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TLR4-dependent adjuvant activity of *Neisseria meningitidis* lipid

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Abstract

The adjuvant activity of *Neisseria meningitidis* serogroup B lipopoly(oligo)saccharide (LOS) from wild-type and genetically-defined LOS mutants and unglycosylated meningococcal lipid A was assessed in C3H/HeN and C3H/HeJ mice. Meningococcal lipid A, a weak agonist for TLR4/MD-2 in human macrophages, was found to have adjuvant activity similar to that of wild-type and KDO₂-lipid A LOS in C3H/HeN mice. All meningococcal LOS structures as adjuvants induced high titers of IgG1, IgG2a and IgG2b but very little IgG3 to OMP compared to no adjuvant PBS controls. In addition, induced OMP antibodies were shown to have high bactericidal activity against serogroup B meningococci. Purified LOS and lipid A structures failed to induce any adjuvant activity in C3H/HeJ mice indicating that meningococcal LOS as an adjuvant was TLR4-dependent. Unglycosylated meningococcal lipid A because of its weak agonist activity for human macrophages and retention of adjuvant activity may be a candidate for use in serogroup B meningococcal OMP and OMV vaccines and for use as an adjuvant in other vaccines.

Keywords

lipid A; IgG titer; adjuvanticity; TLR4

Introduction

Neisseria meningitidis is a leading worldwide cause of rapidly fatal sepsis and meningitis, usually in otherwise healthy individuals [1]. For example, in the African meningitis belt (e.g., subSaharan Africa) major outbreaks of meningococcal disease can occur at rates of up to 1,000/100,000 population [2]. Capsular polysaccharide-based vaccines, including new conjugate vaccines, have been developed for *N. meningitidis* serogroups A, C, Y and W-135

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with considerable success, at least for serogroup C conjugates [3]. However, broadly effective vaccines against serogroup B *N. meningitidis* have proven to be a formidable challenge. The capsular polysaccharide from serogroup B meningococci is an α -2,8-linked polysialic acid moiety mimetic of many human glycoproteins including the neural cell adhesion molecules (NCAM). As an alternative approach, outer membrane vesicles (OMV) vaccines depleted of lipooligosaccharide (LOS) to prevent local severe reactogenicity have been developed [4,5] and other protein vaccines containing overexpressed or tailored OMP(s) are under study but OMV vaccines devoid of endotoxin are poorly immunogenic [6].

Meningococcal LOS structures with less toxicity and reactogenicity are candidates for inclusion in OMV vaccine development since they may retain adjuvant activity, facilitate antibody response against outer membrane proteins (OMP) and possibly be recognized as bactericidal epitopes. Modified meningococcal lipid A, an *lpxL* mutant (penta-acylated fatty acyl structure) retained adjuvant activity similar to a wild-type expressing hexa-acylated lipid A, when used for immunization of mice in combination with LOS-deficient outer membrane complexes (OMC) [6,7]. Interestingly, this *lpxL1* mutant had reduced toxicity as measured in a TNF α induction assay with whole bacteria and OMC [7,8]. The meningococcal serogroup B lipooligosaccharide (LOS) mutant *kdtA* expresses bisphosphorylated hexa-acylated meningococcal lipid A devoid of KDO and other oligosaccharides [9]. Interestingly, this unglycosylated lipid A has weak bioactivity in macrophages compared to wild type or other oligosaccharide truncated meningococcal endotoxin structures [10]. Unglycosylated meningococcal lipid A was a weak inducer of TNF α , IL-1 β and MIP-3 α via the TLR4-MyD88-dependent pathway and nitric oxide, IFN β and IP-10 release via the TLR4-MyD88-independent pathway [11]. While meningococcal KDO₂-lipid A at a low dose of LOS (0.56 pmole /ml ~ 1ng/ml) significantly up-regulated CD80, CD83 and CD86 and released significantly higher amounts of IL-12p70, IL-6, IL-10, TNF α , MCP-1, IP-10 and RANTES from human monocyte-derived dendritic cells (MDDC) [12], unglycosylated meningococcal lipid A as well as the penta-acylated LOS failed to induce DC maturation or activation at the same dose [12]. However, immunogenicity or adjuvant activity of unglycosylated meningococcal lipid A has not been explored. The aim of this study was to investigate adjuvant activity of meningococcal lipid A.

Materials and Methods

Reagents

RPMI 1640 medium, Dulbecco's Eagle medium, fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate and nonessential amino acids were obtained from Cellgro Mediatech (Herdon, VA). Phorbol myristate acetate (PMA) was from GibcoBRL (Grand Island, NY). TNF α , MIP-3 α and MCP ELISA kits were from R&D systems (Minneapolis, MN). RAW 264.7 and THP-1 cell lines were obtained from ATCC (Manassas, Virginia). MM6 cell line was kindly provided by Dr. Geert-Jan Boon (The Complex Carbohydrate Research Center, University of Georgia, Athens, GA), the U937 cell line was kindly provided by Dr. Yusof Abu Kwaik (University of Kentucky School of Medicine, Lexington, KY).

LOS purification and quantitation

Endotoxin from the serogroup B *N. meningitidis* strain NMB (encapsulated, L2/L4 immunotype) and genetically-defined mutants (*gmhB*: KDO₂-lipid A and *kdtA*: unglycosylated lipid A) of this strain were initially extracted by the phenol-water method [13]. These endotoxin preparations were further purified and quantified [10]. Briefly, residual membrane phospholipids were removed by repeated extraction of the dried LOS samples with 9:1 ethanol:water. The expected fatty acyl components of 3-OHC12:0, 3-OHC14:0 and C12:0 and the absence of membrane phospholipids was assessed by mass spectroscopy (GC-MS)

(Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Purified endotoxins were quantified and standardized based on the number of lipid A molecules per sample [14]. LOS was resuspended in pyrogen free water with 0.5% triethylamine, vortexed for at least 5 min, boiled for 1 hr at 65°C, then sonicated for 30 min in a water bath sonicator (L&R, Transistor/Ultrasonic T-14) to enhance solubility. Endotoxin stock solutions were prepared in pyrogen free water at 10 nmole/ml concentration and further diluted with endotoxin free PBS to 1 nmole /ml and 100 pmole /ml with extensive vortex and sonication prior to each dilution.

