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Kdo_2-lipid A: structural diversity and impact on immunopharmacology

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ABSTRACT

3-deoxy-D-manno-octulosonic acid-lipid A (Kdo_2-lipid A) is the essential component of lipopolysaccharide in most Gram-negative bacteria and the minimal structural component to sustain bacterial viability. It serves as the active component of lipopolysaccharide to stimulate potent host immune responses through the complex of Toll-like-receptor 4 (TLR4) and myeloid differentiation protein 2. The entire biosynthetic pathway of *Escherichia coli* Kdo_2-lipid A has been elucidated and the nine enzymes of the pathway are shared by most Gram-negative bacteria, indicating conserved Kdo_2-lipid A structure across different species. Yet many bacteria can modify the structure of their Kdo_2-lipid A which serves as a strategy to modulate bacterial virulence and adapt to different growth environments as well as to avoid recognition by the mammalian innate immune systems. Key enzymes and receptors involved in Kdo_2-lipid A biosynthesis, structural modification and its interaction with the TLR4 pathway represent a clear opportunity for immunopharmacological exploitation. These include the development of novel antibiotics targeting key biosynthetic enzymes and utilization of structurally modified Kdo_2-lipid A or correspondingly engineered live bacteria as vaccines and adjuvants. Kdo_2-lipid A/TLR4 antagonists can also be applied in anti-inflammatory interventions. This review summarizes recent knowledge on both the fundamental processes of Kdo_2-lipid A biosynthesis, structural modification and immune stimulation, and applied research on pharmacological exploitations of these processes for therapeutic development.

Key words: Kdo_2-lipid A, lipopolysaccharide, endotoxin, Gram-negative bacteria, outer membrane.

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I. INTRODUCTION

Gram-negative bacteria have two distinct membranes: an inner membrane and an outer membrane. Lipopolysaccharide (LPS) is a prominent constituent in the outer leaflet of the outer membrane. LPS can be recognized by different immune cells such as dendritic cells, monocytes and mast cells (Matsuguchi, 2012) as a pathogen-associated antigen through a protein complex consisting of Toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2) (Poltorak *et al.*, 1998; Triantafilou & Triantafilou, 2002; Akira, Uematsu & Takeuchi, 2006). LPS binds to a large hydrophobic pocket of MD-2 in one TLR4/MD-2 complex using five of its six lipid chains (see below for structural details) and bridges another TLR4/MD-2 complex using the remaining lipid chain along with its phosphate groups which directly interact with TLR4 in the second TLR4/MD-2 complex. This results in formation of a symmetrical TLR4/MD-2/LPS heterodimer that is active in immune signalling (Park *et al.*, 2009; Bryant *et al.*, 2010; Kang & Lee, 2011; Ohto *et al.*, 2012). After activation by LPS, TLR4 recruits two major signalling pathways that involve different adaptor proteins, myeloid differentiation primary response protein 88 (MyD88) and Toll-interleukin-1 receptor-domain-containing adaptor-inducing interferon-β (TRIF) respectively, within the cytoplasm of cells to propagate a cascade of signal transductions (Yamamoto *et al.*, 2002; Zheng *et al.*, 2012). This leads to the induction or suppression of genes that orchestrate the inflammatory response (Fig. 1).

The LPS molecule consists of three parts: 3-deoxy-D-manno-octulosonic acid-lipid A (Kdo$_2$-lipid A), core polysaccharides and O-antigen repeats (Wang & Quinn, 2010*a*,*b*). Kdo$_2$-lipid A is responsible for displaying LPS on the surface of bacterial cells. It is highly selective for TLR4 and its biological activity is comparable to that of the complete LPS molecule (Raetz *et al.*, 2006). In limited infections, the response to Kdo$_2$-lipid A is beneficial, helping to dispose of invading microbes. However, in overwhelming infections, high levels of circulating cytokines can give rise to the syndrome of septic shock (Parrillo, 1993). The response mounted by the host immune system depends on both the severity of infection and the particular structure of Kdo$_2$-lipid A of the invading bacteria. *Escherichia coli* Kdo$_2$-lipid A, containing two phosphate groups and six acyl chains composed of 12 or 14 carbons (Fig. 2), is a powerful activator of the innate immune system. However, some Gram-negative pathogens such as *Helicobacter pylori* (Ogawa *et al.*, 1997; Suda *et al.*, 2001) and *Francisella tularensis* (Sandstrom *et al.*, 1992; Ancuta *et al.*, 1996) synthesize Kdo$_2$-lipid A molecules that are poorly recognized by human TLR4. The phosphate groups and the length and number of fatty acyl chains of Kdo$_2$-lipid A play important roles in TLR4 activation (Loppnow *et al.*, 1989; Rietschel *et al.*, 1994; Persing *et al.*, 2002; Hajjar *et al.*, 2012).

For decades, LPS has been used for clinical or biological studies of endotoxin activity, but its direct detection and quantification is problematic because of its large size and micro-heterogeneity (Raetz & Whitfield, 2002; Wang *et al.*, 2010). Kdo$_2$-lipid A, however, is a reproducible, well-defined membrane lipid that can be readily detected with great sensitivity by electrospray ionization mass spectrometry (ESI/MS) after uptake by cultured macrophages or administered to animals, permitting quantitative pharmacokinetic and metabolic studies. Kdo$_2$-lipid A, therefore, has a significant advantage over the complete LPS molecule when used for studies of endotoxin activity. The biosynthetic pathway and export mechanisms of Kdo$_2$-lipid A have been well characterized in *E. coli*, and are common to most Gram-negative bacteria. However, the number of Kdo residues connected to lipid A differs among certain bacteria. For example, in *Haemophilus influenzae* and *Bordetella pertussis*, only one Kdo residue is connected to lipid A (Isobe *et al.*, 1999; White *et al.*, 1999), and a phosphate group is incorporated at the same position where the outer Kdo residue is located (White *et al.*, 1999); in *Chlamydia trachomatis* at least three Kdo residues are connected to lipid A (Belunis *et al.*, 1992). Thus the detailed structure of Kdo$_2$-lipid A may vary from one bacterium to another (Trent *et al.*, 2006) and this variation could affect the virulence of the bacterium (Wilkinson, 1996; Raetz *et al.*, 2007). Modified Kdo$_2$-lipid A might act as an immuno-modulator and induce non-specific resistance to both bacterial and viral infections. Therefore, knowledge of the structure of Kdo$_2$-lipid A and its

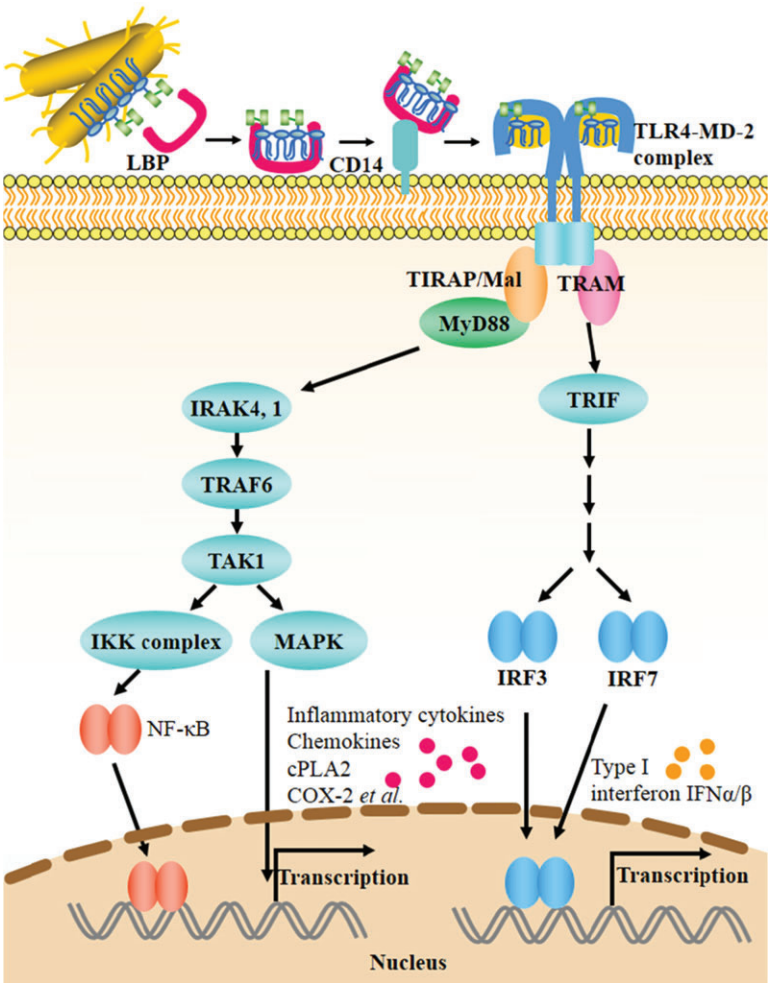

Fig. 1. Host immune response to LPS through TLR4-MD2 signalling. LPS on the outer membrane of Gram-negative bacteria is recognized and extracted by a serum protein LBP. The LBP-LPS complex is then recognized and bound by the leukocyte extrinsic membrane protein CD14 through which LPS is delivered to the TLR4-MD-2 complex. Binding of the LPS by the heterodimer of the TLR4-MD-2 complex triggers the dimerization of the cytoplasmic domain (TIR domain) of TLR4 and recruitment of specific adaptor proteins. Two signal transduction pathways are then activated in the cytoplasm: the MyD88/TIRAP and the TRAM/TRIF pathways which lead to the activation of the transcription factors NF-κB, and IRF3 and 7, respectively. NF-κB-activated gene transcription leads to the production of pro-inflammatory cytokines, chemokines, iNOS etc. which are strong inflammatory signals that lead to the clearance of bacterial pathogens. IRF3 and 7-activated gene transcription mainly causes the production of Type I interferons important for adjuvanticity. Overproduction of inflammatory molecules during overwhelming infection causes septic shock. Only the Kdo_2-lipid A moiety of LPS is shown. See Section VI for abbreviations.

modification is highly relevant to the development of new vaccines.

