Genetic detoxification of an unencapsulated meningococcal vaccine strain enhances potency and cross-reactivity of outer membrane vesicle vaccine responses

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Abstract

Outer membrane vesicle (OMV) vaccines have been used to successfully quell meningococcal serogroup B (MenB) infections in epidemic settings. However, immunodominant responses to the PorA protein limit the potential for utilization of OMV vaccines to prevent invasive disease caused by MenB strains expressing PorA serosubtypes heterologous to the vaccine strain. We previously reported that OMVs isolated from a MenB strain (ΔABR) deleted for expression of PorA and two additional major outer membrane proteins (OMPs), PorB and RmpM, elicited functional antibodies in animals that were more cross-reactive, but less potent, than those induced by wild-type (WT) OMVs. Diminished potency was associated with a decrease in structural membrane integrity of ΔABR OMVs upon detergent detoxification, which is required to limit toxicity of lipooligosaccharide (LOS). Deletion of the acyl transferase LpxL1 has been reported to genetically detoxify LOS by preventing formation of the highly toxic hexa-acylated LOS structure, rendering detergent detoxification unnecessary. In an attempt to examine the impact of detergent detoxification on potency of OMV vaccines, we engineered a genetically-detoxified MenB strain, ΔABRL, that was deleted for expression of PorA, PorB, RmpM, and LpxL1. ΔABRL OMVs were isolated and used to immunize rabbits and mice; sera from animals were tested in human complement serum bactericidal assays (hSBAs) for functional antibody responses against a panel of up to 17 antigenically diverse MenB strains. Functional antibody responses were observed in a greater number of animals immunized with genetically-detoxified ΔABRL OMVs compared to those administered detergent-detoxified WT or ΔABRL OMVs. LpxL1 deletion was also associated with bactericidal activity against a greater number of MenB strains. The effects of LpxL1 deletion were greatly enhanced by additional deletion of the *siaD* gene, which rendered the vaccine strain (ΔABRSL) unencapsulated. Proteomics studies demonstrated that complexes formed when OMV antigens were immunoprecipitated by anti-ΔABRSL OMV sera vs. anti-ΔABRL OMV sera were significantly enriched for putative adhesins and other vaccine candidates, including NMB0586, NMB1125, and Opc. These studies suggest that: (1) deletion of LpxL1 and SiaD from the ΔABR vaccine strain increases OMV vaccine potency, and (2) the presence of residual capsular components in OMV vaccines diminishes serological responses to cross-reactive vaccine antigens, either by decreasing immune recognition of antigens or by dampening overall B cell responses.

Introduction

Detergent-detoxified OMVs (dOMVs) have been employed since the 1980s to quell MenB outbreaks in countries including Cuba, Chile, Norway, France, and New Zealand (1). Although multiple OMPs are expressed on the OMV surface and may initiate contact with the host immune system, the majority of the functional antibody response is directed against the highly variable protein PorA (2), limiting the utility of WT dOMVs in preventing disease caused by heterologous PorA-expressing strains. Deletion of PorA alone or in conjunction with other OMPs from MenB vaccine strains enhances elicitation of cross-reactive anti-dOMV antibodies via a mechanism that is not yet fully understood (3). We previously described the construction of a novel MenB strain, ΔABR, in which PorA was genetically deleted in conjunction with two other major OMPs, PorB and RmpM, by replacement with antibiotic resistance cassettes (4). Immunization of rabbits with ΔABR dOMVs elicited functional anti-MenB antibodies that were more cross-reactive, but of lower potency, compared to WT dOMVs (3). In addition, ΔABR dOMVs consistently and reproducibly enhanced gonococcal clearance in a murine vaginal infection model; significant clearance was only observed in one of two trials when mice were administered WT dOMVs, suggesting the presence of PorA and/or PorB/RmpM in dOMVs impairs strong anti-gonococcal responses (5).

In an effort to understand the basis for the differences in vaccine phenotypes, we previously performed characterization studies on the WT and ΔABR dOMVs. These studies showed that detergent detoxification resulted in a decrease in ΔABR OMV membrane integrity, with a significant loss of vesicular structure that may have contributed to diminished vaccine potency (3). Reports have suggested that detergent detoxification of MenB OMVs may be rendered unnecessary by deletion of genes involved in the lipid A biosynthesis pathway (6). One such gene, *lpxL1*, encodes an acyl transferase that promotes formation of the hexa-acylated lipid A structure (Fig. 1). The hexa-acylated LOS binds strongly to TLR4 and induces a robust pro-inflammatory response (7). In contrast, the less toxic penta-acylated structure is preferentially formed in the absence of *lpxL1*.