KDO acid hydrolysis

Wild-type meningococcal LOS (hexa-acyl lipid A), LOS from the *gmhB*, a KDO₂-lipid A mutant (hexa-acyl lipid A) and LOS from the KDO-deficient mutant *kdtA* with hexa-acyl lipid A were hydrolyzed with 1% acetic acid. Briefly, 50 µl of LOS (stock concentration 10 nmole/ml) was mixed with 450 µl of 1% acetic acid (pH 2.8) or PBS (pH 7.4), all pyrogen free solutions, to give a final lipid A concentration of 1 nmole/ml. After vigorous mixing all tubes were incubated at 90°C for 45 min then dried in a SpeedVac (Savant, Farmingdale, NY). The dried pellets were resuspended in 500 µl of pyrogen-free water, vortexed vigorously and saved for further use to stimulate nitric oxide induction in RAW macrophages or cytokine induction in THP-1 cells. Lipid A structures were confirmed after mild acid hydrolysis using thin layer chromatography [15].

Mice immunization protocol

To assess the adjuvant activity of meningococcal LOS and lipid A structures, 6–8 week-old C3H/HeN and C3H/HeJ mice were used. Eight mice per group were injected subcutaneously outer membrane complexes (5 µg protein) from the LOS-deficient mutant (*lpxA*) of meningococcal strain H44/76 dissolved in 250 µl of PBS with or without 1 µg of purified LOS or lipid A [16]. OMC were prepared from *lpxA* mutant of *N. meningitidis* strain H44/76 as described previously [8]. Briefly, OMP were isolated by sarcosyl extraction after sonic disruption of cells and protein content was quantified using the bichionic acid protein assay reagent (Pierce Chemical Co.) with a BSA standard. Mice were immunized at day 0 and boosted at day 14 and day 28 then bled at day 42. Collected mice sera were saved at 4°C for further testing.

Adjuvant activity and antibody titers determination

Antibody titers in immunized mice sera were determined against H44/76 whole cells in ELISA for each individual serum as described [6]. Briefly, a four-parameter curve fit was made for optical density values obtained with serial dilutions of the sera, and the antibody titers were calculated as reciprocal dilutions that gave 50% of the maximum absorbance. Total IgG titers as well as IgG subclasses IgG1, IgG2a, IgG2b and IgG3 were measured.

Serum bactericidal assay

The serum bactericidal activity (SBA) was performed against *N. meningitidis* H44/76 wild type and the PorA deficient mutant HI5 as described [6]. Sera from mice were heat inactivated for 30 min at 56°C prior to use. Serum samples and meningococci were incubated for 10–15 min at room temperature before the addition of complement. A final concentration of 20% baby rabbit complement was used. The serum bactericidal titer was measured as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

Cell cultures

MM6, U937 and THP-1, human macrophage-like cell lines were grown in RPMI 1640 with L-glutamate supplemented with 10% FBS, 50 IU/ml of penicillin, 50 µg/ml of streptomycin,

1% sodium pyruvate and 1% non-essential amino acids. Culture flasks were incubated at 37°C with humidity under 5% CO₂. Murine macrophage cells (RAW 264.7) were grown in Dulbecco's Eagle medium supplemented and incubated as mentioned above.

Cytokine induction by LOS

THP-1 human monocytes were differentiated into macrophage-like cells using PMA at a final concentration of 10 ng/10⁶ cell and incubated at 37°C for at least 24 hrs. Freshly differentiated macrophages were washed with PBS, counted and adjusted to 10⁶ cell/ml, transferred into a 24 well tissue culture plate (1 ml/well), stimulated with LOS or lipid A at final concentration of 0.56 pmole/ml or increasing LOS and lipid A doses in a dose-response manner and incubated overnight at 37°C with 5% CO₂. Cell culture supernatants were harvested and saved at -20°C.

Cytokines quantitation by ELISA

Human TNF α , MCP and MIP-3 α DuoSet kits (R&D systems, Minneapolis, Minn.) were used for cytokine quantification according to the manufacturer's instructions.

Nitric oxide induction in RAW macrophages

Freshly grown RAW 264.7 macrophages adherent to the flask were washed with PBS and incubated with 5 ml of trypsin for 5 min at 37°C. Harvested cells were washed and re-suspended in Dulbecco's complete media. Approximately 10⁶ macrophages / ml were transferred into a 24 well-tissue culture plate, stimulated with 0.56 pmole /ml final concentration or increasing LOS and lipid A doses in a dose-response manner. Nitric oxide was quantitated using the Griess chemical method [17] was used to detect nitrite (NO₂) accumulated in supernatants of induced RAW 264.7 macrophages. Nitrate was not detected in these experiments, thus nitrite reflected the amount of nitric oxide released.

Statistical analysis

Mean titer values \pm SD and *P* values (Student *t* test, unpaired, two-tail distribution) from eight different mice sera determined individually were calculated in comparison to PBS controls with Microsoft Excel software.

Results

The structural determinants of meningococcal lipid A that influences bioactivity and adjuvanticity were investigated using highly purified LOS or unglycosylated lipid A derived from wild type meningococci or genetically defined mutants (*gmhB*: KDO₂-lipid A; *kdtA*: lipid A) of the serogroup B meningococcal strain NMB (Figure 1). LOS bioactivity was assessed by measuring TNF α and nitric oxide induction in human and murine macrophages respectively. Bactericidal antibody titers in mice sera immunized with meningococcal LOS and lipid A assessed adjuvant activity.