II. BIOSYNTHESIS OF KDO_2-LIPID A

The outer leaflet of the outer membranes of most Gram-negative bacteria is composed primarily of Kdo_2-lipid A, the hydrophobic anchor of LPS. Because Kdo_2-lipid A is essential for the survival of bacteria, its biosynthesis has been intensively studied in order to develop methods to control Gram-negative pathogens. Although Kdo_2-lipid A is displayed on the surface of bacterial cells, its synthesis is initiated in the cytoplasm. The way Kdo_2-lipid A is synthesized in the cytoplasm and exported to the surface of bacteria has been studied most extensively in *E. coli*.

(1) Raetz pathway

The biosynthesis of Kdo_2-lipid A takes place *via* the Raetz pathway (Raetz *et al.*, 2007; Kresge, Simoni & Hill, 2011). The pathway is mediated by nine sequential steps, from initiation in the cytoplasm to completion on the inner surface of the inner membrane (Fig. 2). The

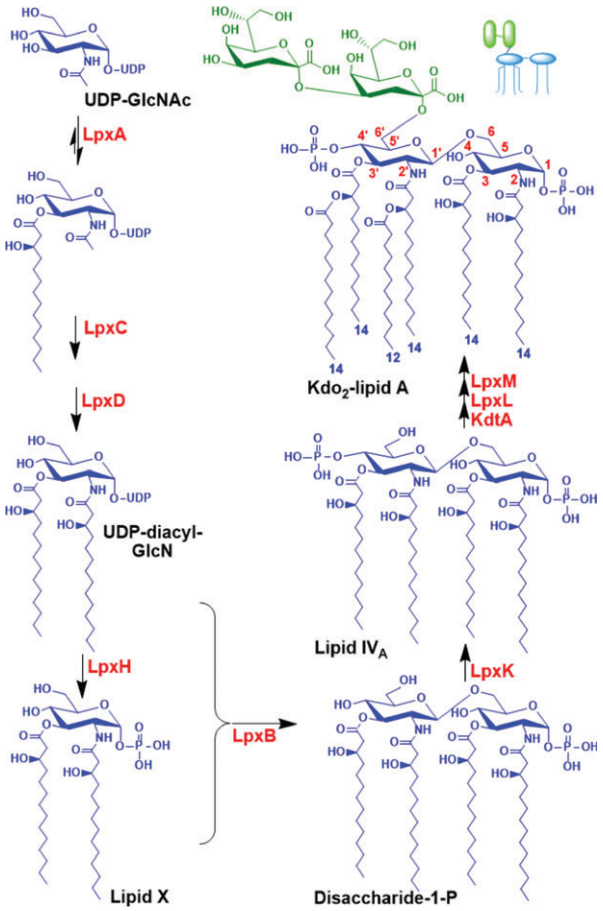

Fig. 2. Structure and biosynthetic pathway of Kdo$_2$-lipid A in *E. coli*. The numbers specify the glucosamine ring positions indicate the predominant fatty acid chain lengths found in *E. coli* lipid A.

evolutionary origin of enzymes of the Raetz pathway involved in the biosynthesis of Kdo$_2$-lipid A has been described (Opiyo *et al.*, 2010).

(*a*) *Reactions catalysed by cytosolic enzymes LpxA, LpxC, LpxD, LpxH and LpxB*

The initial building block for the biosynthesis of Kdo$_2$-lipid A is UDP-*N*-acetylglucosamine (UDP-GlcNAc). The first three reactions of the pathway are catalysed by soluble enzymes LpxA, LpxC and LpxD, resulting in the addition of two 3-OH fatty acid chains to form UDP-diacyl-GlcN. Since the first reaction catalysed by LpxA is reversible, the second reaction catalysed by LpxC becomes the committed step in the pathway. LpxA, LpxC and LpxD have been isolated and their structures characterized by X-ray diffraction and nuclear magnetic resonance (NMR) (Buetow *et al.*, 2007; Barb *et al.*, 2007*a*; Williams & Raetz, 2007; Bartling & Raetz, 2009; Zhang *et al.*, 2012). LpxA and LpxD are structurally similar and both function as homotrimers. The active sites of *E. coli* LpxA and LpxD function as precise hydrocarbon rulers and are manifested by the length of hydroxyacyl chains incorporated. This explains why all the primary fatty acids of Kdo$_2$-lipid A in *E. coli* are the same length (C14). Single amino acid alteration in LpxA in the proximal position of the acyl chain binding site can lead to the incorporation of acyl chains of different lengths at the 3- or 3′- positions (Shah *et al.*, 2013). For instance, replacement of the serine with a leucine in the position corresponding to glycine 176 in *E. coli* LpxA and in *B. pertussis* BP338 LpxA results the incorporation of a shorter acyl chain length (C_{10} or C_{12}) in its lipid A structure (Shah *et al.*, 2013). LpxC is Zn^{2+}-dependent and has no sequence homology with other deacetylases, which makes it a promising target for the development of novel antibiotics. Following the incorporation of the two hydroxyacyl chains, peripheral membrane protein LpxH hydrolyses UDP-diacyl-GlcN to form lipid X (Babinski, Kanjilal & Raetz, 2002*a*; Babinski, Ribeiro & Raetz, 2002*b*). Many Gram-negative bacteria, including all α-proteobacteria and diverse environmental isolates, lack LpxH. A distinct UDP-2,3-diacylglucosamine pyrophosphatase, designated LpxI, was recently discovered. LpxI has no sequence similarity to LpxH despite an enzymatic action that generates the same products as LpxH although by a different mechanism (Metzger *et al.*, 2012). LpxI hydrolyses UDP-diacyl-GlcN by attacking its β-phosphate, whereas LpxH attacks α-phosphate. The gene *lpxI* is located between *lpxA* and *lpxB* in *Caulobacter crescentus*, and could rescue the conditional lethality of *lpxH*-deficient *E. coli* (Metzger & Raetz, 2010; Metzger *et al.*, 2012). Another peripheral membrane protein LpxB condenses lipid X and its precursor UDP-diacyl-GlcN to form disaccharide-1-P (Crowell, Anderson & Raetz, 1986; Crowell, Reznikoff & Raetz, 1987). LpxD, FabZ, LpxA and LpxB are encoded by a gene cluster *lpxD-fabZ-lpxA-lpxB* in *E. coli* and several other species of bacteria (Mohan *et al.*, 1994; Steeghs *et al.*, 1997). Among them, LpxA, LpxB and LpxD catalyse early steps in the Kdo$_2$-lipid A pathway using (3R)-hydroxyacyl-acyl carrier protein (ACP) as a donor; while FabZ catalyses the dehydration of (3R)-hydroxyacyl-ACP to trans-2-acyl-ACP (Heath & Rock, 1996), which is further utilized as a fatty acid donor in the biosynthesis of phospholipids. This gene cluster is important in regulating the proportions of LPS and phospholipids in the membranes of Gram-negative bacteria.

(*b*) *Reactions catalysed by integral membrane proteins LpxK, KdtA, LpxL and LpxM*

The following sequential reactions in the Kdo$_2$-lipid A biosynthesis pathway are catalysed by the integral membrane proteins LpxK, KdtA, LpxL and LpxM, respectively (Fig. 2). Kinase LpxK phosphorylates the 4′-position of the disaccharide-1-P to form lipid IVA (Garrett, Kadrmas & Raetz, 1997; Garrett, Que & Raetz,

1998). The crystal structure of LpxK has been reported (Emptage *et al.*, 2012). The N-terminal domain of the protein is responsible for both catalysing at the P-loop and locating the disaccharide-1-phosphate on the inner membrane where phosphoryl transfer takes place. Lipid IV_A has hitherto been the minimal LPS structure able to sustain *E. coli* viability (Klein *et al.*, 2009). It serves as the acceptor for the Kdo moiety. Transferase KdtA then transfers two Kdo residues from CMP-Kdo to the non-reducing GlcN residue of lipid IVA (Brozek *et al.*, 1989). Crystal structures of the free and CMP-Kdo-bound forms of KdtA from *Aquifex aeolicus* reveal details of the CMP-binding site and implicate a unique sequence motif in Kdo binding. In addition, a cluster of highly conserved amino acid residues was identified which represents the potential membrane-attachment and acceptor-substrate binding site of KdtA (Schmidt *et al.*, 2012). Three acyltransferases LpxL, LpxP and LpxM encoded by paralogous genes catalyse the next reactions in the Raetz pathway in *E. coli,* using ACP-activated fatty acids as co-substrates. At ambient temperatures, a lauroyl residue is first transferred by LpxL to the OH group of the 2′-N-linked (R)-3-hydroxymyristate residue. At low temperature (12°C), however, this catalytic step is partially replaced by LpxP, which transfers palmitoleate to the same position (Vorachek-Warren *et al.*, 2002). The free OH group of the 3′-O-linked (R)-3-hydroxymyristate residue within both types of penta-acylated intermediates is then myristoylated by LpxM to yield a hexa-acylated Kdo_2-lipid A (Fig. 2) (Brozek & Raetz, 1990). Homologous genes for the late acyltransferases LpxL, LpxP and LpxM have also been found in *Cronobacter sakazakii* (Cai *et al.*, 2013).

In most Gram-negative bacteria, the structure of Kdo_2-lipid A and the enzymes involved in the Raetz pathway are highly conserved (Opiyo *et al.*, 2010). Nevertheless the detailed structure of Kdo_2-lipid A is known to be different in some Gram-negative bacteria (Bainbridge *et al.*, 2008; Zhang *et al.*, 2010). The fatty acyl chains of Kdo_2-lipid A are 10, 12 or 14 carbons long in *E. coli* and *Bordetella pertussis* 18-323, and 16 or 18 carbons long in *F. novicida* (Wang *et al.*, 2006; Shaffer *et al.*, 2007; Shah *et al.*, 2013). Two LpxD enzymes LpxD1 and LpxD2 were found in *F. novicida* (Li *et al.*, 2012). LpxD1 adds a 3-OH C_{18} acyl group at 37°C, whereas LpxD2 adds a 3-OH C_{16} acyl group at 18°C. Temperature controls not only the transcription of the two *lpxD* genes, but also the enzymatic activities of LpxD1 and LpxD2. In *Rhizobium etli* Kdo_2-lipid A contains a C_{28} acyl chain which is added by the acyltransferase LpxXL (Basu, Karbarz & Raetz, 2002), using the specialized acyl carrier protein AcpXL as a donor. Compared to the archetypical ACP from *E. coli*, the structure of AcpXL has an extra α-helix, resulting in a larger opening to the hydrophobic cavity that could accommodate the very long-chain fatty acid (Ramelot *et al.*, 2012).

