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Biosynthesis and Structure-Activity Relationships of the Lipid A Family of Glycolipids

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Abstract

Lipopolysaccharide (LPS), a glycolipid found in the outer membrane of Gram-negative bacteria, is a potent elicitor of innate immune responses in mammals. A typical LPS molecule is composed of three different structural domains: a polysaccharide called the *O*-antigen, a core oligosaccharide, and Lipid A. Lipid A is the amphipathic glycolipid moiety of LPS. It stimulates the immune system by tightly binding to Toll-like receptor 4. More recently, Lipid A has also been shown to activate intracellular caspase-4 and -5. An impressive diversity is observed in Lipid A structures from different Gram-negative bacteria, and it is well established that subtle changes in chemical structure can result in dramatically different immune activities. For example, Lipid A from *Escherichia coli* is highly toxic to humans, whereas a biosynthetic precursor called Lipid IV_A blocks this toxic activity, and monophosphoryl Lipid A from *Salmonella minnesota* is a vaccine adjuvant. Thus, an understanding of structure-activity relationships in this glycolipid family could be used to design useful immunomodulatory agents. Here we review the biosynthesis, modification, and structure-activity relationships of Lipid A.

Lipopolysaccharide (LPS) is a complex glycolipid found in the outer membrane of Gram-negative bacteria. It acts as a barrier to entry of foreign molecules into the bacterium, and plays a role in maintaining the integrity of cell membrane. Lipid A, the conserved, lipid component of LPS, anchors LPS to the outer membrane. Canonical Lipid A, produced in *E. coli*, is a β-(1′,6)-linked disaccharide of glucosamine that is hexaacylated and bis-phosphorylated (Compound 2, Figure 4). The low $pK_{a,1}$ value of its phosphate groups results in the compound having at least two negative charges at neutral pH, and contribute towards a net negative surface charge[1]. As a pathogen associated molecular pattern (PAMP), Lipid A induces a range of human innate immune responses upon binding to its receptors, namely Toll-like receptor (TLR4), caspase-4, and caspase-5. These responses result in the recruitment of immune cells and fluids to the site of infection to eliminate the foreign

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pathogen. In some situations, Lipid A can trigger systemic inflammation that cause tissue damage and occasionally death. To evade this immune response, some Gram-negative bacteria, such as *Yersinia Pestis*, have diversified their Lipid A structures to result in attenuated inflammation. In recent years, the structural diversity of Lipid A has been harnessed to develop a vaccine adjuvant (MPL®) that safely enhances a beneficial adaptive immune response against the co-inoculated antigen[2]. This review discusses the biosynthetic diversity of Lipid A, and the sensing of Lipid A analogs by components of the host defense systems. The consolidated information provides a landscape of our current understanding of Lipid A immune response and insights into the potential therapeutic use of this class of molecules.

Biosynthesis of Lipid A in *E. coli*[3,*4]

In *Escherichia coli*, the biosynthesis of Lipid A is catalyzed by nine enzymes (LpxA, LpxC, LpxD, LpxH, LpxB, LpxK, KdtA, LpxL, LpxM), and requires three distinct protein-bound acyl donor substrates along with UDP-*N*-acetylglucosamine (UDP-GlcNAc), ATP, and CMP-3-deoxy-D-*manno*-octulosonic acid (CMP-Kdo). The natural acyl donor substrates are fatty acyl chains attached to the acyl carrier protein (acyl-ACPs), intermediates in fatty acid biosynthesis. Hence, the Lipid A biosynthetic pathway is considered to operate downstream of fatty acid biosynthesis (Figure 1). To facilitate the trafficking of LPS to the outer membrane, Lipid A biosynthetic enzymes (LpxA through LpxM) are either expressed in the cytoplasm or anchored to the inner membrane. After core oligosaccharide-Lipid A biosynthesis, the compound is enzymatically flipped and oriented towards the periplasm for the addition of *O*-antigenic polysaccharide, and it is subsequently transported to the outer membrane (reviewed in [3]).

To initiate the biosynthetic pathway, cytosolic proteins LpxA, LpxC and LpxD catalyze a series of reactions converting UDP-GlcNAc into UDP-2,3-diacyl-glucosamine. First, LpxA transfers the acyl chain from (*R*)-β-hydroxymyristoyl-ACP to the 3-OH group of UDP-GlcNAc. This acyl transfer reaction is thermodynamically unfavorable. To drive the reaction forward, LpxC, a Zn^{2+}-dependent metalloenzyme, hydrolyzes the 2-acetamido functionality, and thus catalyzes the first committed step in the pathway. LpxD then installs another (*R*)-β-hydroxymyristoyl moiety onto the 2-amino group, yielding UDP-2,3-diacyl-glucosamine.