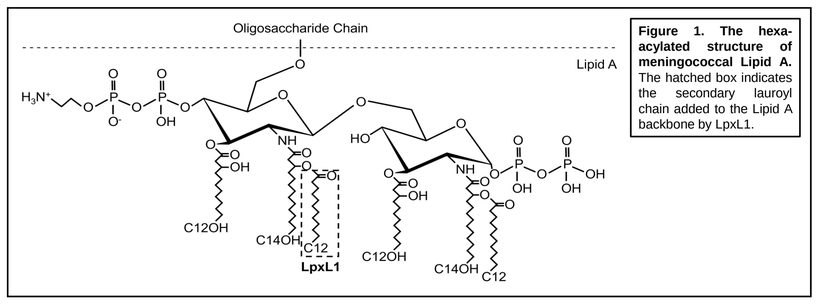

Figure 1. The hexa-acylated structure of meningococcal Lipid A. The hatched box indicates the secondary lauroyl chain added to the Lipid A backbone by LpxL1.

In this study, we sought to examine the impact of detergent detoxification on MenB OMV vaccine potency by engineering markerless deletions of *lpxL1* in conjunction with *porA*, *porB*, and *rmpM*; *siaD* was also deleted to assess the role of capsule on vaccine potency. In electing to use a markerless mutation strategy as opposed to insertional inactivation via antibiotic resistance cassettes, we were able to: (a) decrease the likelihood of generating polar mutations, (b) re-use the same mutation cassette backbone to create all of our gene deletions, and (c) obviate the requirement for addition of and assessment of residual antibiotics during OMV manufacturing.

Construction of Markerless ΔABR Mutant Strains

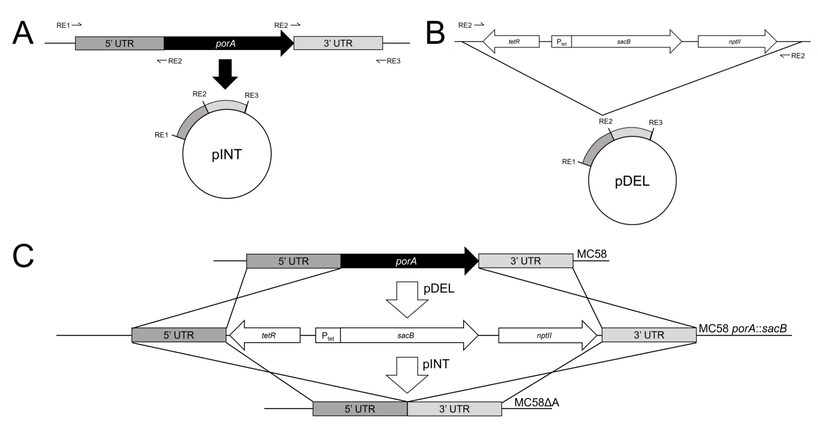

Figure 2. Diagram of the markerless mutation method. (A) Primer pairs engineered to express restriction enzyme digestion sites 1 and 2 (RE1/RE2) and sites 2 and 3 (RE2/RE3) were used to amplify the 5' and 3' untranslated regions (UTRs) upstream and downstream of the *porA* gene, respectively. RE1, RE2, and RE3 were used to clone the 5' and 3' UTR products sequentially into the intermediate plasmid pINT. (B) Primer pairs bearing the RE2 sequence were used to amplify the markerless mutation vector (8), which included genes encoding kanamycin resistance (*nptII*), the TetR repressor (*tetR*), and levansucrase (*sacB* under the control of the P_{tet} promoter). Under stochastic conditions, the *tetR* gene encodes the TetR repressor, which binds to the P_{tet} promoter, inhibiting transcription of *sacB*. In the presence of chlortetracycline (cTc), cTc will bind to TetR, relieving transcriptional inhibition and permitting production of levansucrase, which cleaves sucrose into a product (levan) that is toxic to Gram-negative bacteria. Following PCR of the full mutation cassette, the product was digested with RE2 and cloned into pINT, creating the deletion plasmid pDEL. (C) The WT strain MC58 was transformed with pDEL, replacing the native *porA* gene with the markerless mutation cassette. Clones were positively screened for *porA* deletion on BHI plates containing kanamycin. To generate the markerless mutation, new strain MC58 *porA*::*sacB* was transformed with pINT, creating MC58ΔA. Removal of the markerless mutation cassette was confirmed by negative screening on BHI plates containing cTc and sucrose, followed by colony PCR and whole genome sequencing. The process was repeated to delete genes *porB*, *rmpM*, *lpxL1*, and *siaD* as needed.

