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Synthetic molecules and functionalized nanoparticles targeting the LPS-TLR4 signaling: A new generation of immunotherapeutics*

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Abstract: Toll-like receptor 4 (TLR4), the receptor of bacterial endotoxins in mammalians, plays a pivotal role in the induction of innate immunity and inflammation. TLR4 activation by bacterial lipopolysaccharide (LPS) is achieved by the coordinate and sequential action of three other proteins, the lipopolysaccharide binding protein (LBP), the cluster differentiation antigen CD14, and the myeloid differentiation protein (MD-2) receptors, that bind LPS and present it in a monomeric form to TLR4 by forming the activated $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ complex. Small molecules and nanoparticles active in modulating the TLR4 signal by targeting directly the MD-2·TLR4 complex or by interfering in other points of the TLR4 signaling are presented in this paper. These compounds have great pharmacological interest as vaccine adjuvants, immunotherapeutics, anti-sepsis, and anti-inflammatory agents.

Keywords: immunotherapeutics; lipopolysaccharides; nanoparticles.

LPS STRUCTURE AND TLR4 SIGNALING

Innate immunity is the first line of defense against microbial infections. Defense responses are activated when microbial components are recognized by a variety of pathogen sensors, including toll-like receptors (TLRs) that activate the host defense effector system by rapidly triggering pro-inflammatory processes [1]. Among TLRs, TLR4 selectively recognizes bacterial lipopolysaccharide (LPS) or endotoxin (E) [2], which results in the rapid triggering of pro-inflammatory processes. Among microbial components, LPS and lipooligosaccharides (LOS) and their bioactive portions, the lipodisaccharide lipid A, commonly defined as endotoxins (E), are potent stimulants of immune responses through TLR4 signaling. LPS is the main constituent of the outer leaflet of outer membrane of Gram-negative bacteria. In most bacteria, LPS displays a common structural architecture formed by three domains: a lipophilic moiety termed lipid A, a hydrophilic glycan called the O-specific polysaccharide (also known as O-chain or O-antigen), and a joining core oligosaccharide (OS) (Fig. 1).

Lipid A, the membrane-anchoring moiety of LPS, is essential for bacterial viability and carries the endotoxic properties of the LPSs [3]. As such, lipid A acts as a potent stimulator of the innate immune system via recognition by TLR4. LPS is an amphiphilic molecule and, when released from bacterial cell wall, forms large aggregates in solution whose supramolecular structure depends on the

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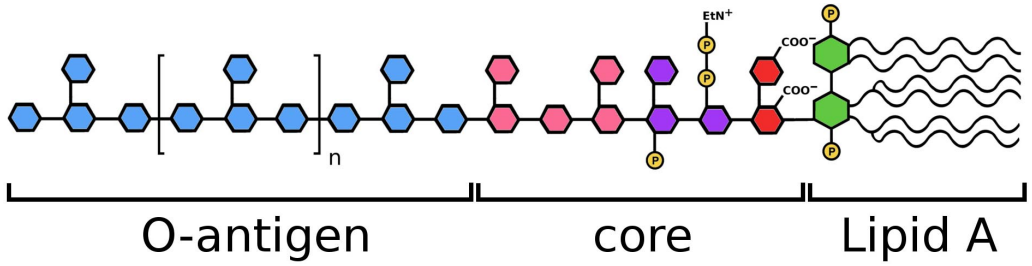


Fig. 1 General structure of the LPS of Gram-negative bacteria.

