Native Outer Membrane Vesicles As Vaccine Candidates Against *Neisseria meningitidis*

Arianna Marini

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Institute of Immunology and Immunotherapy
MRC Centre for Immune Regulation
University of Birmingham
Edgbaston
Birmingham
B15 2TT
UK

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ABSTRACT

An affordable vaccine against all serogroups of *Neisseria meningitidis* causing reoccurring epidemics in sub-Saharan Africa is needed. Native outer membrane vesicles (NOMV) with over-expressed factor H binding protein (fHbp) represent a promising vaccine approach.

We investigated the contribution of fHbp variant 1 amino acidic sequence to the cross-reactivity of the antibody response generated by NOMV OE fHbp. Coupling structure-sequence analysis of fHbp with epidemiological data of meningococcal prevalence in Africa, we selected four fHbp v.1 IDs (1, 5, 9, 74), to generate isogenic mutants of a serogroup W African isolate, over-expressing each of the them. NOMV from the mutants were purified, characterized, and the antibody response generated in mice was investigated, and compared to the corresponding recombinant fHbp.

This is the first study indicating that the amino acid sequence of fHbp influences the specificity of the antibody response generated, not only as recombinant protein, but also when over-expressed on NOMV.

In mice NOMV OE fHbp induced a fast, long-lasting antibody response, with high IgM and IgG antibody levels 7 days after immunization, and germinal centers induced. LipidA modifications do not impact primary antibody response, while precipitation of the antigen on alum attenuated the early antibody response, but enhance its longevity.

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ABBREVIATIONS

APC	Antigen Presenting-Cell
App	Adhesion and penetration protein
ASC	Antibody Secreting Cell
BCR	B Cell-Receptor
BSA	Bovine Serum Albumin
C'	Complement System
CAMP	Cationic Antimicrobial Peptide
CC	Clonal Complex
CCP	Complement Control Protein
CD	Cluster of Differentiation
CEACAM	CarcinoEmbryonic Antigen-related Cell-Adhesion Molecule
CFU	Colony-forming Unit
DC	Dendritic cell
dOMV	detergent-extracted Outer Membrane Vesicles
DZ	Dark Zone
EF	Extra-Folicular
ELISA	Enzyme-Linked Immunosorbent Assay
fHbp	factor H-binding protein
GC	Germinal Center
GMMA	Generalized Modules for Membrane Antigens
GNA33	Genome-derived Neisseria Antigen 33

HSC	Hematopoietic Stem Cell
HSPG	Heparin Sulfate Proteoglycans
ID	Identification Number
IFNγ	Interferon γ
Ig	Immunoglobulin
IL-n	Interleukin-n
IM	Inner Membrane
KO	Knock-Out
LOS	Lipooligosaccharide
Lpp	Braun's lipoprotein
LPS	Lipopolysaccharide
LZ	Light Zone
MAC	Membrane-Attack Complex
MBL	Mannose-Binding Lectin
MenA	*N. meningitidis* serogroup A
MenB	*N. meningitidis* serogroup B
MenC	*N. meningitidis* serogroup C
MenW	*N. meningitidis* serogroup W
MenX	*N. meningitidis* serogroup X
MenY	*N. meningitidis* serogroup Y
MHC	Major Histocompability Complex
MLST	Multilocus Sequence Typing

MspA	Meningococcal serine protease A
MyD88	Myeloid Differentiation Factor 88
MZ	Marginal Zone
NadA	Neisserial adhesin A
NCAM	Neural Cell-Adhesion Molecule
NETs	Neutrophil Extracellular Traps
NhhA	*Neisseria* hia homologue A
NK	Natural Killer cells
NOMV	Native Outer Membrane Vesicles
O-IMV	Outer-Inner Membrane Vesicles
O/N	Over night
OE	Over-expressing
OM	Outer Membrane
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicles
Opa	Opacity protein
PALS	Periarteriolar Lymphoid Sheath
PAMP	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFGE	Pulsed-Field Gel Electrophoresis
PG	Peptidoglycan
PRR	Pattern-Recognition Receptors

SBA	Serum Bactericidal Activity
SCR	Short Consensus Repears
SFU	Spot Forming-Unit
SHM	Somatic Hyperutation
ST	Sequence Type
TCC	Terminal Complement Complex
TCR	T Cell Receptor
TD	T-Dependent Antigen
Tfh	Folliular helper T cell
Th	Helper T cell
TI	T-Independent Antigen
TIR	Toll-Interleukin 1 Receptor
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor α
Treg	Regulatory T cell
VR	Variable Region
WB	Western blot

CHAPTER 1. INTRODUCTION

The project that led to this thesis was in the context of the development of a meningococcal vaccine for Sub-Saharan Africa, based on GMMA (Generalized Modules for Membrane Antigens). The aim of my PhD was to contribute to the generation of such vaccine candidate, and to investigate the immune response, in mice.

I spent the first two years at GVGH, where the attention was focused on the generation and characterization of a panel of vaccine candidates (Chapter 3), in order to then investigate the specificity and cross-reactivity of the antibody response elicited in mice, with particular attention to the key antigen factor H binding protein (fHbp) (Chapter 4). For the last year of the Doctorate I moved to the University of Birmingham, where the aim was to elucidate different aspects of the immune response generated in mice by the vaccine candidates produced in GVGH (Chapter 5).

I am introducing here the value of vaccination, and I am then talking about main aspects of *Neisseria meningitidis*, outer membrane vesicles, the use of aluminium salts for vaccine formulations, and lastly the main features of the immune system. These topics will be the basis for the description and analysis of the results of my PhD work.

1.1. Vaccines

Infectious diseases account for a considerable burden of disease worldwide, and especially in low-income countries: while in high-income countries the most prevalent causes of death are chronic diseases (cardiovascular diseases, cancers, dementia, chronic obstructive lung disease, and diabetes), in developing countries almost one third of all

deaths are caused by lower respiratory infections, HIV/AIDS, diarrhoeal diseases, malaria, and tuberculosis [1]. Vaccination is the most efficient tool and cost-effective measure for preventing infectious diseases from spreading. According to data from the World Health Organization (WHO), active immunization currently avoids an estimated 2 to 3 million deaths every year. Nevertheless, 18.7 million infants worldwide are still missing basic vaccines [2].

Different types of vaccines have been developed through the years, aiming to enhance protection against various diseases, while reducing side effects: live attenuated microorganisms, inactivated microorganisms, subunit vaccines, toxoid vaccines, and DNA vaccines [3, 4]. Live attenuated vaccines are more often used for viruses than for bacteria; they consist of a weakened version of the pathogens, so they can have serious side effects related to the possibility that the microorganism reverts to the pathogenic form. However, this type of vaccine has been used successfully for fighting important diseases, such as polio, and is still in use against diseases such as measles, mumps, and rubella [5]. Inactivated vaccines are killed pathogens, so they are safer than live attenuated vaccines, but, at the same time, could also be less immunogenic. Subunit vaccines include parts of the pathogen, which can evoke a protective immune response; these subunits can be protein, sugars, or sugars conjugated to carrier proteins. Toxoid vaccines are subunit vaccines that are particularly useful when the pathogen secretes toxins; they consist in the "detoxified" toxin [6]. DNA vaccines consist in the genetic sequence of the antigen of interest; once delivered in the immunized hosts, the genetic sequence presented is then translated into the corresponding antigens, and an immune

response is elicited. DNA vaccines are useful not only against infectious diseases [7], but also against cancer, as well as against autoimmune disease.

1.2. Neisseria meningitidis

Neisseria meningitidis was identified for the first time in 1887 as a "diplococcus intracellularis meningitidis", from the cerebrospinal fluid of a sick patient, by the Austrian pathologist and bacteriologist Anton Weichselbaum [8]. Later, it was re-classified and included in the genus *Neisseria*, named after Albert Neisser, the German physician who had discovered the diplococcus *Neisseria gonorrhoeae*. The name *Neisseria meningitidis* was established after meticulous discussions [9].

N. meningitidis is a Gram-negative diplococcus, about 0.6–1.0 μm in diameter. It is a non-sporulating, obligate aerobic, and oxidase-positive microorganism. *N. meningitidis* can be either encapsulated or not; non-capsulated meningococci can colonize human hosts, but invasive disease is almost always caused by encapsulated strains [10, 11]. On the basis of the capsular polysaccharide, 13 serogroups have been identified, and among these 6 are associated with the majority of invasive cases: MenA, MenB, MenC, MenY, MenW, and MenX [12].

Meningococcus is a strict human commensal; it colonizes the upper respiratory tract and has no other known environmental niche [13]. Around 20% of the adult population are asymptomatic carriers, but the carriage rate rises and may reach almost 100% in groups of people living in close contact, such as university students or military recruits [14-17]. This is related to the transmission route of the bacterium, that occurs via droplets [18]. Progression from carriage state to disease is a rare event, and the mechanism is not

completely clear yet. In most people carriage is an immunizing process, and a systemic protective antibody response is developed [19]. Nevertheless, encapsulated strains can penetrate the mucosa and gain access to the bloodstream, causing systemic disease. Many different aspects play a role in the disease development, and their particular combination is crucial for the outcome: bacteria virulence factors, host susceptibility and mucosa integrity, and genetic polymorphism.

Meningococcal diseases have different clinical manifestations (bacteraemia without sepsis, meningococcaemia without meningitis, meningitis with or without meningococcaemia, and meningoencephalitis), and mortality rate depends on the type of disease developed, with fulminant septicaemia showing the highest mortality rate (up to 55%), and meningitis without sepsis the lowest (<5%) [15, 20, 21]. Early symptoms can be undistinguishable from other bacterial and viral infections, but mild symptoms can rapidly change to a terminal disease. The most common symptoms are neck stiffness, high fever, sensitivity to light, confusion, headaches, and vomiting [22]. 5 to 10% of the patients die within 24-48 hours from the onset of the symptoms, even if early diagnosed and appropriately treated. Without treatment, mortality can reach 70-90%. Around 25% of the survivors develop permanent sequelae (epilepsy, mental retardation or subtle cognitive dysfunctions, deafness, hydrocephalus, renal failure, skin scarring, limb amputations) [23-27].

1.2.1. GENETICS

The *N. meningitidis* genome is 2.0-2.2 megabases, and contains around 2,000 genes, 70% of which are involved in essential metabolic functions [28]. 90% of the genome sequence is shared with *N. gonorrhoeae* and *N. lactamica,* with whom *N. meningitidis* is hypothesized to have a common non-capsulated ancestor; meningococcus would have acquired the genes involved in the capsule biosynthesis later, from other bacterial species colonizing human nasopharynx, possibly members of the *Pasteurellaceae* family, by horizontal gene transfer [28]. This fact reflects an important feature of meningococcus genome: its plasticity. *N. meningitidis* is indeed capable of exchanging DNA not only with *Neisseria* spp., but also with other genera, such as *Haemophilus* [28-30].

Based on the DNA sequence of 7 housekeeping genes, multilocus sequence typing (MLST) is the gold-standard for molecular typing and classification of meningococcus, displacing other molecular techniques, such as pulsed-field gel electrophoresis (PFGE) or multilocus enzyme electrophoresis. By MLST, meningococcal strains are divided into different sequence types (STs), that are independent from the serogroup [31]; some of them are significantly over-represented in cases of invasive disease, so they have been identified as hyperinvasive. Clone complexes (CCs) are groups of related STs, which are thought to correspond to lineages of bacteria deriving from a common ancestor [32, 33]; also among CCs there is a different degree of variability in their association with invasive disease. [33].

1.2.2. CELL ENVELOPE

1.2.2.1. CAPSULE

As previously mentioned, meningococci can be either encapsulated or not, and on the basis of the capsule polysaccharide they can be classified into 13 different serogroups. The polysaccharide capsule is an important virulence factor, and it is essential for meningococcus survival in blood and CSF: it confers the bacterium resistance to antibody and complement mediated killing, by inhibiting opsonic and non-opsonic phagocytosis. The capsule also helps meningococci to avoid desiccation and survive in aerosol droplets on inanimate surface (from few hours to few days *in vitro*, depending on both the strain tested and the type of surface), favouring spread and transmission [34, 35].

Composition and structures have been determined for the various serogroups, which possess chemical and immunological specific features. In particular, serogroup A polysaccharide is a homopolymer of (α1→6) N-acetylmannosamine 1-phosphate [36]; serogroup B and C polysaccharides are homopolymers of sialic acid (N-acetylneuraminic acid) with different linkages (α2→8 in serogroup B and α2→9 withpartially O-acetylated sialic acid in serogroup C) [37]; serogroup Y polysaccharide is an alternating of D-glucose and partially O-acetylated sialic acid [38]; serogroup W polysaccharide is an alternating D-galactose and partially O-acetylated sialic acid [38]; serogroup X polysaccharide is a homopolymer of (α1→4) N-acetylglucosamine 1-phosphate.[39, 40]. Sialic acid is commonly present on the host cell surface, so when present on bacteria it contributes to their mimicry and favours meningococci escaping the immune system; the extreme of

this mimicry is seen with MenB serogroup, whose sialic acid is identical in structure to the human fetal neural cell-adhesion molecule (NCAM) [41].

In the meningococcus genome, one single locus *cps* encodes all the process of biosynthesis and translocation of the capsule; all the serogroups have comparable loci, and a common nomenclature has been published [42]. The *cps* locus is divided into 6 regions (A–D, D′, and E); in particular, region A encodes enzymes for the synthesis and polymerization of the capsular polysaccharide, and regions B and C are implicated in the translocation of the high molecular weight polysaccharides from the cytoplasm to the cell surface [42, 43]. *N. meningitidis* has various mechanisms to regulate and alter capsule biosynthesis, such as on-off/off-on phase-variation and capsule switching [44]. The latter happens by horizontal gene transfer and homologous recombination, and it can occur *in vivo* during co-colonization by different serogroups of the same host.

1.2.2.2. ENVELOPE MEMBRANES

As in all Gram-negative bacteria, the cell envelope is composed of an inner membrane (IM) and an outer membrane (OM), separated by a periplasmic space in which are located the peptidoglycan layer (PG) and proteins (Figure 1.1) [25, 45]. The PG cell wall is composed of no more than 2 layers, and it can vary in cross-linking and O-acetylation [46]; it is important for the overall architecture of the cell envelop, as it cross-links the outer and the inner membranes. The IM is a symmetric bilayer of phospholipids and proteins, while the OM is an asymmetric bilayer with the external surface composed of lipooligosaccharide (LOS), phospholipids and proteins, and the inner surface composed of phospholipids and proteins. Various outer membrane proteins (OMPs) ensure bacterial interaction with and adhesion to host cells, as well as the control of

intracellular environment. The periplasmic space (~13 nm) is important for protein folding, and can represent from 7% to 40% of the bacterial cell volume [47, 48].

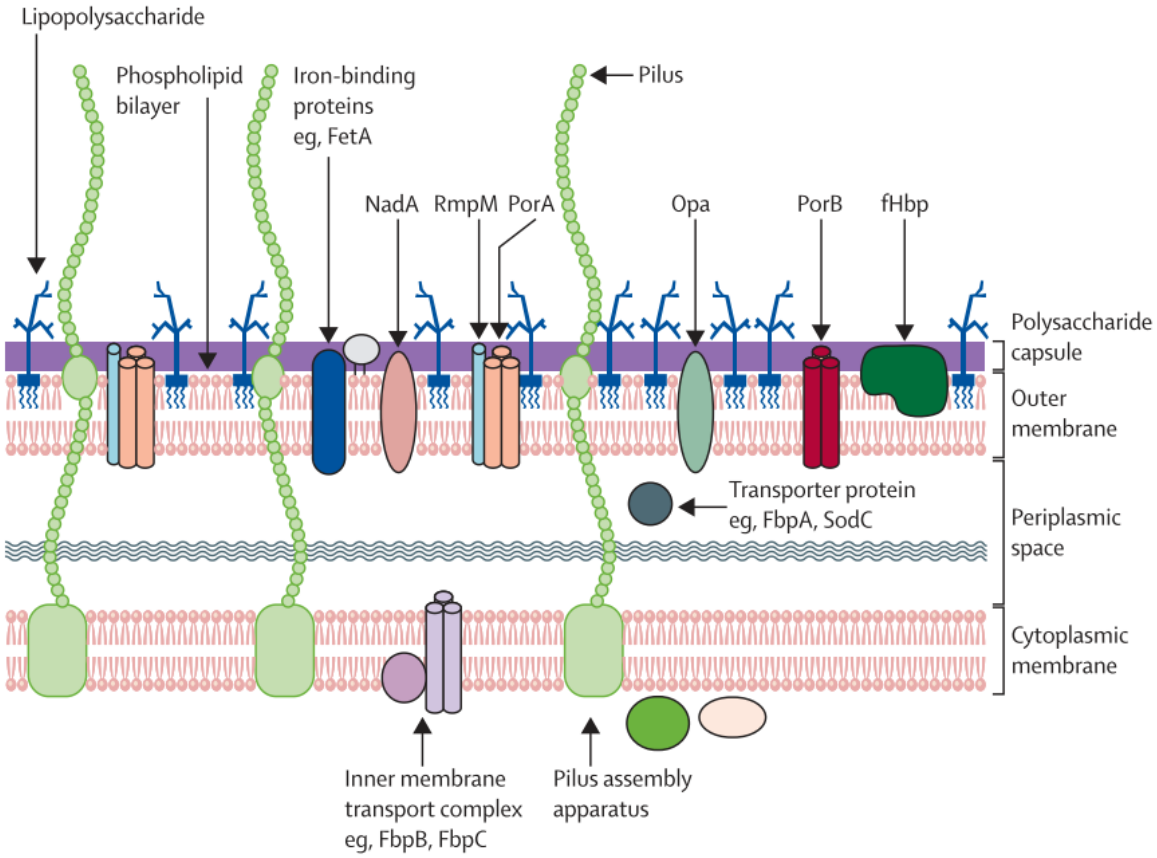


Figure 1.1. Representation of the surface structure of *Neisseria meningitidis*.

Inner (cytoplasmic) membrane, periplasmic space, outer membrane and capsule are represented in this picture, with all the main components indicated. Picture from Sadarangani M. and Pollard A., 2010 [45].

1.2.3. GNA33

Genome-derived *Neisseria* antigen 33 (GNA33, also called NMB0033) was identified during the screening of the *N. meningitidis* serogroup B genome to discover novel vaccine candidates and putative virulence factors [49]. It is a 47kDa lipoprotein, highly conserved among *Neisseria* spp., and homologous to the lytic transglycosylase MltA of *E. coli* [50]. GNA33 is involved in bacteria cell separation, cell membrane assembly, and virulence [51]. Initially, it was observed that recombinant GNA33 induces in mice bactericidal antibodies against meningococcal strains [52]. However, for the production of outer membrane vesicle-based vaccines, the deletion of this protein appeared to be promising [53]: as it is involved in membrane architecture, deletion of *gna33* gene induces physical dissociation of membrane material that leads to enhanced release of membrane vesicles [51].

1.2.4. LIPOPOLY-LIPOOLIGOSACCARIDE (LPS, LOS)

LPS (lipopolysaccharide) is a major component of the Gram-negative bacteria outer membrane, representing 35-45% of the OM. It is important for the structural integrity of bacterial cells, and also as a barrier from chemical compounds; naturally LPS-deficient strains have been isolated only for *A. baumanii* and *N. meningitidis* species [54].

As in *N. meningitidis* this molecule lacks a repeating O-side chain found in enteric Gram-negative bacilli, for this bacterium it is more accurate to use the term lipooligosaccharide (LOS).

LOS is composed of three parts: lipid A, containing hydroxy fatty-acid chains and phosphoethanolamine; an inner core oligosaccharide, containing 3-deoxy-D-manno-oct-

2-ulosonic acid (KDO) and heptose residues; an outer core of highly variable short oligosaccharides (Figure 1.2 A) [29, 55]. These saccharidic chains form the basis for the LOS classification into different immunotype (L1 to L12) [15], are subjected to antigenic and phase variations, and play a key role in interaction with host cells and pathogenesis [56]. Different LOS immunotypes, in fact, are associated with different pathological features and different carriage versus invasive disease establishment [29]. Moreover, immunotype-switching contributes to serum resistance *in vitro* [57, 58].

LOS induces bactericidal antibodies, but it also elicits antibodies against human epitopes [57]. However, it has always received more attention for its role in pathogenesis, rather than as an antigen [59]. In fact, LOS is associated with both pathogenesis and virulence: it is important for meningococcal colonization and invasion of epithelial cells, and plays a crucial role in the induction of septic shock [57, 60]. LOS is also important for the resistance of the bacteria to various host defences, like cationic antimicrobial peptides (CAMPs) [61-63]; moreover, sialylation of LOS in B, C, W, and Y serogroups helps meningococci to escape immune surveillance by mimicking host cell surface components [64, 65].

The major responsible for the biological activity and toxicity of LOS is the lipid core. Lipid A is the hydrophobic part of the LOS that anchors the molecule to the OM. It is composed of a glucosamine disaccharide with four to six acyl chains, and two phosphate groups (Figure 1.2 B) [54]. Biosynthesis of lipid A starts with a single molecule of UDP-N-acetylglucosamine (GlcNAc). The biosynthetic pathway for KDO_2-lipid A is schematized in Figure 1.3; it has been investigated mostly in *E. coli*, but it is a well-conserved pathway

among Gram-negative bacteria. These bacteria share indeed the majority of the enzymes required, even though there are differences related to the variations in the lipid A structure [54, 66]. In particular, diversely from *E. coli*, *N. meninigitidis* has a "*C. violaceum*-type lipid A", that is a symmetrical distribution of the acyloxyacyl chains [67]. Consequently, the final acylation steps in the meningococcal pathway differ from the pathway described for *E. coli*: after acylation of UDP-GlcN by LpxA and LpxD, and dimerization by LpxB, the last two fatty acid chains are not added by LpxL and LpxM, but by the homologous LpxL2 and LpxL1 [68]. LpxL2 adds the C_{12} chain to the N-linked 3-OH C_{14} at the position 2 of GlcN I, and LpxL1 is responsible for adding the last C_{12} chain to the penta-acylated lipid A molecule [68].

Lipid A exercises its endotoxin activity through the Toll-like receptor 4 (TLR4) signalling pathway [63]: through this receptor, it stimulates the release of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α). Further details regarding TLR4 signalling will be elucidated in section *Toll-like receptors*, page 53. The most potent activator of the TLR4 is the complete molecule of lipid A, i.e. the diphosphate hexa-acylated form; alterations of this structure lead to a reduced activation of the Toll-like receptor [54]. Modification of lipid A can be a bacterial strategy for evading host immune system: 10% of meningococcal clinical isolates are found with mutated LOS; in particular, it is rather common for clinical isolates to have the lipid A penta-acylated, due to mutation in the *lpxL1* gene. This form of the molecule has reduced ability to stimulate TLR4 activation and consequent cytokine release [68]. Therefore, compared to isolates with hexa-acylated lipid A, *lpxL1* mutants are associated with less

severe systemic inflammation and coagulopathy, leading to a general less severe meningococcal disease course [54, 69, 70].

Mutations in the biosynthetic pathway of the lipid A can also be exploited for vaccine purposes. In fact, vaccines based on outer membrane vesicles carried the problem of the LOS: it can function as a natural adjuvant, but, on the other hand, its endotoxicity is responsible for high reactogenicity [71]. The first approach for detoxifying outer membrane vesicles, to be used as vaccines, involved their preparation by detergent extraction (dOMV purification process). However, detergent treatment to decrease LOS toxicity has disadvantages, like the removal of lipoproteins that contribute to immunogenicity, and alteration of the membrane structure that can results in aggregation [72]. An alternative to the detergent treatment is the genetic modification of the LOS biosynthesis pathway: inactivation of the *lpxL1* gene results in the penta-acylated lipid A, which correlates with lower endotoxicity, while maintaining adjuvant activity [68, 71]. Further engineering the biosynthetic pathway, inclusion of enzymes from different bacterial species, also led to different forms of lipid A, modified to obtain specific adjuvant properties [73].

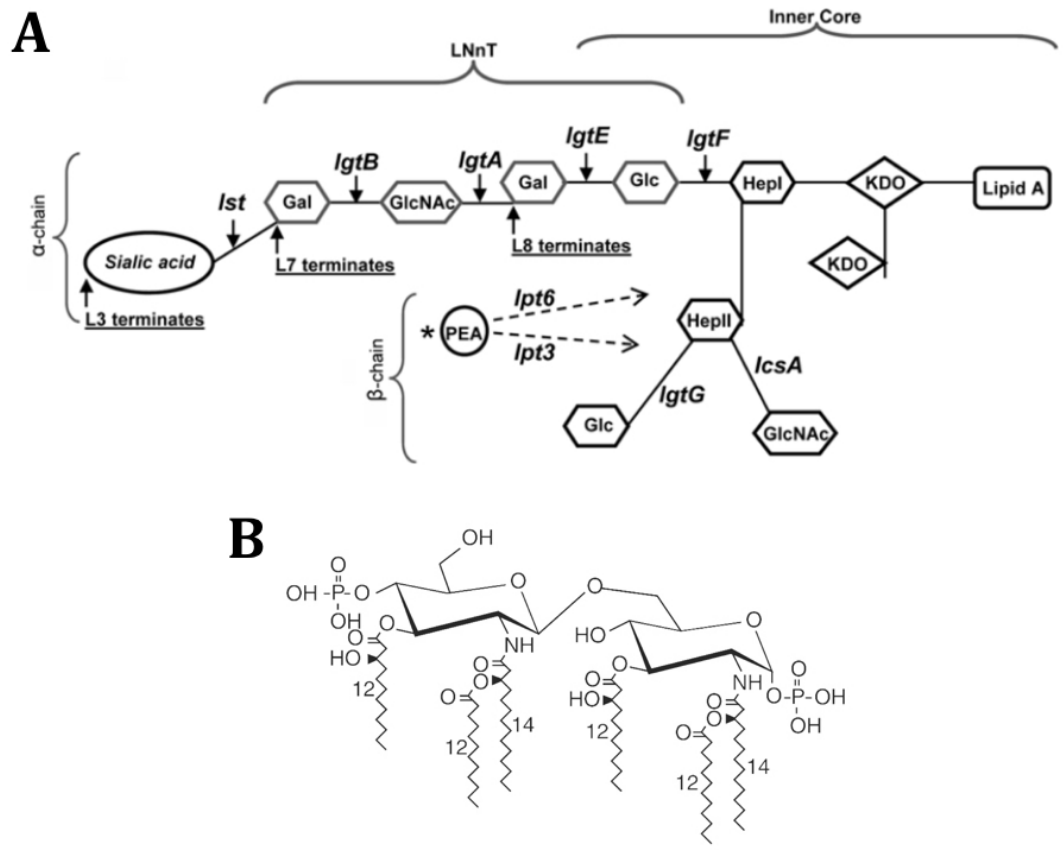


Figure 1.2. Schematic structure of *N. meningitidis* LOS and lipid A.

A) Meningococcal LOS is composed of lipid A (in the membrane) bound to two KDO (2-keto-3-deoxy-d-manno-2-octulosonic acids) and two heptose (HepI and HepII) moieties. Extended from the membrane are α- and β-chains, structurally variable. Glc: glucose; GlcNAc: N-acetyl-D-glucosamine; Gal: galactose; PEA: phosphoethanolamine; LNnT: lacto-N-neotetraose; lst, lgtB, lgtA, lgtE, lgtF: enzymes involved in the biosynthetic pathway for the LOS formation. Picture from Hill D. J. *et al.*, 2010 [15]. **B)** Schematic lipid A structure. Numbers indicate the fatty acid chains length. Picture adapted from Zariri A. and van der Ley P., 2015 [54].

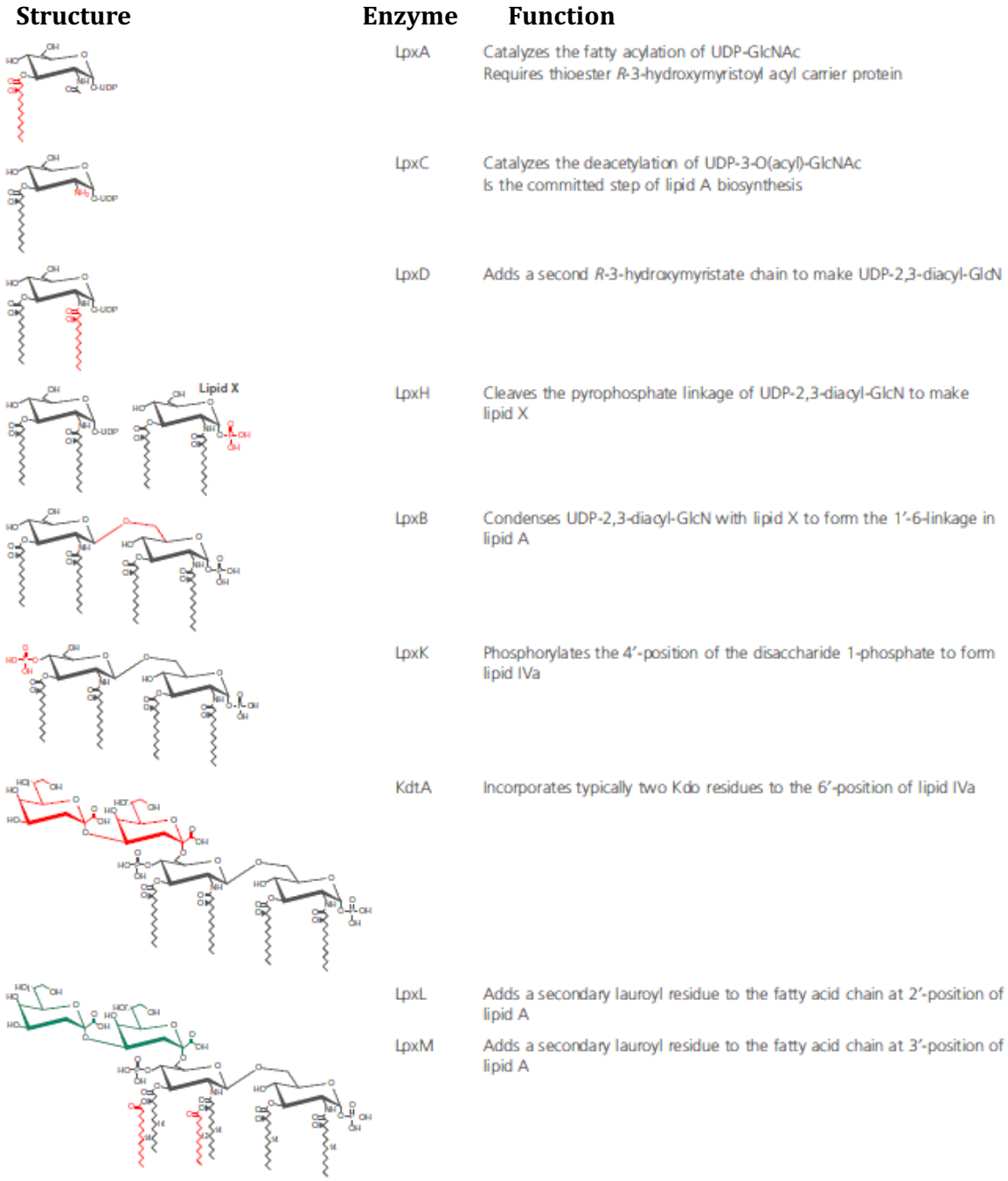


Figure 1.3. Biosynthetic pathway of KDO_2-lipid A in *E. coli*.

Each subsequent addition in the lipid A-Kdo2 structure is indicated in red. The glucosamine backbone is black and the Kdo_2 is in green. GlcNAc: N-acetylglucosamine; Kdo: 3-deoxy-D-manno-octulosonic acid. Picture and legend adapted from Zairi A. and van del Ley P., 2015 [54].

1.2.5. ADHESINS

In order to colonize its host, *N. meningitidis* first needs to take contact with the host cells. Several molecules and proteins are involved in such adhesion. Meningococcal adhesins can be divided into two groups, major and minor adhesins: major adhesins are expressed in abundance on the bacterial surface, and have been extensively studied; minor adhesins are expressed at low level from bacteria grown *in vitro*, and only recently scientists have started to investigate their role in the interaction between meningococcus and host cells [15]. Also porins, even though not classified as adhesins, play an important role in bacteria adhesion.

Major adhesins are pili and opacity proteins.

N. meningitidis pilus belongs to the type IV pilus family: by rapid extension and retraction, it confers meningococcus twitching motility [74]. It measures 6 nm in diameter and several thousand nm in length. It is a homopolymer composed of pilin (PilE) subunits, arranged helically [75, 76]. 23 other proteins are associated with pilus: 15 of them are essential for pilus biogenesis, and 7 are involved in the modulation of its functions [64]. *pilE* gene undergoes sequence variation, by inter- and intra-genomic recombination with silent *pilS* gene [15], and such variation contribute to *N. meningitidis* evasion from the immune system: antigenically different pili from different infection sites of the same patient have been reported [77, 78]. PilE protein also undergoes post-translational modifications at several serine residues: glycosylation (first case of protein glycosylation reported for bacteria), α-glycerophosphate, substitution with phosphate, PEA, or phosphorylcholine [15, 79]. The combination of post-translational modifications and the host target cell type impacts the efficiency of pilus-mediated adhesion of

capsulated meningococcal strains [80]. Pilus molecular target, however, remains elusive: it has been suggested to be CD46 molecule, but without unanimous agreement among researchers [64]. Meningococcal pilus is not only crucial for host cell adhesion: it plays an important role also in external DNA uptake [81].

Opacity proteins, Opa and Opc, are the most abundant adhesins on meningococcal outer membrane. Their name is related to the opaque phenotype they confer to colonies [64]. Opa is expressed also by gonococci, while Opc is peculiar of meningococci [82]. They are similar in size (27-31 kDa) and structure (β-barrel proteins), but while Opa has four surface-exposed loops (three of them variable), Opc has five [64]. Opa can be encoded by different genes (*opaA, opaB, opaD, opaJ*), whose expression shows phase- and antigenic-variability; however, some Opa types are predominantly present among clinical isolates, suggesting that adhesion and virulence properties are different among different Opa types [15, 64]. Opc is, instead, encoded by one single gene and it is invariant [82]. Opa proteins bind to different members of the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM) family, and the specificity for one member or another of this receptors family depends on Opa variable loops [15, 83]. It was suggested that Opa binding to CEACAM1 on $CD4^+$ T cells may inhibit T cell activation and proliferation, but it is not certain [84]. Another target of Opa proteins are the cell-surface-associated heparin sulfate proteoglycans (HSPGs), with different binding specificity between different Opa [15].

Minor adhesins are NadA (Neisserial adhesin A) [85], NhhA (*Neisseria* hia homologue A) [86, 87], App (Adhesion and penetration protein) [88], MspA (Meningococcal serine protease A) [89].

1.2.6. PORINS

Porins are the most abundant proteins in the outer membrane. Differently from other meningococcal proteins, they do not undergo antigenic variations during infection [90]. The two main meningococcal porins are PorA and PorB. They are involved not only in the diffusion of hydrophilic nutrients, but also in interaction with host cells [29]. PorA (~ 45 kDa) was first named as Class 1 outer membrane protein (OMP), while PorB (~ 33 kDa) Class 2 or 3 OMP. *N. meningitidis* and *N. gonorrhoeae* porins share 60-70% homology. PorA, is present only on meningococcus outer membrane; in fact, even though the gene is present in the gonococcus genome, it is not expressed because of inactivating mutations in the promoter and frameshift mutations in the coding region [91]. Interestingly, deletion of both major porins results in vital meningococcus strains, at least *in vitro*, thanks to compensatory expression changes in other proteins involved in the cellular redox state, energy/nutrient metabolism, and membrane stability [92]. *Neisseria* porins organize in trimers (also heterotrimers), and the periplasmic protein RmpM has been suggested to play a role in the stabilization of porin oligomers [93]. These porins have β-barrel structure, similar to the other Gram-negative porins. Conserved portions of the peptide sequence are located in the inner amphipathic β-barrel parts, while the variable regions (VR) form the hydrophilic loops that protrude from the membrane (Figure 1.4) [90, 94, 95]. Among the eight surface-exposed loops of PorA, II and III have structural roles and therefore are not as variable as the others; loops

I and IV, instead, account for the antigenic variability, and constitute the VR1 and VR2 that also represent the rational for PorA nomenclature [94, 95]. Based on the amino acid similarity, in fact, VR1 and VR2 sequences are divided into families, and the name of PorA variant and subvariants is indicated as follows: the prefix "P1" is followed by the VR1 family name, followed by a dash and then the variant number; a comma separate this from the subsequent VR2 variant name in the same format [95]. A precise and shared nomenclature is particular useful, as porins variable regions are used for serological typing, important for epidemiology and vaccine approaches [96]. In fact, PorA elicits bactericidal antibodies, but such anti-PorA bactericidal antibodies are directed toward the variable loops of the protein [97]; therefore PorA-based vaccines offer strain-specific protection, and in the past have been useful mainly to contain clonal outbreaks of serogroup B meningococcal disease [98].

Besides being important for nutrients diffusion, neisserial porins play also different roles in the interaction with host cells; for example, they are known to induce B cells proliferation and antibody secretion [99]. Moreover, PorB modulates cell host apoptosis and activation of Toll-like receptor 2 (TLR2) [99-101]; the latter is important for the adjuvanticity of this porin, which has, in fact, been considered a good candidate as adjuvant for vaccine formulation [99, 102].

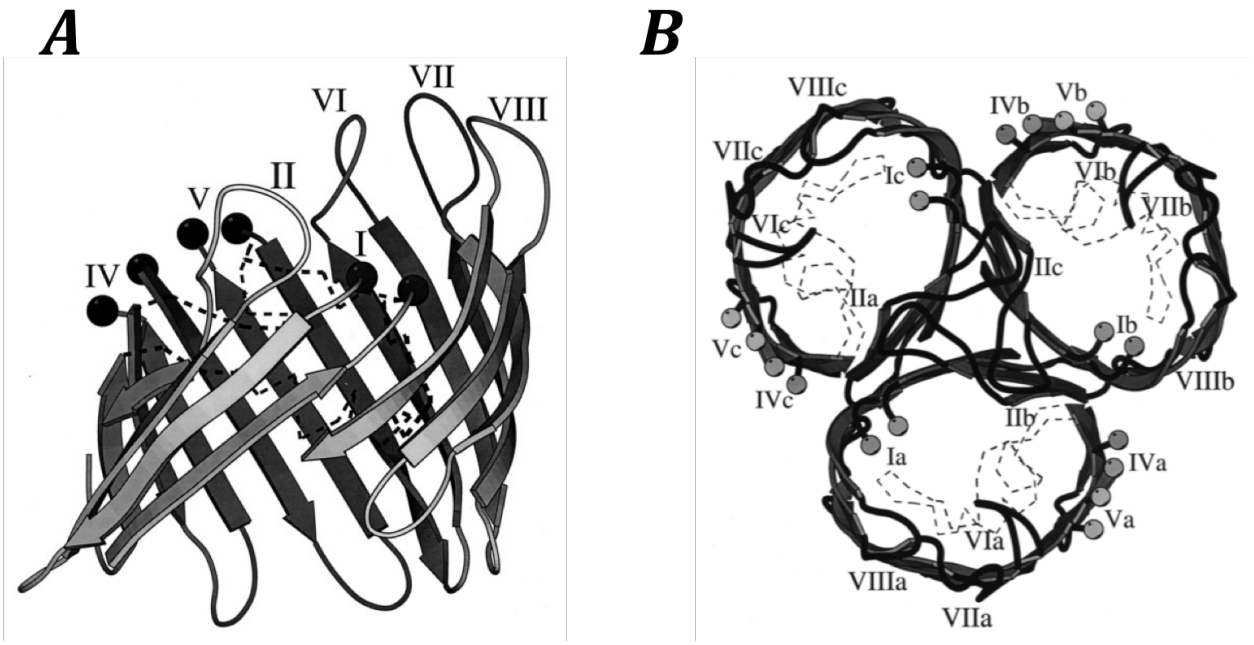


Figure 1.4. Model of the structure of a monomer (A) and trimer (B) of PorA.

A) The positions of the external loop regions are indicated I to VIII, with the exception of loop III, which lies inside the protein and is shown by the broken line. B) Model of a PorA trimer, viewed from above. The positions of the external loop regions are marked as for (A), with the subunits designated a, b, and c. Pictures and legends adapted from Derrik J. P. *et al.*, 1999 [94].

1.2.7. FACTOR H BINDING PROTEIN (FHBP)

Factor H binding protein (fHbp) is a 27 kDa surface-exposed outer membrane lipoprotein. It was detected during studies that aimed to discover vaccine candidate antigens against serogroup B *N. meningitidis*. It was identified by two independent groups for its ability of eliciting bactericidal antibodies in mice, and initially referred to as GNA1870 [103] or as LP2086 [104]. The name fHbp was assigned when its crucial feature of binding human factor H was elucidated [105].

fHbp is expressed by almost all meningococcal strain, regardless for the capsule serogroup, with varying expression levels among them [103, 106-108]. Strains are generally referred to as high-, medium- or low- fHbp expressors. Recently, however, the absolute quantity of fHbp molecules on a panel of clinical *N. meningitidis* clinical isolates was measured by mass spectrometry, confirming that the levels of this lipoprotein differ at least 15-fold among different strains, and do not correlate with serotypes nor with CC or ST classifications [109].

On the basis of amino acid diversity, fHbp was divided into 3 antigenic variants (v.1, v.2, and v.3) [103], or into 2 sub-families (subfamily A, corresponding to v.2 and v.3; subfamily B, corresponding to v.1) [104]. Each variant can be further divided into sub-variants, with small differences in their amino acid sequence, and each sub-variant is identified by a specific identification number (from ID 1 to ID 958 so far [110]). Antibodies against recombinant fHbp show cross-reactivity within each variant group, but limited cross-reactivity between different variants; however, each fHbp ID within

one variant elicits antibodies with diverse specificities [103, 104, 107, 108, 111, 112]. Analysis of the variability of fHbp amino acidic sequence further led to an appreciation of the overall architecture of the protein as a mosaic of five modular variable segments, which can combine into nine different modular groups [113, 114].

Even though there is no correspondence between fHbp sequence variability and serotypes, different distribution of the variants among the various meningococcal serogroups can be appreciated. For example, in USA, variant 1 is predominant among MenB strains, but present in only a small minority of MenY strains [115]. Among African strains, fHbp variability is limited: all MenA isolates express variant 1 (ID 4 or ID 5, whose amino acidic sequences differ by one residue), as well as the majority of MenX strains (ID 74 or ID 73, whose amino acidic sequences show 93.3% identity); among MenW strains all the three variants are expressed, with the majority of isolates expressing variant 2 (ID 22 or ID 23, whose amino acidic sequences differ by one residue), or variant 1 (ID 9) [111].

The crucial feature of this protein is that it binds human factor H, which is a negative regulator of the human complement cascade (C') (1.5.2.3. The Complement System, page 56). By recruiting factor H onto its surface, meningococcus avoids complement-mediated killing [105, 116]. Antibodies raised against fHbp, both in mice and in humans, have bactericidal activity, and determine meningococcal killing in two ways: first, preventing the recruitment of factor H, so that the bacterium is no longer able to escape complement-mediated killing; second, activating the classical C' pathway [105]. The fHbp contributes to immune evasion, however, is not limited to binding fH. In fact, it has been proposed to be important for meningococcal survival in the presence of the

antimicrobial peptide LL-37 [117]. Reported interaction of fHbp with siderophores further suggested other important roles of this protein in the biology of meningococccus [118].

Even though the amino acid sequence is highly variable among the different fHbp variants, the molecule structure is conserved [119]. fHbp is folded into two β-barrels connected by a 15-residues peptide linker, and it is anchored to the outer membrane by the N-terminal Cys residue, modified with a tripalmitoyl-S-Cys moiety (Pam3Cys). The flexible portion between residues 1 and 19 allows fHbp to span the LOS layer of the outer membrane [120, 121]. Both β-barrels are involved in extensive interaction with fH, interaction that is characterized by high affinity, allowing meningococcus to quickly sequestrate the C' inhibitor [122]. Nevertheless, the strength of the binding between the two molecules largely vary with fHbp sequence variations; this, however, does not appear to impact the survival of the bacterium in human blood, which anyway has redundant mechanisms for evading complement-mediated killing [123-125].

Since its discovery, fHbp has been considered a major antigen to be exploited for vaccine design against *N. meningitidis*. Being a protein, it is fundamental for anti-serogroup B meningococcus vaccines, which cannot rely on capsular polysaccharide antigens, because its similarity with human NCAM (see paragraph Cell envelope

Capsule, page 23). Moreover, as it is expressed by almost all *N. meningitidis* strains, and regardless of the serotype, fHbp can be used for generating pan-specific vaccines. As recombinant protein, fHbp has been included in licensed protein-based vaccines [124-

127] and is also under investigation as one of the main target antigens in vaccines based on native outer membrane vesicles [53, 126].

1.2.8. EPIDEMIOLOGY AND VACCINATION IN AFRICA

With around 500,000 cases of infection and up to 50,000 deaths per year globally, *N. meningitidis* accounts for an important portion of bacterial meningitis. Incidence varies according to geography: from rare sporadic outbreaks across Europe and USA (<1case/100.000 population/year), to large-scale epidemics in the area of sub-Saharan Africa (10 to >1.000cases/100.000 population/year) [12, 19, 25, 127]. This area, stretching from Ethiopia to Senegal, bears the largest burden of meningococcal disease worldwide, and this is why it has been called "Africa meningitis belt". Here epidemics show marked seasonality, with peaks during the dry, hot season [128]. Until 2010, in Africa, serogroup A was responsible for the majority of cases and deaths. After the introduction of MenAfriVac®, a group A polysaccharide conjugated vaccine, incidence of MenA cases and carriage dramatically dropped in the countries where the vaccine was introduced for routine immunization [129-131]. W and X serogroups, however, increased their prevalence, and in 2015 also C serogroup had an important impact on the burden of disease, being responsible for over 60% of meningococcal cases [132]. This highlights the need for a vaccine that provides protection against all serogroups causing disease in the area, and postpones the emergence of new ones as far as possible.

Quadrivalent conjugate vaccines based on the capsular polysaccharide are available against strains belonging to capsular groups A, C, W, and Y [133, 134], but are unlikely to be affordable for Africa. The use of unconjugated A, C, W polysaccharide vaccine has been adopted for many years to control epidemics in the African meningitis belt, based on

WHO recommendations, but polysaccharide vaccines are not effective in children under the age of 2 years, can only provide short-term protection, and have little or no impact on pharyngeal carriage. Therefore, polysaccharide vaccines can be useful to control and limit the extent of meningitis outbreaks, but cannot prevent the continuing occurrence of epidemics. Currently, promising affordable vaccine candidates against meningococcal disease for Africa are based on outer membrane vesicles [53, 126, 135].

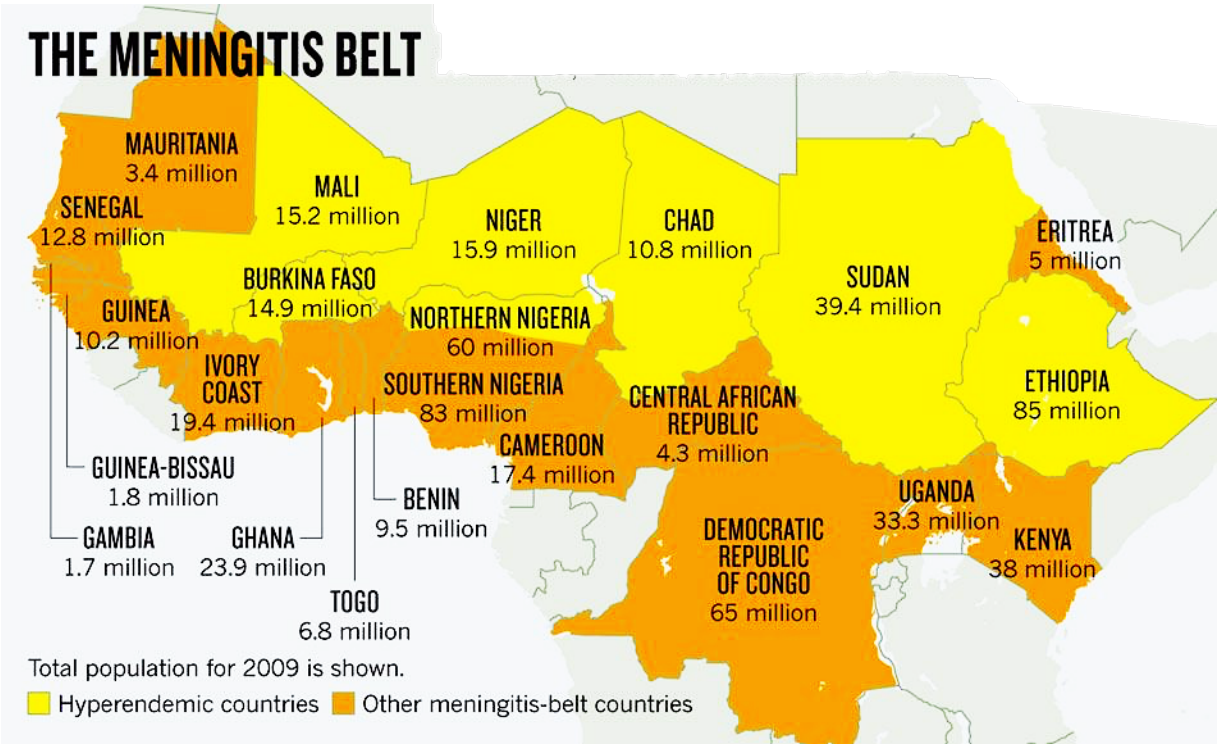


Figure 1.5. Africa meningitis belt.

Countries involved in the African meningitis belt, with population as calculated in 2009. Picture adapted from Butler, 2010 [136].

1.3. Outer Membrane Vesicles (OMVs)

From mycoplasma [137] to Gram-positive bacteria [138] to eukaryotic cells [139-143], all cells release membrane vesicles for several purposes. Similarly, Gram-negative bacteria shed outer membrane vesicles (OMVs), to accomplish different tasks.

OMVs are blebs naturally shed by both pathogenic and non-pathogenic Gram-negative bacteria (Figure 1.6). They have a diameter that varies approximately between 25 and 250 nm, and are not a product of bacterial lysis, but the result of an active process.

Depending on the species and on the environment the bacteria is facing, OMVs have different aims and purposes [47, 48]. Some of the functions of OMVs have been elucidated in relation to their content. In fact, proteomics studies revealed that OMVs composition does not necessary reflect the one of the outer membrane: it is rather enriched or depleted of specific molecules [144], and the specific protein composition helped deducing the reason why these vesicles are released [144-146]. OMVs are generally involved in nutrient acquisition [147], horizontal gene transfer [148], delivery of virulence factors and toxins [149-152], biofilm formation and maintenance [153-155], resistance to antimicrobials and to phages [156], stress response [157, 158], membrane components turnover [47]. OMVs could also be the result of an imbalance between cell growth and OM synthesis, which is therefore released as OMVs [145].

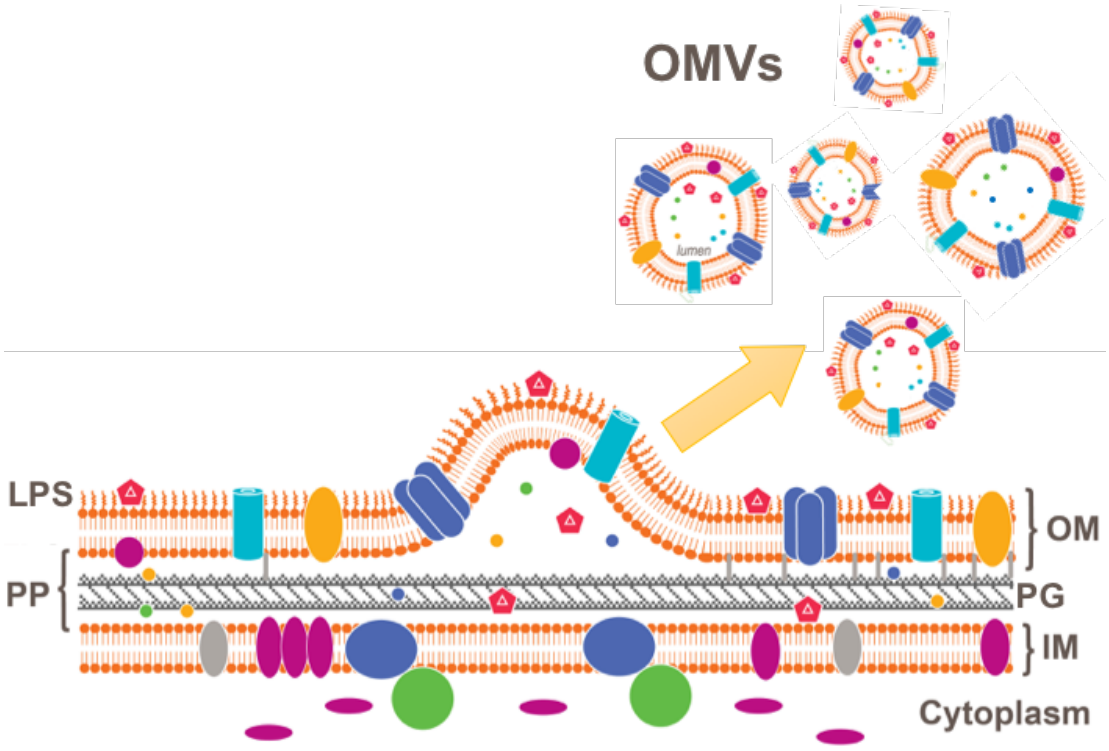


Figure 1.6. Model of outer membrane vesicles release.

