Substrate structure-activity relationship reveals a limited lipopolysaccharide chemotype range for intestinal alkaline phosphatase

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Running title: LPS substrate specificity of alkaline phosphatase

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Keywords: endotoxin, lipopolysaccharide (LPS), metabolic endotoxemia, intestinal alkaline phosphatase, Toll-like receptor (TLR), lipid A, systemic inflammation, metabolic syndrome, gut microbiome, intestinal flora

ABSTRACT

Lipopolysaccharide (LPS) from the Gram-negative bacterial outer membrane potently activates the human innate immune system. LPS is recognized by the Toll-like receptor 4/myeloid differentiation factor-2 (TLR4/MD2) complex, leading to the release of pro-inflammatory cytokines. Alkaline phosphatase (AP) is currently being investigated as an anti-inflammatory agent for detoxifying LPS through dephosphorylating lipid A, thus providing a potential treatment for managing both acute (sepsis) and chronic (metabolic endotoxemia) pathologies wherein aberrant TLR4/MD2 activation has been implicated. Endogenous LPS preparations are chemically heterogeneous, and little is known regarding the LPS chemotype substrate range of AP. Here, we investigated the activity of AP on a panel of structurally defined LPS chemotypes isolated from Escherichia coli and demonstrate that calf intestinal AP (cIAP) has only minimal activity against unmodified enteric LPS chemotypes. Inorganic phosphate was only released from a subset of LPS chemotypes harboring spontaneously labile phosphoethanolamine (PEtN) modifications connected through phosphoanhydride bonds. We demonstrate that the spontaneously hydrolyzed O-phosphorylethanolamine is the actual substrate for AP. We found that the 1- and 4'-lipid A phosphate groups critical in
TLR4/MD2 signaling become susceptible to hydrolysis only after de-O-acylation of ester linked primary acyl chains on lipid A. Furthermore, PEtN modifications on lipid A specifically enhanced hTLR4 agonist activity of underacylated LPS preparations. Computational binding models are proposed to explain the limitation of AP substrate specificity imposed by the acylation state of lipid A, and the mechanism of PEtN in enhancing hTLR4/MD2 signaling.

INTRODUCTION

Low-grade systemic inflammation associated with a number of metabolic syndromes has been proposed to originate from bacterial flora (1-7). Bacterial products (microbe-associated molecular patterns, MAMPs2, or pathogen-associated molecular patterns, PAMPs) from the intestinal flora are normally contained within the lumen by the intestinal epithelium layer. However, high fat diets, obesity, and inflammatory bowel diseases help increase the permeability of the intestinal mucosal barrier, allowing luminal contents to breach containment and enter the blood stream. MAMP engagement by pattern recognition receptors (PRR) on target cells triggers the release of pro-inflammatory cytokines, setting a low-grade systemic inflammatory tone (8,9). Inflammation and oxidative stress is further accelerated by adipose tissue deposits, which can directly respond to MAMPs as well and secrete pro-inflammatory adipocytokines (10,11). A central PRR-MAMP pair in these processes is the Gram-negative bacterial outer membrane (OM) component lipopolysaccharide (LPS or endotoxin) and the Toll-like receptor 4/myeloid differentiation factor-2 (TLR4/MD2) complex. Collectively termed metabolic endotoxemia (ME), elevated serum endotoxin levels and the ensuing aberrant TLR4/MD2 activity have been associated with the complications of metabolic diseases (6,12). So far LPS constitutes the only known bacterial MAMP that can induce obesity as well as insulin resistance when infused subcutaneously into mice (2,13). Thus, preventing ME through degradation and detoxification of LPS offers a potential therapeutic strategy for managing the onset, severity, and progression of ME (14).

According to the endotoxin principle, the 1- and 4’- lipid A phosphates of LPS are critical for biological activity as chemically dephosphorylated lipid A congeners weakly stimulate TLR4/MD2-directed pro-inflammatory cytokine release (15). Alkaline phosphatase (AP) has thus far received the most attention as a potential tool to detoxify LPS and attenuate inflammation through lipid A dephosphorylation (16-19). Recombinant AP is currently being examined in multiple clinical trials to treat a host of chronic (ME) or acute (sepsis) inflammatory conditions where endotoxin-induced inflammation is a suspected component of the underlying pathology (20-22). There are four AP encoding genes in humans, three of which demonstrate tissue specific expression [intestinal AP (IAP, ALP1), placental or embryonic AP (PLAP, ALPP) and germ cell AP (GCAP, ALPL2)] along with a tissue nonspecific AP (TNAP, ALPL) isozyme (23,24). IAP, being a native defense factor within the intestinal mucosal brush border, is a particularly promising therapeutic candidate as the anti-inflammatory mechanism of IAP has been attributed in part to the direct dephosphorylation of LPS (22,25-30). Presumably, IAP hydrolyses critical phosphates from lipid A involved in TLR4/MD2 recognition, although structural characterization of the IAP dephosphorylated LPS product has not hitherto been reported (31,32). IAP activity to this point has instead been indirectly evaluated through the release of inorganic phosphate using variants of the molybdate malachite green assay (33), in tandem with the apparent loss of biological activity. The potential LPS chemotype substrate specificity spectrum is unknown, as is the unanswered question of which of the many phosphate groups present on LPS are actually subject to dephosphorylation by IAP.

Given the complex and structurally heterogeneous nature of LPS (34,35), we determined IAP substrate specificity using a panel of defined LPS chemotypes isolated from Escherichia coli. LPS from E. coli possesses among the highest endogenous endotoxin
activity, and can be non-stoichiometrically modified with a variety of moieties. Variations in LPS acylation state, saccharide core, and phosphorylation patterns could conceivably affect IAP recognition and processing. Of note, *E. coli* converts the 1- and 4'-lipid A phosphate groups from phosphomonoesters into phosphodiester upon addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (Ara4N) residues. PEtN and Ara4N addition are governed by a complex network of alternative transcription factors, two component systems, and regulatory RNAs (36). In *Salmonella enterica*, the regulators controlling LPS remodeling with PEtN/Ara4N are activated upon exposure to the mouse intestinal lumen environment (37). These regulatory systems could thus not only enable bacterial survival, but also block IAP-mediated LPS detoxification.

Herein, we report that IAP and other APs isozymes have minimal activity against unmodified LPS chemotypes (from hexa- to tetra-acylated lipid A) typical to the outer membrane of enteric bacteria such as *E. coli*. Unexpectedly, we have observed that inorganic phosphate only appears to be released from LPS that has been modified with PEtN groups. We demonstrate that PEtN attached to LPS in a phosphoanhydride linkage spontaneously hydrolyzes at neutral to basic pH to release free PEtN, which in turn generates the actual substrate for IAP. Lipid A phosphate groups critical to TLR4/MD2 signaling only become susceptible to IAP activity after de-O-acylation of ester linked primary acyl chains on lipid A. Importantly, LPS with PEtN covalently attached to lipid A on the 1- and 4'-lipid A phosphates through phosphoanhydride bonds stimulates TLR4/MD2 signaling activity in comparison to congeners lacking PEtN. The extent of TLR4/MD2 stimulation by PEtN modifications on lipid A is enhanced with decreasing acylation states, including for the otherwise antagonist tetra-acylated lipid IVα ligand. Computational binding models are applied to explain the substrate specificity of IAP, as well as the effects of PEtN modification on lipid IVα binding to human and murine TLR4/MD2 receptors. The apparent inability for IAP to dephosphorylate the critical lipid A backbone phosphates in fully acylated substrates suggests the physiological role of IAP in suppressing LPS induced inflammation may be more complicated and invoke mechanisms beyond regulating TLR4/MD2 receptor activity.

RESULTS

**Inorganic phosphate is only released from wildtype *E. coli* B LPS chemotype.** To study the effect of LPS modifications on processing by AP, a highly purified *E. coli* B LPS feedstock sample was first isolated using an extended protocol that included specific steps to remove phospholipid and nucleic acids contaminants that could contribute to background phosphate release. We chose *E. coli* B as the parent LPS strain source firstly because of a native insertion sequence element within the outer saccharide core *waaT* gene, encoding for a UDP-galactose:(glucosyl) LPS α1,2-galactosyltransferase glycosyltransferase, that truncates the LPS to a structure of relatively low complexity (38,39), and secondly for the high levels of endogenous PEtN and Ara4N modifications observed when this strain is grown in standard rich medium (Fig. 1A) (40). Analysis by mass spectrometry confirmed a highly PEtN/Ara4N-substituted LPS (Fig. S1A), with an average total phosphate content of between 4 to 5 Pi equivalents per molecule of LPS (Table S2). We initially tested calf intestinal alkaline phosphatase (cIAP) with LPS preparations from wildtype *E. coli* B LPS for phosphate release using the inorganic phosphate specific malachite green assay (Fig. 1B). While phosphate release was readily detected, the amount plateaued well short of the total LPS-associated phosphate input (~25 µM LPS with 100-125 µM total phosphate). Since there are multiple tissue specific AP isoforms (23,24), a panel of commercially available APs was tested to determine if a more robust phosphate release could be realized. However, all APs (including human intestinal alkaline phosphatase, hALP) demonstrated a plateau similar to cIAP even after a prolonged 96-hour incubation period (Fig. 1B). Varying reaction conditions by adding bile salts to act as detergent, bovine serum albumin, 10% whole
serum, or extensive pre-sonication of LPS vesicles did not appreciably enhance the amount of phosphate released (data not shown). Detection of inorganic phosphate was completely dependent on inclusion of AP. This suggested that only a fraction of the total LPS phosphate content was subject to hydrolysis, irrespective of either the AP isofrom or presence of de-aggregation agents.