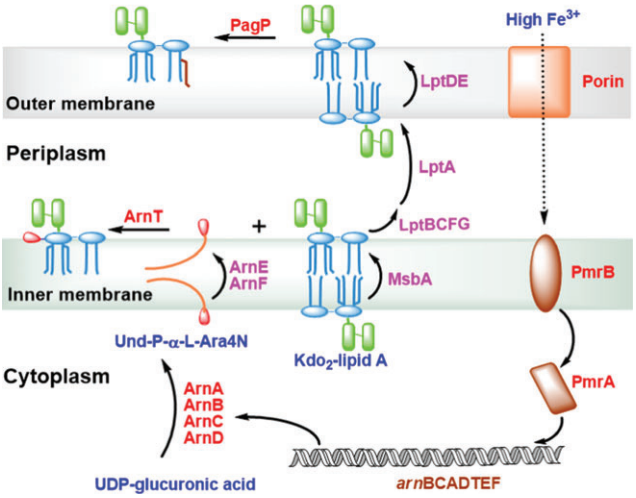

Fig. 3. The transport and structural modification of Kdo_2-lipid A in *E. coli*. At least eight proteins are involved in the export of Kdo_2-lipid A. The ABC transporter MsbA flips newly synthesized Kdo_2-lipid A to the outer surface of the inner membrane, and then the Lpt system transports Kdo_2-lipid A to the outer leaflet of the outer membrane (Bowyer *et al.*, 2011). The Lpt system consists of seven proteins LptABCDEFG (Sperandeo *et al.*, 2007, 2008, 2011; Ruiz *et al.*, 2008). LptC in the LptB CFG complex may act as a dock for the periplasmic protein LptA. LptA shuttles Kdo_2-lipid A from the periplasmic face of the inner membrane to the inner surface of the outer membrane (Chimalakonda *et al.*, 2011; Freinkman *et al.*, 2011), where LptD and LptE control the export and assembly of Kdo_2-lipid A into the outer surface of the outer membrane (Bos *et al.*, 2004; Ma *et al.*, 2008; Chng *et al.*, 2012). Some modifications of Kdo_2-lipid A are regulated by the PmrA/PmrB systems. In the presence of high Fe^{3+}, the PmrA-PmrB system can activate the genes encoding enzymes PagP and ArnABCDEFT. PagP locates in the outer membrane and transfers a palmitate from phospholipid to the 2-position of Kdo_2-lipid A as a secondary fatty acid, resulting in a hepta-acylated structure (Ahn *et al.*, 2004). Soluble proteins ArnABCD convert UDP-glucuronic acid to Und-P-α-L-Ara4N on the cytoplasmic face of the inner membrane. Membrane proteins ArnE and ArnF then transport Und-P-α-L-Ara4N to the periplasmic face of the inner membrane, where the membrane protein ArnT transfers the L-Ara4N moiety to Kdo_2-lipid A. See Section VI for abbreviations.

(2) Transport system

In most Gram-negative bacteria the Kdo_2-lipid A molecules are synthesized in the cytoplasm and exported to the outer leaflet of the outer membrane through dedicated transport systems (Fig. 3). The transport of Kdo_2-lipid A is critical because defects in export are known to be lethal. The enzymes involved in the export of Kdo_2-lipid A can export LPS with different structures.

(*a*) *Transport through the inner membrane: MsbA*

The transport of Kdo$_2$-lipid A through the inner membrane is carried out by a membrane protein MsbA which acts as a flippase (Doerrler & Raetz, 2002; Doerrler, Gibbons & Raetz, 2004). In *E. coli*, MsbA is a homodimer and each monomer contains six transmembrane helices and a cytosolic ATP-binding domain (Ward *et al.*, 2007). MsbA is highly conserved in Gram-negative bacteria and shares homology with the multidrug resistance proteins of eukaryotes. The transport efficiency of MsbA decreases dramatically when the structure of LPS is simpler than Kdo$_2$-lipid A. *E. coli* mutants that only synthesize lipid IV$_A$ grow slowly because lipid IV$_A$ can not be flipped efficiently by MsbA in the inner membrane (Klein *et al.*, 2009). Molecular dynamics simulations of the interaction of MsbA with membrane lipids have provided an insight as to how the protein translocates lipids across bilayer membranes (Ward, Guvench & Hills, 2012).

(*b*) *Transport through the periplasmic space and the outer membrane: Lpt*

The LPS transport system (Lpt) in Gram-negative bacteria is responsible for the export of LPS from the periplasmic surface of the inner membrane, across the periplasm, to the outer leaflet of the outer membrane. The Lpt system consists of seven proteins LptA, LptB, LptC, LptD, LptE, LptF and LptG, spanning the cytoplasmic membrane to the cell surface (Wu *et al.*, 2006; Sperandeo *et al.*, 2007, 2008, 2011; Ruiz *et al.*, 2008). The LptC component of the LptB CFG complex may act as a dock for the periplasmic protein LptA, allowing it to bind Kdo$_2$-lipid A. LptA then shuttles Kdo$_2$-lipid A from the periplasmic face of the inner membrane to the inner surface of the outer membrane, where it binds the integral membrane protein LptD (Bowyer *et al.*, 2011; Chimalakonda *et al.*, 2011; Freinkman, Chng & Kahne, 2011). *E. coli* LptD contains two disulfide bonds and forms a translocon with the lipoprotein LptE, which controls the export and assembly of Kdo$_2$-lipid A into the outer surface of the outer membrane (Bos *et al.*, 2004; Ma, Reynolds & Raetz, 2008; Chng *et al.*, 2012). The seven proteins in the Lpt system are believed to constitute a trans-envelope complex spanning the inner to the outer membrane and operating as a concerted device (Fig. 3).

III. STRUCTURAL MODIFICATION OF KDO$_2$-LIPID A AND ITS REGULATION

In addition to the requirement of numerous gene products to synthesize and transport Kdo$_2$-lipid A, some bacteria have evolved mechanisms to modify the structure of Kdo$_2$-lipid A (Fig. 4). Diverse biochemical structures of Kdo$_2$-lipid A have been identified on the outer surface of different bacteria (Fig. 4) (Wilkinson, 1996). Some modifications to the Kdo$_2$-lipid A structure are regulated by two-component regulatory systems in response to specific environmental stimuli (Guo *et al.*, 1997, 1998) while other bacteria appear to modify their Kdo$_2$-lipid A constitutively (Wang *et al.*, 2006). In recent years, a number of other enzymes able to modify Kdo$_2$-lipid A regions of LPS have been identified in certain bacteria (Table 1).

Modification of Kdo$_2$-lipid A involves the hydrophilic disaccharide and Kdo regions, as well as the hydrophobic acyl chains. Because orthologs of the genes required for the biosynthesis of Kdo$_2$-lipid A in *E. coli* exist in most Gram-negative bacteria which could modify the structure of Kdo$_2$-lipid A, it is believed that Kdo$_2$-lipid A synthesis occurs independently from its modifications *in vivo*. Modifications of Kdo$_2$-lipid A usually take place at the periplasmic face of the inner membrane or in the outer membrane (Fig. 3). In some bacteria, modification of Kdo$_2$-lipid A is likely to be instrumental in aiding resistance of the bacteria to cationic antimicrobial peptides (CAMPs) released by the host immune system, or evading recognition by the innate immune receptor TLR4.

(1) Modification in the hydrophobic region

Since fatty acyl chains of Kdo$_2$-lipid A are located in the hydrophobic domain of the membranes, the enzymes that modify the fatty acyl chains are all intrinsic membrane proteins. Enzymes PagP, PagL, LpxR and LpxO are all known to function in the modification of the fatty acyl chains of Kdo$_2$-lipid A in some bacteria.

PagP is an acyl transferase located in the outer membrane of *Salmonella* Typhimurium and *E. coli* which transfers palmitate from glycerophospholipids to the 2-position of Kdo$_2$-lipid A as a secondary fatty acid (Ahn *et al.*, 2004), resulting in a hepta-acylated structure (Fig. 4). The expression of PagP is regulated by the PhoP-PhoQ system in many bacterial species. PagP has been well characterized in both *E. coli* and *Salmonella* Typhimurium, and its structure has been determined by both NMR spectroscopy and X-ray crystallography (Hwang, Bishop & Kay, 2004; Bishop, 2008). Palmitate is selected specifically by PagP, employing a gating mechanism sensitive to the length of hydrocarbon chains of potential donor lipids (Khan *et al.*, 2010).

PagL is a deacylase located in the outer membrane of *Salmonella* Typhimurium and removes the 3-O-linked acyl chain of Kdo$_2$-lipid A (Kawasaki, Ernst & Miller, 2004). PagL is also under the control of the PhoP-PhoQ system. The *pagL* mutant of *Salmonella* Typhimurium displays no obvious phenotypes in a murine model. PagL might be post-translationally inhibited within the outer membrane because it is not active in *Salmonella* Typhimurium, but could be activated in mutants of *Salmonella* Typhimurium that were unable to modify their Kdo$_2$-lipid A with 4-amino-4-deoxy-α-L-arabinose

