

A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on Generalized Modules for Membrane Antigens (GMMA)



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ABSTRACT

Introduction: *Neisseria meningitidis* causes epidemics of meningitis in sub-Saharan Africa. These have mainly been caused by capsular group A strains, but W and X strains are increasingly contributing to the burden of disease. Therefore, an affordable vaccine that provides broad protection against meningococcal disease in sub-Saharan Africa is required.

Methods: We prepared Generalized Modules for Membrane Antigens (GMMA) from a recombinant serogroup W strain expressing PorA P1.5.2, which is predominant among African W isolates. The strain was engineered with deleted capsule locus genes, *lpxL1* and *gna33* genes and over-expressed fHbp variant 1, which is expressed by the majority of serogroup A and X isolates.

Results: We screened nine W strains with deleted capsule locus and *gna33* for high-level GMMA release. A mutant with five-fold increased GMMA release compared with the wild type was further engineered with a *lpxL1* deletion and over-expression of fHbp. GMMA from the production strain had 50-fold lower ability to stimulate IL-6 release from human PBMC and caused 1000-fold lower TLR-4 activation in Human Embryonic Kidney cells than non-detoxified GMMA. In mice, the GMMA vaccine induced bactericidal antibody responses against African W strains expressing homologous PorA and fHbp v.1 or v.2 (geometric mean titres [GMT] = 80,000–200,000), and invasive African A and X strains expressing a heterologous PorA and fHbp variant 1 (GMT = 20–2500 and 18–5500, respectively). Sera from mice immunised with GMMA without over-expressed fHbp v.1 were unable to kill the A and X strains, indicating that bactericidal antibodies against these strains are directed against fHbp.

Conclusion: A GMMA vaccine produced from a recombinant African *N. meningitidis* W strain with deleted capsule locus, *lpxL1*, *gna33* and overexpressed fHbp v.1 has potential as an affordable vaccine with broad coverage against strains from all main serogroups currently causing meningococcal meningitis in sub-Saharan Africa.

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1. Introduction

Neisseria meningitidis is a major cause of epidemics in sub-Saharan Africa [1]. These were mainly caused by strains belonging

to capsular group A, but there has been an increasing contribution of serogroups W and X strains with epidemic potential in the last two decades [2–5]. A serogroup A polysaccharide conjugate vaccine (MenAfriVac) has been developed for preventive mass immunization in the African meningitis belt [6]. The vaccine is highly effective at prevention of serogroup A invasive disease and carriage [7–9], but group W and X strains remain a persistent problem. This underlines the need for an affordable vaccine that provides protection against the main serogroups causing meningitis in Africa and potentially against serogroups that may emerge in the region in the future.

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GMMA generated from strains engineered to over-express immunogenic antigens that are present across all serogroups, constitute an attractive approach to vaccination. The term GMMA (Generalised Modules for Membrane Antigens) provides a clear distinction from conventional detergent-extracted outer membrane vesicles (dOMV), and native outer membrane vesicle (NOMV), which are released spontaneously from Gram-negative bacteria. GMMA differ in two crucial aspects from NOMV. First, to induce GMMA formation, the membrane structure has been modified by the deletion of genes encoding key structural components, including *gna33* (meningococcus) and *tolR* (*Shigella* and *Salmonella* [10]). Second, as a consequence of the genetic modification, large quantities of outer membrane bud off (the Italian word for bud is 'gemma') to provide a practical source of membrane material for vaccine production, leading to potential cost reduction. While NOMV have been used for immunogenicity studies, the yields are too low for practical vaccines.

The most promising candidate protein vaccine antigen discovered for meningococcus is factor H binding protein fHbp. The extraction process required to make dOMV removes lipoproteins, including fHbp, and increases the cost of production of dOMV relative to GMMA. The fHbp gene is present in most invasive meningococcal isolates independent of the serogroup. fHbp can be divided into three antigenic variants (v. 1, 2 or 3) [11] or into at least nine modular groups based on the combination of five variable α and β fHbp segments [12,13]. Individual peptides within each variant are identified by a unique peptide ID. The outer membrane protein, PorA, is highly immunogenic but antibodies tend to provide subtype-specific protection [14]. African meningococcal isolates are relatively conserved in relation to fHbp variant and PorA subtype [15,16]. Invasive serogroup A and X strains predominantly express fHbp v.1. PorA subtype P1.5.2 is shared by most serogroup W strains and P1.20.9 is expressed by the majority of A strains [15]. This epidemiological pattern makes a protein-based vaccine both a possible and attractive approach for sub-Saharan Africa.

A vaccine for the meningitis belt needs to be affordable and large-scale low-cost production of a GMMA vaccine has to be feasible. Deletions of *gna33* or *rmpM*, that augment the release of these outer membrane particles can reduce costs [17–21]. In this study, we selected a vaccine strain based on a panel of African W strain capsule and *gna33* double knock-out mutants. The isolate with the highest GMMA production was then further engineered for the deletion of *lpxL1* and over-expression of fHbp v.1 (ID1). This genetic approach may form the basis for a broadly-protective, safe and economic vaccine for sub-Saharan Africa.