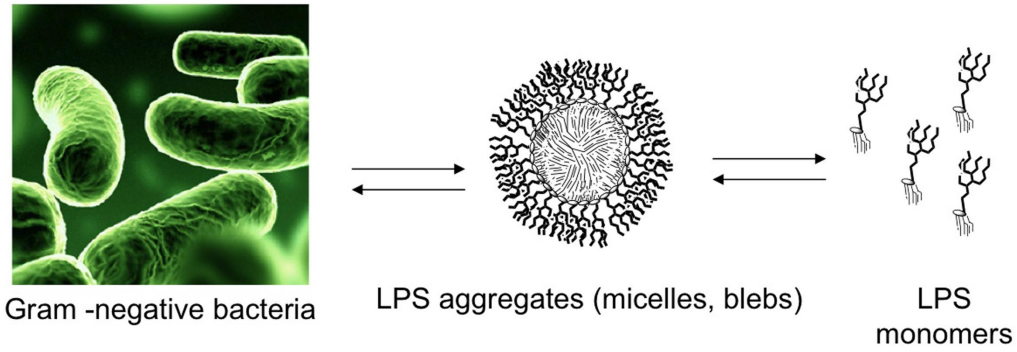


Fig. 2 LPSs either extracted and purified or spontaneously released from Gram-negative bacteria form large aggregates (micelles, membrane blebs) in aqueous environment.

chemical structure of endotoxin and, in particular, on the lipid A moiety [4–6]. However, as for every amphiphilic system, monomers are also present in a dynamic equilibrium, as shown in Fig. 2.

In physiological fluids, LPS aggregates were also found as membrane "blebs", which are constitutively released from growing Gram-negative bacteria [7].

The induction of inflammatory responses by E is not due by direct interaction of LPS with TLR4, but is achieved by the coordinate and sequential action of four principal endotoxin-binding proteins: the lipopolysaccharide binding protein (LBP) [8], the cluster differentiation antigen CD14 [9], the myeloid differentiation protein (MD-2), and finally TLR4 [10] (Fig. 3).

LBP interacts with endotoxin-rich bacterial membranes and purified endotoxin aggregates [11], catalyzing extraction and transfer of LPS monomers to CD14 [12]. LBP preferentially interacts with LPS-rich interfaces and promotes transfer of LPS to CD14, this latter process playing a key role in determining the potency of TLR4 activation by LPS [13]. Monomeric E·CD14 complexes are the most efficient vehicle for transfer of E monomers to MD-2 and to MD-2·TLR4 heterodimer, explaining the importance of LBP and CD14 for LPS signaling at low concentrations of endotoxin. The monomeric E·MD-2 complex is the ligand that, depending on the structural properties of E and MD-2, specifies TLR4 activation or antagonism [14]. Although TLR4 plays a key physiologic role in host response to Gram-negative bacterial infection, an excessively potent and/or prolonged TLR4 response can promote life-threatening pathology such as septic shock [15–17]. The most obvious and important use of TLR4 antagonists is therefore to inhibit LPS-triggered TLR4 activation, which plays a central role in Gram-negative bacterial sepsis and septic shock [15,17].

Recent findings reveal a previously unappreciated role for TLRs and in particular TLR4 in some types of sterile inflammation, namely, inflammation not caused by viruses and microbes that is often the product of tissue injury [18,19]. It is therefore possible to envisage a wider range of possible clini-