The UDP-2,3-diacyl-glucosamine product of LpxA-C-D is transformed by three membrane associated enzymes, LpxH, LpxB and LpxK, into tetraacylated Lipid IV_A (Compound 1, Figure 4). LpxH hydrolyzes UDP-2,3-diacylglucosamine to yield 2,3-diacylglucosamine 1-phosphate. LpxB catalyzes formation of a β-1′,6-glycosidic bond to generate the disaccharide backbone. In the final step leading up to Lipid IV_A, LpxK phosphorylates the 4′-position of this disaccharide.

Prior to late-stage acylation, *E. coli* requires the addition of two 2-keto-3-deoxyoctonate (Kdo) sugars to Lipid IV_A. A bifunctional enzyme KdtA catalyzes two successive glycosyl transfer reactions to form Kdo_2-Lipid IV_A. This tetraacyl tetrasaccharide is further modified by late acyl transferases LpxL and LpxM through the addition of lauroyl and myristoyl secondary acyl chains, respectively, to yield hexaacylated Lipid A.

Biosynthetic Diversity of Lipid A[3,*4]

Gram-negative bacteria have evolved an immense capacity for diversifying Lipid A structure (reviewed in [4]). The modification system typically involves enzymes within the biosynthetic pathway and/or downstream tailoring enzymes that recognize and modify Lipid A.

Within the Lipid A biosynthetic pathway, acyl transferase orthologs have variable substrate specificities, and thus contribute towards alterations in product structure (Table 1). These enzymes recognize fatty acyl chains of different lengths and oxidation patterns. For example, *E. coli* LpxA has 100-1000 times higher specificity for (*R*)-β-hydroxymyristoyl-ACP (C14:0) than C_{12} or C_{10} acyl-ACP substrates. While *E. coli* acyl transferases are chain length specific, orthologs from other Gram-negative bacteria are more tolerant, yielding a heterogeneous Lipid A composition of the outer membrane. These enzymes also show diversity with respect to the identity of their acyl acceptors. For example, *E.coli* LpxA transfers acyl chain to UDP-GlcNAc while *Leptospira interrogans* LpxA specifically modifies UDP 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose (UDP-GlcNAc3N) [5,6].

Beyond the enzymes in Lipid A biosynthesis, a variety of tailoring enzymes can further modify the Lipid A structure (Figure 2). Phosphatases LpxE and LpxF, anchored to the inner membrane, hydrolyze 1- and 4′- phosphate group in the periplasm, respectively. Group transferases such as LpxT and EptA use periplasmically accessible donors, such as undecaprenyl pyrophosphate and phosphatidyl ethanolamine, to further decorate the two phosphates on the disaccharide backbone. Similarly, group transferase ArnT transfers cationic sugars from undecaprenyl phosphate-L-Ara4N (4-amino-4-deoxy-alpha-L-arabinopyranosyl phosphate) to Lipid A. PagP and PagL, located in the outer membrane, catalyze acyl group transfer from phospholipids and deacylation, respectively. Some Gram-negative bacteria express multiple tailoring enzymes that work sequentially to bring yet more variety to Lipid A structures. For example, *Capnocytophaga canimorsus* and *Helicobacter pylori* have LpxE and EptA homologs that process Lipid A sequentially: first LpxE hydrolyzes the 1-phosphate, and then EptA transfers phosphoethanolamine directly onto the disaccharide backbone [7,8].

In an effort to further diversify the Lipid A scaffold, several studies have combinatorially modified this biosynthetic pathway in different host bacteria. For example, *Escherichia coli* [9] and *Neisseria meningitides*[10] have been tested with constructs expressing one to three of the above-mentioned modifying enzymes. Interestingly, most strains expressing one modifying enzyme displayed a single dominant Lipid A species in their outer membranes, whereas strains expressing two or more modifying enzymes displayed a heterogeneous mixture of glycolipids. This observation highlights the substrate specificity of modifying enzymes.

Extracellular sensing of Lipid A by Toll-Like Receptors (TLRs)

Innate immune receptors such as TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) and trigger signaling cascades in response to these PAMPs, often leading to the release of pro-inflammatory cytokines. As a major component of the outer membrane of Gram-negative bacteria, LPS is recognized by Toll-like receptor 4 (TLR4) as a PAMP. A landmark series of experiments by Beutler and coworkers first revealed that a strain of mice harboring a missense mutation in the *TLR4* gene was resistant to otherwise fatal doses of LPS [11]. Subsequent studies reported that LPS recognition involves cooperation of multiple accessory proteins, including the lipopolysaccharide binding protein (LBP), CD14, and the TLR4-MD2 complex [12-14].

