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INTERROGATION OF HUMAN MONOCLONAL ANTIBODIES INDUCED BY 4C-MENB TO IDENTIFY PROTECTIVE ANTIGENS CONTAINED IN THE OMV COMPONENT

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Transparency statement:

Giacomo Vezzani is a PhD student at the University of Bologna Alma Mater Studiorum and participates in a post graduate studentship program at GSK, Siena, Italy. EF is an employee of the GSK group of companies. VS is an employee of University of Bologna, Department of Pharmacy and Biotechnology.

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Human samples:

Human samples obtained from adults' immunization in a Phase I clinical study conducted in Krakow, Poland and sponsored by Novartis Vaccine, now part of the GSK group of Companies, using two doses of multicomponent serogroup B meningococcal vaccines containing recombinant fHbp ID 1. The Clinical trial protocol was approved by the Bioethics Committee of the District Medical Doctors' Chamber in Krakow and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each of the subjects.

1 Introduction

1.1 Biology and typing of *Neisseria meningitidis*

Neisseria meningitidis is a Gram-negative, non-motile, non-sporulating and human restricted diplococcus (Figure 1). It is usually encapsulated and piliated and belongs to the group of the β-proteobacteria [1]. Exploiting its pili this bacterium can colonize the respiratory tract of human hosts, where it is commonly found as a member of the commensals in the microbiota of healthy individuals. Nonetheless, *N. meningitidis* is associated with devastating diseases as meningococcal septicemia and acute meningitidis, called Invasive Meningococcal Diseases (IMD) [2].

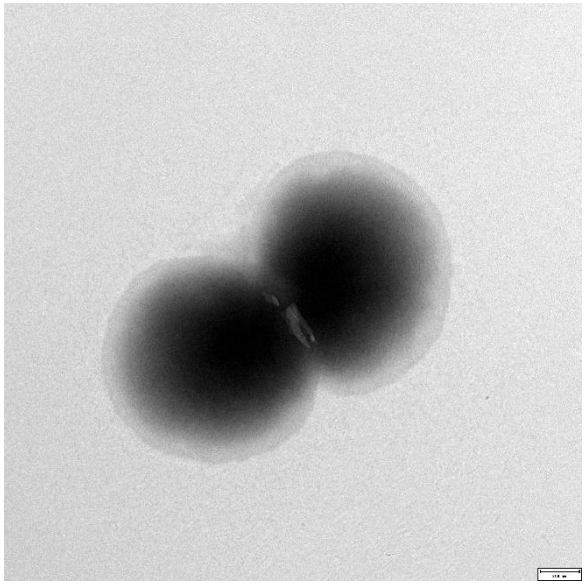


Figure 1. ***Neisseria meningitidis*.** Image obtained from a negative stain made with an electronic microscope.

The causative effects determining invasion of the host are not clear yet, but it is known that to survive into human bloodstream the bacterial capsule is essential, mediating protection from antibody/complement-mediated killing and phagocytosis [3]. For this reason all strains associated with IMD are encapsulated [4], with most of their capsule polysaccharides (CPS) derived from sialic acid, consisting of repeating units of N-acetyl-mannosamine-1-phosphate [5]. The precursor of these CPS is also the most common source of sialic acids in human membranes, explaining why the capsule is determining in masking the bacteria to host immune system, a mechanism known as molecular mimicry [6-8].

There are many different types of Meningococcus strains and in the years different methods to classify them were developed. Typing of these bacteria by CPS structure is considered the traditional approach, but nowadays the gold standard technique for bacterial classification is represented by Multi-Locus Sequence Typing (MSLT). This typing method is based on the analysis of seven different housekeeping genes loci, indexing and combining them to define sequence types (ST) and clonal complexes (CC, when up to 4 loci are identical, meaning a common ancestor between the isolates) [9, 10]. As it was extensively proven during years [11-14], this typing is able to reflect more consistently epidemiological differences between isolates [9, 15, 16], underling some STs disproportionally associated with disease, so termed hyperinvasive lineages [17].

1.2 Epidemiology of main *Neisseria meningitidis* serogroups

Meningococcal infections can occur as epidemic, hyper-sporadic or sporadic diseases, accounting for about 135000 deaths per year worldwide [4]. Despite disease patterns vary widely across geographic regions and age groups, the majority of infections are caused by few Clonal Complexes (CCs) that emerge and spread worldwide [17]. Based on the structure of the capsule polysaccharide (CPS), *N. meningitidis* can be divided in 13 serogroups, six of which (A, B, C, W135, X and Y) are responsible for the 90-99% of the meningococcal infections worldwide [18].

Serogroup A was the main responsible for meningococcal deaths during the first 20 years of 20th century in westernized country, but now it has disappeared in Europe and US while it has become the responsible for devastating outbreaks of meningitidis in sub-Saharan Africa [19]. Serogroup B caused prolonged outbreaks worldwide, compared to serogroups A and C, with significant morbidity and mortality levels. For this reason, this serogroup is the most important cause of endemic disease in developed countries (responsible of up to 80% of disease in Europe and about 30-40% in US) [4]. Serogroup C is accountable for 30% of disease in US and Europe, causing localized or spreading epidemic outbreaks [20]. Serogroup X was reported in some African countries, causing meningococcal isolated cases or outbreaks [21, 22]. Serogroup Y caused more than 25% of meningococcal disease in US in the first years of 21st century [23], causing both meningococcal pneumonia in adults and meningitidis and meningococcemia in infants, and has started to spread in South America [24], Israel and South Africa [25]. Serogroup W-135 has emerged in the last 30 years, causing epidemic meningococcal disease in Saudi Arabia [26], Africa, South America [27] and Burkina Faso [28].

Mortality rate of meningococcal infections vary, depending on type and severity of invasive disease, having up to 55% for fulminant septicemia and from 5% to 25% for meningitidis, depending on its association with septicemia [29]. Even when it is not fatal, meningococcal infection can lead to life-long impairment in physical, cognitive or psychological functioning (such as hearing loss, lower IQ, psychological disorders and major disabling deficits) [30]. To become a threat for humans, the invasion of host through mucosal surfaces is the determining factor for *N. meningitidis* pathogenesis and occurs thanks to bacterial proteins called virulence factors [31].

1.3 *Neisseria meningitidis* pathogenesis

Different strategies are used from this bacterium to invade, survive in the bloodstream and spread in the host. Capsule is fundamental to survive in different environments and its expression undergoes genetic regulation through all the different phases of *Neisseria meningitidis* pathogenesis [32]. While on one hand capsule inhibits biofilm formation, so its expression is reduced during bacterial adhesion, on the other hand its presence is essential to bacterial survival in the bloodstream, making capsule's genes overexpressed during host's invasion [33].