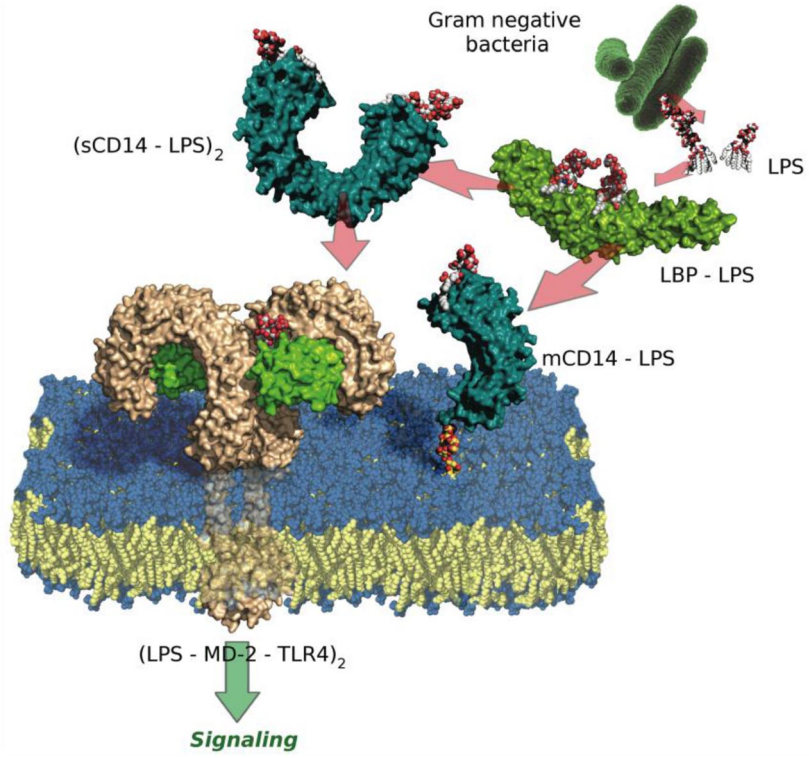


Fig. 3 LPS sequential recognition by innate immunity receptors LBP, CD14, MD-2, and TLR4.

cal settings for application of TLR4 antagonists to contrast certain autoimmune diseases, noninfectious inflammatory disorders, and neuropathic pain [18]. Conversely, agonists of TLR4 can be useful as adjuvants in vaccine development and in cancer immunotherapy [20]. Vaccine adjuvants played a central role in the clinical development of TLR4 agonists [18]. A second clinical application of TLR4 (and TLR9) agonists is in the development of agents against allergic rhinitis and asthma [21–23].

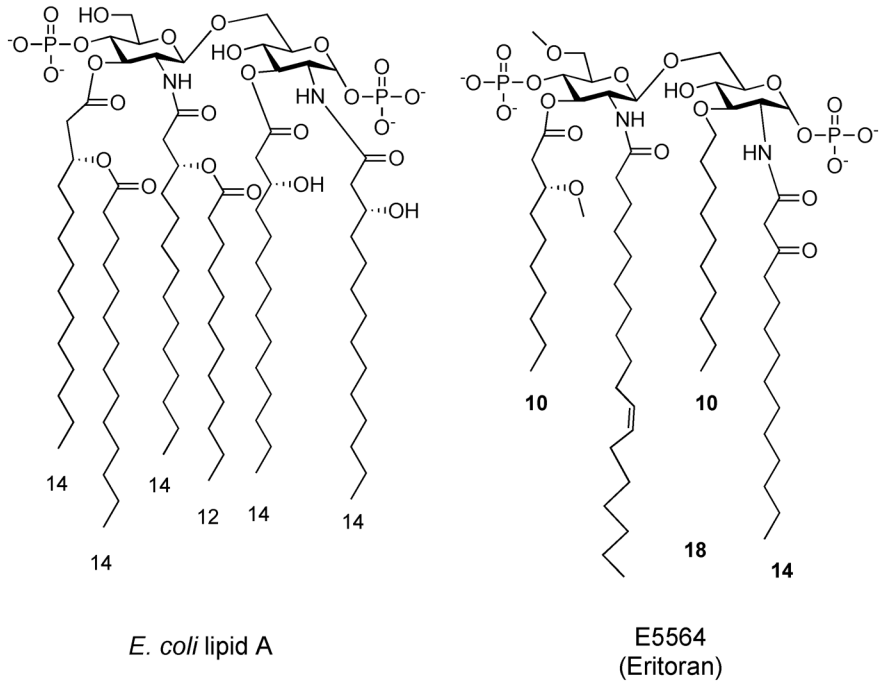
TARGETING TLR4 SIGNALING WITH SMALL MOLECULES

The very recent determination of the crystal structure of $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ complex [24], together with crystallographic data of MD-2 bound to TLR4 antagonists lipid IVa [25] and Eritoran [26] has revealed some fundamental structural aspects of the TLR4 dimerization process and the molecular basis of both TLR4 agonism and antagonism.

The MD-2 cavity volume can readily accommodate four or five lipid chains so that one lipid chain and the disaccharide part with the two phosphates of lipid A would protrude from the MD-2 cavity, thus forming the binding interface for the second TLR4·MD-2 complex [24]. The formation of the binding interface is essential for the interaction of activating LPS·MD-2 from one TLR4·MD-2·LPS ternary complex to TLR4 from a second ternary complex, leading to formation of the $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ dimer.

The majority of TLR4 antagonists, such as the synthetic Eritoran (Eisai's E5564) [27] or the natural lipid IVa, have four lipid chains instead of the six present in lipid A (Fig. 4).