Due to the amphiphilic nature of LPS, it forms aggregates in blood. LBP separates a LPS monomer from its aggregated form and transfers the monomer to CD14, which in turn delivers the glycolipid molecule to the TLR4-MD2 complex [15-18]. The Lipid A portion of LPS binds to the extracellular domain of TLR4-MD2 and recruits another TLR4-MD2 complex to form $\alpha_2\beta_2$ homodimer (Figure 3) [1]. Although TLR4-MD2 complex is sufficient for signaling by Lipid A, it has been discovered that CD14 is essential for more elaborate Lipid A variants, such as “smooth LPS” in which Lipid A is conjugated to the *O*-antigen of *E. coli* [19].

Once activated by Lipid A or LPS, the TLR4-MD2 dimer recruits adapter proteins for functional signaling (Figure 3). Among all TLRs, TLR4 uniquely signals through two distinct pathways, the MyD88-dependent and TRIF-dependent pathways in a spatial and sequential order. The early-stage response, within the first 6 hours, occurs mainly on the cell surface. Activated TLR4-MD2 complex engages MyD88 and TIRAP (TIR-domain-containing adaptor protein); it activates transcription factors including nuclear factor-κB (NF-κB), and activator protein-1 (AP-1) and leads to rapid production of potent inflammatory cytokines such as interleukins -6 and -1β [20-23]. The delayed response involves endocytosis of TLR4-MD2 complex and recruitment of TRIF (TIR-domain-containing adaptor protein inducing IFN-β) and TRAM (TRIF-related adaptor molecule). Interferon regulatory factor 3 (IRF3) is then activated, leading to the production of interferon (IFN)-β [21]. As a type I interferon, IFN-β leads to maturation of antigen presenting cells; thus it provides a critical link between the innate and the adaptive immune responses [24-27].

Although the TLR4-MD2 complex can bind to a broad range of Lipid A variants, its downstream signaling activity is qualitatively and quantitatively influenced by the precise structure of the glycolipid ligand. The differences can be measured at both transcriptional levels as well as the subsequent cytokine profiles. Thus, different glycolipids have alternative cytokine fingerprints characteristic of MyD88-dependent and TRIF-dependent signaling cascades.

The number and length of acyl chains have strong effects, as do the identity and substituent pattern of the disaccharide scaffold. To begin with, tetra-, penta-, and hexa-acylated *E. coli* Lipid A differ in the number of acyl chains, and this results in different signaling activities.

The canonical *E. coli* (4+2) hexa-acylated Lipid A, comprising of two acyl chains on one GlcN and four on the other, is a potent agonist that activates both the MyD88-dependent and TRIF-dependent signaling cascades [28]. In contrast, penta-acylated Lipid A (3+2) and tetra-acylated Lipid IV_A are weaker agonists and antagonists, respectively, compared to the hexa-acylated version (Table 2a) [9,29]. Second, the acyl chain length (Table 2c) can also modulate the severity of TLR4 mediated immune responses [30]. Systematic evaluation of the effect of chain length of secondary fatty acyl substituents (4 to 14 carbons) revealed that a threshold chain length of 10 carbon atoms was required to induce cytokine production by human monocytes, and that Lipid A derivatives with 10 carbon chains have the highest cytokine induction and greatest pyrogenicity [30,31]. Third, phosphorylation state of the 1 and 4′-position on the disaccharide backbone also has an effect on TLR4 activation (Table 2b). For instance, recombinant *E.* coli's LPS containing penta-acylated, 1-phosphorylated Lipid A is more TLR4 stimulatory than LPS containing penta-acylated, 4′-phosphorylated Lipid A [32]. In addition, the 1-dephosphorylated lipid A congeners from *Salmonella minnesota* RE595, a mixture of tetra, penta and hexa-acylated species, promote T-cell response and can be safely used as an adjuvant [33]. Finally, disaccharide backbone variations, usually entailing incorporation of different sugar molecules, impact TLR4 activity. Although most Lipid A species contain a di-glucosamine backbone, *Campylobacter jejuni* and *L. interrogans* Lipid A possess a 2,3-diamino-2,3-dideoxy-D-glucopyranose backbone The presence of the two additional amide linkages compromises TLR-4 agonism, as demonstrated by a decreased level of tumor necrosis factor (TNF-α) induction in THP-1 cells, a human leukemia monocytic cell line [34]. Therefore, these one-factor-at-a-time changes to Lipid A show that the number and distribution of both acyl chains and substituent groups have a profound effect on TLR4 stimulating activity.

Several analogs of Lipid A have been investigated for use in clinical settings (Figure 4). Eritoran, an analog of Lipid IV_A, has undergone extensive evaluation in clinical trials for sepsis [35]. Monophosphoryl Lipid A from *Salmonella minnesota* R595 (MPL®)is gaining widespread use as an adjuvant in modern vaccines, including the hepatitis B virus (HBV) vaccine Fendrix and the human papillomavirus (HPV) vaccine Cervarix [36]. Recently, a structural similar MPLA analog G100 is in several clinical trials for cancer immuno-oncology therapies [37]. The X-ray crystal structures of the TLR4-MD2 complex bound to rough LPS [1] and Eritoran [38], and MD-2 bound to Lipid IV_A [39] have been solved, and provide some insight into the mechanistic logic of the structure-function relationships (reviewed in [40]).