Meningococcal LOS and lipid A bioactivity

Meningococcal lipid A (*kdtA*) lacking KDO was a weak agonist for nitric oxide induction from murine RAW 264.7 macrophages (Figure 2A) and for TNF α release from human THP-1 cells (Figure 2B). The complete and truncated meningococcal LOS structures NMB and *gmhB* (KDO₂-lipid A), respectively, had similar activity in inducing nitric oxide and TNF α even at <1 picomolar concentrations (Figure 2A & B). Similar results were obtained when IP-10, IL-1 β and MIP-3 α were measured (data not shown). The results suggested that meningococcal lipid A alone is significantly less active than complete or truncated LOS for cytokine release from human and murine macrophages. The data also confirmed that KDO₂-lipid A was the

minimal structure required to fully activate both the TLR4-MyD88-dependent and independent signaling pathways.

To confirm the structural requirement of KDO for creating the lipid A configuration necessary for activating macrophages, NMB, *gmhB* (KDO₂-lipid A) LOS and *kdtA* lipid A were subjected to acid treatment with 1% acetic acid (pH 2.8) prior to macrophage stimulation. Previous work has demonstrated that this acid hydrolysis cleaves the linked KDO molecules but not the fatty acyl chains from lipid A [10]. Human THP-1 macrophages stimulated with acid treated LOS failed to induce significant amounts of TNF α (Figure 3) when compared to LOS in PBS alone (pH 7.4), which does not result in hydrolysis of the KDO glycosidic linkage. Thus, KDO removal from meningococcal lipid A resulted in significant attenuation of bioactivity or toxicity of LOS. Synthetic KDO when added to acid hydrolyzed LOS did not induce significant responses in macrophages even at high doses (data not shown). The results indicated that KDO linked to lipid A were structurally required to create a lipid A macromolecular configuration necessary for full macrophage activation via both the TLR4-MyD88-dependent and independent signaling pathways.

Meningococcal LOS and lipid A adjuvant activity

The adjuvant activity of meningococcal LOS and lipid A structures was studied in TLR4-sufficient (C3H/HeN) and TLR4-deficient (C3H/HeJ) mice. Eight mice per group were immunized with 1 μ g of purified LOS or lipid A mixed with 5 μ g of outer membrane proteins (OMP) from an LOS-deficient mutant (*lpxA*) in 250 μ l PBS injected subcutaneously at day 0, boosted at day 14 and 28 and bled at day 42. With all the LOS preparations, a strong increase in anti-meningococcal antibody titers in the C3H/HeN mice was seen as compared to the control group (Figure 4). The control group mice were immunized with LOS-deficient OMP to which no LOS or lipid A was added and was designated as the no adjuvant group. No increase in antibody titers was seen in the TLR4-deficient mice C3H/HeJ immunized with OMP with or without LOS or lipid A adjuvants. These data indicated that both highly purified meningococcal LOS and unglycosylated lipid A utilized a TLR4-dependent pathway to exert adjuvant activity.

Meningococcal lipid A (*kdtA*) with weak agonist activity for TLR4 in human and murine macrophages was found to have adjuvant activity similar to that of the wild type meningococcal LOS and KDO₂-lipid A in C3H/HeN mice (Table 1, Figure 4). Meningococcal LOS and unglycosylated lipid A structures both induced significantly higher titers ($P < 0.01$) of IgG2a and IgG2b (Table 1) but not IgG3 compared to PBS controls. Meningococcal LOS and lipid A also induced higher amounts of IgG1, however the differences were not significant compared to the no adjuvant controls. No LOS adjuvant activity was mounted in the TLR4-deficient mice C3H/HeJ as no difference was seen in IgG1, IgG2 or IgG3 antibody titers between LOS immunized and the no adjuvant controls C3H/HeJ mice (Table 1).

Bactericidal antibody titers were raised in C3H/HeN (TLR4-sufficient) mice immunized with meningococcal OMP with meningococcal LOS or unglycosylated lipid A as adjuvants compared to the no adjuvant control (Table 1). By contrast, no significant bactericidal antibodies were raised in the TLR4-deficient C3H/HeJ mice immunized with OMP with or without meningococcal endotoxin as an adjuvant (Table 1). The induced antibodies in pooled mice sera were PorA specific but not LOS specific, as high titer bactericidal activity was found against viable wild type H44/76 meningococci but not against its PorA-deficient mutant HI-5 (data not shown). Purified LOS and lipid A structures failed to induce significant bactericidal antibody titers in C3H/HeJ mice, again indicating that meningococcal LOS adjuvant activity was TLR4-dependent. The results indicated that meningococcal lipid A with attenuated bioactivity for macrophages retained good adjuvant activity in mice and was similar to wild type KDO₂-lipid A meningococcal LOS structures.

Discussion

Capsular polysaccharide and polysaccharide conjugate vaccines are available for several *N. meningitidis* serogroups except serogroup B. The capsular polysaccharide from serogroup B meningococci is an α -2,8-linked polysialic acid moiety mimetic of human cell components [18,19]. Thus OMV containing meningococcal OMP-derived vaccines have received considerable attention as an alternative approach and have achieved success in control of clonal serogroup B outbreaks [20,21]. Despite reduction in the endotoxin content of OMV vaccines, significant reactogenicity has remained with these vaccines. On the other hand, genetic approaches to eliminate endotoxin (e.g., *lpxA* mutant) have resulted in OMV preparations with poor immunogenicity [6]. Thus, the meningococcal LOS components of these OMV vaccines are a potent TLR4 agonist and adjuvant but elicit unfavorable reactogenicity.

Endotoxin structure plays a crucial role in the induced cellular responses via the TLR4-MD-2 receptor complex. Differences in lipid A structure or conformation are known to influence biological activity by changing the supramolecular conformation of lipid A [22–25]. A bisphosphorylated, C12 and C14, hexa-acylated lipid A structure linked to KDO is the most bioactive when TNF α is the measure of bioactivity [24,26,27]. We have previously shown that meningococcal KDO₂-lipid A was the minimal structure required for full TLR4 agonist activation [10,12]. In contrast, unglycosylated meningococcal lipid A (*kdtA*), although binding to the TLR4 receptor complex, is a very weak agonist for TLR4 in human and murine macrophages and induces much less TNF α and nitric oxide even at high doses. However, if unglycosylated meningococcal lipid A retained adjuvant activity it could be a potential candidate to include in OMV, multivalent protein based meningococcal and other vaccines to avoid or minimize toxicity and reactogenicity.

