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A Meningococcal Outer Membrane Vesicle Vaccine Incorporating Genetically Attenuated Endotoxin Dissociates Inflammation from Immunogenicity

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Background: Group B Neisseria meningitidis, an endotoxin-producing Gram-negative bacterium, causes the highest incidence of group B meningococcus (MenB) disease in the first year of life. The Bexsero vaccine is indicated in Europe from 8 weeks of age. Endotoxin components of outer membrane vesicles (OMVs) or soluble lipopolysaccharide (LPS) represent a potential source of inflammation and residual reactogenicity. The purpose of this study was to compare novel candidate MenB vaccine formulations with licensed vaccines, including Bexsero, using age-specific human in vitro culture systems.

Methods: OMVs from wild type- and inactivated lpxL1 gene mutant-N. meningitidis strains were characterized in human neonatal and adult in vitro whole blood assays and dendritic cell (DC) arrays. OMVs were benchmarked against licensed vaccines, including Bexsero and whole cell pertussis formulations, with respect to Th-polarizing cytokine and prostaglandin E2 production, as well as cell surface activation markers (HLA-DR, CD86, and CCR7). OMV immunogenicity was assessed in mice.

Results: ΔpxL1 native OMVs (nOMVs) demonstrated significantly less cytokine induction in human blood and DCs than Bexsero and most of the other pediatric vaccines (e.g., PedvaxHib, EasyFive, and bacillus Calmette–Guérin) tested. Despite a much lower inflammatory profile in vitro than Bexsero, ΔpxL1 nOMVs still had moderate DC maturing ability and induced robust anti-N. meningitidis antibody responses after murine immunization.

Conclusion: A meningococcal vaccine comprised of attenuated LPS-based OMVs with a limited inflammatory profile in vitro induces robust antigen-specific immunogenicity in vivo.

Keywords: group B meningococci, outer membrane vesicles, vaccine, newborn, dendritic cells
INTRODUCTION

Human newborns and infants suffer a high frequency of infection compared to older children and adults (1), in part due to distinct early life immunity with impaired host defense (2). Early life immunization is desirable, but vaccine-induced responses of newborns and young infants demonstrate slow initiation, low immunogenicity, and reduced persistence of functional antibodies and cell-mediated responses (2). Although the majority of global immunization schedules are focused on the pediatric age group, development of early life vaccines has been hampered by this distinct immunity and an \textit{ad hoc} approach to developing vaccines, relying on adult-derived results that may inaccurately predict infant responses (3).

\textit{Neisseria meningitidis} (meningococcus) is a Gram-negative, endotoxin-producing organism that is a normal commensal of the human nasopharynx and is an important cause of invasive bacterial infection in children worldwide (4). Recent immunization programs with capsular-polysaccharide vaccines have dramatically reduced the incidence of serogroup C and A meningococcal disease in North America, Europe, and Africa (5). However, meningitis and septicemia caused by serogroup B meningococci remain a major health concern in young children, as similar capsule-based vaccines cannot be developed against this serogroup (6). Outer membrane vesicle (OMV)-based vaccines have historically been used, with some success, to control meningococcal disease caused by serogroup B meningococci. OMVs are produced by the blebbing of membranes of clinical-derived live Gram-negative bacteria during \textit{in vitro} growth and are useful vaccine components, as immuno-stimulatory membrane components [lipids, proteins, lipopolysaccharide (LPS), etc.] from meningococci are represented (7). As OMV yield from culture alone is too low for vaccine production, detergent extraction is used to force vesiculation and increase yield and has the added advantage of reducing LPS content to prevent overt reactogenicity. Novartis’ 4-component aluminum hydroxide (Alum)-adjuvanted group B meningococcus (MenB) vaccine (4CMenB, Trade name: Bexsero) has been licensed by both the European Medicines Agency and the U.S. Food and Drug Agency and comprised OMVs and three recombinant immunogenic \textit{N. meningitidis} proteins identified by reverse vaccinology (8). Another meningococcal serogroup B vaccine, Trumenba, contains two recombinant proteins with no OMV component, is approved in the U.S. for use in individuals 10 through 25 years of age, but lacks the potentially broader antigen repertoire inherent to OMVs.