Intracellular sensing of Lipid A by Caspases

For a long time, the TLR pathway remained the only known mechanism by which higher eukaryotes recognized and responded to the Lipid A family of glycolipids. Unexpectedly however, caspase-11-deficient mice showed resistance to LPS-induced septic shock [41]. It took another two decades before two independent groups made the discovery that intracellular LPS (as well as Lipid A) activated caspase-11 in a TLR4-independent manner, thereby adding a caspase to a growing list of high-affinity receptors of this remarkable family of bacterial glycolipids [42,43]. This novel caspase-mediated inflammatory response

to LPS was coined as the “non-canonical inflammasome”[43] (Figure 3), contrasting it to the canonical TLR-mediated pathways.

Both canonical and non-canonical inflammasome signaling exhibit auto-amplificatory behavior. TLR4 priming triggers NF-κB activity, thereby increasing cellular expression of both inflammasome components [44–47]. Subsequent ligand binding triggers proteolytic activation of the caspase, leading to pyroptosis as well as proteolytic maturation and release of IL-1β and IL-18 [48]. Importantly, other TLRs such as TLR2 [43] and TLR3 [42] can also prime the non-canonical inflammasome. Moreover, CD14, MD-1, and MD-2 are dispensable for non-canonical inflammasome activity [43]. Recently, it has been demonstrated that LPS directly binds to mouse caspase-11 and its human counterparts, caspase-4/5, thereby triggering activation in an oligomeric state [49,50]. The binding site is the caspase recruitment domain (CARD), and the K_d of glycolipid binding is exceptionally low [49].

Notably, all of the above studies used non-physiological methods for intracellular delivery of Lipid A, such as transfection, electroporation or cholera toxin subunit B mediated transport (which requires the core oligosaccharide of LPS). As such, the available structure-activity data for non-canonical inflammasome activity must be treated with caution. Nonetheless, it is clear that the Lipid A portion of LPS is a potent ligand of CARD and is sufficient for caspase activation [42,49]. Under-acylated Lipid A species, such as *E. coli* Lipid IV_A, tretra-aclylated *F. novicida* Lipid A and penta-acylated Lipid A from *R. sphaeroides'* LPS (LPS-RS), failed to activate the non-canonical inflammasome pathway [42,43,49]. Subsequent experiments investigating mechanistic logic revealed that Lipid IV_A and LPS-RS could bind caspase 4/11 but fail to induce their oligomerization and activation [49]. Further analysis of both the canonical and non-canonical inflammasome pathways rests critically on the availability of a systematic series of Lipid A analogs.

Conclusions

Gram-negative bacteria have developed an immense capacity to synthesize structurally diverse Lipid A analogs. These analogs differentially modulate human immune response through interactions with extracellular TLR and intracellular caspase pathways. While considerable effort has been directed to investigate their structure-activity relationships, many of these studies go back to the early days of discovery of these biological pathways, and have therefore not taken sufficient advantage of more recent insights into Lipid A chemistry and biosynthesis. Meanwhile, a number of recent studies involving mutant [51,52] or engineered [9,53,54] Gram-negative bacteria have vividly demonstrated the potential for discovery of novel immune-modulatory agents via engineered biosynthesis and semisynthesis. The stage is set for the development of more advanced chemical biology platforms that exploit the resurgence of interest into the biology of one of nature's oldest and most powerful natural products.

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Highlights

- The glycolipid Lipid A is the conserved, amphipathic moiety of LPS
- Lipid A binds to certain toll like receptors and caspases with high affinity
- Bacteria alter their Lipid A structures to modulate immune activities
- Non-toxic Lipid A congeners are effective immunomodulatory agents