To determine which LPS phosphate group(s) was being released, we repeated the assay using a structurally defined Re LPS chemotype as substrate. Re LPS extracted from *E. coli* TXM333 lacks all sugars but Kdo (3-deoxy-α-d-manno-oct-2-ulosonic acid) from the saccharide core due to deletion of the d-sedoheptulose 7-phosphate isomerase gene *lpcA (gmhA)* as well as all types of PEtN/Ara4N modifications on lipid A due to deletions in the respective biosynthetic genes *eptA/arnA* (Fig. 1A). Re LPS was extensively purified as described above for wildtype, and analysis by MS confirmed a nearly homogeneous population of the Re chemotype (Fig. S1B and Table S2). In striking contrast to wildtype LPS, no phosphate was liberated from Re chemotype when incubated under identical conditions (Fig. 1C). This indicated inefficient cleavage of core lipid A phosphates by APs, and that the phosphate liberated from wildtype LPS must originate from either lipid A PEtN or saccharide core modifications attached distal to Kdo residues.

**Released phosphate originates from PEtN added by EptA and EptC.** PEtN groups are added in nonstoichiometric amounts to LPS at three distinct positions by a set of related membrane-bound transferases: i.) EptA onto either lipid A phosphate (41), ii.) EptB onto KdoII (42), or iii.) EptC onto phosphorylated HepI (l-glycero-d-manno-heptose) (43). We first constructed a panel of deletions in *eptA*, since this transferase can add PEtN to both phosphates of lipid A (Fig. 1A). Indeed, the amount of phosphate released was closely correlated with the presence of EptA, and plasmid-borne EptA alone restored phosphate release when testing Re LPS chemotype as substrate (Fig. 2A). Deletion of *arnA*, which modifies lipid A phosphate groups with Ara4N (44), alone or in tandem with *eptA* had minimal influence on phosphate release.

While phosphate installed by EptA accounted for the bulk of the total released, significant amounts of phosphate (up to ~30% of the total) were still liberated from LPS chemotypes isolated from Δ*eptA* strain backgrounds. This suggests some of the detected phosphate originated from the saccharide core as well. We thus tested phosphate liberation from LPS chemotypes produced by strains harboring various combinations of *eptA/B/C* (Fig. 2B). All strains were Δ*arnA* to limit any variability arising from substrate competition by ArnA with EptA. Since the native promoters of each of the PEtN transferases is subject to complex regulation, constructs with constitutive promoters were used to achieve comparable high-level PEtN modification levels as assessed by MS (Fig. S2). Next to Δ*eptA*, the least amount of phosphate release was detected from LPS extracted from strains lacking EptC and WaaP, the HepI kinase that forms the P-Hepl acceptor substrate utilized by EptC (45). Overexpression of EptC, but not EptB, enhanced phosphate release although the amount remained well below EptA. The data collectively suggests EptA installs most of the total labile phosphate pool in *E. coli* wildtype LPS, with EptC making a minor contribution. The phosphate content added by EptB is stable to hydrolysis.

**PEtN release from LPS is spontaneous.** The PEtN groups attached by EptA and EptC are both connected by a phosphoanhydride bond, while EptB installs a typical phosphodiester bond at KdoII (Fig. 1A, inset). The data thus supported one of three scenarios, wherein either: i.) cIAP specifically recognizes PEtN residues attached by EptA/EptC, ii.) cIAP only cleaves phosphoanhydride linked PEtN groups, or iii.) PEtN connected through a phosphoanhydride bond spontaneously hydrolyzes to free O-PEtN monoester that then becomes the actual substrate for cIAP. We initially suspected the third scenario, given the known instability of high-energy phosphoanhydride bonds and because the majority of characterized AP substrates are phosphomonoesters (23,46). To test for non-
enzymatic hydrolysis, Re LPS modified with phosphoanhydride linked PEtN added by EptA (from TXM343) was incubated with or without cIAP, extracted, and analyzed by MS for doubly modified, singly modified, and unmodified Re LPS (Fig. 3). Reactions were performed within dialysis tubing and continuously dialyzed to prevent any potential product inhibition by liberated inorganic phosphate. PEtN hydrolysis was monitored by a mass shift of Δm = 123 u, which corresponds to a single PEtN residue. While the total PEtN content clearly decreased after incubation (buffer pH=7.1, 16 hours) when compared to directly injected samples, the MS profile of mock-treated Re LPS was nearly indistinguishable from cIAP-treated samples (Fig. 3, middle and bottom panels).

To confirm spontaneous (i.e. non-enzymatic) hydrolysis, we examined a second set of PEtN-modified chemotypes with a more homogenous composition since quantitative comparison of Re LPS MS peaks is complicated by multiple glycoforms within the same population. We previously had constructed a mutant E. coli strain that elaborates only lipid IVₐ, a chemotype lacking glycosylation with uniform 3-OH-C₁₄:₀ tetra-acylation, that remains viable due to suppressor mutations in LPS transport systems (47,48). By introducing pEptA into this genetic background, we obtained PEtN-modified lipid IVₐ substrate preparations with 2-PEtN, 1-PEtN, or unmodified lipid IVₐ (Fig. 4A). When assayed for phosphate release, a nonlinear correlation between the amount of released inorganic phosphate and the units of cIAP added was observed well before the total amount of input PEtN-linked lipid IVₐ phosphate should become rate limiting (Fig. 4B). This is consistent with an initial slower, non-enzymatic hydrolytic step preceding cIAP catalysis, namely the putative dephosphorylation of free PEtN. To support this theory, the O-PEtN monoester was directly tested as a substrate for cIAP and hALPI (Fig. S3A). Typical Michaelis-Menten kinetics (Kₘ of 173 ± 27 μM and Vₘₐₓ of 1.09± 0.05 μM/min for cIAP and Kₘ of 197±56 μM and Vₘₐₓ of 1.05±0.1 μM/min for hALPI) were observed for both enzymes with O-PEtN as substrate. Using the crystal structure of the highly homologous rat IAP ortholog (~70% identity with cIAP across 486 non-gapped residues) as a model (49), O-PEtN can be readily accommodated within the substrate binding pocket in the active site (Fig. S3B). This is in contrast to modeling of acylated lipid A as the putative IAP substrate (see below).

**Hydrolysis of phosphoanhydride-linked PEtN from LPS is pH dependent.** We next tested the stability of PEtN linkages attached by EptA on lipid IVₐ by incubation in buffer at defined pH (Fig. 4C). Liberated PEtN was indirectly quantified by adding excess cIAP at the end of the incubation period and measuring inorganic phosphate levels using the malachite green assay. The extent of phosphate released increased as the pH of the pre-incubation buffer was raised, consistent with a base-labile phosphoanhydride bond. Hydrolysis was minimal at acidic pH. Interestingly, a mildly acidic environment naturally induces eptA expression and lipid A modification with PEtN in E. coli and Salmonella enterica (50,51). We repeated the incubation a second time, except the resulting lipid IVₐ population was extracted and separated by TLC before being visualized by sulfuric acid charring (Fig. 4D). Unmodified lipid IVₐ was stable across the entire pH range tested, whereas the dually modified 2-PEtN lipid IVₐ population disappeared with a concomitant increase in free lipid IVₐ as hydrolysis incubation conditions became more basic. Considering no cIAP was utilized, this data in combination with the qualitative MS results using PEtN-modified Re LPS (Fig. 3) supports a two-step mechanism whereby cIAP catalyzes phosphate release from spontaneously hydrolyzed O-PEtN that had initially been bound to LPS in a labile phosphoanhydride linkage.

**cIAP directly releases inorganic phosphate from de-O-acylated lipid A chemotypes.** The recalcitrance of all LPS chemotypes thus far tested to being directly dephosphorylated by cIAP suggested that steric interference may prevent the AP active site from engaging target lipid A phosphate monoesters. To test this hypothesis, we removed all ester linked acyl chains from lipid IVₐ and Re LPS to generate di-N-acetyl de-O-acyl lipid A derivatives (Fig. 5). De-
O-acyl lipid A (the N,N-diacylated) only contains amide linked 3-OH-C14:0 acyl chains at C2 of GlcNI and C2’ of GlcNII. If steric hindrance is indeed problematic, this should facilitate enzyme access to the phosphate groups, particularly at C4’ of GlcNII which is now adjacent to a free hydroxyl group in comparison to the steric bulk of a 3-OH-C14:0 acyl chain in lipid IV_A. Furthermore, decreasing the acyl chain density on the lipid A backbone substrate also increases the conformational flexibility. Unlike the tetra-acylated lipid IV_A parent, de-O-acyl lipid A was rapidly dephosphorylated in an initial phase that was followed by an extended period of slow phosphate release (Fig. 5A). De-O-acylated Re LPS, which has two Kdo residues attached to C6’ of GlcNII (Fig. S4A, inset), was likewise dephosphorylated in a biphasic manner albeit at a slower overall rate (Fig. 5A). MS analysis of cIAP treated products revealed the entire population had lost at least one phosphate group from both de-O-acyl lipid A and Re LPS substrates (Fig. 5B and Fig. S4A). NMR analysis of the residual phosphate remaining after treatment of de-O-acyl lipid A with cIAP indicated the majority (~77%) of the anomeric GlcNI phosphate group was retained under these conditions (Fig. S4B). In sum, the data is consistent with quantitative hydrolysis of a highly cIAP susceptible phosphate group on GlcNII followed by a slower second dephosphorylation event on GlcNI that remains incomplete even after a 48-hour reaction period.

We subsequently generated a model of the cIAP active site using the crystal structure of the highly similar rat IAP ortholog (~70% identity with cIAP across 486 non-gapped residues) as a structural template (49). Whereas de-O-acyl lipid A could be accommodated within the active site, lipid IV_A could not be docked successfully (Fig. 5C). Detrimental van der Waals contacts arise between the protein surface with the two O-acyl side chains at all times when the GlcNII phosphate was positioned to occupy the active site. In particular, the 3’-O-acyl chain on GlcNII clashed with amino acid chains flanking the active site. During simulations, the N-acylated side chains, however, adopted conformations that could avoid steric clashes when docked with the cIAP susceptible de-O-acyl lipid A GlcNII phosphate orientated in the active site. Likewise, docking of the anomic C1-GlcNI phosphate lipid IV_A into the catalytic cleft resulted in multiple steric interferences (Fig. S5). The computed binding models are consistent with the non-anomeric 4’-phosphate of de-O-acyl lipid A being the preferred position for cIAP-mediated dephosphorylation.