The currently licensed meningococcal vaccines have the potential to reduce mortality and morbidity associated with MenB infections, but do have some limitations. Although detergent extraction of OMVs removes the majority of the LPS, the remaining endotoxin, particularly the soluble LPS, may still result in residual reactogenicity, necessitating the use of the Alum to ameliorate excess toxicity (9). Even so, immunization with the OMV-containing Bexsero may correlate with rates of reactogenicity (e.g., fever $\geq 38^\circ$C, tenderness at injection site) as common as 10% in infants less than 1 year of age (10), and severe reactogenicity is reported in some cases (11). Bexsero may also enhance reactogenicity when given together with other vaccines (12), prompting some primary care physicians to prescribe anti-inflammatory agents (13) to prevent potential reactions. Furthermore, as conventional assays for preclinical and release testing of vaccines, such as the rabbit pyrogenicity test and \textit{Limulus} amebocyte lyase (LAL) assay, were developed for vaccines containing no or negligible amounts of endotoxin, predicting the reactogenicity of LPS-containing OMV vaccines is difficult and requires more sophisticated models (14).

To overcome the potential reactogenicity of wild-type (WT) OMV-based vaccines while maintaining the inherent adjuvant activity of vesicles, a vaccine has previously been developed using a \textit{N. meningitidis} strain with genetically attenuated endotoxin (15). This enabled the use of detergent-free manufacturing processes to produce native OMVs (nOMVs) (16). To assess the potential of such OMV vaccines, we reasoned that it would be important to better understand their interactions with human leukocytes, including dendritic cells (DCs). DCs are professional antigen-presenting cells (APCs) that play a vital role in shaping adaptive immunity. DC maturation begins when endogenous or exogenous danger molecules are recognized by pattern recognition receptors [e.g., Toll-like receptors (TLRs)] triggering upregulation of costimulatory molecules and production of immune polarizing cytokines (17). Of note, in addition to interactions \textit{via} TLR4, \textit{N. meningitidis} LPS also directly interacts with DCs through the C-type lectin receptor DC-SIGN and modulates their function (18).

In this study, we benchmarked novel candidate MenB vaccine formulations against licensed vaccines, including Bexsero, using physiologically relevant human neonatal and adult whole blood (WBA) and monocyte-derived dendritic cell (MoDC) \textit{in vitro} culture systems, both of which employ age-specific autologous plasma, a rich source of age-specific immunomodulatory molecules (2, 19). We assessed vaccine-induced production of Th-polarizing cytokines, upregulation of surface activation markers (20), and production of potential vaccine reactogenicity biomarkers such as IL-1$\beta$ and prostaglandin E2 (PGE$_2$) (3, 21, 22). We found that a meningococcal vaccine comprised of attenuated LPS-based OMVs with a relatively low inflammatory profile toward human leukocytes \textit{in vitro} induced robust antigen-specific immunogenicity \textit{in vivo}. Our observations, may inform translational development of MenB vaccines in high-risk populations, such as newborns and infants.

MATERIALS AND METHODS

Ethics Statement

Peripheral blood samples were collected after written informed consent from healthy adult volunteers with approval from the Ethics Committee of Boston Children’s Hospital, Boston, MA, USA (X07-05-0223). Non-identifiable cord blood samples were collected immediately after elective cesarean section delivery (epidural anesthesia) of the placenta. Births to HIV-positive or febrile mothers were excluded. Human experimentation guidelines of the U.S. Department of Health and Human Services, the Brigham and Women’s Hospital, Beth Israel Medical Center, and Boston Children’s Hospital were observed, with approval...
from the Ethics Committee of The Brigham and Women’s Hospital, Boston, MA, USA (protocol number 2000-P-000117) and Beth Israel Deaconess Medical Center Boston, MA, USA (2011P-000118). Mice were obtained from Envigo (Indianapolis, IN, USA), and studies were approved by the Janssen Research and Development, LLC Institutional Animal Care and Use Committee.

