains four (*R*)-3-hydroxydecanoic acid groups, was shown to suppress cell activation elicited by **4**, but did not antagonize *E. coli* LPS. Thus, with its smaller hydrophobic volume, compound **32** is capable of competing with Lipid A for the binding site on hMD-2, but is incapable of displacing LPS, which possesses a higher affinity for the MD-2/TLR4 complex than the corresponding Lipid A.[167] Overall, these results define a crucial role of the inherent plasticity of the carbohydrate backbone of Lipid A—it decides the relocation of a single lipid chain onto the surface of MD-2 in the ligand–receptor structure–function relationships. Replacement of the flexible (1→6)-linked Lipid A backbone by a conformationally constrained trehalose-type scaffold resulted in abrogation of species-specific agonistic activity of lipid IV_A. Consequently, manipulating conformational flexibility of the carbohydrate backbone of Lipid A is a useful tool in the rational design of immunomodulating therapeutics targeting the LPS receptor complex.

Lipid A analogues that lack the disaccharide backbone, that is to say monosaccharide Lipid A mimetics, have also been used to study the structure–activity relationship of the LPS receptor complex. The structural simplicity of these truncated molecules allows for abridged synthetic routes and easier access to a broad library of analogues for chemical genetics. GLA-60 (**33**, Fig. 10)—a synthetic monosaccharide Lipid A analogue having an ester-branched acyl group—has been used extensively as a model structure for structure–activity studies of this type on account of its broad endotoxic activities. To this end, Matsuura and coworkers[168] altered the ester-branched acyl group of GLA-60 into alkyl-branched types (**34–40**, Fig. 10) and evaluated the biological activities of the corresponding analogues to determine the role of a branched side-chain in the expression of endotoxic activities. In terms of ability to induce TNFα production, compounds **34** and **35** were found to be more potent than **33**, but not as potent as *E. coli* Lipid A. Compound **36** was found to be less potent than **33** in inducing production of TNFα, and **37** was even weaker. Similar results were obtained when compounds **33–37** were tested for (1) ability to activate macrophages from LPS-nonresponsive C3H/HeJ mice and (2) tolerance-inducing potency against LPS-stimulated macrophage activation. Compounds **38**–**40**, on the other hand, were found to be more potent than **33** in terms of induction of TNFα expression, mitogenic activity, and LPS lethality. Since the results were comparable to that of **36** and **37**, it can be concluded that branching position has a very slight contribution in the expression of endotoxic activities. These studies demonstrate that the usual ester-branched acyl groups in Lipid A analogues can be replaced by alkyl-branched acyl groups with no great consequence to endotoxic activities.

Subsequently, Matsuura and coworkers proceeded to clarify the structure–activity relationship of monosaccharide Lipid A analogues having different acylation patterns in terms of production of nitric oxide (NO) and cytokine (TNFα, IL-6) in murine macrophages. Briefly, the stimulatory effects of analogues **41–45** (Fig. 10) on the production of NO varied from strongly positive to negative, depending on their structure, and the intensity of activity correlated well with cytokine production. These results strongly suggest the existence of closely related regulatory mechanisms and signaling pathways for the three inflammatory mediators studied. Compound **43** was previously reported[169] to possess the ability to protect mice from LPS lethality under D-galactosamine-sensitized conditions (*in vivo* protective activity) and the potency to induce a hyporesponsive state to LPS stimulation in macrophages. In this study, compound **43** also demonstrated antagonistic activity against LPS, *E. coli* Lipid A, and **33** for induction of NO, TNFα, and IL-6 when it coexisted with the stimulants.

Following the lead of compound **43**,[170] as well as their previous work with nonphosphorylated N(OMe)-linked disaccharides,[165] Peri, Nicotra, and coworkers synthesized monosaccharide Lipid A analogues that featured no phosphate groups, a glucose backbone, and an amine (**46**), ammonium species (**47**), and hydroxylamine (**48**) moiety (Fig. 11).[171] In terms of inhibiting Lipid A-induced TNFα expression in bone-marrow derived macrophages and dendritic cells, compounds **46** and **47** interfered with Lipid A activity in a dose-dependent manner, while compound **48** showed consistently lower activity. Compound **47** was more potent than **46**, which in turn was more potent than **48**. The activity of the most promising lead, compound **47**, was then analyzed in more detail by monitoring the production of another inflammatory cytokine, IL-1β. Thus, **47** inhibited IL-1β and TNFα expression in macrophages and dendritic cells, and the inhibitory effect was proportional to the concentration of the inhibitor. To evaluate the selectivity for TLR4, compound **47** was investigated for TNFα expression in response to stimulation by the CpG motif of bacterial DNA (TLR9 ligand) and trihexadecanoyl cysteine (Pam3Cys-SK4, TLR2 ligand). Production of cytokine was not inhibited by **47** in either the TLR9- or the TLR2-mediated inflammatory cascade, indicating a relevant level of selectivity. Further direct evidence that the activity of compound **47** is selective for the TLR4 receptor was obtained with experiments on a TLR4- and TLR9-transfected HEK 293 cell system. Thus, **47** was able to counteract significantly the effect of Lipid A in TLR4-transfected cells, whereas it was inactive at the maximal dose of 50 mm in influencing the effect of CpG on TLR9-transfected HEK 293.

In another example of rational designs for monosaccharide Lipid A analogues, Fukase and coworkers[134a] synthesized a series of Lipid A analogues to investigate the structure–activity relationships governing the biological activity of triacyl-type Lipid A analogue **49**, which was found to be an LPS antagonist (Fig. 12). In analogues **50–55**, the nonreducing end of Lipid A (3-phosphorylated glucosamine) was substituted with an acidic amino acid, such as aspartic acid or phosphoserine, and the acylation arrangement was varied. These analogues were evaluated for induction of human cytokines and inhibitory activities against LPS. In terms of cytokine induction, most analogues exhibited no induction of IL-6, whereas tetraacylated **54a** exhibited concentration-dependent induction at ≥100 ng/mL. Compound

54b also exhibited IL-6 induction at 10 μg/mL concentration, although the activity was about 100-fold weaker than that of **54a**. As for inhibition of LPS-induced cytokine production, only analogues **53a** and **54a** failed to exhibit inhibitory activities. The phosphoserine-containing analogues showed stronger inhibitory activities than the corresponding aspartic acid-containing analogues that feature the same acylation pattern.

Obvious trends were also observed between the inhibitory activities and the acylation patterns. In either enantiomeric form (D- and L-phosphoserine), the acylation pattern of compound **51** gave the highest inhibitory activity. The acylation pattern in **50** allowed for slightly weaker activity than that of **51**, but it was slightly stronger than that of **52**. For the aspartic acid analogues, the L-form showed stronger inhibition than the D-form, whereas the D- and L-phosphoserine analogues showed no significant differences. In the Limulus test, the expression of Limulus activity was dramatically influenced by the acylation patterns. This result clearly showed that structural requirements for expression of the Limulus activity are different from cytokine-inducing activities or LPS-antagonistic activities.

Following Peri's[171] and Fukase's lead,[134a] Demchenko and coworkers[172] proceeded to synthesize Lipid A analogues containing a glucopyranoside core, hydrophobic ether substituents, and an amino acid moiety to provide ionic character to the constructs. The inhibitory activity of compounds **56** and **57** (Fig. 13) on LPS-induced TNFα expression was investigated *in vitro* using THP-1 macrophages. Compounds **56a**, **57a**, and **57b** exhibited no inhibitory activity against LPS-induced TNFα expression in the concentration range of 0.1–10 mM. Compound **56b**, which has a free carboxylic group, was able to significantly inhibit LPS-induced expression of TNFα at concentrations greater than 10 μM. Unfortunately, cell viability measurements indicated that compound **56b** was toxic to the cells in the 30–100 mM range. Indeed, the similarities between the inhibition and toxicity curves suggested that much of the antagonistic activity by **56b** was related to toxicity. With compound **47** as a positive control, lipidated compound **58** displayed a marked enhancement in LPS-antagonistic ability (550 nM range). It was able to inhibit 80% of the LPS response at a concentration of 5 mM, with no observable toxicity. Cell viability began to be compromised at 10 mM, and some agonist activity was found in the 10–30 mM range. Alkylated compound **59**, on the other hand, demonstrated 70% inhibition of LPS-induced TNFα expression at a concentration of 1 mM and reached 90% inhibition at 40 mM. Compound **59** exhibited no toxicity or agonist activity within the 0.2–40 mM range. These results were better than those obtained with compound **47**, which had an inhibition range of 3–10 mM and began to show agonist activity at concentrations >10 mM. Overall, these studies validate the conclusion that Lipid A analogues that lack the di-glucosamine backbone, phosphate moieties, and typical acylation patters can still demonstrate significant antagonistic activity toward LPS-induced cytokine production.

In a similar effort to define the parameters of antagonistic activity by monosaccharide Lipid A analogues, Johnson and coworkers[134d] identified a new class of potent monosaccharide immunomodulators called aminoalkyl glucosaminide 4-phosphates (AGPs), wherein the less-conserved reducing-sugar unit of Lipid A is substituted with a flexible *N*-acyloxyacyl aglycone unit.[142] The flexible AGP motif permits energetically favored close packing[173] of fatty acid moieties, facilitating intercalation of the lipids into the hydrophobic pocket of

MD-2. In addition, the carboxyl group of seryl-based AGPs serves as a stable bioisostere of the labile anomeric phosphate of Lipid A, which, along with phosphate groups, presumably binds electrostatically to lysine residues along the edge of the hydrophobic pocket of MD-2.[95] Among seryl-based AGPs, compounds **60** and **61** (Fig. 14) containing 10-carbon and 6-carbon secondary acyl residues, respectively, have been found to exhibit potent TLR4 agonist and antagonist activity, respectively, in both murine and human models.[174] In order to overcome the inherent chemical and metabolic instability of the ester-linked secondary fatty acids present in **60** and **61**, and to further evaluate structural modifications in the AGP series, the corresponding ether-linked lipid analogues **62** and **63** (Fig. 14) were synthesized.[134d] Thus, it was found that **60** and its ether analogue **62** exhibited similar abilities in inducing TNFα expression in human monocytes, diminishing splenic bacteria following a Listeria challenge, and providing protection from a lethal influenza challenge. In contrast, neither **61** nor its ether analogue **63** induced detectable cytokines in human cell assays. However, both compounds **61** and **63** were able to inhibit LPS-induced expression of TNFα in human monocytes. Compound **61** effectively inhibited expression of serum TNFα when coadministered intravenously with LPS, but **63** did not demonstrate any antagonist activity. The weak TLR4 agonist activity of ether analogue **63** *in vivo* is further exemplified by its ability to induce TNFα when administered intravenously to mice—a dose–response comparison of serum TNFα levels induced by **60** and **63** showed that **63** was between 10 and 100 times less active than **61**. Overall, ether lipids **62** and **63** exhibited similar TLR4 agonist and antagonist activities, respectively, as compared to their ester analogs **60** and **61**. Unlike the potent TLR4 antagonist **61**, the ether lipid **63** was a weak agonist in murine models, suggesting that one or more of the ester carbonyl groups in **61** play a pivotal role in binding to murine MD-2 and preventing TLR4 activation.

Following Johnson's lead,[134d] Jiang and coworkers[132] synthesized Lipid A mimics (**64**–**66**, Fig. 14) wherein the reducing glucosamine residue of the archetypal disaccharide scaffold in Lipid A has been replaced by an acylated diethanolamine moiety. The design of mimics **64**–**66** was based on the following considerations: (a) conservation of essential functional groups involved in TLR4/MD-2 ligand binding, namely, the phosphate and fatty acid chains; (b) conservation of the glycosidic linkage; and (c) the appropriate location of each functional group through the diethanolamine moiety. Thus, it was found that all three compounds (**64**–**66**) showed no cell toxicity at the highest concentrations tested for each, as measured via Trypan Blue exclusion. Compound **64** and **66** increased ICAM-1 expression in human THP-1 cells, but **66** was found to be less potent than **64**. The maximum ICAM-1 expression level was achieved at a concentration of 2.0 mM for both. In contrast, no significant increase in ICAM-1 expression level is noted for the octa-acylated analogue **65** at the highest concentration tested (4.0 mM). To further characterize the immunostimulatory properties of **64**–**66**, production of TNFα, IL-6, and IL-1β cytokines was also measured. In general, the responses measured for all three cytokines mirror that of the response for ICAM-1 expression—compound **64** showed the greatest potency, compound **66** showed a slightly decreased potency, and compound **65** induced very little to no response. While the octa-acylated analogue **65** is inactive in the induction of ICAM-1 and other cytokines tested, it induces a significant level of IL-1β at the concentration of 9 mM. Building on the results of **64**, Jiang and coworkers[175] synthesized compounds **67** and **68** (Fig. 14) with the view of

improving the potency of **64** as a TLR4 agonist. Thus, compounds **67** and **68** showed increased potency over **64**; mimic **68** showed the greatest potency for stimulating production of all three cytokines (TNFα, IL-6, and IL-1β). In comparison with the terminal free hydroxyl group in **64**, the terminal phosphate group in **67** and the terminal acidic moiety in **68** increased the potency of the immunostimulatory response, with the terminal acidic moiety contributing more toward enhancing the potency. Competitive-inhibition studies with lipid IVa, a known human TLR4 antagonist, confirm TLR4 as the target of diethanolamine-containing Lipid A mimics.