Confirmation of the Deletion Method

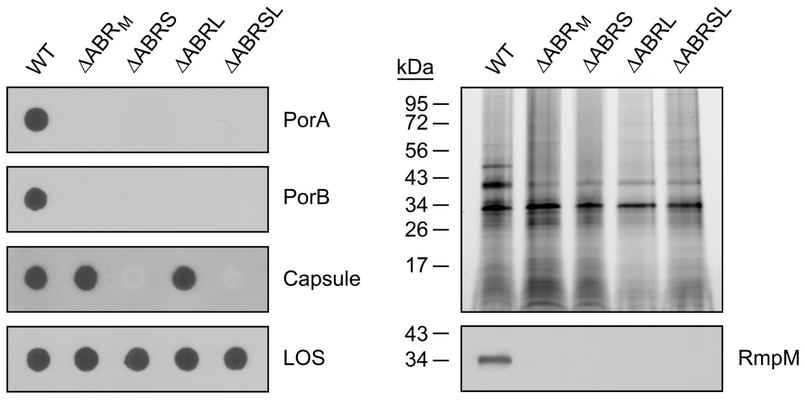

Figure 3. Immunoblots confirming loss of antigen expression. Whole cell lysates of the new markerless ΔABR strain (ΔABR_M) and the markerless ΔABR strains deleted for *siaD* (ΔABRS) or *lpxL1* (ΔABRL) alone, or for both *siaD* and *lpxL1* (ΔABRSL), were tested either in dot blots (left panel) or Western blots (lower right panel) for expression of PorA, PorB, capsule, LOS, and RmpM. Dot blots were probed with monoclonal antibodies specific for each of the antigens and RmpM expression was detected with polyclonal anti-WT dOMV antiserum. Immunoblots showed proper deletion of PorA, PorB, RmpM, and capsule in the expected mutants. The anti-LOS dot blot confirmed that *lpxL1* deletion did not impact production of the overall LOS structure. An image of the strains as fractionated by SDS-PAGE is shown in the upper right panel to confirm roughly equivalent loading of samples for the anti-RmpM Western blot.

Assessment of Mutant Strain Toxicity

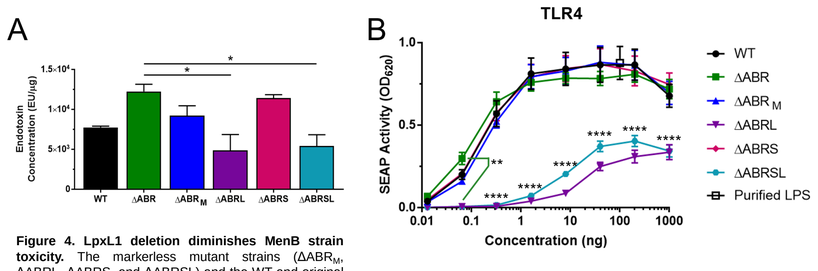

Figure 4. LpxL1 deletion diminishes MenB strain toxicity. The markerless mutant strains (ΔABR_M, ΔABRL, ΔABRS, and ΔABRSL) and the WT and original ΔABR strain (engineered by antibiotic replacement) were cultured in TSB medium; crude membrane extracts were prepared by passing the wet mass through a French press. Equivalent concentrations of membrane preparations were tested in (A) an LAL assay (Thermo Scientific™Pierce™) and (B-C) the HEK-Blue™ reporter cell line system (InvivoGen). For the HEK-Blue™ system, HEK cells overexpressing either TLR4 or TLR2 were incubated with increasing concentrations of the membrane preparations for 10 h or 8 h, respectively, where SEAP activity functioned as a readout of TLR stimulation. The ΔABRL and ΔABRSL preparations exhibited significantly lower toxicity as measured by TLR4 stimulation (B), but not by the LAL assay (A); no difference in TLR2 stimulation was observed (C). Data in A and B-C represent results from two and three independent experiments, respectively. *P < 0.05 for panel A and **P < 0.01 and ****P < 0.0001 for panel B by one-way and two-way ANOVA, respectively, with Tukey's multiple comparisons post test. For B, asterisks represent significance of ΔABRL and ΔABRSL datasets relative to all others unless otherwise noted.