**Human Blood Processing and In Vitro Stimulation**

Human blood was anti-coagulated with 20 U/ml pyrogen-free sodium heparin (American Pharmaceutical Partners, Inc., Schaumberg, IL, USA). All blood products were kept at room temperature and processed within 4 h from collection. Whole blood (WB) was mixed 1:1 with pre-warmed (37°C) RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) and 180 μl plated in a 96-well U-bottom plate (Becton Dickinson, Franklin Lakes, NJ, USA) containing 20 μl of 10x freshly prepared treatment, as described previously (23).

**Human MoDCs**

Blood was layered onto Ficoll-Hypaque gradient (GE Healthcare, Waukesha, WI, USA) to collect cord blood mononuclear cells (CBMCs) or peripheral blood mononuclear cells (PBMCs). Monocytes were isolated by positive CD14 selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). Preparations were routinely >95% pure as assessed by flow cytometry (23–26).

**Production of OMVs and Characterization**

Outer membrane vesicles were provided by Janssen Vaccines and Prevention B.V. Briefly, OMVs were produced from *N. meningitidis* H44/76 (B:P1.7,16;F3-3) WT or derivatives. nOMVs were produced from a H44/76 ΔRΔL strain, resulting in a pentaacylated meningococcal LPS from the lpxL1 mutant *N. meningitidis* strain, lacking the secondary C12:0 acyl chain at the 2’-position of the lipid A. Detergent-extracted OMVs were produced from the WT strain or the ΔRΔL mutant (28). Adsorption to aluminum hydroxide (Sigma-Aldrich) was performed, as described elsewhere (29). Before use, OMVs were diluted in 10 mM Tris pH 7.4/3% (w/v) sucrose to 50 μg total protein/ml. OMV size was determined by dynamic light scattering (DLS) using the Zetasizer nanoseries (Malvern Nano-ZS, 1/4/532 nm, Westborough, MA, USA).

**In Vivo Immunogenicity**

Adult Balb/c mice (6–8 weeks, female, 10/group) were immunized with two doses of OMVs (2.5 μg total protein/dose), two doses of Bexsero [at 2.5 μg total protein/dose (equivalent to 1/10th human dose)], or buffer control, subcutaneously in the scruff of the neck. Doses were administered 4 weeks apart, with terminal bleed taken 2 weeks after the second dose. Serum bactericidal assays (SBAs) were performed, as described previously (30), against the WT H44/76 strain with sera from individual mice using baby rabbit sera (Cedarlane, Burlington, VT, USA) as an exogenous complement source. The H44/76 strain was chosen as our OMV formulations were produced from a H44/76 ΔRΔL (15, 16, 27), resulting in

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**Flow Cytometry**

Monocyte-derived dendritic cells were treated with blocking agent for 10 min (Sigma-Aldrich), transferred to staining buffer [1x PBS, 0.5% (v/v) human serum albumin] and stained for 30 min at 4°C in the dark (1 x 10^6 cells/staining) with fluorophore-labeled antibodies [CD80/Fluorescein isothiocyanate/Clone L307.4, CD86/phycocerythrin/Clone 2331, CCR7/V450/Clone 150503, and HLA-DR/PerCP-Cy5.5/Clone L243 (BD Biosciences)]. Cells were then centrifuged (500 x g, 10 min), washed, fixed [1% (v/v) paraformaldehyde], and acquired using a LSRII flow cytometer employing FACSDiva software (BD Biosciences). Data were analyzed using the FlowJo (Tree Star, Inc., Ashland, OR, USA). Typically, 10,000 events per sample were acquired.

**Statistical Analyses and Graphics**

Statistical significance and graphs were generated using Prism v. 5.0b (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). For data analyzed by normalization to control values, column statistics were conducted using the two-tailed Wilcoxon signed-rank test or unpaired Mann–Whitney test as appropriate. Results were considered significant at *p* < 0.05 and indicated as follows: *p* < 0.05, **p < 0.01, ***p < 0.001, ‘*p < 0.05, ‘**p < 0.01, ‘***p < 0.001, ‘*p < 0.05, ‘**p < 0.01, and ‘***p < 0.001.