These variants are also called underacylated lipid As as their GlcNAc-GlcNAc disaccharide core bears less than six (generally four) fatty acid chains. Comparison of the crystal structure of the $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ complex and that of TLR4·MD-2·Eritoran indicates that the size of the MD-2



E. coli lipid A

Fig. 4 *E. coli* lipid A structure compared to the synthetic TLR4 antagonist Eritoran (E5564). Fatty acid chains lengths are indicated.

pocket is the same whether hexaacylated agonists or tetraacylated antagonists are bound. The four lipid chains of Eritoran occupy the MD-2 cavity and the disaccharide moiety inserts into the cavity and is no more available to form the binding interface for $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ complex formation. Thus, compounds with four fatty acid chains stably occupy MD-2 cavity without inducing the formation of $[TLR4{\cdot}MD\text{-}2{\cdot}LPS]_2$ activated complex, acting as antagonists.

On the contrary, natural and synthetic lipid A variants with six (or seven, as in the case of *Salmonella minnesota*) lipid chains behave as agonists. The toxic effects of heptaacylated *Salmonella minnesota* R595 lipid A can be ameliorated by selective hydrolysis of the 1-phosphate and by removing the acyl chain in position C-3 [28]. The resulting chemically modified lipid A product, monophosphoryl lipid A (MPL), is a mixture of the two differently acylated variants with five or six fatty acid chains, and is an effective adjuvant in prophylactic and therapeutic vaccines (Fig. 5).

MPL agonists with reduced toxicity but increased potency meet the stringent safety criteria required for prophylactic vaccines, and MPL was the first TLR4 agonist approved for use in a human vaccine, FENDrix® vaccine for hepatitis B virus (HBV) in Europe. Currently, two approved HBV vaccines [29] and an almost approved papilloma virus vaccine [30] use MPL as an adjuvant. Other lipid A mimetics with TLR4 agonistic activity are the aminoalkyl glucosaminide 4-phosphates (AGPs) in which the reducing glucosamine residue has been replaced by an acylated amino acid or another acylated function [31,32] (Fig. 5). The AGPs lipid A analogues have six acyl chains and retain significant activity as TLR4 agonists.

The entire disaccharide core can be replaced with a linear linker substituted with six lipophilic chains and with two phosphate obtaining TLR4 agonists (Fig. 5) such as Eisai's ER112022 [33].

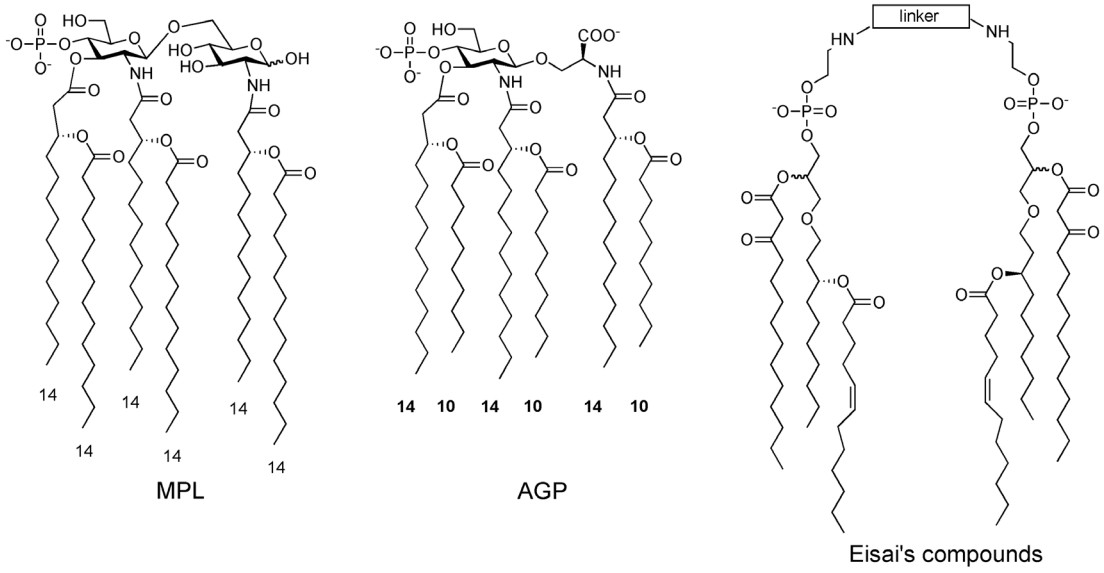


Fig. 5 TLR4 agonists MPL, AGP, and Eisai's compounds lacking the disaccharide core.