The first step in meningococcal pathogenesis is the adhesion to nasopharyngeal epithelial cells, mediated by several different proteins, as Type IV pili [34]. Pili are a widespread colonization factors in bacteria (especially in Gram-negative ones) and Type IV pili promote several functions, such as attachments to surfaces, aggregation, motility and DNA uptake. They are multimeric structures that span both the inner and the outer membrane (PilE) and then create a channel (PilQ) that extrude from the bacterial surface [35]. Through PilE and PilV, a minor pilin protein involved in internalization of bacteria into epithelial cells [36], Type IV pili are also involved in adhesion of *Neisseria meningitidis* to the brain endothelia, through contact with the cell receptor CD147 [37]. Nonetheless, the exact role and function of other pili components still remains unclear [38]. Other proteins involved in the adhesion of meningococcus to host cells are the opacity proteins Opa and Opc, which bind the CarcinoEmbryonic Antigen Cell Adhesion Molecule (CEACAMs) and some components of the extracellular matrix (fibronectin, laminin and plasmatic vitronectin [2, 39]) respectively. Both these proteins are able to bind also to cell-surface-associated heparan sulfate proteoglycans (HSPGs [40, 41]). *Neisseria* adhesin A (NadA) is also involved in the adhesion of meningococcus. Interestingly, transcriptional regulation of NadA gene occurs through the transcriptional regulator NadR (repressor) and binding of NadR to its DNA target is attenuated by a molecule naturally present in human saliva,

determining the expression of NadA *in vivo* when the bacteria need this protein the most to adhere to human epithelia [42]. Lastly, two other proteins involved in the adhesion mechanism are *Neisserial* hia homologue A (NhhA) and Adhesion and Penetration Protein (App) [43].

Once the adhesion of *N. meningitidis* takes place, its internalization happens into non-phagocytic cells, following formation of ezrin and moesin enriched cellular protrusions at the site of bacteria attachment [44]. It has been observed that an inflammatory context favors the internalization of these bacteria and the external loops of PorB are involved in this mechanism, stimulating the toll-like receptors 2 (TLR2), favoring the invasive features of pathogenic *Neisseria meningitidis* strains [45].

In the bloodstream, bacteria survival relies on the capacity to acquire nutrients, through both dedicated transport systems (such as the uptake of L-glutamate by the ABC transporter GltT [46] for the protection from polymorphonuclear neutrophil leukocytes-mediated oxidative stress and killing [47]) or membrane porins (such as the β-barrel proteins PorA and PorB [48], involved mainly in zinc acquisition [49]). The other fundamental characteristic to survive in the bloodstream is to evade complement-mediated killing, for which *N. meningitidis* has evolved different mechanisms, based on:

I) Inhibit the formation of membrane attack complex (MAC) on bacterial surface, acting at different levels of the complement cascade, with factor H binding protein (fHbp), *Neisseria* surface protein A (NspA), Porin B2 (PorB2), Porin A (PorA), NalP and *Neisserial* heparin binding antigen (NHBA) [33];

II) Resist serum-killing by lipooligosaccharide (LOS) present on the outer membrane, with serum killing increasing after LOS disruption [3], and capsule shielding activity, which determine a lower activation of the alternative complement pathway [50, 51].

Once in the bloodstream the meningococcus can reach the Central Nervous System (CNS) through the choroid plexus, where is present the Blood Brain Barrier (BBB). Attachment to endothelial cells of the BBB is allowed by pili and, following attachment, it is possible both that a small number of bacteria is naturally internalized or that the thigh junctions are disrupted by cell signaling mediated by pili, allowing the spreading of *Neisseria meningitidis* through CNS [52].

Due to the high mortality rate of this pathogen and the speed of its pathogenesis, the interest in vaccine development against meningococcus was very high starting from the second half of 20th century.

1.4 Vaccine development against *Neisseria meningitidis*

The first attempts to develop a vaccine specific for *Neisseria meningitidis* was done by the Walter Reed Army Institute of Research (WRAIR) in the mid-60s. The US army was indeed interested in a meningococcus vaccination, because of the frequent outbreaks in their young troops [53]. Taking advantage of the existing knowledge on the protective role of pneumococcus (another capsulated bacteria, responsible for the pneumococcal pneumonia) capsule, WRAIR developed in 1971 the first group C meningococcal (MenC) vaccine based on the capsular polysaccharides of this serogroup [54]. In less than 5 years from the release of the first vaccine for MenC, a group A meningococcus vaccine was also available [55] and in the early 80s a quadrivalent meningococcal polysaccharide vaccine was available too, covering the ACWY strains [53]. Therefore, conjugated vaccines conferred protection to the main pathogenic meningococcal strains, with the exception of group B [56].

The main issue in developing a polysaccharide-based vaccine against serogroup B meningococcus (MenB) resides in the same sialylation of its main capsule polysaccharides, the α(2,8) N-acetyl muraminic acid, with a sialic acid shared with human tissues [57, 58]: a vaccine based on this molecule would have caused auto-immune diseases. For this reason, a vaccine against MenB required a much longer research work. At the end of the 80s and during the 90s different MenB vaccines based on detergent extracted bacterial Outer Membrane Vesicles (OMVs) started to show protection in human clinical trials. Indeed, OMVs-based formulations proved efficacious to protect against MenB in Norway [59, 60], Chile [61], Cuba [62], and New Zealand [63] (Table 1.1).

Country or region	Vaccine	% of cases in country or region of vaccine strain*	Study type	Sample size (n)	Number of doses given	Time interval under observation	Age group	Efficacy** (%)	95% Confidence Interval (CI)
Norway[11,16,17]	Norwegian (NIPH)		Randomised, double-blind placebo-controlled trial	171,800	2	10 months	Secondary school students	87	62-100%
						29 months		57	21-87%
Cuba[18]	Cuban (FI)	95%[18]	Randomised, double-blind, placebo controlled trial	106,251	2	16 months	10–14 yrs	83	42-95%
Sao Paulo, Brazil[19]	Cuban (FI)	44%[19]	Case-control study	112 cases; 409 controls	2	1989–1991	4–6 yrs	74	16-92%
							2–3 yrs	47	-72-84%
							3 months < 2 yrs	-37	<-100-73%
Iquique, Chile[14]	Chilean (WRAIR)		Randomised, double-blind controlled trial	40,811	2	20 months	5–21 yrs	70	14-91%
							1–4 yrs	-23	<-100-73%

* Where available
** Norway, Cuba, and Sao Paulo – Efficacy against group B disease; Chile – Efficacy against group B disease with serotype1 5 and/or subtype P1.3 only

Table 1.1 effectiveness on OMV-based formulations against MenB. Results from the clinical studies of the OMV-based vaccines utilized in different countries (adapted from [64]).