2. Materials and methods

2.1. *N. meningitidis* strains

Three African serogroup W, seven A and seven X strains were the target strains for serum bactericidal assays. Nine African serogroup W strains were screened as potential vaccine production strains (Table 1). Carrier strain 1630 (ST-11) expressing PorA subvariant P1.5.2 and fHbp v.2 (ID23) was chosen for GMMA production [22]. To abolish capsule production, a fragment of the bacterial chromosome containing *synX*, *ctrA* and the promoter controlling their expression, was replaced with a spectinomycin-resistance gene. First, the recombination sites were amplified with primers *ctrAf_Xma*:CCCCCGGGCAGGAAAGCGCTGCATAG and *ctrAr_XbaCGTCTAGAGGTTCAACGGCAAATGTGC*; *Synf_KpnCGG-GGTACCGTGGAAATGTTCTGCTCAA* and *Synr_SpeGGACTAGTCCATTAGGCCCTAAATGCCTG* from genomic DNA from strain 1630. The fragments were inserted into plasmid pComPtac [23] upstream and downstream of the chloramphenicol resistance gene.

Subsequently the chloramphenicol resistance gene was replaced with a spectinomycin resistance cassette. The *lpxL1* gene was deleted by replacement with a kanamycin resistance gene [24], and the *gna33* gene with an erythromycin resistance cassette [25]. fHbp expression was up-regulated using multicopy plasmid encoding fHbp v.1 (ID1) [26].

2.2. GMMA preparation

Bacteria were grown at 37 °C, 5% CO₂ in 50 mL of a modified version of a meningococcus defined medium described previously [27] at 180 rpm until early stationary phase. Cells were harvested (2200 g, 30 min, 4 °C) and the culture supernatant containing the GMMA was filtered through a 0.22 μ m pore-size membrane (Millipore, Billerica, MA, USA). To collect GMMA, the supernatant was ultracentrifuged (142,000 \times g, 2 h, 4 °C). The membrane pellet was washed with phosphate buffered saline (PBS), resuspended in PBS and sterile filtered. GMMA concentration was measured according to protein content by Lowry assay (Sigma–Aldrich, St. Louis, MO, USA). For protein and lipooligosaccharide analysis, GMMA were separated by SDS–PAGE using a 12% gel and MOPS or MES buffer (Invitrogen, Carlsbad, CA, USA). Total proteins were stained with Coomassie Blue stain. The amount of PorA was determined by densitometric quantification of the PorA protein in relation to total measurable protein. Lipooligosaccharide was visualized by treatment of the gel with periodic acid and staining with silver nitrate. The gel was developed with a solution containing 50 mg/L citric acid and 0.05% formaldehyde. fHbp was detected by Western blot using a polyclonal antibody raised in mice against recombinant fHbp ID1.

2.3. IL-6 release by human peripheral blood mononuclear cells (PBMC) stimulated with GMMA

PBMC were separated from whole blood using Ficoll-Paque Plus density gradient (Amersham Pharmacia Biotech), washed with PBS and resuspended in 10% heat-inactivated fetal bovine serum (FBS)/10% Dimethyl sulfoxide and stored in liquid nitrogen until use. For stimulation, PBMCs were thawed, washed with PBS/2.5 mM EDTA and 20 μ g/mL DNase (Sigma–Aldrich, St. Louis, MO, USA) and resuspended in RPMI-1640 complete (with 25 mM HEPES, glutamine, 10% FBS + 1% Antibiotics Pen-Strep). 2×10^5 cells/well were stimulated with GMMA ($1-10^{-6}$ μ g/mL final concentration) for 4 h at 37 °C. Cells were removed by centrifugation and IL-6 in the supernatants was measured by ELISA using 0.1 μ g of an anti-human IL-6 antibody (eBioscience, San Diego, CA, USA). A Biotin-labelled anti-human IL-6 antibody was used for detection (e-Bioscience).

2.4. Measurement of TLR-4 stimulation by NF- κ B luciferase reporter assay

Human Embryonic Kidney 293 (HEK293) cells expressing luciferase under control of the NF- κ B promoter and stably transfected with human Toll-like receptor (TLR) 4, MD2 and CD14 were used. 25,000 cells/well were added to microclear luciferase plates (PBI International) and incubated for 24 h at 37 °C. GMMA ($1-1.28 \times 10^{-5}$ μ g/mL final concentration) were added and incubated for 5 h. Cells were separated from the supernatant and lysed with passive lysis buffer (Promega, Madison, WI, USA). Luciferase assay reagent (Promega) was added and fluorescence was detected using a luminometer LMaxII 384 (Molecular Devices).

2.5. Mouse immunization

Female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Eight mice per group were immunised

Table 1
Characteristics of African *N. meningitidis* wild type strains used for screening of GMMA production strains and in serum bactericidal assays.