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RESULTS

OMVs Incorporating Genetically Attenuated Endotoxin Have Reduced Cytokine Induction Potential in Human Newborn and Adult Blood

Wild-type OMVs were produced from *N. meningitidis* H44/76 (B:P1.7,16:F3-3), while nOMVs (Figure 1A), incorporating a genetically attenuated version of endotoxin, were produced from a H44/76 ΔRΔL mutant strain (16, 27). All OMVs were characterized by DLS for particle size (Figure 1B; Figure S1 in Supplementary Material), with all formulations falling within a range of 80–120 nm in diameter. Next, we tested the ability of (1) the commercially available Bexsero vaccine, (2) detergent-extracted WT OMV (WT dOMV) (i.e., a Bexsero-like OMV formulation), (3) ΔRΔL mutant nOMV strain (ΔlpxLI nOMV) (28), and (4) a ΔlpxLI dOMV formulation (Figure 2A), to induce titration-dependent cytokine production in human neonatal and adult blood (Figures 2A,B). Non-detergent treated WT OMVs containing WT LPS were not tested as these are known to be highly cytotoxic. As indicated by measurement of TNF and IL-1β, both Bexsero and the WT dOMV robustly activated neonatal cord and adult peripheral blood in a titration-dependent manner in all concentrations tested, where levels of these two mediators were significantly increased over baseline (p < 0.001). The mutant OMVs also significantly induced production of TNF and IL-1β, but only at the highest concentration in newborn blood (p < 0.05) (Figures 2A,B; Figure S2 in Supplementary Material). When compared to Bexsero at the highest equivalent volume-to-volume (v/v) dilution tested (1:10), ΔlpxLI nOMV demonstrated reduced TNF production in neonatal (p < 0.001) and adult (p < 0.01) blood. ΔlpxLI nOMV were also less effective in inducing IL-1β production in newborn (p < 0.05) and WBA (p < 0.01). Similar trends were observed when comparing the ΔlpxLI nOMV to WT dOMV.

We next broadened our characterization of the ability of WT dOMV, ΔlpxLI dOMV, and ΔlpxLI nOMV (at v/v 1:1000–1:10) to induce concentration-dependent cytokine production from newborn and WBA using multiplexing assays (Figures 2C,D; Figure S3 in Supplementary Material). When compared head-to-head, the ΔlpxLI dOMV and ΔlpxLI nOMV induced markedly lower cytokine production profiles as compared to the WT dOMV, especially at the lowest concentration tested. The addition of Alum, the most commonly used adjuvant worldwide, and a component of the Bexsero vaccine to the OMV formulations altered the OMV (both wild type and mutant) induced innate cytokine production profiles (Figures S3 and S4 in Supplementary Material). Most notably, Alum reduced the ability of the WT dOMV to induce IFNγ and the chemokine CXCL10 (interferon inducible 10) (Figures S5A,B in Supplementary Material).

**ΔlpxLI nOMV Mature Human Dendritic Cells without Bexsero-Associated Inflammatory Profile**

To further characterize the innate immune effects of OMV formulations, 96-well human MoDC-based arrays were generated after culturing CD14 selected monocytes with IL-4 and GM-CSF in the presence of autologous plasma, a rich source of age-specific soluble immunomodulatory factors (19), as described previously (23, 25). In response to Bexsero, a strong upregulation of CD80, HLA-DR (MHCII), and CCR7 and a modest increase in CD86 were observed in both newborn and adult DCs (Figures 3A,B). Both the ΔlpxLI nOMV and WT dOMV (with or without Alum) produced slightly reduced DC maturation profiles as compared to the Bexsero. Interestingly, the Alum-adjuvanted ΔlpxLI nOMV formulation induced a similar upregulation profile to Bexsero in adult but not newborn MoDCs. Titration-dependent induced MoDC cytokine induction (Figure 3C) supported the flow cytometry results and mostly mirrored those observed in WB. When compared head-to-head, the mutant OMV formulations induced lower cytokine production profiles as compared to the WT formulations, especially at the lowest concentration tested. Interestingly, as seen in the WBA, the addition of Alum selectively enhanced WT dOMV-induced IL-1β production from human DCs (Figure 3D). Moreover, the addition of Alum to the ΔlpxLI
nOMV formulation only induced a slight increase in IL-1β from newborn (Figure 3D; Figure S6A in Supplementary Material) and adult DCs (Figures 3E,F; Figure S6B in Supplementary Material) at the highest concentration tested (1:10 v/v).