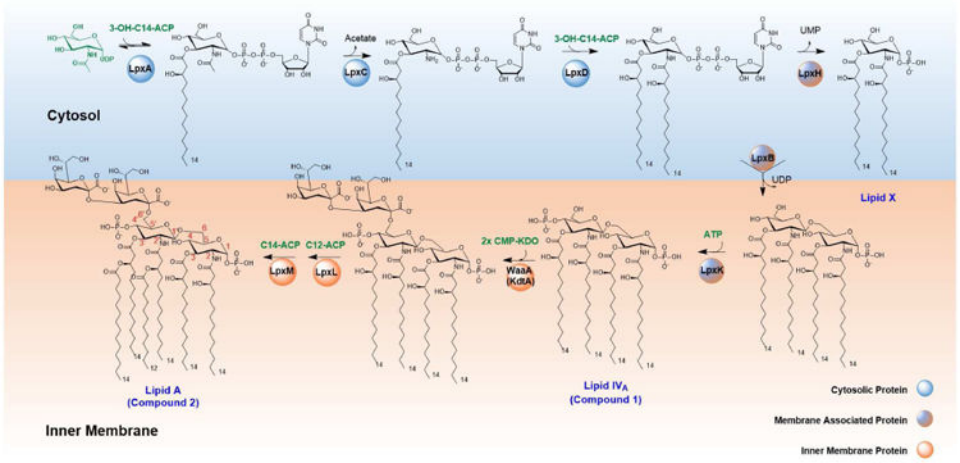

Figure 1. Biosynthesis of Lipid A in *E. coli*

(Adapted from [4]) The enzymes LpxA, LpxC, and LpxD are located in the cytoplasm, whereas LpxH, LpxB, LpxK, WaaA, LpxL and LpxM are anchored to the inner membrane. Substrates for the biosynthetic are highlighted in green: acyl carrier protein (ACP)-bound acyl donors, UDP-N-acetylglucosamine (UDP-GlcNAc), ATP, and CMP-3-deoxy-D-manno-octulosonic acid (CMP-Kdo). The product of the pathway is further modified into variable lipopolysaccharide structures via further glycosylation of the Kdo sugars (not explicitly shown).

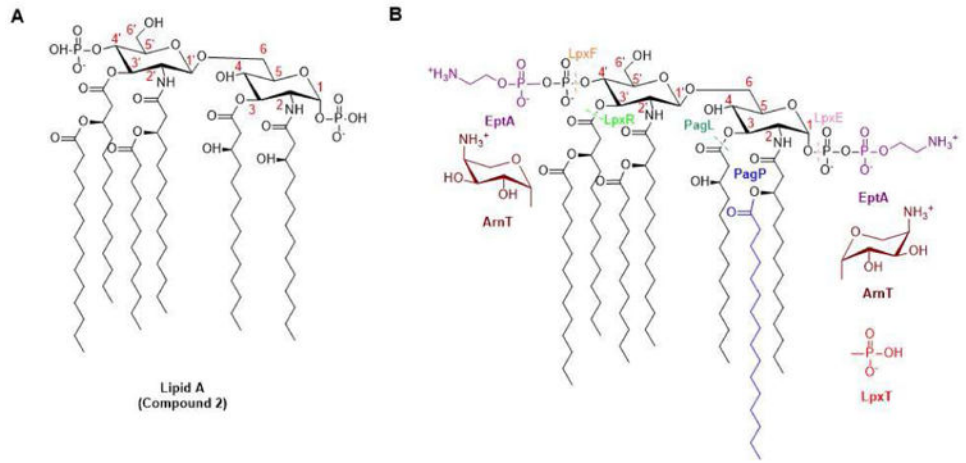

Figure 2. Modification of Lipid A by tailoring enzymes

(Adapted from [4]) (A) The canonical structure of Lipid A from *E. coli* is shown absent the two Kdo sugars, which are not essential for the immunological activities of Lipid A. (B) Modification(s) introduced by each representative tailoring enzyme is indicated in a different color, while components of *E. coli* Lipid A remain in black. Tailoring enzymes such as ArnT, EptA, LpxT, and PagP catalyze group transfer reactions, while enzymes such as PagL, LpxE, LpxF, and LpxR promote hydrolysis (dashed line across scissile bond). LpxE and/or LpxF homologs are observed in *Francisella tularensis*[67], *Helicobacter pylori* [68], *Porphyromonas gingivalis* [69], *Capnocytophaga canimorsus* [7], and *Bacteroides thetaiotaomicron* [70]. LpxR homologs are found in *Salmonella typhimurium* [71], *Yersinia enterocolitica* [72], and *Helicobacter pylori* [73]. PagL is found in *Salmonella typhimurium* [74] and *Pseudomonas aeruginosa* [75]. LpxT is found in *Escherichia coli* [76]. EptA in *Escherichia coli* [77] and *Campylobacter jejuni* [78] has broad specificity for both 1 and 4′ positions, while *P. aeruginosa* EptA only modifies the 4′-phosphate group [79]. PagP in *Pseudomonas aeruginosa* transfers a palmitoyl chain to the 3-OH of the 3′- acyl residue [80], whereas its homologs in *Escherichia coli* [81] and *Salmonella enterica* recognize the 3-OH of the 2-position acyl chain [82,83], and *Bordetella parapertussis* PagP can modify either hydroxyl group [84]. ArnT in *Salmonella typhimurium* can decorate the 1- and 4′-phosphates of Lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) [85,86], whereas ArnT in *Francisella tularensis* adds a galactosamine unit onto the 1-phosphate [87] and ArnT in *Bordetella pertussis* can add a glucosamine unit on either phosphate groups [88].