Strain	Serogroup	Year of isolation	Country	Source	Sequence type	fHbp variant ^a	fHbp peptide ID ^a (% identity to fHbp ID1)	PorA subtype	fHbp expression (%) ^b
<i>N. meningitidis</i> wild type strains used for screening of the GMMA production strain									
1485	W	2003	Ghana	Carrier	11	2	23	5,2	
1630	W	2004	Ghana	Carrier	11	2	23	5,2	
1629	W	2004	Ghana	Carrier	11	2	23	5,2	
1681	W	2004	Ghana	Case	11	2	23	5,2	
1682	W	2004	Ghana	Case	11	2	23	5,2	
1846	W	2004	Ghana	Case	11	2	23	5,2	
1888	W	2004	Ghana	Carrier	11	2	23	5,2	
1973	W	2005	Ghana	Carrier	11	2	23	5,2	
2882	W	2007	Ghana	Carrier	11	2	22	5,2	
<i>N. meningitidis</i> wild type strains used in serum bactericidal assays									
Mali 10/09	W	2009	Mali	Case	11	2	23 (70)	5,2	70
BF2/11	W	2011	Burkina Faso	Case	11	1	9 (94)	5,2	42
N1361	A	2002	Ghana	Case	ND	1	5 (96)	20,9	80
N2008	A	2005	Ghana	Case	ND	1	5	20,9	75
BF6/06	A	2006	Burkina Faso	Case	2859	1	5	20,9	52
N2181	A	2006	Burkina Faso	Case	ND	1	5	20,9	75
Su14/07	A	2007	Sudan	Case	7	1	5	20,9	40
N2602	A	2007	Burkina Faso	Case	ND	1	5	20,9	76
Mali21/10	A	2010	Mali	Case	8639	1	5	20,9	33
BF2/97	X	1997	Burkina Faso	Case	751	1	73 (93)	5-1,10-1	50
BF12/03	X	2003	Burkina Faso	Case	751	1	73	5-1,10-1	53
MRS2006093	X	2006	Ghana	Case	181	1	74 (93)	5-1,10-1	75
Ug9/06	X	2006	Uganda	Case	5403	1	74	19,26	200
BF7/07	X	2007	Burkina Faso	Case	181	1	74	5-1,10-1	160
Ug11/07	X	2007	Uganda	Case	5403	1	74	19,26	4
BF16/10	X	2010	Burkina Faso	Case	181	1	74	5-1,10-1	101

^a Determined by sequencing of the fHbp gene and analysis of the protein sequence using the *N. meningitidis* database on <http://pubmlst.org/neisseria/fhbp/>.

^b fHbp expression was measured by Western blot of whole cell lysates previously described [11]. Percentage expression is in relation to group B strain H44/76, a relatively high expresser of fHbp v.1. Expression of fHbp v.2 in strain Mali 10/09 is expressed as percentage of fHbp expression in group B strain 8047, a relatively high expresser of fHbp v.2.

intraperitoneally three times with 2 weeks intervals. Serum samples were obtained 2 weeks after the third dose. GMMA from the serogroup W Triple KO (*lpxL1*, capsule, *gna33* KO), OE fHbp strain were given at 0.2, 1 and 5 µg doses based on total protein. Two other groups of mice received 5 µg of GMMA from the Double KO (*lpxL1*, *gna33* KO) OE fHbp mutant or 5 µg GMMA from the Triple KO mutant strain. Control mice were immunised with 5 µg recombinant fHbp ID1 or aluminium hydroxide only. All vaccines were adsorbed on 3 mg/mL Aluminium hydroxide in a 100 µL formulation containing 10 mM Histidine and 0.9 mg/mL NaCl. Sera were stored at –80 °C until use. All animal work was approved by the Italian Animal Ethics Committee (AEC project number 14112011).

2.6. Serological analysis

Anti-fHbp IgG antibody titres were measured by ELISA as previously described [28]. The coating antigen was 1 µg/mL non-lipidated recombinant hexa-Histidine-tagged fHbp ID1 [11]. Serial five-fold dilutions of the serum samples starting at 1:100 were analysed. Secondary antibody was a 1:2000 dilution of alkaline phosphatase-conjugated goat-anti mouse IgG (Invitrogen, cat. no 62-6522, Lot 437983A). The titre was defined as the extrapolated dilution resulting in absorbance of 1 at 405 nm after 30 min of incubation with 1 mg/mL 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich) diluted in 1 M diethanolamine and 0.5 mM MgCl₂, pH 9.8.

Serum bactericidal antibody (SBA) activities were measured as described before [28]. Bacteria were incubated at 37 °C, 5% CO₂ in Mueller–Hinton broth containing 0.25% glucose and 0.02 mM Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (Sigma–Aldrich). The cells were washed with Dulbecco's PBS buffer (Sigma–Aldrich) containing 1% BSA. Each reaction mixture contained approximately 400 colony-forming units, 20% human

complement screened for lack of bactericidal activity against the target strain and serial dilutions of the serum samples starting at 1:10. Bactericidal titres were defined as the reciprocal extrapolated dilution resulting in 50% killing of bacteria after 60 min incubation at 37 °C compared to the mean number of bacteria in five control reactions at time 0.