OMVs are vesicles released from the outer membrane of Gram-negative bacteria. OMVs: outer membrane vesicles; LPS: Lipopolysaccharide; PP: periplasm; OM: outer membrane; PG: peptidoglycan; IM: inner membrane. Image property of GVGH.

Independent proteomic studies of OMVs from different bacterial species estimated an amount of outer membrane proteins ranging between 40% and 80% of the total OMVs protein content [159-163]. The lumen of the vesicles may contain not only elements from the periplasm, but also from the cytoplasm, such as proteins, RNA/DNA, and also peptidoglycan [164, 165]. Even though vesicles containing both the inner and the outer membranes, along with cytoplasm, have been described (O-IMVs) [165, 166], these double-layered vesicles represent a too small portion of the membrane vesicles produced by bacteria (0.2-1.2%) to account for the cytosolic components usually detected with OMVs preparation. Instead, the heterogeneity of OMVs composition highlight the active process that leads to their release, and the versatility of their functions. Moreover, the overall content of these vesicles is also dependent on the growth phase and medium composition.

So far, a unique mechanism of OMVs biogenesis has not been elucidated, but it is reasonable to suppose that this reflects the diversity of bacterial species. Nevertheless, one of the most important common aspects involved is the reduction of connections between the OM and the PG: it has been observed that OMVs are shed by sites of OM with a lower amount of cross-links between these two bacterial cell wall components [47, 48]. Among the most relevant players are the non-covalent interactions between the PG and the Tol–Pal (peptidoglycan-associated lipoprotein) system, a cell-division component that spans from the outer to the inner membrane [167-169], and the cross-links between the outer membrane Braun's lipoprotein (Lpp) and the peptidoglycan [170], whose regulation is thought to be implicated in the regulation of OMVs release [47]. In some cases, however, OMVs can stay associated with the producer bacterial cell [47].

OMVs can be easily isolated from supernatant of bacteria liquid cultures. This has made it possible to exploit them for multiple and diverse purposes, such as scaffold for enzymatic cascades [171], or cell-specific drug-delivery tools for cancer treatments [172, 173]. Outer membrane vesicles have also been shown to represent a valid alternative to whole bacterial cultures, as starting material for the purification of membrane components [174]. While these applications are of more recent development, the use of bacterial outer membrane vesicles as vaccine dates back almost 30 years [175]. Outer membrane vesicles have been particularly important for the development of vaccines against serogroup B meningococcus, for which the capsular polysaccharide cannot be used as vaccine antigen (see paragraph Cell envelope

Capsule, page 23) [176-178]. The efficacy, safety and versatility of outer membrane vesicles as vaccine platform, made their field of application expanded to other pathogens, and to the goal of multivalent vaccines [53, 179-181]. The main problem in using OMVs in humans is the presence of LPS. This obstacle was initially overcome by detergent-purification of the vesicles, leading to the production of dOMV (detergent-extracted outer membrane vesicles). As explained in the paragraph Lipopoly-Lipooligosaccaride (LPS, LOS)

, page 26, this approach was subsequently replaced by genetic modification of the LOS biosynthetic pathway, that led to the production of NOMV (non-detergent extracted native outer membrane vesicles) and GMMA (Generalized Modules for Membrane Antigens) [53, 68, 72, 179, 180, 182]. Another issue that could impact the efficiency of OMVs-based vaccines is the level of expression of the different antigens, as important

protective antigens could be masked by other immune-dominant antigens, or be naturally expressed at low levels, as it is indeed the case of *N. meningitidis* fHbp. Again, by genetically modifying the bacteria used for outer membrane vesicle production, the antigenic composition of the vesicles can be modulated and controlled. Such modifications are extremely appealing for the generation of broadly protective vaccines against multiple groups of the same bacterial species, or for multivalent vaccines, based on vesicles presenting heterologous protective antigens against different bacterial species [183]. The attractiveness in the vaccinology field of OMVs as carrier for targeting antigens is also related to their intrinsic ability in stimulating the innate immune system [184]. This also makes OMVs appealing as adjuvant in other vaccines formulation; for example, MenB dOMV were investigated for vaccine development against HIV-1 [185].

1.4. Use Of Alum As An Adjuvant In Vaccines

Adjuvants are compounds used in vaccine formulations for inducing, enhancing, or modifying the immunogenicity of antigens [184]. Vaccines based on live-attenuated or inactivated pathogens contain natural adjuvants (such as pathogen-associated molecular patterns, PAMPs), so they do not always need additional compounds. Purified or recombinant subunits vaccines, on the other hand, can be poorly immunogenic if administered alone, and usually require additional elements to elicit a good immune response. The choice of the adjuvant for vaccine formulation must take into consideration several aspects, such as formulation stability, toxicology, and safety. Several different adjuvants have been licensed for safety use in humans (Figure 1.7) [186]. Aluminium salts have been used as vaccine adjuvants for more than 80 years [187,

188], including in vaccines such as those against HAV, HBV, HPV, Diphteria, Tetanus, Hib, and more [189]. Several aluminium salts can be used, and the first "alum" was prepared as potassium aluminium sulphate, while nowadays "alum" generally indicates either the class of aluminium salts, or, specifically, an aqueous aluminium hydroxide suspension, alhydrogel, included in several licensed vaccines [190, 191]. Despite the widespread use of aluminium salts as vaccine adjuvants, their mechanism of action is not completely understood, and several hypotheses have been postulated over the years [190, 192]. The original hypothesis was that they enabled depot of antigen at the site of the injection, which would allow the antigen to be gradually released [190]. However, this model has been challenged since the 1950s, and recently the role for the depot effect in alum adjuvanticity was investigated *in vivo*, by removing the site of antigen-alum injection two hours after mice immunization; when this is done, there is no effect on humoral and cellular responses [193]. Alum promotes recruitment of immune cells to the site of the injection, the targeting of the antigens to antigen presenting cells (APCs), and the enhancement in the antigen uptake and presentation [194-196], which may require the physical alteration of the antigen by adsorption to alum [196, 197].

Adjuvants not only potentiate, but also modulate the immune response elicited. They can induce changes in the Th1-Th2 balance, and therefore the choice of adjuvant is an important consideration [198]. Aluminium salts can promote a shift towards Th2 response [199].

Licensed vaccine adjuvants

Adjuvant name (year licensed)	Class	Manufacturer	Description
Alum (1926)	Mineral Salt	Various	Improves humoral immune responses and antigen stability. Antigens are adsorbed to the surface. The adjuvant in > 80 % of vaccines licensed for human use. Th2 type immune responses.
MF59 (1997)	Oil-in-water emulsion	Novartis	Improves humoral and cell-mediated immunity. Used in influenza vaccines. Antigen delivery.
AS03 (2009)	Oil-in-water emulsion	GSK	Improves humoral and cell-mediated immunity. Used in influenza vaccine during 2009 H1N1 pandemic.
Virosome (2000)	Liposome	Berna Biotech (Crucell)	Improves humoral and cell-mediated immunity. A virosome is the reconstituted membrane of an enveloped virus. The vaccines for influenza and for Hepatitis A are approved products
AS04 (2005)	Alum-adsorbed TLR4 agonist	GSK	Improves humoral and cell-mediated immunity. Combination of aluminum adjuvant with monophosphoryl lipid A (MPL) co-adsorbed. Used for HPV and HBV vaccine.

Classes of clinically tested vaccine adjuvants

Adjuvant name	Class	Description	Clinical phase
CpG	TLR 9 agonist	Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. CpG oligonucleotides.	Phase 3
Flagellin	TLR 5 agonist	Enhances antibody titer, Th1 and Th2 type immunity. Flagellin linked to antigen.	Phase 1
PolyI:C	TLR3 agonist	Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. Double-stranded RNA analogues	Phase 1
AS01	Combination	Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. Combined with MPL, QS21 and liposomes.	Phase 3
AS02	Combination	Enhances antibody titer and Th1 type immunity. Combined with MPL, QS21 and emulsion.	Phase 3
ISCOMs and ISCOMMATRIX	Combination	Enhances antibody titer, Th1 and Th2 type immunity and CD8 T cell-mediated immunity. Combined with saponin and phospholipid.	Phase 2

Figure 1.7. Adjuvants in licensed vaccine for use in humans, or in clinical trials, up to 2005.

Tables adapted from Lee S. and Nguyen M. T., 2015 [186].

1.5. The Immune System

The immune system defends the body from diseases. It is mainly involved in fighting external threats, but it can also be involved in fighting abnormal self-cells, to prevent the development of cancer. Defence against external microorganisms is mediated in first instance by the non-specific innate immune system, and, if necessary, by the induction of antigen-specific response, via the adaptive immunity.

1.5.1. LYMPHOID ORGANS

Both innate and adaptive immunity rely on multiple cell types, all originating in the bone marrow, where some also mature [200]. They then migrate to peripheral tissues, or circulate in the bloodstream and through the lymphatic vessels. Lymphoid tissues are divided into primary lymphoid organs (the bone marrow and thymus), and secondary (or peripheral) lymphoid organs (lymph nodes, spleen, and mucosal lymphoid tissues), where lymphocytes migrate to, and where adaptive response develops [201].

Located within the central cavity of long bones, the bone marrow is the major hematopoietic organ of the organism, responsible for the generation of erythrocytes, granulocytes, monocytes, lymphocytes, and platelets, all derived form a common hematopoietic stem cell (HSC) progenitor [200]. Moreover, bone marrow is also where long-lived plasma cells reside [202], therefore its importance is not only in the origin of the immune response, but also in its maintenance.

The spleen (Figure 1.8) is not connected to the lymphatic system, as it collects antigens from the blood, and lymphocytes enter and leave the spleen through blood vessels [203].

Surrounded by a capsule of connective tissue, the spleen is organized into a red pulp with white pulp interspersed. The red pulp is an efficient blood-filtering system, where senescent erythrocytes are disposed of, and iron is recycled, thanks to the splenic macrophages [204]. In the red pulp there are also plasma cells and plasmablasts, where dendritic cells (DCs) are associated with their survival [205]. The white pulp is where lymphocytes are organized into T and B cells compartments, surrounding the blood vessels. In particular, a central arteriole passes through the T zone, in the so-called periarteriolar lymphoid sheath (PALS), and lymphoid follicles of B cells surround the T zone [203]. The marginal zone (MZ) surrounds the follicles, and it counts mainly macrophages and non-circulating marginal zone B cells [206]. From the MZ cells transit from blood stream to white pulp, allowing the immune system to efficiently monitor the blood [203]. While white pulp is important for the adaptive responses, the MZ plays a role in both innate and adaptive immunity [203]; the spleen is therefore an important organ for the homeostasis of the immune system.

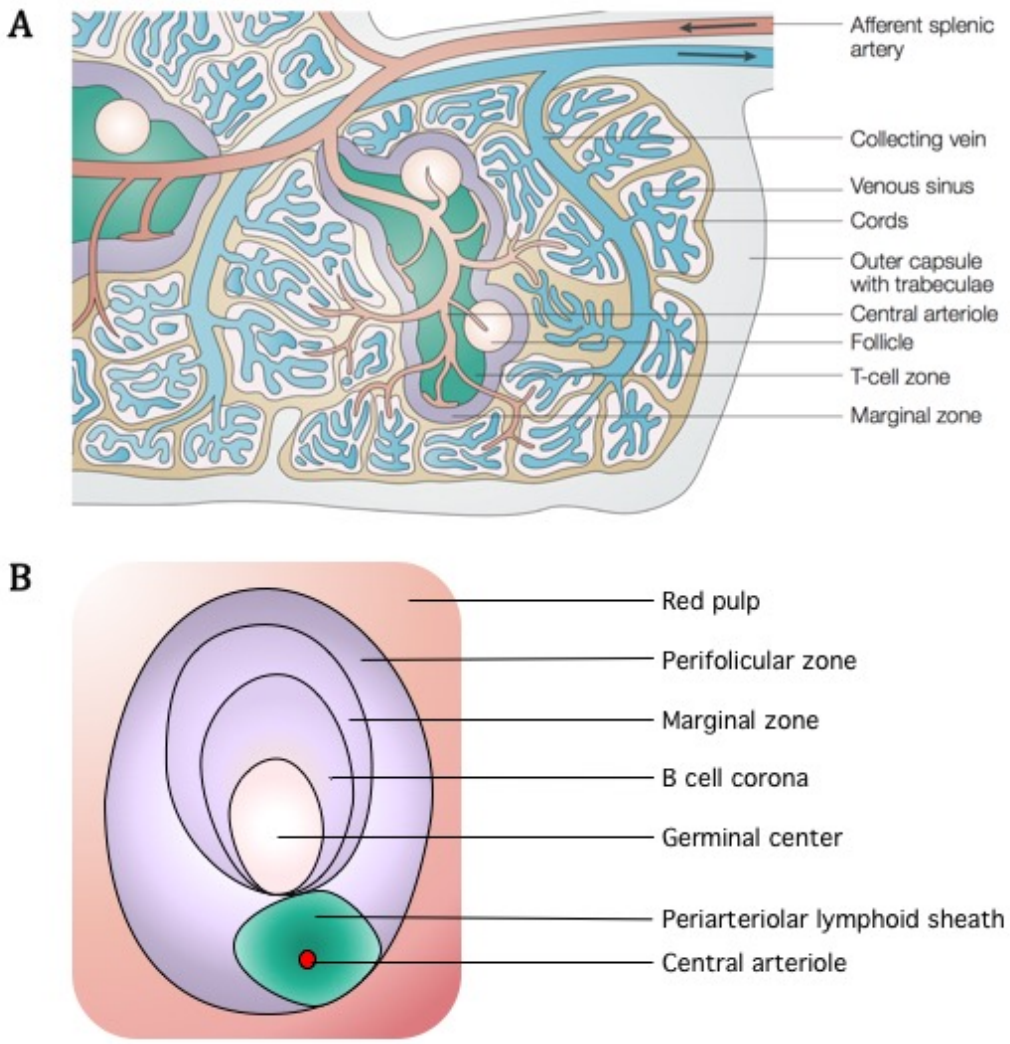


Figure 1.8. Schematic representation of the lymphoid tissue organization in the spleen.

A) White pulp areas are interspersed in the red pulp. White pulp consists of the T cell zone (or "periarteriolar lymphoid sheath", PALS), arterioles, and B cell follicles; the white pulp surrounds central arterioles, which end in cords in the red pulp. The spleen is surrounded by a connective capsule, which is in in continuous with large arteries and veins run together in connective-tissue trabeculae. Image adapted from Mebius R. E. and Kraal G., 2005 [203]. B) Transverse section of the white pulp. Picture loosely drawn from Janesway's Immunobiology [201].

1.5.2. THE INNATE IMMUNE SYSTEM

The innate immune system is composed of physical barriers (mucosae), chemical components (antimicrobial peptides, enzymes, Complement cascade, cytokines), and cellular defences.

1.5.2.1. CELLULAR COMPONENTS

Macrophages are present in almost all tissues and their main function is to engulf and digest microorganisms [207, 208]. They also play an important role in inflammation [209, 210], by producing cytokines like tumor necrosis factor (TNF), Interleukin-1 (IL-1) and IL-12, and contribute to the adaptive immune response by presenting antigens to T cells. They also act as cellular scavengers, by phagocytosis of dead cells and cellular debris [201, 207].

Neutrophils, eosinophils, basophils and mast cells are grouped under the more comprehensive names of polymorphonuclear leukocytes, because of the varying shape of their nuclei, and of granulocytes, because of the characteristic granules present in their cytoplasm. These are vesicles where degradative enzymes and antimicrobial compounds are stored [211]. Neutrophils are the most abundant cellular component of the innate immune system; they are phagocytes, but also release soluble anti-microbial proteins, and generate NETs (neutrophil extracellular traps), webs of granular constituents and nucleic acids that block and kill extracellular microorganisms [212]. Eosinophils, basophils and mast cells are important in clearing parasites, but they are also involved in allergic inflammatory responses [213]. Natural killer cells (NK cells) are cytotoxic lymphocytes involved in the immuno-surveillance of the host organism, killing abnormal cells and virus-infected cells, by releasing proteases and proteins such as perforin [214].

Dendritic cells (DCs) represent the link between innate and adaptive immunity, and their primary role is to process antigens and interact with T cells [215], by displaying processed antigens on their surface to stimulate activation of T cells [216].

1.5.2.2. TOLL-LIKE RECEPTORS

The innate immune system provides a non-specific response to external (and internal) threats. It needs to be able to distinguish self from non-self, and it achieve this by recognising pathogen components that are typical only of microorganisms, called pathogen-associated molecular patterns (PAMPs) [217]. Small molecular motifs are conserved among microbes, but are not found in healthy vertebrate cells. PAMPs include glycans and peptidoglycans, lipoproteins, lipopolysaccharides, bacterial DNA and viral RNA, bacterial flagella. These molecular motifs are recognized by pattern-recognition receptors (PRRs), and among these receptors, Toll-like receptors (TLR) play a key role in fighting infections [218]. Originally discovered in *Drosophila*, homologues of these receptors were found in other animals, including mammals, and named Toll-like receptors [219, 220].

TLRs are transmembrane type I proteins, whose horse-shoe/solenoid shaped ectodomains have leucine-rich repeats (LRRs) that mediate ligands interactions [218]. Upon ligand binding, TLRs monodimers or heterodimers are induced, and the downstream signal transduction begins, due to the intracellular Toll-interleukin 1 receptor (TIR) domains [221]. Most of the TLRs are present on the cell surface, but some are found in intracellular compartments. 10 *TLR* genes are expressed in humans (13 in

mice), and each is specific for a different class of PAMPs [218] (Figure 1.9): TLR2 can complex with TLR1 or TLR6, and binds lipoproteins, lipoglycans, and lipoteichoic acids [222]; TLR3 binds dsRNA, while ssRNA is recognized by TLR7 and TLR8 [223]; TLR4 (with co-receptors MD-2 and CD14) binds LPS of Gram-negative bacteria, and lipoteichoic acids of Gram-positive bacteria [224]; TLR5 binds to flagellin [225]; TLR9 binds unmethylated CpG DNA [226]; the ligand of TLR10 is still unknown, but may be involved in response to viral infections [227], as well as in recognition of bacterial LPS [228].

The downstream signalling triggered by the different TLR differs as well, and this is related to the different adaptor molecules (or different combination of adaptor molecules), with which the TIR domains interact [218]. For example, TLR5, TLR7, and TLR9 interact only with MyD88 (myeloid differentiation factor 88); TLR2 with MyD88 and MAL (MyD88 adaptor-like); TLR3 only with TRIF (TIR domain-containing adaptor-inducing interferon-β); TLR4 can interact with MyD88/MAL, or with TRIF/TRAM (TRIF-related adaptor molecule), and it is the only one that can function along both signalling pathways.

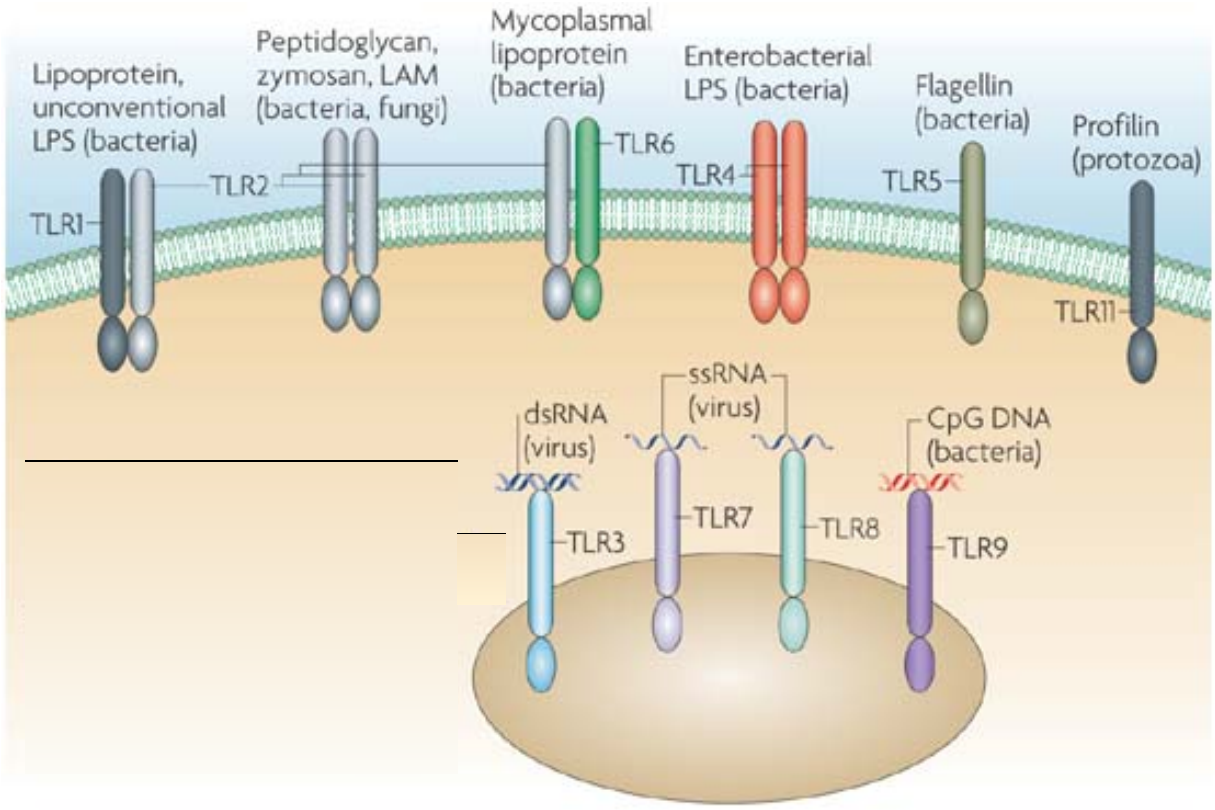


Figure 1.9. Toll-like receptors and their ligands.

Each of the Toll-like receptors (TLRs) is specific for a class of ligands, and its localisation in the cell is related to such specificity. ds: double-stranded; LPS: lipopolysaccharide; LAM: lipoarabinomannan; ss: single-stranded. Adapted from Kauffman, S. H. E., 2007 [229].

1.5.2.2a. TLR4

Different from the other TLRs, TLR4 interaction with its ligand is mediated by co-receptor MD-2 [230]. MD-2 is a secreted glycoprotein, with a hydrophobic pocket that binds the acyl chains of LPS; because of the size of this part of the protein, the sixth acyl chain protrudes outside, and activates TLR4 [231]. Other molecules involved in activation of TLR4 are LPS-binding protein (LBP) and CD14 [232]. LBP is present in the blood and extracellular fluids, and binds LPS that has detached from bacterial membranes; CD14 can be GPI-anchored to the membrane (mCD14), or soluble in serum (sCD14), and it acts as a chaperone for LBP-LPS to TLR4/MD-2 [233, 234].

LPS with hexa-acylated lipid A is the strongest activator of the receptor, while LPS forms with a lower number of acyl chains are poor activators of human TLR4, compared to the hexa-acylated form. However, there are differences in TLR4 activation among different species: studies comparing human, murine, and horse TLR4/MD-2 have highlighted that only the human receptors are not activated by penta- and tetra-acylated lipid A, that actually act as antagonist for human TLR4 [235]. Recent structural studies have shown that this is related to differences in the MD-2 hydrophobic cavity, so that even in the case of tetra-acylated lipid A, acyl chains can still be exposed outside and trigger TLR4 activation [235, 236].

1.5.2.3. THE COMPLEMENT SYSTEM

"Complement" (C') indicates a group of more than 30 plasma proteins that target bacteria, and is one of the first lines of defence of the innate immune system, but it also helps discriminating between healthy host tissues and apoptotic cells and cellular debris [237]. The "functional unit" of this system is a sequential proteolytic cascade, and the

proteins involved are named with a "C" followed by a number, which does not necessarily follow the order of their activation. The products of their cleavage are indicated adding a lower case letter (i.e. a and b) [201]. Different mechanisms can induce the Complement system, and they can be divided into three major pathways of activation (Figure 1.10): the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). All three pathways converge in the activation of the C3 convertase, which cleaves C3 into C3a and C3b. The classical pathway is activated by antibodies bound to target pathogens, or directly by viruses and Gram-negative bacteria, via PRRs [237, 238]. The lectin pathway, is activated by carbohydrates on microbial surfaces, which are recognized by PRRS such as mannose-binding lectin (MBL) and ficolins [239]. The alternative pathway, can be initiated by the lectin or the classical pathways, or can activate by spontaneous hydrolysis of complement component C3; such spontaneous activation allows constant probing of host cells [240].

Two different C3 convertases are involved in the complement cascade: C4bC2b in the classical and lectin pathways, and C3bBb in the alternative pathway [241] (Figure 1.11). As its name suggest, the latter is composed of C3b itself (together with Bb, cleavage fragment of the plasma factor B), and this means that C3b (from whichever pathway is produced) can enhance its own activation, in an amplification loop. C3a modulates inflammation and recruits immune cells to the site of infection, while C3b binds covalently to the pathogen surface and induces opsonisation by phagocytes [242]. Furthermore, C3b together with C3b convertase generates C5 convertase (C3bBbC3b for the alternative pathway and C4bC2bC3b for the classical and lectin pathways), which

cleaves C5 into C5a and C5b [243]. C5a is another inflammatory peptide, while C5b initiates the terminal part of the C' cascade. C5b recruits C6, then, C7, C8, and C9 sequentially and irreversible associate, forming the membrane-attack complex (MAC, also known as terminal complement complex, TCC). The MAC is a hetero-oligomeric pore, that inserts into the target membrane, leading to the lysis of the pathogen (Figure 1.12) [244, 245]. Complement deficiencies cause susceptibility of the host to microbial infections; in particular, late components deficiencies (C5-C9) correlate with a higher susceptibility of individuals to *Neisseria* species [246, 247].

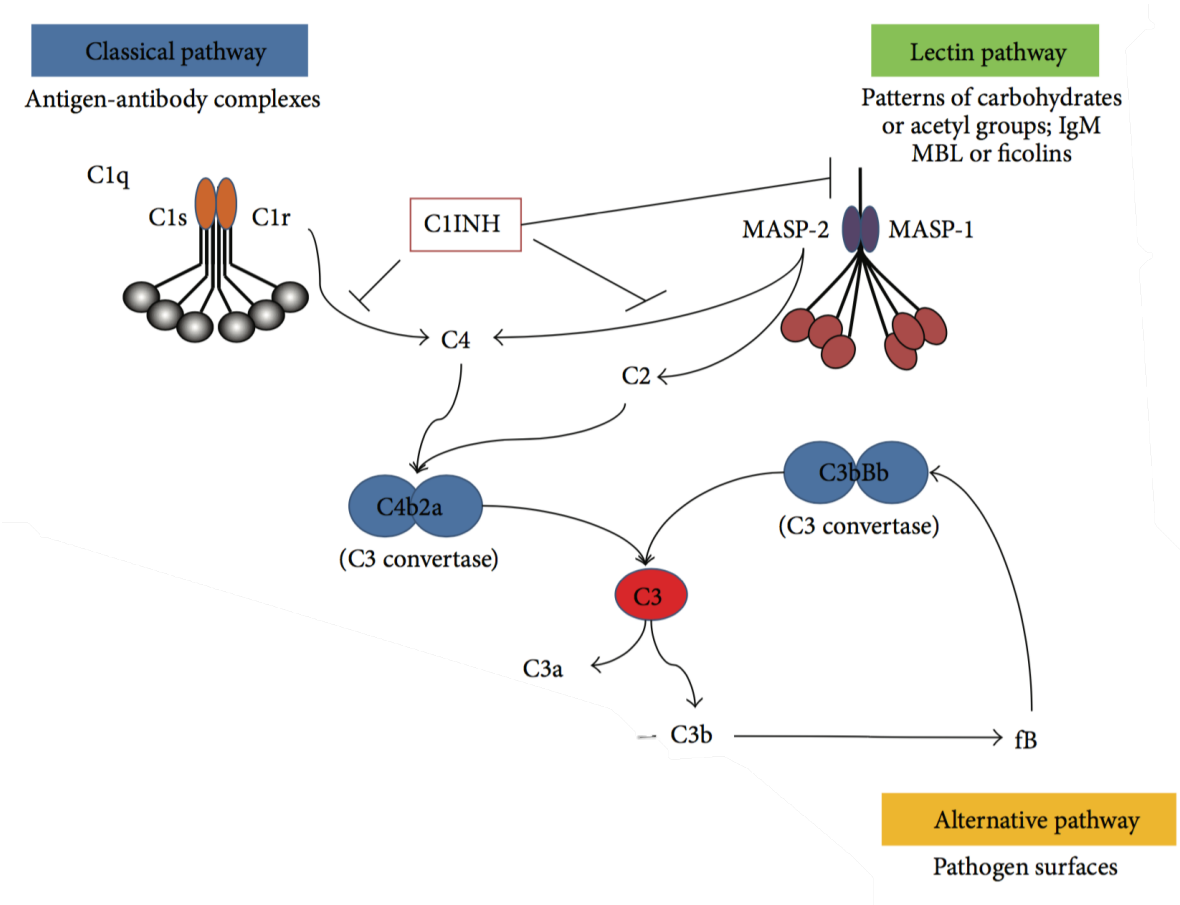


Figure 1.10 Activation of the complement system.

Different stimuli activate the complement cascade via different pathways. The classical pathway is activated when C1q molecule binds to a microbial surface directly, or to antibodies bound to pathogens. The lectin pathway is triggered by soluble proteins that bind carbohydrates (mannose-binding lectin, MBL, and ficolin). The alternative pathway can be activated by spontaneous hydrolysis of C3 molecule. C1INH: C1 inhibitor; MBL: mannose-binding lectin; MASP: mannose-binding lectin associated serine protease. Picture adapted from Osthoff M. and Trendelenburg M., 2013 [248].

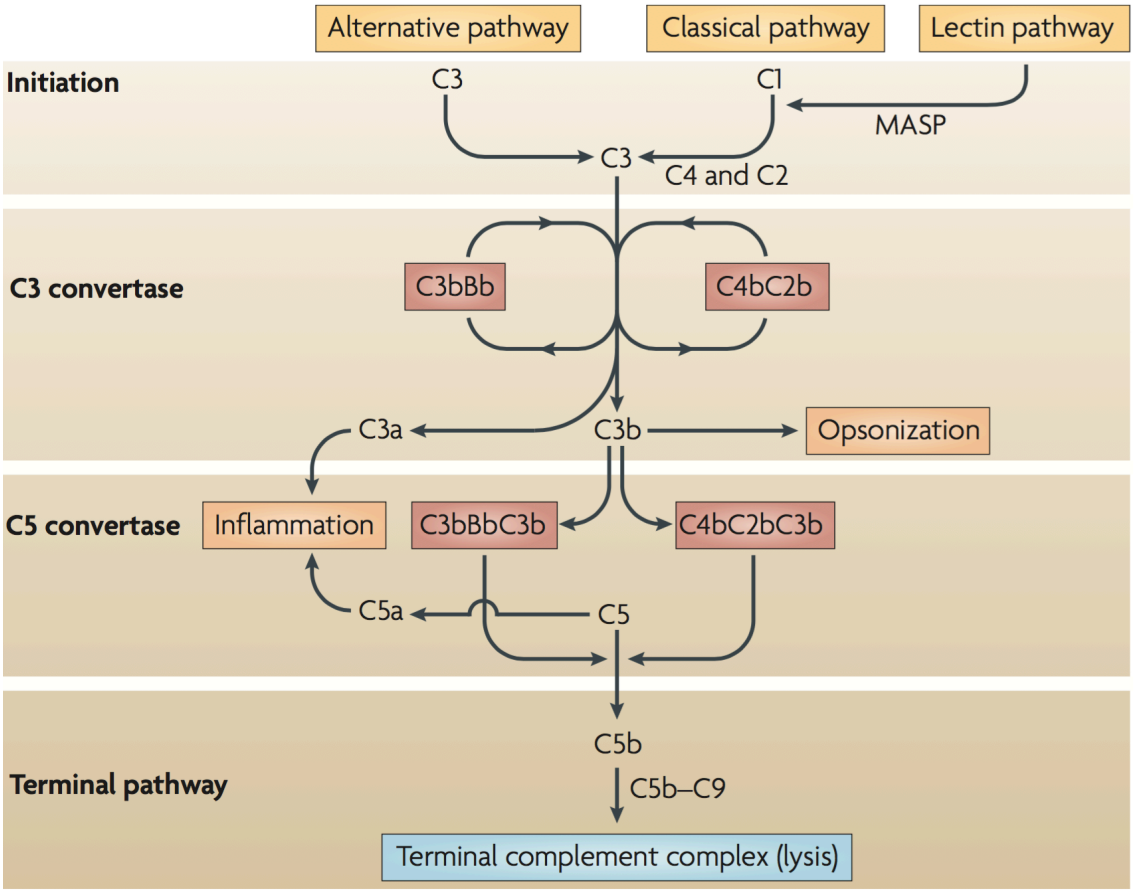


Figure 1.11. Complement cascade.

The three different complement activation pathways converge to the formation of the C3 convertase (C3bBb for the alternative pathway and C4bC2b for the classical and lectin pathways), that cleaves C3 into C3a, a modulator of inflammation, and C3b. C3b induces opsonisation by phagocytes, and, together with C3 convertase, generates C5 convertase (C3bBbC3b for the alternative pathway and C4bC2bC3b for the classical and lectin pathways). C5 convertase cleaves C5 into C5a, another modulator of inflammation, and C5b, which recruits C6, C7, C8, and C9 to form the terminal complement complex, leading to pore formation on the target membrane and subsequent cell lysis. MASP: MBL-associated proteases mannan-binding lectin serine protease 1. Figure from Zipfel P. F. and Skerka C., 2009 [241].

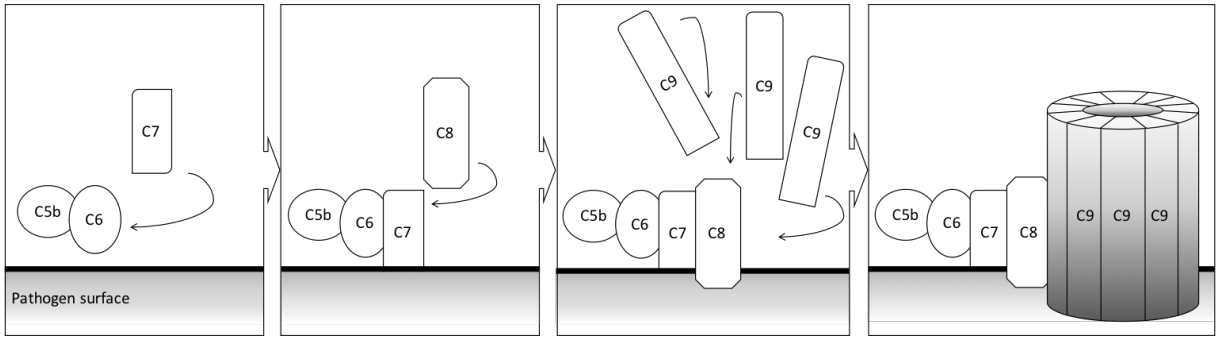


Figure 1.12. Assembly of the membrane attack complex on the pathogen surface.

C5b recruits C6, C7, and C8. The latters expose hydrophobic domains, which allow insertion in the target membrane. This complex recruits C9 molecules, which polymerize and form the pore. The channel leads to the lysis of the pathogen.

1.5.2.3a. FACTOR H (FH)

This lytic pathway needs to be finely regulated, in order not to cause damage to healthy tissues; in fact, dysregulation leads to immunopathologies and autoimmune diseases [249, 250]. One safety mechanism is the short half-life of activated components, which are rapidly inactivated if not bound to their primary target. Nevertheless, the fine-tune regulations is mediated by several C' regulator proteins, both cell-bound and soluble, distributed in all tissues and operating at each step of the cascade [241]. Among the numerous complement regulators, is factor H (fH, also known as β1H), a single polypeptide chain plasma glycoprotein of ~150 kDa, that is involved in the negative regulation of the AP [251]. Factor H is present in human plasma at variable concentrations (110-615 μg/mL), and is composed of 20 globular repetitive units of 60 amino acids, known as short consensus repeats (SCRs) or complement control protein modules (CCP) [252]. It carries out its regulatory roles in different ways: it sequesters

C3b, accelerates the dissociation of the C3 convertase C3bBb, and acts as co-factor in the proteolytic inactivation of C3b by factor I (fI) [253, 254]. Factor H not only blocks C3b in fluid-phase, but it can also inactivate surface-bound C3b. This process is dependent on the composition of the membrane to which C3b is attached, the affinity of fH for C3b decreasing in the presence of sialic acids [251, 255]. Factor H domains 1 to 4 are involved in its active function, while domains 19 and 20 are involved in discrimination between self and non-self surfaces. Nevertheless, C' regulators can be exploited by pathogens against the host itself [256]. One common strategy for bacteria to use is to express on their surfaces proteins that bind Complement negative regulators [257]. Factor H, for example, is the target of several microbial proteins, which, by recruiting it to the bacterial surface, prevent the opsonisation and phagocytosis of the microbes. Such molecules include fHbp of *N. meningitidis* (*see* paragraph 1.2.7 Factor H Binding Protein (, page 37), protein M of *S. pyogenes* [258], OspE (outer surface protein E) of *B. burgdorferi* [259], PspC (pneumococcal surface protein C) of *S. pneumoniae* [260], Tuf from *P. aeruginosa* [261], Sbi (staphylococcal binder of immunoglobulins) of *S. aureus* [262], YadA and Ail of *Y. enterocolitica* [263].

1.5.3. THE ADAPTIVE IMMUNE SYSTEM

Some infections can be controlled and cleared by the native immune system alone, but other pathogens can overcome this, and adaptive immunity is necessary for fighting them. Main differences between the native and the adaptive immune responses are the specificity and the memory: adaptive immunity is acquired by encountering the pathogens, and leads to a more rapid protective response upon re-encounter. The main cell types of the adaptive immune response are T cells (lymphocytes derived from the

thymus), and B cells (lymphocytes derived from the bone marrow), that modulate cell-mediated immunity and humoral immunity, respectively.

1.5.3.1. T CELLS

Cell-mediated immune responses involve T cell activation by antigen-presenting cells (APC), that, upon encountering antigens, process and present them to T cells through the MHC (Major Histocompability Complex) [201]. MHC molecules directly interacts with the T cell receptor (TCR), but for lymphocyte activation a second signal through CD28 is needed, by ligation of CD80 or CD86 on the APC [264] (Figure 1.13). Whilst presentation of antigen via MHC class I is needed to activate CD8+ T cells, MHC class II, present on professional APC such as DCs, macrophages, or B cells, is needed to activate CD4+ T cells [265].

CD4+ T cells can develop toward diverse lineages, each with different characteristics, roles, and cytokines produced [266, 267]. So far T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), T follicular helper (Tfh), and T regulatory (Treg) cells have been described. Th1 cells activate infected macrophages and help B cells for antibodies production; this response is characterized in particular by interferon γ (IFNγ): Th1 differentiation is induced by IFNγ and interleukin-2 (IL-2), and the main products are IFNγ, IL-2, TNFα, and IL-10 [268]. Th2 cells are involved in helping B cells in the humoral response, and in clearing infections of parasites; IL-4 and IL-6 induce this lineages, and among the signals produced are IL-4, IL-5, IL-13, and IL-10 [268]. Th17 cells are involved in inflammation, and are important for mucosal integrity; they are induced by

Transforming Growth Factor-β (TGF-β), IL-6, IL-21, and IL-23, and their main effector cytokines are IL-17, IL-21, and IL-22 [269]. Tfh cells are critical for antibody response and contribute to the induction of IgG response. They reside in the outer T zone, the follicles and germinal centres (GC). Ultimately, they licence the development of plasma cells and B cells isotype switching, and can produce cytokines characteristic of either Th1 or Th2 response [270]. Treg are important for maintenance of the tolerance to self-antigens, to prevent autoimmune diseases; this subset is induced by TGF-β, and its effector cytokine is IL-10 [271, 272].

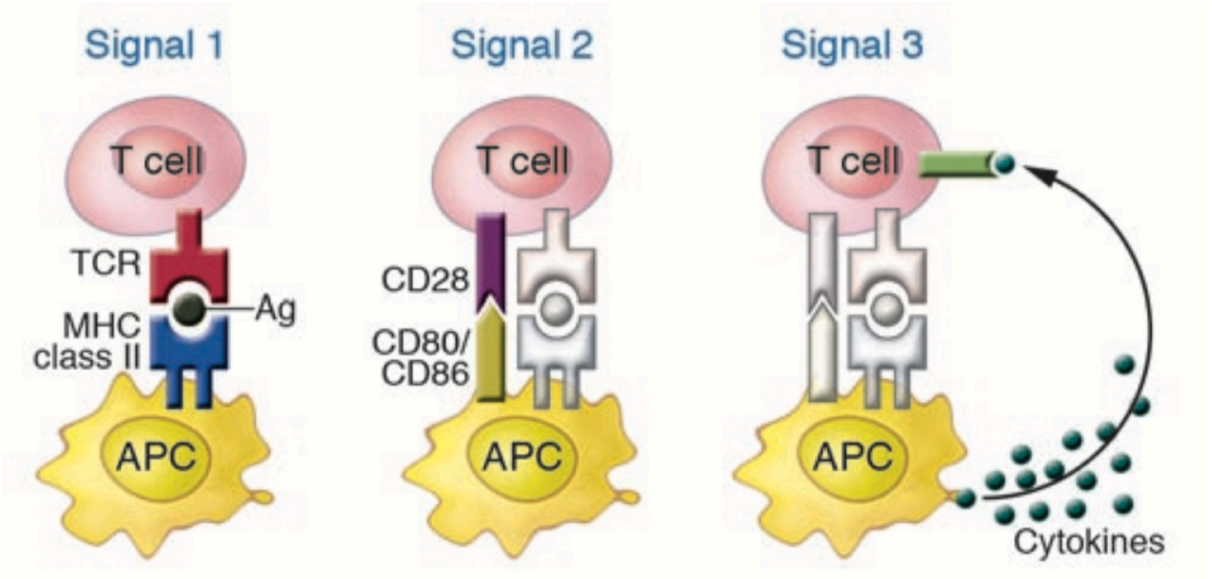


Figure 1.13. Three signals from the antigen-presenting cells (APCs) are required for T cell activation.

The first signal for T cell activation id the binding of the complex antigen-MHC II to the TCR. Co-receptor CD28 must be engaged at the same time, by B7 molecules present on the APC, to sustain survival and proliferation of the T cell; this represent the second signal. The third signal is represented by cytokines released by the APC, which drive the differentiation of the activated T cell toward one of the several lineages possible. Ag: antigen. Picture from Gutcher I. and Burkhard B., 2007 [273].

1.5.3.2. B CELLS AND THE HUMORAL RESPONSE

The humoral response is crucial for protecting the host against infections, and it is the basis for successful vaccination strategies. Antibodies are secreted by plasma cells, which represent the terminally differentiated, non-dividing effector stage of the B cell lineage [274] (Figure 1.14). B cell development starts in the bone marrow, and consists of sequential steps: pro-B, pre-B, and immature B cells. These are characterized by specific surface markers, and rearrangements of the immunoglobulin (Ig) heavy and light chains genes. Immature B cells, expressing surface-bound IgM as B cell receptor (BCR), are tested for autoreactivity before migrating to peripheral lymphoid organs [275]. They can home to the marginal zone of the spleen, originating a non-recirculating B cell population, or circulate through blood and lymph vessels, migrating to follicles of lymph nodes and spleen, and generate germinal center responses [201].

In contrast to TCR, BCR can be activated directly by the antigen [276]. The nature of the latter influences the B cell response established, and the antibody production. Protein antigens usually involve interaction with T cell, and are therefore called Thymus-dependent (TD) antigens; polysaccharides, instead, are able to induce antibody production without involving T cells, and are therefore called Thymus-independent (TI) antigens [277]. TI antigens can be further dived into type I, type II, and type III [278]. TI type I antigens (TI-I), e.g. LPS and CpG, are mitogenic stimuli that activate B cells and induce them to polyclonal proliferation via TLRs [279]. TI type II antigens (TI-II) are those antigens composed of repetitions of the same epitope, and therefore they are able to engage multiple BCRs in parallel, leading to a strong intracellular signalling that does

not need Th cells to produce antibody [280]. Unlike the other types, TI-III response requires additional helper signals, which are provided by bone marrow-derived innate cells [281].

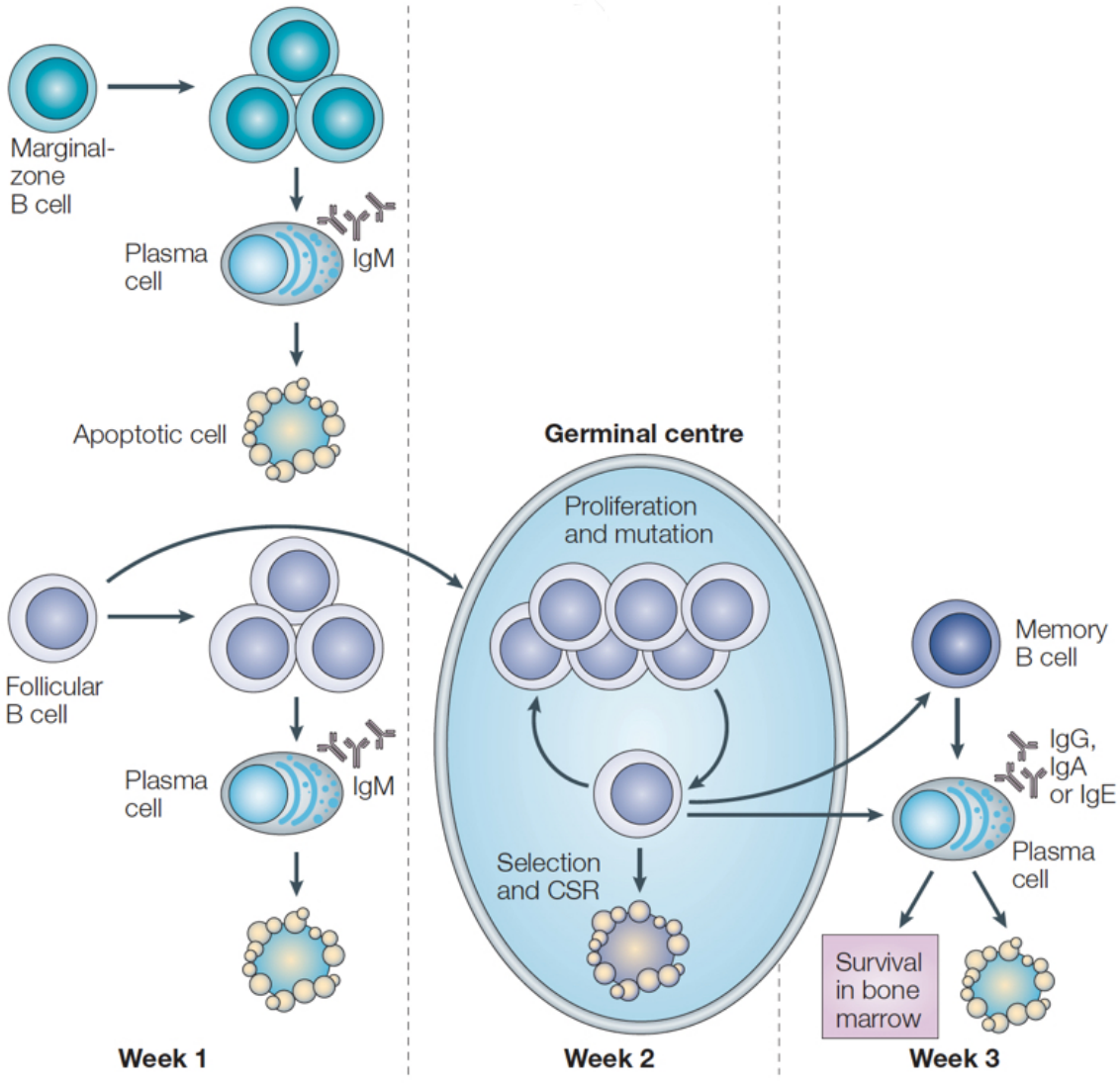


Figure 1.14. Generation of plasma cells.

Upon activation, marginal-zone B cells differentiate into short-lived plasma cells. Differently, follicular B cells can form germinal centers, maturate, and differentiate into both long-lived plasma cells and memory B cells. CSR: class-switch recombination. Picture adapted from Shapiro-Shelef M. and Calame K., 2005 [282].

1.5.3.2a. GERMINAL CENTER RESPONSE

T-dependent response involves the development of germinal centers (GCs), which are transient structures associated with clonal expansion of B cells, antibody class-switching, affinity maturation for the antigen, and a strong long-lasting memory B-cell response [283]. T cells primed and activated by DCs differentiate into Tfh; at the same time, primed B cells also interact with Tfh, and different outcomes are possible [277]: B cells can either die, or differentiate and proliferate. In the second case, they can migrate to extra-follicular (EF) sites, and generate plasmablasts and plasma cells, or can migrate to the follicles and, sustained by Tfh, form GCs. In germinal centers two areas can be distinguished: the dark zone (DZ) and the light zone (LZ) [284]. The DZ is closer to the T cell area, and consists mainly of proliferative B cells ("centroblasts"); this area is the site of somatic hypermutation (SHM) of the genes encoding the variable antigen-binding regions of antibodies [284]. The LZ, closer to the lymph nodes capsule and to the marginal zone in the spleen, is the site of selection of higher-affinity B cells, for which Tfh cells play a crucial role [285]. For affinity maturation to occur, B cells circulated between DZ and LZ several times: 10 to 30% of B cells entering LZ go back to the DZ; the rest either exit the GC or die by apoptosis [286]. The peak of GC response is 10-14 days after antigen encounter; then, it diminishes, and the result is the generation of high-affinity antibody producing plasma cells, and memory B cells [282]. Post-GC memory B cells have high-affinity BCR, do not secrete antibodies, are larger than naïve B cells, and respond more rapidly than the latter to antigen stimulation; they also constitutively express the co-stimulatory molecules CD80 and CD86 [287, 288]. Whilst memory B cells

recirculate through the blood, their main reservoirs are bone marrow and the spleen [289, 290].

1.5.3.2b. ANTIBODIES FUNCTION

Antibodies are immunoglobulins (Ig) composed of two identical heavy chains and two identical light chains. Each of these chains has a constant and a variable part; variable parts form the antigen-binding site, while the constant part of the heavy chains defines the class the antibody belongs to [201], and therefore their effector function. In general, they protect the host against infections in three different ways. First, by neutralization: binding to the pathogen, antibodies can prevent it from entering host cells, and, in the same way, they can block bacterial toxins [291, 292]. Second, by opsonisation: antibodies that coat the surface of extracellular pathogens facilitate their uptake by phagocytes [293]. Third, antibodies coating a pathogen activate the classical pathway of the C' (1.5.2.3. The Complement System, page 56). Different antibody classes are specific for different effector functions. In particular, IgM, which are the first antibodies produced during the humoral response, before class switching and affinity maturation, are effective in activating the complement; this is related to their organization in pentamers, whose avidity compensates the low affinity of the monomers. On the other hand, IgA weakly activate of C', but are important neutralizing antibodies, operating as dimers on epithelial surfaces and mucosae. IgG, present in blood and extracellular fluids, are also potent activators of the C', as well as effective opsonins [294]. However, IgG antibodies are divided into several subclasses, with different specificities [295]; in humans, they are designated with a number, from 1 to 4, indicating their prevalence, with IgG1 being the most abundant, while in mice IgG1, IgG2a, IgG2b, IgG2c, and IgG3 are present, but there

is no direct correspondence with the human numbering. In fact, correspondence between human and mice IgG subclasses can be found the basis of biological and functional activities: human IgG1 and IgG3 and murine IgG2a and IgG2b share the ability to fix complement and bind to protein antigens (25); human IgG2 and murine IgG3 respond predominantly to polysaccharides; human IgG4 and murine IgG1 share the property of binding to mast cells [295, 296].

