Exploring the LPS/TLR4 signal pathway with small molecules

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Abstract
The identification of the bacterial endotoxin receptors for innate immunity, most notably TLR4 (Toll-like receptor 4), has sparked great interest in therapeutic manipulation of the innate immune system. In this mini-review, several natural and synthetic molecules that modulate the TLR4-mediated LPS (lipopolysaccharide) signalling in animals and humans are considered, and their mechanisms of action are discussed. The process of LPS sensing and signal amplification in humans is based on the sequential action of specific receptors located in the extracellular side of the innate immunity cells, which bind and transfer LPS to TLR4: LBP (LPS-binding protein), CD14, MD-2 (myeloid differentiation protein 2). We classified the compounds active on TLR4 pathway depending on the specific molecular targets (LPS, LBP, CD14, MD-2 or TLR4). Small molecules developed by our group are described that inhibit LPS-stimulated TLR4 activation by selectively targeting the LPS-CD14 interaction. These compounds have an interesting anti-inflammatory and anti-neuropathic pain activity in vivo.

The LPS (lipopolysaccharide)/TLR4 (Toll-like receptor 4) signal pathway
Innate immunity is the first line of defence against microbial infections. Host organism responses are activated when microbial components are recognized by a variety of pathogen sensors, particularly TLR4 that activates the host defence effector system by rapidly triggering pro-inflammatory processes [1–3]. Among microbial components, LPS and LOS (lipo-oligosaccharides) and their bioactive portion, the lipodisaccharide lipid A, commonly defined as endotoxins, are potent stimulators of immune responses, and small differences in LPS structure can have a great influence on host immune responses [4–7]. The TLR4 activation caused by endotoxin with subsequent cytokine production is, in principle, beneficial for the organism, but when this process became dysregulated can lead to life-threatening syndromes such as sepsis and septic shock [8,9]. TLR4 has therefore been recognized as an important pharmacological target. Molecules with activity as endotoxin antagonists that are able to inhibit the process of TLR4 activation are interesting hit or lead compounds for antiseptic drug development [10,11], whereas TLR4 agonists can be used as efficient vaccine adjuvants or stand-alone immunotherapeutics [12–14].

Endotoxins are amphiphilic molecules and under physiological conditions are integral constituent of the Gram-negative bacteria outer membrane. After extraction and purification, endotoxins form large aggregates whose supramolecular structure depends on their chemical structure and, in particular, on the structure of the lipid A moiety [15,16]. However, as for every amphiphilic system, monomers are also present in a dynamic equilibrium. The induction of inflammatory responses by endotoxins is achieved by the co-ordinated and sequential action of four principal endotoxin-binding proteins: LBP (LPS-binding protein), CD14, MD-2 (myeloid differentiation protein 2) and TLR4 [17]. LBP interacts with endotoxin-rich bacterial membranes and purified endotoxin aggregates, catalysing extraction and transfer of endotoxin monomers to CD14 that in turn transfers endotoxin monomers to MD-2 and to MD-2–TLR4 heterodimers, explaining the importance of LBP and CD14 for endotoxin signalling at low concentrations of endotoxin [17]. The transfer of LPS from CD14 to MD-2, coupled with the association of MD-2 to TLR4, is required for downstream signalling. Activation includes the formation of a dimer of the ternary complex (TLR4–MD-2–endotoxin)2 [18]. Receptor dimerization leads to the recruitment of adapter proteins to the intracellular domain of TLR4, initiating the intracellular signal cascade that culminates in transcription factors to the nucleus and the biosynthesis of cytokines.

Structural biology of TLR4 activation
The very recent determination of the crystal structure of the (TLR4–MD-2–endotoxin)2 complex [19], together with crystallographic data of MD-2 bound to TLR4 antagonists Lipid IVα [20] and Eritoran [21], has revealed some fundamental structural aspects of the TLR4 dimerization process and the molecular basis of TLR4 agonism and antagonism (Figure 1).

Key words: CD14, inflammation, lipid A, lipopolysaccharide (LPS), myeloid differentiation protein 2 (MD-2), Toll-like receptor 4 (TLR4).
Abbreviations used: FA, fatty acid; HEK, human embryonic kidney; LPS, lipopolysaccharide; [1H]LOSagg., [1H]LOS aggregates; LPS, lipopolysaccharide; LBP, LPS-binding protein; MD-2, myeloid differentiation protein 2; PGα, peptidoglycan; sCD14, soluble CD14; TLR4, Toll-like receptor 4; TNFα, tumour necrosis factor α.