In this study, wild-type meningococcal endotoxin [NeuNAc-Gal β -GlcNAc-Gal β -Glc β -Hep₂ (GlcNAc, Glc α) PEA-KDO₂-lipid A; 1,4' bisphosphorylated; see Figure 1] and truncated LOS structures (*gmhB*: KDO₂-lipid A), as well as hexa-acylated unglycosylated meningococcal lipid A (*kdtA*) retained TLR4-mediated adjuvant activity in mice. Meningococcal lipid A was confirmed as a weak agonist for TLR4 activation in human and murine macrophages even at high doses. In support of these data, Muroi and Tanamoto reported that [28] the *Salmonella minnesota* Re LPS KDO₂-lipid A truncated structure plays an indispensable role in activating THP-1 macrophages. The data also indicate that while the loss of KDO₂ molecules linked to meningococcal lipid A resulted in a dramatic attenuation of TNF α and nitric oxide release from activated human and murine macrophages, adjuvant activity was maintained in a mouse model. Other studies have indicated that although the oligosaccharide chain of endotoxin can influence phagocytosis and internalization by dendritic cells, the oligosaccharide is not essential for adjuvant activity [29,30].

Wild type meningococcal LOS and meningococcal KDO₂-lipid A structures significantly up-regulate CD80, CD83 and CD86 and released significantly higher amounts of IL-12p70, IL-6, IL-10, TNF α , MCP-1, IP-10 and RANTES from human monocyte-derived dendritic cells (MDDC) induced with a very low dose of LOS (0.56 pmole /ml ~ 1ng/ml) [12]. However, unglycosylated meningococcal lipid A as well as the penta-acylated meningococcal LOS, at this dose, did not induce DC maturation and T cell activation when lipid A was removed prior to the MDDC coculture with T cells [12]. Since human macrophages stimulated with very high doses of unglycosylated meningococcal lipid A (1 ng up to 1 μ g) also have a very attenuated response (Figure 2B and data not shown), it might be predicted that meningococcal lipid A would not have an adjuvant effect. However, using monophosphoryl lipid A (MPL), an adjuvant which shares structural similarity to meningococcal lipid A, Ismaili et al showed that at doses less than 50 μ g/ml MPL did not induce human MDDC [31]. In contrast, even low doses of MPL (5 μ g/ml) are sufficient to support optimal activation of allogeneic naïve

CD45RA⁺ T cells. Further, MDDC pulsed for 2 hours with low doses (5 µg/ml) of MPL that was not removed prior to co-culture with T cells results in full T cell activation [31]. This was the result of a direct TLR4-dependent MPL effect on T cells [31]. In further support, when MPL is used as adjuvant in mice, it increases the function of antigen-presenting cells [32] and also switches T cell responses induced by protein antigens from Th2 to Th1 [33]. Th1 responses are preferred because they influence B cell isotype switching to IgG2a production [34]. Thus, TLR4-dependent adjuvant activity of meningococcal lipid A and MPL may be explained by direct priming effect exerted on naïve T cells which greatly enhance immune responses upon interaction with antigen-presenting dendritic cells. The priming of naïve T cells requires only low levels of TLR4 activation that appears to be sufficient for adjuvant activity. In support of lipid A direct priming effect, macrophages pre incubated with unglycosylated lipid A (*kdtA*) prior to respiratory burst trigger by either zymosan or phorbol ester (PMA) released higher oxidative burst responses compared to unprimed cells [10].

LPS has adjuvant activity for protein antigens and enhances antibody responses [35]. In this study both meningococcal LOS and lipid A used as adjuvants induced in mice significantly high titers of IgG2a (10-fold increase) and IgG2b and less IgG1 but not IgG3. The induced antibody titers were bactericidal and were mainly directed against PorA as no bactericidal activity was mounted against PorA-deficient (HI-5) meningococcal strain H44/76. LOS-specific antibodies were not induced in mice sera even when higher doses of meningococcal LOS (5 µg) were used as the adjuvant. Steeghs et al., using various LPSs as adjuvants, demonstrated that the composition and length of the carbohydrate chain of LPS do not influence the induction of bactericidal antibodies against PorA proteins [6,36]. Further, the influence of various adjuvants on the immune response towards LPS-deficient OMPs indicates that 5 µg meningococcal LOS is at least equivalent to 20 µg MPL (monophosphoryl lipid A), 500 µg AIPO₄ (aluminium hydroxide), 20 µg Quil A or 20 µg MF59 [6].

The mechanism by which meningococcal lipid A exerts adjuvant activity is TLR4-mediated since no adjuvant activity was seen in TLR4-deficient mice C3H/HeJ. Antigen-specific antibody responses are enhanced in the presence of TLR ligands such as LPS, zymosan, CpG and poly (I:C) [37]. Durand et al. showed that antibody subclasses IgG1 and IgG2a induction are greatly enhanced by TLR agonists compared to that of mice immunized with protein antigen alone [37]. Meningococcal LPS as an adjuvant increased IgG2a in mice by 7.3-fold (Table 1). In mice, IgG2a is a protective isotype due to its ability to fix complement and induce antibody-dependent cell-mediated cytotoxicity by macrophages [38,39]. Bactericidal activity is a vital correlate of protection against meningococcal disease. In humans, IgG3 is the most effective complement-fixing antibody. Previous studies have shown that protective IgG isotypes raised in mice are different from those raised in humans using the same antigen [40–42].