NEW SYMMETRIC SULFATED DISACCHARIDES AS LIPID A MIMETICS

We recently developed an innovative type of lipid A analogues (compound **D1**, Fig. 6) in which two methyl α-D-glucopyranoside units are bridged through a (6 → 6') succinic diamide linker and two sulfate groups mimic natural phosphates.

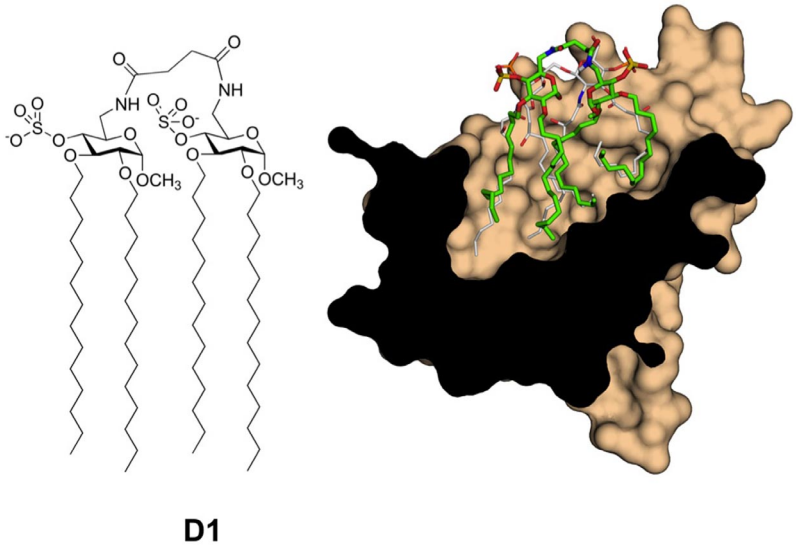


Fig. 6 Left: chemical structure of the symmetric lipid A analogue **D1**. Right: simulation of **D1** (gray) superimposed to lipid IVa (white) into the MD-2 pocket.

The sulfate groups, negatively charged at neutral pH, are bioisosters of phosphates [34]. Unlike natural lipid As, compound **D1** is a symmetric molecules (2-fold rotational symmetry C_2) and therefore its synthesis is greatly simplified. Four linear ether chains ($C_{14}H_{29}$) replace acyl esters ($COC_{13}H_{27}$ or $COC_{11}H_{23}$) found in lipid A. Ether chains are more resistant to enzyme hydrolysis than esters and improve the pharmacokinetic properties of the molecule.

Compound **D1** has been rationally designed according to the recently resolved X-ray structures of lipid A [24], and antagonists lipid IVa [25] and Eritoran [26] bound to MD-2. According to preliminary simulations, disaccharide **D1** can bind to MD-2 in a manner similar to that of lipid IVa [25], with the four fatty acid chains deeply confined in the MD-2 cavity and the phosphate and sugar groups interacting with MD-2 conserved residues at the cavity rim (Fig. 6).

Compound **D1** produced dose-dependent inhibition of cell activation (i.e., extracellular accumulation of IL-8) induced by LOS in HEK293 cells expressing TLR4 (HEK-TLR4 cells). This antagonistic activity is accompanied by a mild TLR4 agonistic activity when **D1** is administered alone to the same cells.

Compound **D1**, much like other tetraacylated lipid A analogues, inhibits activation of TLR4 by endotoxin, but also shows a mild activating properties that make it an interesting hit compound for vaccine adjuvant development.