OMV Isolation Method

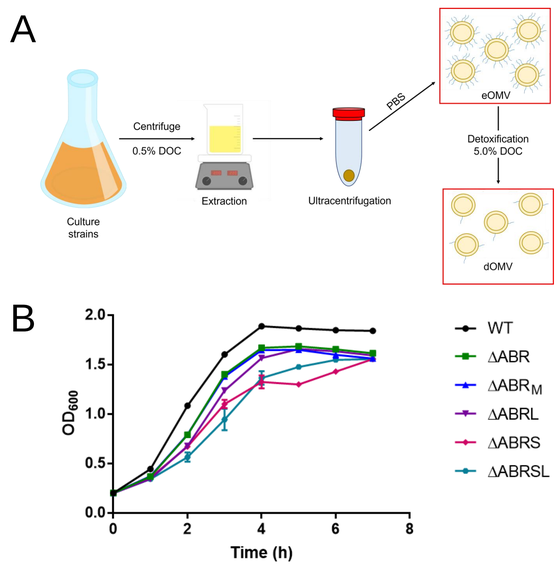

Figure 5. Methods for isolating the genetically-detoxified and detergent-detoxified OMVs. (A) Vaccine strains were cultured in two small batches of TSB for 6-8 h, at which time large batch subcultures were inoculated and incubated for ~16 h at 37°C with shaking. Following heat inactivation and centrifugation, the wet mass was recovered and mixed at room temperature with 0.5% sodium deoxycholate (DOC) in extraction buffer. After centrifugation, the supernatant was ultracentrifuged and extracted OMVs (eOMVs) were recovered and suspended in PBS. If detergent detoxification was required, eOMVs were suspended in detoxification buffer containing 5.0% DOC. The dOMVs were recovered by ultracentrifugation and suspended in PBS. (B) A growth curve over the approximate time course of the primary culture is shown for the WT, original ΔABR, and markerless mutant strains. Data represent results from three independent experiments.

Pilot dOMV Rabbit Immunogenicity Study

	ΔABR dOMV		ΔABR_M dOMV		ΔABRL dOMV		Alum	
Strain (CC, ST)	**KM1**	**KM2**	**KM3**	**KM4**	**KM5**	**KM6**	**KM7**	**KM8**
MC58 (CC32, ST-74)	<4	<4	<4	4	8	4	<4	<4
M09057 (CC32, ST-32)	256	2048	512	256	2048	256	32	256
M14547 (CC32, ST-32)	256	2048	1024	512	1024	512	512	1024
M14223 (CC32, ST-32)	128	4096	512	64	4096	1024	16	2048
Cu385 (CC32, ST-33)	8	16	8	8	32	32	8	32
M49155 (CC32, ST-33)	4	4	32	8	16	32	<4	<4
M14290 (CC35, ST-35)	<4	<4	<4	<4	8	4	<4	<4
M17-240156 (CC213, ST-213)	128	2048	512	256	1024	256	32	256
M15875 (CC162, ST-162)	<4	<4	<4	<4	16	8	<4	<4
M14130 (CC41/44, ST-437)	<4	4	<4	<4	<4	<4	<4	<4
M10566 (CC41/44, ST-437)	<4	4	<4	<4	<4	<4	<4	<4
M28126 (CC41/44, ST-409)	<4	<4	<4	<4	<4	4	<4	<4
NZ98/254 (CC41/44, ST-42)	4	<4	<4	<4	<4	<4	<4	<4
M17-240388 (CC41/44, ST-1097)	<4	4	<4	<4	<4	<4	<4	<4
M12885 (CC41/44, ST-41)	4	<4	4	4	8	4	<4	4
5/99 (CC8, ST-1349)	<4	4	16	<4	<4	<4	<4	<4
Ch501 (CC269, ST-9234)	<4	<4	<4	<4	4	<4	<4	<4
Total Number of Strains Killed	**8**	**10**	**8**	**8**	**11**	**11**	**5**	**6**

Table 1. ΔABRL dOMVs elicit cross-reactive functional antibodies. Two rabbits (KM1-KM8) were administered three doses of either ΔABR, ΔABR_M, or ΔABRL dOMV vaccines (25 μg per dose with Imject™ (alum adjuvant) at one-month intervals; two additional rabbits were administered alum alone as a control. Two-weeks post-3rd immunization, blood samples were collected and sera isolated for testing in hSBAs against a panel of 17 antigenically diverse MenB strains. Clonal complex (CC) and Sequence Type (ST) of test strains are shown, where gradations of the same color in column "Strain (CC, ST)" indicate strains of the same CC.