2.7. Statistical analysis

For statistical analysis, antibody titres were log 10 transformed. ELISA titres <100 were assigned the value 50, SBA titres <10 were assigned the value 5. Mann–Whitney U test was used to compare pairs of values. A probability value of <0.05 was considered statistically significant. The analysis was performed with the Graph Pad Prism software 5.01.

3. Results

3.1. Selection of the serogroup W GMMA production strain and generation of mutants

Nine group W strains (six carrier and three case isolates) with PorA subtype P1.5,2, collected in Ghana between 2003 and 2007, were screened as candidate GMMA production strains. To identify the isolate with highest GMMA production, *gna33* was deleted from all strains. In some isolates, simultaneous deletion of the capsule decreased the GMMA release compared to the *gna33* single knock-out (KO). Therefore, we generated *gna33* and capsule double KO mutants of the nine W strains and compared GMMA production. These double-mutant strains released two to five-fold higher amounts of GMMA than a representative group W wild type strain (Fig. 1A). Strain 1630 (*gna33* KO, capsule KO), which released the

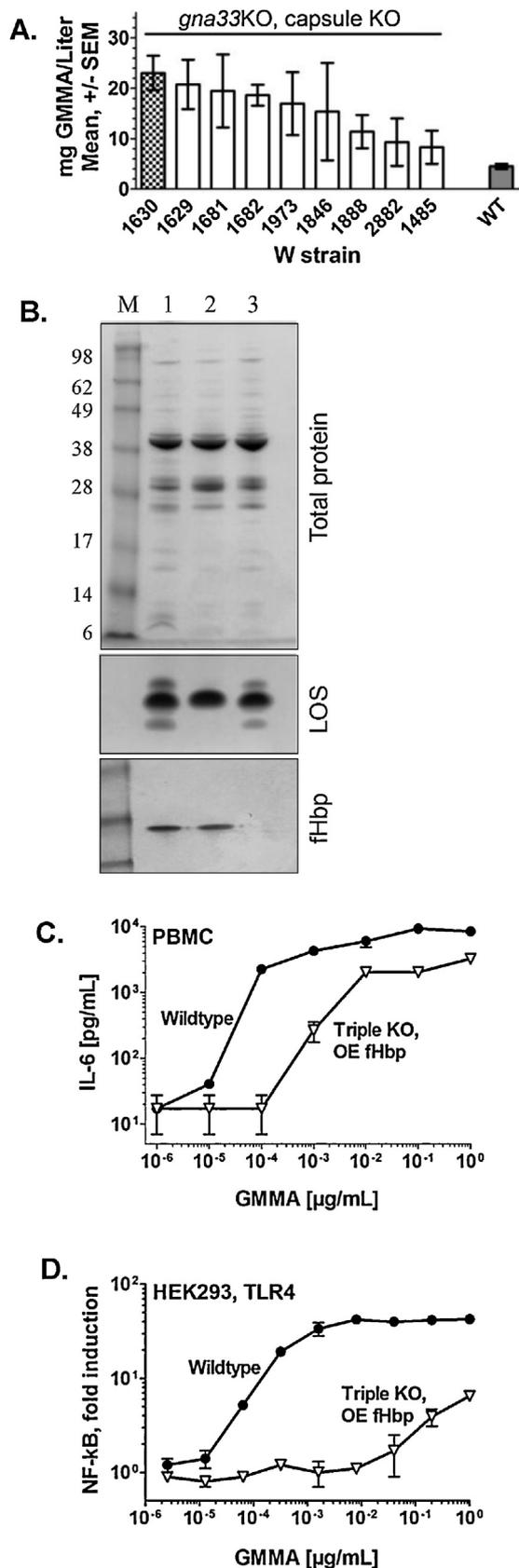


Fig. 1. GMMA release by engineered meningococcal W strains. **A.** W strains engineered to have deleted capsule and *gna33* KO were grown in small-scale shake-flasks. Strain 1630 was selected as the GMMA-production strain for further studies. WT = GMMA release by a representative wild type strain. Bars indicate the mean and standard error of three independent experiments. **B.** Upper panel: SDS-PAGE and Coomassie Blue stain of 5 µg GMMA. Middle panel: Silver stain of

Table 2

N. meningitidis vaccine strains and GMMA vaccines used in the study.

Vaccine strain characteristics	Designation of vaccine strain and GMMA used for immunization
Prototype vaccine candidate Serogroup W, strain 1630 Capsule KO, <i>lpxL1</i> KO, <i>gna33</i> KO, over-expressed fHbp ID1	Triple KO, OE fHbp
Control vaccines Serogroup W, strain 1630 Capsule expressed, <i>lpxL1</i> KO, <i>gna33</i> KO, over-expressed fHbp ID1	Double KO, OE fHbp
Serogroup W, strain 1630 Capsule KO, <i>lpxL1</i> KO, <i>gna33</i> KO	Triple KO

highest quantity of GMMA, was selected for further genetic manipulation.