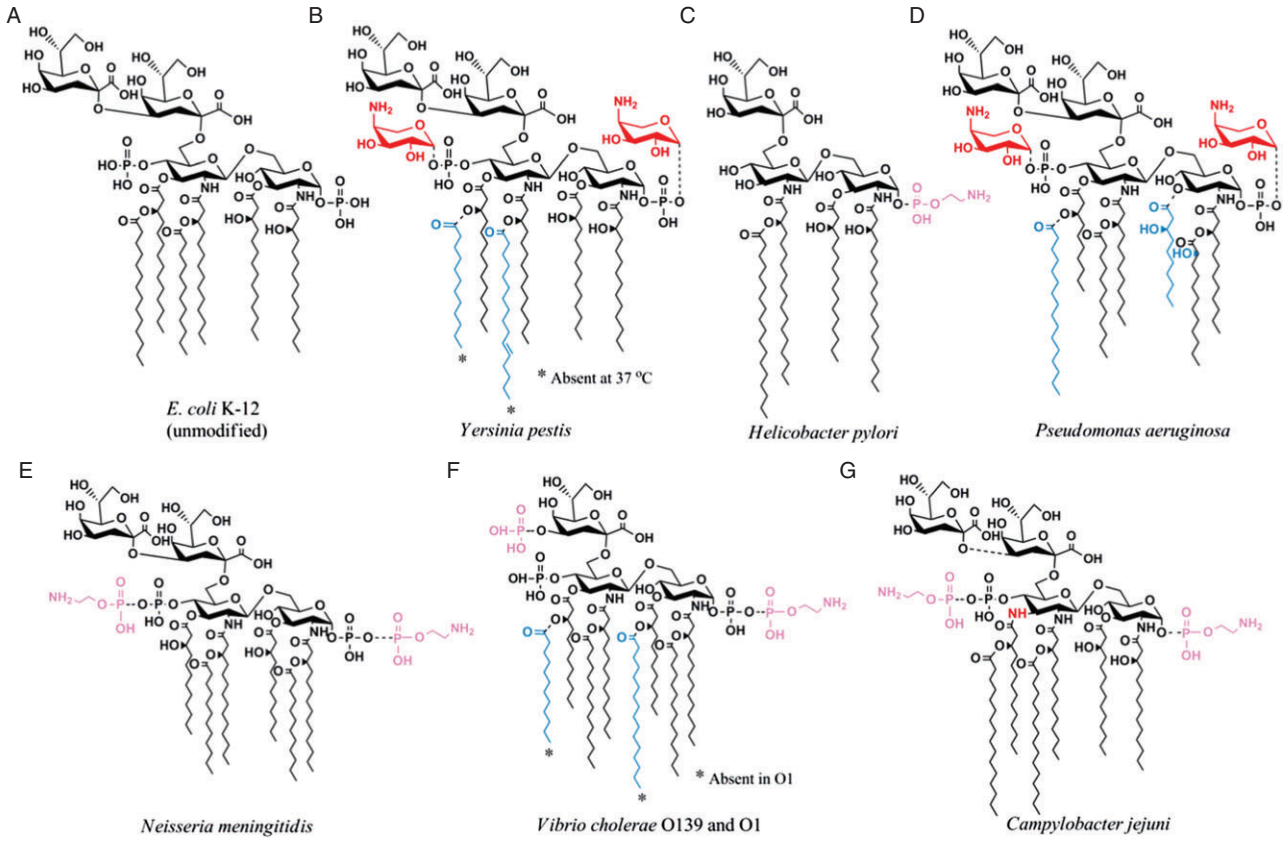

Fig. 4. (A–G) Structures of Kdo_2-lipid A from various Gram-negative bacteria. The figure is modified from Trent *et al.* (2006).

(α-L-Ara4N). PagL from *Pseudomonas aeruginosa* consists of an eight-stranded beta-barrel with the axis tilted by approximately 30° with respect to the lipid bilayer. It contains an active site with a Ser-His-Glu catalytic triad and an oxyanion hole that comprises the conserved Asn (Rutten *et al.*, 2006).

LpxR is another deacylase located in the outer membrane of *Salmonella* Typhimurium and removes the 3′-acyloxyacyl moiety of Kdo_2-lipid A (Reynolds *et al.*, 2006). Orthologs of LpxR can be found in various Gram-negative bacteria such as *H. pylori*, *Yersinia enterocolitica*, *E. coli* O157:H7 and *Vibrio cholerae* (Reynolds *et al.*, 2006). LpxR usually remains inactive in *Salmonella* Typhimurium outer membrane, but appears to be activated in *H. pylori* since the major Kdo_2-lipid A species from *H. pylori* is completely 3′-O-deacylated. *Salmonella* Typhimurium LpxR is regulated by neither the PhoP-PhoQ system nor the PmrA-PmrB system, but requires Ca^{2+} for enzymatic activity. In *Y. enterocolitica*, however, *lpxR* was negatively regulated by the PhoP-PhoQ system and the regulator RovA (Reines *et al.*, 2012). As with other virulence factors of *Y. enterocolitica*, expression of LpxR was also mediated by temperature, and its activity was inhibited in the presence of the aminoarabinose modification of lipid A (Reines *et al.*, 2012). Crystal structure of *Salmonella* Typhimurium LpxR has been resolved. It is a 12-stranded beta-barrel and its active site is located between the barrel wall and an alpha-helix formed by an extracellular loop (Rutten *et al.*, 2009).

LpxO is an inner membrane protein of *Salmonella* Typhimurium that can generate a 2-OH on the secondary fatty acid at the 3′-position of lipid A (Gibbons *et al.*, 2000, 2008). This hydroxylation is independent of MsbA transport, indicating that its active site is on the cytoplasmic surface. LpxO is regulated by neither the PhoP-PhoQ system nor the PmrA-PmrB system in *Salmonella* Typhimurium. In *V. cholerae*, the 2-position on the secondary fatty acid at the 3′-position of lipid A is modified with glycine or diglycine residues by AlmG. This lipid A modification confers polymyxin resistance in *V. cholerae* El Tor (Hankins *et al.*, 2012). *V. cholerae* O1 El Tor mutant lacking *almG* showed a 100-fold increase in sensitivity to polymyxin B.

(2) Modification in the hydrophilic region

In addition to changes in the fatty acid region, the hydrophilic region of the Kdo_2-lipid A molecule can also be modified in some bacteria. Kdo_2-lipid A usually contains two phosphate groups which impart a net negative charge to the molecule. Modification of the hydrophilic

Table 1. Enzymes involved in the structural modification of Kdo$_2$-lipid A in certain Gram-negative bacteria. The structure and numbering scheme for Kdo$_2$-lipid A is shown in Fig. 2

Positions	Enzymes	Function
1	LpxE	Removes the phosphate group from the 1-position of lipid A (Wang *et al.*, 2004).
1	LpxT	Transfers a phosphate group to the 1-phosphate of lipid A (Touze *et al.*, 2008).
1	LpxQ	Oxidizes the proximal glucosamine of lipid A to form an aminogluconate unit (Que-Gewirth *et al.*, 2003).
1	LmtA	Catalyses the methylation of 1-phosphate of lipid A (Hinckley *et al.*, 2005).
1	PmrC	Adds a phosphoethanolamine to the 1-position of lipid A (Lee *et al.*, 2003).
1 or 4′	ArnT	Transfers an L-Ara4N unit to lipid A (Trent *et al.*, 2001*a,b*).
1 or 4′	EptC	Adds a phosphoethanolamine to the flagellar rod protein and the 1 or 4′ position of lipid A (Cullen *et al.*, 2012).
2	PagP	Transfers a palmitate to the 2-position of lipid A (Hwang *et al.*, 2004; Bishop, 2008).
2 or 2′	LpxD2	Adds a shorter 3-OH fatty acid group to 2- or 2′-position of lipid A at low temperature (Li *et al.*, 2012).
2′	LpxP	Incorporates a palmitoleoyl moiety in place of the secondary laurate chain (Vorachek-Warren *et al.*, 2002).
2′	LpxXL	Incorporates a very long fatty acid chain in place of the secondary laurate chain (Basu *et al.*, 2002).
3	PagL	Removes the 3-O-linked acyl chain of lipid A (Rutten *et al.*, 2006).
3′	LpxO	Adds an OH group to the $\alpha_{\beta 3'}$-position (Gibbons *et al.*, 2000, 2008).
3′	AlmG	Adds glycine or diglycine residues to the $\alpha_{\beta 3'}$-position (Hankins *et al.*, 2012)
3′	LpxR	Removes the 3′-acyloxyacyl moiety (Reynolds *et al.*, 2006).
4′	LpxF	Removes the phosphate group from the 4′-position of lipid A (Wang *et al.*, 2006).
Kdo	RgtA	Adds a GalA moiety to the distal unit of Kdo (Kanjilal-Kolar *et al.*, 2006).
Kdo	RgtB	Adds a GalA moiety to the distal unit of Kdo (Kanjilal-Kolar *et al.*, 2006).
Kdo	EptB	Adds a phosphoethanolamine to the distal unit of Kdo (Reynolds *et al.*, 2005).
Kdo	KdoH1/KdoH2	Remove the distal unit of Kdo (Zhao & Raetz, 2010)

region of Kdo$_2$-lipid A focuses on the removal or substitution of the phosphate groups at the 1- and 4′-positions. The negative charges of Kdo$_2$-lipid A allow the binding of positively charged CAMPs. To evade attack by the immune system some bacterial pathogens have evolved a less negatively charged variation of Kdo$_2$-lipid A. The modifications at the 1- and 4′-positions of Kdo$_2$-lipid A include the removal of phosphate groups or the addition of the amine-containing residues such as α-L-Ara4N and phosphoethanolamine. Some of these modifications result in resistance to CAMPs and to polymyxin B, and are primarily controlled by the PmrA-PmrB system.

The removal of phosphate groups to reduce the overall negative charge of Kdo$_2$-lipid A occurs in several bacterial pathogens or endosymbionts. For example, *R. etli* Kdo$_2$-lipid A does not contain phosphate (Que, Ribeiro & Raetz, 2000), while *F. tularensis* and *H. pylori* lipid A contains only one phosphate group (Wang *et al.*, 2006). The absence of the phosphate group would greatly decrease the surface negative charge of these bacteria. Two genes *lpxE* and *lpxF* encoding phosphatases have been identified in *F. novicida* (Wang *et al.*, 2004, 2007; Chen, Tao & Wang, 2011; Han *et al.*, 2013). LpxE selectively removes the phosphate group at the 1-position of Kdo$_2$-lipid A, while LpxF selectively removes the phosphate group at the 4′-position. The *lpxF* deletion mutant of *F. novicida* no longer infects host mice (Wang *et al.*, 2007), suggesting that the phosphate group on Kdo$_2$-lipid A is closely related to the infectivity of bacteria. Orthologs of LpxE also exist in *R. etli* and in *H. pylori* (Karbarz *et al.*, 2003; Tran *et al.*, 2004; Karbarz, Six & Raetz, 2009).