Previous studies by Steeghs et al. have found that adjuvant activity of meningococcal LOS is not dose-dependent [6,8]. Thus, how do modified LOS structures such as those derived from *lpxL1* and *kdtA* mutants or MPL retain adjuvant activity in spite of significantly reduced *in vitro* TLR4-mediated biological activity? As noted above, direct TLR4-dependent priming of naïve T cells by meningococcal lipid A appears to require only low levels of TLR4 activation to enhance T cells immune responses for protein antigens (OMP in this study) presented by dendritic cells or other antigen-presenting cells. Beutler et al. reported that DC activation and costimulatory molecule upregulation by LPS is MyD88-independent and strictly occurs via the TLR4-TRIF-IFNβ axis [43]. Durand et al. (5) demonstrated that LPS adjuvant activity was largely dependent on the IFNα/β signaling pathway. The induction of functional cross-priming to soluble protein antigens by LPS in IFNα/β-deficient mice was abrogated in the absence of IFNα/β signaling [37]. The results of our study suggest that KDO₂ linked to lipid A is crucial for TNFα induction from human macrophages, but is not required for retaining adjuvant activity in mice. TNFα induction is MyD88-dependent [44]. In support, IL-12p70 production

in mice by different microbial ligands does not correlate with IgG2a induction [37]. Macrophages exposed to unglycosylated meningococcal lipid A (*kdtA*) do release lower quantities of nitric oxide in a dose-dependent manner (Figure 2). Nitric oxide induction is MyD88-independent and nitric oxide synthase is one of many IFN β inducible genes [45]. Thus, adjuvant activity of unglycosylated meningococcal lipid A may require the expression of only low levels of TLR4-TRIF-IFN β pathway activation in T cells.

In summary, the results of this study further indicate that KDO linked to lipid A induces conformational changes important in meningococcal LOS agonist activity of both human and murine macrophages. However, unglycosylated meningococcal lipid A, although a very weak agonist for TLR4-MD-2 and thus predicted to have low reactogenicity, was found to have good TLR4-dependent adjuvant activity and induced functional bactericidal antibodies not different from wild-type endotoxin structures. Thus, unglycosylated meningococcal lipid A may be an excellent vaccine adjuvant candidate due to predicted low toxicity and reactogenicity but high adjuvant activity.

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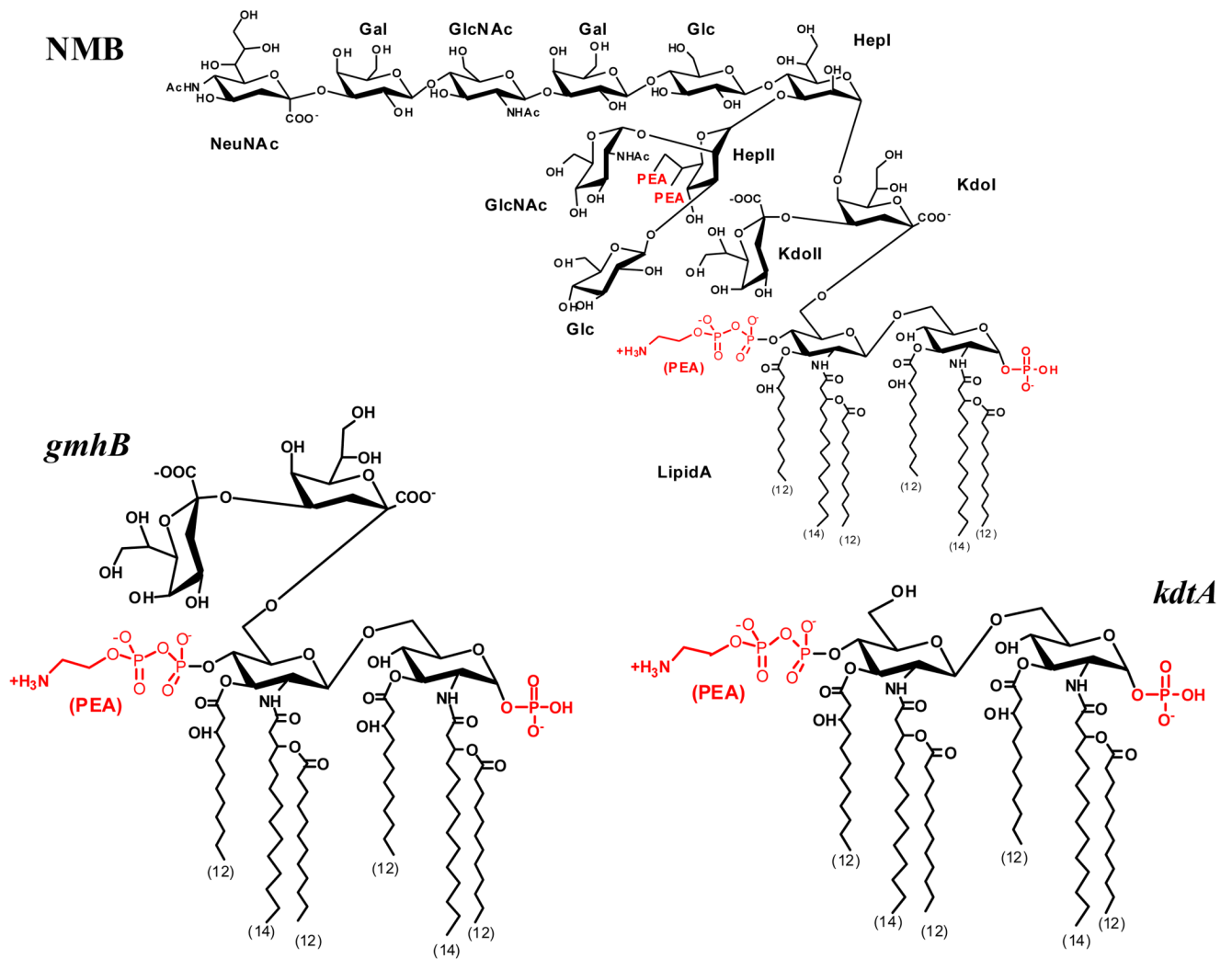


Figure 1.

Meningococcal LOS and lipid A structures. **A:** NMB is complete LOS structure with an alpha lactone tetraose oligosaccharide chain, a complete inner core and hexa-acylated, bisphosphorylated lipid A [46]; **B:** *gmhB* is truncated LOS structure composed only of KDO₂-lipid A (hexa-acylated and bisphosphorylated) [47]; **C:** *kdtA*: is native hexa-acylated, bisphosphorylated lipid A structure [9].