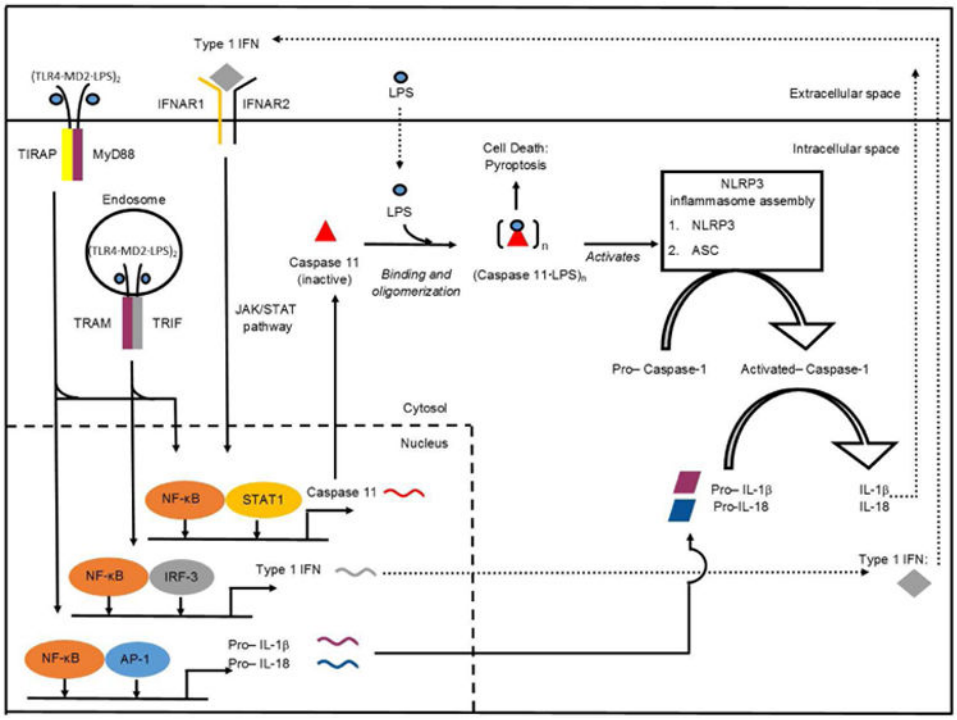

Figure 3. Non-canonical NLRP3 inflammasome activation

Extracellular LPS is recognized by TLR4/MD2 complex, and triggers MyD88- and TRIF-dependent signaling cascades. MyD88- and TRIF- signaling contribute to the transcriptional upregulation of pro-inflammatory cytokines (purple and blue rhombi) and Type I IFN (grey diamond), respectively. Secreted type I IFN binds the IFNAR1/IFNAR2 receptor and leads to the activation of JAK/STAT pathway. Both type I IFN signaling through JAK/STAT pathway, and LPS signaling through TRIF and MyD88 contribute to the expression of pro-caspase 11 (red triangle). Pro-caspase-11 directly binds cytosolic LPS through its CARD domain. Upon LPS binding, pro-caspase-11 oligomerizes to trigger its activation through proximity induced proteolytic cleavage. The activated caspase 11 results in cell pyroptosis and also NLRP3 inflammasome activation to trigger IL-1β and IL-18 release. Abbreviations: TLR, toll-like receptors; MD, myeloid differentiation; LPS, lipopolysaccharide; MyD88, adaptor molecules myeloid differentiation primary response 88; TIRAP, toll-interleukin 1 receptor domain containing adaptor protein;TRIF, toll/ IL-1 receptor homology (TIR)-domain-containing adapter-inducing interferon-β; NF-κB, nuclear factor- κB; TRAM, TRIF- related adaptor molecule; IRF, interferon regulatory factor; IFN, interferon; IL, interleukin; IFNAR, interferon-α/β receptor; STAT, signal transducer and activator of transcription; AP, activator protein; NLRP3, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-contain protein 3; ASC, apotosis-associated speck-like protein containing CARD; JAK/STAT, janus kinase/signal transducers and activators of transcription.

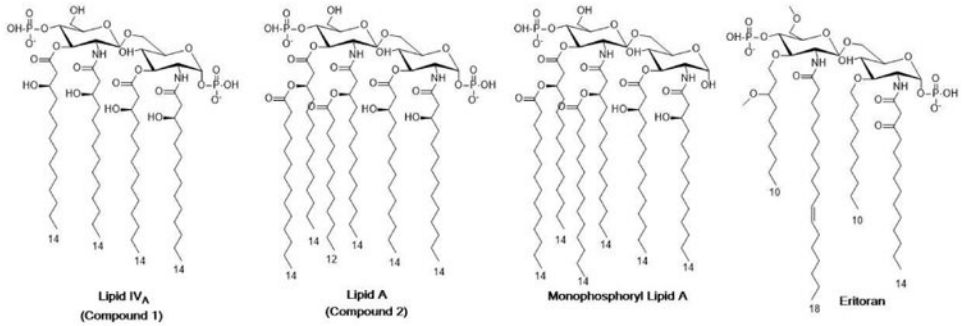

Figure 4. Structures of Lipid IVa, Lipid A, Monophosphoryl Lipid A, and Eritoran

Table 1

Differences in substrate specificity of LpxA, LpxD, LpxL and LpxM contribute to species specific acyl chain and disaccharide backbone variety in Lipid A variants.