In addition to the removal of phosphates, addition of amino-containing groups on Kdo$_2$-lipid A is believed to be another subterfuge that bacteria employ to reduce the overall negative charge of Kdo$_2$-lipid A. ArnT is an amino-arabinose transferase found in *E. coli* and *S. enterica* Typhimurium and transfers L-Arn4N to the 4′-phosphate of lipid A (Trent *et al.*, 2001*a,b*). PmrC (or EptA) encodes a protein necessary for addition of phosphoethanolamine to the 1-phosphate of Kdo$_2$-lipid A (Lee *et al.*, 2003). Under some conditions, the positions of phosphoethanolamine and L-Ara4N substituents are reversed, and lipid A species with two phosphoethanolamine units or two L-Ara4N moieties may be present. Expression of the enzymes ArnT and PmrC is under the control of PmrA. *F. novicida* lipid A contains galactosamine attached to the 1-phosphate group, which is added by an enzyme encoded by *flmK*, an ortholog gene of *arnT* (Wang *et al.*, 2006). A pathway for the synthesis and incorporation of the galactosamine to Kdo$_2$-lipid A has been characterized in *F. novicida* (Song, Guan & Raetz, 2009; Wang *et al.*, 2009). In *B. pertussis* BP338, both the 1- and 4′- phosphates of lipid A are modified by a glucosamine (GlcN) group, and

these modifications increase its ability to activate TLR4 (Shah *et al.*, 2013).

The 1-position of Kdo_2-lipid A can also be modified by enzymes LpxT, LmtA and LpxQ in certain bacteria. Using undecaprenyl pyrophosphate as the substrate donor, LpxT adds a second phosphate group at the 1-phosphate of Kdo_2-lipid A, therefore, some Kdo_2-lipid A molecules in *E. coli* contain a diphosphate unit at the 1-position (Touze *et al.*, 2008). In contrast to the modifications by amino sugars or phosphoethanolamine, addition of a second phosphate group increases the negative charge of Kdo_2-lipid A. This type of modification, however, may enhance the resistance of the bacterium to anionic antimicrobial agents, such as bile acid which is encountered by enteric bacteria in their host gut. LmtA is another enzyme that modifies the phosphate groups of Kdo_2-lipid A using moieties other than amino sugars or phosphoethanolamine. In *Leptospira interrogans*, membrane protein LmtA transfers a methyl group from S-adenosylmethionine to the 1-phosphate of Kdo_2-lipid A (Hinckley *et al.*, 2005). LpxQ found in *Rhizobium leguminosarum*, on the other hand, can oxidize the proximal glucosamine of *R. leguminosarum* Kdo_2-lipid A in the presence of O_2 to form an aminogluconate unit (Que-Gewirth *et al.*, 2003).

Recent studies of *Campylobacter jejuni* identified a gene encoding a novel phosphoethanolamine transferase EptC, that serves a dual role in modifying the flagellar rod protein, FlgG, and the 1 or 4′position of Kdo_2-lipid A of *C. jejuni* with a phosphoethanolamine residue (Cullen *et al.*, 2012). Characterization of EptC and its enzymatic targets expands on the increasingly important field of prokaryotic post-translational modification of bacterial surface structures.

The Kdo group of Kdo_2-lipid A can also be modified. A galacturonic acid (GalA) can be added to the outer Kdo group of Kdo_2-lipid A in *R. leguminosarum* by RgtA or RgtB (Kanjilal-Kolar *et al.*, 2006). A phosphoethanolamine can also be added to the outer Kdo group of Kdo_2-lipid A in *E. coli* by a Ca^{2+}-induced, membrane-bound enzyme EptB (Reynolds *et al.*, 2005). Kdo hydrolase which can remove the outer Kdo unit of Kdo_2-lipid A has been identified in *F. tularensis*, *H. pylori*, and *Legionella pneumophila* (Chalabaev *et al.*, 2010). The membrane-bound Kdo hydrolase consists of two proteins KdoH1 and KdoH2 (or KdhA and KdhB), which are expressed from the adjacent, co-transcribed genes. *F. novicida* KdoH1 has a single predicted N-terminal transmembrane segment, while KdoH2 contains seven putative transmembrane sequences; neither protein alone catalyses the cleavage of Kdo when expressed in *E. coli*. In *E. coli* expressing both KdoH1 and KdoH2, hydrolase activity is detected in the inner membrane fraction (Zhao & Raetz, 2010). In *H. pylori,* Kdo hydrolase activity is dependent upon a putative two-protein complex composed of proteins Hp0579 and Hp0580. Kdo hydrolase mutants were highly sensitive to polymyxin B. Production of a fully extended O-antigen was also diminished in a Kdo hydrolase mutant, with a consequent increase of the content of core-lipid A (Stead *et al.*, 2010). *H. pylori* produces a unique surface LPS characterized by strikingly low endotoxicity. This reduction in endotoxicity may arise from the modification of Kdo_2-lipid A by a series of enzymes including Kdo hydrolase.

In *Shewanella oneidensis*, the Kdo part of the Kdo_2-lipid A was replaced by Kdo8N (8-amino-3, 8-dideoxy-d-manno-octulosonic acid), which is found exclusively in marine bacteria of the genus *Shewanella*. Using bioinformatics, a gene cluster for Kdo8N biosynthesis was identified. Expression of these genes in *E. coli* resulted in $Kdo8N_2$-lipid A, and *in vitro* assays confirmed their proposed enzymatic function. Both the *in vivo* and *in vitro* data were consistent with direct conversion of Kdo to Kdo8N prior to its incorporation into lipid IV_A. A *S. oneidensis* mutant lacking these genes showed increased sensitivity to the CAMP polymyxin as well as bile salts, suggesting a role in outer membrane integrity (Gattis *et al.*, 2013).

(3) Regulation of the modification

Some of the Kdo_2-lipid A modification enzymes identified so far are under the control of the two-component PhoP-PhoQ system and/or PmrA-PmrB system (Guo *et al.*, 1997). The PhoP-PhoQ system governs virulence, mediates adaptation to a Mg^{2+}-limiting environment and regulates numerous cellular activities in Gram-negative bacteria (Soncini *et al.*, 1996; Gibbons *et al.*, 2005). It consists of an inner membrane sensor PhoQ and a cytoplasmic regulator PhoP. PhoQ contains an acidic patch on the surface of its periplasmic domain. Mg^{2+} bridges the acidic patch with anionic phospholipid polar head groups to maintain a repressed regulatory state (Bader *et al.*, 2005; Cho *et al.*, 2006). The PhoP-PhoQ system can also be activated when the bacterium is exposed to CAMPs (Bader *et al.*, 2003; Martin-Orozco *et al.*, 2006). The activation of the PhoP-PhoQ system can lead to the activation or repression of over 40 genes in bacteria (Aranda *et al.*, 1992; Gooderham & Hancock, 2009).

The PmrA-PmrB two-component system is the global regulatory system that controls LPS modification in *E. coli* and *Salmonella* Typhimurium (Gunn *et al.*, 2000). It is usually induced by high Fe^{3+}, the specific signal recognized by the sensor PmrB (Wosten *et al.*, 2000). It can also be induced by low Mg^{2+}, which is detected by the sensor PhoQ of the PhoP-PhoQ system (Vescovi, Soncini & Groisman, 1996). Activation by low Mg^{2+} requires PhoP, PhoQ, PmrA and PmrB proteins (Soncini *et al.*, 1996) as well as the PhoP-activated PmrD protein which connects the PhoP-PhoQ system with the PmrA-PmrB system (Kox, Wosten & Groisman, 2000). In *E. coli*, the PmrA-PmrB pathway cannot be triggered by the

PhoP-PhoQ system because the PmrD is not functional (Winfield & Groisman, 2004).

The best example for PmrA-regulated Kdo$_2$-lipid A modification is the *arn* operon (Gunn *et al.*, 1998; Trent *et al.*, 2001*a*,*b*; Breazeale *et al.*, 2003) and *ugd* gene (Groisman, Kayser & Soncini, 1997) (Fig. 3). Proteins encoded by these genes can synthesize and incorporate a α-L-Ara4N into Kdo$_2$-lipid A (Gunn *et al.*, 1998; Zhou *et al.*, 2001; Breazeale *et al.*, 2003). This modification can assist the bacteria to resist the antibiotic polymyxin B (Roland *et al.*, 1993). The *arn* operon contains *arnB-arnC-arnA-arnD-arnT-arnE-arnF* genes that encode seven enzymes, ArnB, ArnC, ArnA, ArnD, ArnT, ArnE and ArnF, respectively (Breazeale *et al.*, 2005). Ugd initiates the pathway by converting UDP-glucose to UDP-glucuronic acid. Subsequently, the C-terminal domain of ArnA catalyses the oxidative decarboxylation of UDP-glucuronic acid to generate UDP-4-keto-pyranose. ArnB then catalyses a transamination using glutamic acid as the amine donor to form UDP-L-Ara4N. Subsequently, the N-terminal domain of ArnA uses N-10-formyltetrahydrofolate to synthesize N-formylate UDP-α-L-Ara4N, which is, in turn, transferred by ArnC to undecaprenyl phosphate. Then ArnD catalyses deformylation of this substrate to undecaprenyl phosphate-α-L-Ara4N (Und-P-α-L-Ara4N). Following its assembly, ArnE and ArnF flip the Und-P-α-L-Ara4N from the cytoplasmic face to the periplasmic face of the inner membrane (Yan, Guan & Raetz, 2007), where ArnT transfers the L-Ara4N unit to Kdo$_2$-lipid A (Fig. 3).

PmrA-PmrB activation also promotes the addition of phosphoethanolamine to Kdo$_2$-lipid A, and the expression of Wzz$_{fepE}$ and Wzz$_{st}$ required for determining the length of LPS. Wzz$_{st}$ protein is also necessary to maintain the balance of L-Ara4N and phosphoethanolamine modifications to Kdo$_2$-lipid A (Farizano *et al.*, 2012).

In addition to the global regulators PhoP-PhoQ and PmrA-PmrB, recently regulation of the Kdo$_2$-lipid A modification enzymes by small RNAs (sRNAs) and small peptides in certain bacteria has also been reported. These regulatory elements act at a post-transcriptional level, by blocking the translation of the modification enzymes through base-pairing or inhibition of enzyme activity. They often regulate a subset of specific modification enzymes which are also regulated by the PhoP-PhoQ or PmrA-PmrB systems; their regulation often leads to fine-tuned expression of these enzymes rather than simply switching them 'on' or 'off'. For instance, in *E. coli* expression of *eptB* is negatively regulated by sRNAs MgrR which itself is activated by PhoP-PhoQ system and ArcZ whose expression is mediated by the oxygen-sensing ArcA-ArcB two-component systems (Moon *et al.*, 2013). Expression of *eptB* is also subject to the regulation of Sigma E (σ^E) which is a general regulator of envelope stress caused by unfolded or misfolded proteins in the periplasm (Moon *et al.*, 2013).