**PETN modification enhances hTLR4 agonist activity of underacylated LPS chemotypes.** Our data indicate a mechanistic model whereby PETN spontaneously hydrolyzes from phosphaoylhydride linkages on LPS to generate free O-PEtN, a monoester substrate that is processed by cIAP to liberate inorganic phosphate. This interpretation would explain the apparent dependence of cIAP for detection of free inorganic phosphate release from LPS under in vitro reaction conditions since the malachite green assay does not detect organic phosphate as in O-PEtN. Yet that alone does not account for the observed decrease in TLR4/MD2 activity after in vitro cIAP treatment considering the critical role lipid A phosphates at C1-GlcNI and C4’-GlcNII play during binding to TLR4/MD2 (52,53). Our data indicates these key phosphate groups remain intact after exposure to cIAP unless primary O-ester acyl chains on lipid A have first been removed. Transforming lipid A into a good cIAP substrate through prior de-O-acylation would itself abrogate TLR4/MD2 activity, which argues against de-O-acyl glycoform dephosphorylation being relevant to endotoxin neutralization.

Previous studies have, however, demonstrated PEtN modifications of lipid A in *Neisseria meningitidis* (54-57) and *Campylobacter jejuni* (58) increase TLR4/MD2 signaling. Spontaneous hydrolysis of PEtN, which can only be detected by inorganic phosphate assays with added AP, could account for the apparent decrease in biological activity. We therefore determined whether PEtN modification of *E. coli* lipid A, which has an asymmetric lipid A acyl chain distribution unlike in *N. meningitidis*, affects TLR4/MD2 recognition in a similar fashion. We initially
constructed a strain panel that synthesized lipid A glycoforms varying in acylation state and either with or without EptA-appended PEtN modifications (Fig. 6A). As expected, comparable amounts of inorganic phosphate were only detected in the presence of cIAP with LPS substrates that had been isolated from parent strains expressing EptA (Fig. 6B). We next directly compared TLR4/MD2 stimulation using a HEK293/hTLR4/MD2-CD14 whole cell NF-κB reporter assay (Fig. 6C). We utilized a luciferase based reporter assay instead of the secreted embryonic AP (SEAP) HEK-Blue™ colorimetric reporter system, since there are reports that the placental AP (PLAP) isoenzyme dephosphorylates LPS (29). While PEtN addition to hexa-acylated lipid A had minimal impact, PEtN modifications of the penta- and tetra-acylated lipid A glycoforms containing a full saccharide core enhanced TLR4 signaling by ~10-fold. Tetra-acylated lipid IV_A glycoform did not stimulate hTLR4/MD2, consistent with lipid IV_A being a known human TLR4 antagonist (59-62). Surprisingly, PEtN addition by EptA to lipid IV_A imparted low but definite agonist activity (Fig. 6C). Restoration of hTLR4/MD2 activity by PEtN modification of lipid IV_A was suppressed by pre-incubation in buffer in a pH-dependent manner (Fig. S6), as expected considering the labile nature of the lipid IV_A-PEtN phosphoanhydride bond with increasing pH (Fig. 4C and 4D). Collectively this suggests that PEtN addition to lipid IV_A can convert an LPS-like hTLR4 antagonist into a weak agonist. The contribution of PEtN to hTLR4 activity is more determinant with sub-optimal, underacylated E. coli LPS ligands, and agrees with observations made using the N. meningitidis lipid A scaffold (55).

Since key amino acid differences at the TLR4/MD2/LPS interface endow species specific lipid IV_A responses (63), we repeated the assay using the same panel of LPS chemotypes but with mouse TLR4/MD2 expressing NF-κB reporter cells (Fig. 6D). The pattern observed with hTLR4/MD2 was not replicated with the murine receptor complex, as PEtN attachment had minimal affect on signaling for any of the tested lipid A acylation states. This demonstrates that the enhanced signaling observed with hTLR4/MD2 is not an inherent biophysical property of PEtN modified lipid A, but rather due to species-specific ligand recognition and engagement features unique to the respective TLR4/MD2 receptor complexes. **PEtN substitution of both lipid IV_A phosphates is required for maximum hTLR4 agonism.** EptA can covalently add PEtN groups via phosphoanhydride bonds to either of the two lipid A phosphate groups, at C1 of GlcNII or C4' of GlcNII. Hence we sought to determine whether both PEtN groups (2-PEtN) are required or if a single PEtN moiety (1-PEtN) is sufficient to trigger hTLR4/MD2 activity. To accomplish this, we utilized an E. coli B lipid IV_A PEtN producing strain since this genetic background elaborates higher levels of PEtN modified lipid A in comparison to the K-12 strain used in the prior experiments (40). We next developed a lipid IV_A purification protocol to remove any contaminating lipoproteins and phospholipids, as well as to isolate 1-PEtN and 2-PEtN lipid IV_A species to allow for more quantitative comparisons between glycoforms. Established purification methods used prior for LPS chemotypes containing at least part of the saccharide core failed when applied to PEtN-lipid IV_A material extracted using the PCP method (see Experimental Procedures). We thus optimized a pair of nonionic detergent aided lipase pre-treatment steps to remove phospholipids and deacylate interfering lipoproteins, which improved the ensuing chromatographic separation of PEtN-lipid IV_A species in the following step (Fig. S7). Using an adapted anion exchange chromatography protocol developed by Raetz and coworkers to separate Ara4N and PEtN modified chemotypes (64,65), we were able to isolate 2-PEtN and 1-PEtN lipid IV_A species in high purity. A final purification step using HPLC reverse phase chromatography yielded homogeneous 2- and 1-PEtN lipid IV_A samples that were essentially free of contaminating lipoproteins and chemically pure with respect to PEtN content as judged by MS analysis and TLR2 activation (Fig. 7A and 7B). In addition, 31P NMR analysis confirmed PEtN substitution solely at C4' of GlcNII in the purified 1-PEtN fraction (Fig. S8). When comparing purified fractions, the 2-PEtN...
fraction alone accounted for the bulk of the recovered hTLR4/MD2 stimulating activity (Fig. 7C). PEtN substitution at C1 of GlcNI on the lipid IV<sub>A</sub> scaffold (with four symmetrically distributed acyl chains) therefore constitutes a critical determinant of restoring agonistic character.

**Molecular modeling suggests hTLR4/MD2 residues responsible for species specific enhanced recognition of PEtN-modified lipid A.** Signaling assays with TLR4/MD2 reporter cells unveiled that while lipid IV<sub>A</sub> is endotoxically inactive in human receptor complexes as expected, PEtN addition by EptA restores detectable activity (Figs. 6C and 7C). The contribution of PEtN to enhancing activity was more pronounced as the lipid A ligand became increasingly underacylated (Fig. 6). This trend, however, was not observed in murine receptor (mTLR4/MD2) reporter cells. MD2 is highly conserved between species, except for a few key residues (Fig. 8) (63). In the human receptor complex, a non-conserved cationic residue (hLys122 vs. anionic mGlu122) on the rim of MD2 interacts with the negative charge of lipid A phosphate anions and causes the ligand to be buried more deeply within the MD2 cavity in an antagonistic pose. In contrast, mGlu122 forces the ligand’s phosphate groups to move away by charge repulsion into an agonist pose that is well positioned for interactions with other subunits within the complex. Given the constant space in the MD2 binding cleft, underacylated lipid A congeners become more deeply buried than fully acylated ligands in hMD2 until eventually all agonist character is lost as with tetra-acylated lipid IV<sub>A</sub>. Our binding model suggest that as a direct consequence of PEtN substitution, ligand is sufficiently exposed to form contacts with the second TLR4* subunit, triggering dimerization between the [TLR4/MD2] and [TLR4*/MD2*] ectodomains and initiating downstream signaling (Fig. 8). The influence of PEtN groups is thus most evident when needed, i.e. for binding underacylated lipid A ligands in the hTLR4/MD2 complex. This effect is more muted in mTLR4/MD2 since mGlu122 makes the binding contribution of PEtN substitution redundant as mGlu122 already serves a similar function. In addition, there is more potential for favorable electrostatic interaction points between hTLR4 residues (e.g. hAsp294 and hGlu369) with the amino groups of PEtN (Fig. 8B), helping to bridge the space between TLR4 and TLR4*. In contrast, the murine receptor complex with mLys367 in place of hGlu369 is less favorable due to positive charge repulsion.

**DISCUSSION**

The anti-inflammatory properties of IAP and APs in general have generated broad interest as potential therapeutics for treating a number of systemic and acute inflammatory conditions. Dissecting and isolating the relevant phosphorylated molecular targets involved, however, is a complicated endeavor due to the broad substrate specificity of IAP. IAP can dephosphorylate both bacterial derived MAMP/PAMPs as well as pro-inflammatory endogenous damage-associated molecular patterns (DAMPs) of host cell origin produced in response to LPS-mediated TLR4/MD2 inflammation. Along with LPS, flagellin, CpG DNA motifs and extracellular nucleotides have all been proposed as relevant IAP dephosphorylation targets (28). Flagellin and CpG motifs in bacterial DNA are MAMP/PAMP TLR ligands like LPS (66), while extracellular ATP nucleotide binds to purinergic receptors (67) and UDP to the P<sub>2</sub>Y<sub>6</sub> pyrimidinergic receptor (68). The anti-inflammatory mechanism of IAP is further confounded by the difficulty in separating IAP specific effects (i.e. the direct dephosphorylation of MAMP/PAMP/DAMP stimuli) from nonspecific ones resulting from general downregulation of the inflammatory tone (17).