Protein Name	Organism	Related Protein (% identity)	Acyl Donor	Acyl Acceptor	Key Reference
LpxA	*E. coli*	Reference	(R)-β-hydroxymyristoyl-ACP	3-OH group of UDP-GlcNAc	[55]
LpxA	*C. trachomatis*	37.4% *E. coli* LpxA	Myristoyl-ACP	3-OH group of UDP-GlcNAc	[54]
LpxA	*P. aeruginosa*	53.4% *E. coli* LpxA	(R)-β-hydroxydecanoyl-ACP	3-OH group of UDP-GlcNAc	[56]
LpxA	*N. meningitidis*	47.3% *E.coli* LpxA	(R)-β-hydroxylauroyl-ACP	3-OH group of UDP-GlcNAc	[57]
LpxA	*H. pylori*	38.1% *E. coli* LpxA	(R)-β-hydroxypalmitoyl-ACP	3-OH group of UDP-GlcNAc	[58]
LpxA	*L. interrogans*	41.3% *E.coli* LpxA	(R)-β-hydroxylauroyl-ACP	3-NH_2 group of UDP-GlcNAc3N	[59]
LpxD	*E. coli*	Reference	(R)-β-hydroxymyristoyl-ACP	2-NH_2 group of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN	[60]
LpxD	*P.aeruginosa*	48.7% *E. coli* LpxD	(R)-β-hydroxydodecanoyl-ACP	2-NH_2 group of UDP-3-O-(R-3-hydroxydecanoyl)-GlcN	[61]
LpxL	*E. coli*	Reference	Lauroyl-ACP	β -OH group on 2′ acyl chain of Kdo_2-$LipidIV_A$	[62]
LpxM	*E. coli*	Reference	Myristoyl-ACP	β -OH group on 3′ position of Kdo_2-(lauroyl) Lipid IV_A	[63]
LpxP	*E. coli*	54.1% *E.coli* LpxL	Palmitoleoyl-ACP	β -OH group on 2′ acyl chain of Kdo_2-$LipidIV_A$	[64]
LpxXL	*R. leguminosarum*	20.4% *E. coli* LpxL, 23.5% *E. coli* LpxM	27-hydroxyoctacosanoyl-ACPXL	Kdo_2-$LipidIV_A$	[65]
LpxL1	*B. pertussis*	23.9% *E. coli* LpxL, 20.6% *E. coli* LpxM	Not Available [2-hydroxylauroyl-ACP[2]]	Not Available [Observed as a secondary acyl chain at the 2-position of Lipid A]	[66]

[1]The abbreviations used are: UDP-GlcNAc, Uridine diphosphate N-acetylglucosamine; ACP, Acyl carrier protein; UDP-GlcNAc3N, Uridine diphosphate 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose; GlcN, Glucosamine; UDP, Uridine diphosphate; Kdo, 3-deoxy-D-manno-octulosonic acid

[2]Inferred based on observed Lipid A structure

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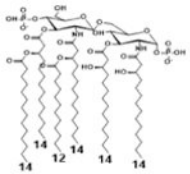
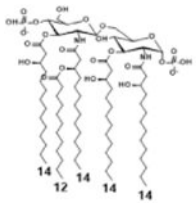
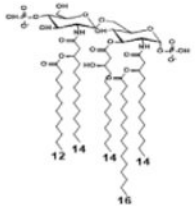
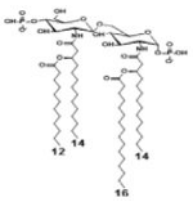
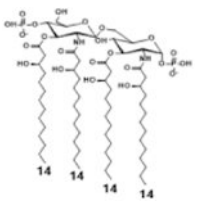
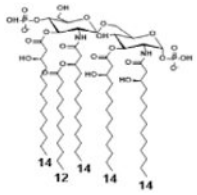
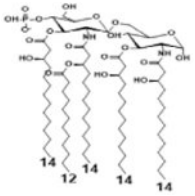
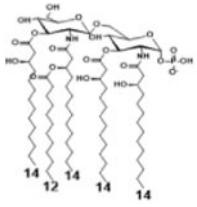
Table 2

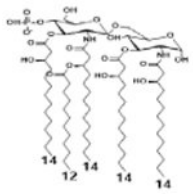
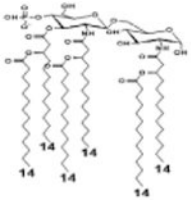
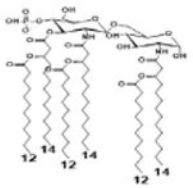
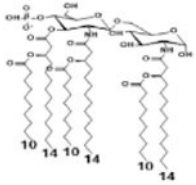
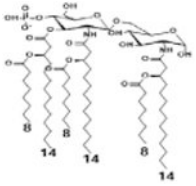
Structure-activity relationships of Lipid A analogs binding to TLR4.