Under-phosphorylated MPLs that have partial TLR4 agonist properties are currently lead compounds as vaccine adjuvants, providing apparently sufficient TLR4-dependent immune boosting while tempering potential TLR4-mediated toxicity [35–37]. In sepsis, where immune dysregulation may be manifest as a systemic inflammatory syndrome and/or subsequent immune paralysis [38], a compound that has both partial agonist and antagonist properties may be more advantageous than the more pure TLR4 antagonists that have been developed and tested to date. Work is in progress to characterize the pharmacodynamic and pharmacokinetic properties of **D1** and to study molecular details of its interaction with the TLR4-MD-2 complex.

A NEW GENERATION OF SMALL-MOLECULE IMMUNOTHERAPEUTICS TARGETING SELECTIVELY CD14

With the aim to develop new TLR4-active compounds, our research group projected and synthesized a series of new molecules formed by a positively charged amine or ammonium ion, a cyclic aromatic or pyranosidic core bearing two C14 lipid chains which inhibited LPS- and lipid A-promoted cytokine production in macrophages and dendritic cells [39,40].

These compounds are now commercially available with the proprietary names **IAXO** and are produced by BIOAXXESS (Tewkesbury Business Park, Oakfield Close, UK) (Fig. 7). Compounds **IAXO101–104** inhibit LPS-induced TLR4 activation in HEK293 stably transfected with TLR4, MD-2, and CD14 genes and containing a secreted alkaline phosphatase SEAP reporter gene, and they also efficiently inhibit LPS-induced septic shock in mice [39]. Structure–activity studies suggest that the pharmacophore consists of glucose or a phenyl ring linked to two C_{14} ether lipid chains and a basic nitrogen. Compounds **IAXO101–104** containing the complete pharmacophore are active in blocking TLR4-mediated cytokine production in innate immunity cells, while very similar compounds **IAXO201** and **IAXO202** lacking the positively charged group are inactive [39]. We have found that compound **IAXO101** can also reduce in vivo neuropathic pain by reversing mechanical allodynia and thermal hyperalgesia in mice [41].

The mechanism of action for molecule **IAXO101** has been investigated by analyzing all possible interactions with LBP, CD14, and MD-2 (free and TLR4-bound) [42]. Using tritiated LOS, we tested **IAXO101** for its ability to inhibit in vitro the formation of the activated TLR4 complex [TLR4·MD-2·LPS]$_2$. We observed that the formation of the activated complex [TLR4·MD-2·LPS]$_2$ was reproducible in the absence of **IAXO101**, but was inhibited in a dose-dependent manner by this compound. Inhibition was associated with the ability of **IAXO101** to selectively block the interaction of LPS with

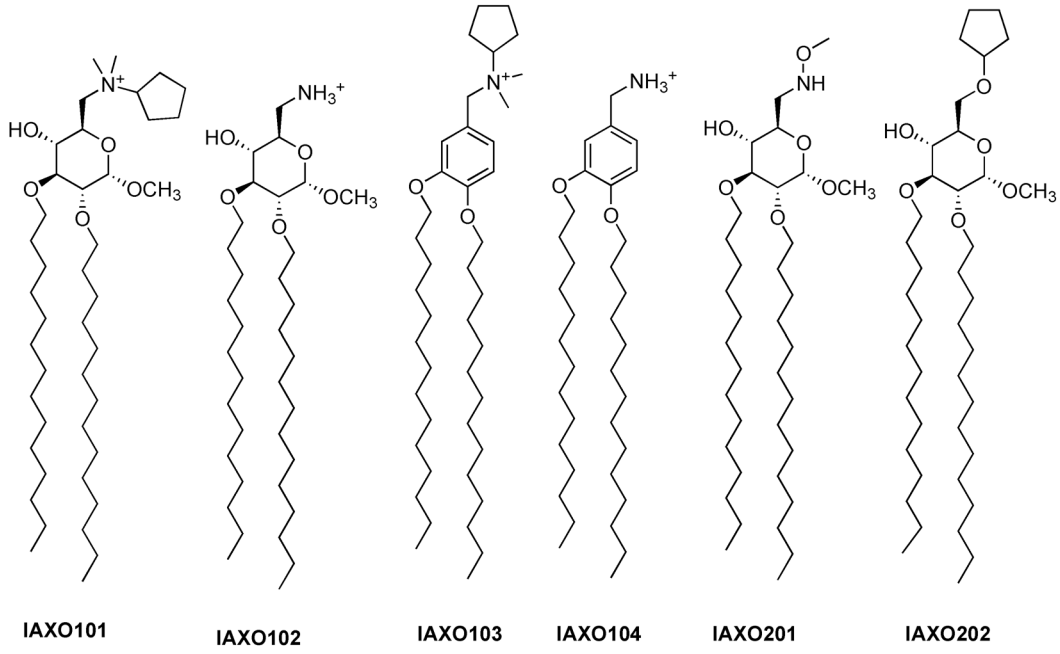


Fig. 7 Synthetic **IAXO** compounds.