1.6. Aims

A pan-protective vaccine against multiple meningococcal serogroups is urgently needed in Sub-Saharan Africa. As outer membrane vesicles with over-expressed fHbp are a promising approach, this thesis aimed to expand the understanding of some aspects of such vaccine candidates, that could contribute to the rational design of an effective vaccine.

One of the aims of this project was to understand if and how the amino acid sequence of the fHbp over-expressed on the vesicle surface, impacts the specificity and cross-reactivity of the antibody response generated. To investigate this aspect, the surface-exposed region of fHbp involved in contact with fH was analysed (Chapter 3). The hypotheses were that this region particularly contribute to the specificity of the response, and that an fHbp ID carrying the most-shared region would induce antibodies with a broader cross-reactivity, compared to other IDs. Therefore, a panel of isogenic meningococcal mutants over-expressing different fHbp ID was generated, for the production of NOMV OE fHbp, and the vesicles were characterized (Chapter 3). Then, the antibody response elicited by the different NOMV, and by the corresponding recombinant fHbp, was tested and compared in mice (Chapter 4).

The other aim of this project was to elucidate specific aspects of the immune response generated in mice (Chapter 5), with particular attention to: i) the impact on the immune response of modifying lipid A in the vaccine candidate; ii) the establishment and quality of the primary and secondary antibody responses; iii) the impact on the immune response of adsorbing the antigen on alum; iv) the persistence of the antibody response.

CHAPTER 2. MATERIALS AND METHODS

2.1. Molecular Cloning

2.1.1. E. COLI STRAINS USED

For plasmid cloning, chemically competent *E. coli* Subcloning Efficiency™ DH5α™ Competent Cells (ThermoFisher Scientific) were used.

For expression and purification of recombinant fHbp production, chemically competent *E. coli* cell BL21 (DE3) strain (Novagen, Madison, WI, US) was used

2.1.2. E. COLI GROWTH CONDITION

E. coli was grown on LB agar plate or in liquid LB cultures, with the appropriate antibiotics. For liquid cultures, 5 mL LB (with the appropriate antibiotic) were inoculated with one single colony and grown at 37°C over-night (O/N).

2.1.3. E. COLI TRANSFORMATION

Transformation was performed by heat-shock. 50 µL of *E. coli* competent cells were thawed in ice (in a 2 mL Eppendorf tube), and 1-5 µL of DNA (1-100 ng) was added. After 30 min incubation in ice, DH5α were heat-shocked at 42°C for 20 sec, and incubated in ice for 2 min, while BL21(DE3) were heat-shocked at 42°C for 10 sec, and incubated in ice for 10 min. Then, 950 µL of LB were added, and bacteria were incubated at 37°C, 180 rpm, for 1 h. After incubation, bacteria were harvested at 6,800 x *g* for 10 min, resuspended in 20-50 µL of LB, and plated onto a LB agar plated containing the selection

antibiotic. Plate was incubated at 37°C O/N. The day after, 10-20 single colonies were streaked onto a new LB agar plate (with the appropriate antibiotic), and screened by PCR. Clones positive by PCR were grown in 5 mL liquid cultures, plasmid DNA was purified with MiniPrep Kit (Qiagen) following manufacturer's protocol, and sent for sequencing (internal sequencing facility).

2.1.4. DNA AMPLIFICATION, DIGESTION, AND LIGATION

DNA was amplified by polymerase chain reaction (PCR), with different polymerases, according to the purpose: amplification of genes and DNA sequences to clone into plasmid was performed with Deep Vent™ DNA Polymerase (New England Biolab); colony PCRs to screen *E. coli* transformed clones were performed with OneTaq® DNA Polymerse (New England Biolab); self-priming PCRs described in Chapter 3 were performed with high-fidelity Phusion® High-Fidelity DNA Polymerase (New England Biolab). Reactions were performed according to manufacturer's protocols, on a 50 µL scale, or in a 20 µL scale in case of colony PCR.

Restriction enzymes were purchased from New England Biolabs. 1 µg of DNA to be digested was incubated with 1 µL of enzyme in the buffer recommended by manufacturer's protocol, in a total volume of 50 µL, and incubated at 37°C O/N.

T4 DNA ligase (New England Biolab) was used to ligate DNA fragments and plasmid fragments, previously digested. The ligase was used according to manufacturer's protocols, and reactions were incubated at room temperature (RT) O/N.

Table 2.1 List of primers used. Restriction site underlined in the sequence.

Purpose	Primer designation	5′-3′ primer sequence	Restriction Site
Amplification of up-stream recombination site of capsule biosynthesis locus	Cps_upF	TCCCCCCGGGTATCGCCAACAAACGGCACAG	XmaI
	Cps_upR	GCTCTAGATTTCGATTAAGTGCTATAATTAGGCC	XbaI
Amplification of down-stream recombination site of capsule biosynthesis locus	Cps_doF	GCCAATGCATCAATATGCTGCCATTACTCC	NsiI
	Cps_doR	GGACTAGTGTTTGTTTGCCGCATGCTAATGCC	SpeI
Amplification of fHbp ID 1, 5, 74	fHbp_fw	CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC	NdeI
	fHbp_rev	CCATTGTGAAAATGCCGTC	
Amplification of fHbp ID 9	ID9_fNde	CGCGGATCCCATATGAACCGAACTACCTTTTTCTGCC	NdeI
	fHbp_rev	CCATTGTGAAAATGCCGTC	
Introduction of R41S mutation in fHbp	fHbp_f41	GGATCAGTCCGTCAGTAAAAACGAGAAAC	/
	fHbp_r41	GTTTCTCGTTTTTACTGACGGACTGATCC	/
Amplification of fHbp for cloning into pET21b	fHbp_tr_F_Nde	CGCGGATCCCATATGGTCGCCGCCGACATCG	NdeI
	fHbp_RV_XhoI	CCCGCTCGAGCTGCTTGGCGGCAAGAC	XhoI
PCR fragment for second transformation as in section 3.2.3, page 104	fHbp_intR	TGGAACTCTCCGCTCTCC	/
PCR fragments for self-priming PCR to transform *N. meningitidis* for fHbp OE	10AA_fw	GACCACTGCCCTGATTCTGACCGCC	/
	10AA_rv	GGCGGTCAGAATCAGGGCAGTGGTC	/

Table 1.1 *Continued from previous page.* List of primers used. Restriction site underlined in the sequence.

Construct of DNA fragment for replacing *lpxL1* gene with kanamycin resistance cassette	lpx_upF	GCATTTGTATTTTGCCGTCTG	
	lpx_doF	ATGCATGCCCCGATTTTTACT GACTACG	
	lpx_doR	CGCCATTTTCTACGCTTTGCC AAG	
	kan_doR	TAGTCAGTAAAAATCGGGCG GGATCCTCATTATTC	
gna33 gene knock-out	U33F	GCTCTAGAGATGAGTCGAAC ACAATGAACAATGTCCTGA	XbaI
	D33R-DUS	ATGCCGTCTGAACCCGCTCGA GAGTAGGGACAACCGG	XhoI

2.2. Neisseria meningitidis strains and growth conditions

Parent wild type strain used for generating *Neisseria meningitidis* mutants, "NVGH0385", was described previously by Koeberling *et al* [53]: it is a serogroup W meningococcus, an ST11 strain isolated in 2004 in Ghana; the native fHbp expressed belongs to variant 2, and PorA subtype is P1.5,2.

Generation of *N. meningitidis* mutants for GMMA and NOMV production is fully described in Chapter 3, page 96.

N. meningitidis strains were grown at 37°C on GC agar plates or in liquid medium (pre-culture of 5 mL in 15 mL falcon tubes, used for inoculating 50 mL cultures in in a 250 mL Erlenmeyer flask) containing yeast extract, casaminoacids, glutamic acid, and lactic acid, in a humid atmosphere containing 5% CO_2.

When required, antibiotics were added to plates and liquid medium at concentration of 5 μg/mL for chloramphenicol, 80 μg/mL for kanamycin, 5 μg/mL for erythromycin, 60 μg/mL for spectinomycin.

Neisseria meningitidis clinical isolates used for serum bactericidal assays are described in details in Table 2.3.

2.2.1. TRANSFORMATION OF NEISSERIA MENINGITIDIS.

The meningococcus strain to be transformed was plated on a GC agar plate from a glycerol stock kept at -80°C. After O/N growth at 37°C, 5% CO_2, 10-15 single colonies were picked and transferred onto a fresh GC agar plate in a single spot. 1-3 µg of DNA to transform meningococcus, in a 20 µL volume, was directly dropped onto bacteria, and then mixed with a 1 µL loop. The plate was incubated at 37°C, 5% CO_2 for 5-6 hours. After incubation bacteria were collected, resuspended in 100 µL of phosphate-buffered saline (PBS) 1X, and plated onto a fresh GC agar plate, containing the appropriate antibiotics for selection. Plates were incubated at 37°C, 5% CO_2 for 18-36 hours, or until transformants appeared big enough to be sub-cultured. Single colonies were sub-cultured on fresh GC agar plates with the respective antibiotics, and incubated at 37°C, 5% CO_2 for 18-36 hours. The day after, glycerol stocks were then prepared resuspending the colonies expanded, in 1 mL medium containing 20% glycerol, and rapidly frozen at -80°C.

2.3. Preparation of NOMV from Neisseria meningitidis

The strain used to prepare NOMV was sub-cultured from glycerol stock on GC agar plates, and incubated at 37°C, 5% CO_2 for 16-24 hours. Single colonies from the plate were pre-cultured in 5 mL of a meningococcal medium, from OD 0.05 to OD 1. The pre-culture was then used to inoculate 50 mL culture in a 250 mL Erlenmeyer flask (baffled, vented), and

incubated at 37°C, 5% CO_2, 150 rpm for 16-18 hours. The OD at the end of the incubation was recorded. The same procedure was followed, changing growing temperature to 30°C, as specified in Chapter 3. The culture was transferred into 50 mL Falcon tubes and centrifuged at 2,200 x *g*, 4°C for 40 minutes. The supernatant was filtered using a Stericup filter with a membrane pore size of 0.22 µm (Millipore, Billerica, MA, USA), to remove residual bacteria. 100 µL of the filtered supernatant were plated on three GC agar plates and incubated at 37°C, 5% CO_2 for 24 hours, to confirm lack of viable *N. meningitidis*. When no growth on plates was observed, the culture supernatant was ultracentrifuged at 142,000 x *g* for 2 hours at 4°C. The pellet containing the NOMV was resuspended in PBS 1X, filtered with a 0.22 µm syringe filter, and stored at 4°C.

2.4. Cloning, expression, and purification of recombinant fHbp

2.4.1. RECOMBINANT FHBP CLONING

Genes encoding for the four fHbp ID types selected (ID 1, ID 5, ID 9, ID 74, all containing the R41S mutation, Chapter 3) were cloned into expression vector pET21b (Novagen, Madison, WI, US), without the signal peptide and with a C-terminal His_6-tag. *E. coli* BL21 (DE3) (Novagen, Madison, WI, US): genes were amplified from the plasmids pACYC184 cps::kanR::Pweak::$fHbp_{R41S}$ (Chapter 3, Figure 3.3, page 106), with primers fHbp_tr_F_Nde and rec_RV_Xho (Table 2.1), and cloned into pET21b with restriction enzymes NdeI and XhoI. For each ID, multiple clones (10-20) were tested for protein expression in 5 mL LB: 5 mL LB with ampicillin 0.1 mg/mL were inoculated with 1 single colony, and when OD reached 0.5 - 0.8, protein expression was induced with 5 µL of Isopropyl-beta-D-thiogalacto-pyranoside (IPTG, Sigma-Aldrich) 1 M, for 4 h. fHbp

production was verified by Western blot on whole cell lysates (2.6.2. Western blot (WB), page 79), using as primary antibody a mouse polyclonal anti-fHbp ID 1 antibody. Clones positive for protein production were used to purify recombinant fHbp on a 1 Lt culture scale.

2.4.2. PROTEIN EXPRESSION

5 mL LB with ampicillin 0.1 mg/mL were inoculated with 1 single colony and grew O/N; the morning after, grew bacteria culture was used for starting 300 mL culture, at OD 0.05. When OD reached 0.5 - 0.8, protein expression was induced with 1 mL of IPTG (Sigma-Aldrich) 1 M, for 4 h. Cells were harvested at 7,000 x *g*, for 30 min at 4ºC. Supernatant was discarded and pellet stored at -20°C O/N.

2.4.3. PROTEIN PURIFICATION

For fHbp purification, cell pellet from 1 L culture was resuspended in 20 mL Buffer 1 (Appendix I. Buffers and media recipes, page 229), and incubated with 0.5 mg/mL lysozyme (Sigma-Aldrich) in ice, on a shaker for 30 min. Cell suspension was then sonicated (30 sec sonicate + 30 sec pause, for 6 times), and centrifuged at 10,000 *g* for 30 min, at 4°C. The cell pellet was discarded, and supernatant was incubated with Ni-NTA agarose resin. IDs 1 and 74 were purified using His-Select Nickel Affinity Gel (Sigma-Aldrich), and IDs 5 and 9 using Ni-NTA Agarose (Invitrogen). All protein purifications were performed according to manufacturers' manuals, with buffers as described in Appendix I. Buffers and media recipes, page 229, using disposable columns (GE Healthcare). Proteins were eluted with 250 mM Imidazole, and dialysed against PBS 1X,

at 4°C O/N, using SnakeSkin Dialysis Tubing, 10K MWCO (ThermoFisher Scientific). Protein purification was confirmed by SDS-PAGE followed by Coomassie staining. Proteins were filtered with a 0.22 μm syringe filter, and stored at 4°C.

2.5. Quantification of NOMV and recombinant fHbp

Concentration of NOMV was calculated as total protein content, determined by Lowry protein assay, in comparison to a standard curve obtained with bovine serum albumin (BSA) (Total Protein Kit, Micro Lowry, Peterson's Modification, Sigma-Aldrich, St. Louis, MO, USA) [297].

Concentration of recombinant fHbp was determined by Lowry protein assay, in comparison to a standard curve obtained with bovine serum albumin (BSA) (Total Protein Kit, Micro Lowry, Peterson's Modification, Sigma-Aldrich, St. Louis, MO, USA).

2.6. Protein gels

2.6.1. SDS-PAGE

Whole cell lysates samples were prepared by centrifuging 1 mL of bacteria liquid culture (OD 1) at 5,000 *x g* for 10 minutes, and resuspending the pellet in 100 μL sample buffer 1X (NuPAGE® Sample Reducing Agent (10X) plus NuPAGE® LDS Sample Buffer (4X) to final concentration 1X); samples were boiled for 10 minutes. 10 μL per sample per well were loaded on a gel. NOMV and recombinant fHbp samples were prepared by mixing equal volumes of samples and sample buffer 2X (NuPAGE® Sample Reducing Agent (10X)

plus NuPAGE® LDS Sample Buffer (4X) to final concentration 2X); samples were then boiled for 10 minutes.

All samples were separated by SDS-PAGE using NuPAGE® Novex® 12% Bis-Tris Protein Gels; gels were run in NuPAGE® MOPS SDS Running Buffer, at 200 V for 1 hour.

2.6.2. WESTERN BLOT (WB)

Western blot was performed with the iBlot® Dry Blotting System (Invitrogen), transferring proteins from the gel to a nitrocellulose membrane (iBlot® Transfer Stack, Invitrogen) for 7 minutes at 20 V. The membrane was blocked in PBS-milk 3% for 2 hours, on a shaker at RT; afterwards it was washed in PBS-Tween 0.1%, and incubated 1 hour with primary antibody. For fHbp detection, primary antibody was a mouse anti-fHbp v.1 polyclonal antibody (in-house made), diluted 1:2,000 in PBS-Tween 0.1%; for GNA33 detection, primary antibody was a mouse anti-GNA33 polyclonal antibody (in-house made), diluted 1:100 in PBS-Tween 0.1%. After 2-hour incubation with primary antibody, membranes were washed 5 times with PBS-Tween 0.1%. Secondary antibody was an anti-mouse IgG conjugated with horseradish peroxidase (Sheep anti-mouse IgG – HRP, GE Healthcare), diluted 1:10,000 in PBS-Tween 0.1%). After 1-hour incubation with secondary antibody, membranes were washed 5 times with PBS-Tween 0.1%. 4 mL of ECL (Pierce ECL Western Blotting Substrate, ThermoFisher Scientific) were added to the membranes for 1 minute; membranes were then removed from the substrates and placed in plastic sheet protectors, exposed to Amersham Hyperfilm ECL film (GE Healthcare) for 20 seconds to 1 minute, and developed.

2.6.3. COOMASSIE STAINING

For Coomassie staining, gels were washed with MilliQ H_2O after run, and stained with SimplyBlue™ SafeStain (Invitrogen) following manufacturer's protocol.

2.7. Negative Staining Transmission Electron Microscopy

For electron microscopy of *N. meningitidis*, 5 mL of liquid culture were started from 5-10 single colonies; when reached OD 1, culture was diluted to OD 0.1 in PBS, and 5 µL were placed on copper formvar/carbon-coated grids (Agar Scientific); after 5 min adsorption, the drop was dried using a Whatman filter paper. 5 µL of formaldehyde (Sigma Aldrich) 2% in PBS were added, and sample was incubated 5 min. Residual drop was dried again using Whatman filter paper.

For electron microscopy of NOMV, 5 µl of vesicles preparation at a concentration of 100 µg/µl in PBS was adsorbed onto 300 mesh copper formvar/carbon-coated grids for 5 min. Grids were washed with 5 µL-drops of MilliQ H_2O, and dried by blotting with Whatman filter paper. Grids were treated with 2% uranyl acetate in MilliQ H_2O for 1 min. Grids were observed with a CM100 transmission electron microscope (Philips, Eindoven, the Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of 105,000x.

2.8. Biophysical analyses of purified NOMV

2.8.1. HPLC-SEC

NOMV preparations were analysed by HPLC-SEC with a TosoHaas TSK gel 6,000 + 4,000 PW columns equilibrated in PBS, and with in-line UV, fluorescence emission, and MALS (Dawn Heleos II, Wyatt) detectors. 80 µL of samples, containing 0.1 mg/mL total protein, were injected and eluted with a flow rate of 0.5 mL/min in PBS. UV, fluorescence emission, and multiangle light scattering (MALS) data were collected and analysed using Empower 3 and ASTRA 6 software (Wyatt Technology). Light scattering analysis was done by using "Zimm" model with fit degree 1.

2.8.2. DYNAMIC LIGHT SCATTERING (DLS)

Dynamic light scattering (dls) measurements were performed with a Malvern Zetasizer Nano ZS (Malvern, Germany), equipped with a 633-nm He-Ne laser and operating at an angle of 173°. The software used for collecting and analysing data was the Setasizer 7.11 from Malvern. 40 µL of each sample were measured in ZEN 0040-disposable micro cuvette (Malvern). The measurements were done with an automatic attenuator, and at controlled temperature of 25°C. For each sample, 10-15 runs of 10 s were performed, with 3 repetitions. The intensity size distribution, the Z-average diameter, and the polydispersity index (PdI) were obtained from the autocorrelation function using the "general purpose mode". Default lower threshold of 0.05 and upper threshold of 0.01 were used. Viscosity and refractive index of PBS at 25 °C were used for data analysis.

2.9. Lipid A analysis

2.9.1. LIPID A EXTRACTION FROM NOMV

To extract lipid A from NOMV, 50 µL of acetic acid 5% were added to 200 µL of NOMV 1 mg/mL. Samples were incubated for 2 hours at 100°C, and then centrifuged at 7,000 x *g* for 15 min; supernatant was discarded and the pellet resuspended in MilliQ H_2O, for washing. Samples were centrifuged once more at 7,000 x *g* for 15 min, and supernatant was again discarded. The resulting pellet was dried by vacuum-concentrator.

2.9.2. MALDI-TOF AND MS/MS ANALYSIS OF LIPID A

Dried lipid A extracted was resuspended in 50 µL of Chloroform-Methanol 4:1, mixed 1:1 with Super DHB solution (Sigma-Aldrich, St. Louis, MO, USA). 2 µL of resulting sample were spotted to the target plate, and analysed by MALDI-TOF in linear ion negative mode using flex control. Spectra reported are the sum of 20 shots and are representative of three independent experiments. Spectra were analysed by Flex Analysis software.

2.10. PBMC isolation, stimulation, and IL-6 detection (Monocyte Activation Assay – MAT)

2.10.1. PBMC ISOLATION AND STIMULATION

Buffy coat from three different human donors were used to isolate PBMC by Ficoll density centrifugation [298, 299]. PBMC were grown in 96-well round bottom plates (Thermo Fisher Scientific), to $2x10^5$ cells/well density, with 180 µL of RPMI-1640 medium, supplemented with 25 mM HEPES, glutamine, 10% FBS (Fetal Bovine Serum), heat-inactivated complement at 56°C for 30 minutes, and 1% penicillin/streptomycin

(p/s) antibiotics. 20 µL of serial 10-fold dilutions (from 10^3 to 10^{-6} ng/mL final concentration; dilution in PBS 1X) of stimulus (i.e. NOMV to be tested) were added to each well. Plates were incubated for 4 hours at 37°C. Plates were then centrifuged at 1,400 x *g* for 5 min, the supernatants were collected and tested for cytokine released by ELISA.

2.10.2.IL-6 DETECTION BY ELISA IN THE SUPERNATANTS OF PBMC STIMULATED WITH NOMV

Nunc MaxiSorp flat bottom 96-well plates (Thermo Fisher Scientific) were coated with 50 µL/well of capture anti-human IL-6 antibodies (eBioscience, 500 µg/mL, cat. 14-7069), diluted 1:250 in PBS 1X, and incubated O/N at 4°C. Plates were washed with PBS-Tween 0.05%, and blocked by incubating with PBS-BSA 1% (200 µL/well) for 1 hour at room temperature (RT). After washing with PBS-Tween 0.05%, 50 µL/well of supernatants from PBMC stimulated with NOMV were added; two-fold dilutions of Recombinant Human IL-6 (eBioscience, cod. 39-8069, 1 µg/mL) in RPMI-FBS 10%, from 4,000 pg/mL (standard 1) to 7,81 pg/mL (standard 10) was included as standard curve. Plates were incubated for 2 hours at RT. Plates were washed with PBS-Tween 0.05%, and 50 µL/well of detection antibody Biotin anti-human IL-6 (e-Bioscience, 500 µg/mL, cat 13-7068), diluted 1:250 in PBS-BSA 0.1% -Tween 0.05%, were added. Plates were incubated for 2 hours at RT. Plates were washed with PBS-Tween 0.05%, and 50 µL/well of Streptavidin-HPR (R&D Systems) diluted 1:200 in PBS-BSA 0.1% -Tween 0.05% were added. Plates were incubated for 20 min at RT, without exposure to light. Plates were washed with PBS-Tween 0.05%, and 100 µL/well of Substrate (R&D Systems) were

added. Plates were incubated for 8 min at RT, without exposure to light. 50 µL/well of H_2SO_4 12.5% were added to stop reaction. Plates were read by ELISA reader (subtracting OD obtained at 450 nm to OD obtained at 630 nm). Data were analysed by Excel and GraphPad Prism 6.

2.11. NF-kB luciferase reporter assay

Human Embryonic Kidney (HEK293) cells expressing luciferase under control of the NF-kB promoter and stably transfected with either TLR4, MD2 and CD14 (TLR4-HEK293) or TLR2 (TLR2-HEK293) were used (cells obtained by Ugo D'Oro, GSK Vaccines RDC – Siena, former Immunology Function Unit in Novartis Vaccines).

HEK293 stable transfected cells were grown in DMEM medium complete (with 4.5 g/L of glucose, HEPES, 10% FBS complement depleted by heating at 56°C for 30 minutes, and 1% Pen-Strep antibiotics). Specific antibiotics for different cell lines were used: for TLR4-HEK293 puramycine 5 µg/mL, blasticidine 10 µg/mL, and hygromycin 250 µg/mL; for TLR2-HEK293 puramycin 5 µg/mL, and hygromycin 250 µg/mL.

For NF-kB luciferase assay $2.5x10^4$ cells/well in 90 µL complete DMEM medium, without specific antibiotics, were grown for 24 hours at 37°C, in 96-well microclear luciferase plates (PBI international). 10 µL of serial 5-fold dilutions (from 10^3 to 10^{-6} ng/mL final concentration) of NOMV diluted in PBS were added; incubation at 37°C for 5 hours followed. Supernatant from each well was discarded, and cells were lysed by adding 20 µL/well of lysis buffer (Promega) and incubating for 20 minutes at RT. Luciferase substrate (Promega) was added, and the plated was read by a luminometer. Data were analysed by Excel and GraphPad Prism 6.

2.12. Mice immunization

2.12.1. ANIMAL STUDY PERFORMED AT GVGH

The animal study performed at the GSK Vaccine Institute for Global Health (GVGH), Siena, Italy, was carried out at the Toscana Life Science animal facility, according to GVGH formal protocols. All animal work was approved by the Italian Animal Ethics Committee.

4-6 weeks old female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Eight mice per group were immunized intraperitoneally (IP) twice, with 4 weeks interval (Day 0 and 28). Mice were immunized with one of the following antigens: NOMV from MenW 2KO, MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74 (Table 3.1, page 115), or recombinant fHbp (ID 1_{R41S}, 5_{R41S}, 9_{R41S}, or 74_{R41S}). All vaccines were adsorbed on 3 mg/mL Alhydrogel (Brenntag) in a total 2,2 mL formulation, containing 10 mM Histidine, and 0.9 mg/mL NaCl. Each mouse was injected with 200 µL, but three extra doses were prepared. For each formulation, 50 µL were plated in triplicate on LB agar plates, for bioburden testing.

Serum samples were obtained from bleeds at 2 and 4 weeks after first dose (Day 14 and 28), and 2 weeks after the second dose (Day 42). Sera were stored at −80 °C until use.

2.12.2. ANIMAL STUDIES PERFORMED AT THE UNIVERSITY OF BIRMINGHAM

Animal studies performed at the University of Birmingham were carried out at the University Animal Facility, according to UK legislation. 6-8 weeks old male C57BL/6 mice were obtained from Charles River Laboratories. Four mice per group were immunized IP at different times and dose, as described in CHAPTER 5, page 180.

2.13. Mice organ isolation and cell suspension preparation (performed at the University of Birmingham)

2.13.1. SERUM

Intermedial bleedings were obtained by tail bleeding. Final bleeds were performed by cardiac puncture, under anaesthesia. Blood was left to clot at RT. After clotting, it was centrifuged for 30 min at 4,000 x *g*. Sera were collected from the supernatant and stored at -80°C until use.

2.13.2. CELLS ISOLATION FORM SPLEEN AND BONE MARROW

2.13.2.1. SPLEEN

Mouse spleens were removed aseptically, and cut: half was used for splenocyte isolation, half was slowly frozen in liquid nitrogen and stored at -80°C until use (see paragraph 2.17. Immunohistochemistry (IHC), page 93). Single cell suspensions were prepared using 70 µm nylon cell strainer filters (BD Biosciences Europe, Erembodegem, Belgium) and RPMI-1640 medium (Gibco), supplemented with 10% FBS and 1% penicillin/streptomycin (p/s) antibiotics. Cells were harvested by centrifugation at 375 x *g* for 5 min at 4°C. Erythrocytes were lysed with 500 µL ammonium-chloride-potassium lysis buffer (gibco Life Technologies) for 5 min at RT. Cells were harvested by centrifugation at 375 x *g* for 5 min at 4°C, and resuspended in RPMI-1640-FBS 10% -p/s 1%. Live cell density was assessed with Trypan Blue staining (Gibco).

2.13.2.2. BONE MARROW

After removal of the muscle tissues from the femurs and tibias, bones were washed with PBS. Both ends of the bones were cut with scissors to expose medullary cavity; the

marrow was flushed out, using a syringe with a 25-gauge needle, with RPMI-1640-FBS 10% -p/s 1%. Cells were resuspended, passed through a70 μm nylon cell strainer filters (BD Biosciences Europe, Erembodegem, Belgium) to remove debris, and harvested by centrifugation at 375 x *g* for 5 min at 4°C. Erythrocytes were lysed with ammonium chloride buffer (BD Bioscience) for 5 min at RT. Cells were harvested by centrifugation at 375 x *g* for 5 min at 4°C, and resuspended in RPMI-1640-FBS 10% -p/s 1%. Live cell density was assessed with Trypan Blue staining.

2.14. ELISpot

Sterile 96-wells filter plates (MultiScreenHTS IP Filter Plate, 0.45 μm, Millipore) were incubated with antigens (50 μL, 5 μg antigen/mL, in PBS) O/N at 4°C. Plates were washed with PBS, and incubated 2 hours at 37°C with RPMI 1640 supplemented with 10% FBS and Penicillin and Streptomycin antibiotics. $0.5x10^6$ cells/well were plated in a total volume of 200 μL of RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics, and incubated at 37°C. After 6 hours, medium was discarded and cells lysed by washing with PBS-Tween 0.1%. Detection antibody (AP-conjugated) used was added in PBS, 50 μL/well. Details of the detection antibody used are listed in Table 2.2. Plates were incubated at 4°C O/N. Plates were washed in PBS-Tween 0.1%, and after a final wash in PBS only, 50 μL /well of develop solution (SIGMA FAST BCIP/NBT, Sigma-Aldrich) was added. After 5 minutes, developing reaction was quenched with MilliQ H_2O. Plates were left drying and spots were counted with the AID

ELISpot Reader System and AID software version 3.5 (Autoimmune Diagnostika, Germany). Spots counted were expressed as Spot-Forming Units per million cells (SFU/10^6 cells). Data were analysed by Excel and GraphPad.

2.15. ELISA

2.15.1. ELISA AT GVGH

Anti-fHbp ID 1_{R41S}, ID 5_{R41S}, ID 9_{R41S}, ID 74_{R41S} IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [300]. Each recombinant fHbp ID_{R41S} was used for coating Nunc MaxiSorp flat-bottom 96-well plate (Thermo Fisher Scientific), at a concentration of 1 μg/mL. Mouse sera were diluted 1:100, 1:4,000, or 1:160,000 in PBS-BSA 0.1% -Tween 0.05%. ELISA units were expressed relative to a mouse anti-fHbp ID 9_{R41S} IgG standard curve: one ELISA unit was defined as the reciprocal of the standard serum dilution that gives an absorbance value of 1 in the assay; each mouse serum was tested in triplicate on different plates. Data are shown as scatter plots of the mean of individual mouse ELISA units, and the geometric mean is shown for each group [300]. Data were analysed by Excel and GraphPad.

2.15.2. ELISA AT UNIVERSITY OF BIRMINGHAM

Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) were coated with antigens (100 μL/well, 5 μg antigen/mL) O/N at 4°C. After blocking for 1 hour at 37°C with 200 μL/well of PBS-BSA 1%, serial dilutions of sera in PBS-BSA 1%-Tween 0.05% were added (100 μL/well), and plates were incubated 1 hours at 37°C. Plates were washed in PBS-Tween 0.1%. Detection antibody (AP-conjugated) was added in PBS-BSA

1% -Tween 0.05%, 100 µL/well, and incubated 1 hour at 37°C. Details of the detection antibodies used are listed in Table 2.2. Reaction was developed with SIGMA-FAST p-Nitrophenylphosphate (bNPP) tablets (Sigma-Aldrich), according to manufacturer's protocol. Plates were incubated with developing solution (200 µL/well) for 1 hour at 37°C, and then read at λ 405 nm by ELISA reader. Relative reciprocal titres were calculated by measuring the dilution at which the serum reached a defined OD_{405}. Data were analysed by Excel and GraphPad.

Table 2.2. Antibodies used for ELISA and ELISpot assays

Antibody	Source	Cat. Ref.	Working Dilution
α-mouse IgM	Southern Biotech	1020-04	1:2000
α-mouse IgG	Southern Biotech	1030-04	1:1000
α-mouse IgG1	Southern Biotech	1070-04	1:1000
α-mouse IgG2b	Southern Biotech	1090-04	1:2000
α-mouse IgG2c	Southern Biotech	1079-04	1:2000
α-mouse IgG3	Southern Biotech	1100-04	1:1000

2.16. Serum Bactericidal Antibody Assay

Serum bactericidal antibody response against meningococcal clinical isolates was measured as described before [53]. *N. meningitidis* strain to be tested in the assay was subculture from glycerol stock on GC agar plate, and incubated O/N at 37°C, 5% CO_2. 7 mL of Mueller-Hinton Broth (MHB) (DIFCO, #275730) supplemented with 0.25% glucose were inoculated with single cells to OD 0.12-0.15. Bacteria were incubated on a shaker at 37%, 5% CO_2 until OD reached 0.6-0.65. 1 mL of culture was transferred into 6 mL warm MHB, supplemented with 0.25% glucose and 0.02 mM CMP-NANA. Bacteria were incubated at 37%, 5% CO_2 until OD 0.6-0.65. The culture was then transferred into 50 mL Falcon tube, and washed with Dulbecco's saline phosphate buffer (Sigma #D8662), supplemented with 1% BSA (DPBS-BSA 1%), and centrifuged for 10 minutes at 3,500 rpm, RT. Supernatant was discarded and pellet resuspended in DPBS-BSA 1% to OD 0.6-0.7. Bacteria were diluted in DPBS-BSA 1% to a final working dilution of 1:12,500. 20% final concentration of Baby Rabbit Complement (CEDARLANE, Canada, lot number 6332)

was used as complement source. The assay was done in sterile 96-well plates, in a final volume of 40 µL/well. In a separate plate ("dilution plate"), sera dilutions were performed, starting from a 1:4 dilution, followed by 7 serial dilutions 1:4. 10 µL of serum sample were added from the dilution plate into the assay plate. In the control wells either DPBS-BSA 1% only, or active complement, or heat-inactivated complement, was added. 10 µL of bacteria at the working dilution were added to each well of the assay plate. Active and inactivated complement was added to control wells. 12 µL of controls were plated on square GC agar plate: 12 µL were loaded on top of the plate, and the drops let run down to bottom of the plate; this control plate was indicated as T0 (time zero), and incubated O/N at 37°C, 5% CO_2. Active complement was added to all the assay wells. Plate was sealed and incubated (37°C, 5% CO_2) for 60 min. After 60 minute (T60) 12 µL of each well were plated on square GC agar plates (as before) and incubated O/N at 37°C, 5% CO_2. Colonies were then counted on T0 plate and T60 plates, and average CFU at T0 from the controls was calculated. The bactericidal titre was defined as the serum dilution that results in 50% decrease of CFU at T60, compared to the average number of CFU at T0.

Origin and characterization of the strains tested are summarized in Table 2.3.

Table 2.3. Characteristics of African *N. meningitidis* strains used in serum bactericidal assay

Abbreviation in the text	Serogroup	Year of isolation	Country	CC [ST][a]	PorA subtype	fHbp variant and ID[b]	fHbp expression (%)[c]
MenA 1	A	2009	Nigeria	5 [7]	P1.20,9	v.1 ID 5	107
MenA 2	A	2006	Burkina Faso	5 [2859]	P1.20,9	v.1 ID 5	52
MenA 3	A	2007	Burkina Faso	5 [6035]	P1.20,9	v.1 ID 5	65
MenW	W	2009	Cameroon	175 [2881]	P1.5-1,2-36	v.1 ID 9	1
MenX 1	X	2006	Niger	181 [181]	P1.5-1,10-1	v.1 ID 74	75
MenX 2	X	2010	Burkina Faso	181 [181]	P1.5-1,10-1	v.1 ID 74	69
MenC	C	2007	Germany	NA[d] [32]	P1.7,16	v.1 ID 1	NDe

[a]CC: Clonal Complx; ST: Sequence Type. [b]Determined by sequencing of the fHbp gene and analysis of the protein sequence using the Neisseria.org database database [180]. [c]fHbp expression was measured by Western blot of whole cell lysates and in relation to group B strain H44/76, as previously described [92].

[d]NA: Not Assigned. [e]ND: Not Determined

2.17. Immunohistochemistry (IHC)

IHC was performed on sections of spleens from immunized mice. Frozen spleens were kept at -80°C until use. Spleens were mounted onto a chuck with OCT TissueTek compound (Dako, Glostrup, Denmark), and 5 µm sections were cut with a cryostat (Bright Instruments, Huntington, UK). Sections were deposited onto four-spot slides (C Hendley Essex Ltd, Essex, UK), and left to dry for 1 hour before fixation. Fixation was carried out by immersing the slides in acetone for 20 min at 4°C; slides were left to dry for 1 hour at RT and then stored in polyethene bags at -20°C.

Before staining, slides were thawed at RT, then washed with Tris-Buffered Saline (TBS), pH 7.6, for 5 min. Slides were incubated with 75 µL of primary antibody (diluted in TBS, according to Table 2.4) for 1 hour at RT in a moist chamber. During incubation time, secondary antibodies were adsorbed to 10% Normal Mouse Serum (NMS), in TBS. After incubation, slides were tilted, and primary antibody removed with filter paper; slides were washed with TBS for 5 min, then incubated 45 min at RT with 75 µL of secondary antibody, in the moist chamber. Following incubation, slides were washed with TBS for 5 min, and incubated with 75 µL of streptavidin-complex with alkaline phosphate (AP), for 40 min in the moist chamber at RT. To develop secondary antibody conjugated to Horse-Radish Peroxidase (HRP), a 15 mg Sigma FAST™ 3,3'-Diaminobenzidine tablet was dissolved in 15 mL TBS pH 7.6, filtered, and added with 1-2 drops of hydrogen peroxidase. 2-3 drops were mounted on each section, which were developed for 1-3

minutes, and washed 5 minutes in TBS before adding the substrate for antibodies conjugated to biotin/AP. The substrate for biotin/AP was prepared dissolving 4 mg of Naphtol AS-MX phosphate in 340 µL dimethyl formamide, that was then added to 8 mg of Levamisol [(-)-tetramisole hydrochloride] dissolved in 10 mL of Tris Buffer pH 9.2. 10 mg of Fast Blue B salt was then mixed and dissolved in the solution, that was then filtered and added in 1-3 drops on the sections. The sections were developed for 5-8 minutes, washed with MilliQ H_2O, and let to dry at RT prior to mounting with coverslips, using Immu-mount (Thermo Electron Corporation). Photographs were taken using a Leica CTR6000 microscope (Leica, Milton Keynes, UK) and QCapture software.

Table 2.4. Antibodies used for IHC

Target	**Species**	**Source**	**Clone/ Reference**	**Working Dilution**	**Final Concentration**
Primary antibody					
Anti-mouse CD3	Rat	Serotec	KT3	1:500	0.1 µg/mL
Anti-mouse IgD	Sheep	Abcam	AB9177	1:1,000	
Anti-mouse IgM	Rat	Serotec		1:500	
Anti-mouse IgG	Goat	Serotec		1::200	
Anti-PNA[1] (Bt[2])	Rabbit	Vector Laboratories	B-1075	1:150	33.3 µg/mL
Secondary antibody					

Anti-Rat (Bt)	Rabbit	Dako	P0450	1:600	1.4 µg/mL
Anti-sheep (PX[3])	Donkey	Jakson Immunoresearch	713-036-147	1:100	

[1]Peanut agglutinin; [2]Biotinylated; [3]Peroxidase-labelled

2.18. Statistical analysis

For statistical analysis of ELISA and SBA, antibody titres were log 10 transformed. ELISA and SBA titres <10 were assigned the value 1. Pearson Correlation test was used to evaluate linear correlation between two sets of values. Mann–Whitney U test was used to compare pairs of groups. A probability value $p < 0.05$ was considered statistically significant. All the analyses were performed with the GraphPad Prism 6 software.

CHAPTER 3. GENERATION OF *NEISSERIA MENINGITIDIS* MUTANTS AND PRODUCTION AND CHARACTERIZATION OF NOMV

3.1. Introduction

Neisseria meningitidis is a leading cause of bacterial meningitis worldwide, and causes recurrent epidemics of meningitis in Sub-Saharan Africa [132, 301]. Until 2010, in Africa, serogroup A was responsible for the majority of cases and deaths. After the introduction of MenAfriVac®, a group A polysaccharide conjugated vaccine, incidence of MenA cases and carriage dramatically dropped in the countries where the vaccine was introduced for routine immunization [129-131]. W and X serogroups, however, increased in prevalence, and in 2015 also serogroup C had an important impact on burden of disease, being responsible for over 60% of meningococcal cases [132]. Even with vaccines against meningococcus serogroups A, C, Y, and W available, an affordable and safe vaccine against all serogroups present in Africa is still needed.

One approach to provide an affordable and broadly-protective vaccine is Generalized Modules for Membrane Antigens (GMMA) [53]. GMMA are non-detergent extracted outer membrane vesicles (NOMV), purified from bacteria genetically-manipulated to over-produce these vesicles. Meningococcal GMMA have been shown to induce a strong antibody response that targets protein antigens: the selection of protein antigens, to be over-expressed on the outer membrane, that are common and conserved across different

serogroups, provides the possibility of achieving protection independently of capsular group.

Our vaccine approach is based on GMMA from an African serogroup W (ST11) isolate, expressing PorA subtype P1.5,2, the predominant PorA subtype expressed by African W strains. Since PorA generates protective antibodies that are subtype-specific, we genetically-engineered the GMMA-producing strain to over-express factor H binding protein (fHbp), which generates variant-specific antibodies. In particular, we over-expressed fHbp variant 1, the predominant fHbp variant carried by A and X African strains. With this combination of PorA subvariant P1.5,2 and fHbp variant 1, we aimed to achieve protection against African W serogroup through the homologous PorA, and against African A and X serogroups through over-expressed fHbp. The most prevalent fHbp v.1 IDs among African meningococcal strains are ID 5 among serogroup A, ID 74 among serogroup X, and ID 9 among serogroup W [111]. However, we aimed to select an ID type capable of providing the broadest coverage possible against meningococcal strains expressing fHbp variant 1 IDs different from that present in the vaccine. Recombinant fHbp induces limited cross-reactivity in the killing of strains carrying different fHbp ID, even though belonging to the same variant [107, 111]; in contrast, GMMA with over-expressed fHbp v.1 ID 1 show broad cross-protection [53]. However, the specific contribution of the fHbp sequence to such cross-protection has not been extensively studied. Therefore, the aim was to investigate whether different fHbp ID sequences have a different impact on the cross-reactivity induced, in the context of outer membrane vesicles. A better understanding would make it possible to achieve the

broadest cross-protection possible (ideally against strains that could emerge in the future, to minimise the risk of strains replacement), by choosing a particular fHbp ID type for over-expression, and how to select this ID.

As a rational approach for the selection of fHbp IDs to investigate their capability of giving broad cross-reaction when over-expressed in NOMV, we examined the portion of fHbp that binds factor H. This part of the protein needs some variability, as it is exposed to the immune selection, but the functional constraint of needing to bind factor H limits this. We therefore hypothesized a strict relationship between this part of the molecule and the cross-reactivity of anti-fHbp antibodies induced. In particular, we hypothesized that the broadest cross-reactivity among fHbp peptides may be present among those with the most shared fH binding sequence.

Coupling our analysis of the fHbp-fH binding site together with epidemiologic data of fHbp v.1 ID distribution [111], we chose four fHbp ID types for over-expression. These four fHbp peptides were to be tested for specificity of antibody response generated (Chapter 4), to evaluate our hypothesis on the relation between cross-protection induced by a specific fHbp ID and its fHbp-fH sequence.

The fHbp genes selected for over-expression in the GMMA-producing strains were engineered to contain the substitution of Arginine with Serine at amino acid position 41 (R41S), known to decrease human factor H (fH) binding [302]. To attenuate virulence and to avoid potential generation of serogroup-specific antibodies, genes of the group W capsule biosynthesis operon were deleted. Furthermore, the lipooligosaccharide (LOS) was genetically detoxified by deleting the *lpxL1* gene. Lastly, *gna33* gene was knocked-out to increase outer membrane blebbing of the mutants [53, 303].

We generated meningococcal mutants, purified GMMA (or, for most experiments, NOMV, as it is explained later in the text) and characterized them. In particular, we analysed the amount of fHbp by Western blot, determined vesicles size and shape by dynamic light scattering (dls), multiangle light scattering (MALS), and Negative Staining Transmission Electron Microscopy (TEM), analysed the mutated lipid A by MALDI-TOF, and examined the ability to induce production of pro-inflammatory cytokine IL-6 and activate toll-like receptor (TLR) 4 and TLR-2.

3.2. Generation of GMMA-producing meningococcal mutants

3.2.1. Pairwise comparison of fHbp-fH contact residues in 383 fHbp v.1 sequence IDs

To analyse the region of fHbp that interacts with human factor H, amino acidic residues of fHbp that are in contact with fH were mapped on the published crystal structure of the fHbp v.1-fH complex [304, 305], using the public available software Cn3D (https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Parameters were set to identify residues on fHbp that are at a maximum distance of 5 Å from the fH molecule, revealing 48 amino acids as points of interaction between the two molecules (Figure 3.1). At the time of the study 383 fHbp v.1 unique sequences were available in the *Neisseria* public database [110]. We aligned these 383 peptides and identified the 48 amino acids of the fH contact site on each sequence, using fHbp ID 1 as reference. The 48 amino acids identified as contact sites with fH in each of the 383 IDs (full list on CD) were compared by sequence alignment and the number of different amino acids between each pair of IDs

were reported in a *mn* matrix. Each cell of the matrix contains the number of amino acid differences between sequence *m* and sequence *n* (Figure 3.2, full matrix on CD).

Among the 383 v.1 peptides, 74 individual sequences of fHbp-fH contact residues were present; 41 of those (55%) were unique to one fHbp ID. The most shared fH binding site sequence was found in 83 out of the 383 peptides (21.7%) and hence represents a fH-binding site consensus sequence. If one difference at any position in the fH binding site consensus sequence was allowed, the number of IDs sharing it increased to 122 (31.9%). The biggest difference in the fH binding site between any pair of IDs was 13 amino acids (27.1%) and was found between 8 different pairs of 8 IDs. The biggest difference compared to the consensus sequence was identified in 5 fHbp IDs: ID 217 and ID 359 both had 10 aa differences (their fH-binding site being identical, and their whole peptide sequences differing by only one amino acid); ID 120 and ID 215 had 8 aa differences (their fH-binding site being identical, and their whole peptide sequences differing only in the N-terminal serine/glycine part); ID 55 similarly had 8 aa differences, with its fH-binding sequence being one of the 41 unique types.

aa residue	38	41	42	43	44	80	84	91	92	98	115	116	117	118	119
ID 1	Q	R	K	N	E	R	V	L	E	V	Q	D	S	E	H

aa residue	120	126	128	129	130	131	132	134	138	197	199	200	201	207	208
ID 1	S	K	Q	F	R	I	G	I	H	D	K	P	D	V	I

aa residue	209	210	211	218	219	220	221	223	224	225	227	234	235	237	238
ID 1	S	G	S	E	K	G	S	S	L	G	F	V	A	S	A

aa residue	240	241	248
ID 1	V	K	H

Figure 3.1. Amino acid residues of fHbp involved in contact with fH.

48 amino acids of fHbp distant no more than 5 Å from bound fH were identified on the crystal structure of the molecules. Here these 48 residues are reported for fHbp ID 1, numbered according to the mature protein sequence of this ID.

ID*m* / ID*n*	ID 1	ID 2	ID 3	ID 4	ID 5	ID 6	ID 7	ID 8	ID 9	ID 10	ID 11	ID 12	…	ID 60	ID 61	ID 62	ID 63	ID 65	ID 66	ID 69	ID 71	ID 73	ID 74	ID 78	ID 80	…	ID 677
ID 1	0	2	3	2	2	2	3	4	4	4	4	4	…	4	2	2	3	5	3	7	3	3	5	2	1	…	2
ID 2	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0
ID 3	3	1	0	3	3	1	5	1	1	1	1	1	…	1	1	1	2	6	5	7	4	4	5	1	4	…	1
ID 4	2	2	3	0	0	2	4	2	2	2	2	2	…	2	2	2	1	5	4	6	3	1	6	2	3	…	2
ID 5	2	2	3	0	0	2	4	2	2	2	2	2	…	2	2	2	1	5	4	6	3	1	6	2	3	…	2
ID 6	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0
ID 7	3	4	5	4	4	4	0	6	6	6	6	6	…	6	4	4	5	7	2	5	4	3	3	4	2	…	4
ID 8	4	2	1	2	2	2	6	0	0	0	0	0	…	0	2	2	1	7	6	6	3	3	6	2	5	…	2
ID 9	4	2	1	2	2	2	6	0	0	0	0	0	…	0	2	2	1	7	6	6	3	3	6	2	5	…	2
ID 10	4	2	1	2	2	2	6	0	0	0	0	0	…	0	2	2	1	7	6	6	3	3	6	2	5	…	2
ID 11	4	2	1	2	2	2	6	0	0	0	0	0	…	0	2	2	1	7	6	6	3	3	6	2	5	…	2
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮		⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮		⋮
ID 60	4	2	1	2	2	2	6	0	0	0	0	0	…	0	2	2	1	7	6	6	3	3	6	2	5	…	2
ID 61	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0
ID 62	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0
ID 63	3	3	2	1	1	3	5	1	1	1	1	1	…	1	3	3	0	6	5	7	2	2	7	3	4	…	3
ID 65	5	5	6	5	5	5	7	7	7	7	7	7	…	7	5	5	6	0	7	11	8	6	9	5	6	…	5
ID 66	3	4	5	4	4	4	2	6	6	6	6	6	…	6	4	4	5	7	0	6	4	3	4	4	2	…	4
ID 69	7	6	7	6	6	6	5	6	6	6	6	6	…	6	6	6	7	11	6	0	6	5	2	6	6	…	6
ID 71	3	5	4	3	3	5	4	3	3	3	3	3	…	3	5	5	2	8	4	6	0	2	6	5	2	…	5
ID 73	3	3	4	1	1	3	3	3	3	3	3	3	…	3	3	3	2	6	3	5	2	0	5	3	2	…	3
ID 74	5	4	5	6	6	4	3	6	6	6	6	6	…	6	4	4	7	9	4	2	6	5	0	4	4	…	4
ID 78	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0
ID 80	1	3	4	3	3	3	2	5	5	5	5	5	…	5	3	3	4	6	2	6	2	2	4	3	0	…	3
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮		⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮		⋮
ID 677	2	0	1	2	2	0	4	2	2	2	2	2	…	2	0	0	3	5	4	6	5	3	4	0	3	…	0

For each ID, number of peptides that diffear for:

0 amino acid	18	29	17	33	33	29	1	83	83	83	83	83		83	29	29	5	3	2	9	1	2	7	29	7		29
1 amino acid	41	64	123	50	50	64	7	39	39	39	39	39		39	64	64	129	8	0	21	4	38	28	64	22		64
2 amino acids	83	158	55	154	154	158	18	78	78	78	78	78		78	158	158	65	6	13	17	18	68	17	158	44		158
3 amino acids	52	24	66	46	46	24	63	41	41	41	41	41		41	24	24	59	6	61	10	157	162	7	24	88		24
4 amino acids	95	27	19	24	24	27	115	42	42	42	42	42		42	27	27	28	28	106	11	78	50	59	27	62		27
5 amino acids	23	44	31	31	31	44	58	46	46	46	46	46		46	44	44	28	97	69	26	59	34	86	44	117		44
6 amino acids	40	32	45	31	31	32	90	39	39	39	39	39		39	32	32	32	59	101	168	31	14	144	32	16		32
7 amino acids	19	2	24	9	9	2	8	10	10	10	10	10		10	2	2	26	98	4	71	19	8	7	2	8		2
8 amino acids	8	3	0	3	3	3	10	3	3	3	3	3		3	3	3	8	14	11	23	10	3	3	3	10		3
9 amino acids	4	0	3	0	0	0	6	0	0	0	0	0		0	0	0	1	21	7	4	2	4	10	0	8		0
10 amino acids	0	0	0	2	2	0	6	2	2	2	2	2		2	0	0	0	29	8	15	4	0	13	0	1		0
11 amino acids	0	0	0	0	0	0	1	0	0	0	0	0		0	0	0	2	14	1	8	0	0	2	0	0		0
12 amino acids	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0		0
13 amino acids	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0		0

Figure 3.2. Fragment of the matrix *m* columns per *n* rows, for pairwise comparison of fHbp-fH contact residues in 383 fHbp v.1 sequence IDs.