To further define structure–activity relationships that influence the LPS-antagonistic activity of compounds **46** and **47** (Fig. 11), Peri and coworkers[176] synthesized compounds **69–73** (Fig. 15). The activity of compounds **46**, **47**, and **69–73** as inhibitors of the TLR4 signal pathway was tested *in vitro* using a HEK-Blue LPS Detection Kit. They found that compounds **47**, **71**, **72**, and **73** exhibited significant inhibition of Lipid A activity in a concentration-dependent manner. The cytotoxic potentials of **47**, **71**, **72**, and **73** were also measured and it was found that **47**, **71** and **72** showed no inhibitory effects on cell viability. Compound **73**, in contrast, showed a diminution of the cell viability of about 17%. Finally, they tested compounds **46**, **47**, **71**, and **73** for protective capacity against LPS-induced lethality *in vivo*. The administration of 10 mg/kg of **47**, **71**, and **73** significantly increased survival of mice against LPS-induced lethality. In particular, compound **47** increased the survival rate from 0% to 25%, compound **73** from 0% to 67%, and all mice that received compound **71** survived. Indeed, at a smaller dose of 3 mg/kg, compound **71** evoked a 100% survival of mice treated with LPS. Compound **46**, which has the weakest TLR4 antagonistic activity *in vitro*, was found to be ineffective in the lethal endotoxin-shock model. In order to clarify the mode of action of **47** and **71**, Peri and coworkers[176b] analyzed possible interactions with the extracellular components that bind and shuttle endotoxin to TLR4, namely, LBP, CD14, and MD-2. Briefly, their data strongly suggest that compounds **47** and **71** inhibit TLR4 activation by competitively occupying CD14, thereby inhibiting the delivery of active endotoxin to the MD-2/TLR4 complex.

VI. Summary and Conclusions

Mortality from Gram-negative sepsis remains a serious problem and the concomitant challenges continue to be intimidating. After several decades of vigorous research in the field, several carefully designed pharmaceutical approaches to modifying the clinical outcome of sepsis have failed. As with any illness, a true understanding of the pathophysiology of disease is a critical step in designing effective remedies. The recent determination of crystal structures of the dimeric TLR4–MD-2 complex with bound endotoxic *E. coli* Lipid A or LPS antagonists (Eritoran, Lipid IV_A) clarified important aspects of the structure–activity relationship in bacterial Lipid A, the endotoxic principle of LPS. Investigations on compounds that can modulate the LPS–receptor complex, such as the synthetic Lipid A analogues outlined in this chapter, not only offer novel pharmacological targets but also contribute to the clarification of basic structural and mechanistic aspects of TLR4 signaling, including the role of LBP, CD14, and MD-2 coreceptors. The identification of new pharmacological targets for LPS-mediated diseases should lead to renewed optimism that effective therapies against Gram-negative sepsis will ultimately be achieved.

Acknowledgments

AVD is thankful to the National Institute of General Medical Sciences (Award GM111835) for providing generous support for synthetic and biomedical studies in his group.

Abbreviations

AGPs	aminoalkyl glucosaminide 4-phosphates
BPI	bactericidal/permeability-increasing protein
CD	cluster of differentiation
CETP	cholesteryl ester transfer protein
CHO	Chinese hamster ovary
DIC	disseminated intravascular coagulation
EGFP	enhanced green fluorescent protein
GPI	glycosyl phosphatidylinositol
HDL	high-density lipoprotein
iNOS	nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
LBP	lipopolysaccharide-binding protein
MCP	monocyte chemoattractant protein
MD	myeloid differentiation antigen
MPL	monophosphoryl Lipid A
MPs	mononuclear phagocytes
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-kappa B
PAI-1	plasminogen activator inhibitor-1 protein
PAMPs	pathogen-associated molecular patterns
PLTP	phospholipid transfer protein
PMNs	polymorphonuclear leukocytes
PRRs	pattern-recognition receptors
SIRS	systemic inflammatory response syndrome
TAT	thrombin–antithrombin
TF	tissue factor
TLR	Toll-like receptor
TRIF	TLR-domain-containing adapter-inducing interferon-β

References

1. Marshall JC. Lipopolysaccharide: An endotoxin or an exogenous hormone? Clin Infect Dis. 2005; 41:S470–S480. [PubMed: 16237650]
2. Dyall SD, Brown MT, Johnson PJ. Ancient invasions: From endosymbionts to organelles. Science. 2004; 304:253–257. [PubMed: 15073369]
3. (a) Veldhuyzen van Zanten SJO, Sherman PM. *Helicobacter pylori* infection as a cause of gastritis, duodenal ulcer, gastric cancer and nonulcer dyspepsia: A systematic overview. CMAJ. 1994; 150:177–185. [PubMed: 8287340] (b) Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. JAMA. 2002; 288:2724–2731. [PubMed: 12460096]
4. Medzhitov R, Janeway CAJ. Innate immunity. New Engl J Med. 2000; 434:338–344. [PubMed: 10922424]
5. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/ Society of Critical Care Medicine. Chest. 1992; 101:1644–1655. [PubMed: 1303622]
6. Pinsky MR. Sepsis: A pro-and anti-inflammatory disequilibrium syndrome. Contrib Nephrol. 2001; 132:354–366. [PubMed: 11395903]
7. Bone RC, Sprung CL, Sibbald WJ. Definitions for sepsis and organ failure. Crit Care Med. 1992; 20:724–726. [PubMed: 1597021]
8. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epdemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. Crit Care Med. 2001; 29:1303–1310. [PubMed: 11445675]
9. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. The dorsoventral regulatory gene cassette spaetzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell. 1996; 86:973–983. [PubMed: 8808632]
10. Michel T, Reichhart J-M, Hoffmann JA, Royet J. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature. 2001; 414:756–759. [PubMed: 11742401]
11. Poltorak A, He X, Smirnova I, Liu M-Y, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/20ScCr mice: Mutations in Tlr4 gene. Science. 1998; 282:2085–2088. [PubMed: 9851930]
12. (a) Pfeiffer R. Untersuchungen über das Choleragift. Z Hyg. 1892; 11:393–411.(b) Centanni E. Untersuchungen über das Infektionsfieber – das Fiebergift der Bakterien. Dtsch Med Wochenschr. 1894; 20:148–153.(c) Rietschel ET, Brade H. Bacterial endotoxins. Sci Am. 1992; 267:54–61. [PubMed: 1641625] (d) Beutler B, Th Rietschel E. Innate immune sensing and its roots: the story of endotoxin. Nature Rev Immunol. 2003; 3:169–176. [PubMed: 12563300]
13. Boivin A, Izard Y. Method for the purification of diphtheria, tetanus and staphylococcus toxins and anatoxins by trichloroacetic acid. C R Soc Biol. 1937; 124:25–28.
14. Westphal O, Lüderitz O. Chemical and biological analysis of highly purified bacterial polysaccharides. Dtsch Med Wochenschr. 1946; 78:17–19. [PubMed: 13067988]
15. Van Amersfoort ES, Van Berkel TJC, Kuiper J. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev. 2003; 16:379–414. [PubMed: 12857774]
16. Tacken A, Rietschel ET, Brade H. Methylation analysis of the heptose/3-deoxy-D-*manno*-2-octulosonic acid region (inner core) of the lipopolysaccharide from *Salmonella minnesota* rough mutants. Carbohydr Res. 1986; 149:279–291. [PubMed: 3756946]
17. Heinrichs DE, Yethon JA, Whitfield C. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol Microbiol. 1998; 30:221–232. [PubMed: 9791168]
18. Imoto M, Kusumoto S, Shiba T, Naoki H, Zähringer U, Rietschel ET, Unger FM. Structural and synthetic study on lipopolysaccharide of *Escherichia coli* Re mutant. Tennen Yuki Kagobutsu Toronkai Koen Yoshishu. 1985; 27:585–592.

19. Weckesser J, Mayer H, Drews G, Fromme I. Lipophilic O-antigens containing D-*glycero*-D-*manno*heptose as the sole neutral sugar in *Rhodopseudomonas gelatinosa*. J Bacteriol. 1975; 123:449–455. [PubMed: 1150623]
20. (a) Schweda EKH, Richards JC, Hood DW, Moxon ER. Expression and structural diversity of the lipopolysaccharide of *Haemophilus influenzae: Implication in virulence*. Int J Med Microbiol. 2007; 297:297–306. [PubMed: 17452015] (b) Sadovskaya I, Brisson JR, Thibault P, Richards JC, Lam JS, Altman E. Structural characterization of the outer core and the O-chain linkage region of lipopolysaccharide from *Pseudomonas aeruginosa serotype O5*. Eur J Biochem. 2000; 267:1640–1650. [PubMed: 10712594] (c) Cox AD, Brisson J, Varma V, Perry MB. Structural analysis of the lipopolysaccharide from *Vibrio cholerae O139*. Carbohydr Res. 1996; 290:43–58. [PubMed: 8805781] (d) Muller-Loennies S, Linder B, Brade H. Structural analysis of oligosaccharides from lipopolysaccharide (LPS) of *Escherichia coli K12 strain W3100 reveals a link between inner and outer core LPS biosynthesis*. J Biol Chem. 2003; 278:34090–34101. [PubMed: 12819207]
21. (a) Stanislavskii ES, Mashilova GM, Dmitriev BA, Knirel YA, Vinogradov EV. The structure and immunochemical specificity of O-antigens of 03 serogroup *Pseudomonas aeruginosa*. J Hyg Epidemiol Microbiol Immunol. 1984; 29:289–295.(b) Richards JC, Leitch RA. Elucidation of the structure of the *Pasteurella haemolytica serotype T10 lipopolysaccharide O-antigen by n.m.r spectroscopy*. Carbohydr Res. 1989; 186:275–286. [PubMed: 2472201] (c) Stanislavsky ES, Kholodkova EV, Knirel YA, Kocharova NA. Saccharides of seven *Pseudomonas aeruginosa immunotypes*. FEMS Microbiol Immunol. 1989; 1:245–251. [PubMed: 2483524] (d) Whitfield C, Richards JC, Perry MB, Clarke BR, MacLean LL. Expression of two structurally distinct D-galactan O antigens in the lipopolysaccharide of *Klebsiella pneumoniae serotype O1*. J Bacteriol. 1991; 173:1420–1431. [PubMed: 1704883]
22. Lerouge I, Vanderleyden J. O-antigen structural variation: Mechanisms and possible roles in animal/plant microbe interactions. FEMS Microbiol Rev. 2002; 26:17–47. [PubMed: 12007641]
23. Wiese A, Gutsmann T, Seydel U. Towards antibacterial strategies: Studies on the mechanisms of interactions between antibacterial peptides and model membranes. J Endotoxin Res. 2003; 9:67–84. [PubMed: 12803879]
24. Lüderitz O, Freudenberg MA, Galanos C, Lehmann V, Rietschel ET, Shaw DH. Lipopolysaccharides of gram-negative bacteria. Curr Top Membr Transp. 1982; 17:79–151.
25. Westphal O, Lüderitz O. Chemical research on lipopolysaccharides of gram-negative bacteria. Angew Chem. 1954; 66:407–417.
26. Imoto M, Yoshimura H, Sakaguchi N, Kusumoto S, Shiba T. Total synthesis of *Escherichia coli* Lipid A. Tetrahedron Lett. 1985; 26:1545–1548.
27. (a) Galanos C, Lüderitz O, Rietschel ET, Westphal O, Brade H, Brade L, Freudenberg M, Schade U, Imoto M, Yoshimura H, Kusumoto S, Shiba T. Synthetic and natural *Escherichia coli* free Lipid A express identical endotoxic activities. Eur J Biochem. 1985; 148:1–5. [PubMed: 2579812] (b) Kotani S, Takada H, Tsujimoto M, Ogawa T, Takashi I, Ikeda T, Otsuka K, Shimauchi H, Kasai N. Synthetic Lipid A with endotoxic and related biological activities comparable to those of a natural Lipid A from an Escherichia coli Re-mutant. Infect Immun. 1985; 49:225–237. [PubMed: 3891627] (c) Takada H, Kotani S. Structural requirements of Lipid A for endotoxicity and other biological activities. Crit Rev Microbiol. 1989; 16:477–523. [PubMed: 2663021]
28. Mitchell JA, Paul-Clark MJ, Clarke GW, McMaster SK, Cartwright N. Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. J Endocrinol. 2007; 193:323–330. [PubMed: 17535871]
29. Freudenberg MA, Freudenberg N, Galanos C. Time course and cellular distribution of endotoxin in liver, lungs, and kidney of rats. Br J Exp Pathol. 1982; 63:56–65. [PubMed: 7039654]
30. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000; 406:782–787. [PubMed: 10963608]
31. Tobias PS, Soldau K, Ulevitch RJ. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J Exp Med. 1986; 164:777–793. [PubMed: 2427635]
32. Ramadori G, Meyer zum Buschenfelde KH, Tobias PS, Mathison JC, Ulevitch RJ. Biosynthesis of lipopolysaccharide-binding protein in rabbit hepatocytes. Pathobiology. 1990; 58:90–94.

33. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. Science. 1990; 249:1429–1431. [PubMed: 2402637]
34. (a) Zweigner J, Schumann RR, Weber JR. The role of lipopolysaccharide-binding protein in modulating the innate immune response. Microbes Infect. 2006; 8:946–952. [PubMed: 16483818] (b) Su GL, Frees-wick PD, Geller DA, Wang Q, Shapiro RA, Wan Y-H, Billiar TR, Tweardy DJ, Simmons RL, Wang SC. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence of extraheptic expression. J Immunol. 1994; 153:743–752. [PubMed: 8021509]
35. Beamer LJ, Carroll SF, Eisenberg D. Crystal structure of human BPI and two bound phospholipids at 2.4 Å ngstrom resolution. Science. 1997; 276:1861–1864. [PubMed: 9188532]
36. Beamer LJ, Carroll SF, Eisenberg D. The BPI/LBP family of proteins: A structural analysis of conserved regions. Protein Sci. 1998; 7:906–914. [PubMed: 9568897]
37. Lamping N, Hoess A, Yu B, Park TC, Kirschning CJ, Pfeil D, Reuter D, Wright SD, Herrmann F, Schumann RR. Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. J Immunol. 1996; 157:4648–4656. [PubMed: 8906845]
38. (a) Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med. 1994; 179:269–277. [PubMed: 7505800] (b) Yu B, Wright SD. Catalytic properties of lipopolysaccharide (LPS) binding protein. Transfer of LPS to soluble CD14. J Biol Chem. 1996; 271:4100–4105. [PubMed: 8626747]
39. (a) Wurfel MM, Hailman E, Wright SD. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide (LPS) by LPS-binding protein and reconstituted high density lipoprotein. J Exp Med. 1995; 181:1743–1754. [PubMed: 7536794] (b) Gutsmann T, Muller M, Carroll SF, MacKenzie RC, Wiese A, Seydel U. Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. Infect Immun. 2001; 69:6942–6950. [PubMed: 11598069]
40. Theofan G, Horwitz AH, Williams RE, Liu PS, Chan I, Birr C, Carroll SF, Meszaros K, Parent JB. An amino-terminal fragment of human lipopolysaccharide-binding protein retains lipid A binding but not CD14-stimulatory activity. J Immunol. 1994; 152:3623–3629. [PubMed: 7511654]
41. Schumann RR, Lamping N, Hoess A. Interchangeable endotoxin-binding domains in proteins with opposite lipopolysaccharide-dependent activities. J Immunol. 1997; 159:5599–5605. [PubMed: 9548502]
42. Martin TR, Mathison JC, Tobias PS, Leturcq DJ, Moriarty AM, Maunder RJ, Ulevitch RJ. Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. J Clin Invest. 1992; 90:2209–2219. [PubMed: 1281827]
43. Tobias PS, Soldau K, Gegner JA, Mintz D, Ulevitch RJ. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. J Biol Chem. 1995; 270:10482–10488. [PubMed: 7537731]
44. Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. J Biol Chem. 1995; 270:5320–5325. [PubMed: 7534294]
45. Ulevitch RJ. Recognition of bacterial endotoxins by receptor-dependent mechanisms. Adv Immunol. 1993; 53:267–289. [PubMed: 7685560]
46. (a) Wright SD, Detmers PA, Aida Y, Adamowski R, Anderson DC, Chad Z, Kabbash LG, Pabst MJ. CD18-deficient cells respond to lipopolysaccharide *in vitro*. J Immunol. 1990; 144:2566–2571. [PubMed: 1969452] (b) Wright SD, Tobias PS, Ulevitch RJ, Ramos RA. Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J Exp Med. 1989; 170:1231–1241. [PubMed: 2477488]
47. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990; 249:1431–1433. [PubMed: 1698311]

48. Golenbock DT, Liu Y, Millham FH, Freeman MW, Zoeller RA. Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. J Biol Chem. 1993; 268:22055–22059. [PubMed: 7691822]

49. Lee JD, Kato K, Tobias PS, Kirkland TN, Ulevitch RJ. Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. J Exp Med. 1992; 175:1697–1705. [PubMed: 1375269]

50. Kirkland TN, Finley F, Leturcq D, Moriarty A, Lee JD, Ulevitch RJ, Tobias PS. Analysis of lipopolysaccharide binding by CD14. J Biol Chem. 1993; 268:24818–24823. [PubMed: 7693705]

51. (a) Bazil V, Horejsi V, Baudys M, Kristofova H, Strominger JL, Kostka W, Hilgert I. Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. Eur J Immunol. 1986; 16:1583–1589. [PubMed: 3493149] (b) Bazil V, Baudys M, Hilgert I, Stefanova I, Low MG, Zbrozek J, Horejsi V. Structural relationship between soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. Mol Immunol. 1989; 26:657–662. [PubMed: 2779588]

52. (a) Simmons DL, Tan S, Tenen DG, Nicholson-Weller A, Seed B. Monocyte antigen CD14 is a phospholipid anchored membrane protein. Blood. 1989; 73:284–289. [PubMed: 2462937] (b) Landmann R, Wesp M, Dukor P. Modulation of interferon-gamma-induced major histocompatibility (MHC) and CD14 antigen changes by lipophilic muramyltripeptide MTP-PE in human monocytes. Cell Immunol. 1988; 117:45–55. [PubMed: 2460255]

53. Ferrero E, Goyert SM. Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. Nucleic Acids Res. 1988; 16:4173. [PubMed: 2453848]

54. Setoguchi M, Nasu N, Yoshida S, Higuchi Y, Akizuki Si, Yamamoto S. Mouse and human CD14 (myeloid cell-specific leucine-rich glycoprotein) primary structure deduced from cDNA clones. Biochim Biophys Acta. 1989; 1008:213–222. [PubMed: 2472171]

55. (a) Juan TSC, Kelley MJ, Johnson DA, Busse LA, Hailman E, Wright SD, Lichtenstein HS. Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular response to LPS. J Biol Chem. 1995; 270:1382–1387. [PubMed: 7530712] (b) Yu W, Soprana E, Cosentino G, Volta M, Lichenstein HS, Viale G, Vercelli D. Soluble CD141-152 confers responsiveness to both lipoarabinomannan and lipopolysaccharide in a novel HL-60 cell bioassay. J Immunol. 1998; 161:4244–4251. [PubMed: 9780199]

56. Stelter F, Bernheiden M, Menzel R, Jack RS, Witt S, Fan XL, Pfister M, Schuett C. Mutation of amino acids 39-44 of human CD14 abrogates binding of lipopolysaccharide and Escherichia coli. Eur J Biochem. 1997; 243:100–109. [PubMed: 9030727]

57. (a) Juan TSC, Hailman E, Kelley MJ, Wright SD, Lichenstein HS. Identification of a domain in soluble CD14 essential for lipopolysaccharide (LPS) signaling but not LPS binding. J Biol Chem. 1995; 270:17237–17242. [PubMed: 7542233] (b) Stelter F, Loppnow H, Menzel R, Grunwald U, Bernheiden M, Jack RS, Ulmer AJ, Schutt C. Differential impact of substitution of amino acids 9-13 and 91-101 of human CD14 on soluble CD14-dependent activation of cells by lipopolysaccharide. J Immunol. 1999; 163:6035–6044. [PubMed: 10570291]

58. (a) Antal-Szalmas P, Van Strijp JAG, Weersink AJL, Verhoef J, Van Kessel KPM. Quantitation of surface CD14 on human monocytes and neutrophils. J Leukoc Biol. 1997; 61:721–728. [PubMed: 9201263] (b) Liu S, Khemlani LS, Shapiro RA, Johnson ML, Liu K, Geller DA, Watkins SC, Goyert SM, Billiar TR. Expression of CD14 by hepatocytes: Upregulation by cytokines during endotoxemia. Infect Immun. 1998; 66:5089–5098. [PubMed: 9784508] (c) Nanbo A, Nishimura H, Muta T, Nagasawa S. Lipopolysaccharide stimulates HepG2 human hepatoma cells in the presence of lipopolysaccharide-binding protein via CD14. Eur J Biochem. 1999; 260:183–191. [PubMed: 10091598] (d) Peterson PK, Gekker G, Hu S, Sheng WS, Anderson WR, Ulevitch RJ, Tobias PS, Gustafson KV, Molitor TW. CD14 receptor-mediated uptake of nonopsonized *Mycobacterium tuberculosis by human microglia*. Infect Immun. 1995; 63:1598–1602. [PubMed: 7534279] (e) Sugawara S, Sugiyama A, Nemoto E, Rikiishi H, Takada H. Heterogeneous expression and release of CD14 by human gingival fibroblasts: Characterization and CD14-mediated interleukin-8 secretion in response to lipopolysaccharide. Infect Immun. 1998; 66:3043–3049. [PubMed: 9632564] (f) Ziegler-Heitbrock HWL, Pechumer H, Petersmann I, Durieux JJ, Vita N, Labeta MO, Stroebel M. CD14 is expressed and functional in human B cells. Eur J Immunol. 1994; 24:1937–1940. [PubMed: 7520002]

59. (a) Marchant A, Duchow J, Delville JP, Goldman M. Lipopolysaccharide induces up-regulation of CD14 molecule on monocytes in human whole blood. Eur J Immunol. 1992; 22:1663–1665. [PubMed: 1376269] (b) Matsuura K, Ishida T, Setoguchi M, Higuchi Y, Akizuki Si, Yamamoto S. Upregulation of mouse cd14 expression in Kupffer cells by lipopolysaccharide. J Exp Med. 1994; 179:1671–1676. [PubMed: 7513013] (c) Ziegler-Heitbrock HWL, Ulevitch RJ. CD14: Cell surface receptor and differentiation marker. Immunol Today. 1993; 14:121–125. [PubMed: 7682078]
60. (a) Landmann R, Fisscher AE, Obrecht JP. Interferon-.gamma. and interleukin-4 down-regulate soluble CD14 release in human monocytes and macrophages. J Leukoc Biol. 1992; 52:323–330. [PubMed: 1381744] (b) Schuett C, Schilling T, Grunwald U, Schoenfeld W, Krueger C. Endotoxin-neutralizing capacity of soluble CD14. Res Immunol. 1992; 143:71–78. [PubMed: 1373513]
61. Tobias PS, Ulevitch RJ. Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage. Immunobiology. 1993; 187:227–232. [PubMed: 7687234]
62. Landmann R, Zimmerli W, Sansano S, Link S, Hahn A, Glauser MP, Calandra T. Increased circulating soluble CD14 is associated with high mortality in gram-negative septic shock. J Infect Dis. 1995; 171:639–644. [PubMed: 7533199]
63. Labeta MO, Vidal K, Nores JER, Arias M, Vita N, Morgan BP, Guillemot JC, Loyaux D, Ferrara P, Schmid D, Affolter M, Borysiewicz LK, Donnet-Hughes A, Schiffrin EJ. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. J Exp Med. 2000; 191:1807–1812. [PubMed: 10811873]
64. (a) Detmers PA, Thieblemont N, Vasselon T, Pironkova R, Miller DS, Wright SD. Potential role of membrane internalization and vesicle fusion in adhesion of neutrophils in response to lipopolysaccharide and TNF. J Immunol. 1996; 157:5589–5596. [PubMed: 8955211] (b) Lichtman SN, Wang J, Lemasters JJ. Lipopolysaccharide-stimulated TNF-alpha release from cultured rat Kupffer cells: Sequence of intracellular signaling pathways. J Leukoc Biol. 1998; 64:368–372. [PubMed: 9738664]
65. (a) Pollack M, Ohl CA, Golenbock DT, Di Padova F, Wahl LM, Koles NL, Guelde G, Monks BG. Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induced proinflammatory functions. J Immunol. 1997; 159:3519–3530. [PubMed: 9317151] (b) Poussin C, Foti M, Carpentier JL, Pugin J. CD14-dependent endotoxin internalization via a macropinocytic pathway. J Biol Chem. 1998; 273:20285–20291. [PubMed: 9685378]
66. Thieblemont N, Wright SD. Transport of bacterial lipopolysaccharide to the Golgi apparatus. J Exp Med. 1999; 190:523–534. [PubMed: 10449523]
67. Vasselon T, Hailman E, Thieringer R, Detmers PA. Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J Exp Med. 1999; 190:509–521. [PubMed: 10449522]
68. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science. 1991; 254:1016–1019. [PubMed: 1719635]
69. Lee JD, Kravchenko V, Kirkland TN, Han J, Mackman M, Moriarty A, Leturcq D, Tobias PS, Ulevitch RJ. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. Proc Natl Acad Sci U S A. 1993; 90:9930–9934. [PubMed: 7694296]
70. Williams MJ, Rodriguez A, Kimbrell DA, Eldon ED. The 18-wheeler mutation reveals complex antibacterial gene regulation in *Drosophila* host defense. EMBO J. 1997; 16:6120–6130. [PubMed: 9321392]
71. (a) Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4). J Exp Med. 1999; 189:615–625. [PubMed: 9989976] (b) Hoshino K, Tsutsui H, Kawai T, Takeda K, Nakanishi K, Takeda Y, Akira S. Cuting edge: Generation of IL-18 receptor-deficient mice: Evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. J Immunol. 1999; 162:5041–5044. [PubMed: 10227969]

72. Anderson KV, Bokla L, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: The induction of polarity by the Toll gene product. Cell. 1985; 42:791–798. [PubMed: 3931919]
73. Imler J-L, Hoffmann JA. Signaling mechanisms in the antimicrobial host defense of *Drosophila*. Curr Opin Microbiol. 2000; 3:16–22. [PubMed: 10679426]
74. Gay NJ, Keith FJ. *Drosophila* toll and IL-1 receptor. Nature. 1991; 351:355–356. [PubMed: 1851964]
75. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homolog of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature. 1997; 388:394–396. [PubMed: 9237759]
76. Jenkin C, Palmer DL. Changes in the titre of serum opsonins and phagocytic properties of mouse peritoneal macrophages following injection of endotoxin. J Exp Med. 1960; 112:419–429. [PubMed: 13789821]
77. Skidmore BJ, Chiller JM, Morrison DC, Weigle WO. Immunologic properties of bacterial lipopolysaccharide (LPS). Correlation between the mitogenic, adjuvant, and immunogenic activities. J Immunol. 1975; 114:770–775. [PubMed: 46249]
78. Underhill DM, Ozinsky A, Haijar AM, Stevens A, Wilson CB, Bassetti M, Aderem A. The toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature. 1999; 401:811–815. [PubMed: 10548109]
79. Heine H, Kirschning CJ, Lien E, Monks BG, Rothe M, Golenbock DT. Cutting edge: Cells that carry a null allele for toll-like receptor 2 are capable of responding to endotoxin. J Immunol. 1999; 162:6971–6975. [PubMed: 10358136]
80. (a) Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, Goddard A, Wood WI, Gurney AL, Godowski PJ. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. Nature. 1998; 395:284–288. [PubMed: 9751057] (b) Da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. J Biol Chem. 2001; 276:21129–21135. [PubMed: 11274165]
81. (a) Kirschning CJ, Wesche H, Ayres TM, Rothe M. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med. 1998; 188:2091–2097. [PubMed: 9841923] (b) Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med. 1999; 189:1777–1782. [PubMed: 10359581]
82. Akashi S, Shimazu R, Ogata H, Nagai Y, Takeda K, Kimoto M, Miyake K. Cutting edge: Cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol. 2000; 164:3471–3475. [PubMed: 10725698]
83. Gangloff M, Gay NJ. MD-2: The Toll “gate-keeper” in endotoxin signaling. Trends Biochem Sci. 2004; 29:294–300. [PubMed: 15276183]
84. Visintin A, Mazzoni A, Spitzer JA, Segal DM. Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. Proc Natl Acad Sci U S A. 2001; 98:12156–12161. [PubMed: 11593030]
85. (a) Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol. 2002; 3:667–672. [PubMed: 12055629] (b) Ohnishi T, Muroi M, Tanamoto K-i. MD-2 is necessary for the Toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. Clin Diagn Lab Immunol. 2003; 10:405–410. [PubMed: 12738639]
86. Viriyakosol S, Tobias PS, Kitchens RL, Kirkland TN. MD-2 binds to bacterial lipopolysaccharides. J Biol Chem. 2001; 276:38044–38051. [PubMed: 11500507]
87. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. Am J Physiol. 2004; 287:L428–L437.
88. Viriyakosol S, Tobias PS, Kirkland TN. Mutational analysis of membrane and soluble forms of human MD-2. J Biol Chem. 2006; 281:11955–11964. [PubMed: 16467306]