CD14, as demonstrated by NMR saturation transfer difference (STD) experiments [42]. Work is in progress to determine the exact structure and stoichiometry of the CD14-**IAXO101** complex.

NANOPARTICLE-BASED IMMUNOMODULATORS

We recently developed novel bioactive LPS-coated magnetic nanoparticles (LMNPs) [43].

LMNPs were designed to mimic natural E micelles or membrane blebs (Fig. 8) that contain an E-rich monolayer on the surface with the fatty acyl chains of lipid A oriented inward away from the aqueous surroundings.

To prepare LMNP, spherical oleylamine-coated iron oxide nanocrystals (hydrophobic brush nanoparticles, HBNPs) were resuspended in hexane and added to an aqueous dispersion of commercial LPS, the biphasic mixture was mildly warmed (40 °C) and sonicated to cause the slow evaporation of the organic solvent while mixing. This process promoted the formation of hydrophilic LMNPs with the polysaccharide chains of LPS forming the outer shell. The coating process resulted in stable particle dispersion with a hydrodynamic diameter of 193 ± 4 nm. LMNPs prepared with the described procedure were very stable and could be purified and isolated by magnetic decantation without apparent loss of water stability once resuspended even after several purification cycles. Moreover, LMNPs could be stored at 4 °C for longer than one month, showing no sign of aggregation as determined by dynamic light scattering (DLS) and no degradation in terms of biological activity.

The bioactivity of LPS bound to LMNPs was assessed by measuring the ability of LMNPs to stimulate TLR4-dependent cell activation. Increasing concentrations of LMNPs (from 4.2 pM to 4.2 nM of nanoparticle-bound LPS) induced a dose-dependent activation of transformed HEK293 cells expressing CD14, MD-2, and TLR4, as manifest by extracellular accumulation of IL-8.

In contrast, LMNPs did not activate the parental HEK293 cells that do not express CD14, MD-2, or TLR4, confirming that activation of HEK-TLR4 cells by LMNPs was CD14, MD-2-TLR4-dependent.

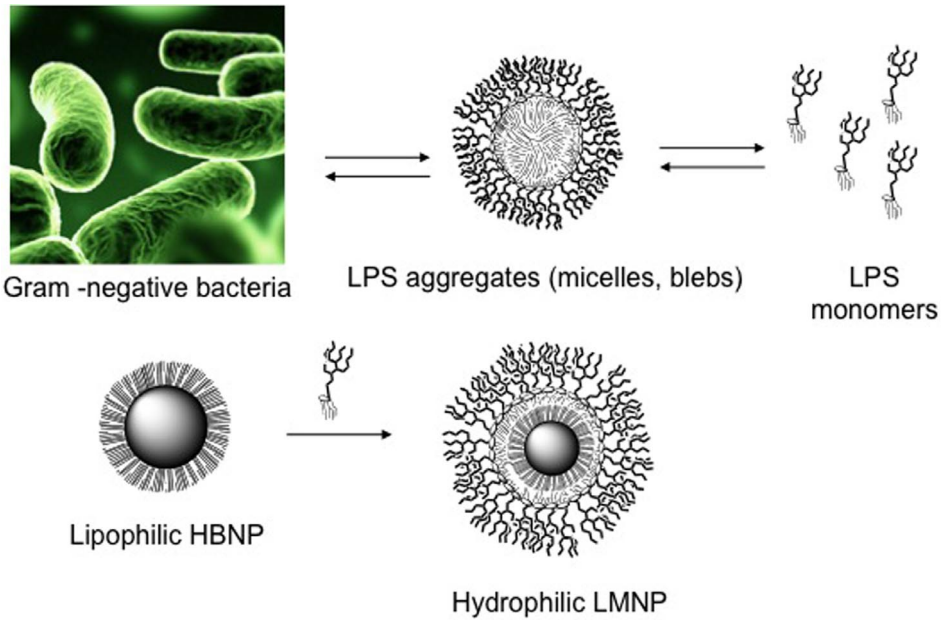


Fig. 8 LMNPs have been designed to mimic LPS aggregates in solution.