Each cell$_{mn}$ contain the number of different amino acids between ID$_m$ and ID$_n$. The darkness of shading of each intersection corresponds to the number of differences, with white = 0 differences. At the bottom of the table, for each ID$_m$ is reported the number of fHbp v.1 peptides differing for number of amino acids, and the average number of different residues between ID$_m$ and all the other v.1 peptides.

3.2.2. SELECTION OF FHBP IDS TO OVER-EXPRESS IN GMMA-PRODUCING STRAINS

To investigate whether there is a correlation between the sequence of fHbp region involved in binding fH, and breadth of cross-protection induced by outer membrane vesicles with over-expressed fHbp, against diverse meningococcal isolates, we selected different fHbp v.1 ID types for overexpression. The hypothesis was that fHbp residues involved in fH contact mostly correlate with the antibodies cross-reactivity induced, and in particular IDs with the most shared fH binding sequence provide broader cross-reactivity, compared to IDs with a less common fH-contact sequence. Therefore, one of the 83 peptides sharing the consensus sequence was included. To combine the amino acidic sequence analysis with epidemiological data, we further selected the fHbp variant 1 peptides identified as the most prevalent among African case-related clinical isolates: ID 5, ID 9, and ID 74 [111, 306]. ID 1 was also included for comparison with previous work [53]. ID 9 is one of the peptides that contain the fH binding site consensus sequence; therefore, no other fHbp v.1 IDs needed to be included. The fHbp-fH binding residues of the four selected fHbp ID types were all different from each other (Figure 3.2). fH-contact sequence of ID 1 is shared by a total of 17 fHbp v.1 peptides (4.4%), the sequence of ID 5 by 33 (8.6%), and sequence of ID 74 by 7 (1.8%) peptides. Among these four selected ID types, the minimum number of differences in the fHbp-fH sequence was 2 amino acids (4.2%) between ID 1 and ID 5, and between ID 5 and the consensus sequence in ID 9, while the maximum number of differences was 6 (12.5%), between ID 74 and ID 5, and

between ID 74 and the consensus sequence in ID 9. The number of amino acid differences between ID 1 and the fH-binding site consensus sequence was 4 (Figure 3.2).

3.2.3. GENERATION OF N. MENINGITIDIS MUTANTS

3.2.3.1. OVER-EXPRESSION OF FHBP V.1 AND DELETION OF CAPSULE BIOSYNTHESIS GENES

In a previous study, as parental strain for generating GMMA-producing meningococcal mutants, a serogroup W meningococcus ("NVGH 0385") ST11 strain was chosen [53]. This was isolated in Ghana in 2004; it expresses PorA subtype P1.5,2 and fHbp v.2 ID 22.

To generate mutants over-expressing fHbp v.1, the native *fHbp v.2* gene was first deleted by replacement with an erythromycin resistance cassette [103]. The resulting fHbp v.2 KO mutant was subsequently used to generate a panel of isogenic strains, over-expressing one out of the four different fHbp v.1 ID types selected.

To over-express the heterologous fHbp peptides, genes encoding the chosen fHbp subvariants (ID 1, 5, 9 and 74) were inserted in the genome of the fHbp v.2 KO mutant, under the control of a strong synthetic promoter [307], with the mutation strategy as follows: the gene sequence was inserted into the capsule biosynthesis locus (*cps*), in place of *synXBCD*. In this way, we abolished capsule biosynthesis and over-expressed fHbp v.1 at the same time, obtaining four mutants, each expressing one of the four fHbp IDs.

To obtain the constructs/plasmids to over-express fHbp, the respective fHbp genes were amplified by PCR from genomic DNA of different meningococcal strains (ID 1 from MenB MC58, ID 5 from MenA, ID 9 from MenW LNP, ID 74 from MenX BuFa1), with primers fHbp_fw/fHbp_rv (ID 1, 5, and 74) and ID9_fNde/fHbp_rv (ID 9), as listed in Table 2.1. Amplified fHbp genes were inserted in a low-copy number plasmid pACYC184. The

plasmid also contained the flanking regions that allow homologous recombination with the target capsule biosynthesis operon, and a kanamycin resistance cassette. It was not possible to generate a plasmid with fHbp under the control of the strong synthetic promoter required for fHbp over-expression in *N. meningitidis*, because high levels of expression of full-length fHbp protein were found to be toxic when clone into *E. coli*. Therefore four plasmids were constructed, containing a weak promoter (from gene nmb1523) that allowed cloning of the full length fHbp in *E. coli* (pACYC184 cps::kanR::P_{weak}::fHbp, represented in Figure 3.3).

Once the fHbp genes had been cloned into this plasmid, the R41S mutation was introduced by site-directed mutagenesis with primers fHbp_f41/fHbp_r41 (Table 2.1) (Figure 3.3). The correct substitution was verified by sequencing.

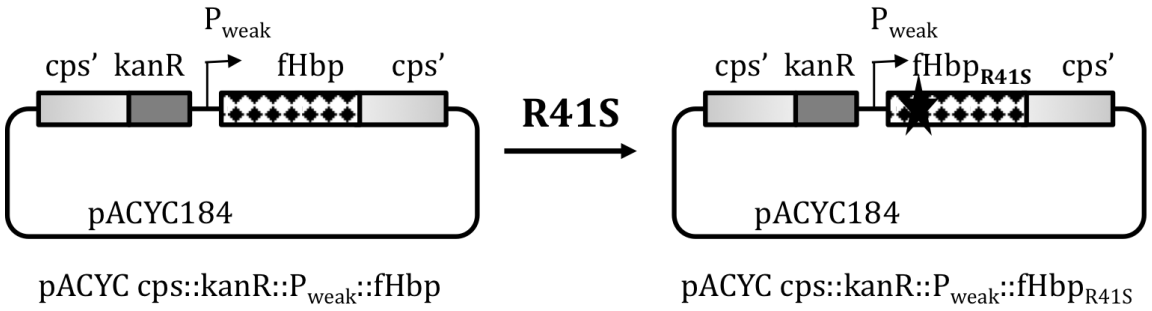


Figure 3.3. Graphic representation of plasmid carrying the fHbp genes.

One plasmid for each of the four fHbp ID types selected was cloned: pACYC184 cps::kanR::Pweak::ID1; pACYC184 cps::kanR::Pweak::ID5; pACYC184 cps::kanR::Pweak::ID9; and pACYC184 cps::kanR::Pweak::ID74. Once cloned, the R41S mutation was introduced into each fHbp ID, resulting in plasmids: pACYC184 cps::kanR::Pweak::ID1$_{R41S}$; pACYC184 cps::kanR::Pweak::ID5$_{R41S}$; pACYC184 cps::kanR::Pweak::ID9$_{R41S}$; and pACYC184 cps::kanR::Pweak::ID74$_{R41S}$.

To over-express fHbp in *N. meningitidis*, we first transformed serogroup W fHbp v.2 KO meningococcus strain with each of the four pACYC184 cps::kanR::Pweak::fHbp$_{R41S}$ constructs (Figure 3.4A). In a second step, we aimed to replace the weak promoter with a strong synthetic promoter, by homologous recombination. To achieve this promoter-substitution, a plasmid was used containing the upstream recombination site, a chloramphenicol resistance cassette, a strong synthetic promoter, and the first 300bp of the fHbp gene (Figure 3.4B). For this second transformation we tried both a linearized plasmid (pACYC cps::cmR::PS$_{trong}$::fHbpN-term) and a PCR product comprising the region from the recombination site to the first 300 base pairs of fHbp, using the last plasmid as template, with primer Cps_upF/fHbp_intR (Table 2.1). We obtained only one mutant correctly over-expressing fHbp ID 5$_{R41S}$ out of more than 20 colonies screened, when transforming with the PCR product.

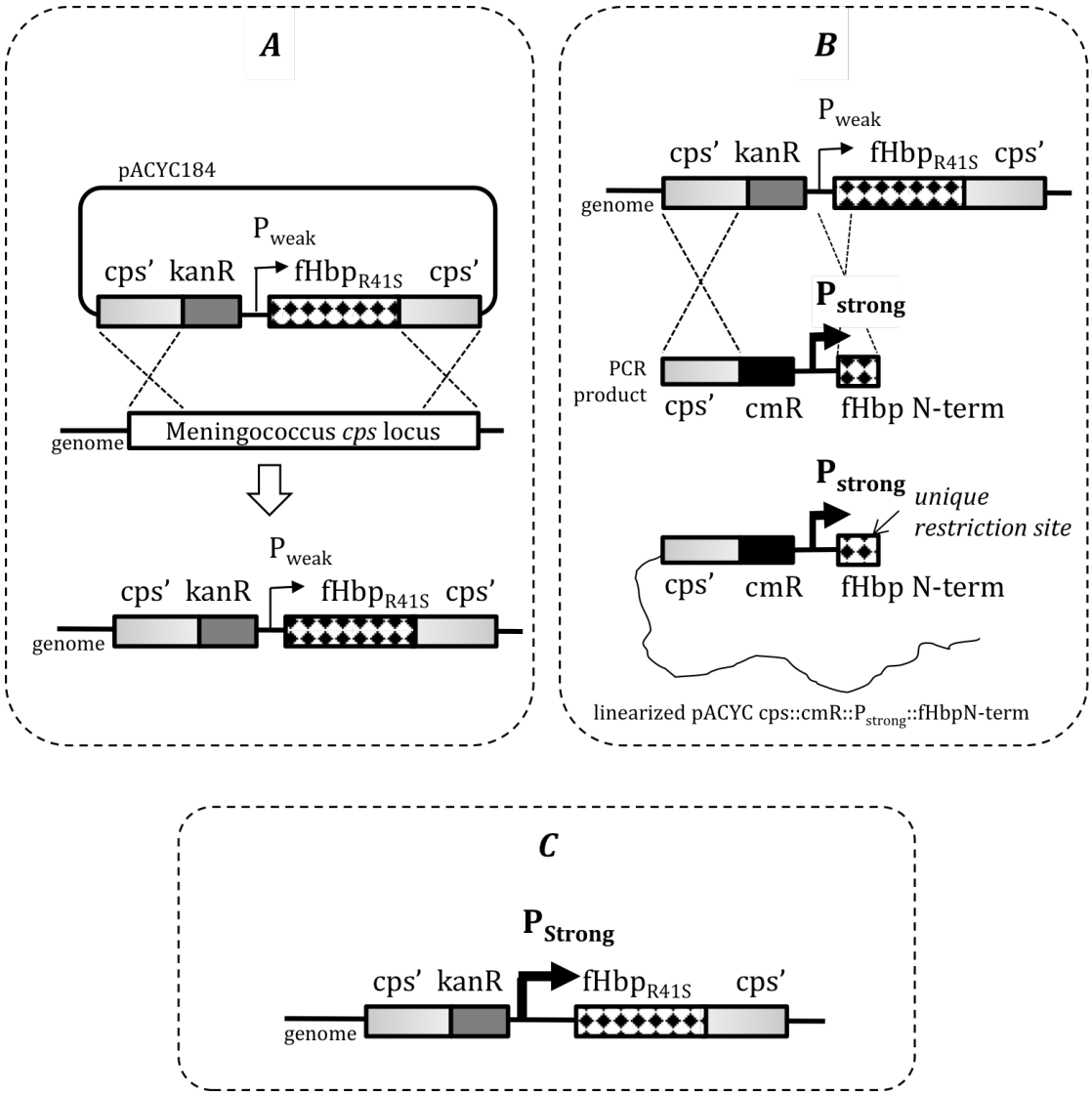


Figure 3.4. "Two step transformation" planned to over-express fHbp in *N. meningitidis*.

A) First transformation to insert the selected $fHbp_{R41S}$ gene in the place of meningococcus capsular biosynthesis locus, under the control of the weak promoter (Pweak). **B)** Second transformation to substitute the weak promoter with the strong synthetic promoter (Pstrong), with either a PCR product or a linearized plasmid. **C)** The engineered meningococcus genome, as final result. cps': sites for homologous recombination in the capsule biosynthesis locus; kanR: kanamycin resistance cassette; cmR: chloramphenicol resistance cassette; fHbp N-term: first 300bp of fHbp gene.

Because the two-step transformation strategy showed low efficiency in generating the correct mutants, we synthesized the DNA sequence containing the recombination sites, the antibiotic resistance cassette, the strong promoter and fHbp ID 1_{R41S}, ID 5_{R41S}, ID 9_{R41S}, or ID 74_{R41S}, by self-priming PCR (Figure 3.5). Using as PCR templates the plasmids previously generated, we amplified two DNA fragments: one fragment was obtained from plasmid pACYC cps::cmR::P_{Strong}::fHbpN-term with primers Cps_upF/10AA_rv (Table 2.1) and contained the upstream recombination site of the *cps* locus, the chloramphenicol resistance cassette, the strong promoter and the first 30 bp of the *fHbp* gene (which are shared by all the sub-variants chosen for over-expression); the second fragment was obtained from plasmid pACYC184 cps::kanR::P_{weak}::$fHbp_{R41S}$ with primers 10AA_fw/Cps_doR (Table 2.1) and contained the full-length *fHbp* gene and the downstream recombination site of the *cps* locus. These two DNA fragments overlapped for the first 30 bp of *fHbp* gene (Figure 3.5). By a self-priming PCR with the two fragments and primers annealing in the *cps* flanking regions (Cps_upF/Cps_doR, Table 2.1), we obtained the full-length DNA sequence with ID 1, 5, 9 and 74 (all containing the R41S mutation). The PCR products were used for transforming MenW *fHbp v.*2 KO strain. With this method 1-2 colonies over-expressing each fHbp v.1 peptide were obtained per μg of DNA used for transformation. Colonies were tested to have the fHbp over-expressed by Western blot, correct fHbp sequence inserted by sequencing, and capsule abolished by Dot blot.

A MenW control strain devoid of both fHbp expression and capsule biosynthesis was also produced, by replacing in the MenW fHbp v.2 KO mutant the *cps* locus with a spectinomycin resistance cassette [53].

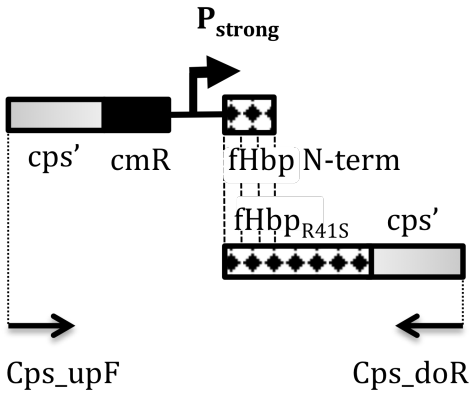


Figure 3.5. "Self-priming PCR" to synthesize the DNA fragment for fHbp v.1 over-expression in *N. meningitidis*.

The two DNA pieces overlap for 30bp of N-term *fHbp*. This allows the generation of a full-length DNA fragment by a self-priming PCR, followed by a PCR with primers annealing at the external extremities (black arrows: Cps_upF and Cps_doR). *cps'*: sites for homologous recombination in capsule biosynthesis locus; *cmR*: chloramphenicol resistance cassette; *fHbp N-term*: first 30bp of fHbp gene.

3.2.3.2. DELETION OF LPXL1 GENE

In the next step, each mutant containing a heterologous *fHbp v.1* gene was engineered to have attenuated endotoxin by inactivating the *lpxL1* gene [68], which was replaced with a kanamycin resistance cassette. To generate the *lpxL1* KO, a DNA fragment containing the recombination sites and the kanamycin resistance cassette was obtained by self-priming PCR between two PCR products amplified from plasmids as shown schematically in Figure 3.6: from plasmid pACYC lpx::cmR::P_{weak}::fHbp, with primer lpx_doF/lpx_doR (Table 2.1), we amplified the *lpxL1* downstream recombination site (Figure 3.6A); from plasmid pACYC lpx::kanR::P_{Strong}::fHbpN-term, with primers lpx_upF/kan_doR (Table 2.1), we amplified the *lpxL1* upstream recombination site and the kanamycin cassette. The two PCR fragments generated overlapped by 26 base pairs between the kanamycin resistance gene and the *lpxL1* downstream recombination site. By a self-priming PCR using the two fragments as template and primers lpx_upF/lpx_doR, generated the DNA sequence containing the *lpxL1* recombination sites and the kanamycin resistance gene. This PCR product was used for *lpxL1* gene KO in the meningococcal mutants MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74, and MenW 2KO, generating the mutants MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, MenW OE 74 ΔL, and MenW 3KO (Table 3.1). Correct replacement of the gene with the antibiotic resistance was verified by PCR.

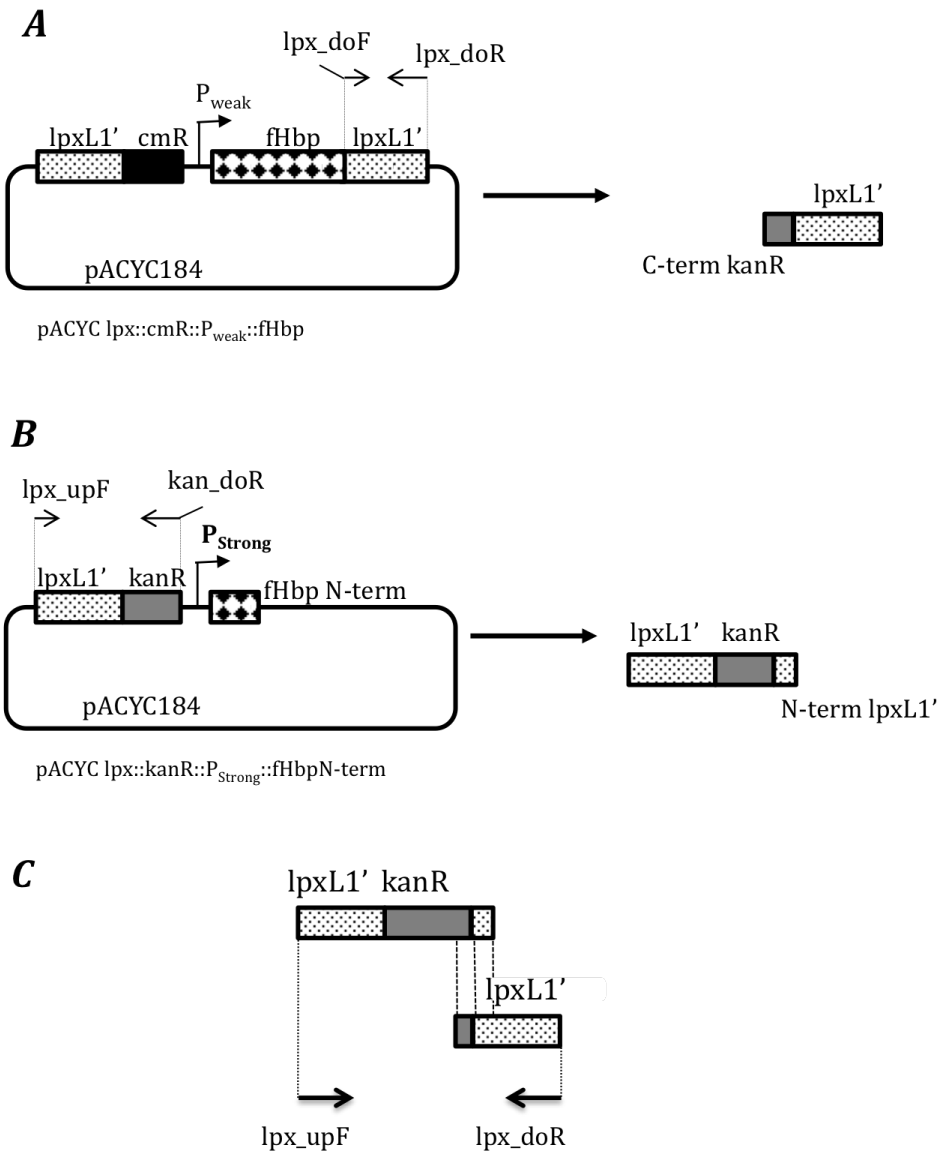


Figure 3.6. Synthesis of the DNA fragment for *lpxL1* KO mutation in meningococcal mutants.

A) Plasmid pACYC lpx::cmR::P_{weak}::fHbp was used as PCR template for amplifying downstream *lpxL1* recombination site, preceded by the C-terminal 26 bp of the kanamycin resistance. **B)** Plasmid pACYC lpx::kanR::P_{Strong}::fHbpN-term was used as PCR template for amplifying upstream *lpxL1* recombination site and kanamycin resistance cassette. **C)** The two DNA fragment amplified were used for a self-priming PCR for obtaining the full DNA sequence for *lpxL1* KO in meningococcal mutant strains.

3.2.3.3. DELETION OF GNA33 GENE

Further modification was the deletion of the *gna33* gene in mutants MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, MenW OE 74 ΔL, and MenW 3KO, to increase GMMA release [53, 303]. For replacing the *gna33* with a spectinomycin resistance cassette, we cloned the upstream and downstream recombination sites, with the antibiotic resistance cassette between them, into pACYC184 plasmid. Transformation of meningococcal mutants with this plasmid did not produce *gna33* KO mutants. One reason could have been the low transformation efficiency of the already engineered meningococcal strains. Therefore, to overcome the problem we amplified from this plasmid (with primers U33F/D33R-DUS, Table 2.1) the DNA sequence for the gene knock-out, adding at the 3'-end the *N. meningitidis* DNA-uptake sequence (DUS). We thus obtained the mutants, and we verified *gna33* KO by western blot and by PCR.

All the mutants generated are listed in Table 3.1.

Table 3.1. List of *N. meningitidis* mutants generated

Strain abbreviation	**Mutations**				
	fHbp v.2 KO	*cps* KO	*lpxL1* KO	*gna33* KO	*fHbp v.1* OE
MenW 2KO	✔	✔			
MenW 3KO	✔	✔	✔		
MenW 4KO	✔	✔	✔	✔	
MenW OE 1	✔	✔			ID 1_{R41S}
MenW OE 5	✔	✔			ID 5_{R41S}
MenW OE 9	✔	✔			ID 9_{R41S}
MenW OE 74	✔	✔			ID 74_{R41S}
MenW OE 1 ΔL	✔	✔	✔		ID 1_{R41S}
MenW OE 5 ΔL	✔	✔	✔		ID 5_{R41S}
MenW OE 9 ΔL	✔	✔	✔		ID 9_{R41S}
MenW OE 74 ΔL	✔	✔	✔		ID 74_{R41S}
MenW OE 1 ΔL Δ*gna33*	✔	✔	✔	✔	ID 1_{R41S}
MenW OE 5 ΔL Δ*gna33*	✔	✔	✔	✔	ID 5_{R41S}
MenW OE 9 ΔL Δ*gna33*	✔	✔	✔	✔	ID 9_{R41S}
MenW OE 74 ΔL Δ*gna33*	✔	✔	✔	✔	ID 74_{R41S}

3.3. NOMV production and characterization

3.3.1. PROTEIN PATTERN AND FHBP LEVEL

Final strains carried all the mutations described. *gna33* KO mutants showed inconstancy in growth, making it difficult to produce GMMA for the subsequent experiments. However, this mutation was required for increasing GMMA production yield, but considered unnecessary for the investigation of immunogenicity of outer membrane vesicles. Therefore, we performed most of the following experiments with NOMV, native outer membrane vesicles purified from *gna33* wild type meningococcal mutants.

NOMV from the mutants generated were used for immunological experiments in mice. Therefore, it was fundamental to characterize the different preparations and demonstrate that they were comparable, in order to be able to correctly interpret results from immunized mice.

We collected NOMV released by wild type MenW and mutant strains MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74, and MenW 2KO during growth, as described in Chapter 2, page 75. SDS–PAGE gel electrophoresis followed by Coomassie Blue staining revealed a similar qualitative protein pattern in NOMV preparations from the different engineered meningococcal strains. These were similar to the protein pattern of NOMV from the serogroup W wild type parental strain, consistent with the protein pattern of meningococcal outer membrane preparation. Predominant bands corresponding to the main porins (PorA and PorB), reduction modifiable protein (Rmp), and Opacity-associated proteins (Opa and Opc) were clearly visible (Figure 3.7). As fHbp runs at the same level as the opacity proteins, it is not visible as a separate band on Coomassie Blue-stained gels.

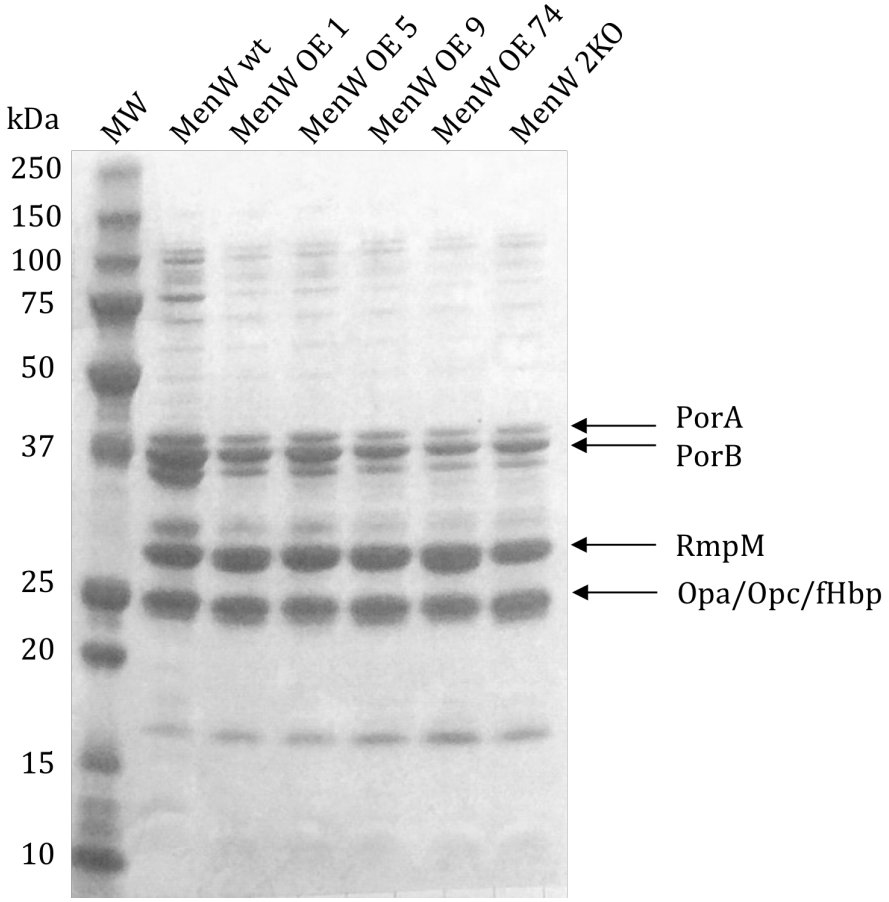


Figure 3.7. SDS-PAGE and Coomassie staining of NOMV.

Samples order from left to right: molecular weight (MW); NOMV from: MenW wt, MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74, MenW 2KO. 5 μg of NOMV were loaded for each sample. The location of different proteins is indicated by the arrows.

Detection of fHbp in NOMV by Western blot, using a mouse polyclonal anti-fHbp v.1 antibody, showed a comparable amount of fHbp in NOMV from the different mutants (Figure 3.8A). We compared by Western blot fHbp levels in NOMV from *lpxL1* KO mutant strains (MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, and MenW OE 74 ΔL), with similar results (Figure 3.8B). In both Western blots, we included, as a control, NOMV from a wild type MenA strain expressing medium levels of fHbp ID 5. We then compared by Western blot the amount of fHbp present in different quantitites of NOMV from the wild type MenA strain and from MenW OE 9 ΔL (Figure 3.9): the quantity of protein detected in 0.05 μg of NOMV from the mutant was higher than the quantity detected in 0.5 μg of NOMV from wild type MenA, suggesting that mutant strains produce NOMV with more than 10-fold the amount of fHbp of this wild type strain chosen for comparison.

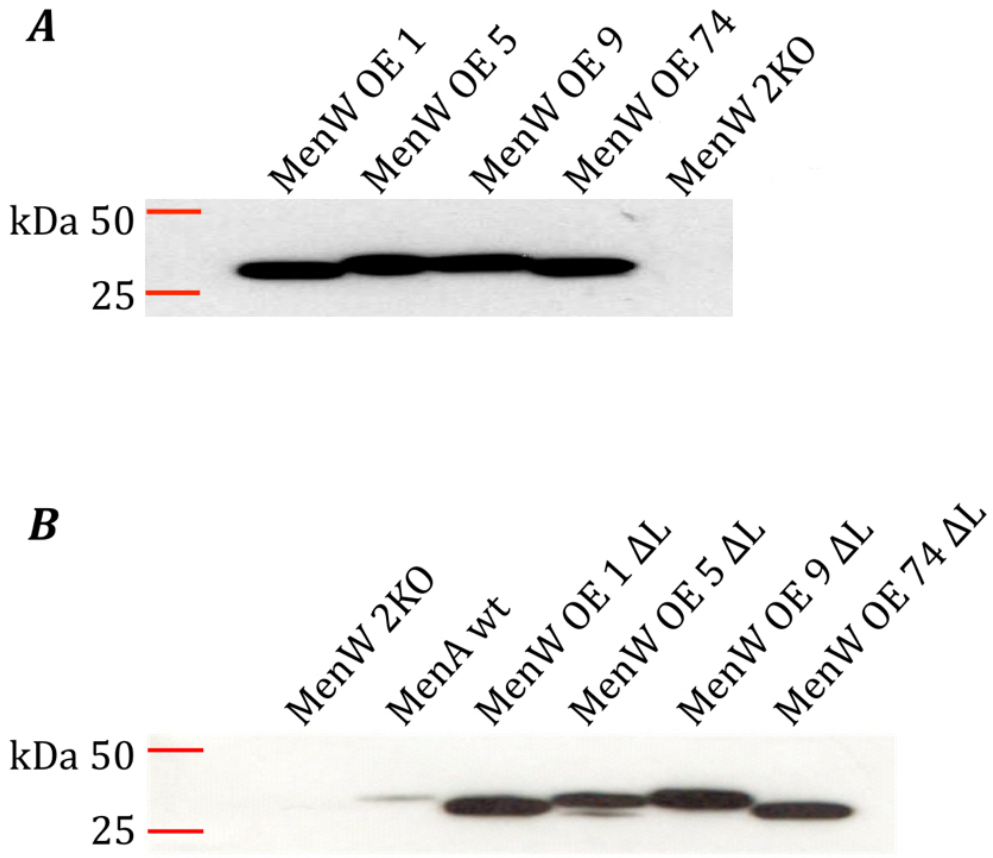


Figure 3.8. Western Blot to detect fHbp v.1 in NOMV.

A) Samples order from left to right: NOMV from MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74, and MenW 2KO. 0.5 µg of NOMV from MenA wt were loaded; 0.2 µg of NOMV from the other strains were loaded. **B)** Samples order from left to right: NOMV from MenW 2KO, MenA wt, MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, MenW OE 74 ΔL. 0.2 µg of NOMV were loaded. Detecting antibody was a polyclonal mouse antibody raised against recombinant fHbp ID 1.

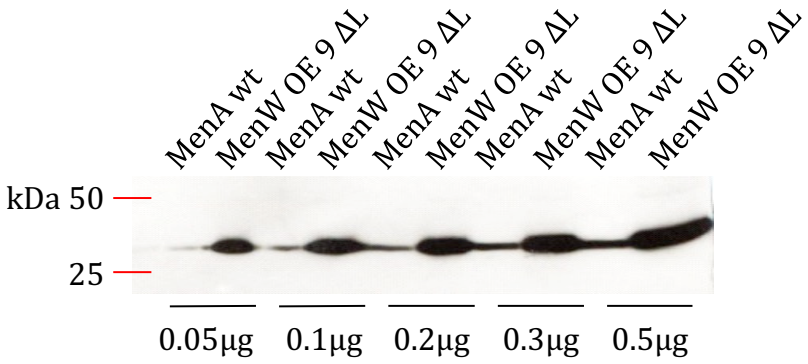


Figure 3.9. Western blot for comparison of fHbp presence in NOMV from a wild type strain MenA (expressing fHbp ID 5) and from mutant strain MenW OE 9 ΔL.

Different quantities of NOMV were compared, as indicated below figures. Detecting antibody was a polyclonal mouse antibody raised against recombinant fHbp ID 1.

Since the mutations we inserted, especially the *lpxL1* KO, can change the structure of the membrane and potentially affect cell integrity, we compared by Western blot the amount of fHbp in NOMV from meningococcal mutants grown at two different temperatures. Expression of fHbp at both temperatures was similar, suggesting that in the mutants the temperature does not affect fHbp expression, translocation to the outer membrane, and its presence in NOMV (Figure 3.10).

As *gna33* encodes a membrane structural component and its deletion affects membrane architecture [51], we wanted to investigate *gna33* KO impact on fHbp levels in vesicles, in particular in combination with *lpxL1* deletion: by Western blot we detected similar amount of fHbp in NOMV from MenW OE 5, in NOMV from MenW OE 5 ΔL, and in GMMA from MenW OE 5 ΔL Δ*gna33*. This suggests that *gna33* and *lpxL1* genes deletion do not have effect on the fHbp levels in the vesicles (Figure 3.11).

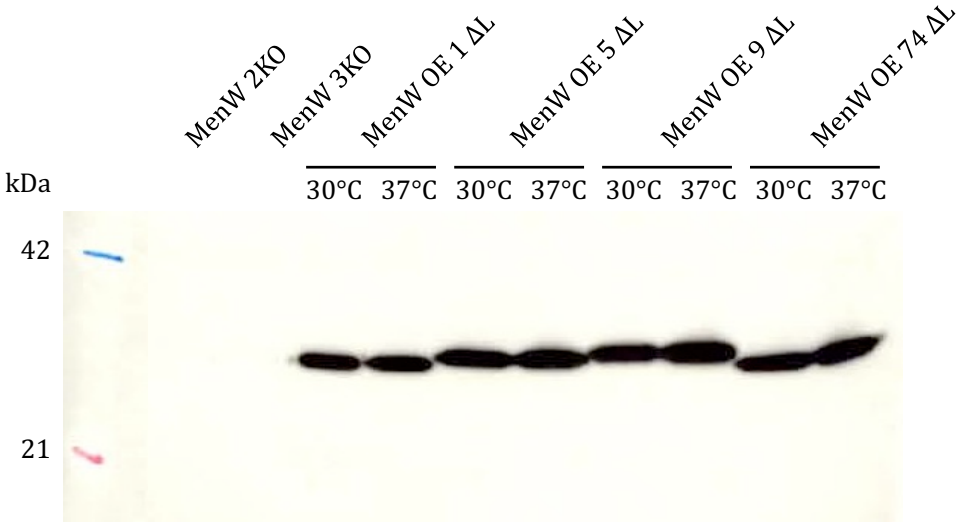


Figure 3.10. Western blot for comparison of fHbp presence in NOMV from mutants grown at either 30°C or 37°C.

As negative control, NOMV from MenW 2KO and MenW 3KO were also loaded. 0.3 µg of NOMV were loaded. Detecting antobody was a polyclonal mouse antibody raised against recombinant fHbp ID 1.

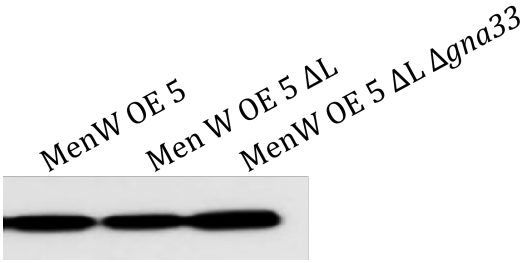


Figure 3.11. Comparison of fHbp levels in NOMV from different mutants.

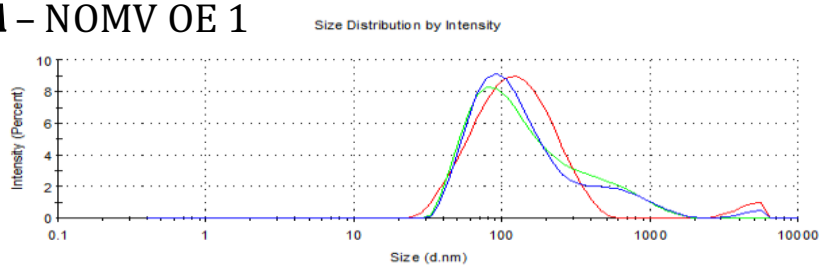
Samples order from left to right: NOMV from MenW OE 5, NOMV from MenW OE 5 ΔL, GMMA from MenW OE 5 ΔL Δ*gna33*. 0.3 µg of NOMV or GMMA were loaded. Detecting antobody was a polyclonal mouse antibody raised against recombinant fHbp ID 1.

3.3.2. SIZE AND MORPHOLOGY OF NOMV

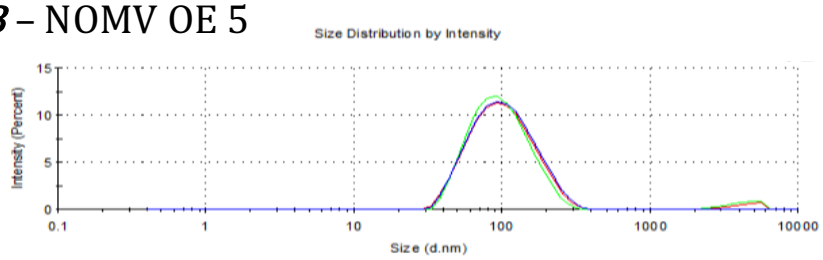
The NOMV from strains MenW OE 1, MenW OE 5, MenW OE 9, MenW OE 74, and MenW 2KO were analyzed by dynamic light scattering (DLS) (Figure 3.12) and by HPLC-SEC/MALS (Figure 3.13), to characterize the average size of the particles. In DLS profiles, the size distribution of all samples showed a single peak around 100 nm, suggesting that each NOMV preparation analyzed cointained a population of vesicles homogeneous in size. The second peak, observed in some samples around 1000 nm, is presumibly a technical artefact, due to dust particles that can be present, without interfering with the analysis, in the nanometer range of the samples. Homogeneicity in size was also evident from HPLC-SEC/MALS profiles, that showed a well characterized single peak, representing a single vescicles population with defined radius. Beside the similar size distribution observed in all the NOMV preparations, there was also good agreement between the values acquired with HPLC-SEC/MALS (radius 53-57 nm) and DLS (diameter 84-109 nm) (Table 3.2).

After the introduction of the *lpxL1* KO mutation, we investigated whether this modification had an impact on vesicles size. Therefore, we analysed by DLS and HPLC-SEC/MALS NOMV from MenW OE 5 ΔL (Figure 3.14). In this case, DLS did not show a single peak, but suggested instead a more heterogeneous distribution of particles size (Figure 3.14 A). Moreover, particle size was around 200 nm in diameter, double that of NOMV from *lpxL1* wild type strains analysed. Again, we found correspondence between analysis by DLS and HPLC-SEC/MALS.

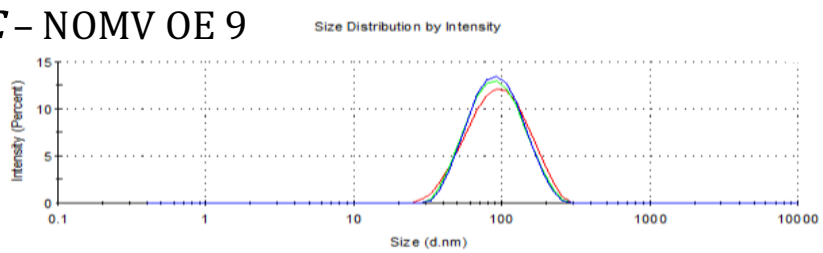
A – NOMV OE 1



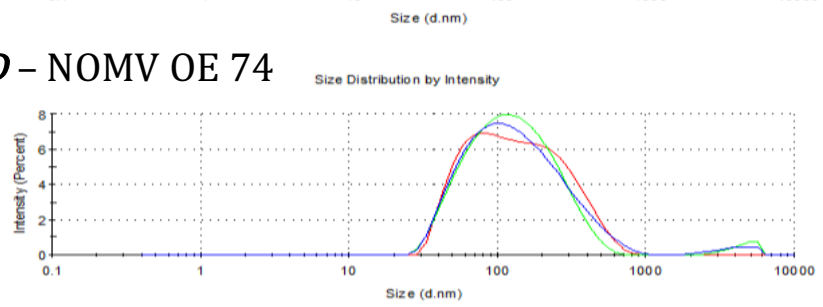
B – NOMV OE 5



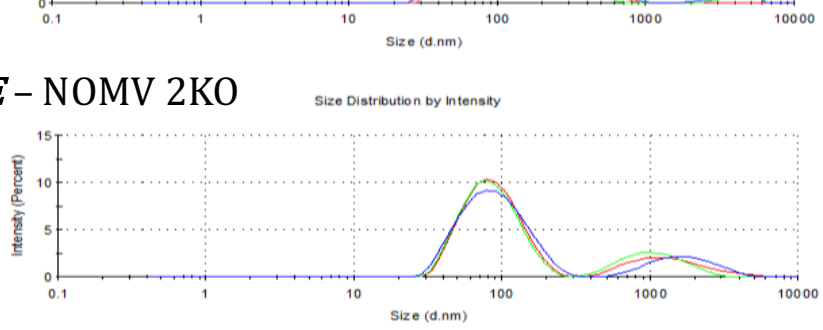
C – NOMV OE 9



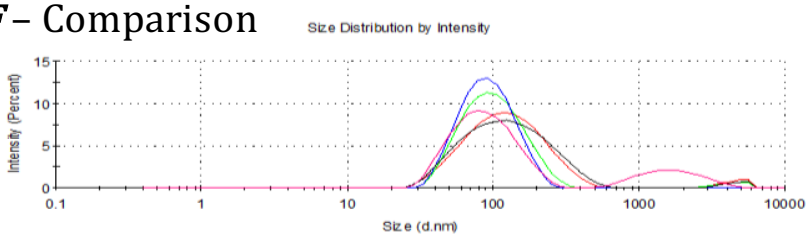
D – NOMV OE 74



E – NOMV 2KO



F – Comparison



MenW OE 1
MenW OE 5
MenW OE 9
MenW OE 74
MenW 2KO

Figure 3.12. Dynamic light scattering (DLS) profiles of NOMV (run in triplicate) and their overlay.

A-E) NOMV from strains: **A)** MenW OE 1; **B)** MenW OE 5; **C)** MenW OE 9; **D)** MenW OE 74; **E)** MenW 2KO. Each curve of a different colour represents one run. **F)** Overlay of the average profile of all samples; colour legend on side.

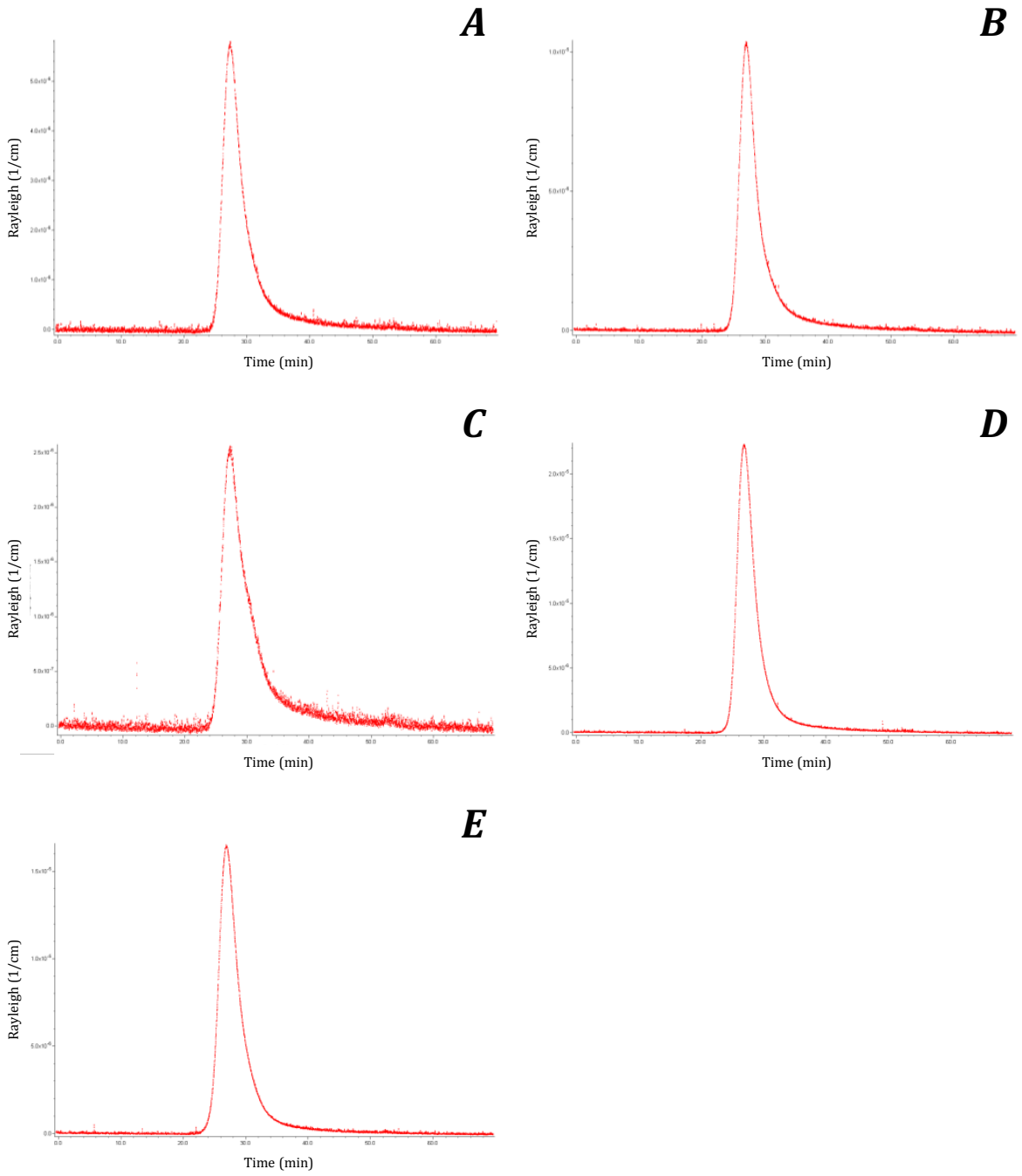
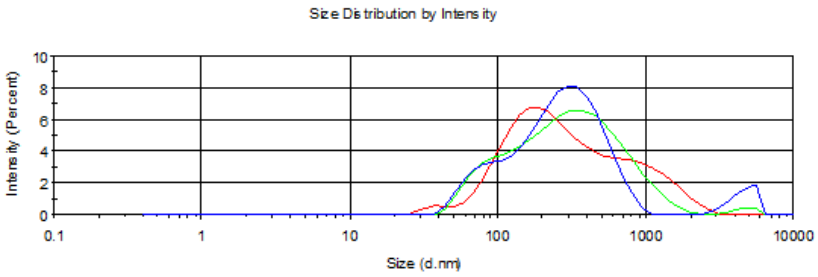


Figure 3.13. HPLC-SEC MALS profiles of NOMV. NOMV from strains: A) MenW OE 1; B) MenW OE 5; C) MenW OE 9; D) MenW OE 74; E) MenW 2KO.

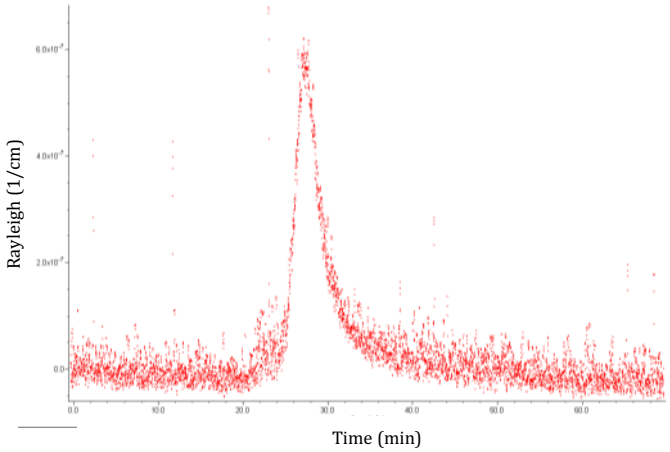
Table 3.2. NOMV characterization by HPLC-SEC/MALS (radius in nm) and DLS (diameter in nm and polidispersity).

NOMV from strain	MALS	dls
	Rw nm	Z-av (d. nm) (PdI)
MenW OE 1	57.3	108.7 (0.331)
MenW OE 5	53.6	91.84 (0.208)
MenW OE 9	52.8	83.36 (0.145)
MenW OE 73	56	106.9 (0.297)
MenW 2KO	50.9	95.32 (0.363)

A



B



C

MALS	dls
Rw nm	Z-av (d. nm) (PdI)
92.9	213.3 (0.432)

Figure 3.14. Size average determination of NOMV from MenW OE 5 ΔL.

A) DLS profile of sample, run in triplicate. **B)** HPLC-SEC/MALS profile. **C)** Table with analysis details**.**

In addition to size characterization by DLS and HPLC-SEC/MALS, we observed integrity and morphology of NOMV by transmission electron microscopy (TEM) (Figure 3.15). We analysed preparations from strains *lpxL1* KO: MenW 3 KO, MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, and MenW OE 74 ΔL; we also included NOMV from MenW wild type as control. As indicated by analytical investigation, we did not find homogeneity in the size of these NOMV preparations, except for NOMV from MenW wild type. In particular, images showed 2 distinct size populations: bigger NOMV around 100 nm in diameter (in line with size characterization of NOMV from *lpxL1* wild type strains), and smaller vesicles around 30-50 nm in diameter. Size of NOMV as observed by TEM micrographs do not correspond with values detected by DLS and HPLC-SEC/MALS.

To visualize NOMV formation, we decided to observe by TEM meningococcal mutants fixed during late exponential phase. We prepared samples from the different *lpxL1* KO strains, but because of technical issues we were able to obtain images only of MenW OE 74 ΔL. In Figure 3.16, MenW OE 74 ΔL diplococcus is clearly visible, and blebbing from the outer membrane is observed.

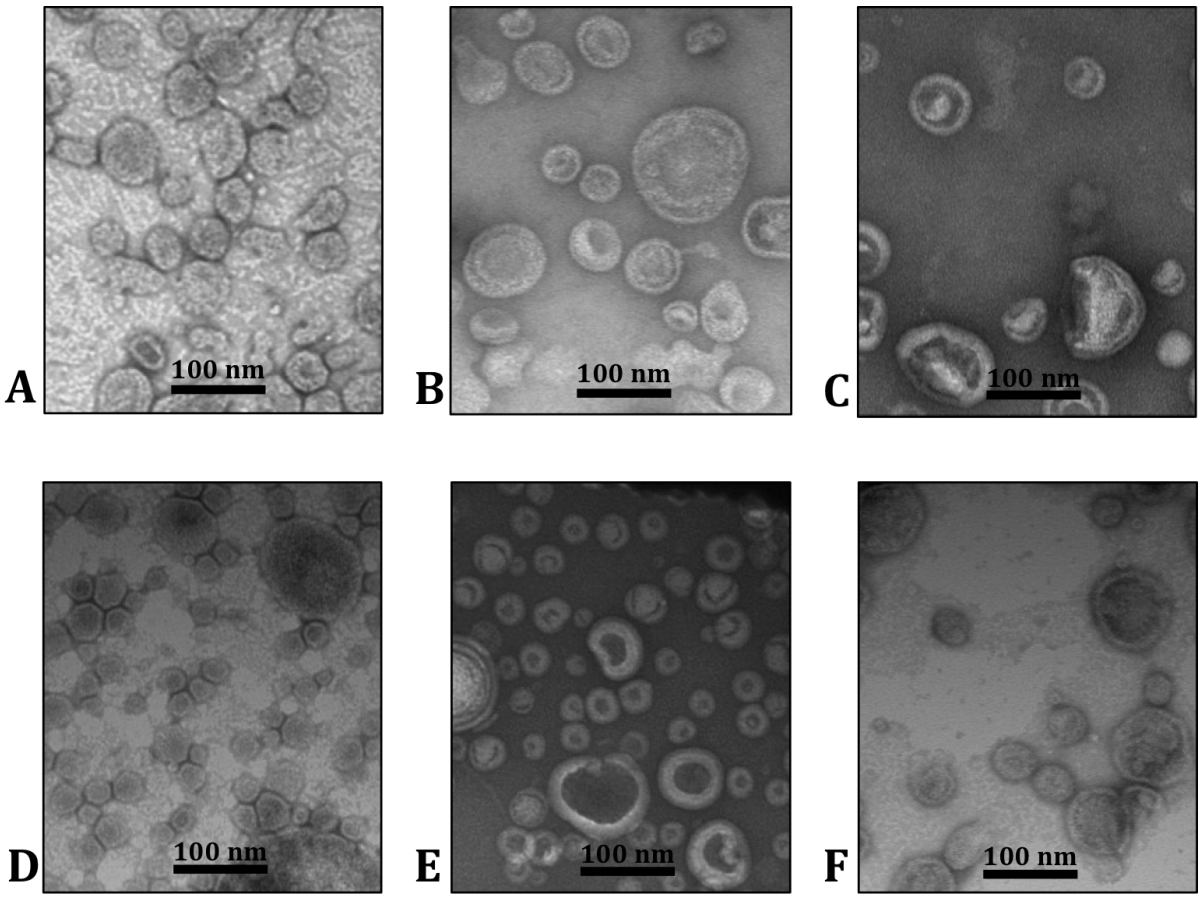


Figure 3.15. Negatively stained transmission electron microscopy of NOMV.