1.5 The next step of the OMV-based vaccine: 4C-MenB vaccine's history

While OMV-based vaccines proved to be fundamental to control outbreaks of MenB throughout the world, it suddenly started to emerge that their major constraint was their limits of efficacy due to serosubtype variability of PorA, the MenB major outer membrane protein [65]. An attempt to overcome this limit was conducted using a multivalent formulation composed of OMV derived from PorA mismatched strains but, due to inconsistency of elicited immune response against all the strains employed, this project was abandoned [66]. The breakthrough in the history of MenB vaccine arrived with the whole-genome sequencing (WGS) techniques, which gave birth to the reverse vaccinology approach [67]. This term indicate the use of computational methods to identify vaccine candidates (mostly surface proteins) that would probably induce a protective response in the vaccinees [68]. Thanks to this new methodology, a broadly cross-protective (i.e. against a vast collection of strains) MenB vaccine was developed and is now available: the 4C-MenB vaccine [67].

The antigens included in 4C-MenB's formulation are the detergent extracted outer membrane vesicles (DOMV) from New Zeland 98/254 MenB epidemic strain and 5 recombinant proteins: NadA (variant 3.1), fHbp (variant 1.1), NHBA (peptide 2), Genome-derived Neisserial antigen (GNA) 2091 and 1030 [69]. In order to facilitate large scale manufacturing, 4 of the 5 recombinant proteins were genetically fused in 2 different constructs (NHBA-GNA1030 and fHbp-GNA2091), while NadA was leaved in its homotrimeric form because studies revealed the loss of its protective role when it was genetically fused to other proteins [70]. DOMVs were included in vaccine's formulation because in clinical trials where 4C-MenB formulation was studied in presence or absence of DOMV, their role in controlling the epidemic of serosubtype 1.4 PorA meningococcal group B epidemic in New Zealand resulted foundamental [71]. NadA, fHbp and NHBA have been deeply characterized structurally, functionally and immunologically, underling their role as key virulence factors of several pathogenic MenB strains [69].

Factor H binding protein (GNA1870) is a surface lipoprotein expressed by most pathogenic MenB strains isolated so far [72]. It was identified as a vaccine candidate both by reverse vaccinology and by the approach of membrane fractionating [73], a method that was utilized to generate the other licensed

vaccine available today against MenB (rLP2086, also known as *Trumemba* that will be briefly discussed later) [74]. This protein binds the human factorH, inhibiting the alternative pathway of the complement cascade and so mediating serum resistance of MenB in the bloodstream [75]. Based on their immunogenicity, fHbp variants are classified into 3 different groups: fHbp-1, fHbp-2 and fHbp-3, that can be further divided in subvariants (fHbp-1.x, fHbp-2.x and fHbp-3.x) [76]. Structure of fHbp, determined both with NMR [77] and X-ray crystallography [78], revealed one N-terminal antiparallel beta-sheet connected by a short linker to a C-terminal antiparallel beta-barrel. These 2 domains are conserved amongst all the different variants of fHbp present in the MenB strains, underlying the importance of this protein [76]. 4C-MenB vaccine contains the fHbp variant 1.1, which is one of the most common in European MenB strains [79].

NHBA (GNA2132) is, like fHbp, a surface lipoprotein ubiquitous in all MenB isolates. It was discovered through reverse vaccinology [80] and protects unencapsulated meningococci from complement-mediated killing, binding glycosaminoglycans (*in vivo*) or heparin (*in vitro*) [81]. The structure of NHBA is resolved only for its C-terminal domain (an antiparallel beta-barrel, similar to the one of fHbp), because its N-terminal region is unstable [82]. Between the N- and the C-terminal regions there is an arginine rich linker which binds heparin [83]. Based on sequence diversity between MenB NHBA proteins, a subdivision into a group of subvariant (or peptides) families was made, indicated as NHBA-x [84]. Amongst NHBA peptides, NHBA-2 (which is contained in 4C-MenB formulation) is the most frequently expressed based on molecular epidemiology studies conducted across the globe [72].

NadA (GNA1994) is a trimeric autotransporter belonging to the oligomeric coiled-coil adhesin (Oca) family [85], which mediates adhesion and entry into epithelial cells [86]. Although its 3D structure is not resolved yet, from studies on Oca members it is thought that NadA has a “head” domain at its N-terminal region, which is the one that mediates binding to epithelial cells, followed by a long homotrimeric coiled-coil regions that anchor the protein to the outer membrane of the bacterium [87]. Differently from fHbp and NHBA, NadA gene is not present in all MenB strains, but it is associated with 3 out of the four hypervirulent serogroups of these bacteria, whereas it is very rare in carriers strains [85]. Of the 5 different variants of this protein, the first 3 (NadA-1, NadA-2 and NadA-3) determine cross-protective immune responses (indeed, recombinant NadA-3 is contained in 4C-MenB vaccine), while the remaining 2 (NadA-4 and NadA-5) are associated with carriers strains [88].

The other licensed vaccine against MenB, rLP2086, is composed of two different variants of fHhp: fHbp-3.45 and fHbp-1-55 [89]. Even if it had a good grade of coverage in different ages groups [90],

concerns were raised on the possible selection of escape mutants that this vaccine could act on a very plastic bacteria as meningococcus [91].

1.6 Role of the DOMV in 4C-MenB induced protection

DOMV from NZ98/254 strain in 4C-MenB formulation are shreds of outer membrane generated treating the bacteria with deoxycholate detergent. Application of this protocol ensure two favorable outcomes:

I) Extractions of DOMV at high yields
II) Removal of toxic LOS from DOMV

The resulting vesicles comprise more than 100 proteins (mostly OMPs, identified by mass spectrometry [92]), with the following 4 that account for about 80% of DOMV's global protein content: PorA, PorB, RmpM and Opa/Opc [93].

Since it is one of the most represented proteins, it was not surprising that in 2006 Martin and colleagues observed that the major target for immune response elicited in human vaccinees by DOMV derived from NZ98/254 strain was the variable region (VR) 2 of the protein PorA [94]. This protein consist of a beta-barrel spanning the outer membrane with 8 surface exposed loops, where loop 1 and loop 4 are highly variable and constitute, respectively, VR1 and VR2 [95]. Based on the sequence in VR1 and VR2, MenB strains can be divided in serogroups with the following nomenclature: P1.VR1-VR2, where P1 stands for PorA [96].