To generate the final vaccine strain, we deleted *lpxL1* and engineered the mutant to over-express fHbp v.1, designated 'Triple KO, OE fHbp'. We also prepared two isogenic group W control strains: one with deleted *lpxL1* and *gna33*, over-expressed fHbp v.1 with the capsule still expressed ('Double KO, OE fHbp'), and another with deleted *lpxL1*, capsule and *gna33*, but no fHbp over-expression ('Triple KO') (Table 2). SDS-PAGE and Coomassie Blue staining of the proteins revealed a similar protein pattern in the three GMMA preparations. Densitometry indicated that in all three GMMA preparations, the relative amount of PorA to total protein is 5%. By silver stain, the GMMA contained similar levels of lipooligosaccharide. By capture ELISA, with recombinant fHbp as standard, approximately 3% of the total protein in GMMA from the Triple KO, OE fHbp was fHbp, and by Western blot, the two GMMA over-expressing fHbp had similar fHbp levels.

3.2. IL-6 release by human PBMC and TLR-4 activation in HEK293 cells after stimulation with GMMA

To assess the endotoxic activity of the GMMA, we measured the release of IL-6 by human PBMC after stimulation with different concentrations of GMMA from the Triple KO, OE fHbp mutant and the parent serogroup W wild type strain (Fig. 1C). Approximately 50-fold higher concentrations of GMMA from the mutant strain were required to stimulate the release of 200 pg/mL IL-6, confirming the decrease in endotoxic activity. We measured the ability of the GMMA to stimulate human TLR-4 in transfected HEK293 cells (Fig. 1D). Low concentrations of GMMA from the wild type bacteria stimulated TLR-4, as measured by increased NF-κB expression. Approximately 1000-fold higher concentrations of GMMA from the Triple KO, OE fHbp mutant were required for equivalent TLR-4 stimulation. These results are consistent with a strongly decreased ability of the LOS in GMMA from the serogroup W mutant to

lipooligosaccharide in 0.5 µg GMMA. Lower panel: detection of fHbp in GMMA by Western blot using a polyclonal anti-fHbp v.1 antibody. M = molecular weight marker. Lane 1, GMMA Triple KO, OE fHbp; lane 2, GMMA Double KO, OE fHbp; lane 3, GMMA Triple KO. **C.** IL-6 release by human PBMCs stimulated with different concentrations of GMMA with deleted capsule, *gna33* and *lpxL1* and over-expressed fHbp v.1 for four hours. IL-6 release into culture supernatants was analysed by ELISA. **D.** Stimulation of TLR-4 in HEK293 cells transfected with human Toll-like receptor (TLR) 4, MD2 and CD14 and luciferase expressed under control of the NF-κB promoter. Cells were stimulated with GMMA for five hours, lysed and emitted light was quantitated with a luminometer. The readings were divided by the control cells stimulated with PBS. Mean results and standard deviations from two independent experiments were plotted. Black circles = GMMA from the group W wild type strain 1630 used to construct the mutants. White triangles = Triple KO, OE fHbp; GMMA from the group W mutant strain with deleted capsule, *lpxL1* and *gna33* and over-expressed fHbp ID1.

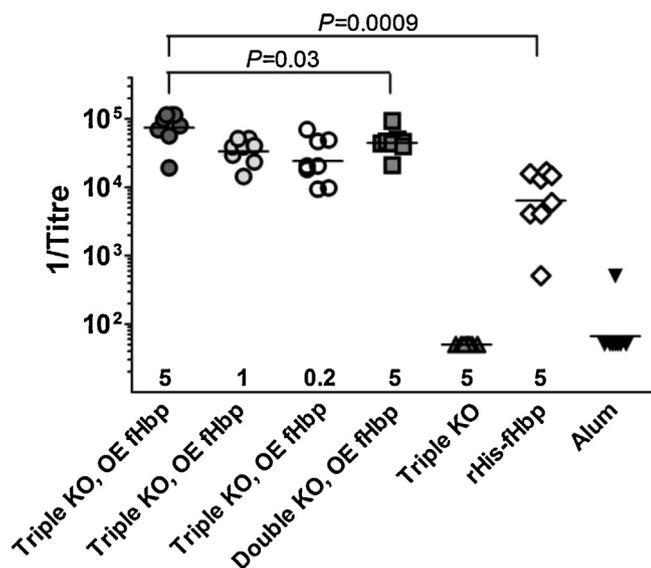


Fig. 2. IgG anti-fHbp antibody responses elicited in mice as measured by ELISA. Groups of eight mice were immunised with three doses of vaccine, 2 weeks apart. The serum samples analysed were obtained 2 weeks after the third dose. Each symbol represents an individual serum sample, the line indicates the geometric mean titre of each vaccine group. GMMA used for immunization: Triple KO, OE fHbp: capsule, *lpxL1* and *gna33* KO with over-expressed fHbp ID1. Double KO, OE fHbp: *lpxL1* and *gna33* KO with over-expressed fHbp ID1 and capsule expression. Triple KO: capsule, *lpxL1* and *gna33* KO without over-expressed fHbp. rHis-fHbp: recombinant hexa-histidine tagged fHbp ID1; Alum: aluminium hydroxide. Numbers above the x-axis show the vaccine dose in μg . Statistical analysis between pairs of groups was by Mann-Whitney U test.

activate TLR-4 compared with GMMA from the non-detoxified parent wild type strain.