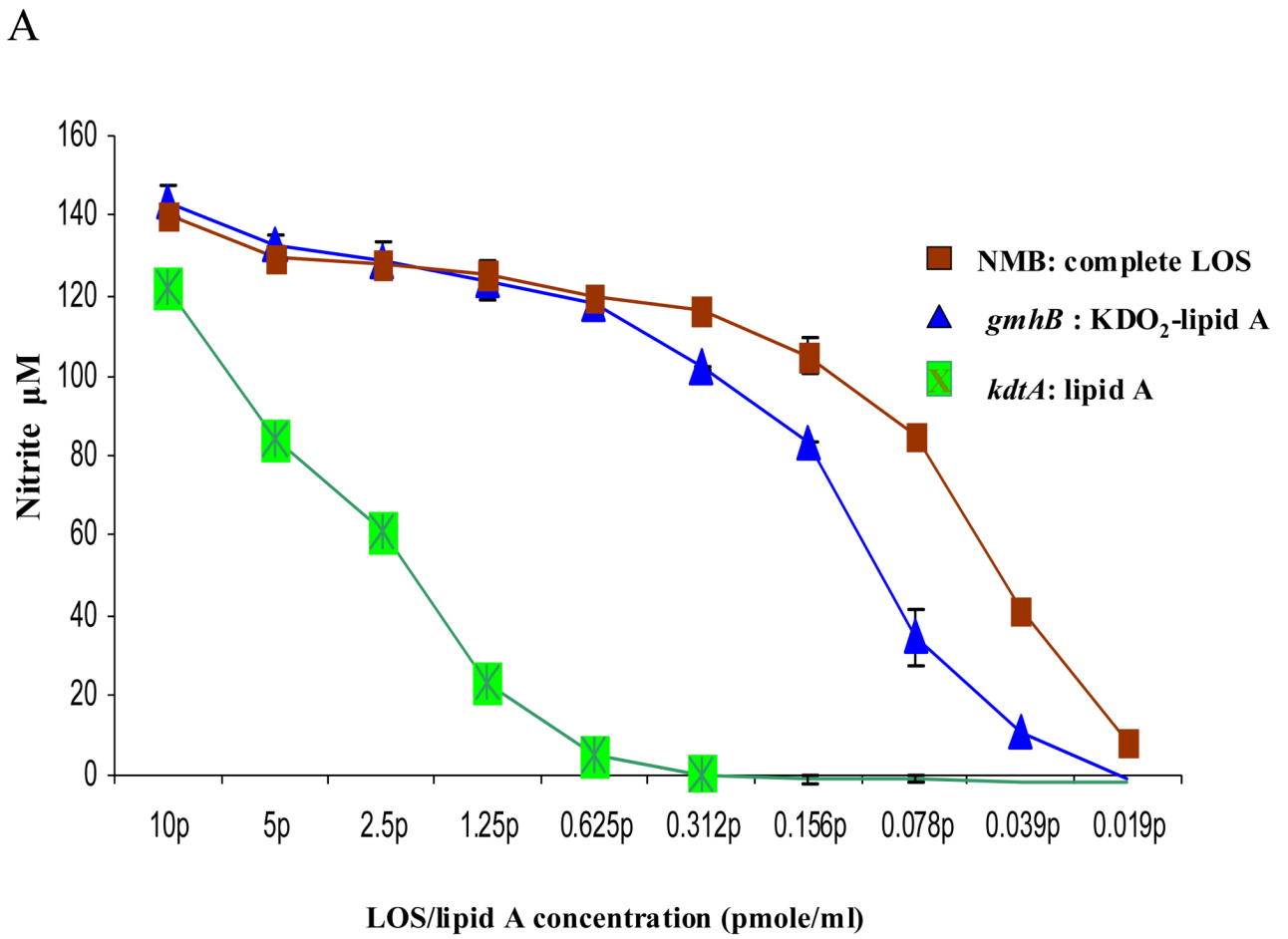
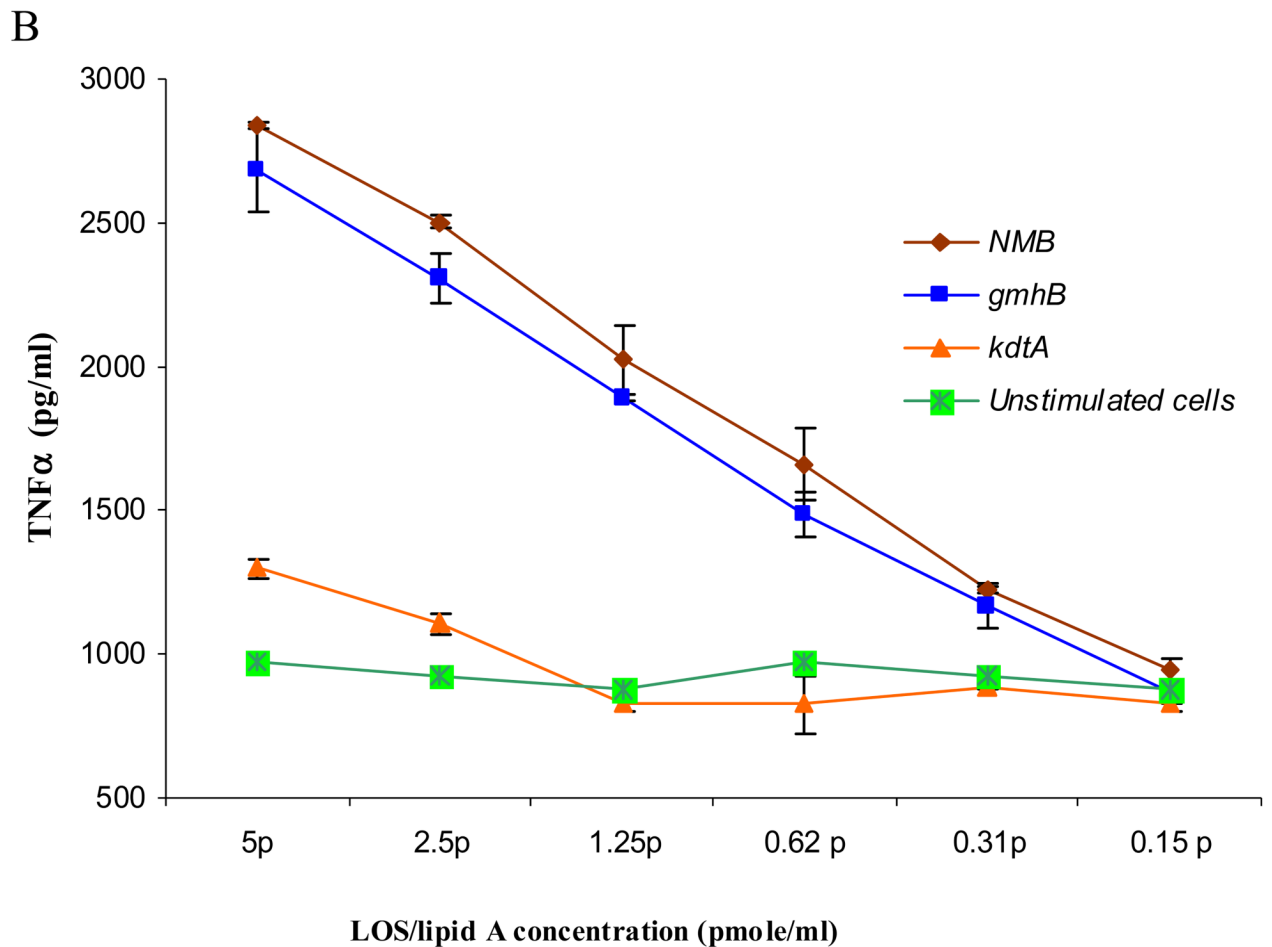
Figure 2A:

Figure 2B:**Figure 2.**

Bioactivity of meningococcal LOS and unglycosylated lipid A structures. **A:** Nitric oxide induction from murine RAW 264.7 macrophages (10^6 cell/ml) stimulated with LOS or lipid A doses (10–0.019 pmole/ml) and incubated 18 hrs. Nitric oxide was measured by the Greiss method as nitrite per μ M in the harvested supernatants. **B:** TNF α induction from THP-1 (10^6) human macrophages stimulated with LOS or lipid A doses (5–0.15 pmole/ml) and incubated 18hrs was measured by ELISA. Error bars represent the \pm SD from the average of 4 readings. The figure is representative of 4 independent experiments. P is picomole/ml

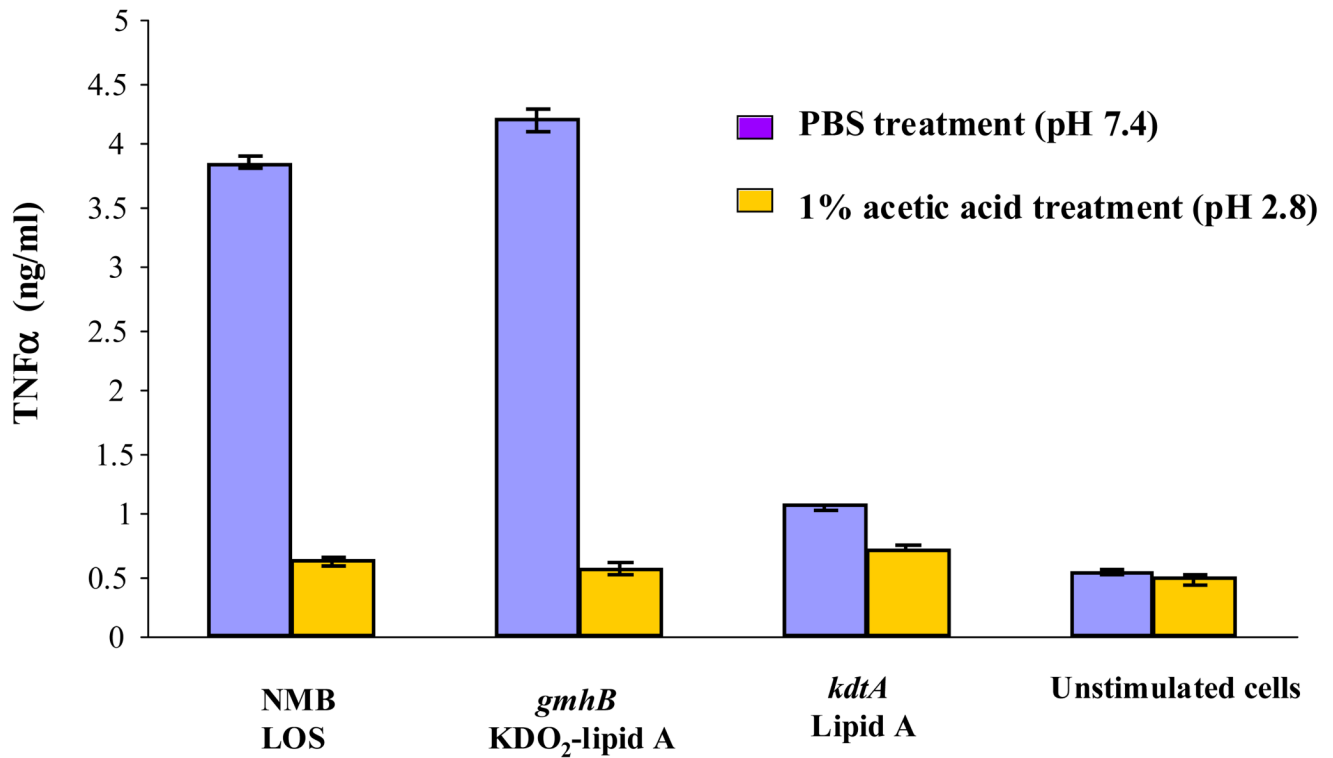


Figure 3.

Effect of removal of meningococcal KDO₂ from lipid A by acid hydrolysis on the release of TNF α by human macrophages. Meningococcal LOS of parent strain NMB, a truncated LOS *gmhB* (KDO₂-lipid A) and *kdtA* (lipid A) were hydrolyzed with 1 % acetic acid (pH 2.8) or as a control treated with PBS (pH 7.4). TNF α release from THP-1 macrophages induced with 0.56 pmole/ml of lipid A was then measured. Unstimulated cells were used as controls. Error bars represent \pm SD from the mean. This figure is representative of three independent experiments.