89. Mancek M, Pristovsek P, Jerala R. Identification of LPS-binding peptide fragment of MD-2, a toll-receptor accessory protein. Biochem Biophys Res Commun. 2002; 292:880–885. [PubMed: 11944896]

90. (a) Visintin A, Latz E, Monks BG, Espevik T, Golenbock DT. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. J Biol Chem. 2003; 278:48313–48320. [PubMed: 12960171] (b) Kawasaki K, Nogawa H, Nishijima M. Identification of mouse MD-2 residues important for forming the cell surface TLR4-MD-2 complex recognized by anti-TLR4-MD-2 antibodies, and for conferring LPS and taxol responsiveness on mouse TLR4 by alanine-scanning mutagenesis. J Immunol. 2003; 170:413–420. [PubMed: 12496426] (c) Schromm AB, Lien E, Henneke P, Chow JC, Yoshimura A, Heine H, Latz E, Monks BG, Schwartz DA, Miyake K, Golenbock DT. Molecular genetic analysis of an endotoxin nonresponder mutant cell line: A point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. J Exp Med. 2001; 194:79–88. [PubMed: 11435474] (d) Re F, Strominger JL. Separate functional domains of human MD-2 mediate toll-like receptor 4-binding and lipopolysaccharide responsiveness. J Immunol. 2003; 171:5272–5276. [PubMed: 14607928] (e) Da Silva Correia J, Ulevitch RJ. MD-2 and TLR4 N-linked glycosylation are important for a functional lipopolysaccharide receptor. J Biol Chem. 2002; 277:1845–1854. [PubMed: 11706042] (f) Ohnishi T, Muroi M, Tanamoto K-i. N-linked glycosylations at Asn26 and Asn114 of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. J Immunol. 2001; 167:3354–3359. [PubMed: 11544325]

91. Gruber A, Mancek M, Wagner H, Kirschning CJ, Jerala R. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. J Biol Chem. 2004; 279:28475–28482. [PubMed: 15111623]

92. Mullen GED, Kennedy MN, Visintin A, Mazzoni A, Leifer CA, Davies DR, Segal DM. The role of disulfide bonds in the assembly and function of MD-2. Proc Natl Acad Sci U S A. 2003; 100:3919–3924. [PubMed: 12642668]

93. Visintin A, Halmen KA, Latz E, Monks BG, Golenbock DT. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. J Immunol. 2005; 175:6465–6472. [PubMed: 16272300]

94. (a) Latz E, Visintin A, Lien E, Fitzgerald KA, Espevik T, Golenbock DT. The LPS receptor generates inflammatory signals from the cell surface. J Endotoxin Res. 2003; 9:375–380. [PubMed: 14733724] (b) Re F, Strominger JL. Monomeric recombinant MD-2 binds Toll-like receptor 4 tightly and confers lipopolysaccharide responsiveness. J Biol Chem. 2002; 277:23427–23432. [PubMed: 11976338]

95. Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, Weiss JP. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc Natl Acad Sci U S A. 2004; 101:4186–4191. [PubMed: 15010525]

96. Kennedy MN, Mullen GED, Leifer CA, Lee CW, Mazzoni A, Dileepan KN, Segal DM. A complex of soluble MD-2 and lipopolysaccharide serves as an activating ligand for toll-like receptor 4. J Biol Chem. 2004; 279:34698–34704. [PubMed: 15175334]

97. Jack RS, Fan X, Bernheiden M, Rune G, Ehlers M, Weber A, Kirsch G, Mentel R, Furll B, Freudenberg M, Schmitz G, Stelter F, Schutt C. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. Nature. 1997; 389:742–745. [PubMed: 9338787]

98. (a) Saitoh, S-i; Akashi, S.; Yamada, T.; Tanimura, N.; Kobayashi, M.; Konno, K.; Matsumoto, F.; Fukase, K.; Kusumoto, S.; Nagai, Y.; Kusumoto, Y.; Kosugi, A.; Miyake, K. Lipid A antagonist, Lipid IVA, is distinct from Lipid A in interaction with Toll-like receptor 4 (TLR4)-MD2 and ligand-induced TLR4 oligomerization. Int Immunol. 2004; 16:961–969. [PubMed: 15184344] (b) Inohara N, Nunez G. ML—A conserved domain involved in innate immunity and lipid metabolism. Trends Biochem Sci. 2002; 27:219–221. [PubMed: 12076526]

99. (a) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124:783–801. [PubMed: 16497588] (b) Beutler B, Rietschel ET. Timeline: Innate immune sensing and its roots: The story of endotoxins. Nat Rev Immunol. 2003; 3:169–176. [PubMed: 12563300]

100. Karin M, Lin A. NF-kB at the crossroads of life and death. Nat Immunol. 2002; 3:221–227. [PubMed: 11875461]

101. (a) Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, Panoskaltsis N. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. New Engl J Med. 2006; 355:1018–1028. [PubMed: 16908486] (b) Reitsma PH, Branger J, Van Den Blink B, Weijer S, Van Der Poll T, Meijers JCM. Procoagulant protein levels are differently increased during human endotoxemia. J Thromb Haemost. 2003; 1:1019–1023. [PubMed: 12871371] (c) Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry SF. A network-based analysis of systemic inflammation in humans. Nature. 2005; 437:1032–1037. [PubMed: 16136080]

102. Lopez-Bojorquez LN, Dehesa AZ, Reyes-Teran G. Molecular mechanisms involved in the pathogenesis of septic shock. Arch Med Res. 2004; 35:465–479. [PubMed: 15631870]

103. Nathan CF. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J Clin Invest. 1987; 80:1550–1560. [PubMed: 2445780]

104. Taveira da Silva AM, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL. Brief report: Shock and multiple-organ dysfunction after self-administration of *Salmonella* endotoxin. New Engl J Med. 1993; 328:1457–1460. [PubMed: 8479465]

105. (a) Madge LA, Pober JS. TNF signaling in vascular endothelial cells. Exp Mol Pathol. 2001; 70:317–325. [PubMed: 11418010] (b) Schlayer HJ, Karck U, Ganter U, Hermann R, Decker K. Enhancement of neutrophil adherence to isolated rat liver sinusoidal endothelial cells by supernatants of lipopolysaccharide-activated monocytes. Role of tumor necrosis factor. J Hepatol. 1987; 5:311–321. [PubMed: 3429839] (c) Varani J, Ward PA. Mechanisms of endothelial cell injury in acute inflammation. Shock. 1994; 2:311–319. [PubMed: 7743355]

106. Baud V, Karin M. Signal transduction by tumor necrosis factor and it relatives. Trends Cell Biol. 2001; 11:372–377. [PubMed: 11514191]

107. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature. 1987; 330:662–664. [PubMed: 3317066]

108. Peters K, Unger RE, Brunner J, Kirkpatrick CJ. Molecular basis of endothelial dysfunction in sepsis. Cardiovasc Res. 2003; 60:49–57. [PubMed: 14522406]

109. (a) Rajalingam D, Kacer D, Prudovsky I, Kumar TKS. Molecular cloning, overexpression and characterization of human interleukin 1alpha. Biochem Biophys Res Commun. 2007; 360:604–608. [PubMed: 17618910] (b) Brody DT, Durum SK. A plasma membrane anchoring mechanism for IL-1. Prog Leukoc Biol. 1988; 8:101–107.(c) Wingfield P, Payton M, Graber P, Rose K, Dayer JM, Shaw AR, Schmeissner U. Purification and characterization of human interleukin-1.alpha produced in *Escherichia coli*. Eur J Biochem. 1987; 165:537–541. [PubMed: 3297693] (d) Gubler U, Chua AO, Stern AS, Hellmann CP, Vitek MP, Dechiara TM, Benjamin WR, Collier KJ, Dukovich M. Recombinant human interleukin 1alpha: Purification and biological characterization. J Immunol. 1986; 136:2492–2497. [PubMed: 3485152]

110. Dinarello CA. Biological basis for interleukin-1 in disease. Blood. 1996; 87:2095–2147. [PubMed: 8630372]

111. Kumar A, Thota V, Dee L, Olson J, Uretz E, Parrillo JE. Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum. J Exp Med. 1996; 183:949–958. [PubMed: 8642298]

112. Hazelzet JA, Kornelisse RF, van der Pouw Kraan TCTM, Joosten KFM, van der Voort E, van Mierlo G, Suur MH, Hop WCJ, de Groot R, Hack CE. Interleukin 12 levels during the initial phase of septic shock with purpura in children: Relation to severity of disease. Cytokine. 1997; 9:711–716. [PubMed: 9325021]

113. (a) Van Zee KJ, DeForge LE, Fischer E, Marano MA, Kenney JS, Remick DG, Lowry SF, Moldawer LL. IL-8 in septic shock, endotoxemia, and after IL-1 administration. J Immunol. 1991; 146:3478–3482. [PubMed: 2026876] (b) Bossink AWJ, Paemen L, Jansen PM, Hack CE, Thijs LG, Van Damme J. Plasma levels of the chemokines monocyte chemotactic proteins-1 and -2 are elevated in human sepsis. Blood. 1995; 86:3841–3847. [PubMed: 7579352]

114. Dinarello CA. Proinflammatory cytokines. Chest. 2000; 118:503–508. [PubMed: 10936147]
115. (a) Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA Jr. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J Exp Med. 1984; 160:618–623. [PubMed: 6332168] (b) Gregory SA, Morrissey JH, Edgington TS. Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Mol Cell Biol. 1989; 9:2752–2755. [PubMed: 2503712]
116. Maruyama I. Biology of endothelium. Lupus. 1998; 7:S41–S43. [PubMed: 9814671]
117. Ryu J, Pyo H, Jou I, Joe E. Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase, and NF-kappaB. J Biol Chem. 2000; 275:29955–29959. [PubMed: 10893407]
118. McGilvray ID, Rotstein OD. Signaling pathways of tissue factor expression in monocytes and macrophages. Sepsis. 1999; 3:93–101.
119. (a) Suffredini AF, Harpel PC, Parrillo JE. Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. New Engl J Med. 1989; 320:1165–1172. [PubMed: 2496309] (b) Van Deventer SJH, Buller HR, ten Cate JW, Aarden LA, Hack CE, Sturk A. Experimental endotoxemia in humans: Analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood. 1990; 76:2520–2526. [PubMed: 2124934] (c) Van der Poll T, Bueller HR, ten Cate H, Wortel CH, Bauer KA, Van Deventer SJH, Hack CE, Sauerwein HP, Rosenberg RD, ten Cate JW. Activation of coagulation after administration of tumor necrosis factor to normal subjects. New Engl J Med. 1990; 322:1622–1627. [PubMed: 2188129] (d) Van Der Poll T, Levi M, Buller HR, Van Deventer SJH, De Boer JP, Hack CE, ten Cate JW. Fibrinolytic response to tumor necrosis factor in healthy subjects. J Exp Med. 1991; 174:729–732. [PubMed: 1714936]
120. (a) Fourrier F, Chopin C, Goudemand J, Hendrycx S, Caron C, Rime A, Marey A, Lestave P. Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. Chest. 1992; 101:816–823. [PubMed: 1531791] (b) Mesters RM, Mannucci PM, Coppola R, Keller T, Ostermann H, Kienast J. Factor VIIa and antithrombin III activity during severe sepsis and septic shock in neutropenic patients. Blood. 1996; 88:881–886. [PubMed: 8704245]
121. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med. 1986; 163:740–755. [PubMed: 3753996]
122. (a) Senior RM, Skogen WF, Griffin GL, Wilner GD. Effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide B. J Clin Invest. 1986; 77:1014–1019. [PubMed: 3005361] (b) Bick RL. Disseminated intravascular coagulation. Hematol Oncol Clin North Am. 1992; 6:1259–1285. [PubMed: 1452511] (c) Bick RL. Disseminated intravascular coagulation and related syndromes: A clinical review. Semin Thromb Hemost. 1988; 14:299–338. [PubMed: 3057630] (d) ten Cate H, Brandjes DP, Wolters HJ, van Deventer SJ. Disseminated intravascular coagulation: Pathophysiology, diagnosis, and treatment. New Horiz. 1993; 1:312–323. [PubMed: 7922411] (e) Taylor FB Jr. The inflammatory-coagulant axis in the host response to gram-negative sepsis: Regulatory roles of proteins and inhibitors of tissue factor. New Horiz. 1994; 2:555–565. [PubMed: 7804804] (f) Carrico CJ, Meakins JL, Marshall JC, Fry D, Maier RV. Multiple-organ-failure syndrome. Arch Surg. 1986; 121:196–208. [PubMed: 3484944] (g) Shibayama Y. Sinusoidal circulatory disturbance by microthrombosis as a cause of endotoxin-induced hepatic injury. J Pathol. 1987; 151:315–321. [PubMed: 3585589] (h) Schlag G, Redl H. Morphology of the microvascular system in shock: Lung, liver, and skeletal muscles. Crit Care Med. 1985; 13:1045–1049. [PubMed: 4064714]
123. Rangel-Frausto MS, Pittet D, Costigan M, Hwang T, Davis CS, Wenzel RP. The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. JAMA. 1995; 273:117–123. [PubMed: 7799491]
124. (a) Bulusu M, Hildebrandt J, Lam C, Liehl E, Loibner H, Macher I, Scholz D, Schutze E, Stutz P, Vyplel H, Unger F. Enzymic synthesis of analogs of bacterial lipid A and design of biologically active LPS-antagonists and -mimetics. Pure Appl Chem. 1994; 66:2171–2174.(b) Cohen PS, Nakshatri H, Dennis J, Caragine T, Bianchi M, Cerami A, Tracey KJ. CNI-1493 inhibits monocyte/macrophage tumor necrosis factor by suppression of translation efficiency. Proc Natl Acad Sci U S A. 1996; 93:3967–3971. [PubMed: 8632999] (c) Rice GC, Brown PA, Nelson RJ, Bianco JA, Singer JW, Burstein S. Protection from endotoxic shock in mice by pharmacologic

inhibition of phosphatidic acid. Proc Natl Acad Sci U S A. 1994; 91:3857–3861. [PubMed: 8171002] (d) Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heyes JR. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature. 1994; 372:739–746. [PubMed: 7997261] (e) Bianchi M, Ulrich P, Bloom O, Meistrell MI, Zimmerman GA, Schmidtmayerova H, Bukrinsky M, Donnelley T, Bucala R. An inhibitor of macrophage arginine transport and nitric oxide production (CNI-1493) prevents acute inflammation and endotoxin lethality. Mol Med. 1995; 1:254–266. [PubMed: 8529104]