**ΔlpxLI nOMV Are Less Inflammatory toward Human Leukocytes than the Majority of Pediatric Vaccines**

To gain further insight into the inflammatory potential of the ΔlpxLI nOMV formulation, we next benchmarked it against a number of conventionally licensed pediatric vaccines (Table S1 in Supplementary Material) in both WB and MoDC assays. We have previously demonstrated that several of these licensed vaccines, such as the EasyFive (DTwP-HepB-Hib), induce distinct reactogenicity biomarker profiles in vitro (31–33). Of note, newborn DCs demonstrated significantly reduced ΔlpxLI nOMV-mediated PGE2 responses as compared to both Bexsero (p < 0.01) and WT dOMV (with Alum) (p < 0.05) (Figure 5A). A similar pattern was observed for adult DCs, but with significance only observed between Bexsero and ΔlpxLI nOMV (p < 0.01) (Figure 5B). Newborn (Figure 5C) and adult DCs (Figure 5D) responses to ΔlpxLI nOMV were also benchmarked against conventional licensed pediatric vaccines (at 1:10 v/v, Tables S1 and S2 and Figure S8 in Supplementary Material) in the MoDC array. Here,
we focused on the correlation of both PGE2 and IL-1β production, as co-production of both in human monocytic assays may predict rabbit pyrogenicity (i.e., fever) in vivo (33). Overall, a common trend was observed. The bacillus Calmette–Guérin (BCG) vaccine, Bexsero and WT dOMV consistently induced the highest production of both PGE2 and IL-1β. ΔlpxLI nOMV conversely induced a lower MoDC PGE2/IL-1β profile than WT OMV-based vaccines, suggesting similarity to low reactogenicity pediatric vaccines such as PCV13 and HBV (Figures 5C,D).

**ΔlpxLI nOMV Dissociates Inflammation from Immunogenicity**

Having characterized the relatively low reactogenicity potential of ΔlpxLI nOMV toward human leukocytes in vitro, we next...
assessed the ability of ΔlpxLI nOMV to induce immunogenicity in mice in vivo. Four cohorts of 10 mice each (Figure 6) were immunized subcutaneously with Tris/Sucrose (buffer control), ΔlpxLI nOMV or WT dOMV (2.5 µg total protein/dose, dOMVs formulated with Alum), or a 1/10th dose of Bexsero (equivalent to 2.5 µg dOMV). Two doses were administered 4 weeks apart, with blood collected 2 weeks after the second dose to obtain serum for SBA. The buffer control alone failed to induce meningococcal bactericidal antibody responses. In marked contrast, Bexsero (p < 0.05), WT dOMV (p < 0.01), and ΔlpxLI nOMV (p < 0.01) all induced robust and significant antibody responses as compared to baseline (Figure 6). Remarkably, even though ΔlpxLI nOMVs demonstrated reduced inflammation potential toward human leukocytes in vitro, there was no significant difference in immunogenicity as compared to either Bexsero or WT dOMV.

