Improving the immunogenicity of a trivalent *Neisseria meningitidis* native outer membrane vesicle vaccine by genetic modification

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Trivalent native outer membrane vesicles (nOMVs) derived from three genetically modified *Neisseria meningitidis* serogroup B strains have been previously evaluated immunologically in mice and rabbits. This nOMV vaccine elicited serum bactericidal activity (SBA) against multiple *N. meningitidis* serogroup B strains as well as strains from serogroups C, Y, W, and X. In this study, we used trivalent nOMVs isolated from the same vaccine strains and evaluated their immunogenicity in an infant Rhesus macaque (IRM) model whose immune responses to the vaccine are likely to be more predictive of the responses in humans. IRMs were immunized with trivalent nOMV vaccines and sera were evaluated for exogenous human serum complement-dependent SBA (hSBA). Antibody responses to selected hSBA generating antigens contained within the trivalent nOMVs were also measured and we found that antibody titers against factor H binding protein variant 2 (fHbpv2) were very low in the sera from animals immunized with these original nOMV vaccines. To increase the fHbp content in the nOMVs, the vaccine strains were further genetically altered by addition of another fHbp gene copy into the *porB* locus. Trivalent nOMVs from the three new vaccine strains had higher fHbp antigen levels and generated higher anti-fHbp antibody responses in immunized mice and IRMs. As expected, fHbp insertion into the *porB* locus resulted in no PorB expression. Interestingly, higher expression of PorA, an hSBA generating antigen, was observed for all three modified vaccine strains. Compared to the trivalent nOMVs from the original strains, higher PorA levels in the improved nOMVs resulted in higher anti-PorA antibody responses in mice and IRMs. In addition, hSBA titers against other strains with PorA as the only hSBA antigen in common with the vaccine strains also increased.

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1. Introduction

*Neisseria meningitidis* is a Gram-negative diplococcus and an obligate human pathogen. Meningococcal strains can be divided into multiple serogroups based on chemically and antigenically distinct polysaccharide capsule expression. Six capsular polysaccharide serogroups of *N. meningitidis* (A, B, C, W, X, and Y) cause nearly all cases of invasive meningococcal disease [1]. These infections have different clinical consequences including meningitis and septicemia. For serogroups A, C, Y and W, capsular polysaccharide vaccines [such as Menomune® [2]] and capsular polysaccharide conjugate vaccines [such as Menactra® [3]] have been licensed, based on the induction of a functional antibody response only (serum bactericidal activity, SBA) [4]. A polysaccharide approach is not being pursued for serogroup B (MenB) due to poor immunogenicity and potential cross-reactivity between the MenB polysaccharide capsule and human tissue antigens [5–7]. Vaccine development efforts for MenB have therefore focused on non-capsular polysaccharide immunogens, particularly outer membrane proteins either in the form of purified recombinant proteins or outer membrane vesicles (OMVs) which are prepared from outer membrane “blebs” spontaneously produced by the bacteria. Recently, two recombinant protein containing MenB vaccines, Bexsero® and Trumenba®, have been approved [8,9].

The meningococcus has an outer membrane which contains numerous protein types. Some of these proteins are used for further classification of meningococci into serotypes and sereosubtypes, based on antigenic differences in their major outer membrane proteins, PorB and PorA, respectively [10,11]. The
lipopolysaccharide (LOS), embedded in the outer membrane, determines 12 different immunotypes [10,12]. OMV-based vaccines have the advantage of including several outer-membrane proteins in properly folded conformations which might help to elicit a protective immunological response. OMVs may be prepared by known methods comprising a detergent extraction of the bacterial cells, which has the benefit of reducing LOS endotoxin and most of the capsular polysaccharide from the vaccine. However, detergent extraction also reduces or eliminates conserved protective lipoproteins such as factor H binding protein (fHbp) [13]. In children under two years of age, OMV vaccines produced from detergent extraction generate a predominately PorA-specific SBA response and will thus only protect against strains with the same PorA serosubtype [14]. Therefore, vaccines based on extracted OMVs from epidemic outbreak MenB strains were used with success to control local clonal outbreaks [15–18] but did not offer broad protection against heterologous strains with different PorA subtypes. This lack of broad protection restricts the ability of these OMVs to be used as a universal MenB vaccine.