125. (a) McGeehan GM, Becherer JD, Bast RCJ, Boyer CM, Champion B, Connolly KM, Conway JG, Furdon P, Karp S, Kidao S, McElroy AB, Nichols J, Pryzxansky KM, Schoenen F, Sekut L, Truesdale A, Verghese M, Warner J, Ways JP. Regulation of tumor necrosis factor-.alpha. processing by a metalloproteinase inhibitor. Nature. 1994; 370:558–561. [PubMed: 8052311] (b) Galloway CJ, Madanat MS, Mitra G. Monoclonal anti-tumor necrosis factor (TNF) antibodies protect mouse and human cells from TNF cytotoxicity. J Immunol Methods. 1991; 140:37–43. [PubMed: 2061612] (c) Pauli U, Bertoni G, Duerr M, Peterhans E. A bioassay for the detection of tumor necrosis factor from eight different species: Evaluation of neutralization rates of a monoclonal antibody against human TNF-.alpha. J Immunol Methods. 1994; 171:263–265. [PubMed: 8195595] (d) Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science. 1985; 229:869–871. [PubMed: 3895437]
126. Hale KK, Smith CG, Baker SL, Vanderslice RW, Squires CH, Gleason TM, Tucker KK, Kohno T, Russell DA. Multifunctional regulation of the biological effects of TNF-.alpha. by the soluble type I and type II TNF receptors. Cytokine. 1995; 7:26–38. [PubMed: 7749064]
127. (a) Dinarello CA, Gelfand JA, Wolff SM. Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. JAMA. 1993; 269:1829–1835. [PubMed: 8459516] (b) Redmond HP, Chavin KD, Bromberg JS, Daly JM. Inhibition of macrophage-activating cytokines is beneficial in the acute septic response. Ann Surg. 1991; 214:502–508. [PubMed: 1659339] (c) Natanson C, Hoffman WD, Suffredini AF, Eichacker PQ, Danner RL. Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis. Ann Intern Med. 1994; 120:771–783. [PubMed: 8147551]
128. Ohto U, Fukase K, Miyake K, Satow Y. Crystal structures of human MD-2 and its complex with antiendotoxic Lipid IVa. Science. 2007; 316:1632–1634. [PubMed: 17569869]
129. (a) Takayama K, Qureshi N, Beutler B, Kirkland TN. Diphosphoryl lipid A from *Rhodopseudomonas sphaeroides ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide*. Infect Immun. 1989; 57:1336–1338. [PubMed: 2784418] (b) Christ WJ, McGuinness PD, Asano O, Wang Y, Mullarkey MA, Perez M, Hawkins LD, Blythe TA, Dubuc GR, Robidoux AL. Total synthesis of the proposed structure of *Rhodobacter sphaeroides Lipid A resulting in the synthesis of new potent lipopolysaccharide antagonists*. J Am Chem Soc. 1994; 116:3637–3638.
130. (a) Hawkins LD, Christ WJ, Rossignol DP. Inhibition of endotoxin response by synthetic TLR4 antagonists. Curr Top Med Chem. 2004; 4:1147–1171. [PubMed: 15279606] (b) Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM, Karp CL, McAlees J, Gioannini TL, Weiss J, Chen WH, Ernst RK, Rossignol DP, Gusovsky F, Blanco JCG, Vogel SN. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature. 2013; 497:498–502. [PubMed: 23636320]
131. (a) Gmeiner J, Lüderitz O, Westphal O. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. Eur J Biochem. 1969; 7:370–379. [PubMed: 4307215] (b) Hase S, Rietschel ET. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur J Biochem. 1976; 63:101–107. [PubMed: 770165]
132. Lewicky JD, Ulanova M, Jiang Z-H. Synthesis and immunostimulatory activity of diethanolamine-containing lipid A mimics. R Soc Chem Adv. 2012; 2:1917–1926.
133. (a) Zughaier SM, Zimmer SM, Datta A, Carlson RW, Stephens DS. Differential induction of the Toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. Infect Immun. 2005; 73:2940–2950. [PubMed: 15845500] (b) Trent MS, Stead CM, Tran AX, Hankins JV. Invited review: Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res. 2006; 12:205–223. [PubMed: 16953973]

134. (a) Akamatsu M, Fujimoto Y, Kataoka M, Suda Y, Kusumoto S, Fukase K. Synthesis of lipid A monosaccharide analogs containing acidic amino acid: Exploring the structural basis for the endotoxic and antagonistic activities. Bioorg Med Chem. 2006; 14:6759–6777. [PubMed: 16828560] (b) Fujimoto Y, Adachi Y, Akamatsu Masao, Fukase Yoshiyuki, Kataoka Mikayo, Suda Yasuo, Fukase Koichi, Kusumoto S. Synthesis of lipid A and its analogues for investigation of the structural basis for their bioactivity. J Endotoxin Res. 2005; 11:341–347. [PubMed: 16303089] (c) Zhang Y, Gaekwad J, Wolfert MA, Boons GJ. Modulation of innate immune responses with synthetic lipid a derivatives. J Am Chem Soc. 2007; 129:5200–5216. [PubMed: 17391035] (d) Bazin HG, Murray TJ, Bowen WS, Mozaffarian A, Fling SP, Bess LS, Livesay MT, Arnold JS, Johnson CL, Ryter KT, Cluff CW, Evans JT, Johnson DA. The 'Ethereal' nature of TLR4 agonism and antagonism in the AGP class of lipid A mimetics. Bioorg Med Chem Lett. 2008; 18:5350–5354. [PubMed: 18835160]

135. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature. 2009; 458:1191–1195. [PubMed: 19252480]

136. (a) Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol. 2002; 3:354–359. [PubMed: 11912497] (b) Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR, Golenbock DT. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest. 2000; 105:497–504. [PubMed: 10683379] (c) Poltorak A, Ricciardi-Castagnoli P, Citterio S, Beutler B. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. Proc Natl Acad Sci U S A. 2000; 97:2163–2167. [PubMed: 10681462]

137. Fukase K, Fukase Y, Oikawa M, Liu W-C, Suda Y, Kusumoto S. Divergent synthesis and biological activities of Lipid A analogues of shorter acyl chains. Tetrahedron. 1998; 54:4033–4050.

138. Qureshi N, Mascagni P, Ribi E, Takayama K. Monophosphoryl lipid A obtained from lipopolysaccharides of *Salmonella minnesota* R595. Purification of the dimethyl derivative by high performance liquid chromatography and complete structural determination. J Biol Chem. 1985; 260:5271–5278. [PubMed: 3988753]

139. Johnson DA, Keegan DS, Sowell CG, Livesay MT, Johnson CL, Taubner LM, Harris A, Myers KR, Thompson JD, Gustafson GL, Rhodes MJ, Ulrich JT, Ward JR, Yorgensen YM, Cantrell JL, Brookshire VG. 3-O-desacyl monophosphoryl lipid a derivatives: Synthesis and immunostimulant activities. J Med Chem. 1999; 42:4640–4649. [PubMed: 10579826]

140. Funatogawa K, Matsuura M, Nakano M, Kiso M, Hasegawa A. Relationship of structure and biological activity of monosaccharide lipid A analogs to induction of nitric oxide production by murine macrophage RAW264.7 cells. Infect Immun. 1998; 66:5792–5798. [PubMed: 9826356]

141. Kiso M, Tanaka S, Fujita M, Fujishima Y, Ogawa Y, Ishida H, Hasegawa A. Synthesis of the optically active 4-*O*-phosphono-D-glucosamine derivatives related to the nonreducing-sugar subunit of bacterial lipid A. Carbohydr Res. 1987; 162:127–140. [PubMed: 3594476]

142. Johnson DA, Sowell CG, Johnson CL, Livesay MT, Keegan DS, Rhodes MJ, Ulrich JT, Ward JR, Cantrell JL, Brookshire VG. Synthesis and biological evaluation of a new class of vaccine adjuvants: Aminoalkyl glucosaminide 4-phosphates (AGPs). Bioorg Med Chem Lett. 1999; 9:2273–2278. [PubMed: 10465560]

143. Rietschel ET, Kirikae T, Schade FU, Ulmer AJ, Holst O, Brade H, Schmidt G, Mamat U, Grimmecke HD, Kusumoto S, Zähringer U. The chemical structure of bacterial endotoxin in relation to bioactivity. Immunobiology. 1993; 187:169–190. [PubMed: 8330896]

144. (a) Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL. Molecular basis of reduced potency of underacylated endotoxins. J Immunol. 2005; 175:4669–4676. [PubMed: 16177114] (b) Rossignol DP, Lynn M. TLR4 antagonists for endotoxemia and beyond. Curr Opin Investig Drugs. 2005; 6:496–502.

145. Kim HM, Park BS, Kim J-I, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist eritoran. Cell. 2007; 130:906–917. [PubMed: 17803912]

146. Netea MG, Deuren Mv, Kullberg BJ, Cavaillon J-M, Meer JWMVd. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? J Immunol. 2002; 23:135–139.

147. Jeyaretnam B, Glushka J, Kolli VSK, Carlson RW. Characterization of a novel lipid-A from *Rhizobium* species sin-1: A unique lipid-A structure that is devoid of phosphate and has a glycosyl backbone consisting of glucosamine and 2-aminogluconic acid. J Biol Chem. 2002; 277:41802–41810. [PubMed: 12193590]

148. Zhang Y, Wolfert MA, Boons G-J. The influence of the long chain fatty acid on the antagonistic activities of *Rhizobium* sin-1 lipid A. Bioorg Med Chem. 2007; 15:4800–4812. [PubMed: 17513113]

149. Vasan M, Wolfert MA, Boons G-J. Agonistic and antagonistic properties of a *Rhizobium* sin-1 lipid A modified by an ether-linked lipid. Org Biomol Chem. 2007; 5:2087–2097. [PubMed: 17581652]

150. (a) Ogawa T. Chemical structure of lipid A from *Porphyromonas (Bacteroides) gingivalis lipopolysaccharide*. FEBS Lett. 1993; 332:197–201. [PubMed: 8405442] (b) Ogawa T. Immunobiological properties of chemically defined lipid A from lipopolysaccharide of *Porphyromonas (Bacteroides) gingivalis*. Eur J Biochem. 1994; 219:737–742. [PubMed: 8112323]

151. (a) Darveau RP, Pham TTT, Lemley K, Reife RA, Bainbridge BW, Coats SR, Howald WN, Way SS, Hajjar AM. *Porphyromonas gingivalis* lipopolysaccharide contains multiple Lipid A species that functionally interact with both Toll-like receptors 2 and 4. Infect Immun. 2004; 72:5041–5051. [PubMed: 15321997] (b) Reife RA, Coats SR, Al-Qutub M, Dixon DM, Braham PA, Billharz RJ, Howald WN, Darveau RP. Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity: Differential activities of tetra- and penta-acylated lipid A structures on E-selectin expression and TLR4 recognition. Cell Microbiol. 2006; 8:857–868. [PubMed: 16611234]

152. (a) Sawada N, Ogawa T, Asai Y, Makimura Y, Sugiyama A. Toll-like receptor 4-dependent recognition of structurally different forms of chemically synthesized lipid As of *Porphyromonas gingivalis*. Clin Exp Immunol. 2007; 148:529–536. [PubMed: 17335558] (b) Zhang Y, Gaekwad J, Wolfert MA, Boons GJ. Synthetic tetra-acylated derivatives of lipid A from Porphyromonas gingivalis are antagonists of human TLR4. Org Biomol Chem. 2008; 6:3371–3381. [PubMed: 18802645]

153. (a) Demchenko AV, Wolfert MA, Santhanam B, Moore JN, Boons GJ. Synthesis and biological evaluation of Rhizobium sin-1 Lipid A derivatives. J Am Chem Soc. 2003; 125:6103–6112. [PubMed: 12785841] (b) Vandenplas ML, Carlson RW, Jeyaretnam BS, McNeill B, Barton MH, Norton N, Murray TF, Moore JN. Rhizobium Sin-1 lipopolysaccharide (LPS) prevents enteric LPS-induced cytokine production. J Biol Chem. 2002; 277:41811–41816. [PubMed: 12193596]

154. Takayama K, Ribi E, Cantrell JL. Isolation of a nontoxic Lipid A fraction containing tumor regression activity. Cancer Res. 1981; 41:2654–2657. [PubMed: 7018667]

155. Vuopio-Varkila J, Nurminen M, Pyhälä L, Mäkelä PH. Lipopolysaccharide-induced non-specific resistance to systemic *Escherichia coli* infection in mice. J Med Microbiol. 1988; 25:197–203. [PubMed: 3279215]

156. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003; 21:335–376. [PubMed: 12524386]

157. Okemoto K, Kawasaki K, Hanada K, Miura M, Nishijima M. A potent adjuvant monophosphoryl lipid A triggers various immune responses, but not secretion of IL-1 beta or activation of caspase-1. J Immunol. 2006; 176:1203–1208. [PubMed: 16394010]

158. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, Towne E, Tracey D, Wardwell S, Wei F-Y, Wong W, Kamen R, Seshadri T. Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell. 1995; 80:401–411. [PubMed: 7859282]

159. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science. 2007; 316:1628–1632. [PubMed: 17569868]

160. Cekic C, Casella CR, Eaves CA, Matsuzawa A, Ichijo H, Mitchell TC. Selective activation of the p38 MAPK pathway by synthetic monophosphoryl Lipid A. J Biol Chem. 2009; 284:31982–31991. [PubMed: 19759006]

161. (a) Kitchens RL, Ulevitch RJ, Munford RS. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. J Exp Med. 1992; 176:485–494. [PubMed: 1380063] (b) Golenbock DT, Hampton RY, Qureshi N, Takayama K, Raetz CRH. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J Biol Chem. 1991; 266:19490–19498. [PubMed: 1918061] (c) Kovach NL, Yee E, Munford RS, Raetz CR, Harlan JM. Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human whole blood ex vivo. J Exp Med. 1990; 172:77–84. [PubMed: 2193101]

162. (a) Christ WJ, Asano O, Robidoux ALC, Perez M, Wang Y, Dubuc GR, Gavin WE, Hawkins LD, McGuinness PD. E5531—A pure endotoxin antagonist of high potency. Science. 1995; 268:80–83. [PubMed: 7701344] (b) Ingalls RR, Monks BG, Savedra R Jr, Christ WJ, Delude RL, Medvedev AE, Espevik T, Golenbock DT. CD11/CD18 and CD14 share a common Lipid A signaling pathway. J Immunol. 1998; 161:5413–5420. [PubMed: 9820516] (c) Lien E, Chow JC, Hawkins LD, McGuinness PD, Miyake K, Espevik T, Gusovsky F, Golenbock DT. A novel synthetic acyclic Lipid A-like agonist activates cells via the lipopolysaccharide/Toll-like receptor 4 signaling pathway. J Biol Chem. 2001; 276:1873–1880. [PubMed: 11032843]

163. (a) Shiozaki M, Watanabe Y, Iwano Y, Kaneko T, Doi H, Tanaka D, Shimozato T, Kurakata Si. Synthesis of lipid A analogues containing glucose instead of glucosamine and their LPS-antagonistic activities. Tetrahedron. 2005; 61:5101–5122.(b) Shiozaki M, Doi H, Tanaka D, Shimozato T, Kurakata S-i. Syntheses of glucose-containing Lipid A analogues and their LPS-antagonistic activities. Bull Chem Soc Jpn. 2005; 78:1091–1104.

164. Shiozaki M, Doi H, Tanaka D, Shimozato T, Kurakata S-i. Syntheses of glucose-containing E5564 analogues and their LPS-antagonistic activities. Tetrahedron. 2006; 62:205–225.

165. Peri F, Marinzi C, Barath M, Granucci F, Urbano M, Nicotra F. Synthesis and biological evaluation of novel lipid A antagonists. Bioorg Med Chem. 2006; 14:190–199. [PubMed: 16203155]

166. Artner D, Oblak A, Ittig S, Garate JA, Horvat S, Arrieumerlou C, Hofinger A, Oostenbrink C, Jerala R, Kosma P, Zamyatina A. Conformationally constrained Lipid A mimetics for exploration of structural basis of TLR4/MD-2 activation by lipopolysaccharide. ACS Chem Biol. 2013; 8:2423–2432. [PubMed: 23952219]

167. Gaekwad J, Zhang Y, Zhang W, Reeves J, Wolfert MA, Boons G-J. Differential induction of innate immune responses by synthetic Lipid A derivatives. J Biol Chem. 2010; 285:29375–29386. [PubMed: 20634284]

168. Matsuura M, Shimada S-I, Kiso M, Hasegawa A, Nakano M. Expression of endotoxic activities by synthetic monosaccharide lipid A analogs with alkyl-branched acyl substituents. Infect Immun. 1995; 63:1446–1451. [PubMed: 7890408]

169. Matsuura M, Kiso M, Hasegawa A, Nakano M. Multistep regulation mechanisms for tolerance induction to lipopolysaccharide lethality in the tumor necrosis factor-.alpha.-mediated pathway. Application of non-toxic monosaccharide lipid A analogs for elucidation of mechanisms. Eur J Biochem. 1994; 221:335–341. [PubMed: 8168521]

170. Matsuura M, Kiso M, Hasegawa A. Activity of monosaccharide lipid A analogues in human monocytic cells as agonists or antagonists of bacterial lipopolysaccharide. Infect Immun. 1999; 67:6286–6292. [PubMed: 10569739]

171. Peri F, Granucci F, Costa B, Zanoni I, Marinzi C, Nicotra F. Inhibition of lipid A stimulated activation of human dendritic cells and macrophages by amino and hydroxylamino monosaccharides. Angew Chem Int Ed Engl. 2007; 46:3308–3312. [PubMed: 17387663]

172. Kaeothip S, Paranjape G, Terrill SE, Bongat AFG, Udan MLD, Kamkhachorn T, Johnson HL, Nichols MR, Demchenko AV. Development of LPS antagonistic therapeutics: Synthesis and evaluation of glucopyranoside-spacer-amino acid motifs. R Soc Chem Adv. 2011; 1:83–92.

173. Seydel U, Labischinski H, Kastowsky M, Brandenburg K. Phase behavior, supramolecular structure, and molecular conformation of lipopolysaccharide. Immunobiology. 1993; 187:191–211. [PubMed: 8330897]

174. (a) Cluff CW, Baldridge JR, Stöver AG, Evans JT, Johnson DA, Lacy MJ, Clawson VG, Yorgensen VM, Johnson CL, Livesay MT, Hershberg RM, Persing DH. Synthetic Toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. Infect Immun. 2005;

73:3044–3052. [PubMed: 15845512] (b) Stöver AG, Correia JDS, Evans JT, Cluff CW, Elliott MW, Jeffery EW, Johnson DA, Lacy MJ, Baldridge JR, Probst P, Ulevitch RJ, Persing DH, Hershberg RM. Structure-activity relationship of synthetic Toll-like receptor 4 agonists. J Biol Chem. 2004; 279:4440–4449. [PubMed: 14570885] (c) Fort MM, Mozaffarian A, Stöver AG, Correia JdS, Johnson DA, Crane RT, Ulevitch RJ, Persing DH, Bielefeldt-Ohmann H, Probst P, Jeffery E, Fling SP, Hershberg RM. A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. J Immunol. 2005; 174:6416–6423. [PubMed: 15879143]

175. Lewicky JD, Ulanova M, Jiang Z-H. Improving the immunostimulatory potency of diethanolamine-containing lipid A mimics. Bioorg Med Chem. 2013; 21:2199–2209. [PubMed: 23490149]

176. (a) Piazza M, Rossini C, Della Fiorentina S, Pozzi C, Comelli F, Bettoni I, Fusi P, Costa B, Peri F. Glycolipids and benzylammonium lipids as novel antisepsis agents: Synthesis and biological characterization. J Med Chem. 2009; 52:1209–1213. [PubMed: 19161283] (b) Piazza M, Yu L, Teghanemt A, Gioannini T, Weiss J, Peri F. Evidence of a specific interaction between new synthetic antisepsis agents and CD14. Biochemistry. 2009; 48:12337–12344. [PubMed: 19928913]

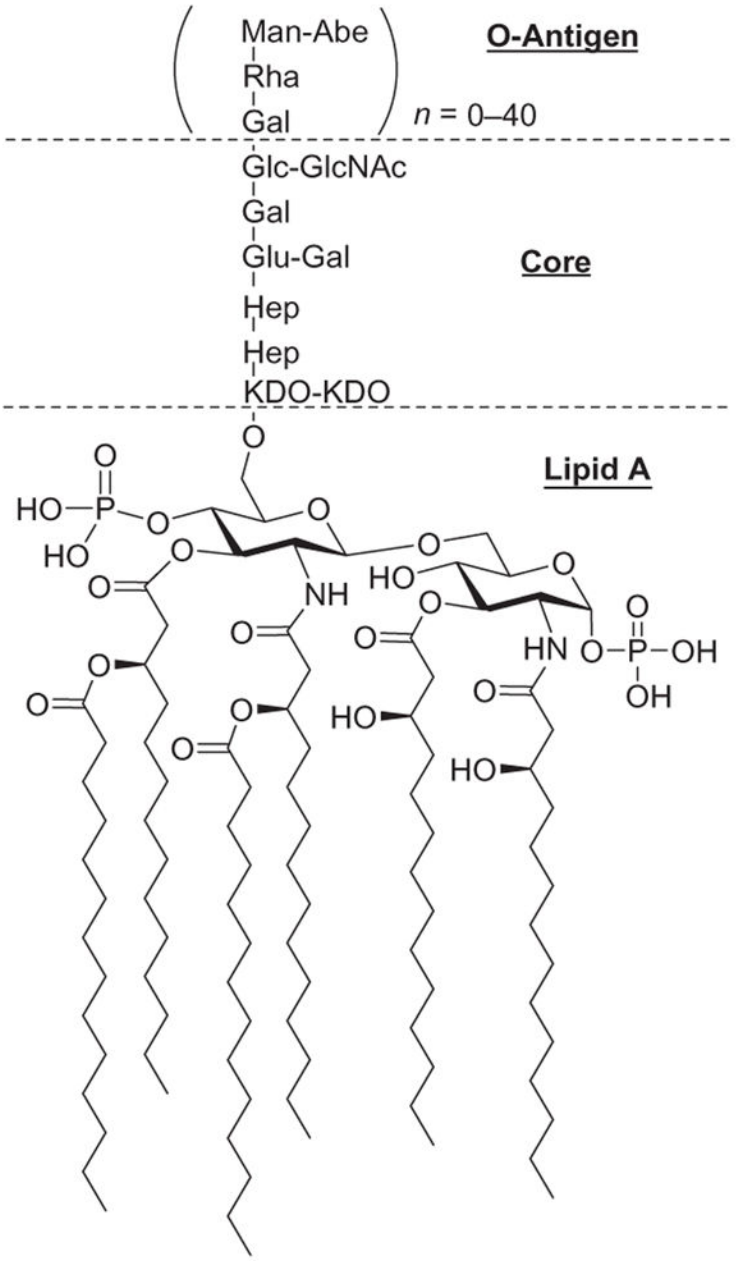

Fig. 1.

Structure of LPS and its typical antigenic regions: Lipid A, core oligosaccharide, and O-antigen.

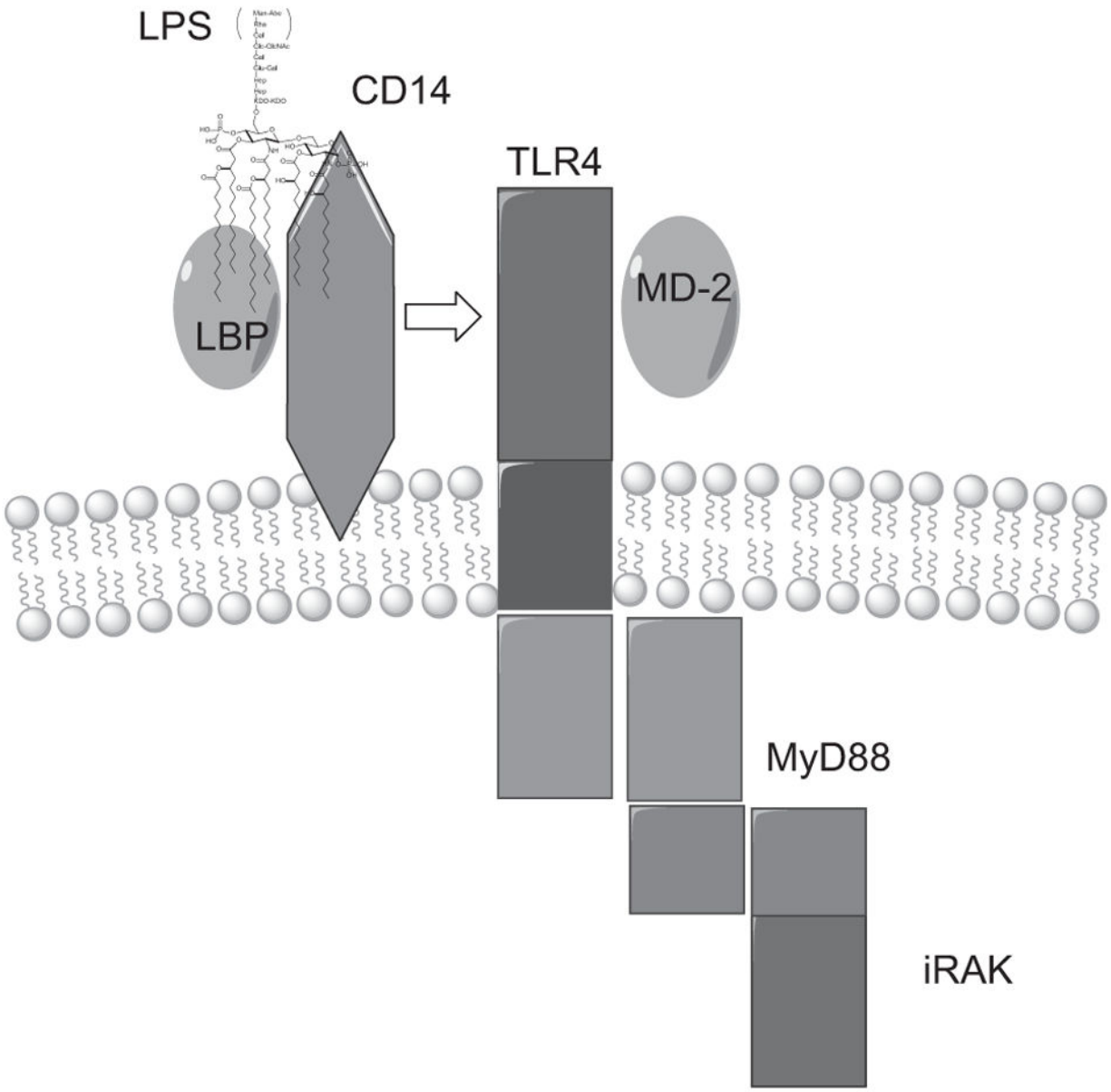

Fig. 2.