Mouse Immunogenicity Study: OMV Vaccines Elicit Serum and Vaginal Antibodies

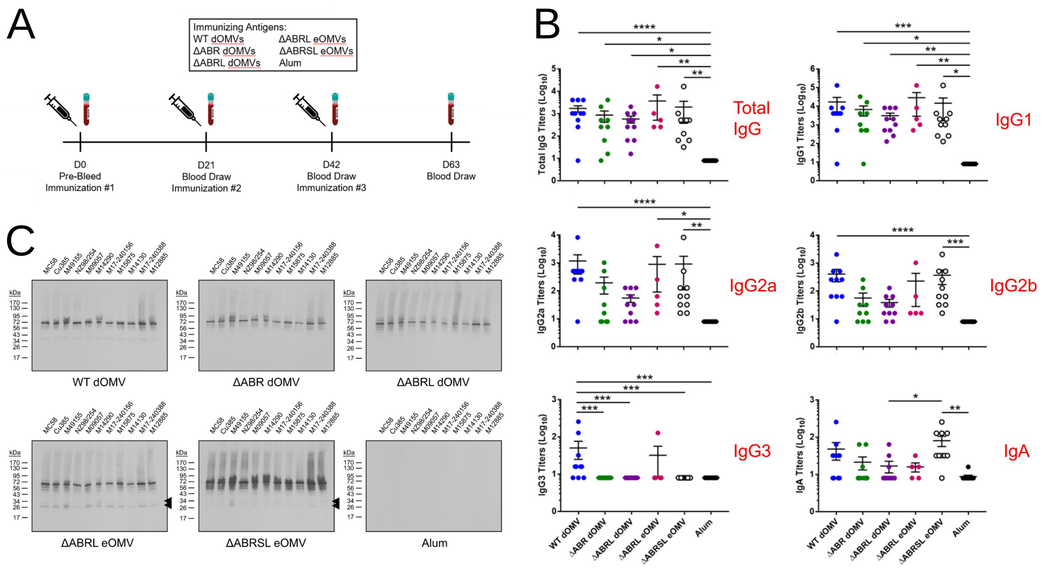

Figure 6. ΔABRSL eOMV-immunized mice produce significant levels of IgG1, IgG2a, IgG2b, and IgA serum and vaginal antibodies that are associated with binding to unique antigens in Western blots. (A) Mouse immunization schedule. Mice (n=10 per group) were administered OMV vaccines (12.5 μg OMVs with Alhydrogel® adjuvant), including WT dOMVs, ΔABR dOMVs, ΔABRL dOMVs, ΔABRL eOMVs, and ΔABRSL eOMVs, three times at three-week intervals; Alhydrogel® (alum) was administered alone as a control. Three-weeks post-3rd immunization, vaginal lavages and blood samples were collected for assessment of antibody levels and bactericidal activity. (B) Anti-MC58 OMV antibody titers in vaginal lavages as determined by ELISA. Serum samples were also tested for antibody levels, though minimal differences were observed among vaccine groups (data not shown). (C) Serum IgG immunoblots of whole cell MenB lysates. Unique banding patterns were observed with anti-ΔABRL eOMV and anti-ΔABRSL eOMV pooled sera (arrows) compared to pooled sera obtained from mice immunized with dOMV vaccines.

Mouse Immunogenicity Study: ΔABRSL eOMVs Elicit Robust, Cross-Reactive Bactericidal Antibodies