NOMV from: **A)** MenW wild type; **B)** MenW 3KO; **C)** MenW OE 1 ΔL; **D)** MenW OE 5 ΔL; **E)** MenW OE 9 ΔL; **F)** MenW OE 74 ΔL. 105,000x magnification; the length of the bar in the micrographs is 100 nm.

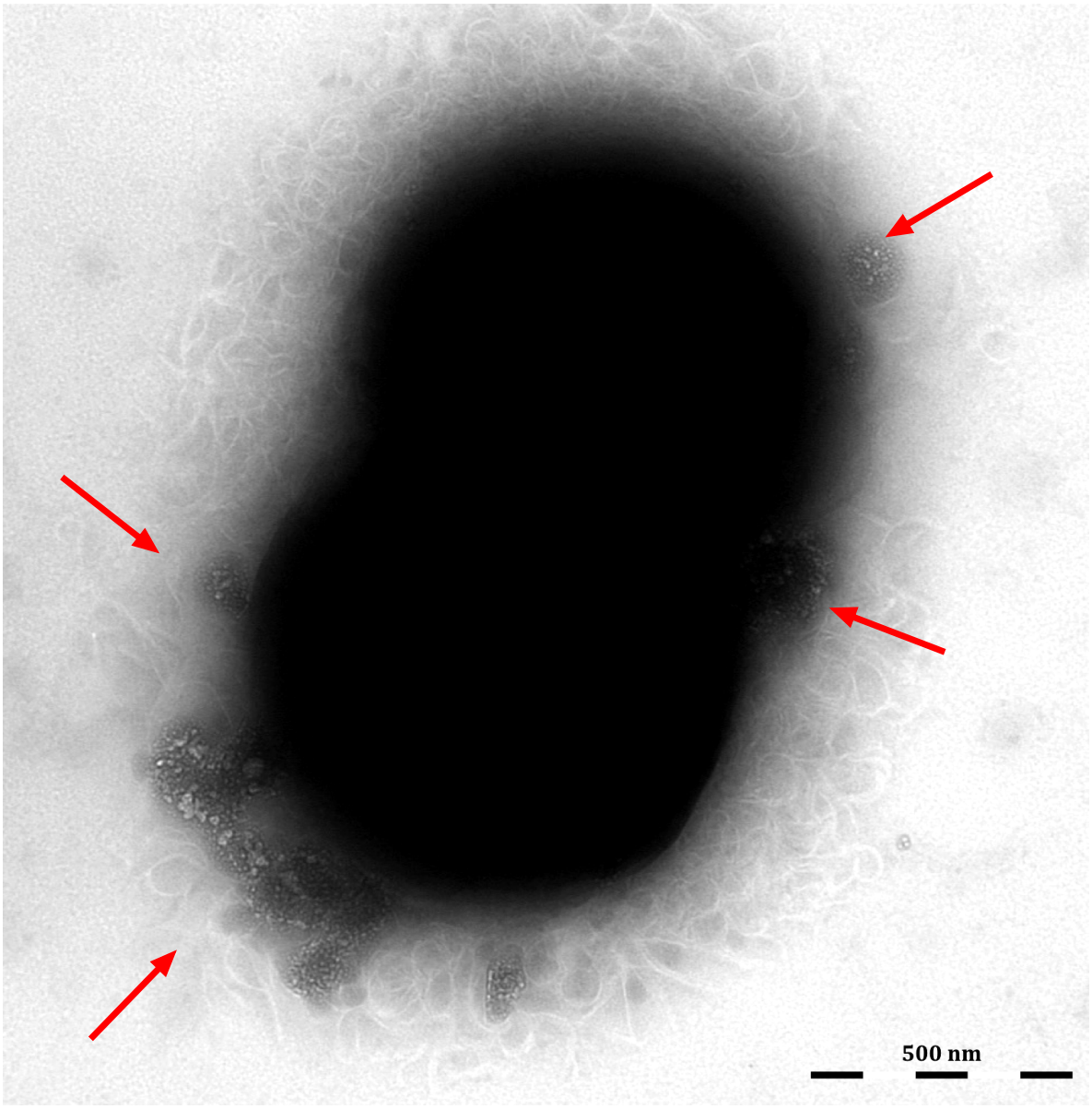


Figure 3.16. Negatively stained transmission electron microscopy of *N. meningitidis* MenW OE 74 ΔL.

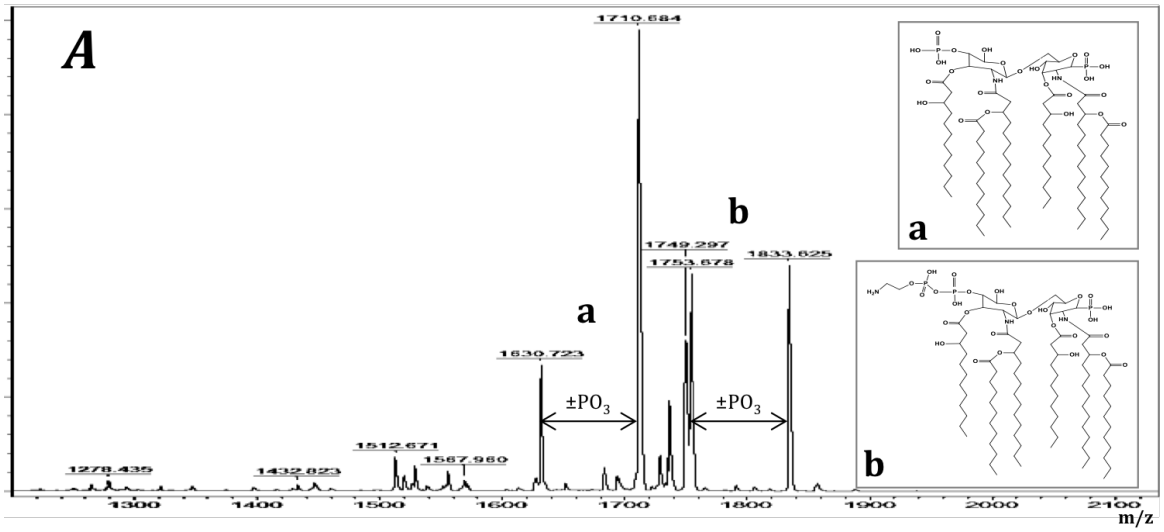
Red arrows indicate blebbing of vesicles. Length of the bar 500 nm.

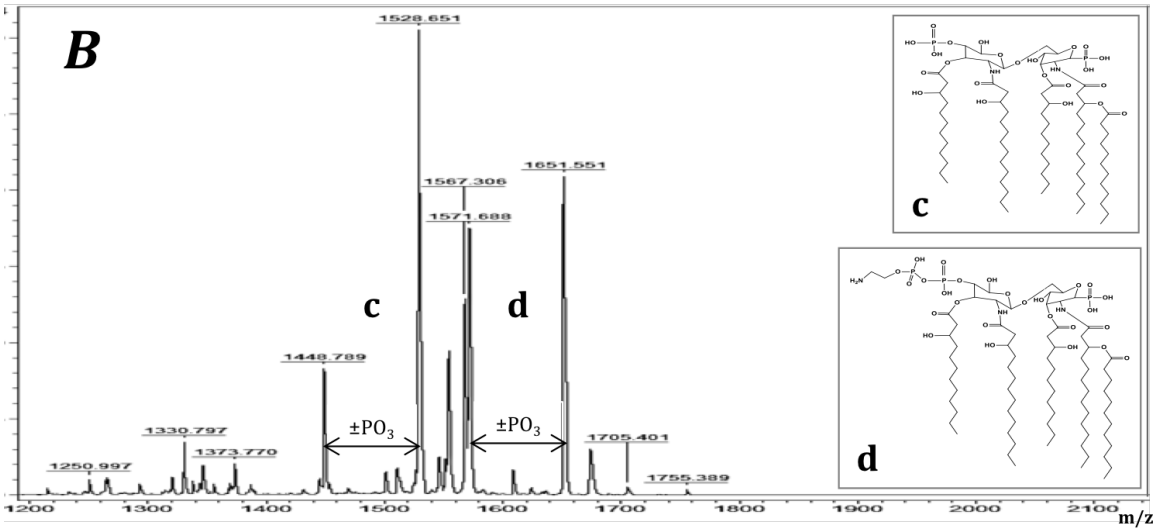
3.3.3. LIPID A ANALYSIS BY MALDI-TOF

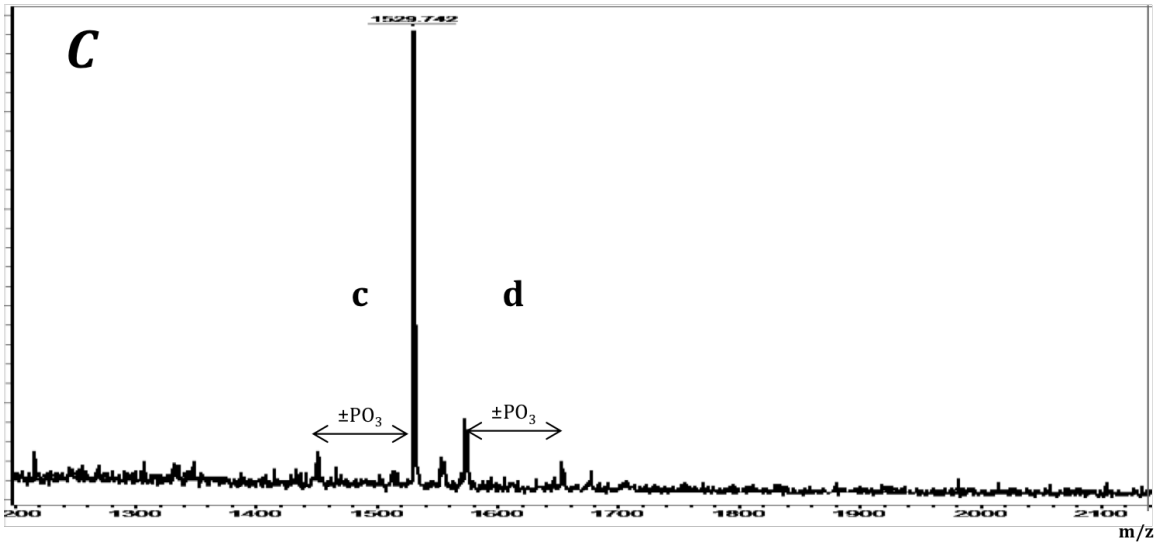
To decrease the endotoxicity of NOMV, we deleted the *lpxL1* gene in each of the four MenW strains over-expressing $fHbp_{R41S}$ v.1. Lacking this gene, meningococcus LOS has penta-acylated lipid A, instead of the wild type hexa-acylated form. This modification is associated with a decreased reactogenicity of LOS, compared to the wild type LOS [53, 68], as tested by *in vitro* interleukin-6 release from cells stimulated with NOMV.

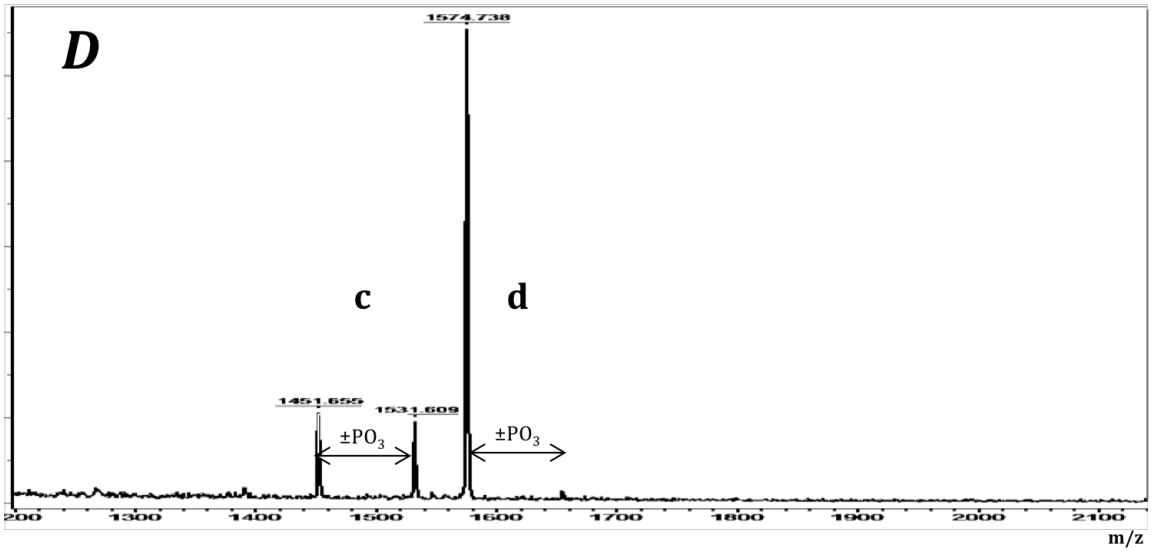
To confirm the penta-acylated structure of lipid A of the *lpxL1* KO strains, lipid A was extracted by acid hydrolysis as described in Chapter 2, from NOMV of these strains, and also of a *lpxL1* wild type strain (MenW 2KO) for control. Lipid A from these mutants was analysed by MALDI-TOF (spectra obtained are shown in Figure 3.17). The main peaks in the mass spectra of lipid A of NOMV from non-detoxified meningococcus (Figure 3.17A) corresponded to the theoretical mass of the hexa-acylated lipid A. In particular, we can observe four main peaks: these represent the hexa-acylated lipid A with (1837 Da, Figure 3.17 A, b) or without (1714 Da, Figure 3.17 A, a) the phosphoethanolamine chain, and both structures are present as double peaks, corresponding to the lipid A with or without the phosphate group. Lack of phosphoethanolamine and phosphate group in the MALDI-TOF spectra can be due to either the real presence of lipid A species without these groups, or to technical reasons, as phosphoethanolamine and phosphate can be lost during analysis. Compared with spectra of wild type lipid A, the main peaks in the mass spectra of lipid A extracted from NOMV from *lpxL1* KO strains (Figure 3.17 B-F) were all shifted to a lower molecular weight by 182 Da, the difference corresponding to the mass

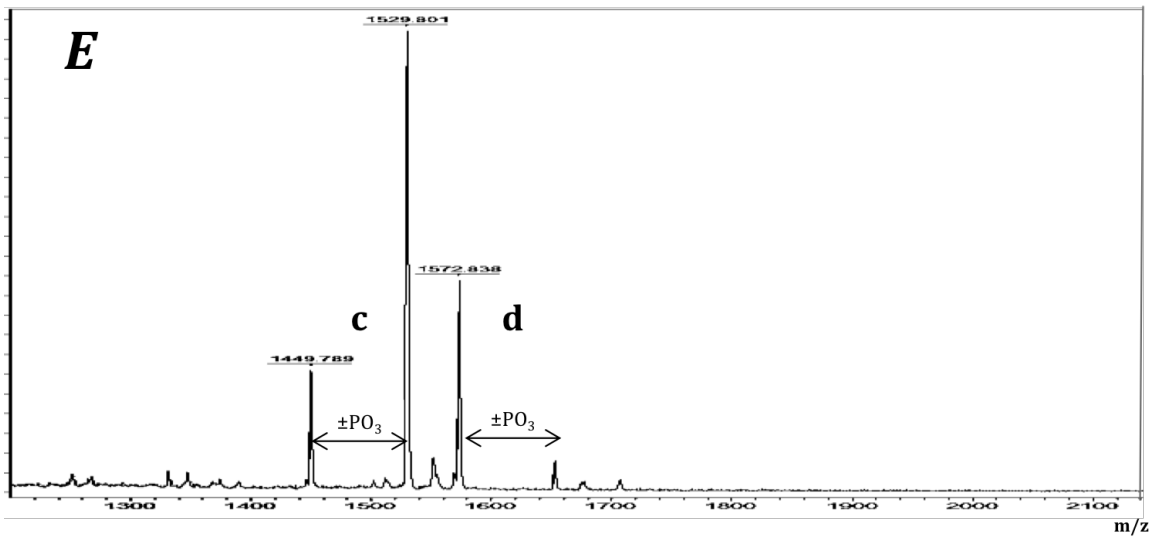
of the missing lauroyl (C12) chain. These results confirmed that the meningococcus *lpxL1* KO mutants express only penta-acylated lipid A species, as the lipid A species of highest molecular weight.


A
1710.684
1749.297
1753.678
1833.625
1630.723
1512.671
1567.960
1278.435
1432.823
a
b
±PO3
±PO3
1300
1400
1500
1600
1700
1800
1900
2000
2100
m/z


B
1528.651
1567.306
1571.688
1651.551
1448.789
1330.797
1373.770
1250.997
1705.401
1755.389
c
d
±PO3
±PO3
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
m/z


C
1529.742
c
d
±PO3
±PO3
200
1300
1400
1500
1600
1700
1800
1900
2000
2100
m/z


D
1574.738
c
d
1451.655
1531.609
±PO3
±PO3
200
1300
1400
1500
1600
1700
1800
1900
2000
2100
m/z


E
1529.801
1572.838
c
d
1449.789
±PO3
±PO3
1300
1400
1500
1600
1700
1800
1900
2000
2100
m/z

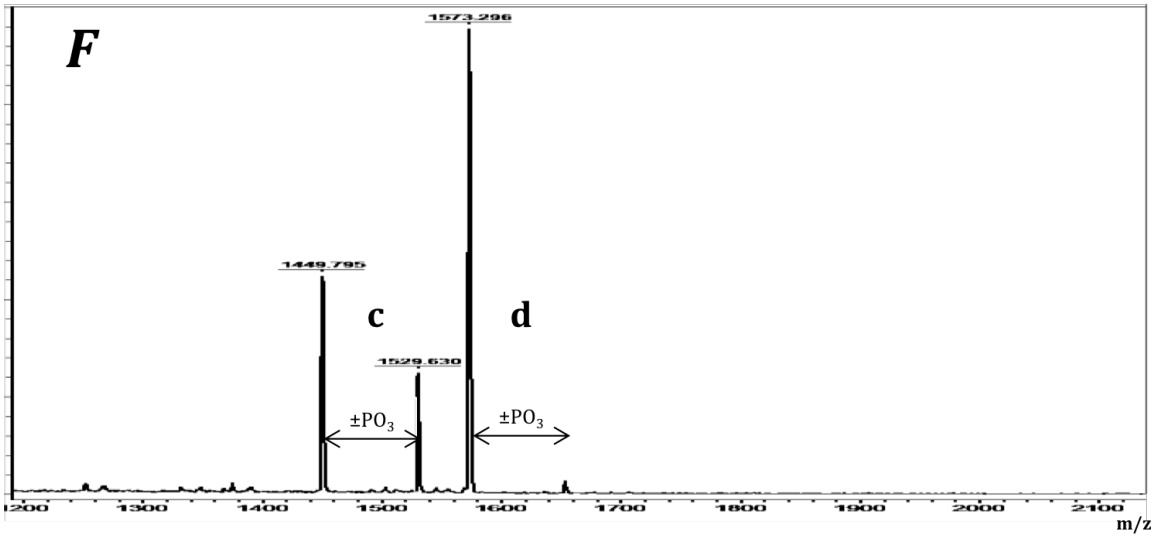


Figure 3.17. MALDI-TOF spectra obtained analysing lipid A extracted from NOMV, in reflection ion negative mode.

Analysis of lipid A extracted from NOMV of: **A)** MenW *cps* KO; **B)** MenW 3KO; **C)** MenW OE 1 ΔL; **D)** MenW OE 5 ΔL; **E)** MenW OE 9 ΔL; **F)** MenW OE 74. In **A)** and **B)** in the boxes are represented the chemical structures of the predicted hexa-acylated (a and b) and penta-acylated (c and d) lipid A, with or without the phosphoethanolamine chain.

3.3.4. *In vitro analysis of reactogenicity of NOMV with attenuated LOS*

To assess the reactogenicity of NOMV from *lpxL1* KO mutants, we analysed the release of cytokine interleukin 6 (IL-6) by human PBMC, induced by stimulation with NOMV from strains with wild type and mutated lipid A. To dissect the effectors of the overall reactogenicity of NOMV, we than analysed the specific activation of two different human Toll-like receptors (TLRs): TLR4, known to bind to LPS of Gram-negative bacteria and to lipoteichoic acids of Gram-positive bacteria, and TLR2, which has several ligands, including lipoproteins [63, 201, 218]. For analysing TLRs activation, Human Embryonic Kidney cells (HEK293) (naturally lacking these receptors) were stably transfected to express only either TLR4 (with co-stimulatory molecules MD2 and CD14) or TLR2, and a luciferase report gene induced by NF-kB. In this way, the activation of the TLR signalling cascade results, through activation of NF-kB, in the expression of the luciferase reporter gene. The activation of the specific TLR was assessed with a luminometer measuring luciferase activity.

3.3.4.1. *Analysis of IL-6 release by human PBMCs after incubation with NOMV*

We stimulated human peripheral mononuclear blood cells (PBMC) for four hours with serial 10-fold dilutions of NOMV concentrations (from 10^{-4} to 10^{3} ng/mL) purified from meningococcus mutants either carrying the *lpxL1* KO mutation or not. We tested NOMV from MenW 3KO, MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, and MenW OE 74 ΔL. As controls we included NOMV from MenW *cps* KO and MenW 2KO, expressing the wild type hexa-acylated lipid A. MenW *cps* KO was used, for safety reasons, as representative of the wild type strain, as the only mutation is the abolition of the capsule. We analysed the release of IL-6 from these cells by ELISA, as described in Chapter 2.

Around 10^{-2} ng/mL of NOMV with wild-type lipid A resulted in the release of IL-6 at a concentration 10-fold over background; the same concentration of IL-6 release was induced by around 10^{0} ng/mL of NOMV with modified lipid A (Figure 3.18). Such difference indicates that NOMV from *lpxL1* KO strains have 100-fold lower ability to stimulate IL-6 release, compared to NOMV from *lpxL1* wild type strains.

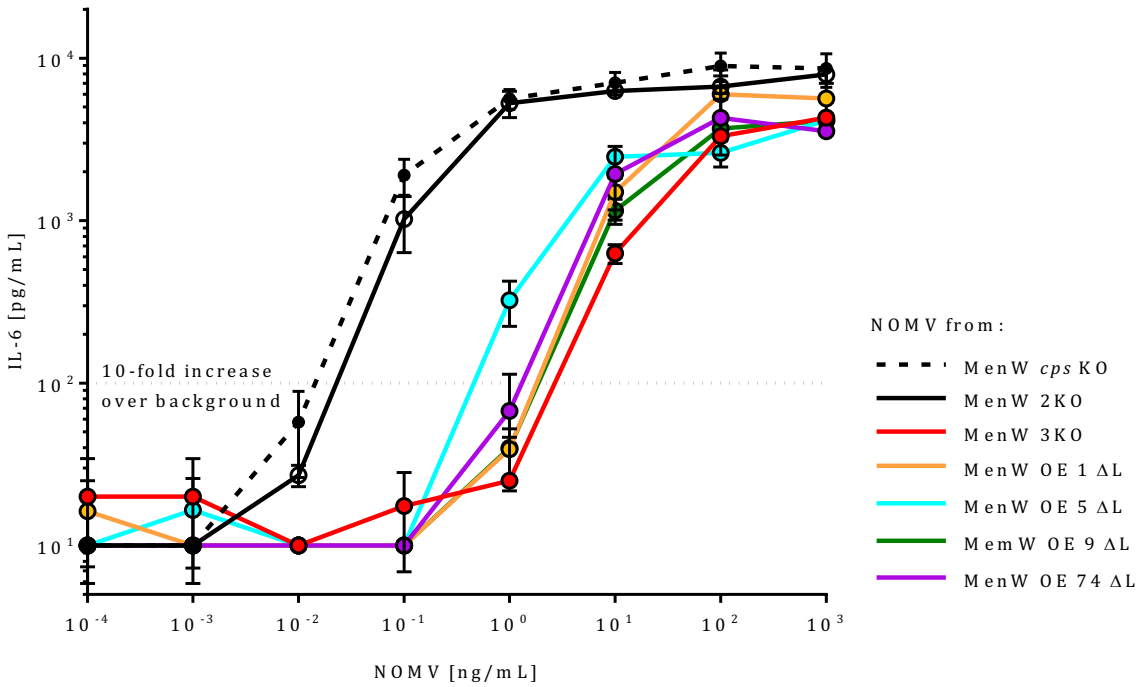


Figure 3.18. Cytokine release by human PBMC after stimulation with 10-fold serial dilution of NOMV concentration, as measured by ELISA.

Human PBMC were isolated using a Ficoll density gradient and 200,000 cells/well were added in a 96 well plate; PBMC were stimulated for 4 hours with 10-fold serial dilution of NOMV concentrations, from 10^{3} to 10^{-4} ng/mL; supernatants were recovered and IL-6 released was measured by ELISA. Each sample was tested in triplicate; data are shown as mean with SD (vertical bars).

3.3.4.2. Analysis of stimulation of TLR4 and TLR2 by NF-kB luciferase reporter assay, after stimulation with NOMV

HEK293 stably transfected to express only either TLR4 (with co-stimulatory molecules MD2, and CD14), or TLR2, both in combination with a luciferase report gene induced by NF-kB, were stimulated with serial 10-fold dilutions of NOMV concentrations (from 10^3 to 10^{-4} ng/mL) purified from meningococcus mutants either carrying the *lpxL1* KO mutation or not. We tested NOMV from MenW 3KO, MenW OE 1 ΔL, MenW OE 5 ΔL, MenW OE 9 ΔL, and MenW OE 74 ΔL. As controls we included NOMV from MenW *cps* KO and MenW 2KO, expressing the wild type hexa-acylated lipid A. Luciferase activity, related to the activation of the specific TLR, was measured with a luminometer, as described in Chapter 2.

10^{-2}-10^{-1} ng/mL of NOMV with wild type lipid A induced TLR4 activation; in contrast, 10^1-10^2 ng/mL of NOMV with modified lipid A were required for activation (Figure 3.19A). Such difference indicates that NOMV from *lpxL1* KO strains have 1000-fold lower ability to stimulate TLR4 activation, compared to NOMV from *lpxL1* wild type strains.

TLR2 was activated by all NOMV at a concentration between 10 and 10^1 ng/mL (Figure 3.19B), without differences related to any of the mutation inserted. This suggests that the modifications we introduced in these vesicles, including *lpxL1* KO and fHbp OE, do not impact on the interaction between NOMV and TLR2.

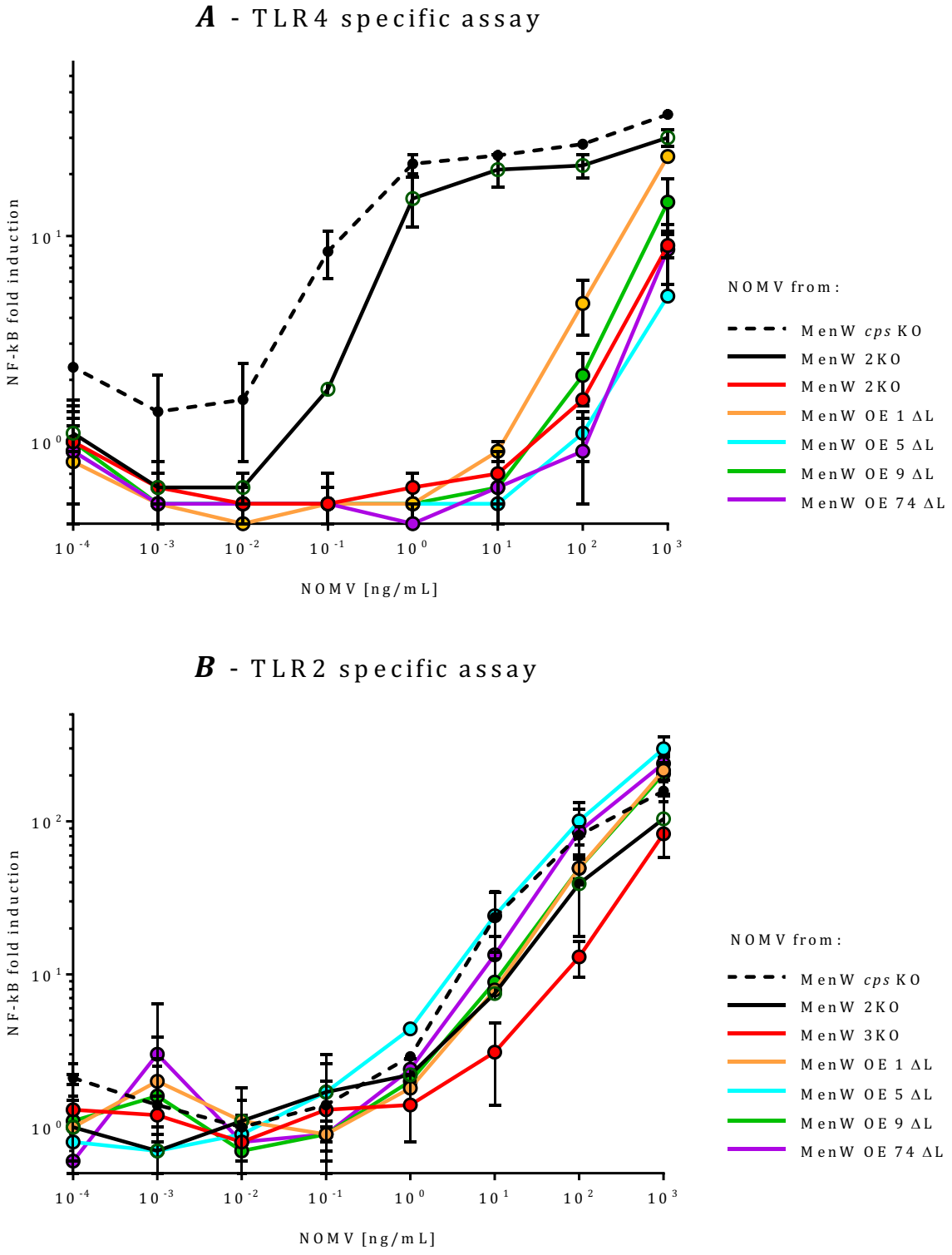


Figure 3.19. NF-kB luciferase report assay for TLR4 and TLR2 activation after stimulation with 10-fold serial dilution of NOMV concentration.

A) TLR4 specific assay: 25,000 cells/well of HEK293 TLR4, MD2, CD14 were stimulated for five hours with 10-fold serial dilutions of NOMV concentrations, from 10^{3} to 10^{-4} ng/mL. NF-kB activation measured as described in Chapter 2. Each sample was tested in triplicate; data are shown as mean with SD (vertical bars). **B)** TLR2 specific assay: 25,000 cells/well of HEK293 TLR2 were stimulated for five hours with 10-fold serial dilutions of NOMV concentrations, from 10^{3} to 10^{-4} ng/mL. NF-kB activation measured as described in Chapter 2. Each sample was tested in triplicate; data are shown as mean with SD (vertical bars).

3.4. Discussion

A broadly protective vaccine against *N. meningitidis* is needed in Sub-Saharan Africa. Native, non-detergent extracted outer membrane vesicles from *N. meningitidis* engineered to over-express fHbp variant 1, as the main cross-protective antigen, represent a valid approach for a vaccine that protects against all the serogroups present in Africa [53].

Current variation among fHbp carried by meningococcal strains circulating in Africa is limited, but new IDs may emerge in the future, especially after introduction of new vaccines. Therefore, in this study we searched for a rational approach to identify the fHbp v.1 ID type, capable of providing the broadest cross-reaction possible against meningococcal strains, when over-expressed on NOMV. We used a novel approach to examine fHbp sequence, to further relate its variability to the cross-reaction induced by NOMV OE fHbp. In the past, this relationship was investigated for recombinant fHbp: bactericidal activity induced by the protein was observed to decrease while the genetic distance between fHbp sub-variant in the vaccine and on the strain tested increased [308]. However, we postulated that analysing the entire fHbp sequence, and its variability among the different IDs, would not generate adequate information for our investigation: with such analysis, variations at positions not necessarily immunologically relevant are taken into consideration. Without excluding that other portions of the protein, and their variations, can be relevant, we chose to focus on the surface-exposed part of fHbp involved in factor H (fH) binding.

We recognized as the fH-binding portion on fHbp molecule those residues distant no more than 5 Å from bound fH, and obtained a 48 amino acids sequence. This result

includes all the fHbp residues that make hydrogen bonds with fH, and 1 of the 2 residues indicated to be involved in salt-bridges with fH [122]; it also includes 28 amino acids not previously reported, that we considered nevertheless important for the interaction between the two molecules, because of their proximity to fH.

Compared to the entire protein sequence, for which 87-100% of amino acids site identity was observed among variant 1 peptides [309], a higher degree of variability in the part of fHbp involved in binding factor H was observed in our study: only 37.5% of the residues were identical in all the 383 v.1 IDs analysed. This is likely to indicate the immune selection that influences this portion of fHbp and the relevance of the epitopes underling in these residues. Therefore, our hypothesis was that a fHbp v.1 peptide carrying the most shared fH-binding sequence, will provide broader cross-reactivity against meningococcal strains with different fHbp variant 1 IDs, compared to that induced by fHbp IDs with less common fH-binding sequences. This hypothesis is tested in the following chapter (Chapter 4, page 149).

On the basis of both epidemiological data and fH-binding site analysis, ID 5, ID 9, and ID 74 were chosen for over-expression on NOMV; ID 1 was also included, for comparison with previous studies [53].

Native, non-detergent extracted, outer membrane vesicles (NOMV) were produced from meningococcal mutants over-expressing one of the four fHbp ID types selected. SDS-PAGE protein profiles of the different NOMV preparations showed homogeneity among them, and consistency with the protein pattern of NOMV from the wild type strain, suggesting that the genetic modifications inserted did not alter the overall protein

content of meningococcal outer membrane vesicles. By Western blot, we observed similar level of fHbp in NOMV from all the preparations, confirming the reliability of the over-expression system used. Because fHbp is an outer membrane lipoprotein, it was possible that mutations that could impact membrane stability, such as *lpxL1* and *gna33* KO, could alter its presence in the vesicles. Nevertheless, NOMV from MenW OE 5, NOMV from MenW OE 5 ΔL, and GMMA from MenW OE 5 ΔL Δ*gna33* showed all similar amounts of fHbp, as detected by Western blot. This observation is of good relevance for further vaccine development, indicating that the genetic background of vaccine-producing meningococcal mutants does not negatively impact the presence of the major cross-protective antigen.

While studies on GMMA from *Shigella sonnei* and *Shigella flexneri*, found that lowering temperature for bacteria growth from 37°C to 30°C had a positive impact on GMMA production, and it was a specific requirement with some mutants [182], we did not observe such differences with our meningococcal NOMV, and all the mutants tested were able to grow at both temperatures. Moreover, growing meningococcal NOMV-producing strains at either 30°C or 37°C did not make any difference in fHbp levels, as detected by Western blot, suggesting stability of the protein over-expression. This marks an important difference between the over-expression system used in this study and the regulation of the native fHbp gene expression: the latter is temperature-regulated, being activated at 37°C, but downregulated at 30°C [310]. From a manufacturing point of view, such difference can be relevant and advantageous, because unwanted temperature alterations that might happen if problems occurred during the production process, would not impact the level of one of the major antigens in the NOMV purified.

The appearance and size of the vesicle preparations did not differ from the homogenous, 80-90 nm in diameter, non-detergent extracted, meningococcal outer membrane vesicles, that have been previously observed [72, 311]. Nevertheless, we noted NOMV OE 5 ΔL were bigger compared to NOMV from *lpxL1* wild type mutants, and had a more heterogeneous size distribution, with two distinct size populations observed. Dual-size population was also observed with GMMA from *Shigella sonnei,* and different size of the vesicles did not have any particular relevance for further application, since, however, still in the optimal range for optimal vaccine design [312]. However, particles size deducted from TEM images were smaller compared to what was detected by DLS and HPLC-SEC/MALS. A possible explanation for the difference observed with the different techniques could be aggregation of the vesicles, which are therefore recorded as bigger by light scattering. Further investigation that also takes in account NOMV samples from the other *lpxL1* KO meningococcal mutants would be advantageous: direct comparison of analytical size determination and TEM imaging for each of the different NOMV preparations, would definitively help the understanding of size distribution of these vesicles.

By MALDI-TOF analysis, we confirmed that the introduction of the *lpxL1* KO mutation resulted in the presence of only penta-acylated lipid A, consistent with what was previously reported for the lipid A of serogroup B meningococcus [68]. This modification allowed the production of NOMV which are around 100-fold less capable than NOMV from *lpxL1* wild type strains, to induce the release of IL-6 in PBMC. Such a decrease in pro-inflammatory cytokines induced is in line with what was observed for MenB whole

cells, when the *lpxL1* mutation was initially described [68], and slightly higher than what achieved with GMMA from a MenW similar mutant [53]. In the past it was shown that human Toll-like receptor 4 (TLR4), but not human TLR2, is activated by LPS [63], and the lower ability of *lpxL1* KO LPS in inducing pro-inflammatory cytokines release *in vitro* was associated with its the lower ability to activate TLR4 [313]. Here we confirmed the decreased ability to stimulate TLR4 of NOMV with modified lipid A, compared to NOMV with wild type lipid A, in line with previous observations [53]. However, not only TLR4, but also TLR2 was demonstrated to play an important role in pro-inflammatory cytokines release induced by GMMA from *Shigella* strains [182]; moreover, we over-expressed in NOMV fHbp, a lipoprotein, and TLR2 is recognized to respond to lipoproteins [201, 218]. Nevertheless, we did not observe different stimulation of this receptor by NOMV from the different mutants. This appears to exclude the possibility that the modifications we introduced in the outer membrane of the meningococcus alter the interaction between vesicles and TLR2.

The characterization described in this chapter, on NOMV from the different mutants generated, demonstrates the reproducibility and consistency of these vesicles preparations. These consistency and similarity allows a direct comparison of the immune response they induce in mice. These vesicle preparations are indeed investigated for specific immunological aspects in the next chapters.

CHAPTER 4. CROSS-REACTIVITY OF ANTIBODIES INDUCED IN MICE BY NOMV WITH OVER-EXPRESSED DIFFERENT FHBP VARIANT 1 PEPTIDES

4.1. Introduction

NOMV have been reported to induce bactericidal antibodies with broader cross-reactivity than those induced by recombinant fHbp [53, 135]. Studies have shown that variations of the sequence of the recombinant protein, even within a variant, induce antibodies with different specificity. Nevertheless, the contribution of the fHbp sequence over-expressed on NOMV to the cross-reactivity of the bactericidal response induced has not been investigated so far. As discussed in the previous chapter, we hypothesized that amino acid in the surface-exposed portions of fHbp have a major impact on the cross-binding breadth of anti-fHbp antibody generated, and, in particular, we focused our attention on the portion of the protein involved in the interaction with factor H. Among fHbp v.1 peptides, an ID carrying the most shared fH-binding sequence may provide a broader cross-reactivity against meningococcal strains with different fHbp variant 1, compared to IDs with a fH-binding sequence less common, and to test this hypothesis, the antibody response elicited in mice by NOMV presenting diverse fHbp variant 1 sequences was compared. Mice were immunized with the NOMV preparations previously purified and characterized as described in Chapter 3: NOMV from MenW OE 1 (NOMV OE 1), NOMV from MenW OE 5 (NOMV OE 5), NOMV from MenW OE 9 (NOMV OE 9), NOMV

from MenW OE 74 (NOMV OE 74), and NOMV from MenW 2KO (NOMV fHbp KO) as control. To better discern the specific contribution of fHbp to the antibody response generated, and to directly compare the different responses elicited by fHbp either over-expressed on NOMV or synthesized as recombinant protein, mice were also immunized with recombinant fHbp ID 1, ID 5, ID 9, and ID 74; all fHbp sequences contained the substitution of Arginine to Serine at position 41, known to decrease binding of fH [314]. Immunization was performed IP, twice (4 weeks apart), with either 1 μg or 5 μg of NOMV OE fHbp, or 5 μg of NOMV 2KO, or 20 μg of recombinant fHbp, as described in Chapter 2. Even though in a previous study [53] 1 or 5 μg of NOMV OE fHbp induce no statistically significant different anti-fHbp IgG responses, we tested both the doses, to appreciate possible differences due to the different fHbp sub-variant over-expressed; on the other hand, only the higher dose of 5 μg was used for immunization with NOMV fHbp KO, to enhance the negative control; 20 μg of recombinant fHbp was the dose chosen to get the maximum response. Serum from blood was collected 2 and 4 weeks after the first dose, and 2 weeks after the second dose. The anti-fHbp antibody response of individual mice was evaluated in all the immunization groups, by measuring IgG ELISA units against specific antigens, as described in Chapter 2. For each immunization group, anti-fHbp IgG antibodies were determined against each of the four fHbp v.1 IDs used for immunization (i.e. ID 1, ID 5, ID 9, ID 74, all carrying the R41S substitution).

Serum bactericidal activity (SBA) elicited in individual mice from each immunization group was evaluated against a panel of seven meningococcal isolates (carrying each of the fHbp IDs used for immunization, Table 2.3, page 92), by SBA assay. SBA is considered the gold-standard for assessing the protection against meningococcal disease, when

analysing human sera [315-317], and it is normally used to evaluate the functionality of the antibody response elicited in mice by candidate vaccines.

4.2. Anti-fHbp IgG antibody response induced by NOMV OE fHbp and by recombinant fHbp

To investigate the specificity and cross-reactivity of the anti-fHbp IgG antibodies induced by NOMV OE the different fHbp sequences, IgG levels specific to each of the four proteins were measured in single sera from mice of all immunization groups, two and four weeks after the first dose, and two weeks after the second dose (Figure 4.1, Figure 4.2, and Figure 4.3).

NOMV fHbp KO induced no detectable, or very low (likely due to technical artefacts), anti-fHbp signal (Figure 4.1); in the other immunization groups significantly higher IgG levels two weeks after the second dose, compared to antibodies after the first dose, were detected ($p \leq 0.01$), suggesting an evident booster effect.

Against each ID, anti-fHbp IgG levels induced by 1 μg and 5 μg of NOMV OE the same fHbp were not significantly different; exceptions were anti-fHbp ID 1 antibodies induced by NOMV OE 5 and NOMV OE 9, and anti-fHbp ID 9 induced by NOMV OE 9, significantly higher in mice immunized with 1 μg of antigen, than 5. These observations suggest that under our experimental conditions, that maximum anti-fHbp antibody response is obtained with 1 μg of NOMV (Figure 4.2). Therefore, subsequent analyses were only performed with data from mice immunized with the lower dose of NOMV.

All the NOMV preparation induced geo mean anti-fHbp IgG levels of $>10^3$ ELISA units, (Figure 4.4), but differences were observed among the different immunization groups, and, within each, among the different antigens tested. NOMV OE 1 induced anti-fHbp IgG levels significantly higher when measured against homologous ID 1, than against the heterologous sub-variants; NOMV OE 5 induced IgG anti-fHbp ID 1, anti-fHbp ID 5, and anti-fHbp ID 9 that were not significantly different from each other, but were higher than IgG anti-fhbp ID 74 ($p < 0.01$); NOMV OE 9 and ID 74 induced no significant differences among IgG levels against the homologous fHbp, and fHbp ID 1.

Compared to mice immunized with NOMV OE fHbp, mice immunized with the recombinant fHbp had IgG antibodies levels significantly higher against the respective homologous fHbp IDs, than against the heterologous fHbp proteins. (Figure 4.4). Direct comparison of anti-fHbp IgG antibodies induced by NOMV OE fHbp and by recombinant fHbp, showed that the recombinant proteins induced equal or statistically significant higher ($p< 0.05$) antibody levels, compared to the corresponding NOMV, with the exception of anti-fHbp ID 1 IgG antibody level induced by recombinant fHbp ID 5 being lower than that induced by NOMV OE 5.

To investigate the specificity of anti-fHbp antibody elicited, we analysed whether there was a correlation between IgG levels against pairs of different fHbp IDs, within each immunization group (Figure 4.5). Linear correlation was observed for 18 out of 24 comparisons of mice groups immunized with NOMV OE fHbp, and for 20 out of 24 comparisons of mice groups immunized with recombinant fHbp. Correlation was not always reciprocal; for example, in mice immunized with NOMV OE 74, IgG antibodies against recombinant fHbp ID 74 and against recombinant fHbp ID 5 showed linear

correlation (Pearson $r = 0.76$; $p < 0.05$), while this was not true for mice immunized with NOMV OE 5, when tested against the same antigens (i.e. fHbp ID 5 and ID 74). Correlation between anti-fHbp IgG, within the same immunization group, against different antigens, was observed also in those cases where the antibody levels were significantly different; for example, among mice immunized with NOMV OE 1, linear correlation was present between all the combination of anti-fHbp IgG antibodies even though IgG levels against the homologous fHbp ID 1 were significantly higher than against the heterologous peptides. These data suggest that the different fHbp IDs induce antibodies with different specificities in targeting fHbp epitopes.

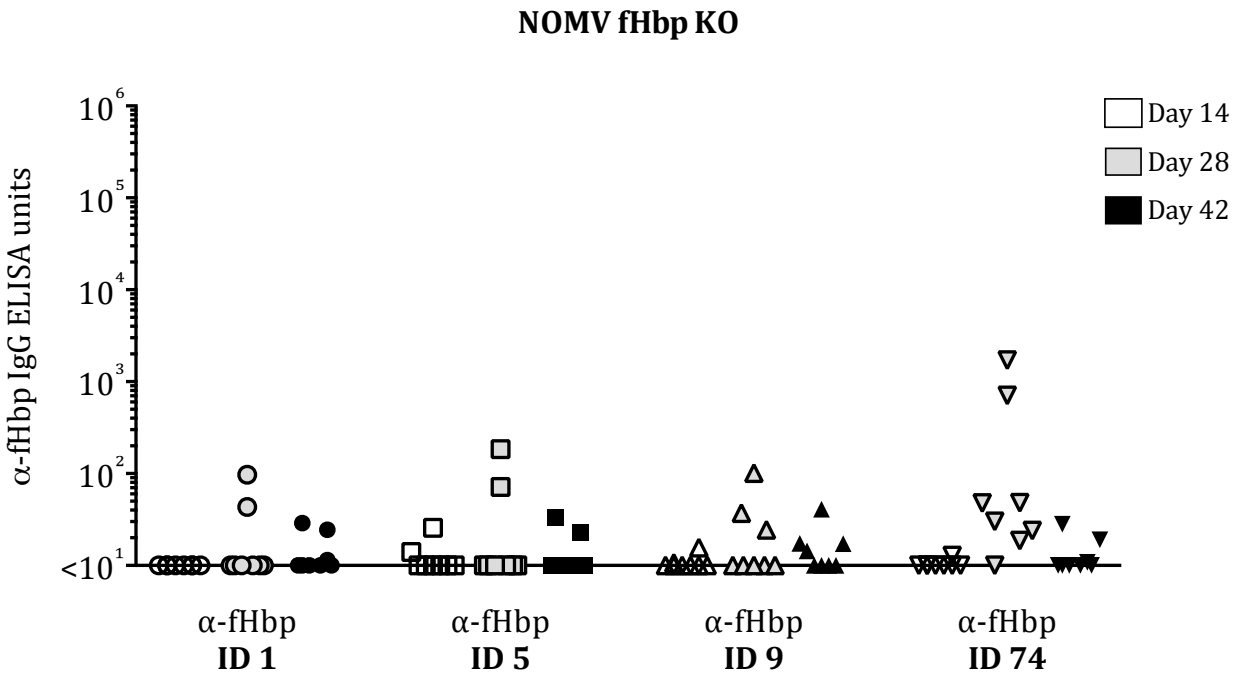
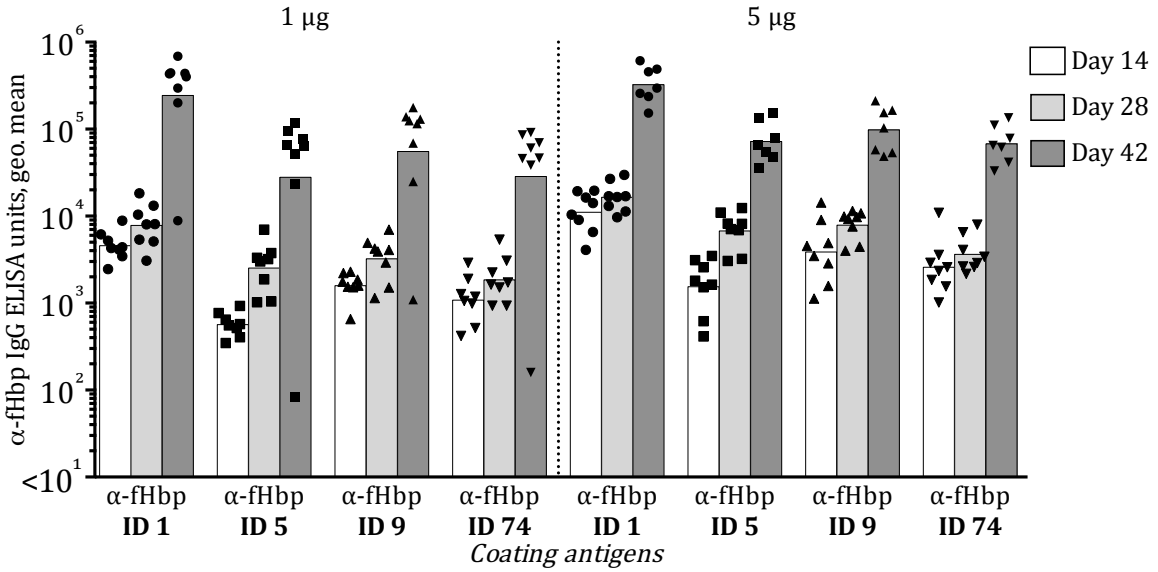


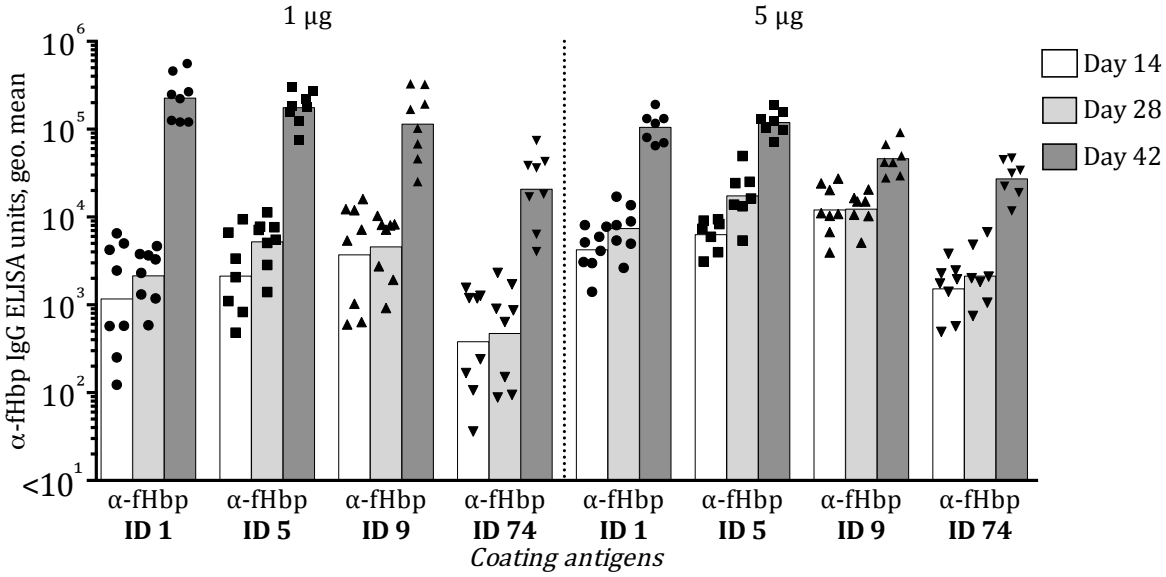
Figure 4.1. Serum anti-fHbp IgG antibodies in mice immunized with NOMV fHbp KO.