Recognition of LPS on the surface of phagocytes. LPS is opsonized by lipopolysaccharide-binding protein (LBP), and the LPS–LBP complex is recognized by cluster of differentiation 14 (CD14). CD14 is incapable of generating a transmembrane signal, and subsequently, the LPS–LBP–CD14 ternary complex activates toll-like receptor 4 (TLR4). TLR4, in turn, signals through the Myeloid differentiation primary response gene (88) (MyD88) and the interleukin-1 receptor-associated kinase (IRAK). Myeloid differentiation 2 (MD-2) is a secreted protein that binds to the extracellular domain of TLR4 and is an important component of its signaling pathway.[30] (See the color plate.)

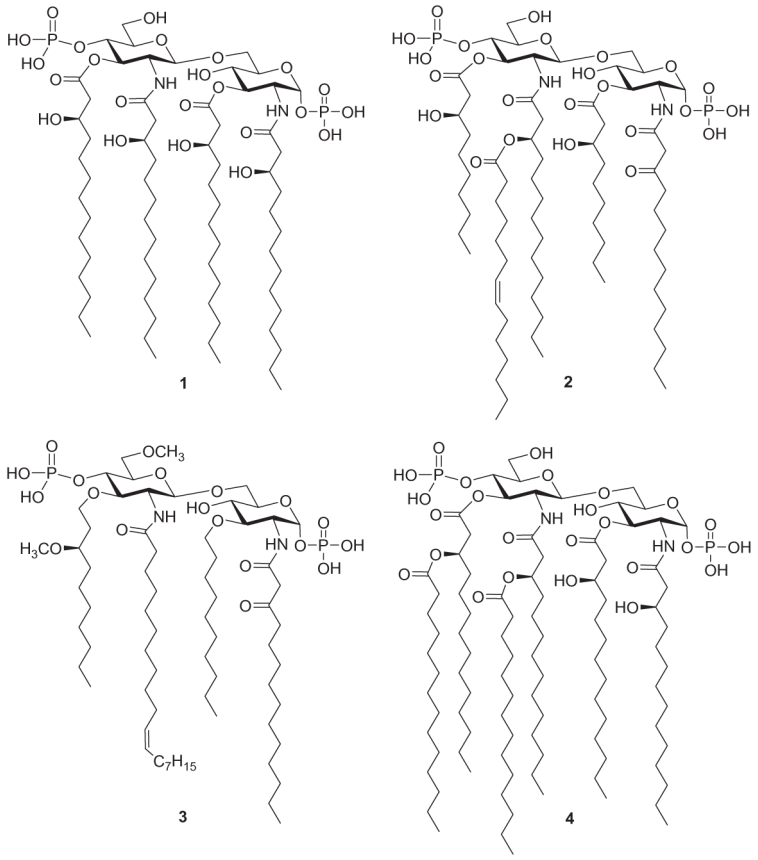

Fig. 3.

Structure of Lipid IV_A (**1**), Lipid A of *R. sphaeroides* (**2**), Eritoran (**3**), and Lipid A of *E. coli* (**4**).

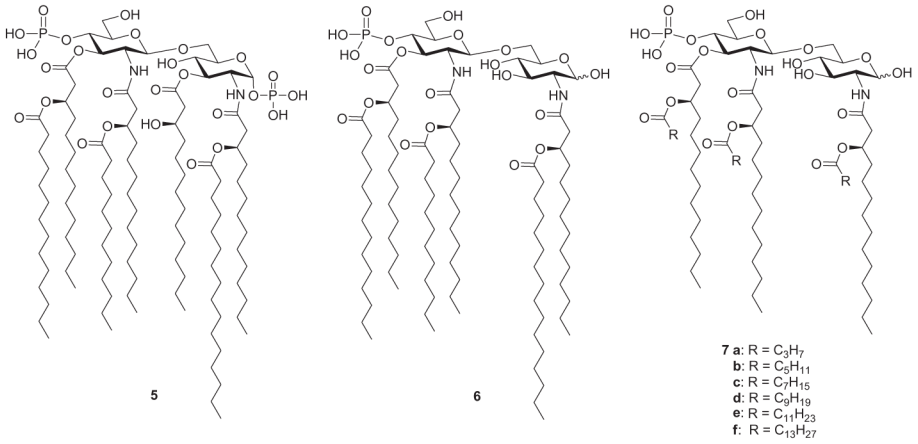

Fig. 4.

Monophosphoryl Lipid A from *S. minnesota* **5**, and its chain-length homologues **6** and **7a–f**.

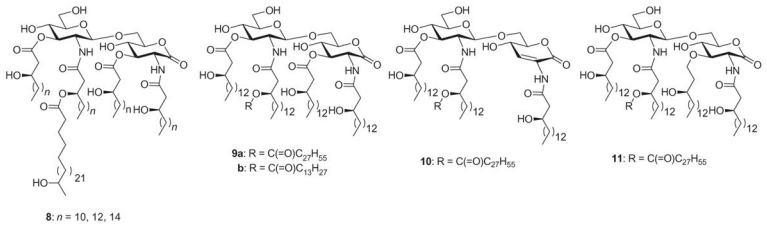

Fig. 5.
Rhizobium sin -1 Lipid A **8** and its derivatives **9–11**.

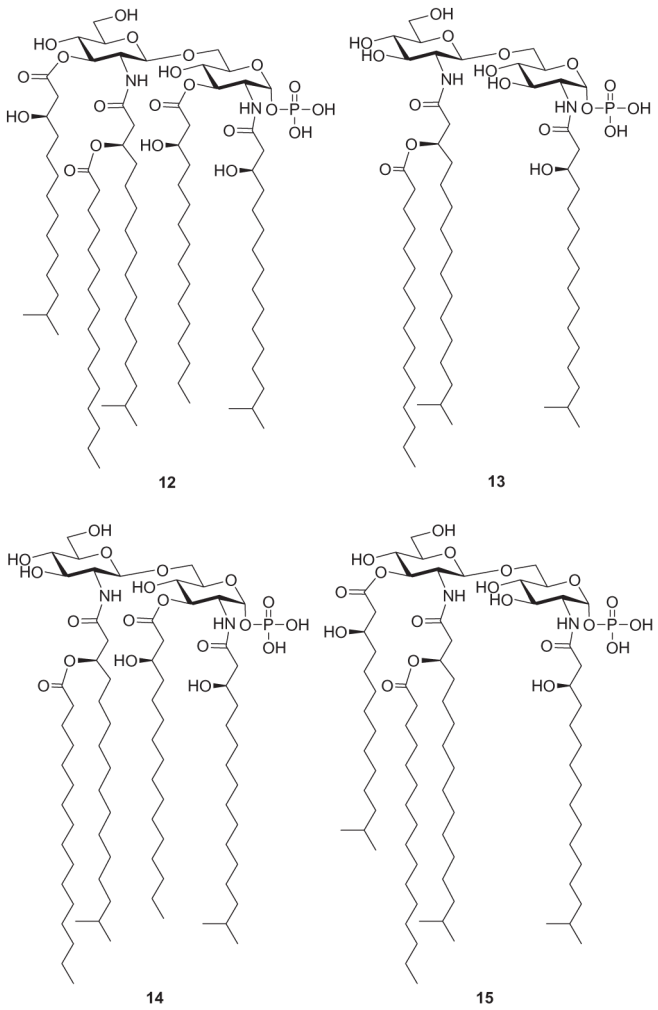

Fig. 6.
Lipid A structures from *Porphyromonas gingivalis* **12–15**.

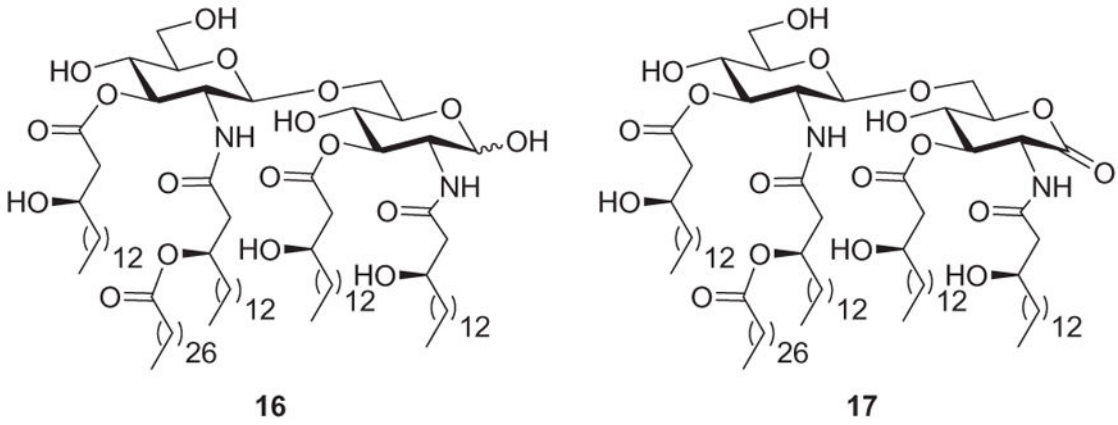

Fig. 7.
R. sin-1 Lipid A derivatives.

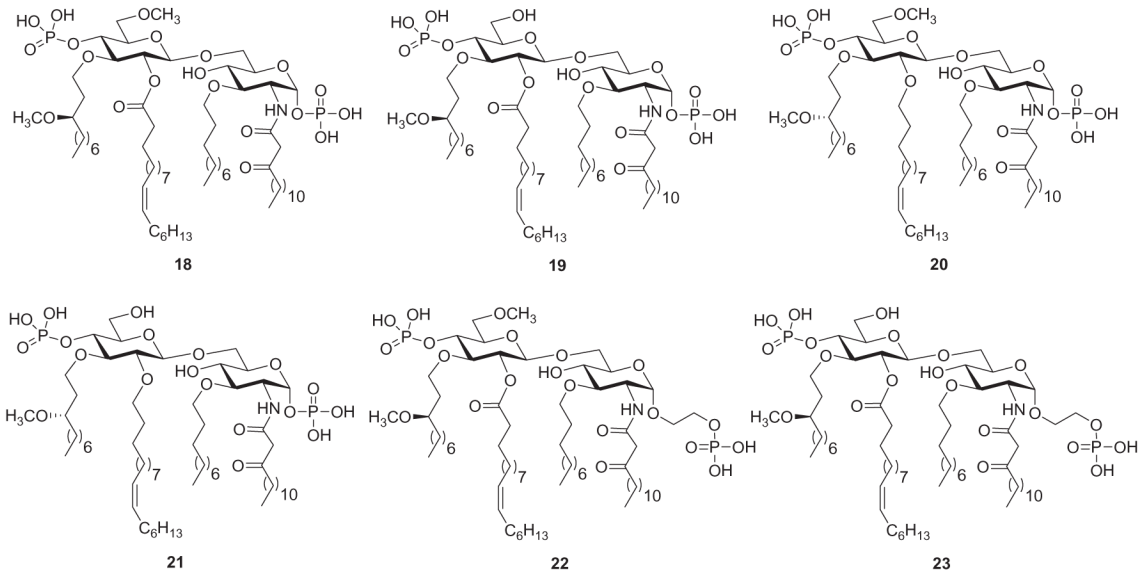

Fig. 8.

Unnatural Lipid A derivatives **18–23** based on Eritoran (**3**).

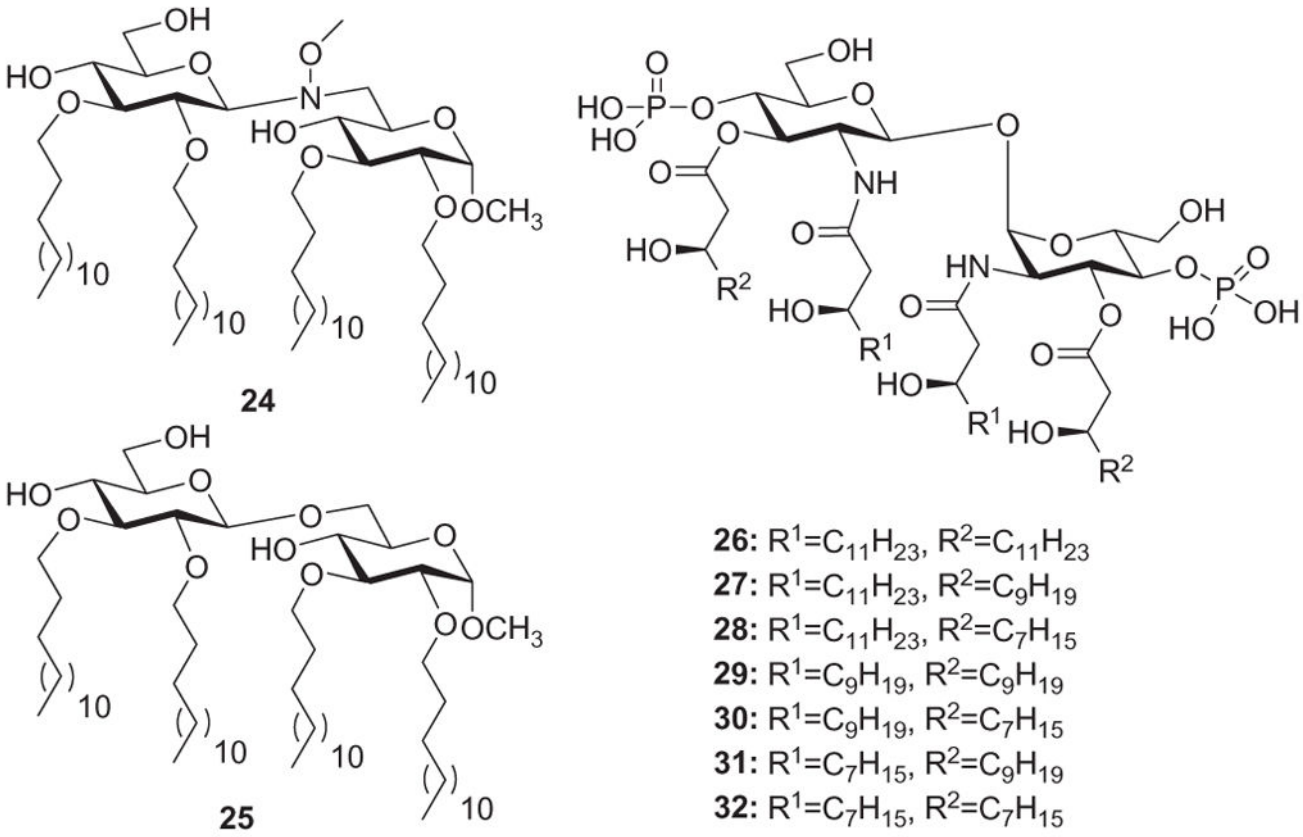

Fig. 9.
Nonnatural Lipid A derivatives.

