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Characterization of TRIF selectivity in the AGP class of lipid A mimetics: Role of secondary lipid chains

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ABSTRACT

TLR4 agonists that favor TRIF-dependent signaling and the induction of type 1 interferons may have potential as vaccine adjuvants with reduced toxicity. CRX-547 (**4**), a member of the aminoalkyl glucosaminide 4-phosphate (AGP) class of lipid A mimetics possessing three (*R*)-3-decanoyloxytetradecanoyl groups and D-relative configuration in the aglycon, selectively reduces MyD88-dependent signaling resulting in TRIF-selective signaling, whereas the corresponding secondary ether lipid **6a** containing (*R*)-3-decyloxytetradecanoyl groups does not. In order to determine which secondary acyl groups are important for the reduction in MyD88-dependent signaling activity of **4**, the six possible ester/ether hybrid derivatives of **4** and **6a** were synthesized and evaluated for their ability to induce NF-κB in a HEK293 cell reporter assay. An (*R*)-3-decanoyloxytetradecanoyl group on the 3-position of the D-glucosamine unit was found to be indispensable for maintaining low NF-κB activity irrespective of the substitutions (decyl or decanoyl) on the other two secondary positions. These results suggest that the carbonyl group of the 3-secondary lipid chain may impede homodimerization and/or conformational changes in the TLR4–MD2 complex necessary for MyD88 binding and pro-inflammatory cytokine induction.

Toll-like receptors (TLRs) are a family of pattern recognition receptors on innate immune cells that recognize pathogen-specific components of microbial invaders. The recognition of microbial ligands by TLR receptors triggers intracellular signaling via the cytoplasmic Toll-interleukin receptor (TIR) domain common to all TLRs, resulting in the release of pro-inflammatory cytokines, chemokines, and anti-microbial defensins, and in the expression of co-stimulatory molecules. Expression of these factors drives the innate immune response to infection as well as the recruitment and activation of antigen-presenting cells and effector B and T cells involved in adaptive immunity.[1,2]

Lipopolysaccharide (LPS, endotoxin), the main cell surface component of Gram-negative bacteria, is the natural glycolipid ligand that binds TLR4 and its accessory molecule, MD-2, to form a stable TLR4–MD-2 receptor complex, triggering an initial innate immune response.[3] Although cellular activation through the TLR4–MD-2 receptor is architecturally complex[4] and involves many signaling elements, TLR4–MD-2 receptor signaling proceeds mainly through two intracellular pathways: the MyD88-dependent pathway, and the TRIF-dependent pathway, also known as the MyD88-independent pathway.[5] Signaling through the MyD88-dependent pathway involves binding of two adaptor proteins, MyD88 and the TIR-adaptor TIRAP (Mal), to the cytoplasmic domain of TLR4 and induces early NF-κB activation and the release of pro-inflammatory cytokines such as TNF-α and IL-1β.[6] The TRIF-dependent pathway, on the other hand, relies on cytoplasmic adaptor proteins TRIF (TIR domain-containing adapter inducing IFN-β) and the TRIF-related molecule TRAM, and induces later and lower levels of NF-κB activation, resulting in lower expression of mediators of inflammation and toxicity. The TRIF-dependent pathway also activates the nuclear translocation of the transcription factor IRF-3, resulting in expression of type I interferons (IFN-α/β) and IFN-inducible genes.[7] IFN-dependent signaling downstream of TRIF, in turn, is involved in the up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules on dendritic cells, mediators of antigen stimulation and T-cell activation and proliferation that are crucial for an effective adaptive immune response to infectious agents and heterologous vaccine antigens.[8]

Although LPS is a potent stimulator of host defense systems via its interaction with the TLR4–MD-2 receptor complex, the pathophysiology of LPS and its active principle, lipid A, preclude their use as adjuvants in human vaccines. The toxicity of the LPS from

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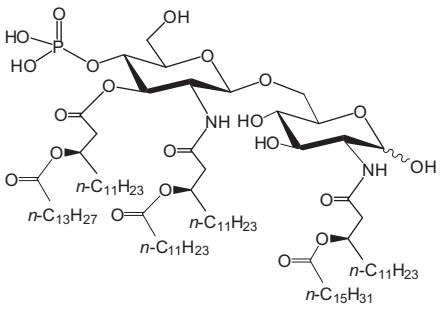
Salmonella minnesota R595, however, has been ameliorated by the selective hydrolysis of certain groups, including the anomeric phosphate, to produce a TLR4-active product approved for human use known as monophosphoryl lipid A (MPL). MPL, which comprises several less highly acylated compounds in addition to the major, hexa-acyl component **1** (Fig. 1), is an effective adjuvant in prophylactic and therapeutic vaccines and shows an excellent safety profile in humans.[9] The reduced toxicity of MPL has been attributed to selective induction of the TRIF signaling pathway and MyD88-independent factors such as IP-10 and MCP-1 coupled with threshold levels of MyD88-dependent cytokines.[10] By the same token, the lower virulence of some bacterial strains as well as the decreased toxicity of certain LPS molecules, including *Salmonella* LPS, has also been attributed to selective TRIF signaling.[5,11] However, the structural variability within individual lipid A or LPS preparations and the potential presence of other bioactive substances often make it difficult to draw definite conclusions about which structural features are responsible for a particular immune response. Thus, considerable effort has been directed towards the synthesis of not only individual natural lipid A components[12] but also subunit analogs of lipid A in which the disaccharide backbone of lipid A has been replaced with a structural motif more amenable to systematic structure–activity relationship (SAR) and mechanism of action investigations.[13,14]