Mice were immunized IP with two doses of 5 μg of NOMV fHbp KO, four weeks apart. Serum samples were obtained 14 days (white symbols), 28 days (grey symbols), and 42 days (black symbols) after the first immunization. Each symbol represents serum sample from an individual mouse.

A - NOMV OE ID 1



B -NOMV OE ID 5



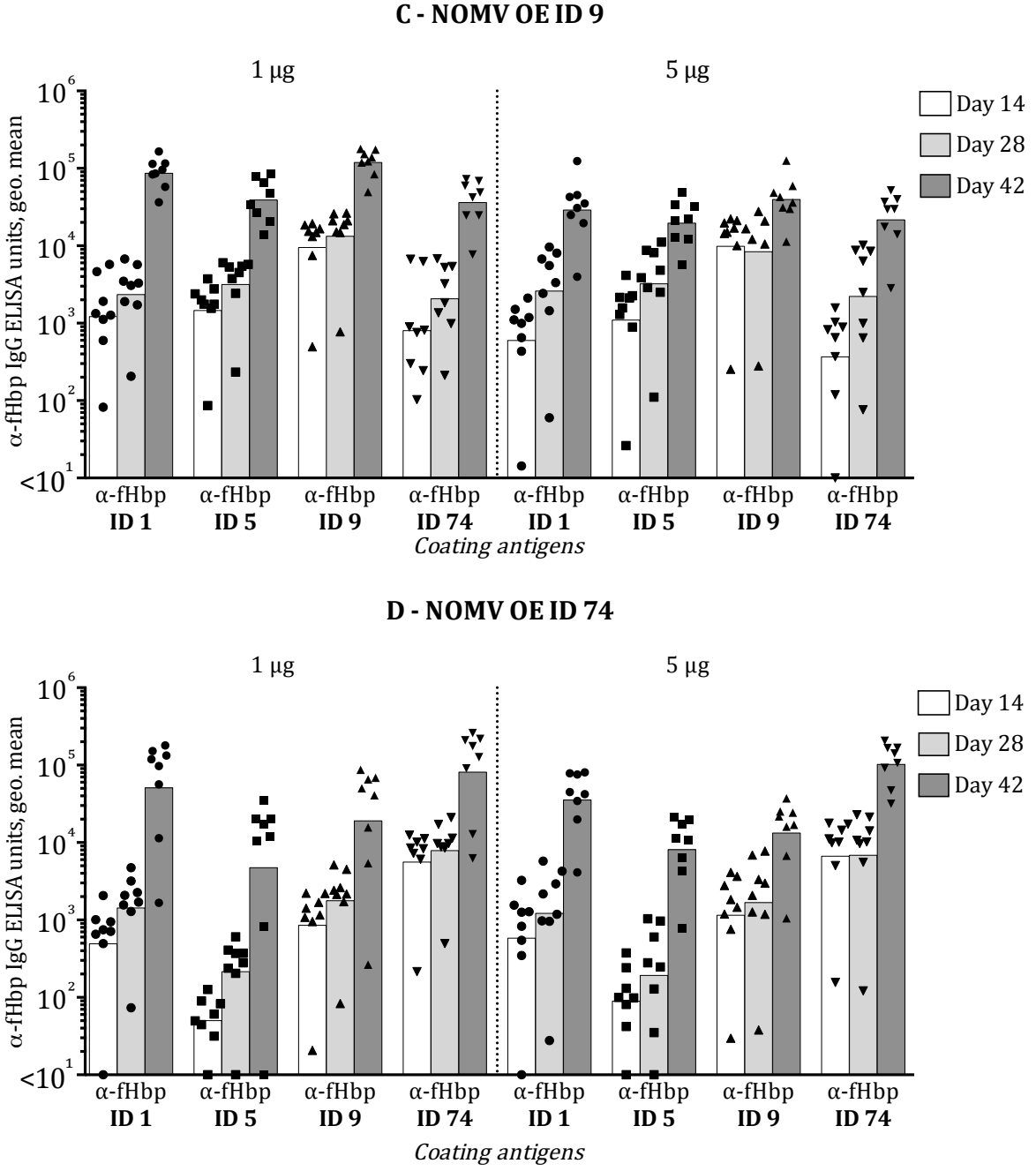


Figure 4.2. Serum anti-fHbp IgG antibodies in mice immunized with NOMV OE fHbp.

Mice were immunized IP with two doses (4 weeks apart) of either 1 or 5 µg of A) NOMV OE 1, B) NOMV OE 5, C) NOMV OE 9, D) NOMV OE 74. Immunization dose is indicated on top of the graphs. Serum samples were obtained 14 days (white bars), 28 days (light grey bars), and 42 days (dark grey bars) after the first immunization, and were tested against recombinant fHbp ID 1 (●), ID 5 (■), ID 9 (▲), and ID 74 (▼); coating antigens indicated

on the X axis. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group.

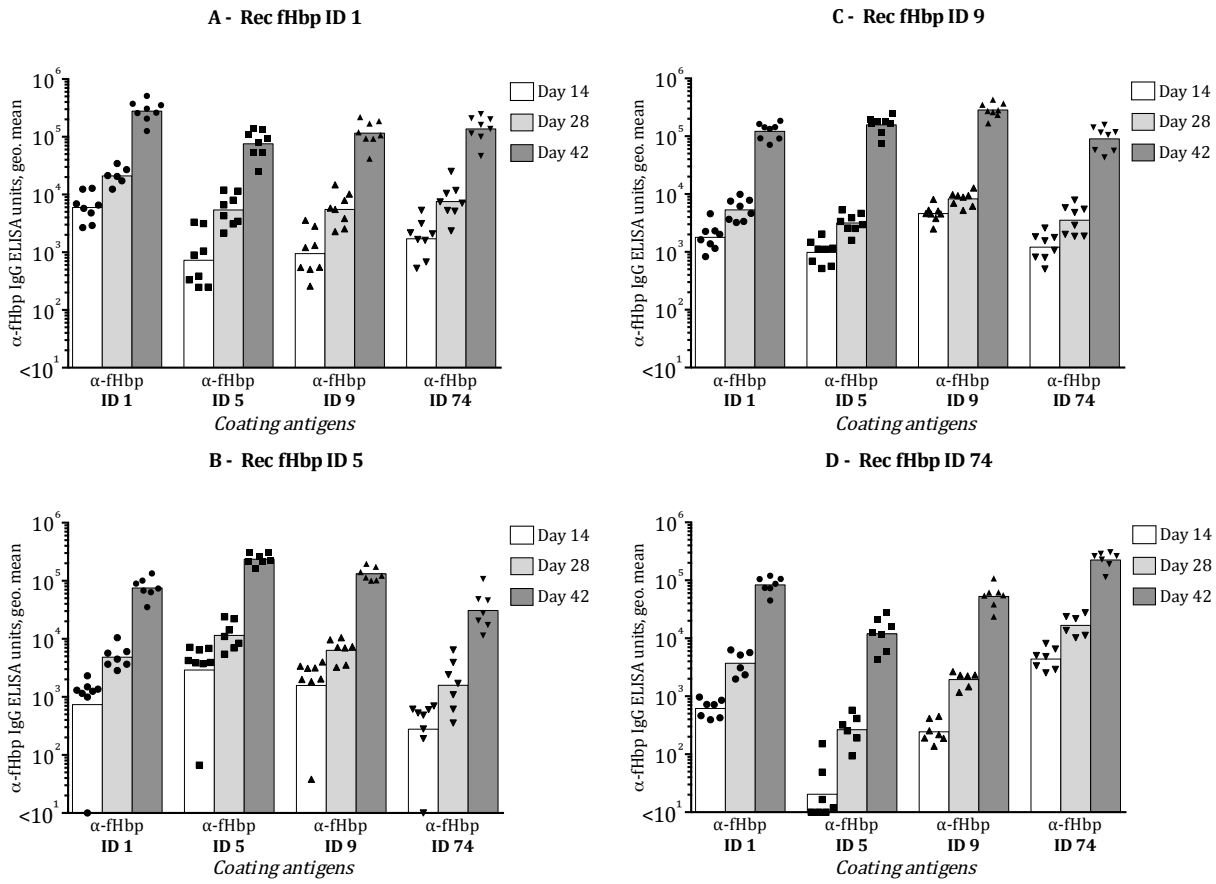


Figure 4.3. Serum anti-fHbp antibodies in mice immunized with recombinant fHbp.

Mice were immunized IP twice (4 weeks apart) with 20 μg of rec fHbp A) ID 1, B) ID 5, C) ID 9, D) ID 74, all carrying the R41S mutation. Serum samples were obtained 14 days (white bars), 28 days (light grey bars), and 42 days (dark grey bars) after the first immunization, and were tested against recombinant fHbp ID 1 (●), ID 5 (■), ID 9 (▲), and ID 74 (▼). Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group.

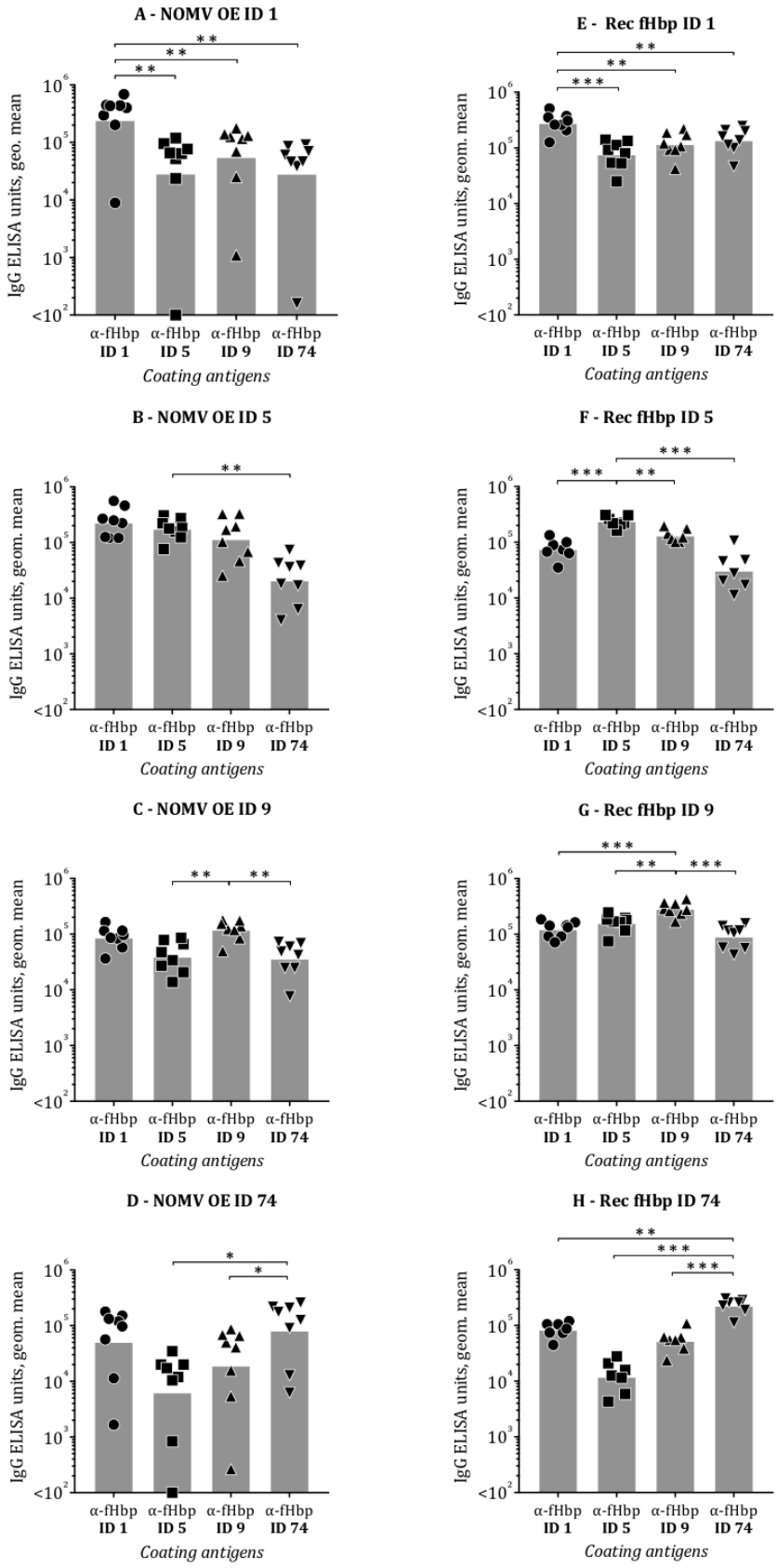
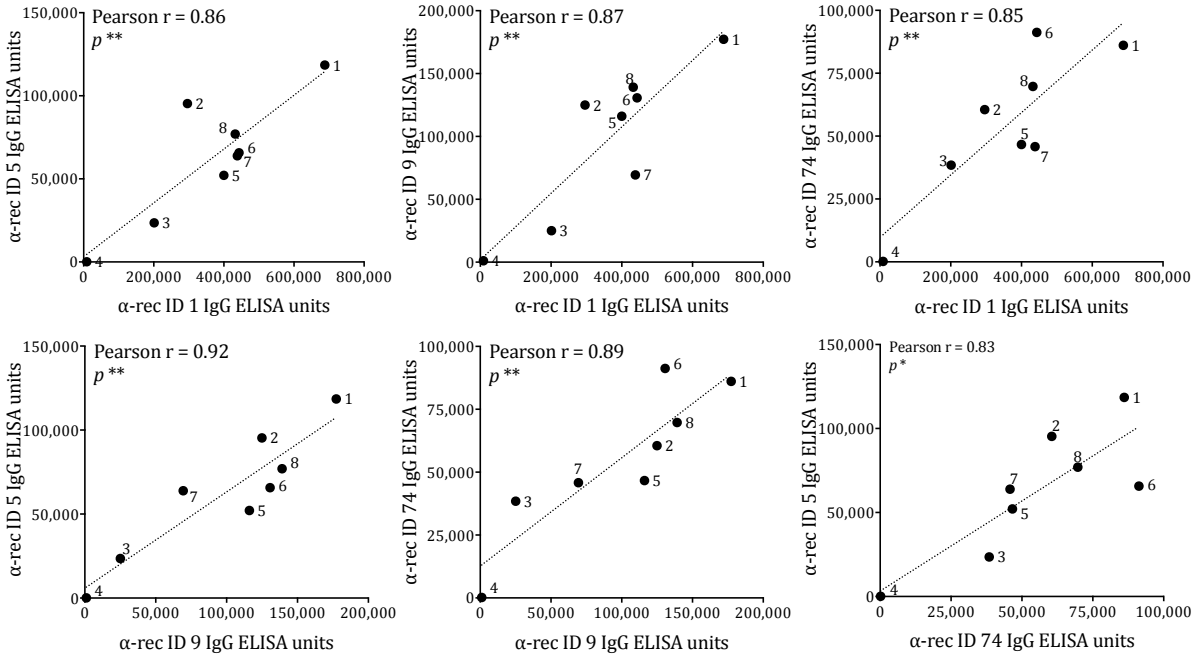

A - NOMV OE ID 1
E - Rec fHbp ID 1
B - NOMV OE ID 5
F - Rec fHbp ID 5
C - NOMV OE ID 9
G - Rec fHbp ID 9
D - NOMV OE ID 74
H - Rec fHbp ID 74
IgG ELISA units, geo. mean
IgG ELISA units, geom. mean
α-fHbp ID 1
α-fHbp ID 5
α-fHbp ID 9
α-fHbp ID 74
Coating antigens

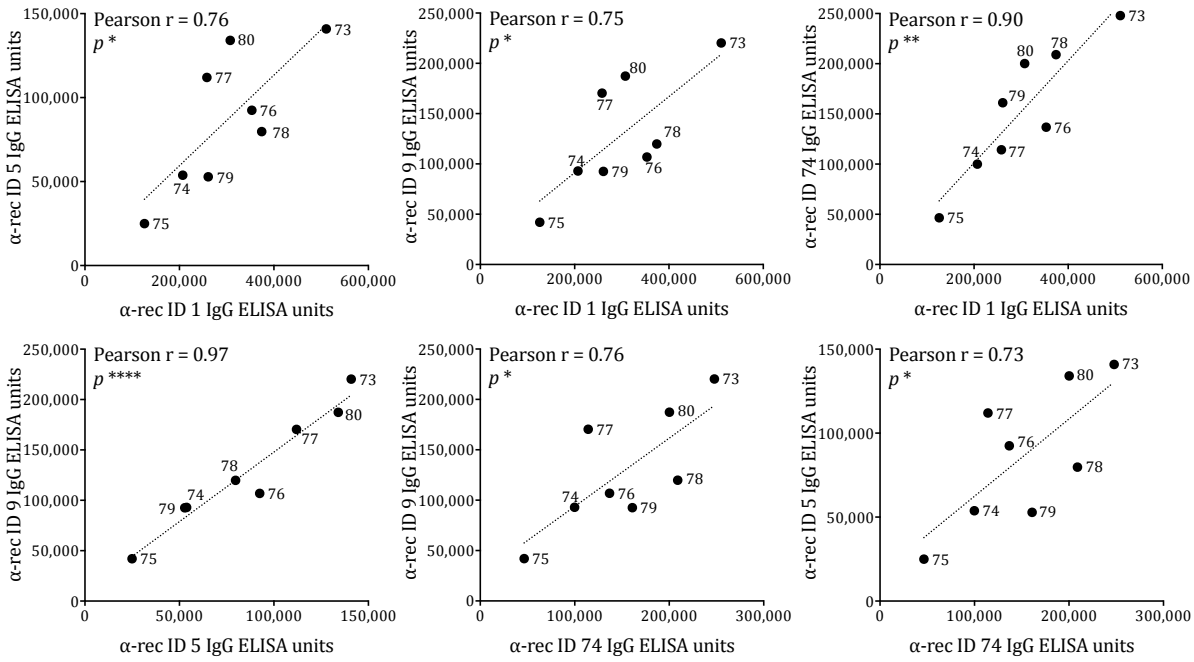
Figure 4.4. Serum anti-fHbp IgG levels in mice immunized with NOMV OE fHbp or recombinant fHbp, two weeks after the second dose.

A-D) Mice were immunized with 1 µg NOMV OE one of the four different fHb IDs. **E-G)** Mice were immunized with 20 µg recombinant fHbp. All mice received two doses, 4 weeks apart; serum samples analysed were obtained 2 weeks after second dose, and were tested against recombinant fHbp ID 1 (●), ID 5 (■), ID 9 (▲), and ID 74 (▼). Each symbol represents serum from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups; $p < 0.05$ was considered significant: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

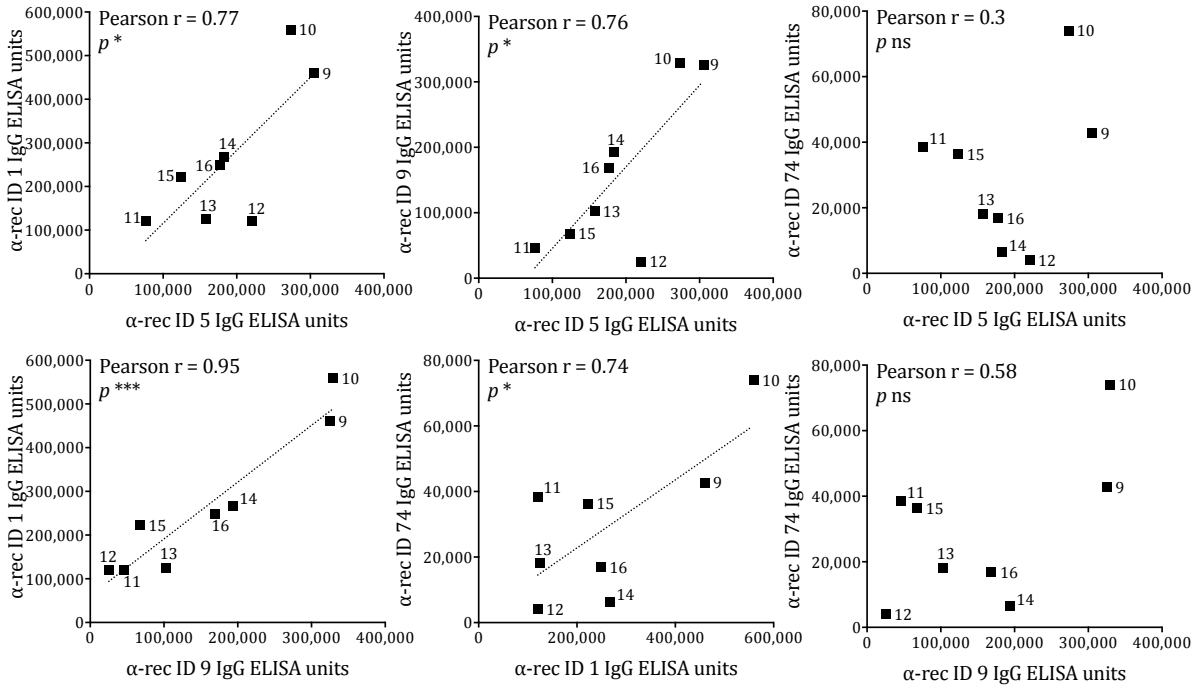
Mice immunized with NOMV OE ID 1



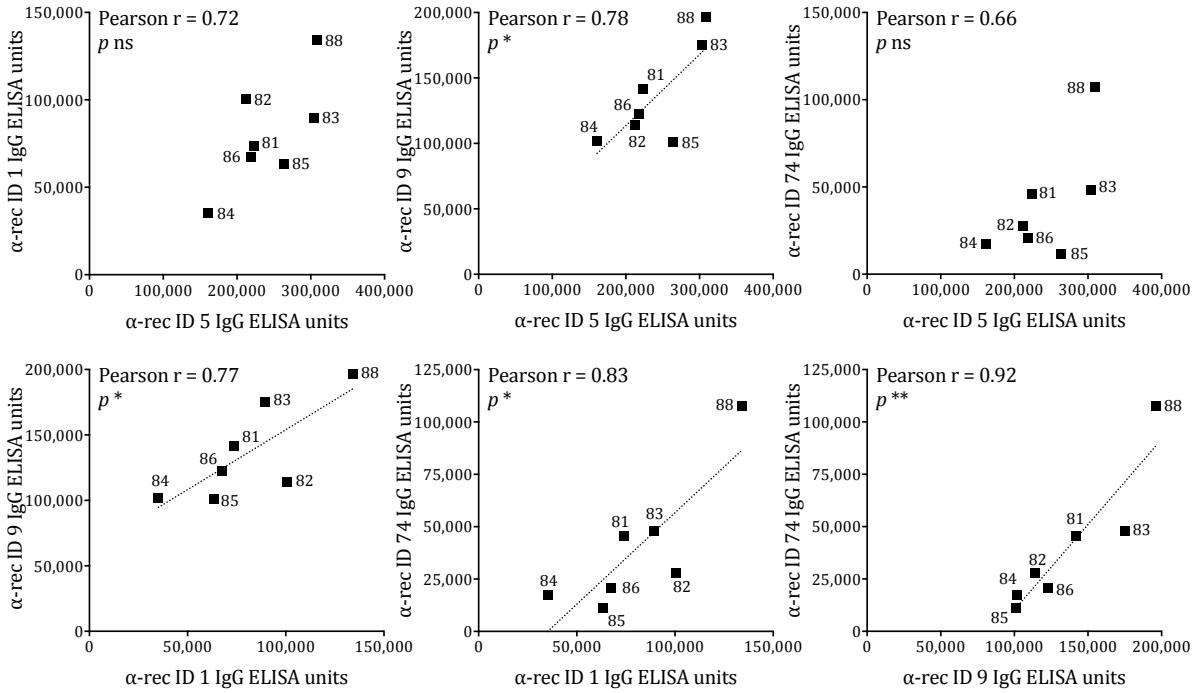
Mice immunized with recombinant fHbp ID 1



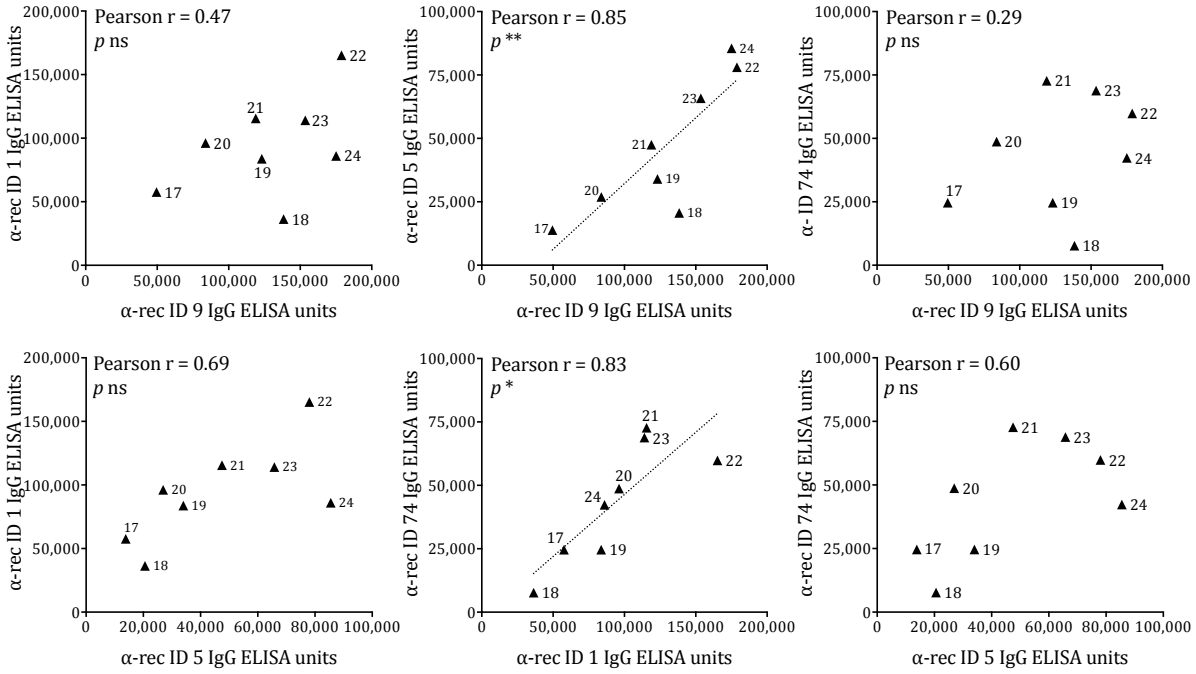
Mice immunized with NOMV OE ID 5



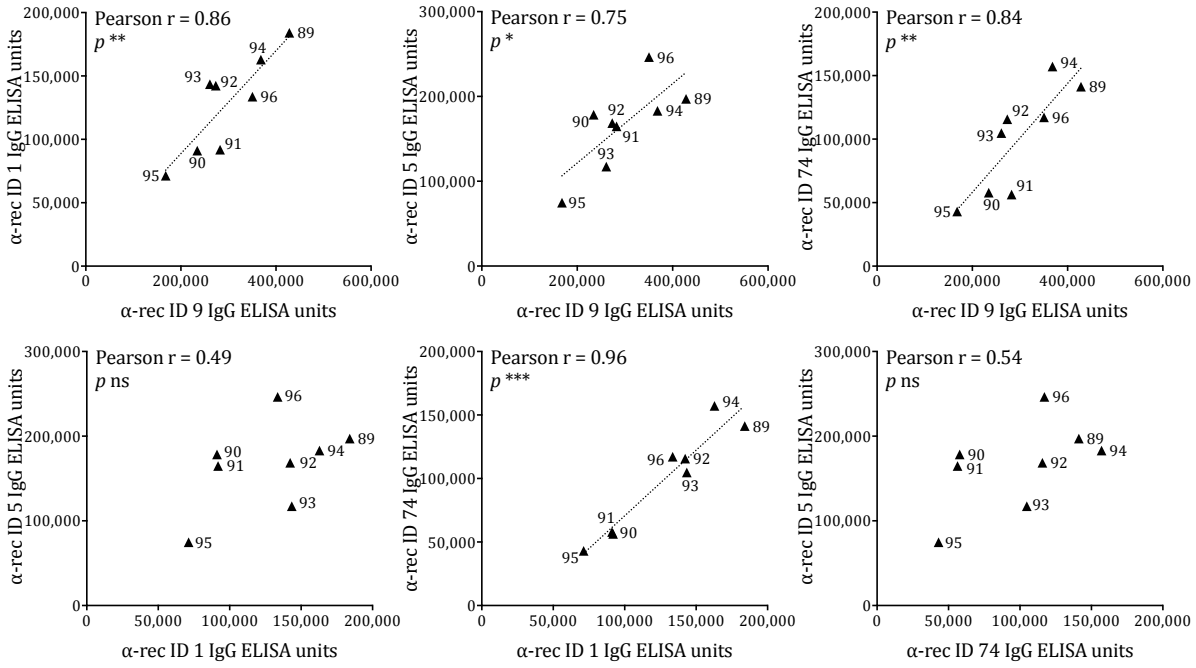
Mice immunized with recombinant fHbp ID 5



Mice immunized with NOMV OE ID 9



Mice immunized with recombinant fHbp ID 9



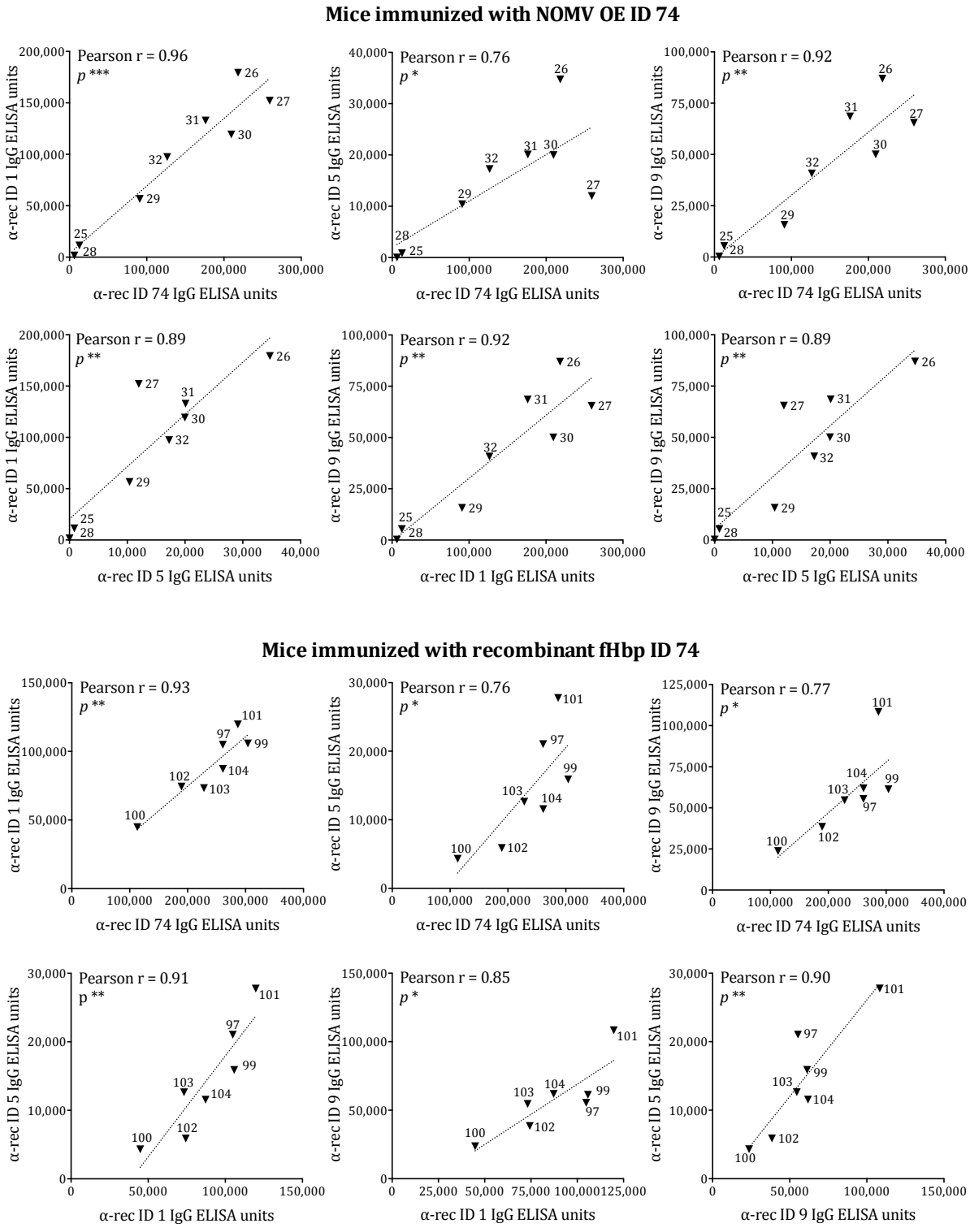


Figure 4.5. Analysis of correlation between IgG antibody titres against different fHbp$_{R41S}$ IDs of individual mice, within each immunization group.

Mice were immunized with NOMV OE fHbp, or recombinant fHbp, as indicated in the picture. Each symbol represents the reciprocal titre of an individual mouse, identified by a number. The Pearson Correlation test was performed to assess the presence or absence of linear correlation; a p value < 0.05 was considered significant: *p < 0.05; **p < 0.01; ***p < 0.001.

4.3. Serum bactericidal activity of mice immunized with NOMV with over-expressed different fHbp variant 1 IDs or recombinant fHbp

Serum bactericidal activity of immunized mice was tested against a panel of invasive meningococcal strains (Table 2.3), carrying each of the fHbp IDs over-expressed on NOMV or synthesised as recombinant protein, and expressing a heterologous PorA with respect to that present in the NOMV vaccines. SBA titres were measured in single sera from mice immunized with 1 μg of NOMV OE fHbp, or 5 μg of NOMV fHbp KO, or 20 μg of recombinant protein, and data are reported grouped per immunization antigen (Figure 4.6 and Figure 4.7), or per strains tested (Figure 4.8).

The MenC (ID 1) and MenA (ID 5) strains were susceptible to killing by sera raised against NOMV fHbp KO (Figure 4.6), indicating that antigens present in NOMV other than fHbp and PorA are able to induce bactericidal antibodies. The antigenic targets of these bactericidal antibodies were not investigated. However, against strains carrying fHbp ID 5 (MenA 1, MenA 2, and MenA 3) the highest SBA titres were induced by NOMV OE the homologous fHbp, with geometric mean titres (GMT) > $2*10^4$, and the lowest by NOMV fHbp KO, with GMT < $4*10^3$.

Strains carrying fHbp ID 74 (MenX 1 and MenX 2) were resistant to sera raised against NOMV fHbp KO, and susceptible to killing by sera from mice immunized with NOMV OE each of the fHbp sub-variants tested; an exception was MenX 2, resistant to 6/8 sera raised against NOMV OE ID 5. Against both MenX strains SBA titres induced by NOMV OE the homologous fHbp and fHbp ID 1 were not significantly different, and significantly higher ($p < 0.05$) than those induced by NOMV OE 5.

MenW strain, a low expressor of fHbp ID 9, was resistant to killing by most of the mice sera tested. The MenC strain, carrying fHbp ID 1, was susceptible to almost all of the sera tested raised against the NOMV.

In general, mice immunized with recombinant fHbp developed lower SBA responses, compared to mice immunized with NOMV OE fHbp, with a higher number of mice showing no detectable bactericidal activity of sera. MenC represented an exception, being susceptible to killing by all sera raised against recombinant fHbp.

MenX strains were resistant to sera induced by NOMV fHbp KO, meaning that there killing was dependent only on anti-fHbp antibodies. Therefore, we investigated whether there was a correlation between SBA titres against these two strains and the anti-fHbp ID 74 IgG levels, in mice immunized with NOMV OE 74. We found no correlation, suggesting that anti-fHbp IgG levels do not reflect the bactericidal activity of the sera analysed (Figure 4.9).

Within each group immunized with the different NOMV OE fHbp, we compared SBA titres of individual sera against the different meningococcal isolates. We mainly find linear correlation between SBA titres against strains expressing the same, rather than different, fHbp IDs. For example (Figure 4.10), in mice immunized with NOMV OE 1, SBA titres against two meningococcal strains both carrying either fHbp ID 5 or fHbp ID 1 correlated, but this was not true for SBA titres against MenX 1 and MenA 3 strains carrying diverse fHbp. This might suggest that bactericidal antibodies induced by NOMV OE fHbp target fHbp epitopes that are ID-specific.

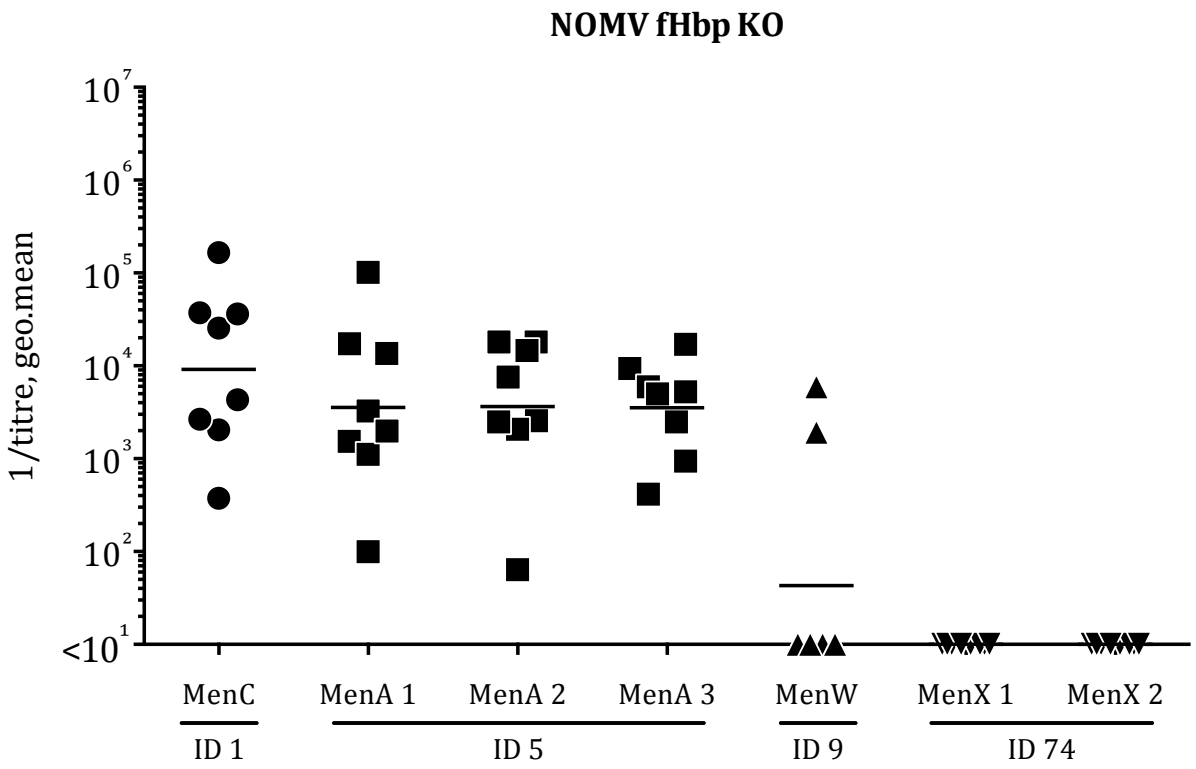


Figure 4.6. Serum bactericidal activity in mice immunized with NOMV fHbp KO.

Mice were immunized twice with 5 μg of NOMV fHbp KO, four weeks apart, as described in the materials and methods. Serum samples analysed were obtained two weeks after the second dose. Mice sera were tested against a panel of seven meningococcal strains, characterized as in Table 2.3, expressing the four different fHbp ID types: ID 1 (●), ID 5 (■), ID 9 (▲), or ID 74 (▼). Each symbol represents the reciprocal titre of an individual mouse; horizontal bar represents geometric mean titres of the group.

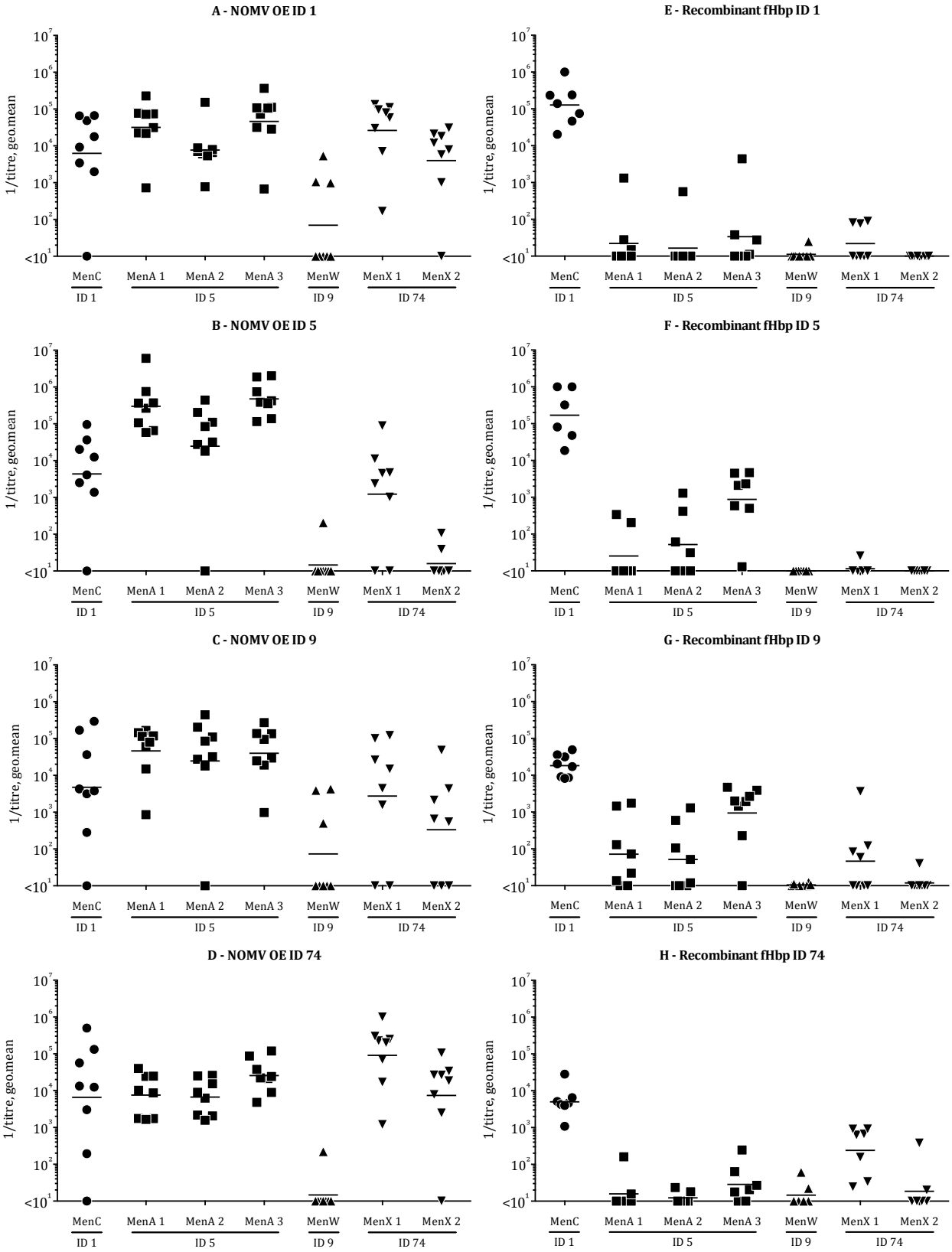

A - NOMV OE ID 1
E - Recombinant fHbp ID 1
B - NOMV OE ID 5
F - Recombinant fHbp ID 5
C - NOMV OE ID 9
G - Recombinant fHbp ID 9
D - NOMV OE ID 74
H - Recombinant fHbp ID 74
1/titre, geo.mean
<10¹
MenC
MenA 1
MenA 2
MenA 3
MenW
MenX 1
MenX 2
ID 1
ID 5
ID 9
ID 74

Figure 4.7. Serum bactericidal responses induced in individual mice immunized with NOMV over-expressing fHbp.

Mice were immunized twice with 1 μg of NOMV OE 1 (A), NOMV OE 5 (B), NOMV OE 9 (C), or NOMV OE 74 (D), or with 20 μg of recombinant fHbp ID 1 (E), ID 5 (F), ID 9 (G), or ID 74 (H) four weeks apart. Serum samples analysed were obtained two weeks after the second dose, and tested against a panel of seven meningococcal strains, characterized as in Table 2.3, expressing the four different fHbp ID types: ID 1 (●), ID 5 (■), ID 9 (▲), or ID 74 (▼). Each symbol represents the reciprocal titre of an individual mouse; horizontal bars represent geometric mean titres of the group.

A - MenA 1
B - MenA 2
C - MenA 3
D - MenC
E - MenW
F - MenX 1
G - MenX 2
1/titer, geo.mean
<10
10
10
10
10
10
10
10
NOMV fHbp KO
NOMV OE 1
NOMV OE 5
NOMV OE 9
NOMV OE 74
rec fHbp ID 1
rec fHbp ID 5
rec fHbp ID 9
rec fHbp ID 74
*
**
***

Figure 4.8. Serum bactericidal responses against each of the seven meningococcal strains, induced by the different immunization.

SBA titres presented in Figure 4.6 and Figure 4.7 are here represented highlighting how the seven meningococcal strains tested are differently killed by all the sera analysed. Legend of immunization groups in the picture. Each symbol represents the reciprocal titre of an individual mouse; horizontal bar represents geometric mean titres of the group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups; $p < 0.05$ was considered significant: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; if nothing is indicated, difference between groups is not statistically significant.

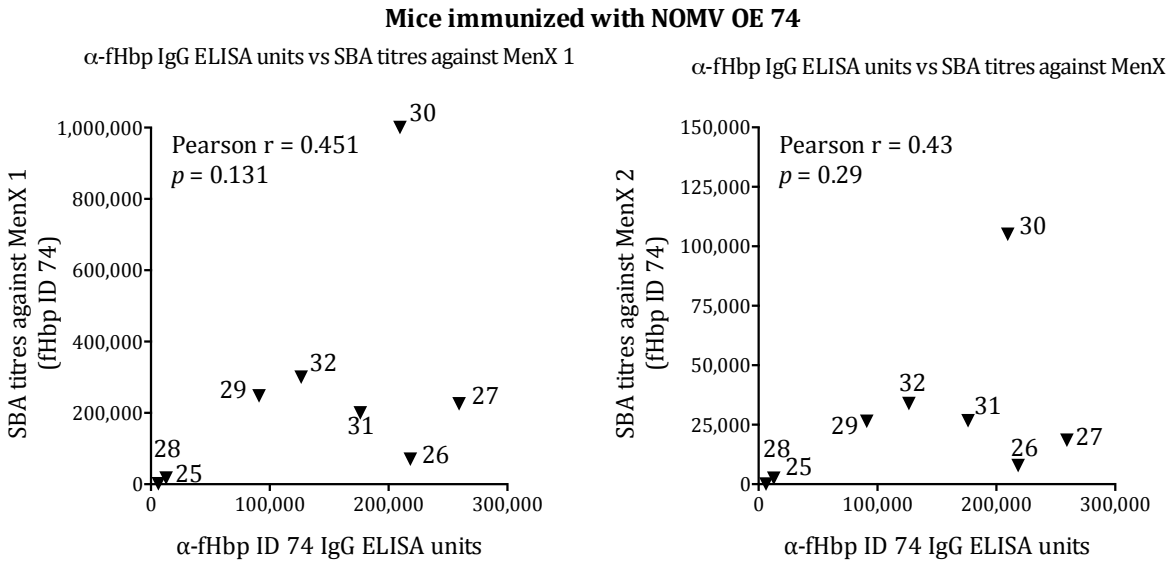


Figure 4.9. Analysis of correlation between IgG antibody levels anti-fHbp ID 74$_{R41S}$ and SBA titres against MenX strains carrying fHbp ID 74, in individual mice immunized with NOMV OE 74.

Each symbol represents the reciprocal titre of a mouse, identified by a number. No linear correlation was evident according to the Pearson Correlation test.

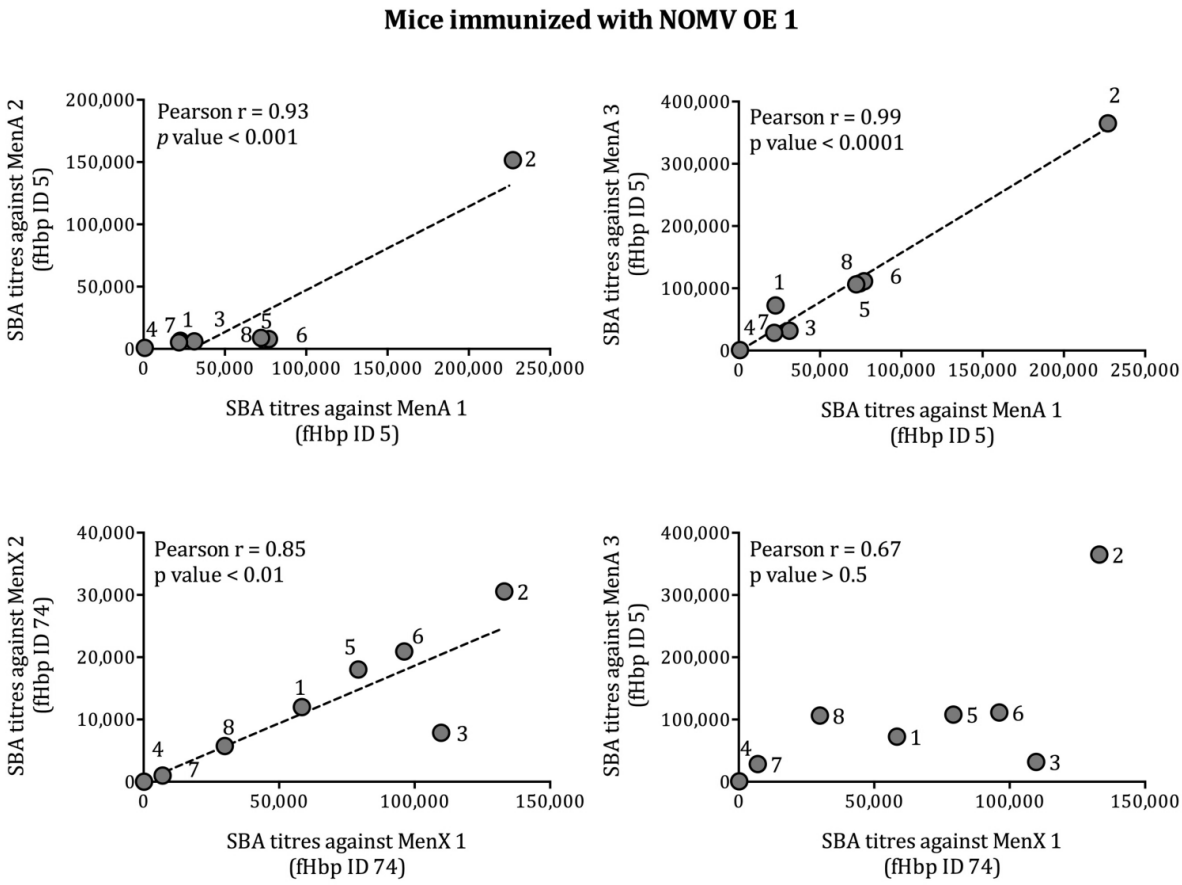


Figure 4.10. Analysis of correlation between SBA titres against different strains, in individual mice immunized with NOMV OE 1.

Each symbol represents the reciprocal titre of a mouse, identified by a number. According to the Pearson Correlation test, linear correlation is present between SBA titres against strains carrying the same fHbp ID, but not between SBA titres against strains carrying different fHbp ID.