Nonetheless, DOMV surface contains many other proteins and there are studies on their capacity to induce antibody-mediated protection. The proteins identified so far, by screening of vaccinees sera, are: ferric enterobactin receptor (FetA [97]), PorB, OpcA, PilQ, RmpM (which are all OMP) and the two lipoproteins BamC and GNA1162 [98]. Intriguingly, the importance of other antigens on DOMV surface in eliciting protective immune response was already emerging since the early phase II trials of 4C-MenB. Indeed, in this study a cross-killing effect of vaccinees sera on different MenB strains was observed only when sera were obtained from immunization with the formulation containing the DOMV of NZ98/254 and not when the same formulation was depleted of DOMV [99].

Overall, evidences collected in the last decade suggest a protective role played by different proteins contained in the DOMV component of 4C-MenB vaccine, but their exact contribution still needs to be fully understood and characterized.

1.6.1 The reverse vaccinology 2.0

We are now in middle of the so-called Reverse Vaccinology 2.0 age. This terms refer to the new approaches and technologies that are being utilized to interrogate the immune response to identify new and more effective vaccine candidates [100].

The main technological breakthroughs that enabled this advance in the vaccinology field are:

- The ability to isolate mAbs from single B-cells, coupled with the deep knowledge of pathogenesis of viruses and bacteria;
- Conformational epitope mapping, which allows the deep investigation of the protective epitopes of selected antigens, studying the binding characteristics of functional mAbs [101];
- New computational approaches able to use the information of the previous points to design novel immunogens to elicit targeted and more potent immune responses [102].

Focusing on the first point, it is fundamental to consider that most vaccines against infectious diseases works thanks to their ability to stimulate the B-cell response to a specific pathogen's antigen over time. Thus, the isolation of mAbs coming from B-cells derived from human vaccinees is allowing the deep understanding of the immune system response to vaccine antigens [100]. mAbs identification can be performed both from memory B cells (MBCs) and plasmablasts (PBs), thanks to the possibility to screen the mAbs for binding and functionality without knowing their sequence [103, 104]. The power of these techniques is shown by the fact that many vaccine candidates were identified with these methods, such as: the pentameric complex of Human Cytomegalovirus [105] or the pre-fusion protein of the Respiratory Syncitial Virus [106].

The combination of knowledge acquired on mAbs, computational methods and microbial genetics will open the road to the application of Reverse Vaccinology 2.0 to pathogenic bacteria, such as *Neisseria meningitidis* [107].

1.7 Structural and functional overview on human antibodies

Antibodies are Y-shaped proteins, belonging to the immunoglobulin superfamily (IgSF), produced by the B-cells of the immune system [108]. They are made of two identical Ig heavy chains (H) and two identical Ig light chains (kappa, or lambda) [109]. Each chain is composed of a variable (V) N-terminal IgSF domain (consisting 2 sandwiched β-pleated sheets pinned together by a disulfide bridge between 2 conserved cysteines) and one (for light chain) or more (for heavy chain) constant C-terminal IgSF domains (Figure 1.2) [110].

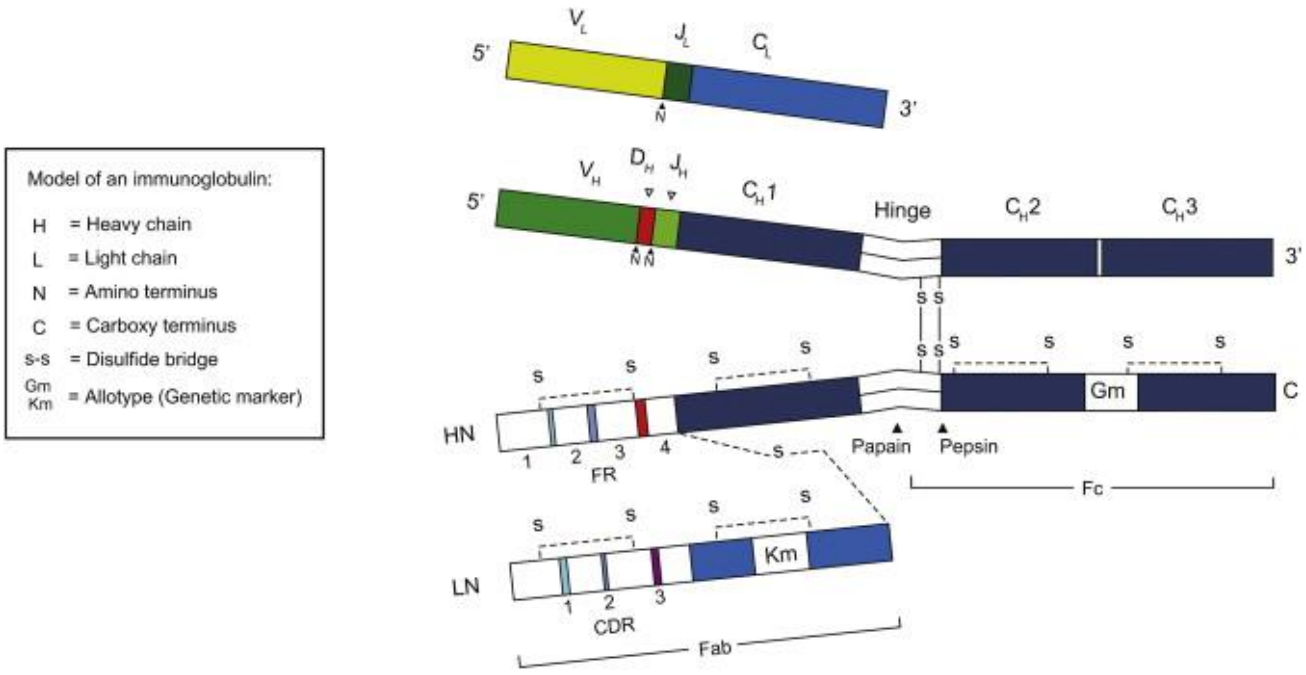


Figure 1.2. Schematic representation of an antibody. A view of the Ab structure at the nucleotide level (H and L chain at the top) or protein sequence level (H and L chain at the bottom). Arrows indicate cutting sites of listed enzymes. CDR is for complementary determining region and FR is for framework region, which are the constant parts into the V region (figure adapted from [109]).