The activity of *Salmonella* Typhimurium LpxT was recently found to be inhibited by a small membrane peptide PmrR (Kato *et al.*, 2012) which is in the PmrA-PmrB regulon and is activated by PmrA in response to high Fe^{3+} in the growth environment (Kato *et al.*, 2012). This regulation has been shown to be necessary for the reciprocal control of the modification status of cell-surface LPS and the dynamic levels of Fe^{3+} in the growth environment. With the advancement of genome annotations, more sRNAs and small peptides may be identified in the regulation of Kdo$_2$-lipid A modification enzymes, which will expand the current regulatory network of these genes.

(4) Effect of Kdo$_2$-lipid A structural modification on the virulence of bacteria

Although a viable mutant of *Neisseria meningitidis* deficient in Kdo$_2$-lipid A biosynthesis has been isolated (Steeghs *et al.*, 1998), most Gram-negative bacteria lacking Kdo$_2$-lipid A are not viable. *E. coli* mutants deficient in Kdo biosynthesis are conditionally lethal because they only synthesize lipid IV$_A$ which cannot be efficiently flipped by MsbA (Meredith *et al.*, 2006). Kdo-lipid IV$_A$ provides the ready substrates for LpxL and LpxM, resulting in the synthesis of penta- and hexa-acylated Kdo$_2$-lipid A, which is optimal for the MsbA flippase (Reynolds & Raetz, 2009). *E. coli* mutant Δ*kdtA* could accumulate lipid IV$_A$ and its penta-acylated and hexa-acylated derivatives at 21°C, indicating that *in vivo* late acylation can occur without Kdo. By contrast, *E. coli* mutant strain Δ(*waaC lpxL lpxM lpxP*) showed primarily Kdo$_2$-lipid IV$_A$, indicating that Kdo$_2$-lipid IV$_A$ is sufficient to support the growth of *E. coli* under slow-growth conditions at 21°C. These lipid IV$_A$ derivatives could be modified biosynthetically by phosphoethanolamine, but not by L-Ara4N, indicating export defects of such minimal LPS (Klein *et al.*, 2009) because L-Ara4N addition occurs only in the periplasm. *E. coli* mutant strains Δ*kdtA* and Δ(*waaC lpxL lpxM lpxP*) exhibited cell-division defects with a decrease in the levels of FtsZ and outer membrane protein (OMP)-folding factor PpiD, and led to strong constitutive additive induction of envelope responsive CpxR/A and σ^E signal transduction pathways (Klein *et al.*, 2009).

Some bacterial pathogens can survive in the host by modifying the structure of Kdo$_2$-lipid A. *Francisella novicida* can synthesize LPS without core-oligosaccharide and O-antigens (Wang *et al.*, 2006). *F. novicida* lipid A is a disaccharide of glucosamine, acylated with primary 3-hydroxystearoyl chains at the 2-, 3-, and 2′-positions, and a secondary palmitoyl residue at the 2′-position. The 4′- and 3′-positions of lipid A are not modified. *F. novicida* lipid A cannot activate TLR4. The *lpxF* mutant of *F. novicida* is avirulent in a mouse infection model and is hypersensitive to CAMPs (Wang

et al., 2007). The lipid A of *Francisella novicida lpxF* mutant also does not activate TLR4. The hypersensitivity of the *lpxF* mutant to CAMPs may cause damage to the bacterial envelope and expose other ligands. Mice infected with the *lpxF* mutant of *F. novicida* survived primary infection and subsequently developed protective immunity against a lethal wild-type *F. novicida* challenge. The role of innate immune components, including Toll-like receptor 2 (TLR2), TLR4, caspase-1, MyD88, alpha interferon (IFN-α), and gamma interferon (IFN-γ), was examined using knockout mice. Interestingly, only the IFN-γ-knockout mice succumbed to a primary *F. novicida lpxF* mutant infection, highlighting the importance of IFN-γ production (Kanistanon *et al.*, 2012).

The number and the length of fatty acyl chains of Kdo_2-lipid A are also related to the infectivity of bacteria. *Yersinia pestis* causes infection through flea bites. At 21–27°C, the body temperature of the flea, *Y. pestis* synthesizes lipid A containing six fatty acid chains, but at 37°C, human body temperature, *Y. pestis* synthesizes lipid A containing four fatty acid chains (Montminy *et al.*, 2006). The lipid A with six fatty acid chains can activate the immune system through TLR4, but the lipid A with four fatty acid chains cannot. Therefore, *Y. pestis* can escape attack from the human immune system. A similar relationship between the pattern of acylation and immune activation was also observed in the pathogen *Y. enterocolitica* (Reines *et al.*, 2012).

The acylation patterns of Kdo_2-lipid A are also related to the infectivity of certain bacteria. Modification of the acylation pattern of *Salmonella* Typhimurium Kdo_2-lipid A by either PagP or PagL results in attenuation of lipid A signalling through the TLR4 pathway and, therefore, may promote evasion of the innate immune system during infection (Kawasaki *et al.*, 2004). PagP-dependent palmitoylation of *Klebsiella pneumoniae* lipid A plays an important role in reducing the engulfment of the bacterium by alveolar macrophages, and the virulence of the *pagP* deletion mutant of *K. pneumoniae* is attenuated (March *et al.*, 2013).

F. novicida LpxD1 adds the 3-OH C_{18} acyl group to lipid A at 37°C (Li *et al.*, 2012). The lpxD1-null mutant, containing lipid A with shorter acyl chains, was attenuated in mice and subsequently exhibited protection against a lethal wild-type challenge. Kdo_2-lipid A molecules of the legume symbiont *Sinorhizobium meliloti* and the phylogenetically related mammalian pathogen *Brucella abortus* are unusually modified with a very-long-chain fatty acid, which could be crucial for the chronic infection of both bacteria (Haag *et al.*, 2009).

The two-component Kdo hydrolase KdhAB also plays an important role in *F. tularensis* pathogenesis. The *kdhAB* mutant of *F. tularensis* LVS strain not only was significantly attenuated in mice, but also induced an early innate immune response both *in vitro* and *in vivo*. Immunization of mice with this mutant provided protection against the highly virulent *F. tularensis* strain Schu S4 (Okan *et al.*, 2013).

LpxE from *Francisella tularensis* has been used to construct recombinant, plasmid-free strains of *E. coli* and *Salmonella* Typhimurium that produce predominantly 1-dephosphorylated lipid A (Chen *et al.*, 2011; Han *et al.*, 2013). LpxE expression in *Salmonella* Typhimurium reduced its virulence in mice. Mice inoculated with the detoxified strains were protected against wild-type challenge. After inoculation by the candidate *Salmonella* Typhimurium vaccine strain expressing LpxE and synthesizing pneumococcal surface protein A (PspA), mice produced robust levels of anti-PspA antibodies and showed significantly improved survival against challenge with wild-type *Streptococcus pneumoniae* WU2 compared with vector-only-immunized mice, validating *Salmonella* Typhimurium synthesizing 1-dephosphorylated lipid A as an antigen-delivery system (Kong *et al.*, 2011). When *Salmonella* Typhimurium *msbB pagL pagP lpxR* mutant, carrying penta-acylated lipid A, was used as the parent strain to construct a series of mutants synthesizing 1-dephosphorylated, 4′-dephosphorylated, or nonphosphorylated penta-acylated lipid A, the dephosphorylated mutants exhibited increased sensitivity to deoxycholate and showed increased resistance to polymyxin B. Removal of both phosphate groups severely attenuated the mutants when administered orally to BALB/c mice, but the mutants colonized the lymphatic tissues and were sufficiently immunogenic to protect the host from challenge with wild-type *Salmonella* Typhimurium. Mice receiving *Salmonella* Typhimurium with 1-dephosphorylated or nonphosphorylated penta-acylated lipid A exhibited reduced levels of cytokines. Attenuated and dephosphorylated *Salmonella* vaccines were able to induce adaptive immunity against heterologous and homologous antigens (Kong *et al.*, 2012).

IV. IMMUNOPHARMACOLOGICAL EXPLOITATION OF KDO_2-LIPID A BIOSYNTHESIS AND ITS IMMUNE ELICITATION

Owing to its indispensible biological and physiological roles in virtually all Gram-negative bacteria and its capabilities of eliciting host innate immune responses, the biosynthesis, modification, and host immune response to Kdo_2-lipid A has proved to be a promising target for immunopharmacological exploitation. In recent years, with the elucidation of the three-dimensional (3D)-structures of many key enzymes in these processes, there have been exciting advances in the development of novel antibiotics, anti-inflammatory drugs, and vaccine adjuvants.

(1) Key enzymes of Kdo$_2$-lipid A biosynthesis: targets for novel antibiotics

With the increasing emergence of multidrug-resistant bacterial infections in the past several decades, there has been a critical need for the identification of novel antibiotics directed towards previously unexplored targets, such as those other than cell wall, nucleic acid, or protein biosynthesis. The Kdo$_2$-lipid A biosynthetic pathway provides a promising target for this purpose because it is indispensabe to the viability of almost all Gram-negative bacteria. In recent years, with increasing knowledge of 3D structures of most of the Kdo$_2$-lipid A biosynthetic enzymes, development of novel antibiotics targeting the key enzymes in the pathway has been the research focus of many industrial groups and academic laboratories (Brown *et al.*, 2012; Lee *et al.*, 2013; Loppenberg *et al.*, 2013). Although inhibition of any one of the first six enzymes of Kdo$_2$-lipid A biosynthesis has been shown to be lethal to *E. coli*, the most prominent target to date is LpxC, the UDP-3-*O*-(acyl)-*N*-acetylglucosamine deacetylase that catalyses the second step of the Kdo$_2$-lipid A biosynthetic pathway (Onishi *et al.*, 1996; McClerren *et al.*, 2005; Mdluli *et al.*, 2006). Several unique features of LpxC underscore its feasibility as an antibiotic target: (*i*) *lpxC* gene is conserved and essential in virtually all Gram-negative bacteria and it catalyses the first committed step of Kdo$_2$-lipid A biosynthesis; (*ii*) LpxC is a zinc-dependent metallo-enzyme the 3D structure of which displays a unique protein fold; and (*iii*) LpxC does not possess sequence homology to other deacetylases or eukaryotic proteins, suggesting that it can be highly selectively targeted with minimal toxicity to the mammalian host.