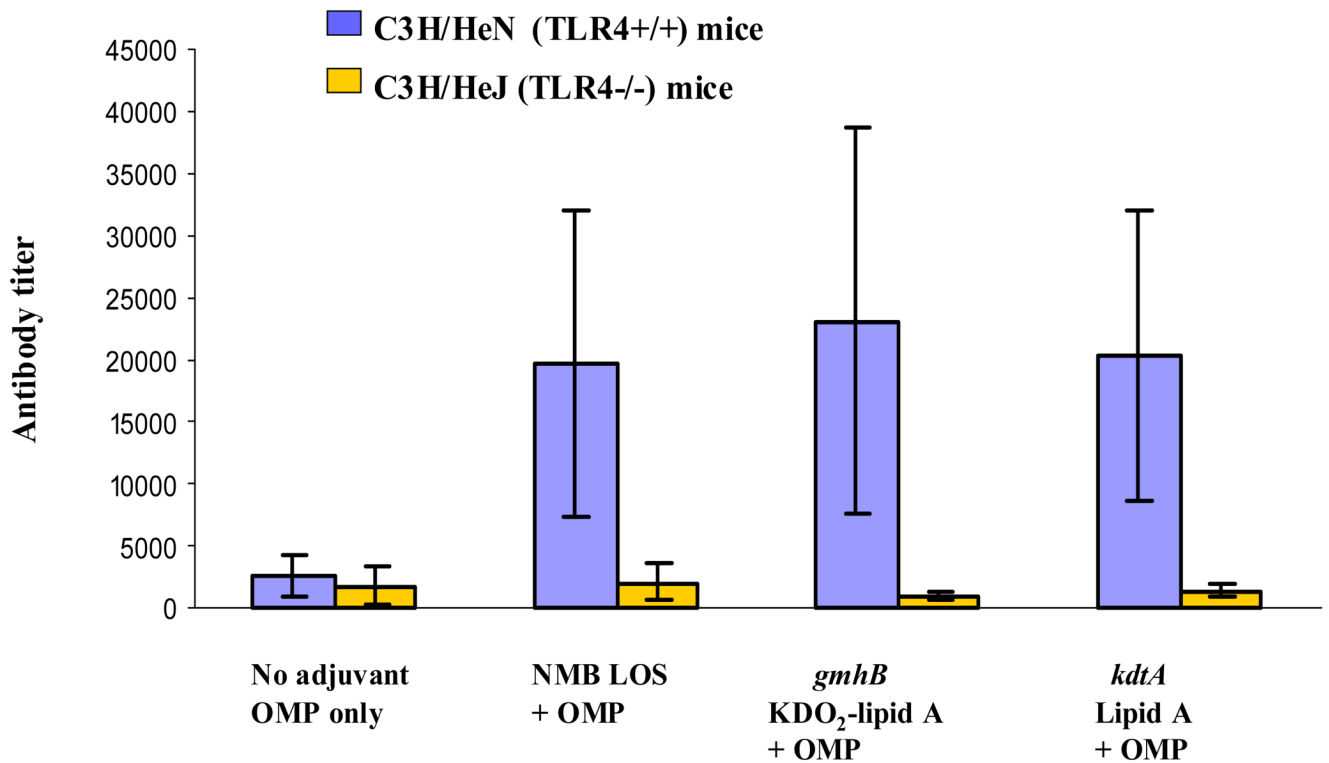


Figure 4.

TLR4-dependent LOS and lipid A adjuvant activity. TLR4-sufficient (C3H/HeN) and TLR4-deficient (C3H/HeJ) mice (8 mice/group) were immunized with 5 μ g of OMP from the LOS-deficient *lpxA* meningococcal mutant of strain H44/76 with or without 1 μ g of purified LOS or lipid A as the adjuvant. Induced antibody titers were measured in whole cell ELISA using meningococcal strain H44/76.

Table 1

Average antibody titers in C3H/HeN (TLR4+/+) and C3H/HeJ (TLR4-/-) mice sera (8 mice/group) induced against LOS-deficient OMP when meningococcal LOS and lipid A were used as adjuvants.

		ELISA titers																							
		Bactericidal titers against H4476				Total IgG				IgG1				IgG2a				IgG2b				IgG3			
Adjuvant	p value	TLR4+/+		TLR4-/-		TLR4+/+		TLR4-/-		TLR4+/+		TLR4-/-		TLR4+/+		TLR4-/-		TLR4+/+		TLR4-/-		TLR4+/+		TLR4-/-	
		No adjuvant		925	1,501	2,535	1,818	4,514	2,388	2,364	1,050	610	499	148	100										
	*		**	*	**	*	**	*	**	*	**	*	**												**
NMB		12,960	925	19,670	2,093	14,939	3,811	17,261	2,283	9,172	343	110	100												
	**	*0.0012	**0.244	*0.0027	**0.361	*0.073	**0.203	*0.007	**0.164	*0.010	**0.110	*0.376	**0.118												
gmhB		S	NS	S	NS	NS	NS	S	NS	S	NS	NS	NS												NS
	*	5,120	524	23,164	990	9,171	1,339	15,365	1,144	9,225	308	445	122												
	**	*0.0058	**0.105	*0.0033	**0.078	*0.096	**0.008	*0.015	**0.411	*0.009	**0.116	*0.071	**0.118												
kdta		S	NS	S	NS	NS	NS	S	NS	S	NS	NS	NS												NS
	*	7,540	260	20,416	1,418	6,603	2,477	18,255	1,341	5,148	658	100	122												
	**	*0.026	**0.05	*0.0012	**0.27	*0.272	**0.468	*0.013	**0.307	*0.004	**0.26	*0.257	**0.18												

* p values were compared to no adjuvant C3H/HeN (TLR4+/+) control mice;

** p values were compared to no adjuvant C3H/HeJ (TLR4-/-) control mice. S: significant difference; NS: not significant difference.