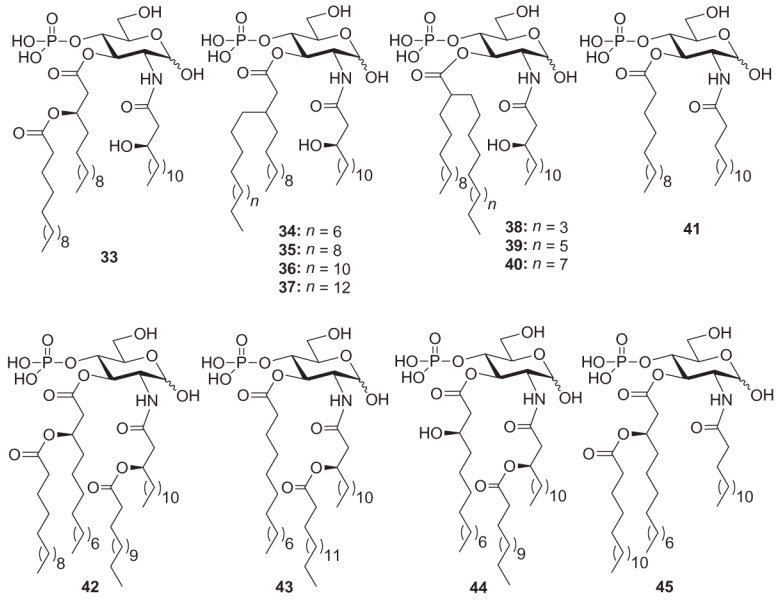

Fig. 10.

Monosaccharide Lipid A analogues **34–45** based on GLA-60 (**33**).

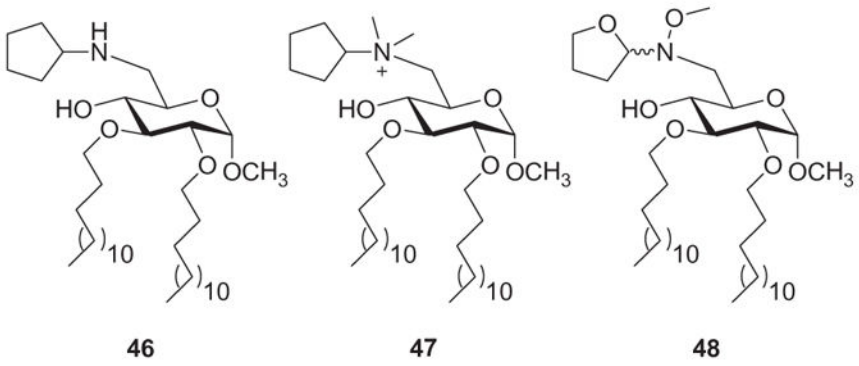

Fig. 11.
Methyl α-D-glucopyranoside-derived monosaccharide Lipid A analogues **46**–**48**.

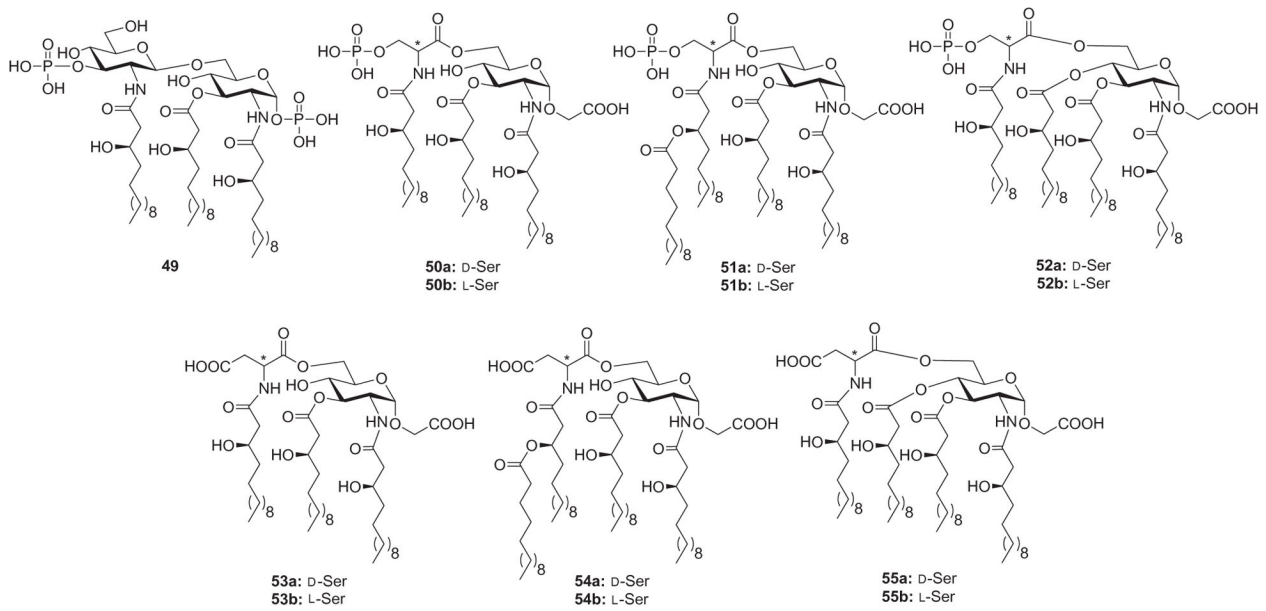

Fig. 12.

Triacylated Lipid A **49** and its monosaccharide analogues **50–55** containing acidic *N*-acyl amino acids.

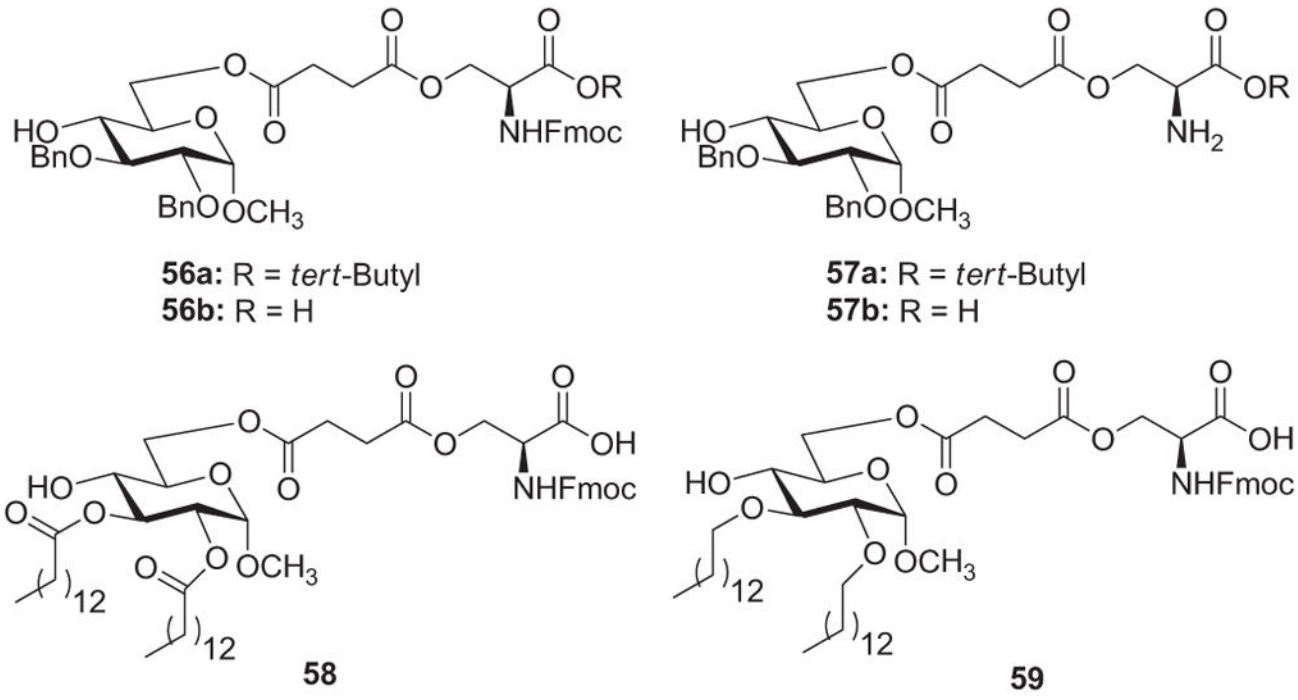

Fig. 13.

Monosaccharide Lipid A analogues **56–59** with glucopyranoside-spacer–amino acid motifs.

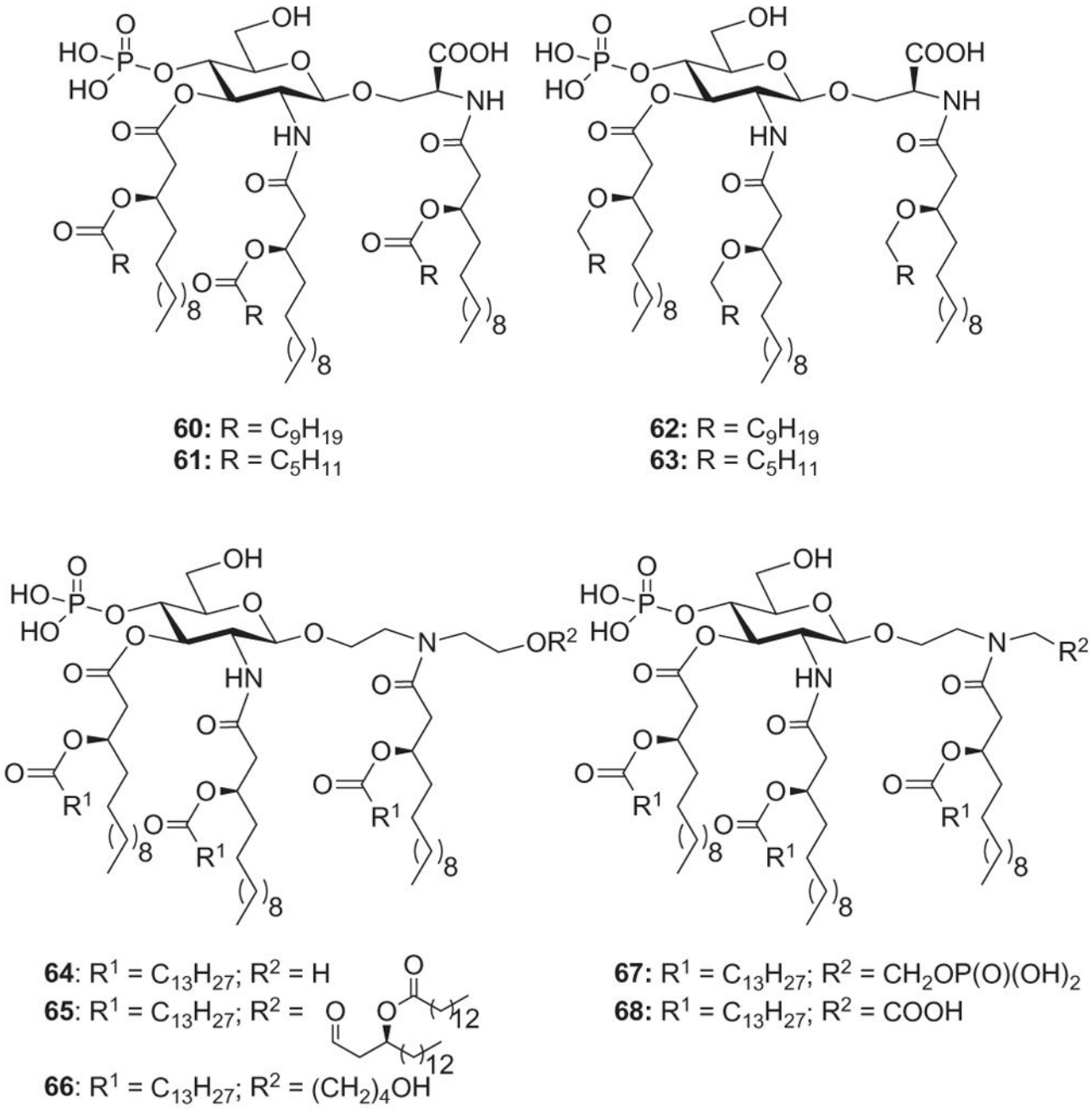

Fig. 14.
AGP class of Lipid A mimetics **60–68**.

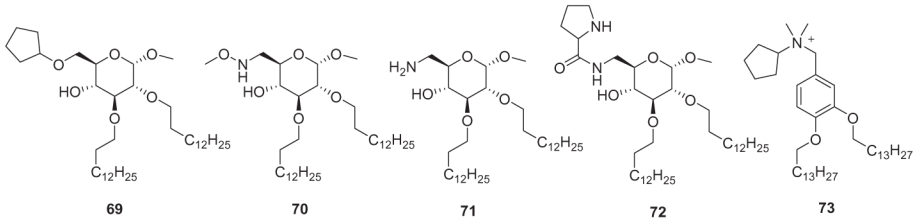

Fig. 15.
Glycolipid-based (**69–72**) and benzylammonium lipid-based (**73**) Lipid A mimics.