In most cases, interaction of IgH with IgK/L generates two identical binding sites (called paratopes), located in the fragment variable region (Fab). Paratopes are specific for a defined region of a single antigen (called epitope)[111]. Ag-specificity is mainly driven by genetic recombination of 3 different genetic regions, coding for the variable part of the antibodies which is determined by: germline variable (V), diversity (D, present only for IGH) and joining (J) gene segments [112]. The fragment constant

(Fc) portion of the Ab defines the isotype, which allows the grouping of Abs into different classes, named: IgA, IgM, IgD, IgG, IgE [113].

1.8 Serum bactericidal assay (SBA) as correlate of protection for MenB infection

The *in vitro* serum bactericidal assay (SBA) is the correlate of protection to evaluate vaccine efficacy in first clinical trials of vaccines against MenB, as incidence of meningococcal infections are not so frequent to measure by dedicated clinical trials the efficacy of a new licensed vaccine [114]. This technique relies on the activation of the classical pathway of the complement cascade, in the presence of antibodies specific for *N. meningitidis*. This activation starts with the interaction of the complement component 1q (C1q) with the Fc portion of antibodies bound to the surface of the meningococcal strain of interest and determines bacteriolysis through the formation of the Membrane Attack Complex (MAC) in the bacterial membrane. SBA titre of hyperimmune serum, or mAbs, is the reciprocal of the last dilution where more than 50% of bacteria were killed in comparison to the control with no sera/mAb added (Figure 1.3) [115].

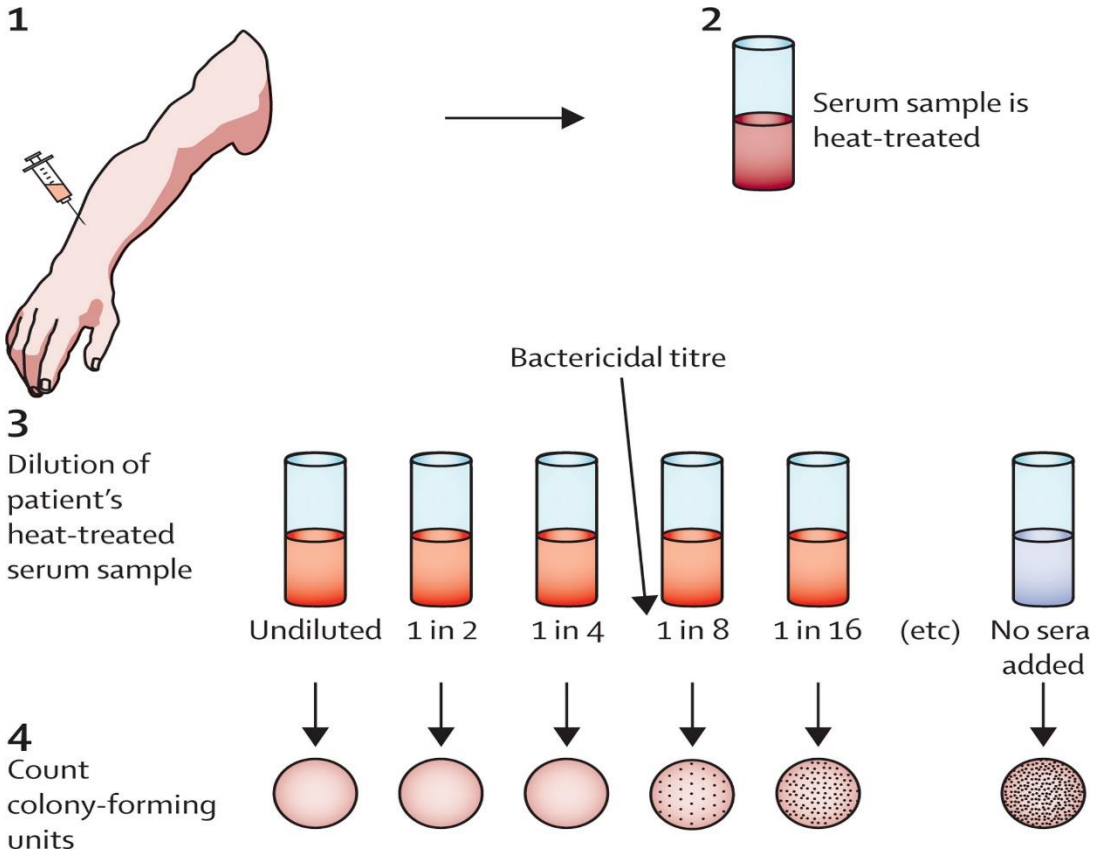


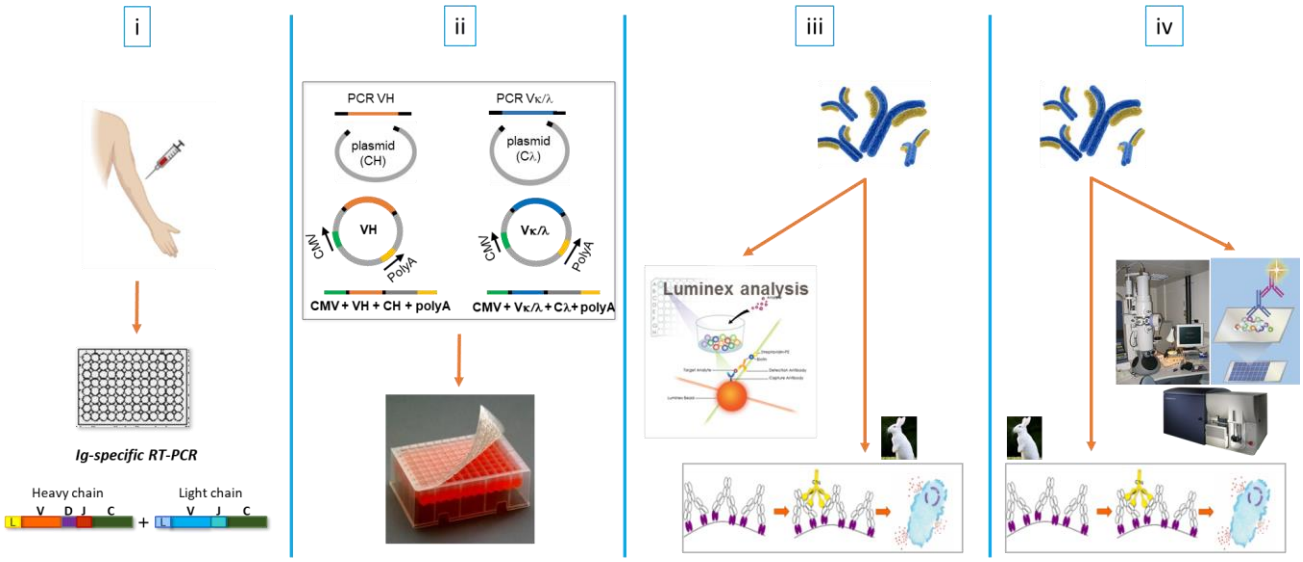
Figure 1.3 schematic illustration of SBA. Complement of patient's sera is heat-inactivated and then serially diluted. Dilutions are incubated with bacterial strain of interest and exogenous complement source and plated on specific plates to allow colonies growth. Colonies are then counted and SBA titre is assessed as the last dilution able to kill more than 50% of colonies that are counted in the control plate (figure adapted from [116]).