4.4. Discussion

Meningococcal virulence factor fHbp has been successfully exploited as a vaccine antigen [318-320]. In mice, recombinant fHbp proteins generated antibodies with functional fine specificities against strains carrying fHbp IDs with a different sequence than the one used for immunization; non-detergent extracted outer membrane vesicles (NOMV) with over-expressed fHbp, instead, induced antibodies with higher cross-reactivity [53, 103, 104, 107, 108, 111, 112, 135]. Immunogenicity of NOMV with over-expressed fHbp ID 1 or ID 9 has been tested in separate studies [53, 135, 321, 322]. However, no extensive analysis has been conducted so far to understand the specific contribution of the fHbp amino acid sequence over-expressed on outer membrane vesicles, on the generation of cross-reactive antibodies. This aspect, which is important to drive the rational design of future broadly protective meningococcal vaccines, was the aim of this study: we investigated the specificity and cross-reactivity of anti-fHbp antibody response induced by NOMV carrying different fHbp variant 1 IDs.

As discussed in the previous chapter, on the basis both of the epidemiological distribution of fHbp sub-variants among meningococcal African isolates, and of the analysis we performed on the fH-binding site of fHbp, four different fHbp v.1 IDs were chosen for over-expression in NOMV, and the breadth of the cross-reaction they induced were tested in mice, and compared to that induced by recombinant fHbp.

NOMV OE fHbp and recombinant fHbp induced comparable anti-fHbp IgG levels, even if the amount of fHbp administered with the NOMV vaccines was much lower than that

administered with the recombinant protein. This confirms the intrinsic adjuvanticity of outer membrane vesicles: the immune response against protective antigens on NOMV is likely to be enhanced due to the stimulation of innate receptors [184, 185]. Nevertheless, each of the NOMV OE fHbp vaccines were able to induce a higher and broader bactericidal antibody response against the meningococcal strains tested, compared to the corresponding recombinant fHbp. It has been previously reported [111] that recombinant fHbp induced in mice elevated SBA responses against strains expressing the identical ID used for immunization, but lower cross-reactivity against strain with different IDs, even if belonging to the same variant. In the present study, we observed similar trends against MenA and MenX strains, although recording lower SBA titres. In fact, it is remarkable that a considerable number of mice immunized with recombinant fHbp did not develop antibodies with serum bactericidal activity against most meningococcal strains tested. From ELISA data, it is evident that these mice are not non-responder: they have developed high anti-fHbp IgG antibody levels, but these antibodies are unable to kill meningococci efficiently. However, MenC strain was effectively killed by sera raised against recombinant proteins, meaning that these sera do not lack bactericidal activity. Rather, intrinsic differences in the ability of the strains to survive in sera, especially combined with their different fHbp expression levels, may contribute to explain the difference we report here with, compared other studies, in which, however, serum bactericidal activity were tested for pools of sera. Nevertheless, the present study indicates that anti-fHbp IgG antibody levels do not reflect the bactericidal activity of the sera, even when such activity appears to be dependent on anti-fHbp antibodies. This lack of correlation contrasts to what has been observed in adult humans for anti-dOMV IgG

[323, 324] and anti-serogroup C capsule IgG [325]. Several aspects may account for these different outcomes: i) differences between human and murine immune system; ii) wider range of antigens present on NOMV compared to dOMV; iii) the different nature of the antigens themselves, particularly in the case of MenC polysaccharide and fHbp (a polysaccharide and a protein). The fact that anti-fHbp IgG antibodies elicited by NOMV do not necessarily reflect the bactericidal activity of sera following immunization with NOMV, may highlight the breadth of protective antigens present on NOMV. In contrast, it is surprising this lack of correlation between the ELISA for fHbp and SBA, when bacterial killing appears to be dependent only on anti-fHbp antibodies. This suggests that, even when presented on NOMV, fHbp can elicit a functional IgG antibody response against non-protective epitopes. Nevertheless, this study suggests that compared to recombinant fHbp, fHbp exposed on NOMV is able to elicit antibodies directed against more protective epitopes, not only present on the homologous ID peptide, but also on heterologous IDs. Moreover, also when comparing the immune response to recombinant fHbp and to NOMV OE fHbp, an important element to take in consideration is the intrinsic adjuvanticity of outer membrane vesicles [185, 326, 327].

Because of time constraints, it was not possible to test SBA also for the other groups; nevertheless, especially against MenW and MenX 2 strains, more resistant to killing compared to the others, it would be interesting to analyse the serum bactericidal activity of mice immunized with 5 μg of NOMV OE fHbp, to evaluate if a higher dose of the vaccine could result in a more effective bactericidal response, enhancing the antibodies response towards more protective epitopes. Furthermore, understanding the different

impact on the bactericidal response of different NOMV doses, if present, would be advantageous for an optimal vaccine formulation.

Most meningococcal vaccines studies reported in literature analyse serum bactericidal response against *N. meningitidis* testing pools of sera [53, 328, 329]. However, with this kind of analysis, it is not possible to appreciate individual differences, which become hidden in the pools, resulting in a potentially misleading immune response readout; in particular, a single bactericidal serum in a pool of non-killing sera, would give a positive result, and statistical analysis would not be reliable. Therefore, SBA assays were performed in this study by testing single mouse sera. This approach, however, had the consequence of only being able to test a limited number of strains in SBAs, because of time limitation. Nevertheless, we can now appreciate small differences in the anti-fHbp antibody response generated by similar but different vaccine antigens, as well as the individual variations in response within groups of animals. These results suggest that even when over-expressed on NOMV, where plenty of other antigens contribute to the bactericidal response induced (e.g. PorA, PorB, Opa/Opc, FbpA, FetA, NadA [330-333]), the fHbp amino acid sequence influences the cross-reactivity of the antibodies induced. These data suggest that each fHbp ID over-expressed on NOMV elicits bactericidal antibodies directed towards ID-specific epitopes on the fHbp molecule, that differ for the different ID types. This is consistent with ELISA results, which indicate that some fHbp IDs induce antibodies against more conserved and shared epitopes of the fHbp molecule, while others induce antibodies against more variable epitopes.

No obvious correlation was found in this study between similarity in the fH-contact sequence of the fHbp over-expressed on NOMV, and the breadth of cross-reactive

antibodies generated. However, the overall high antibody responses induced by NOMV OE fHbp and serum bactericidal activity elicited by NOMV fHbp KO represent relevant limitations: they mask eventual small differences in magnitude and functionality of antibody responses to fHbp, generated among immunization groups. Moreover, the fHbp IDs investigated differ not only in the fH-binding site, but also in other parts of the protein, and this makes the discrimination of the specific relevance of fH-contact residues not straightforward. Therefore, a suitable tool for better investigating this aspect could be the generation of investigational NOMV vaccines, expressing fHbp IDs with different fH-binding sites, but identical with respect to the rest of the protein sequence, or vice versa. Another interesting approach would be to take in consideration residues of fHbp surrounding fH-contact site. In this regard, however, our analysis already has considered, among fH-contact residues, extra amino acids, with respect to previous studies [122].

In conclusion, this study confirms that, in contrast to recombinant fHbp, NOMV OE fHbp generate a broader bactericidal antibody response that is able to kill heterologous strains of meningococcus. Differences in the fHbp amino acid sequence over-expressed on NOMV can contribute to different fine specificities in the antibody response generated. The new structure-sequence approach for the rational selection of fHbp IDs, focusing attention on functional surface-exposed regions, could be expanded to other vaccine antigens, to elucidate their contribution to the cross-reactivity against meningococcal strains, induced by outer membrane vesicles. This will be valuable for the design of future native outer membrane vaccines, as well as for the development of other vaccines.

CHAPTER 5. MURINE RESPONSES TO DETOXIFIED NOMV WITH AND WITHOUT ALUM

5.1. Introduction

Detergent-extracted OMV (dOMV) have been used safely and effectively in humans for more than 30 years [175]. Recently, non-detergent extracted native OMV (NOMV) released by genetically modified bacteria have also been authorized for use in human clinical trials, e.g. the use of GMMA ("Generalized Modules for Membrane Antigens") as a vaccine against *Shigella sonnei* infection [179]. Therefore, deeper understanding of how the immune system responds to vaccination with NOMV would be valuable for improving vaccine design. Hence, we investigated specific aspects of the murine immune response to NOMV-based meningococcal vaccines.

Lipid A is the moiety responsible for the endotoxin activity of LOS. Detoxification of early outer membrane vesicle vaccines was achieved by their preparation with detergent extraction (dOMV purification process), which removed the majority of the LOS. However, detergent treatment has disadvantages, such as the removal of lipoproteins that contribute to immunogenicity, most importantly fHbp, and alterations in the membrane structure can result in aggregation [72]. To avoid detergent extraction, but retain immunogenicity for use in humans, LOS can be made less reactogenic, by genetically modifying its biosynthetic pathway: deletion of the *lpxL1* gene results in a penta-acylated lipid A, instead of the wild type hexa-acylated lipid A, and this form has lower potential to induce pro-inflammatory cytokines release, in MAT assays [68, 71].

When this study was designed, there had not been a direct comparison of the immune response to OMV antigens in the context of OMV having WT or mutated lipid A. Previous studies have examined the adjuvanticity of penta-acylated lipid A-LOS investigating the immune response in mice to recombinant proteins, co-administered with purified LOS (either wild type or modified) or with outer membrane vesicles (either from wild type or from *lpxL1* KO strains) [68, 334, 335]. However, we have shown the immune response to a protein administered as recombinant protein, or presented as part of an outer membrane vesicle can differ (Chapter 4). Therefore, modification of an important component of the outer membrane such as LOS, could affect the overall immune response to antigens presented on NOMV. In Chapter 3, the decreased ability of NOMV with mutated lipid A to induce IL-6 release *in vitro* was shown; here the immune response in mice to NOMV from a *lpxL1* wild type strain and from a *lpxL1* KO strain are compared. Moreover, formulations that will be used to administer NOMV-based vaccines in humans require the adsorption of the outer membrane vesicles on aluminium hydroxide. However, how alum impacts the immunogenicity of NOMV has not been explored so far.

In this chapter, the antibody response to meningococcal NOMV-based vaccine in mice is investigated, with a focus on these key areas: i) the early primary response; ii) the persistence of the response; iii) how the number of doses in the immunization schedule influences the persistence of the antibody response; iv) how lipid A modification alters the immune response; v) and the impact of formulating NOMV with alum.

5.2. Immunization with NOMV induces a rapid antibody response in mice

The primary immune response to meningococcal outer membrane vesicles was assessed in mice 7 days after immunization with 1 μg of NOMV, administered in PBS or adsorbed on alum, intraperitoneally (IP). To examine the impact of lipid A modification, mice were injected with NOMV purified from either a *lpxL1* wild type strain, or a *lpxL1* KO strain. Specifically, the NOMV used as model antigens were from MenW OE 5 and from MenW OE 5 ΔL (Table 2 in Chapter 3). Since they were chosen before testing the hypothesis related to fHbp cross-reactivity in Chapter 4, the selection criterion was based on the most common variant 1 ID among African isolates [111]. In the present chapter, the two different NOMV types are identified as NOMV OE 5 and NOMV OE 5 ΔL, respectively. If no distinction is made between mice that received NOMV with wild type or mutated lipid A, the immunization group will be indicated simply as NOMV.

7 days after immunization with NOMV OE 5 and NOMV OE 5 ΔL in PBS, NOMV-specific IgM antibody secreting cells (ASC) (Figure 5.1 A), and NOMV-specific IgG ASC (Figure 5.1 B), were detected in the spleen, at similar frequency in the two immunization groups. ASC specific to fHbp ID 5 were low, but still detectable. Results from ELISpot reflected the antigen-specific serum antibody titres for IgM and IgG against NOMV, and against fHbp ID 5, detected in both immunization groups (Figure 5.2). IgG2b and IgG3 antibodies were present in sera from immunized mice, while IgG1 and IgG2c were largely undetectable (Figure 5.3). No difference in the antigen-specific IgG subclasses distribution was observed between the two immunization groups.

Histological analysis of the spleen showed both types of NOMV induced germinal centre (GC) responses, and typically each follicle had at least one GC. In the two immunization

groups the frequency of GCs was the same, but those induced by NOMV with modified lipid A were generally smaller (Figure 5.4).

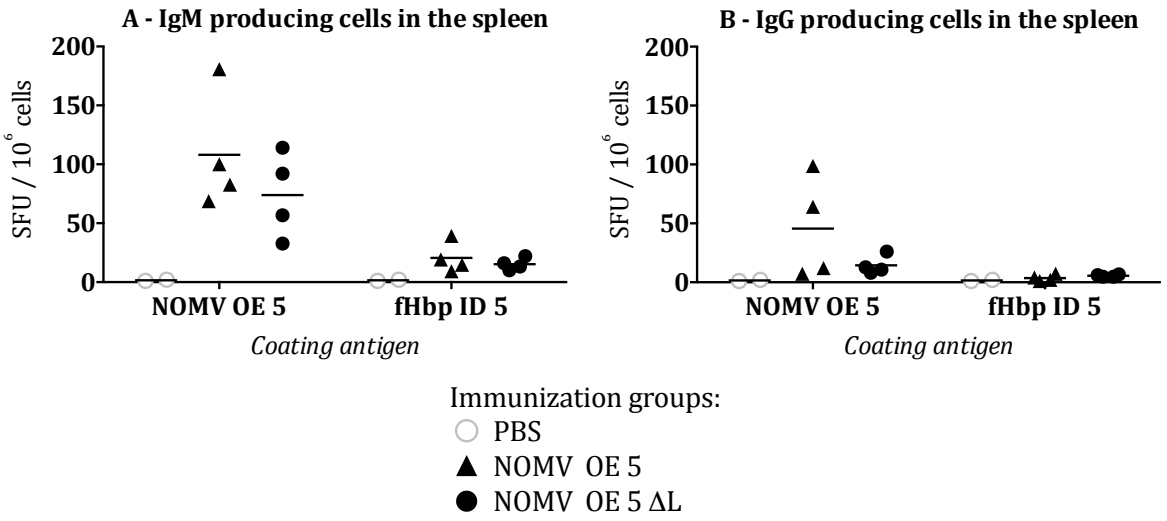


Figure 5.1. No significant difference in antigen-specific ASC between mice immunized with NOMV OE 5 and with NOMV OE 5 ΔL was observed 7 days after immunization.

IgM **(A)** and IgG **(B)** antibody secreting cells specific to NOMV OE 5 and to fHbp ID 5 were measured, by ELISpot, in the spleen of mice immunized IP with 1 μg of either NOMV OE 5 (▲) or NOMV OE 5 ΔL (●). Two mice received PBS (○), as negative control. Organs were collected 7 days after immunization. Data are indicated as spot-forming unit (SFU) per 10^6 cells (Y axis). Each symbol represents sample from an individual mouse; bars represent arithmetic mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

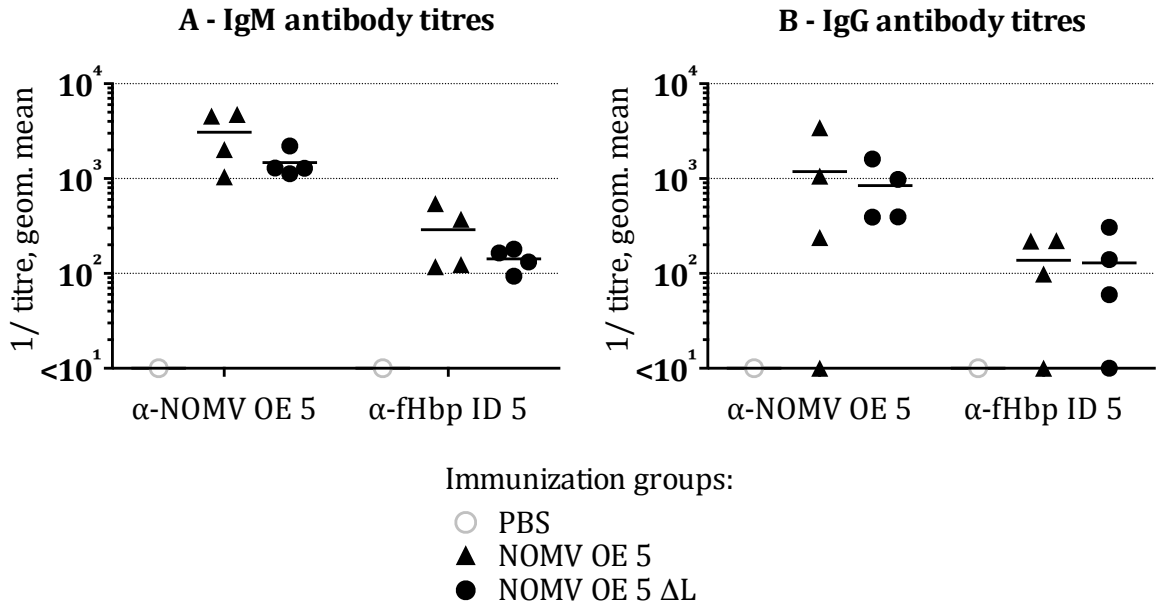


Figure 5.2. Serum antigen-specific IgM and IgG antibody titres are detectable 7 days after immunization.

Serum IgM (A) and IgG (B) antibody titres against NOMV OE 5 and against fHbp ID 5 were measured by ELISA. Mice were immunized IP with 1 μg of either NOMV OE 5 (▲) or NOMV OE 5 ΔL (●), or received PBS (○). Serum samples were obtained 7 days after immunization. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

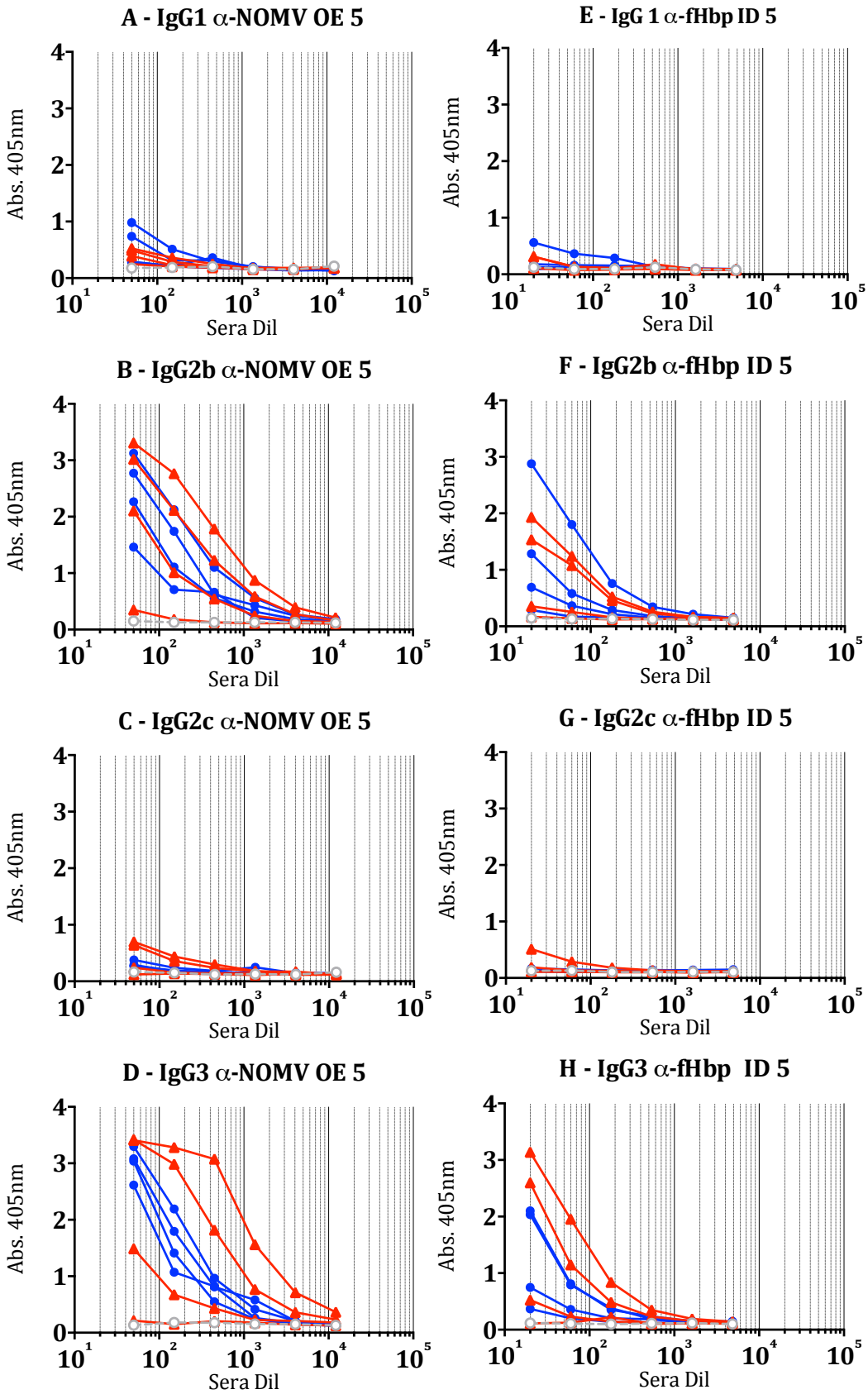

A - IgG1 α-NOMV OE 5
E - IgG 1 α-fHbp ID 5
B - IgG2b α-NOMV OE 5
F - IgG2b α-fHbp ID 5
C - IgG2c α-NOMV OE 5
G - IgG2c α-fHbp ID 5
D - IgG3 α-NOMV OE 5
H - IgG3 α-fHbp ID 5
Abs. 405nm
Sera Dil
Immunization groups:
PBS
NOMV OE 5
NOMV OE 5 ΔL

Figure 5.3. Serum antigen-specific IgG subclasses levels, 7 days after immunization.

Serum IgG1, IgG2b, IgG2c, and IgG3 antibody levels anti-NOMV OE 5 **(A-D)** and anti-fHbp ID 5 **(E-H)** were analysed by ELISA. Mice were immunized IP with 1 μg of either NOMV OE 5 or NOMV OE 5 ΔL, or received PBS. Sera analysed were obtained 7 days after immunization. Each curve represents serum sample from an individual mouse.

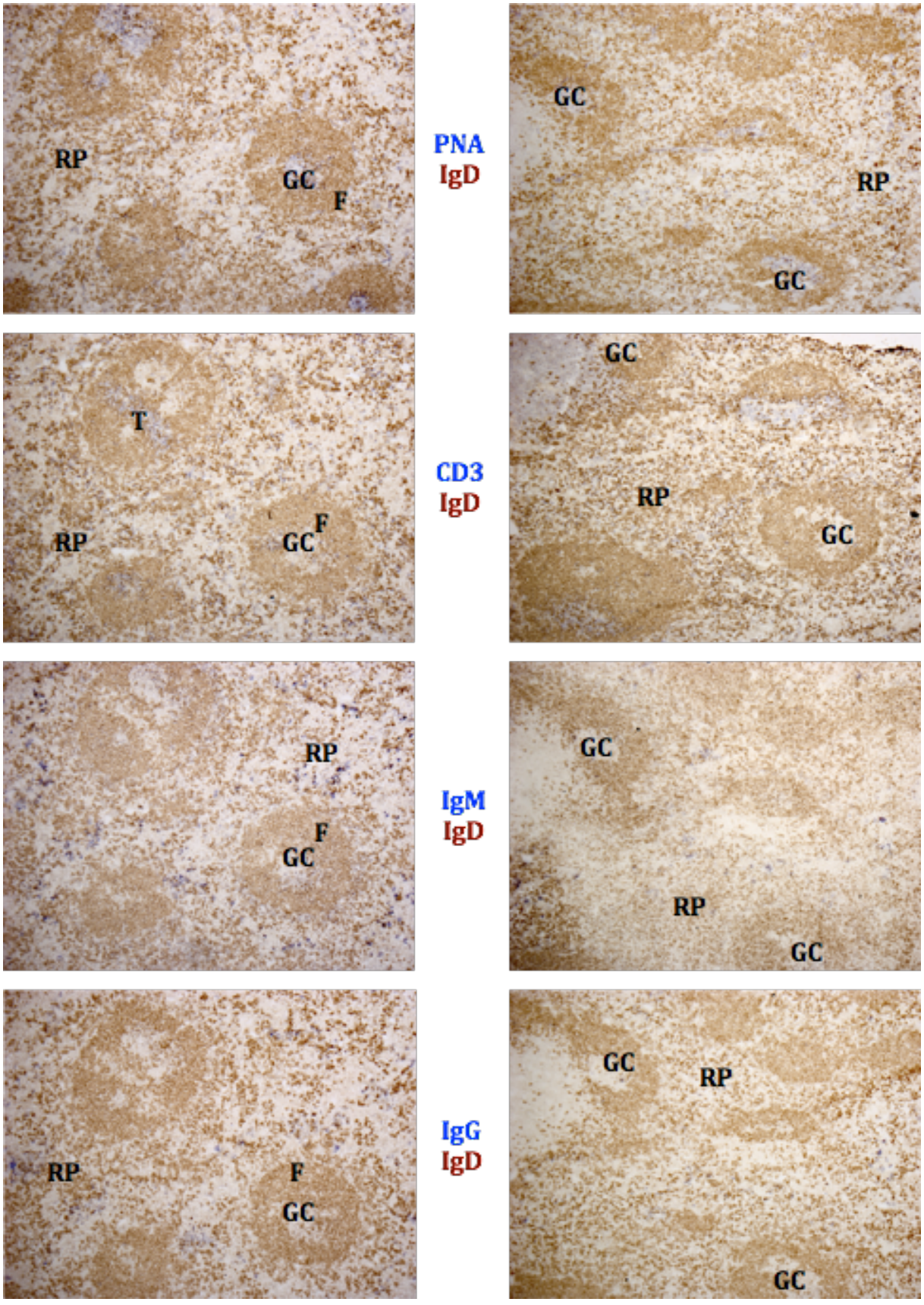

RP
GC
F
PNA
IgD
GC
RP
GC
T
RP
GC
F
CD3
IgD
GC
RP
GC
RP
F
GC
IgM
IgD
GC
RP
GC
RP
F
GC
IgG
IgD
GC
RP
GC

Figure 5.4. Immunohistological assessment of spleens after immunization with 1 μg of either NOMV OE 5 or NOMV OE 5 ΔL.

Spleens from mice immunized were collected 7 days after immunization, frozen in liquid nitrogen. Serial sections were double-stained by IHC as indicated in the panel of figures: with PNA (blue, to visualize germinal centres) and for IgD (brown); for CD3 (blue) and IgD (brown); for IgM (blue) and IgD (brown); for IgG (blue) and IgD (brown). F: Follicle; GC: Germinal Centre; RP: red Pulp; T: T-zone.

5.2.1. Alum-Precipitation Decreases The Extent of The Primary Antibody Response to NOMV

To evaluate the impact on the primary antibody response of formulating NOMV with alum, mice were immunized IP with 1 μg of meningococcal outer membrane vesicles either in PBS or adsorbed on alum. The antibody response elicited by the two different formulations was examined 7 days after immunization. A higher number of NOMV-specific IgM ASC was detected by ELISpot in the spleens of mice immunized with NOMV suspended in PBS, compared to those of spleens from mice immunized with NOMV adsorbed on alum (Figure 5.5). Anti-NOMV and anti-fHbp ID 5 IgM and IgG antibody titres were measured by ELISA (Figure 5.6), and mice immunized with NOMV suspended in PBS showed higher antibody titres than mice immunized with NOMV adsorbed on alum. Between the two immunization groups there was a similar distribution of IgG subclasses, as observed in the previous experiment (Figure 5.3), with antigen-specific IgG2b and IgG3 antibodies most readily detected, while IgG1 and IgG2c were just above detectable levels (Figure 5.7).

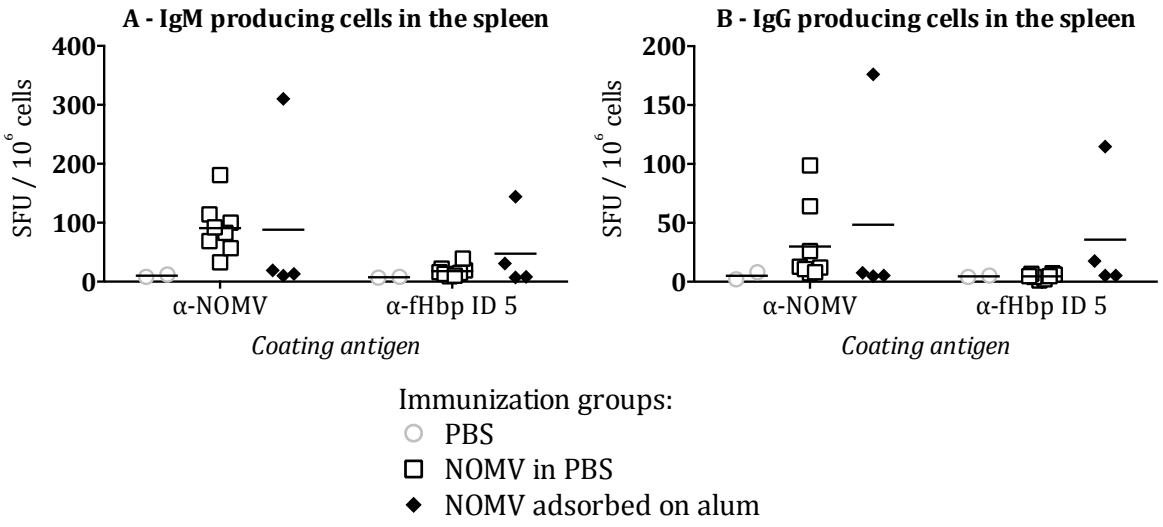


Figure 5.5. Quantification of antigen-specific IgM and IgG ASC in the spleen, 7 days after immunization with NOMV in PBS or adsorbed on alum.

IgM **(A)** and IgG **(B)** antibody secreting cells specific to NOMV and to fHbp ID 5 were measured in the spleen of immunized mice, by ELISpot. Mice were immunized IP with 1 μg of NOMV either suspended in PBS (□) or adsorbed on alum (◆), or received PBS (○). Organs were collected 7 days after immunization. Specific coating antigen is indicated on X axis; data are indicated as SFU/106 cells (Y axis). Each symbol represents sample from an individual mouse; bars represent arithmetic mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

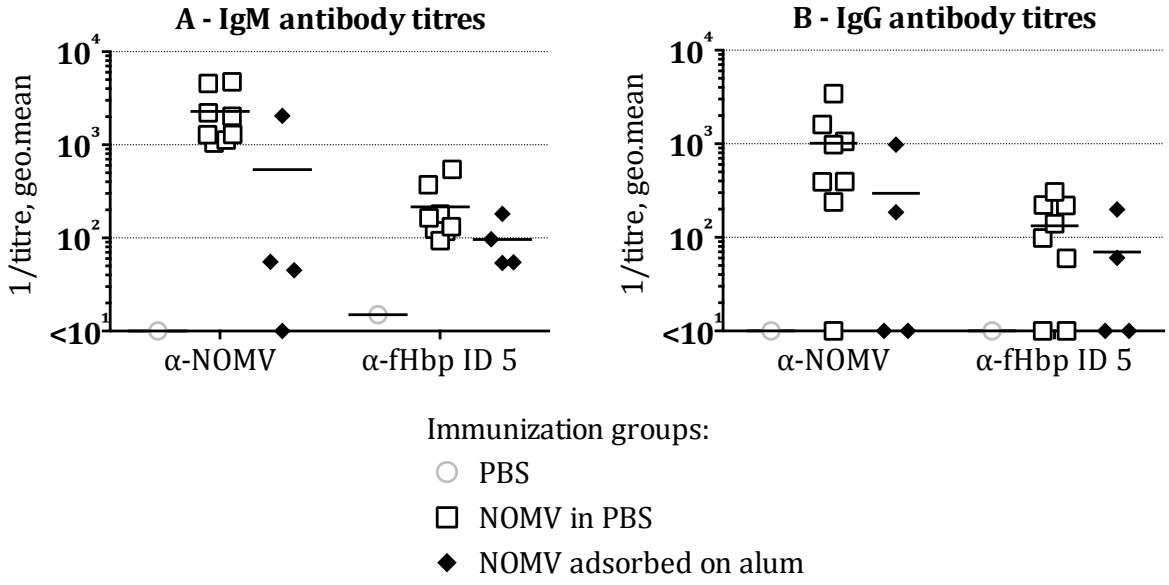


Figure 5.6. Higher antibody titres in mice immunized with NOMV suspended in PBS, compared to mice immunized with NOMV adsorbed on alum.

Serum anti-NOMV and anti-fHbp ID 5 IgM **(A)** and IgG **(B)** antibody titres were measured by ELISA. Mice were immunized IP with 1 μg of NOMV either suspended in PBS (□) or adsorbed on alum (◆), or received PBS (○). Serum samples were obtained 7 days after immunization. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

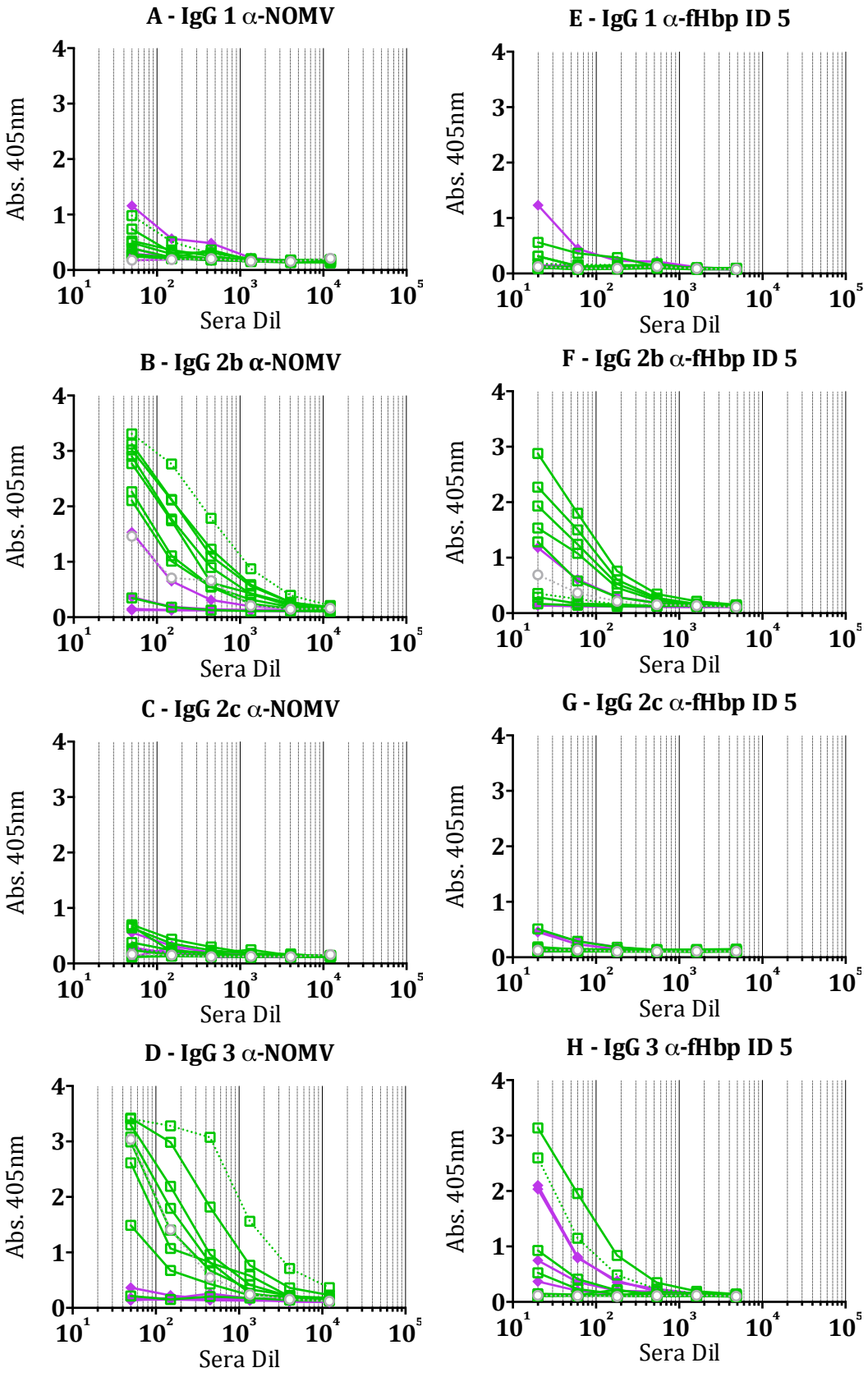

A - IgG 1 α-NOMV
E - IgG 1 α-fHbp ID 5
B - IgG 2b α-NOMV
F - IgG 2b α-fHbp ID 5
C - IgG 2c α-NOMV
G - IgG 2c α-fHbp ID 5
D - IgG 3 α-NOMV
H - IgG 3 α-fHbp ID 5
Abs. 405nm
Sera Dil
Immunization groups:
PBS
NOMV in PBS
NOMV adsorbed on alum

Figure 5.7. Alum does not impact serum antigen-specific IgG subclasses levels, 7 days after immunization

Serum IgG1, IgG2b, IgG2c, IgG3 antibody levels anti-NOMV **(A-D)** and anti-fHbp ID 5 **(E-F)** were analysed by ELISA. Mice were immunized IP with 1 µg of NOMV either suspend in PBS or adsorbed on alum, or received PBS. Serum samples were obtained 7 days after immunization. Each curve represents serum sample from an individual mouse.

Even though we observed no differences induced by immunization with either NOMV OE 5 or NOMV OE 5 ΔL, we investigated if alum could differently impact the primary immune response to meningococcal outer membrane vesicles in the context of lipid A modification. Therefore, mice were selectively immunized with either NOMV from the *lpxL1* wild type or from the *lpxL1* KO strain, suspended in PBS or adsorbed on alum. Mice immunized IP with 1 μg of NOMV OE 5 in PBS and sacrificed after 7 days later tended to have higher numbers of antigen-specific IgM and IgG antibody producing cells in the spleen, than the groups with alum (Figure 5.8). These data reflected the antigen-specific IgM and total IgG antibody titres detected by ELISA: higher titres were detected in mice that had received NOMV in PBS, than in mice that had received NOMV adsorbed on alum (Figure 5.9). Similar response was observed when NOMV OE 5 ΔL were used as immunising antigen (Figure 5.10 and Figure 5.11).

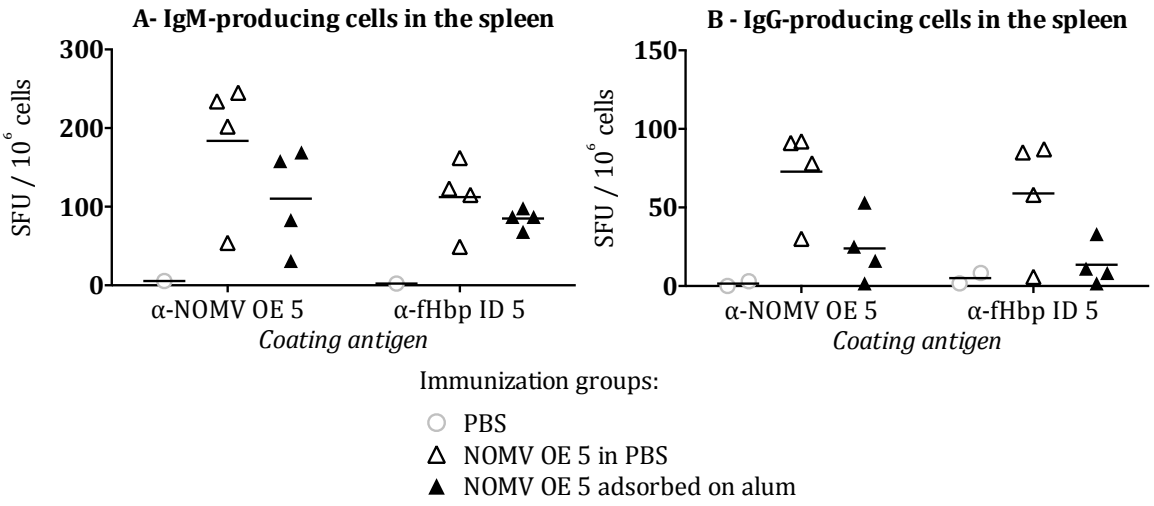


Figure 5.8. Quantification of antigen-specific antibody-producing cells in the spleen, 7 days after immunization with NOMV OE 5 in PBS or adsorbed on alum.

IgM **(A)** and IgG **(B)** antibody secreting cells specific to NOMV OE 5 and anti-fHbp ID 5 were measured in the spleen by ELISpot. Mice were immunized IP with 1 μg of NOMV OE 5 either suspended in PBS (Δ) or adsorbed on alum (▲), or received PBS (○). Organs were collected 7 days after immunization. Data are indicated as SFU/10^6 cells (Y axis). Each symbol represents sample from an individual mouse; bars represent arithmetic mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

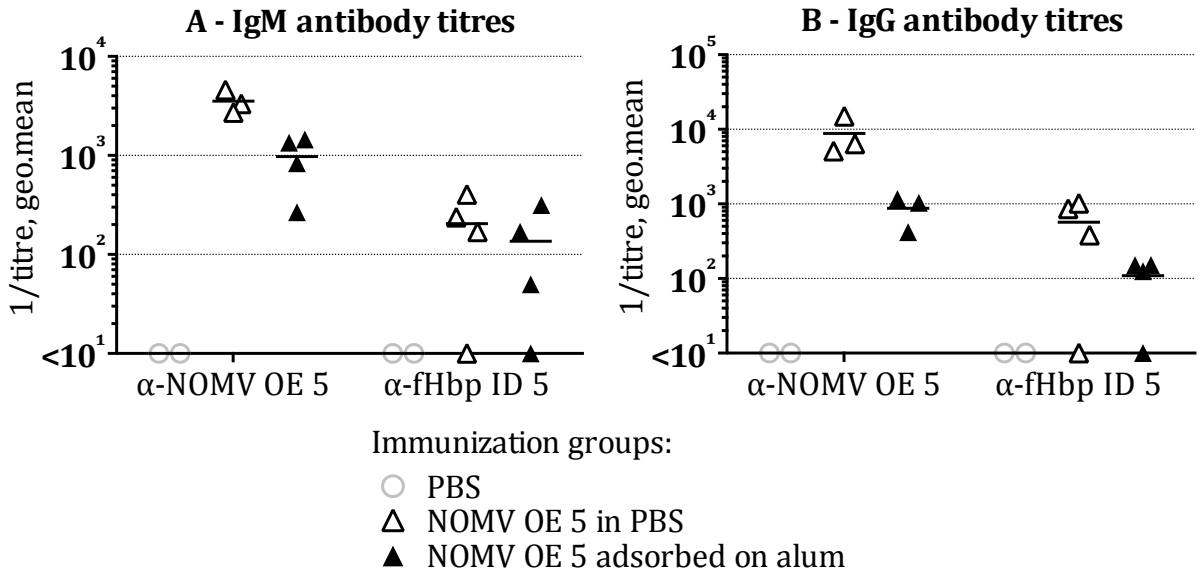


Figure 5.9. Serum IgM and IgG antibody titres against NOMV OE 5 and fHbp ID 5, 7 days after immunization with NOMV OE 5 in PBS or adsorbed on alum.

Serum anti-NOMV OE 5 IgM and IgG, and anti-fHbp ID 5 IgM and IgG antibody titres were measured by ELISA. Mice were immunized IP with 1 μg of NOMV OE 5 either suspended in PBS (Δ) or adsorbed on alum (▲), or received PBS (○). Serum samples analysed were obtained 7 days after immunization. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

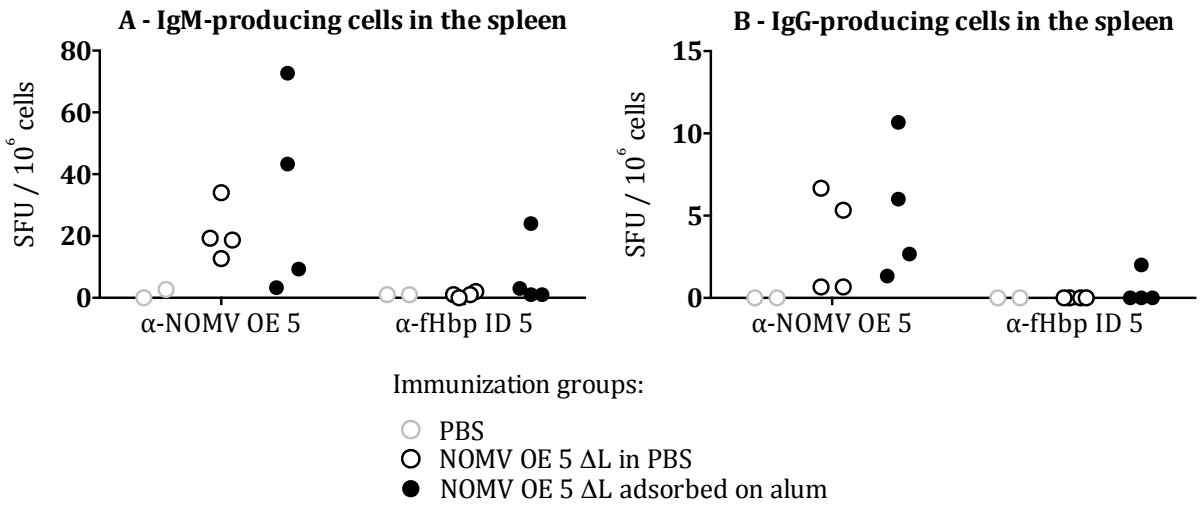


Figure 5.10. Quantification of antigen-specific antibody-producing cells in the spleen, 7 days after immunization with NOMV OE 5 ΔL in PBS or adsorbed on alum.

Specific anti-NOMV OE 5 and anti-fHbp ID 5 IgM **(A)** and IgG **(B)** antibody-producing cells were measured in the spleen, by ELISpot. Mice were immunized IP with 1 μg of NOMV OE 5 ΔL either suspended in PBS (○) or adsorbed on alum (●), or received PBS (○). Organs were collected 7 days after immunization. Data are indicated as SFU/10^6 cells (Y axis). Each symbol represents sample from an individual mouse. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

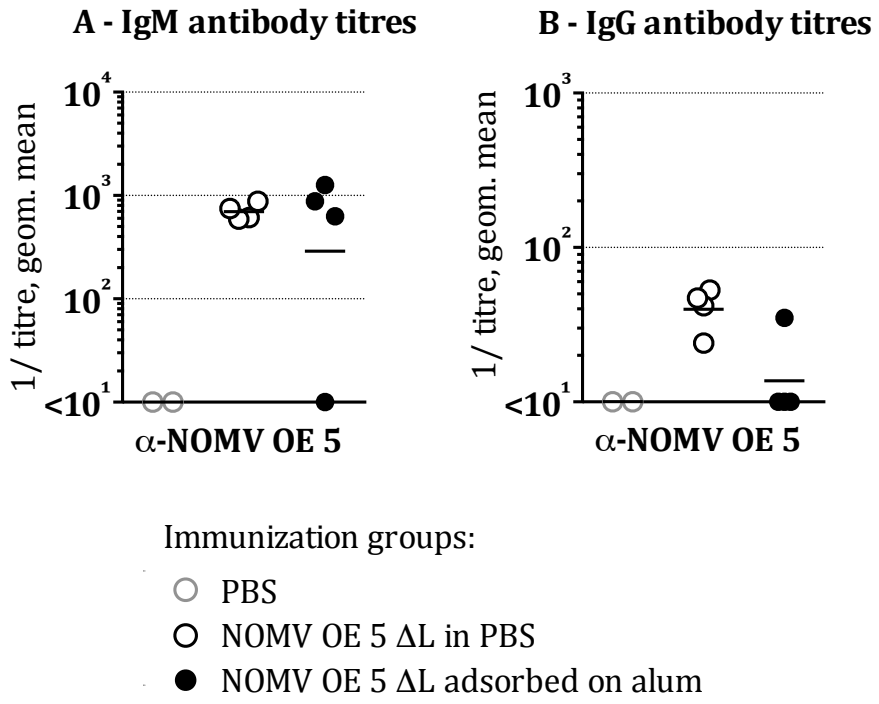


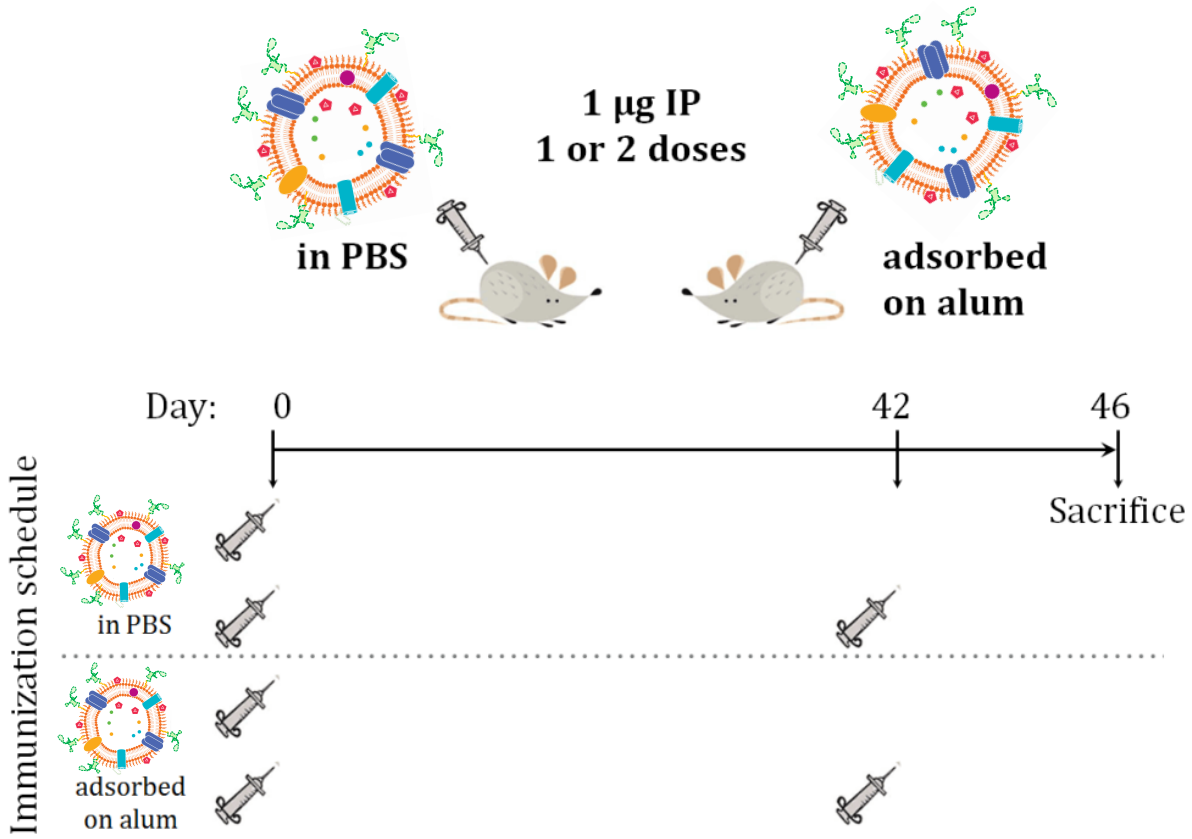
Figure 5.11. Serum IgM and IgG antibody titres against NOMV OE 5 ΔL, 7 days after immunization with NOMV OE 5 ΔL in PBS or adsorbed on alum.

Serum anti-NOMV OE 5 ΔL IgM and IgG antibody titres were measured by ELISA. Mice were immunized IP with 1 μg of NOMV OE 5 ΔL either suspended in PBS (○) or adsorbed on alum (●), or received PBS (○). Serum samples were obtained 7 days after immunization. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

5.3. Secondary response to meningococcal NOMV

Subunit vaccines containing proteins are typically given multiple times to exploit immunological memory and enhance long-term antibody responses. Therefore, we investigated the effect of a second immunization on the immune response generated by meningococcal outer membrane vesicles, in the presence or absence of alum. Since there was no significant difference in the specific antibody titres and IgG subclasses detected in the primary response elicited by wild type NOMV or NOMV with mutated lipid A, only NOMV from the *lpxL1* KO strain, i.e. NOMV OE 5 ΔL, were tested. This is because the lipid A modification is likely to be necessary before NOMV generated from native non-detergent extracted outer membrane vesicles can be used as vaccines in humans.

Mice were primed with 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum. To investigate what happens in mice following a second dose, forty-two days after priming, half of the mice received a booster dose of 1 μg of NOMV OE 5 ΔL, formulated as per the first dose, and 4 days after boosting mice were sacrificed. Details on the immunization schedule are illustrated in Figure 5.12.