2 a) TLR4 activity as a function of number and distribution of acyl chains						
						
Description		**Lipid A [Benchmark] Compound 2**	**Penta-acylated Lipid A**	**Penta-acylated Lipid A**	**Tetra-acylated Lipid A**	**Lipid IV_A Compound 1**
NF-κB	Activation	+++++	ND	+++++	++++	ND
MyD88	TNF-α	+++++	ND	++	++	N/A
	IL-1β	+++++	+	+++	++	N/A
	IL-8	+++++	++	+++++	+++++	N/A
TRIF	G-SCF	+++++	ND	++	+	N/A
	RANTES	+++++	+	++	++	N/A
	MCP-1	+++++	+	+++	+++	N/A
Conditions	Lipid A analog cone.	100ng/mL	100ng/mL	100ng/mL	100ng/mL	100ng/mL
	Cell type	THP-1	THP-1	THP-1	THP-1	THP-1
	Reference	[9]	[9]	[9]	[9]	[29]

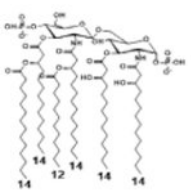
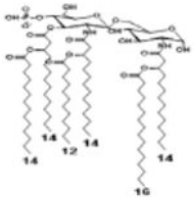
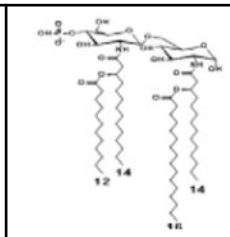
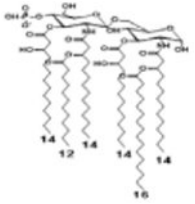
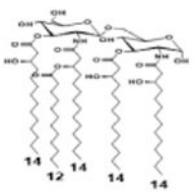
2 b) TLR4 activity as a function of number and distribution of phosphate substituents				
				
Description		Bis-phosphorylated Lipid A [Benchmark]	4′- phosphorylated Lipid A	1- phosphorylated Lipid A
NF-κB	Activation	++	++	+++

Conditions	Lipid A analog cone.	100ng/mL	100ng/mL	100ng/mL
	Cell type	HEK293 hTLR4/hMD-2	HEK293 hTLR4/hMD-2	HEK293 hTLR4/hMD-2
	Reference	[32]	[32]	[32]

2 c) TLR4 activity as a function of secondary acyl chain length

						
Description	Secondary acyl chain length	4′- phosphorylated Lipid A [Benchmark]	14-14-14	12-12-12	10-10-10	8-8-8
MyD88	TNF-α	+	+	+++	++	ND
	IL-1β	+	++	++	+++++	ND
Conditions	Lipid A analog conc.	10μg/mL	10μg/mL	10μg/mL	10μg/mL	10μg/mL
	Cell type	Human whole blood	Human whole blood	Human whole blood	Human whole blood	Human whole blood
	Reference	[30]	[30]	[30]	[30]	[30]

2 d) TLR4 activity as a function of combined structural changes

						
Description		Lipid A [Benchmark] Compound 2	Hexa-acylated, monophosphorylated Lipid A	Tetra-acylated, monophosphorylated Lipid A	Hexa-acylated, monophosphorylated Lipid A	Penta-acylated Lipid A without phosphates
Modification enzymes involved		Benchmark	PagP, PagL, LpxE	LpxE, PagL, LpxR	LpxM, LpxE, PagP	LpxM, LpxE, LpxF
VF-κB	Activation	+++++	+++++	+++++	++++	++
MyD88	TNF-α	+++++	++	+	+	ND
	IL-1β	+++++	+++	+	+	ND

	IL-9	+++++	+++++	++++	+++++	+++
TRIF	G-SCF	+++++	++	+	ND	ND
	RANtes	+++++	++	+	+	ND
	MCP-1	+++++	++	+	++	+
Conditions	Lipid A analog conc,	100ng/ml	l00ng/mL	100ng/mL	100ng/mL	100ng/mL
	Cell type	THP-1	THP-1	THP-1	THP-1	THP-1
	Reference	[9]	[9]	[9]	[9]	[9]

[a] '+' symbols correspond to the signaling output intensity. All activity intensities are normalized with respect to the benchmark (Column 1, Tables (2a, 2b, 2c, 2d)). The greater the number of '+' symbols, the greater the signaling intensity.

[b] Signaling intensity of 'ND' corresponds to baseline/ negligible activity output

[c] N/A: not available