To license a vaccine, generally more than 90% of vaccinated individuals must have a SBA titre higher than 4 (hSBA, performed with human complement, used as the gold-standard technique for correlate of protection against meningococcus) or 8 (rSBA, performed with rabbit complement, often used in preclinical studies) [117].

The combination of a modern techniques, as the Reverse Vaccinology 2.0, and a classical assay, as the SBA, can help the identification of protective antigens in new vaccines and a better understanding of the already licensed ones (as the 4C-MenB vaccine).

2 Aim of the study

In the present work, we aimed to expand our knowledge on immunogenic and protective antigens presented by the DOMV component in 4C-MenB vaccine as schematized in the figure underneath. To address this question, we single-cell sorted Plasmablasts (PBs) from peripheral blood mononuclear cells (PBMCs) derived from blood of 4C-MenB vaccinees (i). Variable regions of heavy and light chains encoded in each sorted PB were amplified and cloned into specific expression plasmids, containing the constant fragments of IgG to reconstitute a complete antibody chain, which were used to transfect mammalian cell lines to produce mAbs in raw culture supernatant (ii). Each mAb was screened for binding to 4C-MenB single components and recombinant PorA (rPorA) derived from NZ98/254 strain, by Luminex assay, and for functional activity by rSBA (iii). DOMV-positive mAbs were expressed in larger quantities, purified and characterized for binding by OMV/protein array, electron microscopy (EM) and fluorescent-activated cell sorter (FACS) and for functionality by rSBA (iv).



3 Materials & Methods

Human peripheral blood mononuclear cells (PBMCs) isolation from sera of 4C-MenB-vaccinated subjects

Human samples were obtained from adults immunization in a Phase I clinical study conducted in Krakow, Poland and sponsored by Novartis Vaccine, now part of the GSK group of Companies, using two doses of multicomponent serogroup B meningococcal vaccines 4C-MenB. Blood to isolate PBMCs was collected 7 days after the second immunization performed on the vaccinated subjects. The Clinical trial protocol was approved by the Bioethics Committee of the District Medical Doctors' Chamber in Krakow and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each of the subjects.

Single cell sorting of PBs

Frozen human PBMCs were thawed at 37°C in a water bath and stained for 20 minutes at room temperature (RT) with Live/Dead Fixable Acqua (Invitrogen; ThermoFisher Scientific) in a final volume of 100 µl. After a wash with phosphate buffer saline (PBS), unspecific binding were saturated with 50 µl of 20% rabbit serum in PBS with an incubation of 20 minutes at 4° C. Cells were washed with PBS and stained, for 30 minutes at 4° C, with 50 µl of the following mix: CD19 v421 (L.8270584; BD) 1:160, CD20PerCP-Cy5.5 (L.7167885; BD) 1:80, CD27 PE (L.8332701; BD) 1:15, CD38 APC (L.B255162; Biolegend) 1:40, IgD Alexa700 (L.8249618; BD) 1:10 in PBS, 1% FBS. After a wash with PBS 1% FBS cells were resuspended in PBS/EDTA 2,5 mM and single-cell sorted with FACSAria III instrument (BD) in 96-well plates containing the reaction mix to lysate cells and perform the reverse transcription PCR (RT-PCR).

Plasmids generation with amplified variable heavy (VH) and variable light (VL) from sorted single cells

RT was performed on PBs sorted in the following mix: RNaseOUT (10777019; ThermoFisher Scientific), FS buffer (4480724; ThermoFisher Scientific), DTT (P2325; ThermoFisher Scientific), NP40 cell lysis buffer (FNN0021; ThermoFisher Scientific), SuperScript IV (11756500;

ThermoFisher Scientific), $MgCl_2$ (AM9530G; ThermoFisher Scientific), dNTP (R0186; ThermoFisher Scientific) and the primers listed as CH, CK and CL in table 1.

After the RT step, 2 subsequent nested PCRs were performed, using a mix composed by: Q5 polymerase master mix 2X (M0492L; New England Biolabs), DEPC treated nuclease-free water (9601-OP; Sigma) and primers (called 1a for the first PCR and 1b for the second one) as listed in Table 3.2.

Amplified products were then purified using the Pro-nex size-selective purification system (NG2001; Promega), following producer's guidelines, quantified by Nanodrop2000 and ligated at 50°C for 20 minutes with the open and linearized plasmids of interest (pcDNA3.1H and pcDNA3.1L for VH and VL, respectively, obtained from TWIST), utilizing the NEBuilder HiFi DNA assembly master mix (E2621X; New England Biolabs).

Ligation products were either amplified with primers annealing on the plasmid or used to transform *E. coli* TOP10 competent cells (C404010; Invitrogen). In the first case the PCR products generated (called TAP, Transcriptionally Active PCR) included the CMV promoter, the complete antibody heavy or light chain sequence and the terminator. PCR cicle utilized is shown in Table 3.1. In the second case, bacteria were grown o.n. in LB + Amp [100µg/mL] and plasmids extracted with the EZNA plasmid DNA Mini kit I (D6942; OMEGA Bio-Tek), following the manufacturer instructions.