LPS has emerged as an often cited and most likely MAMP/PAMP-related molecular target for AP, due to its potent inflammatory potential when present at low concentrations. The conclusion that IAP dephosphorylates LPS in vitro predominantly rests on the observation that LPS-derived inorganic phosphate is only detectable if AP is added when using either the molybdate complex or tissue histology based assays as readouts. The corresponding LPS preparations treated with AP have diminished capacity to induce inflammation through
TLR4/MD2 stimulation, as would be expected for hydrolysis of the lipid A backbone phosphate groups since these groups are central in TLR4/MD2 recognition. Direct evidence pertaining to which phosphate groups are indeed removed by AP is lacking, however. LPS preparations can also have multiple phosphate groups on the inner saccharide core in addition to those on lipid A. In E. coli for instance, WaaP and WaaY heptose kinases phosphorylate HepI and HepII, respectively (45), while KdkA kinase modifies Kdo in bacteria with phosphorylated mono-Kdo residues such as Haemophilus influenzae (69). We thus sought to determine the origin of the inorganic phosphate released by AP. While our results are consistent with inorganic phosphate release being contingent on AP activity for structurally heterogeneous LPS preparations, we found defined substrates such as Re LPS chemotype that only have lipid A phosphate monooester groups to be completely inert to dephosphorylation (Fig. 1B and 1C). In previous studies where defined LPS chemotypes were tested for AP activity, a decrease in phosphate release has also been noted. Tuin et al. recorded lower phosphate release when histology scoring rat liver sections challenged with Re LPS from S. enterica sv. Minnesota (70), while Pettengill et al. observed low phosphate release from S. enterica sv. Minnesota Re LPS and no measurable phosphate release from monophosphoryl lipid A (MPLA) substrate using recombinant TNAP (31). Re LPS lacks the Hep acceptor for the inner core PETN transferase EptC, which we have now shown is an apparent source of cIAP-released phosphate along with the lipid A PETN transferase EptA (Fig. 2). PETN groups are common in many Gram-negative bacteria where they are added to both lipid A and the LPS inner saccharide core to increase the net positive charge (71). The increased positive charge imparts resistance to cationic peptides and enhances outer membrane integrity, particularly under more challenging growth environments. Both EptC and EptA enzymes add PETN in a phosphoanhydride linkage using phosphatidylethanolamine phospholipid head groups as PETN donors, forming a rather unique bond type in the process considering the extracellular location (Fig. 1A, inset). While other bacterial surface phosphoanhydride bonds such as in bactoprenol glycosyl donors do exist, these are generally fleeting intermediates whose high energy character is used to drive the polymerization of peptidoglycan, O-antigen, and other cell surface polymer pathways as intracellular ATP is not available. Considering the inherent reactivity of phosphoanhydride bonds, we suspected spontaneous hydrolysis might play a role since phosphate was only released from LPS substrate containing phosphoanhydride linked PETN groups and not from phosphodiester linked PETN groups appended to Kdo by EptB (Figs. 1, 2, and 6B). Indeed, phosphodiester-linked PETN remained stable across a wide pH range, whereas PETN connected by phosphoanhydride bonds became labile in neutral to basic pH and was only stable under mildly acidic conditions (Fig. 4C and 4D). Intriguingly, acidic environmental conditions induce EptA (72), suggesting that phosphoanhydride linked PETN may be hydrolytically susceptible by design so as to transiently tailor the LPS layer provided acidic conditions persist. Certain bacteria do not add phosphoanhydride linked PETN to lipid A, but rather hydrolyze lipid A phosphate first before transferring PETN to form phosphodiester bound PETN (73,74). Presumably such PETN phosphodiesterases are more stable, as observed here for phosphodiester-bound Ara4N-modified lipid A and PETN attached by EptB (Fig. 2). Apparently, cIAP is unable to directly release PETN or phosphate from LPS, but does dephosphorylate spontaneously hydrolyzed PETN phosphomonoesters (Figs. 3 and 4). Free O-PETN is a characterized substrate for PLAP (75), and experiments here confirm it is a good substrate for the cIAP isozyme as well (Fig. S3).

In this study, we used commercially purified cIAP as AP enzyme source. In the intestinal lumen, IAP is enriched within lumenal vesicles secreted by enterocytes (76), a native environment which could enhance IAP catalytic activity towards LPS. When the apparent phosphate released from LPS by lumenal vesicles and purified IAP was directly
compared, however, activities were comparable (77). A second consideration for any in vitro based LPS assays is substrate presentation. Highly aggregated LPS may not be bioavailable to IAP without accessory proteins to expose the lipid A phosphate groups. In serum, lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14), and albumin all have integral roles in disaggregating LPS and enhancing presentation to the TLR4/MD2 receptor complex (78,79). Likewise, the activity of the LPS acyloxyacyl hydrolase, which cleaves secondary acyl chains on lipid A (80,81), is stimulated up to 100-fold when substrate is presented by either sCD14 or LBP (82). While we tested native preparations of AP enzymes extracted from tissue to capture relevant post-translational modifications (Fig. 1), glycosylation can influence activity in certain AP isozymes (83). It should be noted that even with tetra-acylated lipid IV\textsubscript{A}, which lacks secondary acyl chains and is more polar than hexa-acylated lipid A, there was no appreciable phosphate released by cIAP (Fig. 6). If LPS is not a relevant IAP substrate, then the bulk of the anti-inflammatory effects observed with AP would seem more likely to arise from dephosphorylation of ATP and UDP rather than through detoxification of LPS (18,19,84). IAP has been reported to shape the microbiota composition in a mouse IAP A\textsubscript{kp}-3 knockout model (85) and to directly inhibit E. coli growth in culture (86), so that an indirect role of IAP in modulating the composition of the endotoxin load present in the intestinal lumen need also be considered.

Although the exact structure of LPS and lipid IV\textsubscript{A} was unknown at the time, initial studies investigating AP activity also concluded that LPS was completely resistant to bacterial AP (87-89). However, ~50% of the total phosphate could be released from "lipid A precursor" (i.e. later named lipid IV\textsubscript{A}) if first de-O-acylated by treatment under mild alkaline conditions (88,89). The authors concluded that the C4'-GlcNII phosphate is recognized by AP, and that the C1-GlcNII anemonic phosphate remains intact. The resistance of C1 to hydrolysis was attributed to steric hindrance by the C2 amide linked 3-OH-C14:0 acyl chain, which unlike the 3-OH-C14:0 ester acyl chain neighboring the C4' phosphate of GlcNII, remains intact after base treatment. It was also suggested that de-O-acylation may decrease the aggregation state, facilitating enzyme access to substrate. Since these studies focused on an AP enzyme of bacterial origin with low sequence similarity, we replicated their study using cIAP with highly purified lipid IV\textsubscript{A} preparations (Fig. 5). As reported for bacterial AP, cIAP rapidly dephosphorylates de-O-acylated lipid A in comparison to tetra-acylated lipid IV\textsubscript{A}. Bacterial phosphate monoesterases (EC 3.1.3.1) such as E. coli alkaline phosphatase (EcAP) resemble mammalian IAP in catalytic site topology (catalytic residues, trimetallo core, Zn\textsuperscript{2+} and crown-like flap) (90,91). Bacterial and mammalian mono-phosphoesterases with a trimetallo core (here: EcAP and rat IAP) share highly conserved amino acids for Zn\textsuperscript{2+} and Mg\textsuperscript{2+} complexation (49,90). Computational docking of lipid IV\textsubscript{A} ligand into the active site of mammalian IAP (Fig. 5C) as well as bacterial AP (Fig. S9A) revealed steric hindrance by neighboring 3'-OH C14:0 acyl chains that prevent positioning of the C4'-phosphate of lipid A at the active site. Likewise, the approach of the anemonic phosphate appears hindered by the amide acyl chain at C2 of GlcNII (Fig. S5 and Fig. S9B for mammalian and bacterial AP, respectively), in keeping with our experimental data for cIAP (Fig. 5) and data using bacterial AP (88,89). The anemonic phosphate can be removed after de-O-acylation, as a small but completely dephosphorylated population is clearly observed by MS and NMR (Fig. 5 and Fig. S4). The increased conformational flexibility of the remaining N-acyl chains after de-O-acylation likely accounts for the appearance of weak phosphatase activity at C1-GlcNII. The presence of Kdo residues in de-O-acyl Re LPS substrate slowed but did not prevent dephosphorylation (Fig. 5A), indicating prior hydrolysis of the saccharide core is not necessarily required for cIAP activity.

While de-O-acylated lipid A is rapidly dephosphorylated, the direct relevance in endotoxin detoxification is questionable given de-O-acylated lipid A is already a weak TLR4/MD2 agonist. However, many of the more
populous gut-associated bacteria (as from bacteria in the order Bacteroidales) remodel their lipid A to underacylated chemotypes (92,93). Underacylated chemotypes not only have lower intrinsic endotoxin activity, but also dampen TLR4/MD2 signaling and inflammation from more potent lipid A congeners with higher acylation states as found in Enterobacteriaceae through competition for TLR4/MD2 receptor. It is conceivable that endogenous underacylated chemotypes from gut associated bacteria may be selectively subject to further detoxification by IAP, or that IAP may play an indirect role in lowering the endotoxin burden by processing the underacylated antagonistic lipid A population pool.