3.3. Antibody responses elicited in mice immunised with GMMA

We measured anti-fHbp v.1 antibody responses in individual serum samples by ELISA. GMMA from all mutants with over-expressed fHbp elicited high anti-fHbp antibody responses, even at the lowest dose of 0.2 μg (Fig. 2). 5 μg Triple KO, OE fHbp GMMA induced significantly higher geometric mean titres than 5 μg Double KO, OE fHbp GMMA ($P=0.03$) or 5 μg of recombinant fHbp v.1 ($P<0.001$). GMMA from the Triple KO mutant without fHbp over-expression induced no measurable anti-fHbp antibody responses.

3.4. SBA responses of mice immunised with GMMA from recombinant serogroup W strains

The three serogroup W test strains were isolated in Ghana, Mali and Burkina Faso and expressed PorA subtype P1.5.2, which is identical to that expressed by the GMMA vaccine strains. Strain BF2/11 expressed fHbp v.1 (ID9) and the two other strains expressed fHbp v.2 (ID23). The seven group A strains tested were collected in Ghana, Burkina Faso, Sudan and Mali. They expressed a heterologous PorA compared to that in the GMMA, and fHbp v.1 (ID5). fHbp expression in these test strains ranged from 33 to 80% of that of a reference group B strain H44/76 with relatively high fHbp expression [11]. The seven group X strains were isolated in Burkina Faso, Ghana and Uganda. Two strains from Burkina Faso expressed fHbp ID73, the other isolates expressed ID74. The strains from Burkina Faso were sequence type 751 and 181, respectively. The two strains from Uganda were ST5403 and expressed PorA subtype P1.19.26, while the other five group X strains were P1.5-1,10-1. The two strains from Uganda differed from each other by the level of fHbp

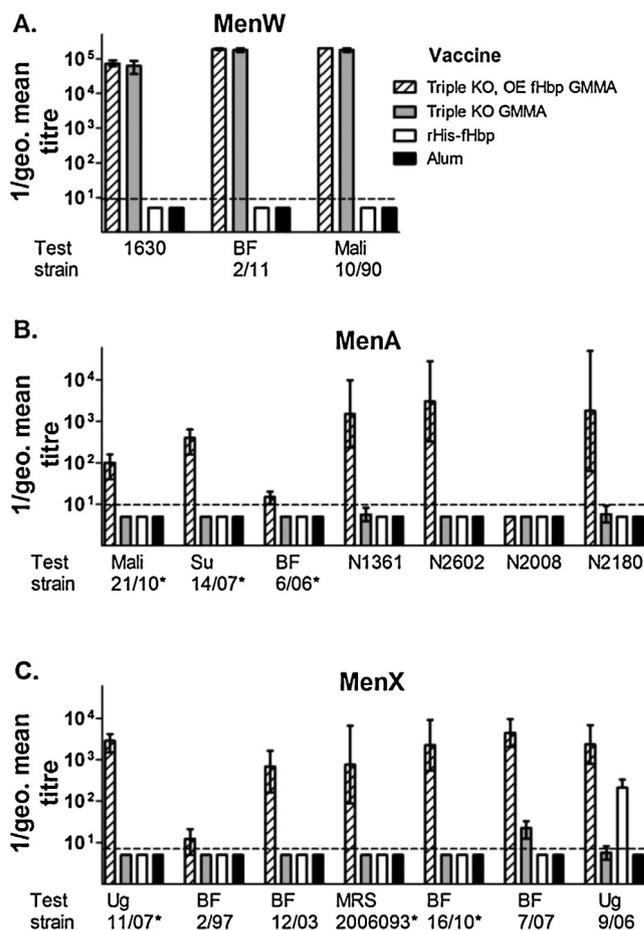


Fig. 3. Serum bactericidal antibody responses of immunised mice against African meningococci group W (panel A), group A (panel B) and group X strains (panel C) measured with human complement. Group A and X strains were ordered based on their relative fHbp expression, increasing from the left to the right. Serum samples analysed were obtained 2 weeks after the third dose (see legend to Fig. 4). The bars show reciprocal geometric mean titres ($\pm 95\%$ confidence interval) from four serum samples per GMMA vaccine group, containing sera from two mice each. For the strains labelled with an asterisk and the negative control group (Alum) two serum pools were analysed containing sera from four mice each and error bars indicate the standard error of the mean. GMMA vaccines: Triple KO, OE fHbp: capsule, *lpxL1* and *gna33* KO with over-expressed fHbp ID1, hatched bars. Triple KO: capsule, *lpxL1* and *gna33* KO without over-expressed fHbp, grey bars. rHis-fHbp: recombinant hexa-histidine tagged fHbp ID1, white bars; Alum: aluminium hydroxide, black bars. Dotted lines indicate the lowest serum dilution tested (1:10).

expression. Strain Ug11/07 had 4% and Ug9/06 has 200% of the fHbp expression level compared to the reference strain (Table 1).