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The majority of antisepsis agents designed to be TLR4 antagonists, such as Eritoran [10,11], comprise a β(1→6) N-acetylglucosamine disaccharide scaffold with two phosphates in the 1 and 4′ positions and four lipid chains instead of the six present in lipid A. Comparison of the crystal structure of the (TLR4–MD-2–LPS)² complex [19] and that of TLR4–MD-2–Eritoran [21] indicates that the size of the MD-2-binding cavity is the same whether hexa-acylated agonists or tetra-acylated antagonists are bound (Figure 1). The MD-2 cavity volume can readily accommodate the four lipid chains of antagonists, and additional space for lipid binding is generated, at least in the case of hexa-acylated Escherichia coli LPS, by displacing the glucosamine backbone upwards by approx. 5 Å (1 Å = 0.1 nm) [19]. This shift of the anomeric phosphate and resulting rearrangement of the lipid A acyl chains seems to be essential for the formation of a new surface which allow the interaction of two TLR4–MD-2–LPS ternary complexes, thus leading to the formation of a (TLR4–MD-2–LPS)² dimer. In contrast, in the case of antagonists with four lipid chains, the disaccharide part and the phosphates are more inside the MD-2-binding pocket and are not able to interact with the residues of the second TLR4. In this way, the antagonist would occupy the receptor without triggering the formation of the activated complex.

**Natural and synthetic modulators of LPS/TLR4 signalling**

Many other compounds with chemical structures unrelated to lipid A or LPS have also been described to interfere with TLR4-dependent LPS signalling. We briefly present some of the known small-molecule modulators of the TLR4 signalling by grouping them into broad categories depending on the specific molecular target (LPS ligands, compounds targeting CD14, MD-2 or the MD-2–TLR4 complex, and ligands of TLR4 or MD-2).

**LPS ligands (sequestrants)**

Both synthetic and natural (host) polycationic amphiphiles have biological activity as LPS sequestrants: these compounds bind LPS with high affinity, sequestering it from the CD14/MD-2/TLR4 pathway and protecting animals against endotoxin-induced lethality [22–24]. The anionic and amphiphilic nature of lipid A enable the interaction with a wide variety of cationic amphiphiles such as small peptides (polymyxin B), amine dendrimers, pentamidines, polyamines (spermine) or gemini surfactants. The optimization in the design of cationic amphiphiles allows the maximization of LPS affinity while minimizing the toxicity associated with the surfactant properties.

**Molecules targeting CD14**

CD14 has long been considered to have an important, yet non-essential, function in TLR4 signalling, acting to concentrate LPS and thereby increasing the sensitivity of the receptor complex to its ligands. CD14 is essential for the activation of TLR4–MD-2 complex when LPS is in the smooth form [5]. Although LPS is the major ligand, CD14 can also interact with other molecules, including lipoteichoic acid [25], soluble PGNs (peptidoglycans), lipoarabinomannan and lipoproteins [26]. Interestingly, whereas the majority of CD14 ligands that activate the TLR4 pathway are negatively charged molecules (LPS and PGN), positively charged triacylated lipopeptides (di- or tri-palmitoylated cysteiny1-polylysines) also bind to CD14 with high affinity and activate TLR1–TLR2 signalling [27,28].