The influence of lipid A acylation state on attenuating hTLR4/MD2 activity has long been recognized. However, the importance of PEtN modification on lipid A is just beginning to be appreciated. Indeed, the structure-hTLR4/MD2 activity relationship of EptA modified lipid A is nearly as equipotent as the removal/addition of secondary acyl chains (Fig. 6C). The PEtN effect is not unique to E. coli, as the potency of other lipid A scaffolds in N. meningitidis (54-57) and C. jejuni (58) is likewise enhanced by PEtN. The influence of PEtN-modified lipid A on TLR4/MD2 engagement demonstrates marked species dependent effects, as the degree of mTLR4/MD2 stimulation was agnostic to the presence of EptA in the producing strain across all the tested lipid A acylation states (Fig. 6D). Of note, EptA expression in a lipid IV<sub>A</sub> producing strain restored some hTLR4/MD2 agonist character (Fig. 7). This raises the possibility that detoxification by acyloxyacyl hydrolase through removal of secondary acyl chains may be less effective when PEtN modified LPS is abundant. Our computational binding model suggests this is due to key residues specific to hTLR4/MD2 (Fig. 8). The weak activity of 2-PEtN lipid IV<sub>A</sub> is particularly interesting, considering this is a lipid A-based hTLR4/MD2 agonist with inherently self-limiting biological activity as the phosphoanhydride linked PEtN groups are unstable at physiological pH (Fig. 4C and 4D). These properties suggest a potential design for engineering safe next generation adjuvants. Utilizing lipid A analogs agonists to bolster immune responses to synthetic vaccines antigens without inducing toxicity is a major challenge (55,94-96). Chemically unstable 2-PEtN lipid IV<sub>A</sub> type analogs will spontaneously convert over time from an agonist to antagonist under physiological conditions, offering an intriguing adjuvant strategy that warrants further investigation.

We cannot definitively rule out a direct LPS dephosphorylating activity for select AP-LPS chemotype pairs, as the E. coli LPS chemotypes-AP pairs studied here is a far from exhaustive list. The complement of respective intestinal AP isoforms varies widely among vertebrates, so that biological roles may be species specific. It has been proposed that the intestinal AP dephosphorylation activities have co-evolved to complement the substrates being produced by the particular resident microbiota (97). If hexa-acylated LPS is a bona fide substrate however, there is clearly an integral missing component from the reconstituted in vitro system. Regardless, the labile nature of phosphoanhydride linked PEtN modifications on LPS and the ensuing diminished TLR4/MD2 signaling precludes using phosphate release and decreased endotoxin activity as sole determinants of AP-mediated LPS detoxification. It also emphasizes the advantages of using chemically defined LPS chemotypes, given that the PEtN content will not only depend on the particular source strain, but will also vary according to both the growth media and the extraction and purification methods. Defined chemotypes will allow more meaningful comparisons between studies and ultimately a better understanding of the role of AP in ameliorating LPS-induced inflammation.

**EXPERIMENTAL PROCEDURES**

**Reagents.** Calf intestinal alkaline phosphatase (cIAP) was purchased from New England BioLabs. Human placenta (PLAP) and human liver alkaline phosphatases (TNAP) were ordered from Lee Biosolutions Inc., while porcine kidney alkaline phosphatase was from Sigma. Human intestinal alkaline phosphatase (ALPI) was obtained from Sino Biological. All
Chemicals were purchased from Sigma Millipore unless noted otherwise.

**Bacterial strain construction.** Gene deletions were introduced into *E. coli* strains using the λ-Red recombinase system as described (98). Targeting cassettes were obtained by PCR amplification using P1-P2 primer pairs (Table S1). Each primer contained a 5′ 42-bp homology extension arm and a 3′ 18-bp sequence specific for the indicated antibiotic selection marker of the plasmid template. For the *arnA::kanR* and *lpxM::kanR* cassettes, genomic DNA was purified from the Coli Genetic Stock Center strains CGSC#9813 and #9540 and used as a template to flank the antibiotic cassette with FRT sites for subsequent marker excision using the FLP recombinase plasmid pCP20 (99). Integration cassettes were purified and electroporated into recipient strains harboring the arabinose-inducible λ-Red recombinase plasmid pKD46 (98). Plasmids were cured by passaging at 37°C, colonies were checked for loss of plasmid, and cassette insertion was confirmed using check primers (Table S1). For construction of tetracylated LPS strains containing a complete saccharide core (GKM499 and GKM502), plasmid pMMW52-msbA was first introduced to enhance LPS transport and improve fitness. In this background, deletion cassettes were then introduced by generalized transduction using P1vir. Strain genotypes are listed in Table 1.

Plasmids expressing either EptA, EptB, or EptC were constructed using the Infusion Cloning kit (Clontech). PCR primer pairs (Table S1) were used to amplify inserts from *E. coli* BL21DE3 genomic DNA and then cloned into the vector pSEVA434 (100) that had been digested with *EcoR*I/BamHI. Plasmids were maintained with spectinomycin (50 µg/ml) and used without induction as basal expression was sufficient for phenotypic conversion for all three constructs.

**LPS purification.** Bacteria were harvested from stationary phase cultures grown at 37°C in either Lysogeny Broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) or TB media (39) (10 g tryptone, 5 g yeast extract, 3 g NH₄Cl, 12 g Na₂HPO₄, 6 g KH₂PO₄, 0.5 g Na₂SO₄, 7.5 g glucose per liter) supplemented with the necessary antibiotics for plasmid selection. Dried bacterial cell biomass was obtained by sequentially stirring in ethanol overnight and then two rounds of acetone (12 hours each) at 4°C, collecting biomass between incubations by centrifugation (6,000 x g, 10 min, 4°C). All LPS and lipid IVₐ chemotypes were initially extracted from cell powders via the phenol/chloroform/petroleum ether (PCP) method (101). Briefly, dried biomass was resuspended in PCP solution (90% phenol/chloroform/petroleum ether in 2:5:8 v/v/v ratio) and incubated for one hour on tube rotator. Biomass was pelleted and the supernatant collected, with the extraction being repeated twice. Pooled supernatant extract was rotavaped to remove chloroform and petroleum ether, and 100 µl of 3 M sodium acetate (pH 7.0) was added to the phenol phase. Here treatment of the samples varied depending on the chemotype. For LPS samples, precipitation was carried out via dropwise addition of water. Pelleted LPS was washed once with 80% phenol and then a second time with acetone. For lipid IVₐ samples, five volumes of acetone were added to precipitate lipid IVₐ from the phenol phase and then the pellet was washed once with acetone.

To remove co-extracting phospholipids from LPS, samples underwent a modified chloroform/methanol wash (102). Briefly, pellets were resuspended in chloroform/methanol/3 M sodium acetate (pH 7.0) (85:15:1, v/v/v) and 2 to 3 volumes of methanol were added to precipitate LPS. Phospholipid containing supernatant was decanted and this process was repeated twice. Removal of contaminating lipoprotein was achieved via phenol/sodium deoxycholate extraction as described by Hirschfeld et al. (103). Lipid IVₐ samples could not be efficiently recovered using either the chloroform/methanol wash or the phenol/DOC extraction, hence these steps were omitted.

All chemotypes underwent ultracentrifugation to remove any malachite green reactive nucleic acid material. LPS or lipid IVₐ pellets were resuspended in 3 ml of water and added to 30 ml of a Tris-saline buffer.
Inorganic phosphate release and dephosphorylated LPS product assay. LPS samples (100 µg/ml) were incubated in alkaline phosphatase buffer [50 mM Tris-HCl at the indicated pH (pH = 8.25, 7.4, or 7.1), 100 mM NaCl, 1 mM MgCl2, 20 µM ZnCl2] with either cIAP (4 U/ml), human liver phosphatase (0.1 U/ml), human placenta phosphatase (1 U/ml), human intestinal phosphatase (0.17 µg/ml) or porcine kidney phosphatase (1 U/ml) at 37 °C. Aliquots were taken at different time points and phosphate release was measured with the malachite green assay as described previously (33). Briefly, one volume of malachite green solution (0.1% malachite green, 14% sulfuric acid, 1.5% ammonium molybdate, and 0.18% Tween-20) was mixed with four volumes of LPS solution and the mixture was incubated at room temperature for 10 min. Absorbance was read at 630 nm on SpectraMax Plus 384 plate reader (Molecular Devices). Sodium phosphate was used for standard curve determination.

For direct assay of dephosphorylated LPS, 10-ml reactions were assembled as described above in dialysis tubing (0.5-1 kDa MWCO). Reactions were continuously dialyzed against 500 ml of buffer to remove any released inorganic phosphate. LPS products were isolated by extensive dialysis against water and lyophilized before analysis by mass spectrometry as detailed below.

Buffer composition for assays conducted at varying pH values was 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS)-50 mM Tris adjusted to either pH 6.5, 7.4, or 8.5 along with 100 mM NaCl, 1 mM MgCl2 and 20 µM ZnCl2. In the experiments where 10 units of cIAP was added after pre-incubation in the buffer alone, samples were incubated for 30 min at 37°C to release inorganic phosphate from spontaneously hydrolyzed PETN.

**TLR4 stimulation assay.** HEK293/hTLR4-MD2-CD14, HEK293/mTLR4-MD2-CD14, and parental HEK293/Null2 control cells were grown as specified by the supplier (InvivoGen). For the stimulation assay, the cells were plated at 50,000 cells per well in a white 96 well plate with clear bottom (Costar™ 3610, Corning Incorporated) in 200 µl of growth medium (DMEM, 2 mM L-glutamine, 10% heat inactivated fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin™). The pNiFty-Luc plasmid (InvivoGen) encoding five NF-κB repeated transcription factor binding sites in front of the luciferase reporter gene was mixed with transfection reagent LyoVec™ (InvivoGen) at a concentration of 1 µg of plasmid per 100 µl of LyoVec™, and after incubation at room temperature for 20 min, the mixture (10 µl per well) was added to cells in a 96 well microplate. The next day the medium was removed and replaced with 180 µl of fresh growth medium. Various LPS chemotypes were added at different concentrations in a 20-µl volume per well. Endotoxin-free water was used as a negative control and TNF-α (200 ng/well) was used as a positive control. Pierce™ Firefly Luciferase One-Step Glow Assay Kit was used according to manufacturer’s instructions with luminescence being measured after 20 hours of stimulation. All LPS and lipid IVα preparations were confirmed to be negative for NF-κB induction when challenging HEK293/Null2 control cells (InvivoGen) up to the highest tested LPS concentration (100 ng/ml, data not shown).