Alternatively, native OMV (nOMV) have been purified from meningococci without detergent extraction [19–21]. These vesicles are not depleted of LOS or lipoproteins and the outer membrane proteins are likely to be present in their native conformation. Zollinger et al. designed a genetically engineered trivalent nOMV vaccine from three parent strains H44/76, 8570 and B16B6 [21] (Table 1). The vaccine was designed to include multiple outer membrane antigens such as PorA, fHbp, NadA, Opc and LOS, each with the capability to induce SBA. The trivalent nOMV vaccine was immunogenic in mice and rabbits, and elicited functional SBA antibody responses against strains from not only MenB, but also other serogroups, due to conserved cross-reactive antigens [21–23]. A small phase I study in healthy adults with a monovalent nOMV vaccine (from strain B2) was also conducted and the vaccine was shown to be safe and immunogenic [24]. However, the trivalent nOMV vaccine has not been tested in either humans or non-human primates.

To enhance safety of the trivalent nOMV vaccine, the lpxL1 gene was deleted from the vaccine strains, resulting in mutant LOS with penta-acylated lipid A which has attenuated endotoxin activity [21]. Previous studies have shown that mouse dendritic cells were stimulated by the penta-acylated LOS, and the cytokine responses might result in a potent adjuvant effect [25], where human dendritic cells showed marked decreased responses to penta-acylated LOS [26]. The adjuvant effect of penta-acylated LOS in immunized non-human primates were much lower and likely to be similar to that in humans, suggesting that a non-human primate model might be more suitable to predict the antibody responses to these modified nOMV vaccines in humans [27]. Because human infants are the primary target for a MenB vaccine, we sought to evaluate the immunogenicity of this trivalent nOMVs vaccine in an infant non-human primate model. Infant Rhesus macaques (IRMs) were immunized with trivalent nOMV vaccines and sera were evaluated for hSBA and antigen-specific antibody responses. We found that antibody responses against fHbp variant 2 (fHbpv2) were very low, even though the vaccine strain B3 was genetically engineered to increase its expression level [21]. Therefore, we further genetically modified the vaccine strains by adding another fHbp gene copy into the porB locus of each vaccine strain to increase the fHbp content in all the three nOMVs. We showed here that genetic modification can further improve the immunogenicity and breadth of the nOMV MenB vaccines.

2. Material and methods

Details on material and methods can be found in Supplemental material section.
Table 2
Serum bactericidal antibody titers induced by the original trivalent nOMV vaccines in infant Rhesus macaques.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Number of animals</th>
<th>Immunogen</th>
<th>Test strain</th>
<th>44/76 hSBA geomean titers</th>
<th>ED50 ELISA Titters, Log10</th>
<th>Sero-conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>9</td>
<td>Trivalent nOMV/AIPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td></td>
<td>13</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Group 2</td>
<td>9</td>
<td>Trivalent nOMV/AIOH</td>
<td></td>
<td>14</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>Group 3</td>
<td>9</td>
<td>Trivalent nOMV/no adjuvant</td>
<td></td>
<td>5</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>Group 4</td>
<td>9</td>
<td>Trivalent nOMV/AlOH</td>
<td></td>
<td>15</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sero-conversion = >4 fold rise in hSBA titer for post vs. pre immunization sera.

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3. Results

3.1. Immunogenicity of a trivalent nOMV vaccine in IRMs

We intramuscularly immunized three groups of IRMs (2–4 months of age at the start of the study) with the prepared trivalent nOMV vaccines on weeks 0, 8 and 16. Groups of IRMs were given nOMV vaccines without adjuvant or with an aluminum adjuvant, at a dose of 37.5 µg of total nOMV proteins. Serum was collected on week 20 and evaluated in hSBA and antigen-specific ELISA titers. The trivalent nOMV vaccines were immunogenic in IRMs and elicited hSBA (Table 2). We showed that with an aluminum phosphate adjuvant (AIPO<sub>4</sub>), IRMs were 100% sero-converted against all three vaccine parent strains. Animals receiving nOMV vaccines with aluminum hydroxide adjuvant (AIOH) were 100% sero-converted against two of the parent strains, and only one out of nine animals did not generate hSBA antibody against the 44/76 strain. nOMVs without adjuvant elicited lower immune response, as expected. In the phase 1 clinical trial, the monovalent nOMV vaccine formulated with AIOH adjuvant was shown to be safe in healthy adults [24]. Our IRM data suggested that an adjuvant such as an aluminum adjuvant might be necessary for use with this vaccine in infants.