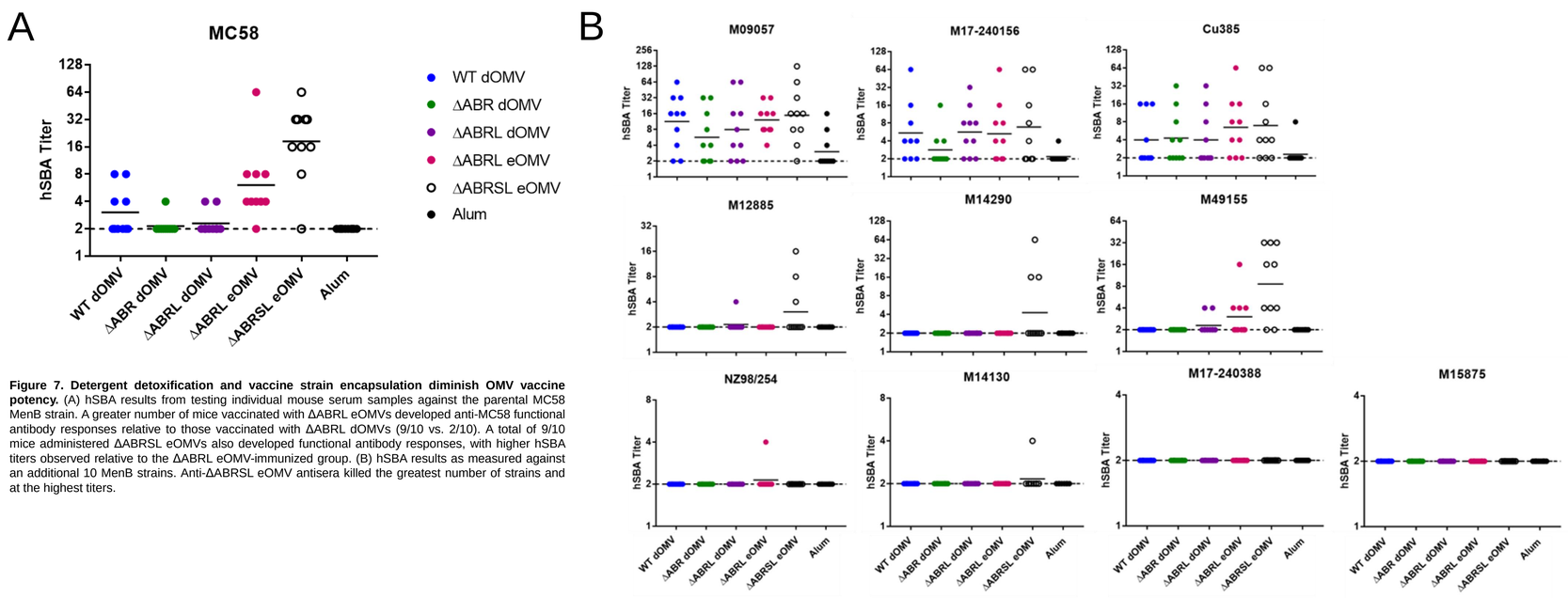

Figure 7. Detergent detoxification and vaccine strain encapsulation diminish OMV vaccine potency. (A) hSBA results from testing individual mouse serum samples against the parental MC58 MenB strain. A greater number of mice vaccinated with ΔABRL eOMVs developed anti-MC58 functional antibody responses relative to those vaccinated with ΔABRL dOMVs (9/10 vs. 2/10). A total of 9/10 mice administered ΔABRSL eOMVs also developed functional antibody responses, with higher hSBA titers observed relative to the ΔABRL eOMV-immunized group. (B) hSBA results as measured against an additional 10 MenB strains. Anti-ΔABRSL eOMV antisera killed the greatest number of strains and at the highest titers.

Mouse Immunogenicity Study: Impact of Encapsulation and Detergent Detoxification on Anti-Protein Immune Responses

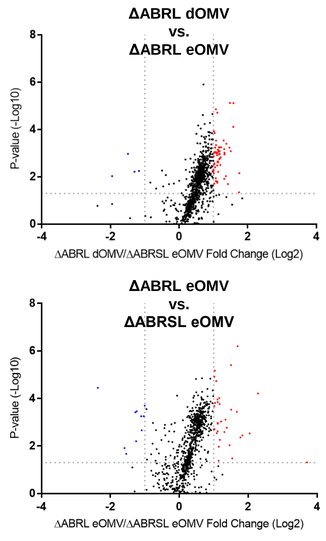

Table 2. Peptides showing differential enrichment when immunoprecipitated with anti-ΔABRL dOMV antisera relative to anti-ΔABRL eOMV antisera.