**TLR2 stimulation assay.** HEK-Blue™ hTLR2 cells (InvivoGen) were propagated as specified by the supplier. For the stimulation assay, the cells were plated at 25,000 cells per well in a 96 well plate in 180 µl of growth medium (DMEM, 2mM L-glutamine, 10% heat inactivated fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin™). LPS was added at different concentrations in a 20 µl per well volume. Endotoxin-free water was used as a negative control. QUANTI Blue™ (InvivoGen) reagent was used, according to manufacturer’s
instructions, 20 hours later to detect NF-κB-dependent secreted embryonic alkaline phosphatase (SEAP) activity. Absorbance at 620 nm was read following incubation of the samples with QUANTI Blue™ substrate for 3 hours at 37°C.

**Lipase treatment of lipid IVₐ extracts.** Crude lipid IVₐ (with or without PEtN) was treated with lipase via two sequential incubations. Both 12-hour reactions were conducted at 45 °C in a 20 mM phosphate buffer (pH 7.0). Each reaction contained 40 mg of crude PCP extracted lipid IVₐ, *Thermomyces* Lipase (TL, Sigma), and Novozyme® 51032 (Strem Chemicals, Newburyport, MA) at respective final concentrations of 0.1 mg/ml, 90 µg/ml, and 25 µg/ml. The initial reaction included 3.4 mM BIG CHAP (SolTec Bio Science, Beverly, MA) as a nonionic detergent additive. Immediately following this reaction, lipid IVₐ was recovered by conversion to a 2:2:1:8 (v/v/v) chloroform/methanol/water Bligh-Dyer biphasic mixture. Lipid IVₐ was isolated from the lower organic phase by rotary evaporation, resuspended in endotoxin-free water, and then lyophilized. Recovered lipid IVₐ was treated again in a second lipase reaction with 20 mM octyl β-D-glucopyranoside as the detergent additive, and re-isolated as described above. PEtN-modified lipid IVₐ was stored at -20 °C until further use.

**Chromatographic purification of lipid IVₐ species.** Ion exchange chromatography was performed using a 5-ml HiTrap™ SP HP cation exchange column connected in tandem to a 20-ml HiPrep™ DEAE FF 16/10 anion exchange column with the AKTA™ Pure FPLC system. Lipase-treated PEtN lipid IVₐ prepared as described above was loaded in 15 ml of a 60% n-propanol solution adjusted to pH 5 with acetic acid. The system was then washed with 10 ml of the same buffer, after which the cation exchange column was removed. The anion exchange column was subsequently washed with 4.5 column volumes of 60% n-propanol (pH 5), before elution using a linear gradient of non-pH adjusted 0 to 80 mM ammonium acetate in 60% n-propanol over 20 column volumes. To identify fractions containing non-volatile organic compounds, 20 µl of each fraction was spotted onto an Analtech Silica gel G TLC plate and visualized by charring using a 10% sulfuric acid-ethanol solution with heating at 160 °C for 10 min. Fractions containing organic material were further analyzed by spotting 20 µl on an Analtech Silica gel H TLC plate, and developed using a pyridine/chloroform/formic acid/water (50:50:16:5, v/v/v/v) mobile phase before sulfuric acid charring (104). Fractions containing lipid IVₐ species were pooled, rotovaped to dryness, and subjected to two rounds of lyophilization to remove residual traces of ammonium acetate. The resulting powder was kept at -20 °C until further purification by reversed-phase high performance liquid chromatography (RP-HPLC). For this, lipid IVₐ samples were subjected to RP-HPLC essentially as described (105,106), but with some modifications. A semi-preparative Kromasil C18 column (5 µm, 100 Å, 10 × 250 mm, MZ Analysentechnik, GmbH, Mainz, Germany) was used and samples [resuspended at 5 mg/ml in chloroform/methanol/0.1 M acetic acid (8:2:1, v/v/v)] were eluted using a gradient consisting of methanol/chloroform/water (57:12:31, v/v/v) containing 10 mM ammonium acetate as mobile phase A and chloroform/methanol (70.2:29.8, v/v) with 50 mM ammonium acetate as mobile phase B. The initial solvent system consisted of 2% B and was maintained for 10 min, raised from 2 to 15% B (10–20 min), kept at 15% B for 20 min, raised from 15 to 25% B (40–50 min), kept at 25% B for 20 min, and raised from 25 to 100% B (70–100 min). The solvent was held at 100% B for 20 min, followed by re-equilibration of the column to 2% B for 10 min and held there for an additional 10 min prior to the next injection. The flow rate was 2 ml/min using a splitter between the evaporative light-scattering detector equipped with a low-flow nebulizer (Sedex model 75C ELSD, S.E.D.E.R.E., France). Nitrogen (purity 99.996%) was used as gas to nebulize the post column flow stream at 3.5 bar into the detector at 50 °C setting the photomultiplier gain to 11. The detector signal was transferred to the Gilson HPLC.
De-O-acylation and dephosphorylation assays of lipid IV_{A} and Re LPS with cIAP. Lipid IV_{A} was dissolved (1 to 4 mg/ml) in a 1 M NaOH aqueous solution and incubated for 20 hours at room temperature. Reactions were neutralized via addition of glacial acetic acid while stirring until pH 7.0. Neutralized reactions were extensively dialyzed against water (MWCO: 500-1000 Da) and lyophilized. Re LPS was likewise de-O-acylated, but was recovered by precipitation from neutralized solution using five volumes of ethanol. De-O-acylated products were further purified via anion exchange chromatography as described above. Fractions were pooled, concentrated by rotary evaporation, and dialyzed against water (100-500 Da MWCO) followed by lyophilization.

Mass spectrometry. LPS samples were measured on a 7-tesla APEX Qe Electrospray Ionization Fourier Transform Ion Cyclotron Resonance (ESI-FT-ICR) mass spectrometer (Bruker Daltonics). Measurements were performed in negative ion mode. Samples (approximately 0.03 mg/ml) were dissolved in a water/2-propanol/trimethylamine/acetic acid mixture (50:50:0.06:0.02, v/v/v/v). Spectra were acquired in broadband acquisition mode with nano-ESI using the Triversa Nanomate (Advion, Ithaca, NY) as ion source with a spray voltage set to −1.1 kV. For positive ion-mode, samples were dissolved in water/propan-2-ol/30 mM ammonium acetate/acetic acid mixture (15:15:1:0.04, v/v/v/v) with a spray voltage set to +1.1 kV. Both mass spectrometers were calibrated externally with glycolipids of known structure. All mass spectra were charge deconvoluted and given mass values refer to the monoisotopic masses of the neutral molecules, if not indicated otherwise.

NMR spectroscopy. NMR spectroscopic measurement of PEtN-lipid IV_{A} was performed in CDCl_{3}/MeOH-d_{4}/D_{2}O (60:35:8, v/v/v) and de-O-acyl Re LPS after treatment with cIAP in CDCl_{3}/MeOH-d_{4}/D_{2}O (2:3:1, v/v/v) (107), respectively, at 300 K on a Bruker AvanceIII 700 MHz (equipped with an inverse 5-mm quadruple-resonance Z-grad cryoprobe). Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). TMS was used as an external standard for calibration of 1H (δH 0.0) and 13C (δC 0.0) NMR spectra, and 85% of phosphoric acid was used as an external standard for calibration of 31P NMR spectra (δP 0.0). All data were acquired and processed by using Bruker's TOPSPIN V 3.0 software. 1H NMR assignments were confirmed by 2D 1H,1H COSY and total correlation spectroscopy (TOCSY) experiments. 13C NMR assignments were indicated by 2D 1H,13C HSQC, based on the 1H NMR assignments. Inter-residue connectivity and further evidence for 13C assignment were obtained from 2D 1H,13C heteronuclear multiple bond correlation and 1H,13C HSQC-TOCSY. Connectivity of phosphate groups were assigned by 2D 1H,31P HMOC and 1H,31P HMOC-TOCSY.

Molecular modeling. Standard modeling tools and protocols were conducted according to published protocols (53,63). Modeling software [Autodock 4.2 (108), Chimera 1.13.1 (109), SPDBV 4.10 (110), VEGA ZZ 3.1.2 (111)] was licensed for academic use to generate and visualize three-dimensional model structures of lipid IV_{A}, TLR4/MD2, and phosphatase enzymes, in addition to partial charges, electrostatic molecular surfaces or fitted active site conformers (108,109,112,113). The TLR4/MD2 docking protocol first presented by
Meng et al. (53) and later adapted (63) using the hTLR4/MD2-E. coli LPS crystal structure [3FXI, (114)] was utilized to model LPS-like congener binding. The large number (52) of freely rotatable bonds at the 4 side chains of lipid IV\(_A\) had to be reduced and were limited to the first three chain members including the four H-O bonds as well as the two ester bonds, while excluding the two more rigid \(\text{trans}\)-amide bonds. To visualize the steric hindrance associated to nonbinders (no valid docking/scoring solutions found) we superimposed them onto the final docked poses of those ligand types with favorable side chain patterns for successful cavity binding – here called the binders (Fig. 5C). Whereas for nonbinders any of the calculated conformations under docking conditions would show detrimental van der Waals contacts, binders provide (some not all) conformational solutions for successful binding in the micromolar range. Lipid IV\(_A\) (115) and all enzymes and receptors were retrieved from PDB repository server (116) with the exception of hitherto structurally unknown cIAP that was generated by homology modeling using described methodology (117). The cIAP target (GenBank entry code: AAA30571.1) shares more than 70\% identity with rat IAP across 486 non-gapped residues, including all active sites residues. Using the experimentally determined rat IAP crystal structures (4KJD, 4KJG) (49), a cIAP homodimer structure was generated under Swiss PDB Viewer (110). Multiple sequence alignments were carried out with built-in Clustal X under Vega ZZ (111). Of note, all phosphate groups were charged and modeled as monoanionic, e.g. bearing one -OH group. In some figures hydrogen atoms were not displayed for visual simplicity (118). All model figures were generated with Chimera (109).