To further define the ability of LMNPs to stimulate TLR4-dependent cellular responses, LMNP-induced activation of cells of the innate immune system was investigated using wild-type (wt), $CD14^{-/-}$ and $TLR4^{-/-}$ murine bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMDMs). LMNPs produced dose-dependent activation of both cell types derived from wt mice. The potency of the LMNPs was, however, reduced about 10-fold toward $CD14^{-/-}$ cells and little or no cell activation was induced by LMNPs in $TLR4^{-/-}$ cells. Thus, activation of BMDCs and BMDMs by LMNPs was TLR4-dependent and promoted by CD14, consistent with action of the bound LPS in the LMNPs.

The hydrophobic coating of the spherical HBNPs has made possible binding of LPS molecules on the nanoparticle surface in an orientation that likely resembles the natural presentation of LPS in the outer membrane of Gram-negative bacteria as well as in aggregates of extracted and purified LPS. The LMNPs are stable in solution, and the LPS is not spontaneously released in solution because it is stably immobilized on the nanoparticles. LMNPs trigger cell activation in an LPS- and TLR4-dependent manner that is promoted by CD14, making it likely that cell activation is dependent on mobilization of LPS monomers from the surface of the LMNPs to MD-2-TLR4. Work is in progress to further investigate the in vitro and in vivo effects of controlled LPS release from LMNPs and the possible application of these reagents as new, nanoparticle-based vaccine adjuvants or immunotherapeutics.

CONCLUSIONS AND PERSPECTIVES

Investigations on small molecules that can modulate the TLR4 pathway not only offer novel pharmacological targets but they contribute to the clarification of basic structural and mechanistic aspects of TLR4 signaling, including the role of LBP, CD14, and MD-2 co-receptors. This method of investigation of biological signal pathways through the use of small-molecule ligands is the so-called "chemical genetics" approach [44], and is complementary to the classical forward (mutagenesis) and reverse (gene knockout) genetic approaches. Some compounds presented in this article have been rationally designed to target MD-2 or the $[TLR4{\cdot}MD\text{-}2]_2$ complex. These compounds are mainly lipid A analogues with

agonist or antagonist activity on the LPS-TLR4 signal pathway, such as MPLs and AGPs that mimic the entire lipid A or part of its structure. The recent determination of crystal structures of the $[TLR4{\cdot}MD\text{-}2]_2$ complex with bound lipid A (agonist) or lipid A antagonists (Eritoran, lipid IVa) clarified important aspects of the structure–activity relationship in natural lipid As or synthetic lipid A analogues. Accordingly, it is possible today to use rational rules for the design of TLR4 agonists and antagonists with a lipid A-derived structure.

A big deal for the future would be to determine and characterize allosteric sites on TLR4 so that specific ligand can be designed to modulate TLR4 activity through non-classical CD14 and MD-2-mediated ligand presentation. This would also allow specific targeting of sterile inflammations or autoimmune diseases not caused by the presence of a pathogen. Several research groups, included ours, are involved in the development of nonclassical TLR4 antagonists as lead compounds for the development of innovative and selective drugs against chronic pain, neuropathic pain, and other syndromes caused by microglial TLR4 activation. On the other hand, the development of non-lipid A TLR4 mild agonists would provide innovative compounds as nontoxic vaccine adjuvant and immunotherapeutics.

The selective CD14 targeting by molecules such as **IAXO** compounds that bind to CD14 and not to MD-2 or MD-2-TLR4 is also an innovative way to inhibit the whole TLR4 pathway and to elude bacterial resistance in the development of a new generation of antisepsis agents as well as agents to target TLR4-mediated noninfectious inflammatory conditions such as certain forms of neuropathic pain.

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