We next evaluated antibody responses against individual vaccine antigens including PorAs, NadA, fHbpv1 and fHbpv2, as those antigens were either added or designed to be over-expressed in the vaccine strains. We observed significant serum antibody titers in immunized animals (group 1, nOMV with AIPO<sub>4</sub>) against all six PorAs, as well as NadA and fHbpv1 (Fig. 1), suggesting that these antigens are presented in the nOMV vaccines at immunogenic levels. Using a quantitative mass spectrometry (QMS) method [28], we determined that nOMV from strain B2 consists of ~0.53% of fHbpv1 antigen while nOMVs from strain B3 consists of only ~0.13% of fHbpv2 antigen (Fig. 2B). Consistently, the antibody response against fHbpv2 in the immunized IRM sera was much lower compared to the response to fHbpv1 (Fig. 1). The lower antibody responses against fHbpv2 might also result from sequence differences between the two fHbp types. Zollinger et al. reported increased anti-NadA and anti-fHbpv1 antibody titers in mice immunized with genetically engineered nOMVs, compared to the unmodified strains, however, they did not report increased anti-fHbpv2 antibody responses in mice [21]. A previous study suggested that a critical threshold of fHbp expression is required in broad protective antibody responses elicited by nOMV vaccines [29], therefore, we sought to further genetically modify the vaccine strains to increase the expression level of fHbpv2.

3.2. Genetic modification and verification

PorB protein is the most abundant protein on the surface of all three nOMVs (Fig. 2C, for strain B3; data not shown for strains B1 and B2). Although PorB does induce SBA antibodies in an SBA assay using baby rabbit serum as the source of exogenous complement [30], it appears to be less immunogenic, especially in the infant population [14,31,32]. Therefore, it might be dispensable in the vaccine strains. We chose to introduce an fHbpv2 gene (fHbpv2.16) into the porB locus of both strain B1 and strain B3 by homologous recombination. As fHbpv1 has been shown to be an important vaccine antigen and is included in both of the licensed MenB vaccines, Bexsero<sup>a</sup> and Trumenba<sup>a</sup>, we also introduced an fHbpv1 gene (fHbpv1.1) similarly into the porB locus of strain B2 to further increase its expression level. The final genetically modified vaccine strains are designated as NB1, NB2 and NB3 (Table 1). These improved vaccine strains grow well in cell culture medium, suggesting the porB gene is not required for fitness of the bacteria in vitro and these strains are suitable to use for vaccine manufacture.

We evaluated surface expression of the antigens in these improved vaccine strains using a whole cell flow cytometry antibody binding assay (Fig. 2A). As expected, we detected no surface expression of PorB in the improved NB3 strain, as opposed to the original B3 strain. We also obtained much higher levels of fHbpv2 for the improved NB3 strain, compared to the original strain. These data verified the genetic modification we performed and results for the NB1 and NB2 strains were similar (data not shown). We next purified and characterized nOMVs derived from these improved vaccine strains, and measured fHbp levels in the purified nOMVs using QMS [28]. Consistent with our whole bacteria antibody binding data, the improved nOMVs indeed consist of much higher levels of fHbp antigens (Fig. 2B), suggesting that exogenous genes
are expressed to a high level from the porB locus. On a SDS–PAGE gel (Fig. 2C), nOMV from the NB3 strain clearly shows the absence of the PorB band, as compared to the nOMV from the original strain. Interestingly, it appeared that the amount of PorA antigen was increased in the NB3 strain. To verify this finding, we performed whole cell flow cytometry antibody binding experiments on strains B3 and NB3 using antibodies against PorAs and indeed observed higher surface expression levels of both native (nPorA) and recombinant (rPorA) PorAs (Fig. 2A). Similarly, NB1 and NB2 strains also contain increased levels of PorA expression (data not shown).

3.3. Immunogenicity of the improved trivalent nOMV vaccine in animals

nOMVs from the original vaccine strains or the improved strains were formulated with AAHS and evaluated individually as monovalent vaccines in CD-1 mice. Immunized mouse sera were tested in ELISA assays for antigen specific antibody responses (Fig. 3). Overexpression of fHbpv2 in the porB locus resulted in at least 2–3 logs higher antigen-specific antibody titers in mice immunized with either NB1 or NB3 nOMV vaccine. nOMV from the original strain B2, with an fHbpv1 level of ~0.53% of total nOMV protein, already induced high anti-fHbpv1 antibody titers. However, we showed that overexpression of fHbpv1 in the porB locus further increased the antibody response about 3 fold higher. Although the amount of fHbpv1 and fHbpv2 are similar in the improved strains as measured by QMS, fHbpv1 was able to elicit at least 10 fold higher antibody titers in CD-1 mice. The reason for this
difference is not clear, but one possibility is that the sequence differences might render fHbpv1 more immunogenic in mice.