**ACKNOWLEDGEMENTS**

We would like to thank Tania Laremore (Proteomics and Mass Spectrometry Core Facility, Penn State University) for help with experiments. We gratefully acknowledge Birte Buske, Dörte Grella, Manuel Hein, Heiko Käßner and Brigitte Kunz (all Research Center Borstel) for their excellent technical assistance.

**FOOTNOTES**

This work was supported by National Institutes of Health Grants NIAID R21 AI138152 (to TCM). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Supplementary Information (Table S1-S2 and Figures S1-S9).

\(^2\) The abbreviations used are:

- **hALPI** human intestinal alkaline phosphatase
- **AP** alkaline phosphatase
- **Ara4N** 4-amino-4-deoxy-L-arabinose
- **cIAP** calf intestinal alkaline phosphatase
- **DAMP** damage-associated molecular pattern
- **EcAP** *E. coli* alkaline phosphatase
- **Hep** \(\ell\)-glycerol-\(\alpha\)-manno-heptose
- **IAP** intestinal alkaline phosphatase
- **Kdo** 3-deoxy-\(\alpha\)-D-manno-oct-2-ulosonic acid
- **LA** lipid A
- **LBP** lipopolysaccharide binding protein
- **LPS** lipopolysaccharide
- **MAMP** microbe-associated molecular pattern
- **MD2** myeloid differentiation factor-2
- **ME** metabolic endotoxemia
- **MS** mass spectrometry
- **OM** outer membrane
- **PAMP** pathogen-associated molecular pattern
- **PCP** phenol/chloroform/petroleum ether
- **PETN** phosphoethanolamine
- **PLAP** placental/embryonic alkaline phosphatase
- **PRR** pattern recognition receptors
- **sCD14** soluble CD14
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### Table 1
Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant genotype or phenotype | Source or reference |
|-----------------------------|-------------------------------|---------------------|
| **E. coli strains**         |                               |                     |
| TXM319 E. coli strains      | Wildtype BL21 (DE3); E. coli B F− ompT hsdS6(rB− mB−) gal dcm lon λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) | Lab stock           |
| TXM322 BL21 (DE3) arnA::kanR; Kanr | | This study |
| GKM329 BL21 (DE3) eptA::catR; Catr | | This study |
| TXM331 BL21 (DE3) eptA::catR arnA::kanR; Catr Kanr | | This study |
| TXM333 BL21 (DE3) lpcA::gentR eptA::catR arnA::kanR; Gentr Catr Kanr | | This study |
| TXM343 BL21 (DE3) lpcA::gentR eptA::catR arnA::kanR [pSEVA434-eptA]; Gentr Catr Kanr Specr | | This study |
| GKM357 BL21 (DE3) eptA::catR arnA::kanR waaP::gentR; Catr Kanr Gentr | | This study |
| GKM358 BL21 (DE3) eptA::catR arnA::kanR waaP::gentR [pSEVA434-eptA]; Catr Kanr Gentr | | This study |
| GKM373 BL21 (DE3) eptA::catR arnA::kanR eptB::gentR; Catr Kanr Gentr | | This study |
| GKM374 BL21 (DE3) eptA::catR arnA::kanR eptC::gentR; Catr Kanr Gentr | | This study |
| GKM380 BL21 (DE3) eptA::catR arnA::kanR eptB::gentR [pSEVA434-eptC]; Catr Kanr Gentr Specr | | This study |
| GKM381 BL21 (DE3) eptA::catR arnA::kanR eptC::gentR [pSEVA434-eptB]; Catr Kanr Gentr Specr | | This study |
| TXM402 BL21 (DE3) eptA::catR arnA::kanR eptC::gentR [pSEVA434-eptA]; Catr Kanr Gentr Specr | | This study |
| TXM418 BL21 (DE3) eptA::catR arnA::FRT eptC::gentR lpxM::kanR; Catr Kanr | | This study |
| TXM419 BL21 (DE3) eptA::catR arnA::FRT eptC::gentR lpxM::kanR [pSEVA434-eptA]; Catr Gentr Kanr Specr | | This study |
| GKM445 ClearColi® K-12 F−, λ− ΔendA ΔrecA msbA52 frr181 ΔgutQΔksdsDΔlpxLΔlpxMΔlpxPΔeptA | Lucigen |
| GKM446 GKM445 (pSEVA434-eptA); Specr | | This study |
| GKM499 BL21 (DE3) eptA::catR arnA::FRT eptC::gentR lpxM::kanR lpxL::aprP pagP::hygR [pMMW52-msbA]; Catr Gentr Kanr Aprr Hygr Carbr | | This study |
| GKM502 BL21 (DE3) eptA::catR arnA::FRT eptC::gentR lpxM::kanR lpxL::aprP pagP::hygR [pMMW52-msbA, pSEVA434-eptA]; Catr Gentr Kanr Aprr Hygr Carbr Specr | | This study |
| TXM843 ClearColi® BL21 (DE3) F− ompT hsdSB (rB− mB−) gal dcm lon λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) msbA148 ΔgutQ ΔksdsD ΔlpxLΔlpxMΔpapPΔlpxPΔeptA | Lucigen |
| TXM844 TXM843 [pSEVA434-eptA]; Specr | | This study |

### Plasmids

| Plasmid | Source or reference |
|---------|---------------------|
| pSEVA434 | pBBR1 ori lacIq- Ptr Specr (100) |
| pSEVA434- Pirc eptA from E. coli BL21 (DE3) | This study |
|   | pET  | Source | Notes |
|---|------|--------|-------|
| pSEVA434-epfB | $P_{trc} \text{epfB}$ from *E. coli* BL21 (DE3) | This study |
| pSEVA434-epfC | $P_{trc} \text{epfC}$ from *E. coli* BL21 (DE3) | This study |
| pMMW52-msbA | pMBL19 carrying a subcloned 3.5-kb insert with *ycaI*, *msbA*, and *lpK*; Carb<sup>r</sup> | (48) |
| pKD3 | Cat<sup>r</sup> template | (98) |
| pCP20 | FLP recombinase expression plasmid; Carb<sup>r</sup> Cat<sup>r</sup> | (99) |
| pKD46 | λ-Red recombinase expression plasmid; Carb<sup>r</sup> | (98) |
| pEXG2 | Gent<sup>r</sup> template | (119) |
| pSET152 | Apr<sup>r</sup> template | (120) |
| pUC19-oriT-hyg | Hyg<sup>r</sup> template | Lab stock |