Enrichment Relative to ΔABRSL eOMV	Number of Significantly Enriched Proteins	Surface Proteins with 1.5-Fold to 2.0-Fold Change (% of Significantly Enriched)	Surface Proteins with >2-Fold Change (% of Significantly Enriched)	List of Surface Proteins with >2-Fold Change in Enrichment
Decreased	14	0 (0.00%)	1 (7.14%)	FrpA/C
Increased	426	28 (7.03%)	3 (1.82%)	CtrA, NMB0586, NMB1797

Table 3. Peptides showing differential enrichment when immunoprecipitated with anti-ΔABRL eOMV antisera relative to anti-ΔABRSL eOMV antisera.

Enrichment Relative to ΔABRSL eOMV	Number of Significantly Enriched Proteins	Surface Proteins with 1.5-Fold to 2.0-Fold Change (% of Significantly Enriched)	Surface Proteins with >2-Fold Change (% of Significantly Enriched)	List of Surface Proteins with >2-Fold Change in Enrichment
Decreased	43	6 (13.95%)	1 (2.33%)	Opc
Increased	198	12 (6.06%)	4 (2.02%)	FrpA/C, LolA, NMB0039, NMB1470

Figure 8. Robust anti-ΔABRSL eOMV hSBA titers are associated with elicitation of antibodies against a discrete number of individual surface proteins. To identify immunogenic proteins in each of the OMV vaccines, pooled serum samples from immunized mice were incubated with Elugent™ (EMD Millipore)-treated membrane preparations and Protein G-conjugated Dynabeads® (Invitrogen). Beads were washed and immunoprecipitated proteins identified by data-independent acquisition mass spectrometry. A comparison of the proteins identified in samples immunoprecipitated with pooled mouse anti-ΔABRL dOMV vs. anti-ΔABRL eOMV sera (upper panel) and anti-ΔABRL eOMV vs. anti-ΔABRSL eOMV sera are depicted. Blue and red dots in volcano plots (left) represent decreased and increased protein enrichment relative to the normalizing sample, respectively. An analysis of the percentage of significantly enriched surface proteins is shown on the tables on the right, where surface proteins were identified as OMPs or extracellular proteins by PSORTb and BUSCA localization tools. A list of all identified surface proteins with >2-fold change in enrichment is included. Notable vaccine candidates that were significantly enriched ≥1.5-fold in anti-ΔABRSL eOMV samples vs. anti-ΔABRL eOMV samples include the adhesin NMB0586, the multidrug efflux pump channel protein MtrE, the copper-containing nitrite reductase AniA, and lipoproteins NMB1125, NMB1212, and NMB1468; no significantly enriched vaccine candidates other than FrpA/C (enriched >2-fold) were identified in the ΔABRL eOMV group vs. the ΔABRL dOMV group. Results represent data from five independent experiments.

Conclusions

- LpxL1-deficient strains demonstrated lower toxicity than LpxL1-sufficient strains, permitting immunogenicity comparisons of detergent-detoxified dOMVs with non-detergent-detoxified eOMVs
- A greater number of mice immunized with ΔABRL eOMVs elicited bactericidal antibodies relative to ΔABRL dOMV-vaccinated mice, with higher hSBA titers observed, confirming that detergent detoxification diminishes OMV vaccine potency in animal immunogenicity studies
- ΔABRSL eOMVs elicited functional antibodies in immunogenicity studies that killed the greatest number of strains with consistently higher titers observed relative to all other groups, including ΔABRL eOMV-vaccinated animals, suggesting that the presence of residual capsular components in OMV vaccines diminishes anti-OMV cross-reactivity and vaccine potency
- ΔABRSL eOMV-immunized mice produced significantly higher titers of IgG1, IgG2a, IgG2b, and IgA serum and vaginal antibodies relative to alum-immunized controls, while ΔABR dOMV-immunized mice produced only significantly higher titers of IgG1; mucosal antibody responses suggest that ΔABRSL eOMVs may function as an improved gonococcal vaccine compared to the ΔABR dOMV vaccine
- Unique banding patterns were observed in whole cell MenB immunoblots probed with anti-ΔABRL and anti-ΔABRSL eOMV pooled mouse sera; the detergent detoxification procedure may have removed cross-reactive antigens from the OMV or diminished presentation to the host immune response by disruption the OMV structure
- Despite exhibiting seroresponses of lower potency, a greater number of surface-associated proteins were enriched in samples immunoprecipitated with anti-ΔABRL eOMV pooled mouse sera relative to those immunoprecipitated with anti-ΔABRSL eOMV pooled serum; potency of OMV responses may be associated with elicitation of robust immune responses to specific surface antigens rather than responses to a breadth of antigens

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