**Molecules targeting MD-2 or the TLR4–MD-2 complex**

The majority of lipid A analogues so far synthesized as both TLR4 agonists or antagonists interact with the MD-2-binding site with a spatial orientation very similar to that of natural lipid A or LPS. The switch from agonism to antagonism would be determined by the different number and spatial distribution of lipid chains. In agonists, the FA (fatty acid) chains number six or five and the hydrophobic part has a conical shape, whereas, in antagonists, the number of FA chains is fewer than six, and the hydrophobic region is more cylindrical [6]. Both synthetic and natural underacylated lipid A analogues therefore act as antagonists by occupying the MD-2 cavity without triggering TLR4 activation [19–21]. Lipid IVa and Eritoran are the most known examples.
of natural and synthetic TLR4 antagonists respectively, and Eritoran is currently in Phase III clinical trials as an anti-inflammatory agent. The dephosphorylated Salmonella Minnesota lipid A, known as MPL (monophosphoryl lipid A) [29], its 3-O-desacyl variant [30], monosaccharide lipid A analogues [31] and lipid A mimetics with a linear non-carbohydrate structure [14] have been developed as TLR4 agonists. These molecules stimulate the production of inflammatory cytokines, enhances both antibody production and T-cell responses and are effective adjuvants in prophylactic and therapeutic vaccines [32]. The behaviour of the AGP (aminosalkyl glucosaminide phosphate) class of lipid A analogues is dual: these compounds can switch from potent TLR4 antagonism to agonism by simply varying the length of the ‘secondary’ lipid chains [33].

Recently, a peptide antagonist of the MD-2–TLR4 interaction was synthesized [34]. This 17-residue peptide reproduces the TLR4-binding region of MD-2 and contains all of the critical residues for binding, according to the model derived from the recently determined crystal structure of the MD-2–TLR4 complex.

Compounds that directly bind to TLR4 or MD-2

Some non-LPS molecules interfere with the TLR4 pathway by binding TLR4 or MD-2 receptors directly. These compounds do not generally require the canonical LPS presentation sequence to activate TLR4 (that is, sequentially, binding to LBP then to CD14 and finally to MD-2 alone or MD-2 in complex with TLR4). The small-molecule cyclohexene derivative TAK242 suppresses production of multiple cytokines by inhibiting TLR4 signalling [35]. TAK242’s mechanism of action has been very recently clarified at the molecular level: it binds directly to Cys747 in the intracellular domain of TLR4 [35].

The anti-tumour drug Taxol (Paclitaxel®) binds directly to human MD-2. The binding site for Paclitaxel® overlaps with the binding site for LPS, which results in the ability of taxanes to modulate LPS signalling in human receptor models. Molecular docking between taxanes and MD-2 suggest that hydrophobic interactions are the main driving forces in the binding [36]. As a consequence of its interaction with the TLR4–MD-2 complex, it has been shown that Paclitaxel® can regulate tumour survival and chemoresistance in ovarian cancer [37] and has an anti-proliferative effect by acting on TLR4 expressed in meningiomas [38].

A previous study showed that haemin [iron(III) haem] activates macrophages from wild-type, but not from TLR4−/−, CD14−/− or MyD88−/− (myeloid differentiation factor 88) mice, indicating that haemin-induced secretion of TNFα (tumour necrosis factor α) by mouse macrophages is TLR4- and CD14-dependent [39]. However, whereas the TLR4 antagonist Eritoran and an anti-TLR4–MD-2 antibody inhibited TNFα secretion induced by LPS, these molecules did not inhibit cell activation by haemin. These data suggest that haemin can interact with MD-2–TLR4 in a CD14-dependent manner, but in a different site compared with the one used for LPS binding [39]. Our group also investigated the biochemical basis of the TLR4 pathway modulation by haemin and its metabolite, the oxidated derivative coprohaemin [40]. High concentrations of haemin triggered TLR4-mediated IL (interleukin)-8 production in human HIEK (human embryonic kidney)-293/TLR4 cells in the absence of the co-receptors CD14 and MD-2. Differences between haemin and endotoxin in the requirement for MD-2 and CD14 suggest that haemin and endotoxin activate TLR4 by different mechanism.

Targeting CD14: new therapeutic perspectives

Besides being an essential chaperone, assisting TLR4–LPS recognition and binding (particularly in the case of smooth LPS) [5,41], CD14 probably has an important role in cellular pathways not necessarily related to TLR4. It has been discovered recently that CD14 regulates the life cycle of dendritic cells after LPS exposure through a signal pathway based on NFAT (nuclear factor of activated T-cells) activation, totally independent from the TLR4-activated intracellular pathway [42]. Moreover, although the role of CD14 in LPS response is well-established, the participation of CD14 to other TLR-dependent ligands has been less studied. In particular, it has been shown that CD14 contributes to innate immune response activation also by non-endotoxin ligands, including many TLR2–TLR1 ligands [27,28]. It is also suggested that targeting CD14 may also interfere with TLR3 activation by viral nucleic acids, thus holding out the perspective these agents may be effective in the control of viral as well as bacterial diseases in which excessive immune responsiveness damages the host [43].