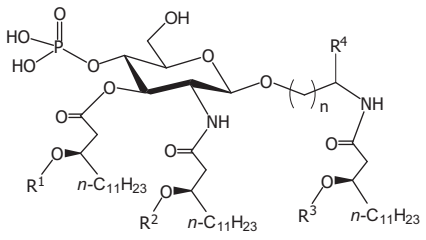
In the course of our own SAR studies on lipid A, we identified a new class of TLR4-active glycolipids known as aminoalkyl glucosaminide 4-phosphates (AGPs).[15] The immunostimulatory activity of the AGP class of synthetic lipid A mimetics, which have the general structure **1** (Fig. 1), was found to depend greatly on the length of the secondary lipid chain length (R^1–R^3) as well as the structure of the aglycon moiety.[15,16] Maximum TLR4 agonist activity in human in vitro models was observed with seryl-based AGPs ($R^4 = CO_2H$, $n = 1$) containing 10-carbon secondary acyl or alkyl groups (R^1, R^2, R^3 = decanoyl or decyl), whereas the corresponding seryl derivatives possessing 6-carbon secondary lipid chains were potent TLR4 antagonists in human systems.[17] CD14, a protein involved in the shuttling of LPS to MD-2, was not required for MyD88-dependent agonist activity in the AGP series in vitro but did enhance responses, particularly for lower potency agonists.[16] Site-directed mutagenesis studies[18] and structural studies with secondary acyl hybrid AGP molecules[16] pointed to the particular importance of secondary lipid chain R^1 in determining TLR4 activity. These observations are consistent with a TLR4–MD-2–AGP complex in which the terminal methylene units of secondary lipid chain R^1 of the AGP molecule interact with the dimerization interface created by hydrophobic patches on MD-2 and the TLR4 ectodomain to form a symmetrical 'm'-shaped TLR4–MD-2–AGP homodimer.[18] Dimerization of ligand-complexed TLR4–MD-2 is thought to be if not prerequisite to TLR4 activation[19] at least promotive of more rapid signaling.[20]

Such an orientation of the AGP molecule in the MD-2 hydrophobic pocket with the R^1 terminus interacting with the TLR4 ectodomain corresponds to that determined crystallographically for TLR4 antagonists eritoran (E5564) bound to a hybrid human TLR4–MD-2 heterodimer[21] and lipid IVa bound to human MD-2,[22] but is opposite (i.e., rotated 180 degrees) to that shown crystallographically for hexa-acyl *Escherichia coli* LPS bound to the hybrid TLR4–MD-2 heterodimer, wherein the lipid chain amide-linked to the reducing sugar interacts with hydrophobic residues of both MD-2 and the TLR4 ectodomain at the dimerization interface.[4] While the latter 'agonist' or 'LPS-like' orientation may be favored by crystallization conditions and/or the presence of a divalent counter ion, as well as by structural changes made to the TLR4 molecule to permit solubilization/co-crystallization,[21] the above data suggest that the AGP class of lipid A mimetics bind in an 'antagonist' or 'eritoran-like' orientation to the human TLR4–MD-2 heterodimer to induce dimerization and signaling. Nonetheless, given the C_2 symmetry of certain TLR4 agonists[14] and antagonists,[23] the pseudosymmetry of the receptor itself, as well as the striking effect of different metal counter ions on LPS activity,[24] it is likely that some TLR4–MD-2 ligands modulate immune responses by inserting into MD-2 in both orientations.

Because MPL's beneficial adjuvant effects have been associated with a bias toward the TRIF-dependent pathway, members of the AGP class of lipid A mimetics were also screened for differential induction of MyD88- and TRIF-dependent signaling pathways in the hope of identifying a TRIF-selective AGP for potential use as a vaccine adjuvant with improved safety and efficacy profiles. A comparison of seryl-based AGPs CRX-527 (**3**) and CRX-547 (**4**) possessing 10-carbon secondary acyl chains and differing only in the configuration of the seryl stereocenter (Fig. 2) showed that the D-seryl-based AGP **4** ('D-isomer') induced significantly lower levels of MyD88-dependent cytokines relative to the L-isomer **3** in human primary PBMC-derived monocytes and dendritic cells but similar levels of TRIF-dependent chemokines.[25] The relative responses of CRX-527 and CRX-547 in these cell-based assays correlated strongly with their MyD88-dependent NF-κB activity in a human embryonic kidney (HEK) cell based reporter assay, using either MD2/TLR4 or MD2/TLR4/CD14 receptor transfectants (data not shown; see also reference 25). The inverted configuration of the seryl carboxyl group, a bioisostere of the anomeric phosphate of lipid A, in CRX-547 likely disrupts electrostatic binding to positively charged amino acids of MD-2 or TLR4 and results in altered receptor dimerization and/or conformational changes in TLR4, which affect adaptor protein (MyD88 and/or Mal/TIRAP) binding and subsequent intracellular signaling. In fact, it was recently shown that the TRIF-selectivity of congeneric MPL is due to impaired CD14-dependent homodimerization of the TLR4–MD-2–MPL complex at the cell surface and concomitant reduction in MyD88-dependent signaling.[26]



1 major component in MPL



2 AGP generic structure

Figure 1. Synthetic and naturally derived lipid A mimetics.