Searching for LpxC inhibitors as potential antibiotics initiated from screening a metallo-enzyme inhibitor library which led to the identification of a series of sulphonamide derivatives of α-(R)-amino hydroxamic acids which inhibit LpxC activity by chelating the Zn^{2+} ion at its active site, such as the compound BB-78485 (Fig. 5) (Clements *et al.*, 2002). The first hydroxamic acidic compound which can inhibit LpxC at nanomolar concentrations (the inhibitory constant $K_i \sim 50$ nM) is L-161,240 (Fig. 5) was reported by researchers at Merck (Onishi *et al.*, 1996; Chen *et al.*, 1999). However, although L-161240 displays a comparable antibacterial activity to *E. coli* as that of ciprofloxacin, it does not inhibit LpxC orthologs from other Gram-negative species, such as that of *P. aeruginosa* which is the primary cause of fatality in cystic fibrosis patients. Based on this, studies by the research team of Chiron/University of Washington identified a diphenyl-acetylene-containing hydroxamic acid compound CHIR-090 (Fig. 5), a very potent, slow, tight-binding inhibitor against both *E. coli* and *P. aeruginosa* LpxC (McClerren *et al.*, 2005; Barb *et al.*, 2007*b*). X-ray 3D-structure of *Aquifex aeolicus* LpxC bound with CHIR-090 revealed that in addition to the essential hydroxamate-zinc interaction, the diphenyl-acetylene group of CHIR-090 also contributes to the inhibitory effect by occupying the hydrophobic substrate-binding passage of LpxC, with the first phenyl group (proximal to the hydroxamate group) located close to the active site and the second phenyl ring (distal to the hydroxamate group) emerging from the hydrophobic passage (Barb *et al.*, 2007*a*). However,

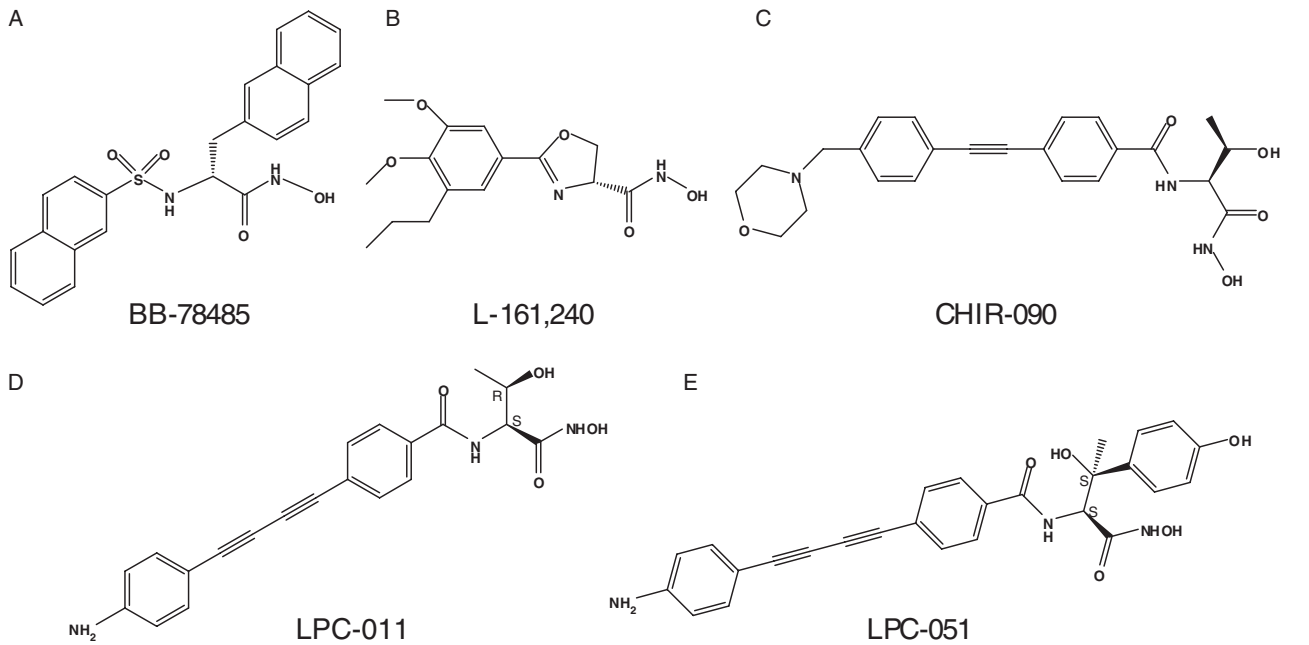

Fig. 5. (A–E) Structures of various potent LpxC inhibitors.

CHIR-090 is ~60-fold less effective against LpxC from the *Rhizobium* family (RLpxC) than to *E. coli* LpxC (EcLpxC). X-ray crystal structure revealed that this is because of a species-specific residue located at the exit of the hydrophobic passge of LpxC occupied by CHIR-090. In the Gram-negative bacterial species which are susceptible to CHIR-090, such as EcLpxC and *P. aeruginosa* LpxC (PaLpxC), a conserved glycine residue is located at the exit of the substrate-binding hydrophobic passge, whereas in RLpxC, it is replaced by a serine residue which causes van der Waals clashes with the distal phenyl ring of CHIR-090 and decreases its accessibility to the substrate binding site of LpxC (Lee *et al.*, 2011, 2013).

The elucidation of the 3D structure of LpxC bound with its competitive inhibitors further facilitated the rational design of LpxC inhibitors. One class of molecules developed was hydroxamate compounds with a linear diacetylene scaffold, such as LPC-009 and LPC-011 (Liang *et al.*, 2011). The narrow diacetylene group in these compounds has been shown effectively to diminish the intrinsic resistance of previous coumpounds to RLpxC. Among them, LPC-011 (Fig. 5) has been shown to inhibit EcLpxC with a K_i of ~0.039 nM and exhibits a general enhancement of potency (2- to 64-fold) over CHIR-090 against a wide range of Gram-negative bacteria. Further efforts towards enhancing their antibacterial activity focus on improving the membrane permeability of these diacetylene scaffold compounds (Liang *et al.*, 2011).

(2) Antagonists of TLR4/LPS signalling: anti-inflammatory drugs

In addition to the Kdo_2-lipid A biosynthesis which has been an emerging target for antibiotic development, key protein receptors involved in the LPS-elicited TLR4 signalling pathway provide an additional category of therapeutic targets which have significant potential for the development of anti-septic shock and anti-inflammatory drugs. The recognition and initiation of LPS/TLR4 signalling is dependent on the sequential and coordinated action of specific receptors including LPS-binding protein (LBP), cluster of differentiation 14 (CD14), myeloid differentiation protein 2 (MD-2), and eventually TLR4 situated on the extracellular surface of the innate immunity cells (Fig. 1). Upon activation, the dimerized TLR4 recruits specific adaptor proteins which amplify the LPS-initiated signal and lead to the production of a large amount of pro-inflammatory cytokines and interleukin-1β (IL-1β). The signalling can be remarkably sensitive and robust, stimulating prompt and potent host defence responses. However, excessive activation of the pathway generates life-threatening syndromes such as acute sepsis and septic shock, accounting for ~45% of deaths on intensive care units. Disregulation of the pathway also causes other chronic inflammatory diseases including atherosclerosis, rheumatoid arthritis, and inflammation caused by tissue damage (Keogh & Parker, 2011). Thus, immune mediators that can effectively antagonize the LPS/TLR4 pathway have significant therapeutic and pharmacological value.

Initial efforts towards the development of TLR4 antagonists largely focused on structural analogues of Kdo_2-lipid A which can bind competitively to the LPS binding sites of the key receptors in the pathway and antagonize LPS/TLR4-dependent signalling. In recent years, many natural and synthetic small molecules which are structurally unrelated to lipid A were found to have anti-inflammatory activities through antagonizing the LPS/TLR4 signalling pathways, expanding the spectrum of these therapeutic and pharmacological molecules.

(*a*) *Kdo_2-lipid A analogues*

Antagonists of lipid A analogues were developed based on the structures of lipid A molecules isolated from non-pathogenic bacterial species such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. Lipid A molecules from these species are under-acylated compared with that of *E. coli* lipid A and contain shorter, unsaturated acyl chains. Using *R. capsulatus* lipid A as the template, the compound Eisai (E5531) was developed which displayed antagonistic activity to TLR4 signalling. It was replaced later by a second-generation lipid A analogue, Eritoran (E5564), owing to its undesirable interaction with plasma proteins in blood in *in vivo* experiments, causing loss of the activity as a function of time. Eritoran was derived from the lipid A of *R. sphaeroides* and was shown to be a potent antagonist of endotoxin in *in vitro* experiments. The mechanistic action of Eritoran was elucidated and it was shown to bind competitively to the hydrophobic pocket of the lipid A binding site of MD-2. However, since Eritoran has four acyl chains instead of six all of its lipid chains are completely submerged inside the pocket and cannot provide the interaction contacts with TLR4 for the second TLR4-MD-2 complex. Consequently, its binding fails to induce the formation of the TLR4-MD-2-LPS heterodimer and the associated downstream signal transduction (Kim *et al.*, 2007). Although acting as a potent LPS antagonist *in vitro*, Eritoran was recently discontinued in phase III clinical trials because of the lack of statistically significant activity when tested on a panel of 2000 septic patients (Peri & Piazza, 2012).

(*b*) *Drug development targeting to LBP, MD2 and CD14*

Natural and synthetic LPS/TLR4 antagonists with structures unrelated to lipid A were developed in two ways: fortuitous screening of dietary and phytochemicals that already have other beneficial effects on humans and rational design of molecules targeting the specific sites of key receptors in the signalling pathway. Here we

classify these compounds based on the targets of their action.

The first class is LPS-binding compounds which mimic cationic antimicrobial agents such as polymyxin B. These compounds include synthetic acyl and sufonamido homospermines which are cationic amphiphiles that bind to the anionic amphiphilic Kdo$_2$-lipid A to block TLR4 signalling. These compounds have been shown to sequester LPS effectively with low effective dose (ED) values and inhibit the production of antimicrobial nitric oxide in a murine model and tumor necrosis factor alpha (TNF-α) production in human blood (Miller *et al.*, 2005). Another class of synthetic cationic amphiphiles that inhibit LPS-stimulated TLR4 activation in a dose-dependent manner include 3,4-bis(tetradecyloxy)benzylamine and its derivatives (Piazza *et al.*, 2010). However, their effect was through the competitive occupation of CD14, thereby reducing the delivery of endotoxin to MD-2/TLR4 rather than directly sequestering LPS (Piazza *et al.*, 2010).