Our data suggested that the improved nOMV vaccines consisted of higher levels of both nPorA and rPorA. As nPorAs at their normal levels presented in OMV vaccines are able to elicit functional SBA antibodies, we hypothesized that the increased amount of nPorAs in our vaccine strains would elicit sufficient anti-nPorA bactericidal antibodies. Thus, we focused on evaluation of immunogenicity of rPorAs, P1.7-1, 1 in NB1, P1.22, 14 in NB2 and P1.22-1, 4 in NB3, that were introduced into these strains by genetic modification (Table 1, [21]). We showed here that all three improved vaccine strains derived in the present study resulted in higher levels of anti-rPorA antibody responses (Fig. 3), consistent with the higher expression of NadA but was unable to be killed with serum generated from either mice or IRMs and with either the original nOMVs or the improved nOMVs, even though it does not match the PorA types contained in the vaccine strains.

Table 3

<table>
<thead>
<tr>
<th>Test strain</th>
<th>Antigens expressed by the test straina</th>
<th>hSBA titer</th>
<th>Original nOMVs</th>
<th>Improved nOMVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3576</td>
<td>P1.22-1, 4; fHbpv1</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>6557</td>
<td>P1.22-1, 14</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>M4720</td>
<td>P1.22, 14</td>
<td>16</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>1901</td>
<td>P1.18, 25</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td></td>
</tr>
</tbody>
</table>

a Only antigens with increased expression levels in the improved trivalent nOMVs are listed.
b This is the negative control strain, and its porA subtype is listed here even though it does not match the PorA types contained in the vaccine strains.

given against a few test strains, possibly due to either insufficient levels of antibodies (e.g. anti-fHbp2 as we showed here) and/or low level of antigen expression in the test strains [21]. Three of these strains were chosen to be tested here because they express surface antigens that are in common with the antigens of increased levels in our improved nOMV vaccine (Table 3). Strain 3576 expresses fHbpv1 and PorA subtype P1.22-1, 4 and strain M4720 expresses PorA subtype P1.22, 14 ([21] and data not shown), and the improved nOMV vaccine elicited higher antibody response to all three of these antigens in IRMs. Strain 6557 expresses PorA subtype P1.22-1, 4 (data not shown) – this PorA subtype is similar to P1.22, 14 and the two subtypes might be cross-reactive [33]. Strain 1901 was included as a negative control strain, as it does not have detectable surface expression of fHbp and has a PorA subtype that is different from the vaccine strains. As expected, there is no improvement on the hSBA titer against strain 1901. The improved nOMV vaccine elicited higher hSBA titers against two of the test strains (3576 and 6557) as compared to the original nOMV vaccine, suggesting that antigen specific functional antibody titers were indeed increased, even though the improvement was not dramatic as the total IgG titers observed. Increased antibody levels against fHbp and PorA might both contribute to the increase hSBA titer against strain 3576. Strain 6557 does not have detectable surface expression of fHbp [21], therefore, increased anti-PorA antibody response is the only driver for the higher hSBA titers. However, we did not observe an increased hSBA titer against strain M4720, in contrast with the result obtained for strain 6557, although both strains express P1.22, 14 or a variant of this subtype. It is not clear what results in this discrepancy but strain M4720 might be particularly difficult to kill, as it also has good surface expression of NadA but was unable to be killed with serum generated from either mice or IRMs and with either the original nOMVs or the improved nOMVs, even though the vaccines induced significant anti-NadA antibody response (Fig. 1 and [21]).

4. Discussion

The original trivalent nOMV vaccine was designed to induce broad cross protection against MenB strains [21]. The vaccine has multiple surface antigens, therefore, it could avoid dependency on a single antigen or antigen type and potentially induce synergy of antibodies to different antigens in cases where the surface expression level of a particular antigen on the N. meningitidis strain is low [34,35]. Bactericidal titers in immunized rabbits are relatively low, compared to those in immunized mice for both the homologous MenB strains and the non-serogroup B strains [21–23]. This difference could result from species differences, suggesting that the titers in humans may be different from those in either mice or rabbits. Human infants are the primary target population for a MenB vaccine and a non-human primate model might be more suitable than a mouse model to predict the antibody responses to these modified nOMV vaccines in humans [25–27]. Our IRM immunogenicity data showed that the trivalent nOMV vaccines were immunogenic in IRMs and induced functional antibody responses. hSBA titers in IRMs are lower compared to those in mice [21,22], however, 100% sero-conversion was achieved with an aluminum adjuvant. This data, together with the data from a phase I trial of a monovalent nOMV vaccine in healthy adults [24], suggest that the nOMV vaccine with an appropriate adjuvant might generate effective hSBA titers in human infants.