Mouse	Antigen	Immunization schedule
1-2	PBS	Day 0
3-6	NOMV OE 5 ΔL in PBS	Day 0
7-11	NOMV OE 5 ΔL adsorbed on alum	Day 0
12-15	NOMV OE 5 ΔL in PBS	Day 0; day 42
16-18	NOMV OE 5 ΔL adsorbed on alum	Day 0; day 42

Figure 5.12. Immunization scheme to assess the impact of a booster dose on the immune response to NOMV OE 5 ΔL.

Mice were immunized with 1 µg of NOMV OE 5 ΔL either suspended in PBS or adsorbed on alum. Four mice (3-6) received 1 dose of NOMV OE 5 ΔL in PBS, on day 0; five mice (7-11) received 1 dose of NOMV OE 5 ΔL adsorbed on alum, on day 0; four mice (12-15)

received 2 dose of NOMV OE 5 ΔL in PBS, on day 0 and day 42; three mice (16-18) received 2 dose of NOMV OE 5 ΔL adsorbed on alum, on day 0 and day 42; two control mice (1-2) received PBS; all immunization were performed intraperitoneally (IP). Mice were sacrificed 4 days after second dose.

The number of antigen-specific IgM and IgG antibody producing cells was measured in the spleen and bone marrow of each mouse (Figure 5.13). IgG ASC specific for NOMV in the bone marrow of mice that had received the booster dose were slightly higher than those of mice immunized only once, while there was no difference in IgG ASC specific for fHbp. IgG ASC to fHbp, however, were higher in the spleen of mice immunized twice, than in those of mice immunized only once. The antigen-specific IgM ASC detected in non-immunized mice, is likely to be an artefact of cross-reacting ASC; in fact, by ELISA no antigen-specific antibodies were detected in the corresponding sera (Figure 5.14).

Antigen-specific IgM antibodies were detected by ELISA only in sera from mice that had received the second dose 4 days before (Figure 5.14 A). Anti-NOMV total IgG titres, instead, were still elevated in mice that had been immunized once, and did not differ from titres of mice that had received the booster dose (Figure 5.14 B). Different from what was observed early in the primary response (Figure 5.3 and Figure 5.7), IgG1 and IgG2c antibodies were detectable in most mice, in addition to IgG2b and IgG3 (Figure 5.14 C-F). Alum precipitation of antigen had no effect on antigen-specific total IgG, IgG1, and IgG2b antibody titres. When anti-NOMV total IgG antibody titres were compared in the same mice before and after boosting, little change was observed (Figure 5.15). For mice with poor responses (numbered 14 and 15, Figure 5.14 and Figure 5.15) the most logical interpretation is that these were miss-injected on day 0, and therefore, when sacrificed, they actually had received only one single dose, four days before.

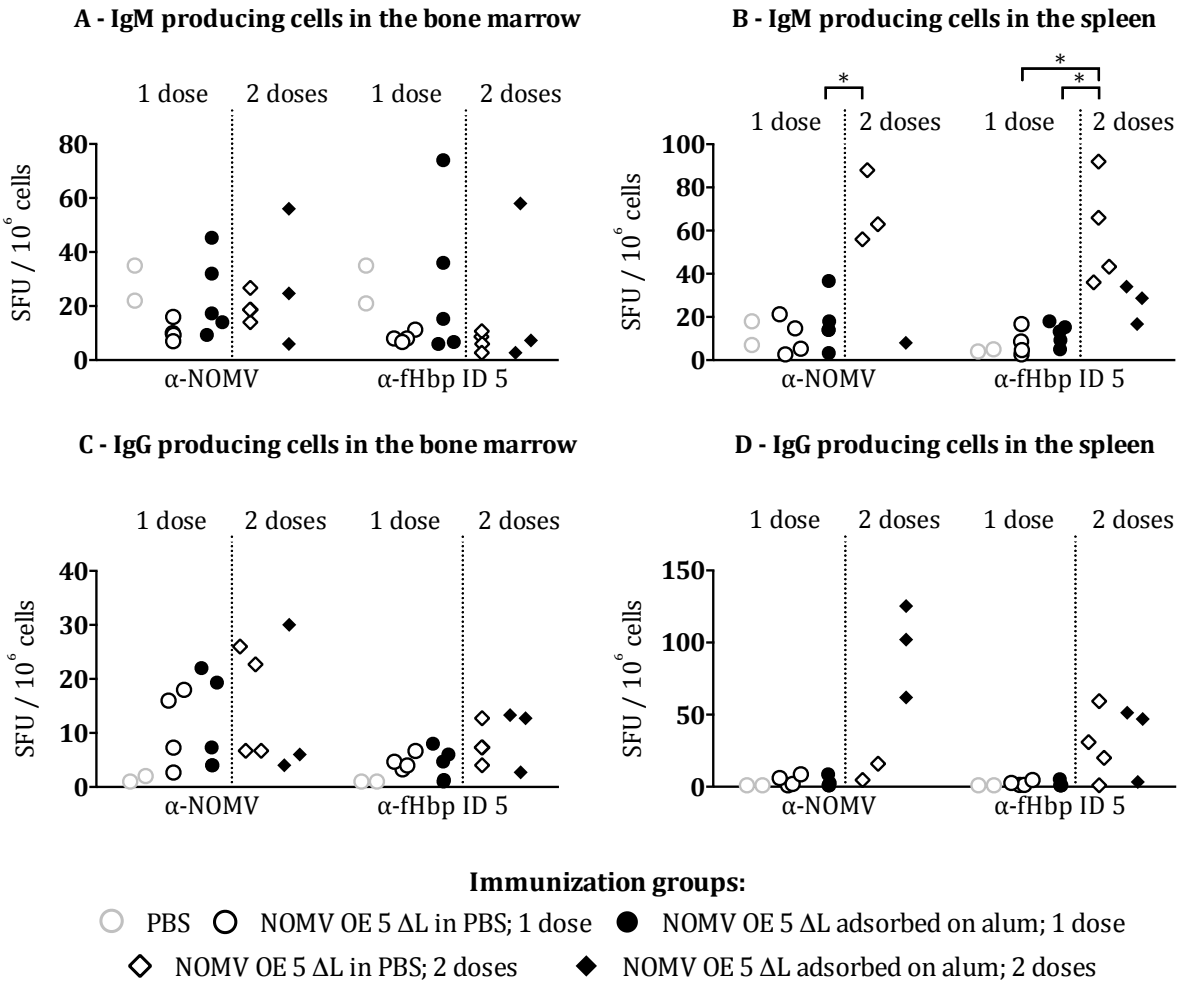


Figure 5.13. Quantification of antigen-specific IgM and IgG antibody-producing cells in the spleen and bone marrow, by ELISpot.

Specific anti-NOMV IgM and IgG antibody-producing cells and anti-fHbp ID 5 IgM and IgG antibody-producing cells were measured by ELISpot in the spleen and bone marrow. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS. Organs were collected 4 days after second dose. Data are indicated as SFUs/10^6 cells (Y axis). Each symbol represents sample from an individual mouse. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

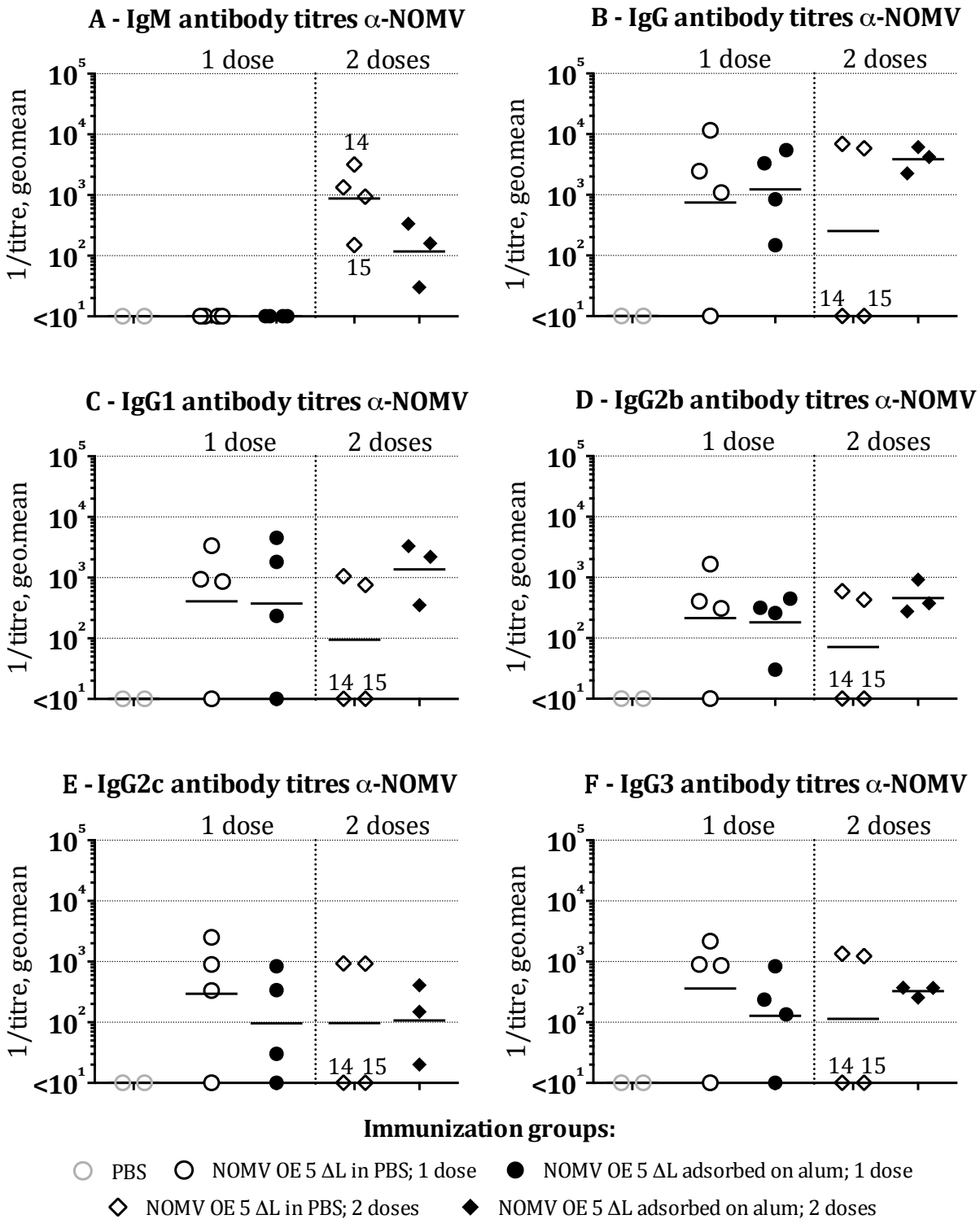


Figure 5.14. Serum antibody titres against NOMV, as measured by ELISA.

Serum anti-NOMV IgM, IgG, IgG1, IgG2b, and IgG3 antibody titres were measured by ELISA. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS. Serum samples analysed were obtained 4 days after second dose. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. Mice 14 and 15 are identified by the number next to their symbol. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups; statistical analysis was performed not taken in consideration mice number 14 and 15; $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

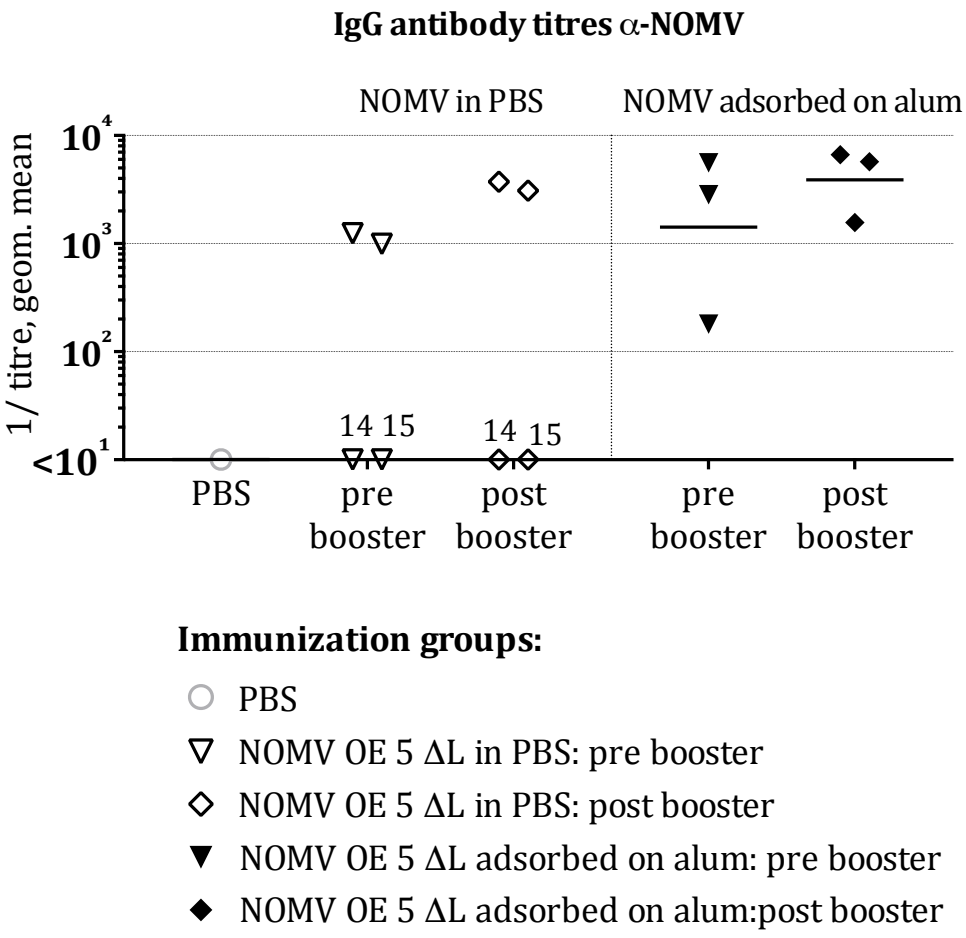


Figure 5.15. Serum anti-NOMV IgG antibody titres, before and after the booster dose.

Anti-NOMV IgG antibody titres were measured by ELISA in serum of mice immunized with two doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum; control mice received PBS. Serum samples analysed here were obtained before the second dose (day 42, "pre booster" on X axes), or four days after the booster dose ("post booster" on X axes). Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. Mice 14 and 15 are identified by the number next to their symbol. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups; statistical analysis was performed not taken in consideration mice number 14 and 15; $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

5.4. Meningococcal NOMV induce a long-lasting antibody response in mice

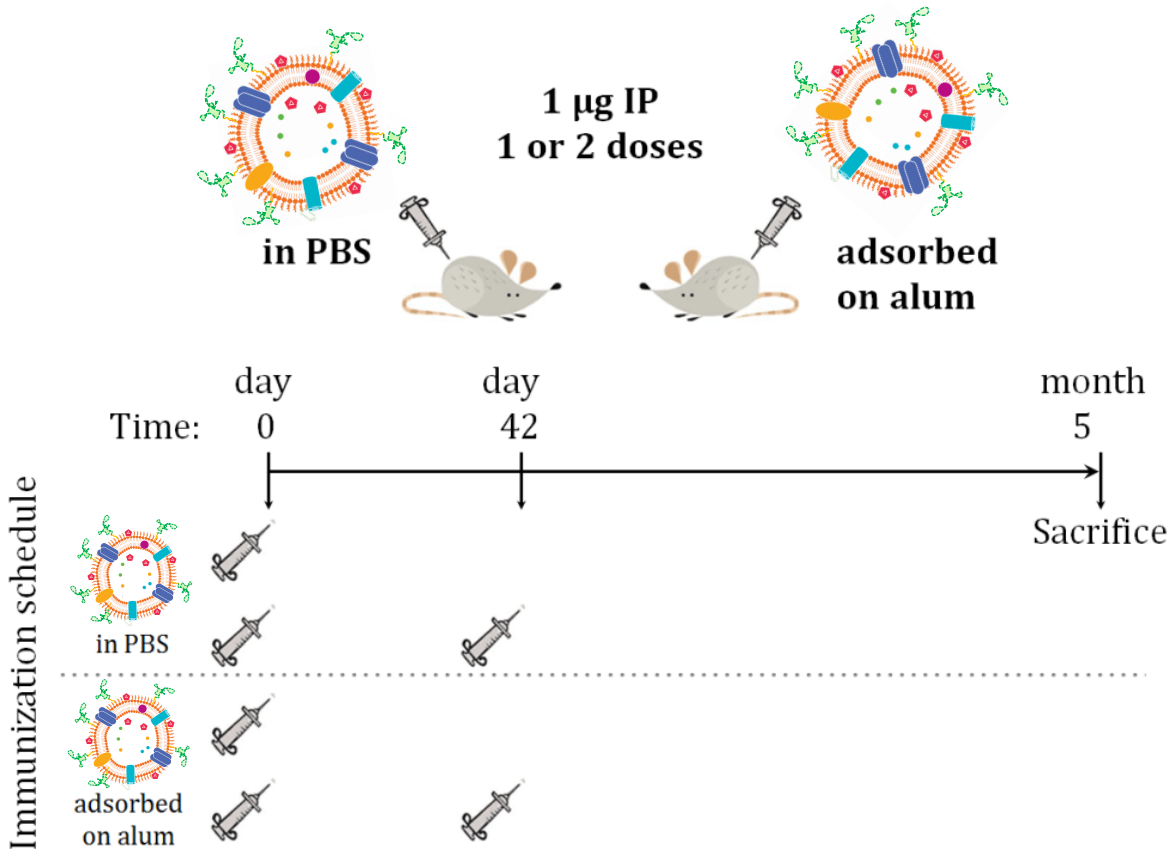
The last key element to be investigated was the antibody persistence in mice of the immune response to meningococcal outer membrane vesicles, and the impact on such persistence of a second immunization. Whether the use of alum had any impact on the endurance of the antibody response was also evaluated. As shown in Figure 5.16, mice were immunized with 1 μg of the antigen either suspended in PBS, or adsorbed on alum. After 42 days, half of the mice received a second dose formulated as per the first injection. All mice were sacrificed 5 months after priming.

The number of IgM and IgG ASC specific for NOMV and fHbp was measured in the spleen and bone marrow of each mouse by ELISpot (Figure 5.17). In both spleen and bone marrow antigen-specific IgM ASC and IgG ASC were detected. No statistically significant difference between mice immunized with one single dose or two doses was found. Data suggest a possible association between the presence of alum and a higher number of IgM and IgG antibody secreting cells specific for NOMV, in the bone marrow.

Anti-NOMV and anti-fHbp ID 5 IgM antibodies were detected by ELISA in sera from mice immunized twice, but were almost absent in sera from mice that had received one single dose (Figure 5.18 A). In mice that received two immunizations, the antigen-specific IgG antibody titres tended to be higher (Figure 5.18 B), although not statistically significantly so. However, the highest IgG titres among groups were associated with the use of alum, especially in mice that had received one single dose. The presence of alum on IgG antibody response was seen most prominent for to the subclass IgG1 (Figure 5.19 A and B): mice immunized with meningococcal vesicles adsorbed on alum showed higher antigen-specific IgG1 titres, compared to mice immunized with the antigen suspended in

PBS. The presence of alum was not associated with such a pronounced effect on the other IgG isotypes (Figure 5.19).

Another aspect examined was the relative affinity of antigen-specific IgG antibodies elicited by meningococcal outer membrane vesicles. For each immunization group a comparison was performed between anti-NOMV IgG titres against either 5 μg/mL NOMV, or 0.5 μg/mL NOMV (Figure 5.20). Antibody induced was of high affinity for each preparation of antigen, and there was little difference in the affinity among the groups.



Mouse	Antigen	Immunization schedule
1-2	PBS	Day 0
3-6	NOMV OE 5 ΔL in PBS	Day 0
7-10	NOMV OE 5 ΔL adsorbed on alum	Day 0
11-14	NOMV OE 5 ΔL in PBS	Day 0; day 42
15-18	NOMV OE 5 ΔL adsorbed on alum	Day 0; day 42

Figure 5.16. Immunization scheme to assess the persistence of the antibody response in mice to meningococcal NOMV OE 5 ΔL.

Mice were immunized with 1 µg of NOMV OE 5 ΔL either suspended in PBS or adsorbed on alum. Four mice (3-6) received 1 dose of NOMV OE 5 ΔL in PBS, on day 0; four mice (7-10) received 1 dose of NOMV OE 5 ΔL adsorbed on alum, on day 0; four mice (11-14) received 2 dose of NOMV OE 5 ΔL in PBS, on day 0 and day 42; four mice (15-18) received 2 dose of NOMV OE 5 ΔL adsorbed on alum, on day 0 and day 42; two control

mice (1-2) received PBS; all immunization were performed intraperitoneally (IP). Mice were sacrificed 5 months after first dose.

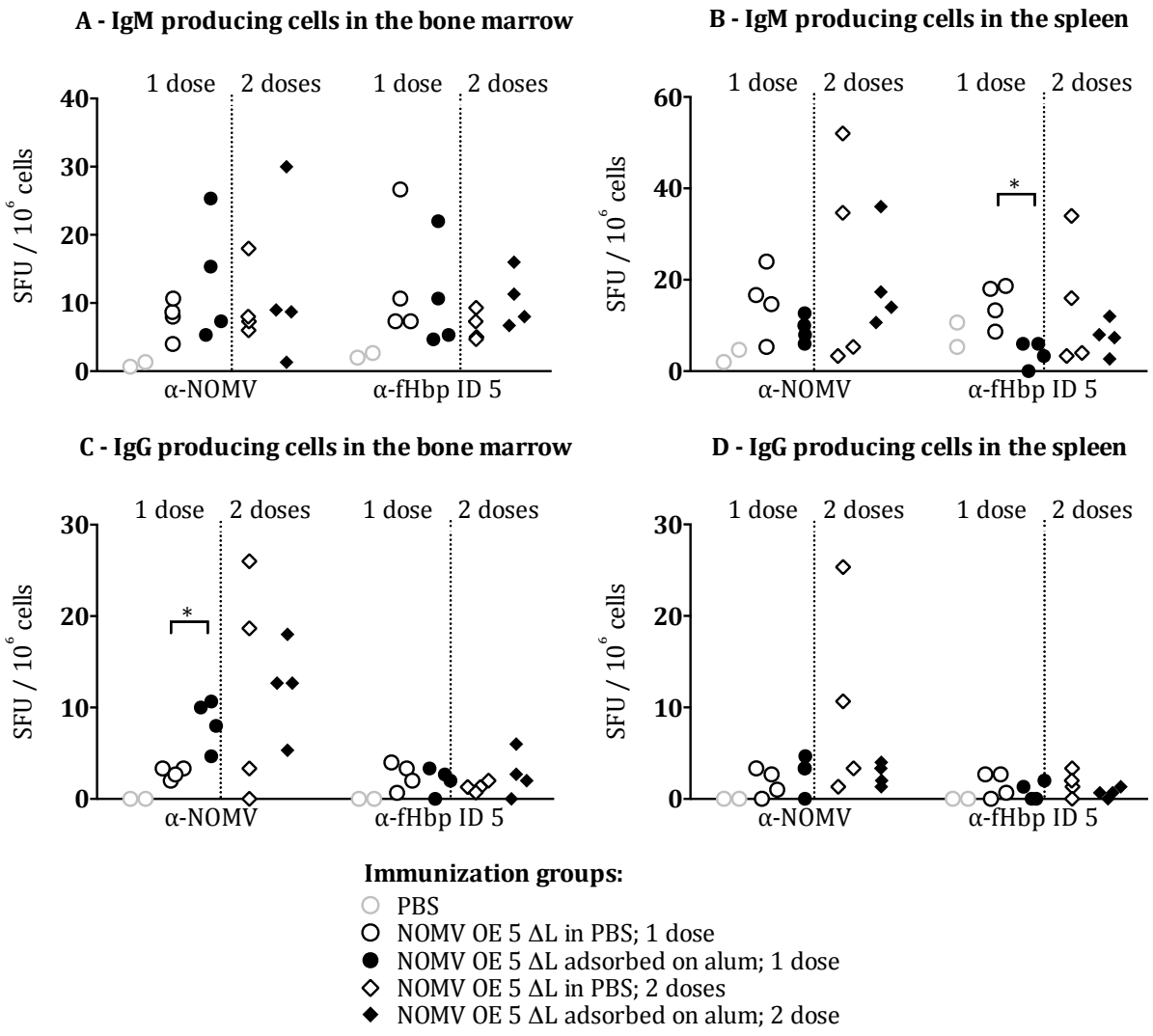


Figure 5.17. Quantification of antigen-specific IgM and IgG antibody-producing cells in the spleen and bone marrow, five months after first immunization.

Specific anti-NOMV and anti-fHbp ID 5 IgM and IgG antibody-producing cells were measured by ELISpot in the bone marrow **(A, C)**, and in the spleen **(B, D)**. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS. Organs were collected 5 months after first immunization. Data are indicated as SFU/10^6 cells (Y axis). Each symbol represents sample from an individual mouse. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

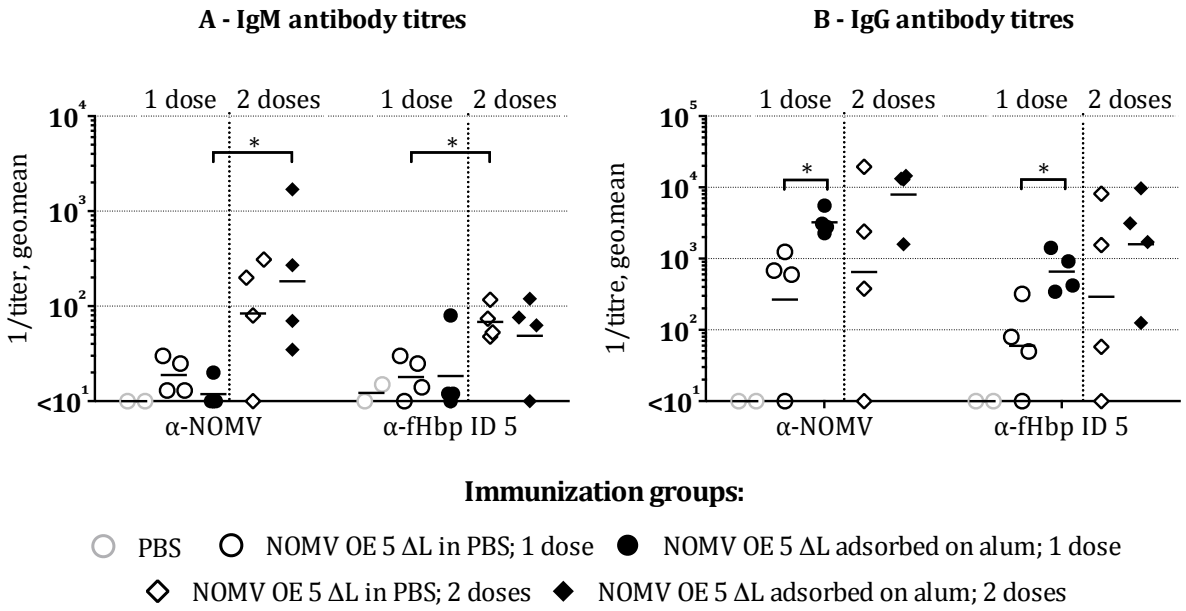


Figure 5.18. Serum IgM and IgG antibody titres against NOMV and fHbp ID 5, five months after first immunization.

Serum anti-NOMV and anti-fHbp IgM **(A)** and IgG **(B)** antibody titres were measured by ELISA. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS. Serum samples analysed were obtained 5 months after first dose. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

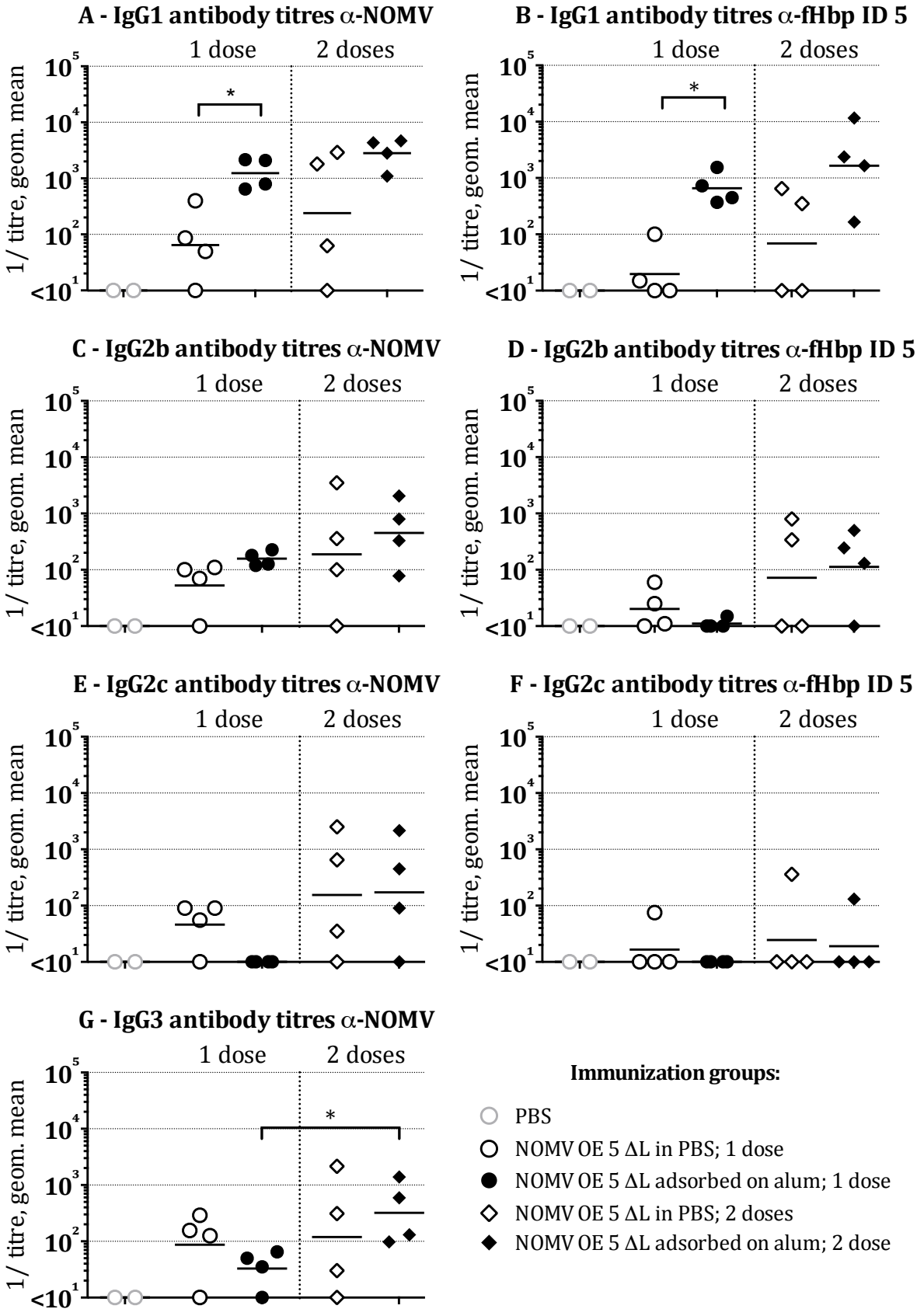
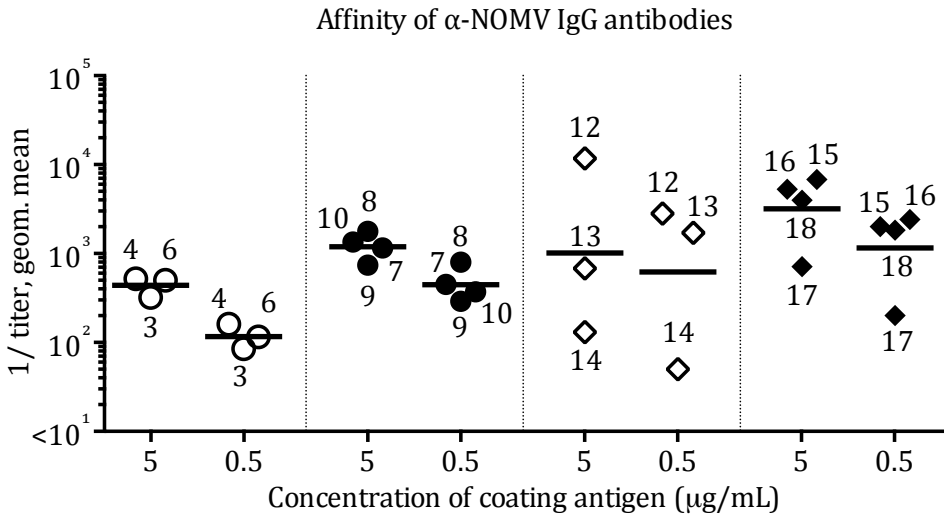

A - IgG1 antibody titres α-NOMV
B - IgG1 antibody titres α-fHbp ID 5
C - IgG2b antibody titres α-NOMV
D - IgG2b antibody titres α-fHbp ID 5
E - IgG2c antibody titres α-NOMV
F - IgG2c antibody titres α-fHbp ID 5
G - IgG3 antibody titres α-NOMV
1 dose
2 doses
1/ titre, geom. mean
10^5
10^4
10^3
10^2
$<10^1$
*
Immunization groups:
PBS
NOMV OE 5 ΔL in PBS; 1 dose
NOMV OE 5 ΔL adsorbed on alum; 1 dose
NOMV OE 5 ΔL in PBS; 2 doses
NOMV OE 5 ΔL adsorbed on alum; 2 dose

Figure 5.19. Serum IgG sub-classes antibody titres against NOMV and fHbp ID 5, five months after first immunization.

Serum anti-NOMV IgG1, IgG2b, IgG2c, and IgG3, and anti-fHbp ID 5 IgG1, IgG2b, IgG2c, and IgG3 antibody titres were measured by ELISA. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS. Serum samples analysed were obtained 5 months after first dose. Each symbol represents serum sample from an individual mouse; bars represent the geometric mean of each group. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.



Ratio of titres at antigen concentration 5 μg/μL to titres at ratio concentration 0.5 μg/μL			
○ NOMV OE 5 ΔL in PBS; 1 dose		● NOMV OE 5 ΔL adsorbed on alum; 1 dose	
Mouse	*Ratio*	*Mouse*	*Ratio*
3	3.76	7	2.56
4	3.25	8	2.21
6	4.35	9	2.54
		10	3.62
◇ NOMV OE 5 ΔL in PBS; 1 dose		◆ NOMV OE 5 ΔL adsorbed on alum; 2 dose	
Mouse	*Ratio*	*Mouse*	*Ratio*
12	4.16	15	3.41
13	0.40	16	2.19
14	2.60	17	3.55
		18	2.19

Figure 5.20. Meningococcal NOMV induce antibody with high affinity for the antigen.

The affinity for the antigen of anti-NOMV IgG antibodies was evaluated in the different immunization groups. Mice were immunized IP with 1 or 2 doses of 1 μg of NOMV OE 5 ΔL, either suspended in PBS or adsorbed on alum, or received PBS; serum samples analysed were obtained 5 months after first dose. In each group, anti-NOMV IgG titres were measured by ELISA with two different concentration of the coating antigen (indicated on X axis): 50 μL of either 5 μg/mL or 0.5 μg/mL of NOMV. Each symbol represents serum sample from an individual mouse, identified by a number; bars represent the geometric mean of each group. The ratio between the ELISA titre

measured with 5 μg/mL of coating antigen and the ELISA titre measured with 0.5 μg/mL of coating antigen is indicated, per each mouse, in the table. The Mann-Whitney test 2-tailed test was performed to compare pairs of groups, and $p \leq 0.05$ was considered significant; where nothing is indicated, comparison was not statistically significant.

5.5. Discussion

This study investigated the immune response elicited in mice by vaccine candidates against *Neisseria meningitidis* based on outer membrane vesicles. We show here that meningococcal NOMV OE fHbp induce a rapid primary response in mice. Whilst IgM and IgG responses are detectable early after immunization, there is a selective induction of isotypes, with IgG1 not detected, but with IgG2b and IgG3 present. Efficient killing of *N. meningitidis* is associated with these latter complement (C')-fixing IgG subclasses [184, 336]; therefore, this pattern of response may indicate effective protection offered early by immunization with NOMV OE fHbp. The rapid induction of C'-fixing IgG subclasses was found against NOMV, as well as against fHbp, key protective antigen in the vaccine candidate. In the early response, germinal centres were found in the spleens of immunized mice, suggesting the engagement of the immune system for memory and high affinity antibody production [283], and high affinity antibodies were found 5 months after immunization.

In Chapter 3, the different ability of NOMV with wild type or mutated lipid A to induce pro-inflammatory cytokine was assessed *in vitro*. Compared to NOMV from *lpxL1* wild type strains, NOMV from *lpxL1* KO strains had an impaired capacity to stimulate IL-6 release by PBMC, and of activating TLR4 in transfected HEK cells. Nevertheless, the presence of wild type or mutated lipid A in NOMV surprisingly did not appear to differently impact the establishment of the immune response in mice. A potential issue is that these observations were made in wild type mice, while there is evidence that murine TLR4, in contrast to human TLR4, is not able to fully differentiate between hexa-acylated and penta-acylated lipid A [313, 337]. This is related to species-specific structural

differences, and consequent discrepancies in the interaction between the various endotoxins forms and both the TLR4 receptor and the MD2 co-receptor [235, 236].

An ideal vaccine would provide a long-term memory immune response; therefore, a key point to evaluate was the persistence of the antibody response following immunization with meningococcal NOMV. Moreover, it is valuable to know how many vaccine doses are needed for a long-term persisting immune response. Ideally, single dose-vaccines are preferable, especially in low-income countries: due to the need of keeping the vaccine cost down, but also to the many difficulties in addressing vaccination in these countries (e.g. recruiting local population for mass vaccination), vaccines able to afford life-long protection following a single dose would be preferred [338, 339]. Surprisingly, in this study meningococcal NOMV OE fHbp appeared to induce IgG antibody titres that were similar in mice immunized once or twice, particularly in the presence of alum. Five months after priming, antigen-specific IgG antibody titres in mice receiving two doses were slightly higher than in mice receiving one single dose, but the major contribution in increasing antibody levels was associated with alum, rather than with multiple immunizations. In the longer term, all IgG subclasses were present, both in mice immunized once and in mice immunized twice. This is in contrast with the dose-dependent development of anti-dOMV IgG1 induced by detergent-extracted meningococcal OMV [340, 341]: in the present study the detection of IgG1 was rather time-dependent. Nevertheless, the levels of the different antigen-specific IgG subtypes are comparable, especially in mice receiving two doses of the vaccine candidate. This is also a different outcome compared to what was observed in humans vaccinated with

dOMV: in that case IgG1 and IgG3 (that share murine IgG2b ability in fixing complement [296]) were induced, while IgG2 and IgG4 (similar to murine IgG3 and IgG1, respectively [296]) were weakly detected [342-345]. Interestingly, in mice immunized twice, IgM titres were higher than in mice that received a single immunization, whose IgM titres were barely above detection limit. Again, this is different from the weak IgM response observed in humans immunized with dOMV, which are not enhanced by the booster doses [346, 347]. Altogether these data suggest a broader antibody spectrum induced by NOMV in mice, compared to dOMV in humans, and an antibody response, which is less dependent on the number of injections. This is encouraging for planning an immunization schedule with a limited number of doses.

The use of adjuvants is common for reducing dosage and/or the number of doses of vaccines given, and alum is one of the most widely adjuvants used. This study investigated how formulating meningococcal NOMV with alum may impact the antibody response elicited in mice. A previous study from another group suggested that the adsorption on alum would slightly increase the bactericidal activity of antibodies elicited by meningococcal NOMV [348]. However, the different experimental designs of their and our experiments, make the two works not comparable: they immunized mice three times (at 0, 4, and 8 weeks) with 3 μg of NOMV (adsorbed or not on alum), and reported the geometric mean serum bactericidal titres against different meningococcal strain, for sera obtained 10 weeks after the first dose. Here instead, we investigated the impact of alum on the primary antibody response, on the recall antibody response, and on the long-term persistence of the response, by analysing the levels on the IgG elicited, and the isotypes distribution. The use of alum resulted in a reduced primary antibody response to NOMV.

However, even though alum can alter the immune response to a Th2-response [349], no change in the IgG subclasses distribution was observed. Forty-six days after priming, alum was associated with lower IgG2c and IgG3 levels, both in mice receiving one dose and in mice receiving a booster dose. In mice receiving two immunizations, alum was also associated with reduced IgM and increased IgG1. In the long-term alum was associated with augmented antibody responses, as after months, mice receiving NOMV adsorbed on alum had higher IgG antibody titres compared to mice receiving NOMV in PBS. Interestingly, alum increased the persistence of antigen-specific IgM in boosted mice. Together these data suggest that the kinetics of the early and the long-term antibody response to meningococcal NOMV are differently influenced by the use of alum. This would be valuable for the rational formulation of vaccines, which could then be tailored to induce specific characteristics, e.g. a rapid massive response to fight epidemic outbreaks, or a long-lasting protection that ideally does not require booster doses, to prevent and conceivably eradicate diseases.

The main limitation of this study was the short time available for investigating all these aspects of the immune response in mice to the vaccine candidates. This work, in fact, was done within the last year of PhD candidature, when a considerable amount of time was also dedicated to learning the immunological techniques, and eight months were then dedicated to the experiments described in this chapter. Therefore, it was not possible to repeat mice experiments, in order to confirm all the observations. Another consequence of the limited time was the impossibility of testing the bactericidal activity of sera from immunized mice. This would have helped correlating the type, potency, and persistence

of the antibody response observed, to its functional efficacy. Nevertheless, this study proved the ability of NOMV OE fHbp in eliciting a fast, strong, and long-lasting antibody response in mice.

CHAPTER 6. GENERAL DISCUSSION

An affordable vaccine against multiple meningococcal serogroups is urgently needed in Africa. Previous work has shown that native outer membrane vesicles (NOMV) with over-expressed fHbp represent a promising vaccine candidate [53, 126, 350, 351]. However, as outer membrane vesicles are complexes of multiple antigens [159], it is challenging to understand the specific contribution of single components to the cross-protection induced; yet, this is fundamental for a rational vaccine design approach. In particular, as fHbp is the main broadly-protective antigen in our vaccine candidate, and for this protein three variants and almost a thousand of sub-variants (IDs) have been described [103, 110], it is important to understand the impact of the different fHbp IDs over-expressed on NOMV, to the breadth of the antibody response generated. This was therefore one of the aim of this thesis, that led to the investigation of an approach for selecting fHbp sub-variants, different from the traditional epidemiologic approach. In fact, while analysing the prevalence and distribution of disease-causative agents, and their phenotypic and genotypic characteristics, is the traditional approach to select vaccine candidates [111, 128, 352], it cannot predict which strains may arise in the future, especially after the introduction of a new vaccine. As a complementary approach, we propose in Chapter 3 an analysis based on the sequence-structure of fHbp, independent from its prevalence among circulating meningococcal strains. We hypothesized that variations of surface-exposed regions are more relevant in generating cross-reacting antibodies, than non-exposed ones, and, among these, fH-binding site is

crucial, because of its function. Consequently, we analysed the amino acids involved in the interaction with factor H, and found a higher degree of variability among these residues, compared to the whole protein sequence. Thus, we hypothesized that the fH-binding site most shared among IDs would induce the most broadly-protective anti-fHbp antibodies. To test our hypothesis, we coupled this analysis with epidemiologic data, in order to restrict the number of fHbp IDs to investigate; as a result, we chose 4 fHbp variant 1 IDs for over-expression in NOMV (Chapter 3), and we tested the different antibody responses induced in mice (Chapter 4). A basic precondition for our investigation, is that the different NOMV preparations used for immunizing mice only differ for the fHbp ID over-expressed, and this is why we have extensively characterized NOMV (Chapter 3), showing that the mutated genetic background of the meningococcal strain did not impact the overall protein pattern of the NOMV purified, nor the level of fHbp. The reproducibility and consistency of the NOMV preparations is essential for further investigation of the immune response induced in mice, as we can exclude that differences observed in mice were caused by structural variations among the antigens administered.

When tested in mice, NOMV fHbp KO were able to induce killing against 4 of the 7 meningococcal strains tested. This was unexpected, and represents a limitation to our ability to understand the specific contribution of the different fHbp IDs to the bactericidal response from this work; on the other hand, it underlines the complexity of the antigenic content in NOMV, and the presence of broadly protective antigens other than fHbp. Their presence is extremely valuable for a vaccine that needs to induce the broadest coverage possible, and further investigation of such antigens should be

prioritised. It is likely that some of these antigens have already been considered for vaccine design (PorA, Tbp, NadA, FetA, NhhA, Opa/Opc, NspA [125, 330-333, 353, 354]), but a study discerning the contribution of each of them to the bactericidal response induced by NOMV is lacking. A first approach could be to evaluate by ELISA the levels of antibodies specific for all meningococcal antigens known to elicit bactericidal response, in sera from mice immunized in this study.

At the moment, we are not able to confirm the relevance of the fH-binding site region of fHbp, in eliciting broadly bactericidal antibodies. Nevertheless, this is the first study where NOMV from isogenic mutants, differing only for the fHbp ID over-expressed, are compared, and we demonstrated for the first time that over-expressing different fHbp sequences influence the cross-reactivity and specificity of the antibody response induced by NOMV. These findings highlight the value of a rational approach to select the fHbp ID to include in a future vaccine, beyond just the protein prevalence among circulating strains. Further experiments involving SBA against a panel of meningococcal isogenic strains, whose fHbp only differ for the fH-binding site, would help to understand better the specific contribution of this surface-exposed part of the antigen to the bactericidal response induced. Moreover, it will also be interesting to compare the bactericidal response induced in mice by vesicles with fHbp IDs selected only on the basis of structure-sequence analysis. In particular, comparing fHbp IDs that share the whole amino acid sequence, except for the fH-binding site, or, *vice versa*, IDs that have the same fH-binding site, but variations in the rest of the molecule, would help clarifying the specific contribution of different parts of fHbp to the cross-protection induced.

As no extensive immunological studies have been conducted so far, another crucial aspect we wanted to investigate was the immune response elicited by meningococcal NOMV vaccine candidate. Understanding the mechanisms underlying the efficacious immune response they induce would have significant implications in improving NOMV-based vaccines; for example, it would help to improve and modulate formulations and dosages. Therefore, in Chapter 5, we investigated the immune response elicited in mice by meningococcal NOMV over-expressing fHbp.

In our experiments, NOMV with wild type hexa-acylated and mutated penta-acylated lipid A induced comparable responses, despite the dramatic difference in their ability to stimulate IL-6 release by PBMC observed *in vitro* in Chapter 3. Even though this is a really important observation regarding the immunogenicity of NOMV with mutated lipid A, it would be interesting to repeat the experiments in transgenic mice, expressing human TLR4. In fact, there is evidence that in mice TLR4 does not distinguish between hexa- and penta-acylated lipid A, as the human receptor does [235, 236].

Our data indicate a fast, strong antibody response seven days after immunization, with complement-fixing IgG subclasses rapidly induced. We observed germinal centres in the spleens of immunized mice after 7 days, and high affinity antibodies 5 months after immunization; it would be interesting to test the affinity of the early antibody response. Moreover, observation of the evolution of the splenic germinal centres during the 5 months following immunisation would contribute to elucidating the mechanistic development of the immune response. This would be possible by imaging HIC-stained spleen sections, as done in Chapter 5, after multiple time points.

Studies conducted with sera from adult humans immunized with detergent-extracted OMV, indicated that the long-term persistence of the antibody response is dependent on the number of injections. In contrast, our data suggest that the longevity of the antibody response induced by NOMV in mice does not strictly require multiple doses, and it can be enhanced by the use of alum can enhance. Confirmation of such results by repetition of the animal study could be encouraging for future optimization of vaccination schedule. In addition, it would be valuable to correlate the longevity and persistence of the antibody response, with its functionality, by testing the bactericidal activity of these sera against diverse meningococcal isolates.

In conclusion, this project contributes to the current knowledge of important elements in the field of meningococcal vaccines, which can be exploited for improvement of vaccines and vaccine design. It is also noteworthy that one of the meningococcal mutants generated within this project was selected for further development as GMMA-producing strain for potential use in Africa.

6.1. Limitations

Immunological studies conducted *in vivo* are generally performed in mice; however, this can represent a limitation, because of the differences in the murine and human physiology [355]. In this project as well, one of the limitations was the evaluation of a human vaccine in mice, and, in particular, in wild type mice. In some cases, in fact, such

constraint could be overcome, in part, by the use of transgenic mice, which would contribute in outcomes closer to what would happen in a human host. For example, transgenic mice expressing human TLR4 would be the ideal animal model to test the response to NOMV with wild type or mutated lipid A [356]. Transgenic mice expressing human factor H would also better represent the result in human administration of a vaccine containing fHbp, even though mutated to decreased binding to factor H [357].

The other major limitation, in particular in Chapter 5, was the restricted number of mice analysed. In this case, repetition of key experiments would overcome this issue.

6.2. Future work

This project led to the generation of a vaccine-production meningococcal strain, for potential use in Africa; a GMP master cell bank has been produced and released, and further development will be needed prior to its use in human.

The generation of a panel of isogenic strains, from a serogroup C meningococcus, expressing different fHbp IDs has been started in the PhD project of Dr Maria Grazia Aruta (GVGH). A first future step would be to test the bactericidal activity of sera induced by NOMV OE fHbp in this project, against those strains. Generation of further and diverse strains to test could also be evaluated. Generation of diverse NOMV-producing meningococcal mutants, OE different fHbp ID will then be taken in consideration.

APPENDIX I. BUFFERS AND MEDIA RECIPES

This appendix lists buffers and growth media used that have not been described in previous sections.

PBS 1X

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2HPO_4	1.8 mM

pH 7.4

LURIA-BERTANI (LB) BROTH

20 g of LB broth (Lennox) powder (Sigma-Aldrich) resuspended in 1 Lt MilliQ H_2O, equivalent to:

Tryptone	10 g/Lt
Yeast Extract	5 g/Lt
NaCl	5 g/L

Broth was autoclaved for 15 minutes at 121ºC.

LB AGAR PLATES

35 g of LB broth with agar (Lennox) powder (Sigma-Aldrich) resuspended in 1 Lt MilliQ H_2O, equivalent to:

Agar	15 g/Lt

Tryptone	10 g/Lt
Yeast Extract	5 g/Lt
NaCl	5 g/L

Solutes were dissolved in MilliQ H_2O, and autoclaved for 15 minutes at 121ºC.

GC AGAR PLATES

36,25 g/Lt of GC medium base (BD Difco™) resuspended in 1 Lt MilliQ H_2O, equivalent to:

Proteose Peptone nº2	15 g/Lt
Corn Starch	1 g/Lt
K_2HPO_4	4 g/Lt
KH_2PO_4	1 g/Lt
NaCl	5 g/Lt
Agar	10 g/Lt

After autoclaved (15 min, 121ºC), medium was let cooled down to 50ºC, and Supplement I (10 mL/Lt) and II (1 mL/Lt) were added.

Supplement I

Glucose	400g/L
L-Glutammine	10 g/Lt
Cocarboxylase	20 mg/Lt

Supplement II

$Fe(NO_3)_3 \bullet 9H_2O$	5 g/Lt

NEISSERIA MENINGITIDIS LIQUID GROWTH MEDIUM

L-Glutamic acid	1 g/Lt
L-cystein*	0.003 g/L

$MgSO_4 \bullet 7H_2O$*	0.06 g/Lt
$Na_2HPO_4 \bullet 7H_2O$	3.25 g/Lt
KCl	0.09 g/Lt
Casaminoacids (BD Bacto™)	10 g/Lt
Lactic acid**	7.5 g/Lt
Yeast Extract (BD Bacto™)***	2.5 g/Lt

pH 7.2, adjusted with NaOH.

* Stock solution: 1.5 g/Lt L-cystein and 30 g/Lt $MgSO_4 \bullet 7H_2O$

** Stock solution: Lactic acid 25% v/v

*** Stock Solution: Yeast extract 100 g/Lt

BUFFER FOR PURIFICATION OF RECOMBINANT FHBP

Buffer 1

NaCl	300 mM
Na_2HPO_4	50 mM
Imidazole	10 mM

pH 8

Washing buffer

NaCl	300 mM
Na_2HPO_4	50 mM
Imidazole	20 mM

pH 8

Elution buffer

NaCl	300 mM
Na_2HPO_4	50 mM
Imidazole	250 mM
pH 8	

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