Sugar-derived small molecules that selectively target CD14

With the aim of developing new lipid A mimetics containing a non-natural enzymatically stable glycosidic bond, we serendipitously found that amino glycolipids with the formulae shown in Figure 2 are active in inhibiting endotoxin-promoted cytokine production in innate immunity cells such as macrophages or dendritic cells [44]. We have found that molecule 1 and similar compounds (Figure 2) inhibit LPS-induced TLR4 activation on HEK-293/TLR4 cells and prevent LPS-induced septic shock in mice [45].

Our structure–activity studies pointed out that glycolipids composed of a glucose unit linked to two C14 hydrophobic chains and with a basic nitrogen on C-6, such as compounds 1, 2 and 3, are active in blocking TLR4-mediated endotoxin stimulus, whereas very similar compounds lacking a positive charge in C-6 (such as molecule 4) are totally inactive. These molecules are effective in inhibiting in vivo LPS-induced lethality [45] and are also able to inhibit other pathologies caused by TLR4 activation, such as inflammation and neuropathic pain [46,47]. Interestingly, if the cyclic...
The pyranose scaffold of the sugar is replaced by an aromatic group, the biological activity is retained, and compound 3 (Figure 2) was shown to be highly potent as an anti-sepsis agent [45].

The mechanism of action of molecules 1, 2 and 3 has been investigated by analysing all possible interactions with the extracellular components that bind and shuttle endotoxin to TLR4, namely LBP, CD14 and MD-2 (free and TLR4-bound) [48]. We tested the ability of glycolipid 1 to inhibit LBP/CD14-dependent transfer of triitated endotoxin ([3H]LOS) monomers from [3H]LOSagg to [3H]LOS–sCD14 followed by transfer of [3H]LOS monomers from [3H]LOS–sCD14 to MD-2–TLR4. Thus the chromatographic profile of [3H]LOSagg incubated first with LBP and sCD14 and then with conditioned medium containing soluble MD-2–TLR4 suggests that, under these experimental conditions, there is nearly complete extraction and transfer of triitated endotoxin monomers from solution aggregates to [3H]LOS–sCD14 followed by transfer of [3H]LOS monomers from about half of the [3H]LOS–sCD14 formed to MD-2–TLR4.

In contrast, in the presence of 10 μM molecule 1, accumulation of both [3H]LOS–sCD14 and ([3H]LOS–MD–2–TLR4)2 was markedly reduced, and most [3H]LOS was recovered in the void volume, presumably as large [3H]LOSagg (Figure 3). The inhibition of the accumulation of [3H]LOS–sCD14 suggested a primary effect of molecule 1 on LBP/sCD14-dependent extraction and transfer of [3H]LOS monomers from solution aggregates to [3H]LOS–sCD14.

Our findings strongly suggest that these compounds inhibit TLR4 activation by endotoxin by competitively occupying CD14 and thereby reducing the delivery of activating endotoxin to MD-2–TLR4. This was also confirmed by STD (saturation transfer difference) NMR measurements, indicating that glycolipid 1 binds to CD14 and that the lipid chains interact directly with the binding site of the protein [48].

Future perspectives

We developed a panel of new molecules that modulate (inhibit) TLR4 signalling by competing with endotoxin for CD14 binding. Interestingly, these compounds do not interact with other receptors, namely TLR4 and MD-2. Whereas the elucidation of the binding mode of these compounds to CD14 is under investigation, their activity is reminiscent of that of triacylated glycopeptides acting on CD14 and interfering with the TLR1/TLR2 pathway [27,28].

Besides being interesting leads for the development of new antisepsis and anti-inflammatory agents, glycolipid 1 and related compounds 2 and 3 are important chemical tools for dissecting the TLR4 and other TLR pathways in which CD14 is involved. Increasing evidence is emerging that TLR4 and CD14 are implicated in other important pathologies, such as neuropathic pain, related to the microglial activation of TLR4 [49] or Alzheimer’s disease [50]. The development of new molecules based on selective CD14 binding would therefore provide access to innovative and hopefully efficient therapies to combat not only septic shock, but also neuropathic pain, Alzheimer’s disease and inflammation.
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References

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