CYCLE		
98°	30''	
98°	10''	X35
62°	20''	
72°	1'30''	
72°	5'	
10°	∞	

Table 3.1: TAP PCR cycle utilezed to amplify mAbs coding regions. The column on the right represents the number of cycle for each step

NAME	SEQUENCE
IgG CHrevRT	GGAAGGTGTGCACGCCGCTGGTC
IgA CHrevRT	CCTGGGGGAAGAAGCCCTGGACC
IgM CHrevRT	GGGAATTCTCACAGGAGACGA
CK revRT	CCTCTAACACTCTCCCCTGTTGAAG
CL revRT	CATTCTGYAGGGGCMACTGTCTTCTC
L-VH1_VH7 fw	CACTCCCAGGTGCAGCTGGTGCAG
L-VH2 fw	TGGGTCTTRTCCCAGGTCACCTTG
L-VH 3fw	AAGGTGTCCAGTGTSAGGTGCAG
L-VH4_6 fw	GTCCTGTCCCAGGTGCAGCTGCAG
L-VH5 fw	GAGTCTGTTCCGAGGTGCAGCTGG
IgG CH rev	GTGCCAGGGGGAAGACCGATG
IgA CH rev	GCMGAGGCTCAGCGGGAAGAC
IgM CH rev	GAGACGAGGGGGAAAAGGGTTG
L-VK1 fw	CAGGTGCCAGATGTGHCATCCAG
L-VK2 fw	CTGGATCCAGTGSGGATATTGTGATG
L-VK3 fw	CCCAGATACCACCGGAGAAATTGTG
L-VK4 fw	CTCTGGTGCCTACGGGGACATCGTG
L-VK5 fw	CTGATACCAGGGCAGAAACGACAC
CK rev 1st	GAACACTCTCCCCTGTTGAAGCTCTTTG
L-VL1 fw	GGTCCTGGGCCCAGTCTGTGCTG
L-VL2 fw	GGTCCTGGGCCCAGTCTGCCCTG
L-VL3 fw	TCTGTGRCCTCCTATGAGCTGAC
L-VL4_VL5_VL9 fw	CTCTCGCAGCCTGTGCTGACTCA
L-VL6 fw	GTTCTTGGGCCAATTTTATGCTG
L-VL7 fw	GGTCCAATTCTCAGGCTGTGGTG
L-VL8 fw	GAGTGGATTCTCAGACTGTGGTG
L-VL10 fw	GTCAGTGGTCCAGGCAGGGCTGAC
CL rev 1st	GTGCTCCCTTCATGCGTGACC
C134_VH1_5_7	GAAGGCGTGCACTGCCAGGTGCAGCTGGTGCAGTCTG
C134_VH2	GAAGGCGTGCACTGCCAGGTCACCTTGAAGGAGTCTGGTC
C134_VH3	GAAGGCGTGCACTGCGAGGTGCAGCTGGTGGAGTCTGGGGGAG
C134_VH4_6_a	GAAGGCGTGCACTGCCAGGTGCAGCTGCAGGAGTCGGG
C134_VH4_6_b	GAAGGCGTGCACTGCCAGGTGCAGCTGCAGCAGTGGGG
IgG CH rev	CAGGGGGAACACGCTTGGGCCCTTGGTGGARGC
IgA CH rev	CAGGGGGAACACGCTCTTGGGGCTGGTCGGGGA
IgM CH rev	CAGGGGGAACACGCTTGGGGCGGATGCACTCCC
C135_VK1	CCGGACACCACCGGTGCCATCCAGATGACCCAGTCTCCATC
C135_VK2_a	CCGGACACCACCGGTGATATTGTGATGACCCAGACTCCACTCTC
C135_VK2_b	CCGGACACCACCGGTGATATTGTGATGACTCAGTCTCCACTCTC
C135_VK3_a	CCGGACACCACCGGTGAAATTGTGTTGACACAGTCTCCAG
C135_VK3_b	CCGGACACCACCGGTGAAATTGTGATGACGCAGTCTCCAG
C135_VK4	CCGGACACCACCGGTGACATCGTGATGACCCAGTCTCCAG
C135_VK5	CCGGACACCACCGGTGAAACGACACTCACGCAGTCTCCAG
C135_VK-Rev2	GTCGCTGGGGGGGAAGATGAAGACAGATGGTGCAGCCACAGTTC
C080_VL1	CCGGACACCACCGGTCAGTCTGTGCTGACTCAGCCGCCCTCAG
C080_VL2	CCGGACACCACCGGTCAGTCTGCCCTGACTCAGCCTGCCTCCG
C080_VL3_a	CCGGACACCACCGGTTCCTATGAGCTGACACAGCCAC
C080_VL3_b	CCGGACACCACCGGTTCCTATGAGCTGACTCAGGACC
C080_VL4	CCGGACACCACCGGTCAGCCTGTGCTGACTCAATCGTCCTCTG
C080_VL5-9	CCGGACACCACCGGTCAGCCTGTGCTGACTCAGCCRACTTC
C080_VL6	CCGGACACCACCGGTAATTTTATGCTGACTCAGCCCCACTC
C080_VL7	CCGGACACCACCGGTCAGGCTGTGGTGACTCAGGAGCCCTC
C080_VL8	CCGGACACCACCGGTCAGACTGTGGTGACCCAGGAGCCATC
C080_VL10	CCGGACACCACCGGTCAGGCAGGGCTGACTCAGCCACCCTCGG
C080_CL1_rev	GGAGCTGGGGGGGAACAGAGTGACCGTGGGGTTGGCCTTGGGCTGACC
C080_CL1_rev	GGAGCTGGGGGGGAACAGAGTGACCGAGGGGGCAGCCTTGGGCTGACC

Table 3.2: Primers list for RT-PCR (light yellow), PCR1a (light blue) and PCR1b (light green).

Expression of HumAbs from Transcriptionally Active PCR fragments (TAPs) or purified plasmids

Two similar protocol were followed, depending on small scale expression (1 ml) and large scale expression (30 or 60 ml). Briefly, for small scale expression TAP fragments were generated by a PCR performed as in the Table 1.1 and purified, while for large scale expression purified plasmids were utilized as transfecting DNA. Each purified TAP/plasmid was diluted in adequate volume of Opti-MEM (31985062; Gibco) and incubated for 15-30 minutes with the same volume of medium with addition of 5% Expifectamine (A14524; Gibco). Following this incubation, the mixture was added to 800 µl of Expi293 Expression medium (A1435104; Gibco), containing 2,0 x 10^6 Expi293F cells (100044202; Gibco) in a 96/w plate (small scale expression), or to 25 ml of 3,0 x 10^6 cells in a T250 flask (large scale expression), that was incubated overnight at 37°C, 5% CO_2, 1000 rpm (96w plate) or 125 rpm (flasks) shaking. 18-22 hours after transfection Enhancer I and Enhancer II (A14524; Gibco) were added to each plate/flask that was put back in the incubator.

From 5 to 7 days from transfection, culture supernatant containing the expressed HumAbs was collected, after a centrifugation at 1000 x g for 10 minutes at RT, and stored at -20°C for the subsequent analysis.

Luminex binding screening of HumAbs in colture supernatants

In order to perform the screening, recombinant proteins and DOMVs included in 4C-MenB formulation were obtained in PBS and coupled to different Luminex beads (MC12XXX; MagPlex). Beads utilized in the screening with the respective antigens (Ags) are listed in table 3.3.