In an initial evaluation of the antigen specific antibody responses, we found that serum antibody response against fHbp2 was low in immunized IRMs. In both of the recently approved MenB vaccines, Trumenba™ and Bexsero™, fHbp was primarily responsible for inducing a broadly protective antibody response.
Another minor outer membrane antigen, NadA, can elicit protective bactericidal antibodies and is included in Bexsero®. However, NadA is known to be phase variable and prone to frame-shift [36–38], and the NadA gene is found in certain clonal complexes and only present in a small subset of the strains analyzed [39]. Therefore, we sought to increase the fHbp content in the trivalent nOMVs by further genetic modification of the vaccine strains. By adding another fHbp gene copy into the porB locus, we have shown here that nOMVs from the improved vaccine strains possessed higher fHbp antigen levels and elicited higher fHbp specific antibody responses in both mice and IRMs.

We showed that genetic modification can further improve the immunogenicity and breadth of the nOMV MenB vaccines. The number of test strains used in this study is limited, given the limited volume of sera obtained from IRMs. These test strains were not chosen to represent the current circulating group B strains, but rather to test the effectiveness of the particular vaccine antigens. We did not observe increased hSBA titer against strain M4720, although it has a matched PorA subtype to our vaccine. Similarly, it was reported that strains with moderate to high NadA and/or NHBA (neisserial heparin-binding antigen) expression can be resistant to anti-NadA or anti-NHBA bactericidal antibodies elicited by 4CMenB vaccination [40]. A study involving a larger panel of MenB strains, as well as other serogroups, may offer a better insight of the potential of this experimental trivalent nOMV vaccine to induce a broadly protective antibody response. Previous studies have demonstrated that killing in the SBA assay can be predicted by the levels of surface expression of the antigens on the test strain [41,42]. McNeil et al. reported positive fHbp surface expression on about 60–70% of the strains tested using a flow cytometry based assay [43]. Previous studies showed positive fHbp expression on about 60–70% of the strains tested using MATS-ELISA [38,42]. The different percentages of strains that have positive fHbp surface expression might result from different strain collections, and/or different assays/reagents used. Nonetheless, increased fHbp levels in the nOMV vaccine would elicit better hSBA against the strains with positive surface expression of fHbp. For those strains with low fHbp surface expression, the trivalent nOMV vaccine with multiple outer membrane antigens would possibly offer broader strain hSBA coverage through other surface antigens. In addition to the previous study that showed the trivalent nOMV vaccine’s potential to elicit SBA against multiple serogroups [22], it was reported recently that an nOMV vaccine with overexpressed fHbp has greater potential to confer serogroup-independent protection in Africa than the licensed 4CMenB vaccine (Bexsero®), and inclusion of fHbpv2 into the nOMV vaccine would further improve strain coverage for serogroup C strains in certain areas of Africa [44]. Therefore, we speculate that the improved trivalent nOMV vaccine with higher fHbpv1 and fHbpv2 contents would potentially confer better hSBA killing against strains from multiple serogroups as well, compared to the original trivalent nOMV vaccine.

*N. meningitidis* expresses two distinct porins, PorA and PorB [45]. Both porins are β-barrel proteins, which associate into trimers in the bacterial outer membrane through which small hydrophilic nutrients diffuse into the cell. These porins also appear to have multiple roles in helping colonization and survival of meningococci in the blood [46]. Interestingly, we found that fHbp insertion into the *porB* locus resulted in higher PorA expression in all three modified vaccine strains and showed that the modified nOMV vaccine elicited better hSBA titers against a strain with PorA as the only hSBA antigen in common with the vaccine strains. It is not clear why the expression of PorA was increased with porB gene knockout. As both porins allow nutrient diffusion into cells, one hypothesis is that during in vitro cell culture following genetic modification, clones with higher PorA expression levels were positively selected to compensate for the loss of PorB. In addition, both nPorA and rPorA in the vaccine strains are under the control of their native promoters [21], therefore, porB gene knockout might result in up-regulation of both genes via similar signal transduction pathways.

A genetically optimized OMV approach might have utility for other bacterial pathogens as well [47]. We show here that the major surface antigen PorB can be replaced with a heterologous protein without affecting the fitness of the cells or the ability of cells to produce outer membrane vesicles. The strong *porB* promoter resulted in good surface expression of the heterologous antigens, therefore, this approach could be applied to generate other OMV based vaccines that express different antigens of interest.

### Authors’ contributions

LZ, ZW, JL, HK, PH, XMW, JTM, PLA, LD and RR performed the experiments. LZ, ZW, JTM, MJK and CTP analyzed the data. LZ and CTP wrote the manuscript. All authors critically reviewed the manuscript.

### Conflict of interest statement

All co-authors were employees of Merck & Co., Inc., Kenilworth, NJ, as indicated on the title page of the manuscript.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.05.049.

### References