<sup>a</sup> Kan<sup>r</sup>- kanamycin; Cat<sup>r</sup>- chloramphenicol; Gent<sup>r</sup>- gentamycin; Spec<sup>r</sup>- spectinomycin; Carb<sup>r</sup>- carbenicillin; Apr<sup>r</sup>- apramycin; Hyg<sup>r</sup>- hygromycin.
Fig 1. Inorganic phosphate released from LPS chemotypes in the presence of various alkaline phosphatases. (A) Schematic of hexa-acylated wildtype LPS chemotype from *E. coli* BL21 (DE3) with the nonstoichiometric phosphoanhydride (EptA/EptC) and phosphodiester (EptB) linked PEtN modifications indicated. 4-Amino-4-deoxy-L-arabinose (Ara4N) modifications (not shown) share a common site of attachment with PEtN added by EptA to lipid A substrate. The structure of Re LPS resulting from *lpcA* deletion is indicated. LPS was extracted from either wildtype (Wt) (B) or unmodified Re LPS (TXM333 ΔlpcAΔeptAΔarnA) (C) producing strains and incubated with the indicated AP [100 µg/ml substrate, 50 mM Tris-HCl (pH = 8.25), 100 mM NaCl, 1 mM MgCl₂, 20 µM ZnCl₂ at 37 °C]. Phosphate release was measured using the malachite green assay and the data plotted as the mean ± the standard deviation of three independent replicates.
Fig. 2. Phosphate released during incubation of (A) BL21 (DE3) (wt), TXM322 (ΔarnA), GKM329 (ΔeptA), TXM331 (ΔeptAΔarnA), TXM333 (Re LPS, ΔlpcAΔeptAΔarnA), TXM343 (Re LPS, ΔlpcAΔeptAΔarnA + pEptA), and (B) TXM331 (ΔeptAΔarnA), GKM373 (ΔeptAΔarnAΔeptB), GKM374 (ΔeptAΔarnAΔeptC), GKM357 (ΔeptAΔarnAΔwaaP), GKM380 (ΔeptAΔarnAΔeptB + pEptC), GKM381 (ΔeptAΔarnAΔeptC + pEptB), TXM402 (ΔeptAΔarnAΔeptC + pEptA), and GKM358 (ΔeptAΔarnAΔwaaP + EptA) with cIAP [100 µg/ml substrate, 4 U/ml, 50 mM Tris-HCl (pH = 8.25), 100 mM NaCl, 1 mM MgCl2, 20 µM ZnCl2 at 37 °C]. Released phosphate was measured after 48 hours of incubation using the malachite green assay and normalized to BL21 (DE3) (wt). Data are representative of three independent experiments conducted in duplicates and the error bars show SDs. +/− indicates chromosomal genes, (P) denotes gene introduced on a plasmid.
Fig 3. MS analysis of EptA-modified Re LPS. Re LPS was extracted from TXM343 [lpcA::gentR eptA::catR arnA::kanR (pSEVA434-eptA)] and analyzed by MS. Samples were either directly analyzed (top panel), or incubated for 16 hours at 37 °C in buffer [100 µg/ml substrate, 50 mM Tris-HCl (pH = 7.1), 100 mM NaCl, 1 mM MgCl₂, 20 µM ZnCl₂] alone (mock) or with cIAP (4 U/ml). Reactions were assembled within dialysis tubing and continuously dialyzed against buffer to remove potentially inhibitory inorganic phosphate product. Masses in italic type represent sodium adducts (Δm = 22 u), while those marked with a green star account for a difference of Δm = 14 u, consistent with a methylene unit (-CH₂-). Chemical composition assignments (red) for unmodified Re LPS species are listed in Table S2.
Fig 4. Hydrolysis of phosphoanhydride linked PEtN-lipid IV$_A$ is spontaneous and pH dependent. (A) MS analysis in the negative anion mode of lipid IV$_A$ isolated from GKM446 ($\Delta$eptA$\Delta$gutQ$\Delta$kdsD$\Delta$lpxL$\Delta$lpxM$\Delta$lpxP$\Delta$pagP + pEptA). The structure of lipid IV$_A$ modified with two PEtN residues in non-stoichiometric amounts at C1-GlcNI and C4'-GlcNII when EptA is expressed is indicated (purple). Masses in italic style represent sodium adducts ($\Delta m = 22$ u), differences marked with a green star account for a difference of $\Delta m = 14$ u, consistent with a methylene unit (-CH$_2$-). (B) Phosphate release was measured as a function of increasing concentrations of cIAP after incubation for 6 hours at 37 °C [100 $\mu$g/ml PEtN-lipid IV$_A$ substrate, 50 mM Tris-HCl (pH = 8.25), 100 mM NaCl, 1 mM MgCl$_2$, 20 $\mu$M ZnCl$_2$] using PEtN-lipid IV$_A$ substrate isolated from GKM446. Phosphate was measured using the malachite green assay, and data are representative of two independent experiments conducted in triplicate with the error bars showing SDs. (C) Either lipid IV$_A$ alone (ClearColi® K-12 GKM445 $\Delta$eptA$\Delta$gutQ$\Delta$kdsD$\Delta$lpxL$\Delta$lpxM$\Delta$lpxP$\Delta$pagP) or with PEtN added by EptA (GKM446 $\Delta$eptA$\Delta$gutQ$\Delta$kdsD$\Delta$lpxL$\Delta$lpxM$\Delta$lpxP$\Delta$pagP + pEptA) was incubated for 48 hours at 37 °C in MOPS-Tris buffer [100 $\mu$g/ml substrate, 50 mM MOPS/50 mM Tris (adjusted to pH 6.5, 7.4, or 8.5), 100 mM NaCl, 1 mM MgCl$_2$, 20 $\mu$M ZnCl$_2$], and then treated with cIAP (10 U/ml cIAP) to release inorganic phosphate form spontaneously hydrolyzed PEtN. Phosphate was quantified using the malachite green assay. (D) Lipid IV$_A$ species were hydrolyzed for 48 hours in MOPS-Tris buffer at the indicated pH as described in (C) except samples were isolated by extraction before separation by TLC. Total lipid was visualized by sulfuric acid charring.
Fig. 5. De-O-acylated lipid A is rapidly dephosphorylated by cIAP. (A) Phosphate released by cIAP from either de-O-acylated lipid IVₐ or Re LPS (100 µg/ml substrate, 4 U/ml cIAP, 50 mM Tris-HCl (pH = 7.4), 100 mM NaCl, 1 mM MgCl₂, 20 µM ZnCl₂) at 37 °C was quantified at the indicated times using the malachite green assay. The data is plotted as the mean ± standard deviation of three independent replicates. (B) MS analysis in the positive anion mode of de-O-acyl lipid IVₐ before (top panel, calculated mass 952.467 u; recorded in negative ion mode) and after treatment with cIAP (bottom panel, calculated masses of 872.501 u and 792.535 u for monophosphoryl and non-phosphorylated products, respectively; recorded in positive ion mode). The highly cIAP susceptible phosphate at C4’-GlcNII is colored orange. Masses resulting from dephosphorylation events are indicated (P), while masses in italic style represent sodium adducts (Δm = 22 u). (C) Binding model of tetra-acylated lipid IVₐ with the C4’-GlcNII phosphate at the active site of cIAP. The phosphorylated catalytic serine covalent intermediate (bottom-right, deeply buried in the cleft) is aligned with the C4’-GlcNII lipid IVₐ phosphate as reference (Fig. S3). Proximal atoms of the two O-acylated side chains (with the terminal Ω, Ω-1, Ω-2 carbon atoms colored in yellow) permeate the protein surface. The two N-acylated side chains can occupy the cleft in their entire length without any steric clashes (green caps). The anomeric 1-phosphate group of GlcNII lies to the front.
most, right) and is only slowly cleaved in de-O-acyl lipid A (N,N-di-acylated lipid IVₐ derivative, modeled in Figure S5). Red, blue, and white surface colors indicate negative, positive, and neutral partial charges, respectively. Hydrogen atoms are not displayed.
**Fig. 6.** LPS modification with PEtN by EptA induces NF-κB activity through hTLR4/MD2 signaling. (A) The structure of hexa-acylated lipid A modified with PEtN at both the C1-GlcNI and C4'-GlcNII phosphates. The major lipid A acylation chemotype being produced in paired pEptA−/− E. coli constructs GKM374/TXM402 (ΔeptAΔarnAΔeptC), TXM418/TXM419 (ΔeptAΔarnAΔeptCΔlpxM), GKM499/TXM502 (ΔeptAΔarnAΔeptCΔlpxLΔlpxMΔpagP), and ClearColi® K-12 GKM445/GKM446 (ΔeptAΔgutQΔkdsDΔlpxLΔlpxMΔlpxPΔpagP) are indicated. (B) The inorganic phosphate released during incubation with cIAP [4 U/ml, 100 µg/ml substrate, 50 mM Tris-HCl (pH = 8.25), 100 mM NaCl, 1 mM MgCl₂, 20 µM ZnCl₂] was measured using the malachite green assay. Data are representative of three independent experiments done in duplicates and the error bars show SDs. (C) HEK293/hTLR4-MD2-CD14 NF-κB reporter cells were stimulated with the indicated LPS chemotypes and the luciferase activity was measured. (D) The bioactivity of the LPS chemotypes with murine TLR4/MD2 receptor was re-tested using stably transfected HEK293/mTLR4-MD2-CD14 reporter cells. For both hTLR4 and mTLR4 assays, data are representative of three independent experiments conducted in duplicates with the error bars showing SDs.
Fig. 7. MS analysis and TLR activity of purified 2-PEtN and 1-PEtN modified lipid IV_\text{A} samples. (A) MS analysis of the purified PEtN-lipid IV_\text{A} species isolated from E. coli strain TXM844 (msbA148ΔeptAΔgutQΔkdsDΔlpxLΔlpxMΔpagPΔpxP + pEptA). (B) Relative NF-κB induction in HEK-Blue hTLR2 reporter cells stimulated with crude LPS, 2-PEtN lipid IV_\text{A}, 1-PEtN lipid IV_\text{A}, and unmodified lipid IV_\text{A}. Data are from experiments done in triplicate and the error bars show SDs. (C) Relative NF-κB induction in HEK293/hTLR4-MD2-CD14 reporter cells stimulated with crude PEtN-lipid IV_\text{A} (TXM844) and the purified 2-PEtN lipid IV_\text{A}, 1-PEtN lipid IV_\text{A}, and unmodified lipid IV_\text{A} fractions. Data are from experiments conducted in triplicate and the error bars show SDs.
**Fig. 8.** Simulated hTLR4/MD2 binding of 2-PEtN modified lipid IVA. (A) Computational model of hTLR4/MD2 with 2-PEtN modified lipid IVA substrate bound at the hTLR4 homodimer interface. Surface color codes are *grey* for TLR4 (A chain, right), *turquoise* for the second TLR4* subunit (B chain, left), and *pink* for MD2 (C chain, bottom). Key substrate binding determinants on each subunit chain are indicated as follows. For MD2, hLys122 and hLys58 (bottom) are indicated by two *purple* patches. Patches on TLR4* to the left highlight hGlu369 (*yellow*, equivalent to mLys367), hLys388 (*blue*, behind hGlu369), and hSer415 (*green*, below hGlu369). Highlighted mid-section patches on TLR4 are hLys362 (*powder blue*) and hLys341 (*cyan*), while top right-side patches include hTyr296 (*green*), hAsp294 (*orange*), and hArg264 (*blue*). Atom colors for the 2-PEtN modified lipid IVA ligand: *black* C-H, *orange* P, *red* O, *blue* N, *white* polar H. The GlcNII ring is visible in the cleft while the GlcNI moiety is occluded by hLys122. The two cationic H₂N⁻ head groups of PEtN contact anionic hGlu369 (anomeric C1-phosphate of GlcNI) and hAsp294 (non-anomeric C4'-phosphate of GlcNII). The MD2 lipophilic cavity buries all four acyl chains of lipid IVA. Tyr296 is positioned to contact the 6'-C-OH group or the 4'-pyrophosphate group of PEtN on GlcNII. (B) Binding map schematic highlighting critical residues that vary between the human (colored text to match panel 8A) and murine (black text) TLR4/MD2 receptor complex. To bind LPS-like ligands, the dimerized receptor complex provides a binding site contoured by TLR4*/MD2/TLR4. When projected onto a plane from a certain perspective, the three proteins (B/C/A chains) form a triangle (wedge). Amino acids potentially serving as favorable electrostatic contact points for the cationic amino head groups of PEtN moieties are noted, including anionic hAsp294 (TLR4) and hGlu369 (TLR4*) residues. Of note, the latter is replaced by a non-homologous lysine (mLys367) residue in the mTLR4 receptor. As in panel (A), hTyr296 (green) is interacting with the C6-OH group of GlcNII (green) or with the adjacent pyrophosphate group (orange). The amino acid residue numbering scheme has been described (63).