GMMA with or without fHbp over-expression elicited high bactericidal titres that were not significantly different from each other against the three W strains expressing either fHbp v.1 or v.2 (Fig. 3A). This is consistent with previous observations that bactericidal activity against strains sharing the same PorA as the GMMA-production strain is predominantly mediated by anti-PorA antibodies [26].

GMMA from the Triple KO, OE fHbp strain induced antibodies that were able to kill six out of seven serogroup A strains (geometric mean titres [GMT] ranging from 20 to 2500) (Fig. 3B). The only isolate that was resistant to killing was readily killed by a mouse serum raised against group A polysaccharide conjugate vaccine. The antibodies induced by the GMMA from the Triple KO, OE fHbp strain were able to kill all serogroup X strains tested (GMT = 18–5500) (Fig. 3C). GMMA produced from the W strain which lacked fHbp v.1 over-expression (Triple KO), induced antibodies that were only able to kill one X strain (BF7/07), consistent with the majority of

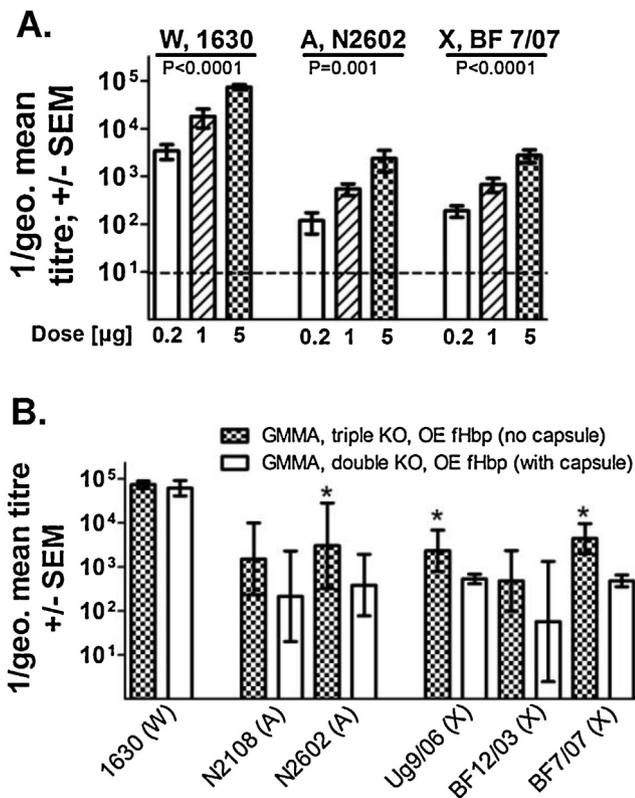


Fig. 4. Serum bactericidal antibody responses induced by meningococcal W GMMA. A. Dose-dependent serum bactericidal antibody responses induced by group W triple KO, OE fHbp GMMA (capsule, *lpx11* and *gna33* KO, over-expressed fHbp ID1) measured with human complement. Mice were immunised three times 2 weeks apart with 0.2 μg (white bars), 1 μg (hatched bars) or 5 μg (squared bars). Serum samples were obtained 2 weeks after the third dose and four pools containing sera from two mice each were measured against African serogroup W strain 1630, serogroup A strain N2602 and serogroup X strain BF7/07. The dotted line indicates the lowest serum dilution tested (1:10). Spearman Rank test was used for the statistical analysis of the correlation between the dose and geometric mean titres of each strain. B. Serum bactericidal antibody responses elicited by GMMA from W with or without expression of capsule against one African serogroup W, two serogroup A and three serogroup X strains. GMMA vaccine groups: Triple KO, OE fHbp: capsule, *lpx11* and *gna33* KO with over-expressed fHbp ID1, checked bars. Double KO, OE fHbp: *lpx11* and *gna33* KO with over-expressed fHbp ID1 and capsule expression, white bars. Statistical analysis between pairs of groups was done by Mann–Whitney U test and asterisks indicate probability values of <0.05. The bars show reciprocal geometric mean titres (±standard error of the mean) from four serum samples per GMMA vaccine group, containing sera from two mice each.

bactericidal antibodies induced by the GMMA vaccine being directed against fHbp. Antibodies made against the recombinant fHbp ID1 were only bactericidal against serogroup X strain Ug9/06 with the highest fHbp expression.

We investigated the dose-dependent bactericidal antibody response against one W (1630), A (N2602) and X (BF7/07) isolate (Fig. 4A). Sera raised against GMMA with over-expressed fHbp were bactericidal against these strains in a dose-dependent manner (Spearman Rank $P=0.001$ for group A and $P<0.0001$ for group W and X) with killing occurring at all three doses (0.2, 1 and 5 μg). GMMA from the triple KO, OE fHbp mutant was prepared from a mutant with deleted capsule expression in order to attenuate virulence of the vaccine strain and reduce serogroup-specific antibody production. To test the latter, we investigated whether maintaining capsule expression in the GMMA-producing strain affects the bactericidal antibody response. Sera from mice immunised with GMMA prepared from the Triple KO, OE fHbp vaccine strain had significantly higher SBA activity against three of five A and X strains tested than GMMA from the isogenic mutant that expressed the

capsule (Fig. 4B). These data are consistent with the hypothesis that deletion of capsule biosynthesis in the GMMA-production strain not only decreases virulence, but also increases antibody responses towards non-capsular antigens, such as fHbp.