With knowledge of LPS recognition and transfer at the extracellular surface of cells of the innate immune system, as well as the X-ray structure of the (TLR4-MD-2-LPS)$_2$ complex (Park *et al.*, 2009), small molecule antagonists that target each of the components in the pathway have been developed. The first class include those that inhibit the interaction of LPS with its binding protein LBP and CD14. Asai, Hashimoto & Ogawa (2003) found that a heterogeneous glycoconjugate preparation from *Treponema spirochetes* (Tm-Gp) displays an antagonistic effect on TLR4 or TLR2-dependent pathways. The mechanism of its action was to inhibit the interaction of LPS with LBP and CD14. A series of synthetic glycosylamino- and benzylammonium lipids were subsequently developed and found to have antagonistic effect on TLR4 signalling through a similar mechanism of inhibiting the interaction of LPS with LBP and CD14. Structural activity research revealed that the optimal activity of these glycolipids requires the presence of a glucopyranose or a phenyl ring linked to two C14 ether lipid chains and a positively charged nitrogen atom (Peri & Piazza, 2012).

A very active target in the LPS/TLR4 pathway to which a broad class of molecules can bind or react and consequently inhibit the activation of TLR4 is MD-2, the co-receptor of TLR4. MD-2 is indispensible for the recognition and binding of LPS by the TLR4/MD-2 complex which specifically recognizes the lipid A domain of LPS. Of the six *E. coli* lipid A acyl chains, five are bound to MD-2 in its hydrophobic pocket (Park *et al.*, 2009). The hydrophobic ligand-binding cavity of MD-2 has a large volume (1720 Å^3) with an internal surface lined with hydrophobic residues and positively charged residues situated at the opening rims of the pocket, responsible for the interaction with the phosphate groups of the lipid A (Park *et al.*, 2009). Within the hydrophobic cavity, six cysteine residues form disulfide bridges to help determine the shape of the binding cavity and one free cysteine (Cys133) is situated at a location in close vicinity with the bound lipid A moiety. Both the hydrophobic pocket and the Cys133 have been shown to be the effective target sites for TLR4 signalling antagonists and MD-2 has been proposed to be a more suitable therapeutic target for pharmacological intervention than TLR4 (Mancek-Keber *et al.*, 2009). Several dietary phytochemicals have been shown to have anti-inflammatory activities through their binding to the hydrophobic cavity of MD-2 and consequently inhibit the LPS-mediated TLR4 signalling pathway. Examples include curcumin, a polyphenol compound enriched in the spice turmeric used in diets and traditional medicine (Gradisar *et al.*, 2007), and xanthohumol (XN) (Peluso *et al.*, 2010) and 2′,4-dihydroxy-6′-isopentyloxychalcone (JSH) (Roh *et al.*, 2011), chalcone-type flavonoids found in hops and beer. These dietary phytochemicals represent a rich source of potential drugs owing to their reliable safety record and have received increased attention in recent years as novel anti-inflammatory drugs. Additionally, endogenously oxidized phosphatidylcholine, 1-palmitoyl-2-(5-keto-6-octenedioyl)-sn-glycero-3-phos phocholine (KOdiA-PC), was found to have anti-inflammatory activity by inhibiting the binding of LPS to MD-2 (Kim *et al.*, 2013*a*).

Another class of LPS/TLR4 antagonists targeting MD-2 are thiol-reactive electrophiles which react with the C133 in the hydrophobic ligand-binding cavity of MD-2 and thus irreversibly inhibit the activation of the TLR4-mediated signal. Dietary phytochemicals bearing this property are especially promising drug candidates. Examples include sulforaphane [SFN, 1-isothiocyanato-4-(methylsulfinyl)-butane], an organosulfur compound obtained from cruciferous vegetables such as broccoli or cabbages (Koo *et al.*, 2013); caffeic acid phenethyl ester (CAPE), the active phenolic compound found in propolis (Kim *et al.*, 2013*b*), as well as the existing drugs JTT-705 and auranofin which has already shown safety in clinical trials (Mancek-Keber *et al.*, 2009). Natural phytochemicals which can both bind to the hydrophobic lipid A binding cavity of MD-2 and react with C133 represent future directions in screening the novel and potent anti-septic shock and anti-inflammatory drugs that specifically inhibit the LPS/TLR4 pathway.

(3) Agonists of TLR4/LPS signalling: vaccine adjuvants

Another class of immunopharmacological molecules with activity based on the exploitation of TLR4/LPS signalling are agonists of the system which can act as vaccine adjuvants (Mata-Haro *et al.*, 2007; Casella & Mitchell, 2008; Kong *et al.*, 2011; Bowen *et al.*, 2012). The first TLR4/LPS agonist developed as a vaccine adjuvant is a non-toxic derivative of LPS known

as monophosphoryl lipid A (MPLA) isolated from *Salmonella minnesota* rough strain (Kong *et al.*, 2011). Alum-absorbed MPLA has been approved for use as an adjuvant for hepatitis B virus (HBV) and human papillomavirus (HPV) vaccines (Kundi, 2007; Didierlaurent *et al.*, 2009). It is known that LPS-stimulated TLR4 signalling leads to the activation of two major downstream signal transduction pathways: the MyD88/TIRAP pathway which results in the generation of various pro-inflammatory cytokines and chemokines and the TRAM/TRIF pathway which causes the production of Type I interferons important for adjuvanticity (Fig. 1). The vaccine adjuvant activity of MPLA is belived to be associated with its selective activation of TRIF-dependent signalling following its binding to the TLR4/MD-2 complex, resulting in greatly reduced toxicity while maintaining most of the beneficial immunostimulatory activity of LPS (Mata-Haro *et al.*, 2007). Recent studies (Casella & Mitchell, 2013) suggested that the agonist activity of MPLA is attributable to its inefficient induction of TLR4/MD-2 heterotetramerization owing to the lack of the 1-phosphate which contributes to the dimerization of the two TLR4/MD-2 complexes in the diphosphorylated lipid A.

In addition to MPLA, two further classes of LPS derivatives have been developed recently as potential adjuvants. These include CRX-547, a member of the aminoalkyl glucosaminide 4-phosphate class of synthetic monosaccharide lipid A mimetics (Bowen *et al.*, 2012) and deacylated lipooligosaccharide (dLOS) which consists of a core oligosaccharide lacking the terminal glucose residue, a glucosamine disaccharide with two phosphate groups, and two N-linked acyl groups (Han *et al.*, 2014). Although structurally different, both CRX-547 and dLOS act as a comparable or even more potent activator of human monocytes and dentritic cells with substantially reduced production of proinflammatory mediators associated with the MyD88 pathway. Current studies focus on the structure–function relationships of these agonisits and the development of more potent vaccine adjuvants.

V. CONCLUSIONS

(1) Kdo_2-lipid A is an essential component for the survival of most Gram-negative bacteria.

(2) Kdo_2-lipid A biosynthesis is catalysed by a series of conserved enzymes located in both the cytoplasm (LpxA, LpxC, LpxD, LpxH and LpxB) and cytoplasmic membrane (LpxK, KdtA, LpxL and LpxM) of Gram-negative bacteria that are organized in the Raetz pathway.

(3) Kdo_2-lipid A molecules are synthesized in the cytoplasm and exported to the outer leaflet of the outer membrane through a dedicated transport system, MsbA, which transports the Kdo_2-lipid A molecules through the cytoplasmic membrane, and the Lpt system which transports the lipid through the periplasmic space and the outer membrane.

(4) A diverse range of Kdo_2-lipid A modification enzymes are present in various Gram-negative bacteria. Modification is tightly regulated and often plays an important role in the virulence and adaptation of these bacteria.

(5) The biosynthesis, modification, and the immune response pathways of Kdo_2-lipid A provide multiple emerging targets for immunopharmocological exploitation.

(6) A series of hydroxamic acid compounds containing a hydrophobic scaffold which inhibits the Kdo_2-lipid A biosynthetic enzyme LpxC with high potency are a class of promising novel antibacterial compounds.

(7) A series of Kdo_2-lipid A analogues and natural phytochemicals which target the essential component MD-2 in the host innate immune response pathway to Kdo_2-lipid A have the potential to be developed as novel anti-inflammatory drugs.

(8) MPLA and other Kdo_2-lipid A mimietics which agonize TLR4/LPS signalling by selectively activating the TRIF-dependent pathway are candidates for novel vaccine adjuvants.

VI. ABBREVIATIONS

α-L-Ara4N	4-amino-4-deoxy-α-L-arabinose
ABC	ATP binding cassette
ACP	acyl carrier protein
CAMP	cationic antimicrobial peptide
CD14	cluster of differentiation 14
COX-2	cyclooxygenase-2
cPLA2	cytosolic phospholipases A2
ESI/MS	Electrospray Ionisation Mass Spectrometry
GalA	galacturonic acid
IFN	interferon
IKK	inhibitory κB kinase
IL-1β	interleukin-Iβ
iNOS	inducible nitric oxide synthase
IRAK4	IL-1 receptor-associated kinase 4
IRF3	interferon regulatory factor 3
Kdh	Kdo hydrolase
Kdo	3-deoxy-D-manno-octulosonic acid
Kdo8N	8-amino-3,8-dideoxy-D-manno-octulosonic acid
LBP	LPS-binding protein
LPS	lipopolysaccharide
Lpt	LPS transport system
MAPK	mitogen-activated protein kinase
MD-2	myeloid differentiation protein 2
MPLA	monophosphoryl lipid A
MyD88	myeloid differentiation primary response protein 88

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
OMP = outer membrane protein
PspA = pneumococcal surface protein A
sRNA = small RNA
TAK1 = transforming growth factor-β-activated kinase 1
TIR = Toll-IL-1 receptor
TIRAP = TIR-domain-containing adaptor protein
TLR4 = Toll-like receptor 4
TNF-α = tumor necrosis factor alpha
TRAF6 = TNF receptor-activated factor 6
TRAM = TRIF-related adaptor molecule
UDP-GlcNAc = UDP-*N*-acetylglucosamine
Und-P-α-L-Ara4N = undecaprenyl phosphate-α-L-Ara4N

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