**DISCUSSION**

The majority of global immunization schedules are pediatric, with particular focus on newborns and young infants, yet most vaccine discovery programs do not rationally design vaccine formulations for use in humans in early life. Indeed, even when employed, in vitro modeling of human responses often occurs in late stage vaccine development. Such current pre-clinical approaches may contribute to vaccine formulations inducing sub-optimal responses in the very young (3). To more completely evaluate an OMV-based *N. meningitidis* vaccine, we took a rational vaccine design approach, in which we combined a relevant in vivo model and age-specific human in vitro culture systems that together may better model OMV-induced innate immunomodulatory capacity and immunogenicity potential. Overall, when compared to the WT detergent-extracted OMV (WT dOMV), mutant detergent-extracted OMV (ΔlpxLI dOMV), and multiple licensed vaccines with respect to innate signaling toward human newborn and adult leukocytes in vitro, a prototype ΔlpxLI nOMV vaccine generated a pattern of response suggestive of low reactogenicity potential while still demonstrating robust immunogenicity in mice in vivo. Demonstration of non-inferior in vivo immunogenicity of the ΔlpxLI nOMV vaccine, as compared to Bexsero, is noteworthy, especially as the in vitro DC maturation profiles are divergent. Such a result may indicate that the inclusion of native endotoxin molecules into traditionally designed OMV-based vaccines may not be as essential for immunogenicity as often assumed.

A key concern regarding modern vaccine development is reactogenicity (33), the propensity of a formulation to cause acute inflammatory events either locally, such as erythema or tenderness...
at the injection site, or systemically, such as fever. In this context, in vitro assays that provide potential reactogenicity biomarker data may be highly advantageous in de-risking formulation selection for in vivo use (21). By employing an in vitro human DC array, we benchmarked the immunomodulatory abilities of an ΔlpxLI nOMV-based vaccine against several licensed pediatric vaccines. DCs are logical targets for such in vitro studies as they efficiently process antigens for induction of immunity against pathogens and their components (34). The few published studies that assessed the activating effects of OMV on human leukocytes in vitro (35–40) evaluated fixed concentrations with limited cytokine measurements. One study evaluated concentration-dependent OMV responses in human WB and PBMC assays, solely from adult donors (39). Accordingly, our study is the first to comprehensively investigate (a) the ontogeny of OMV-induced immune responses and (b) the responses of human DCs to OMVs as benchmarked against licensed vaccines. ΔlpxLI nOMV induced cytokine responses significantly lower than those induced by either Bexsero or licensed vaccines (i.e., PedvaxHIB and EasyFive). As demonstrated by the stronger induction of costimulatory molecule expression by the ΔlpxLI nOMV with Alum in adult but not newborn MoDCs, the dramatically enhanced human DC IL-1β production in response to Alum-adjuvanted WT dOMV, as well as our prior studies demonstrating distinct ontological effects of Alum on in vitro DC IL-1β production (24), it is reasonable to suggest that our age-specific in vitro systems may be useful tools with respect to characterizing candidate vaccine formulations with or without Alum.

Our study has multiple strengths including (a) robust age-specific human in vitro modeling, (b) extensive benchmarking to multiple licensed vaccines, and (c) confirmation of immunogenicity in vivo. That said, our study also has some limitations including (a) the unavoidable use of different species for in vitro (human) and in vivo (mouse) studies, (b) that in vitro systems, no matter how carefully developed, are imperfect models for responses in vivo, (c) the lack of full concordance in the antigens profile found in Bexsero- which contains added recombinant protein- and our ΔlpxLI nOMV formulations that do not, and (d) the lack of non-detergent treated- WT strain-derived OMVs that contain WT LPS. While such a formulation was initially not evaluated as part of our current study due to the known cytotoxic effects in humans, based on our results, such WT OMVs may provide a benchmark in future studies employing human in vitro systems. Of note, our experiences to date with in vitro benchmarking suggest that our in vitro studies provide insights relevant in vivo (2). Additionally, studies evaluating OMV vaccine-based reactogenicity in vivo using similar strategies to ours in mice (41) and humans (42) support these conclusions.

Several aspects of our results will prompt future investigations. For example, nucleotide-binding oligomerization domain (NOD)-like receptor-mediated IL-1β production is greater in human newborn cord than adult peripheral blood (43, 44) and slowly decreases to adult levels over the first years of life (45). A similar phenomenon for basal PGE2 levels in WB has been
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00562/full#supplementary-material.

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