4. Discussion

The group A polysaccharide conjugate vaccine, MenAfriVac, is highly effective at prevention of serogroup A invasive disease and carriage [7–9]. However, other serogroups, in particular W and more recently X, are increasingly contributing to the burden of meningococcal disease in sub-Saharan Africa [3,29–32]. Additionally, other meningococcal serogroups, e.g. group C, that, although not having caused outbreaks in recent years, may become a threat in the future. The challenge for future vaccine approaches for the meningitis belt is to develop a meningococcal vaccine that is not only affordable, but provides broad cross-serogroup protection against meningococcus, and complements the roll out pneumococcal vaccination to deal with the problem of pneumococcal meningitis in the region.

GMMA from recombinant meningococcal strains offer a promising option. They contain protein antigens (e.g. fHbp) which induce antibodies with serogroup independent cross protection. In addition, a simple, economic and scalable procedure for their preparation has been developed with minimal downstream processing required, which enables large quantities of GMMA vaccine to be produced at low cost [10]. While strains containing deletions of *lpx11* and capsule synthesis genes with up-regulated fHbp expression have been described [33,34], our approach incorporates the additional deletion of *gna33* in order to enhance the level of GMMA production, and consequently the potential affordability of the vaccine for use in Africa. The mechanism of up-regulation of GMMA production is not fully understood. Our findings indicate that GMMA release by different *gna33* KO strains is variable, indicating a requirement to screen multiple strains for high level GMMA release.

We tested bactericidal activity of sera from immunised mice against 17 group A, W and X strains. Five μg of the GMMA from the Triple KO, OE fHbp group W strain induced SBA responses against 16 (94%) of these isolates. Ability to kill the A and X strains was attributable to fHbp which comprises only about 3% of the total GMMA protein. In comparison, 5 μg recombinant fHbp ID1 induced a detectable bactericidal antibody response only against one X strain which had the highest level of fHbp expression. This is consistent with previous studies with NOMV demonstrating that fHbp expressed in the native membrane environment induces antibodies with greater functional activity than vaccines containing recombinant fHbp [15,35,36].

Previous studies have demonstrated broad cross-protection of NOMV vaccines against a panel of diverse African strains [15,34,37]. We did not compare our GMMA vaccine directly with NOMV. Nevertheless, the strong bactericidal activity of the GMMA-induced antibodies against strains with homologous or heterologous PorA and different fHbp ID types (ID 5, 73 and 74), suggests that the new combination of mutations, including deletion of *gna33*, that all affect the outer surface, does not impair the immunogenicity of the main antigens, fHbp and PorA. It has been shown that decreased SBA titres are induced when mice expressing human factor H are immunised with NOMV over-expressing wild type fHbp [38]. This can be overcome by introducing the R41S mutation into the fHbp gene of the vaccine-producing strain [38,39]. The aim of the current study was to serve as a first proof of concept in mice for a GMMA meningococcal candidate vaccine and the R41S mutation was not incorporated into our vaccine design. We are currently investigating the utility of this mutation in GMMA vaccines.

For safety and immunological reasons, we engineered the vaccine strain to have deleted *lpxL1* and be non-encapsulated which is associated with the inability to cause invasive disease [40]. As described for group B strains, deletion of *lpxL1* resulted in decreased ability of the group W GMMA to stimulate IL-6 release by human PBMC and activate TLR-4. These data indicate that genetic detoxification of meningococcal LOS by inactivation of *lpxL1* is a common mechanism among different serogroups.

Consistent with our hypothesis that removal of the capsule would enhance the level of bactericidal activity induced against non-W serogroups, GMMA produced by the non-encapsulated mutant W strain induced higher bactericidal titres against A and X strains, than the isogenic encapsulated control. The underlying mechanisms require further investigation. Capsular polysaccharide on GMMA may mask fHbp epitopes from the immune system, particularly from fHbp-specific B cells. An alternative explanation is that capsular polysaccharide on GMMA may serve as an antigenic competitor, interfering and decreasing the immune response to common protein antigens such as fHbp, although addition of external group A polysaccharide conjugate did not impair antibody responses to protein antigens in a meningococcal NOMV vaccine [34]. Thermostability is also highly desirable for any new vaccine targeted at the African meningitis belt and we are currently investigating this quality in our GMMA vaccine.

In conclusion, the findings of this study provide support for a GMMA-based vaccine approach as an affordable and broadly-protective vaccine strategy against meningococcal meningitis for Africa.

Conflict of interest

OK, OR, AS and CAM are employees of the Novartis Vaccines Institute for Global Health. CAM is the recipient of a clinical research fellowship from GlaxoSmithKline.

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