5 µl of transfection supernatant were incubated with 5 µl of the mix of Ags diluted 1:7 in PBS - 0,05% Tween20 (P1379; Sigma-Aldrich) - 1% BSA (A2153; Sigma-Aldrich) for 1 hour in agitation (1200 x rpm). Beads were washed 3 times with PBS - 0,05% Tween20 and incubated for 45 minutes with R-Phycoerythrin-AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Fcγ Fragment Specific (109-116-098; Jackson Immunoresearch). After 3 washes, beads are resuspended in PBS - 0,05% Tween20 and signals are acquired by BioPlex 3D suspension array system (#BioPlex3D; BIO-RAD).

Antigen	Region	Catalog #
NadA	12	MC10012
fHbp-GNA2091	27	MC10027
NHBA-GNA1030	30	MC10030
PorA MenB NZ	45	MC10045
OMV MenB NZ	26	MC10026

Table 3.3: List of antigens utilized in this study with corresponding beads regions to which were coupled.

SBA assays

Bacteria strains of interest were streaked on a round chocolate agar plate (43101; Biomerieux) with a 1µl inoculating loop and incubated for 15-20 hours at 37°C with 5% CO_2. The bacteria grown from the agar plates were collected and inoculated into 7 ml of Mueller-Hinton Broth (MHB, produced in house, containing 0.25% (w/v) glucose (G8270; Sigma-Aldrich)) in a 14 ml polypropylene tubes at a starting OD_{600} = 0,05-0,06. Bacteria were incubated at 135 rpm, 37°C with 5% CO_2 and the incubation was stopped when it reached OD_{600} = 0,24-0,26 (= 10^9 CFU/ml). A working suspension of bacteria was prepared by diluting bacteria in working buffer (PBS 1% BSA 0,1% glucose) at 10^5 CFU/ml (1:10.000). A 96/w round-bottom plate was prepared with serial dilutions (step 2, starting from 1:2) of either Expi293 culture supernatants or purified mAbs, inserting in the last 2 wells these controls:

1) Complement Dependent Control (CDC), to measure killing by complement alone in the absence of antibodies,

2) Complement Independent Control (CIC), to measure killing by sample alone in presence of heat inactivated complement and either Expi293 culture supernatants or purified mAbs

These controls were used to calculate the average of colonies after 60 minutes of incubation (T_{60}). The 50% of this value corresponds to the number of colonies considered to define the higher bactericidal sample dilution. Bacteria working dilution (10^5 CFU/ml) was added to each well, together with baby rabbit complement (excluded CIC wells), mixed gently for few seconds and incubated at 37°C with 5% CO_2 for 60 minutes. At T_{60} 7µl of each well were spotted on squared Petri dishes with MH agar produced in house. All wells were plated in duplicate and plates were incubated overnight at 37C° with 5% CO_2. The following day the number of colonies in each spot were counted and the last reciprocal

sample dilution that gives more than 50% decrease in the number of bacterial colonies counted in the spot was considered as the bactericidal titer (SBA) of the sample tested.

mAbs expression and purification from colture supernatants

The sequence of VH and VL encoding for mAbs of interest were obtained by Sanger or next generation sequencing (NGS), allowing their order from an external provider (GeneArt or TWIST Biosciences) as codon-optimized, full length immunoglobulins encoding expression plasmids. For the subsequent expression in larger quantities, transfection volume was scaled up to 60 ml for each HumAb of interest, according to manufacturer's instructions.

Culture supernatants were then sterile-filtered with a 0,22 um filter and HumAbs were purified with Protein G–Sepharose Fast Flow (P3296; Merck). Briefly, 1,5 ml of resin was placed into a 15 ml column, washed with 15 ml of sterile water and equilibrated with 30 ml of PBS by gravity flow. Colture supernatant was then passed through the resin and , after 2 washing steps in PBS, mAbs were collected in acidic conditions using 0.3 M Glycine buffer (pH=2,1) to elute them from the resin. pH was restored to 7,4 with Tris buffer (pH=9) and then buffer was exchanged by PD10 columns (GE17-0435-01; Merck), according to manufacturer's instructions, to store mAbs in PBS. mAbs concentration was quantified by Nanodrop2000.

Protein array protocol

Slides were prepared as described in Scietti et al. (2016 Scientific reports) with few modifications.

Nitrocellulose was removed from the border of the slide, allowing to mark them with a liquid blocker super PAP pen (ab2601; Abcam), marking the slide's perimeter. Each mini-array was saturated with 500 µl of BlockIt blocking buffer (BKT; ArrayIt) and incubated for 1 hour at RT in a dark humid chamber. After this incubation, 300 µl of BlockIt blocking buffer with diluted Ab of interest were added on the mini-array and incubated for 1 hour at RT in the dark humid chamber. Slides were washed 3 times with PBS 0,1% - Tween20 (PBST, 5 minutes each wash) and 300 µl of 1:800 Alexa fluor 647 AffiniPure rabbit anti-human IgG, Fc fragment specific (309-605-008; Jackson Immunoresearch) in BlockIt blocking buffer were added for 1 hour at RT in the dark humid chamber. Slides were washed 2 times with PBST, for 5 minutes each, followed by 2 washes in milliQ water (1

time for 10 minutes and 1 time for 5 seconds). Slides were dried with compressed nitrogen flow, then the image was acquired with InnoScan 710-AL (Innopsys LifeSciences).

nOMVs production from MenB strains

To produce natural OMVs (nOMVs), each MenB strain was plated on LB + isovitalex (produced in house) and incubated overnight at 37°C without CO_2. The strains grown to produce nOMVs are the following: NZ98/254 (NDV000049), M07576 (NDV000926), M08389 (NDV004127), M14569 (NDV001502), M12898 (NDV001404), M09929 (NDV001148), M07-0240184 (NDV002240), LNP24651 (NDV000340), M18711 (NDV001912), M13547 (NDV001455), M08129 (NDV001060), M07463 (NDV000918), M13520 (NDV001453), ARG3054, ARG3175, ARG3222, ARG3191 and ARG3753. The following day, bacteria were collected from the plate and 10 ml of Mueller-Hinton Broth (MHB, produced in house) were inoculated in 50 ml falcon tubes to have a starting OD_{600} = 0,4-0,6. Tubes were then incubated at 37°C 5% CO_2, in agitation, until OD_{600} = 1,0-1,5 was reached and then the 10 ml were put in 50 ml of prewarmed MHB, in 500 ml flasks, and incubated at 37°C 5% CO2 in agitation.

OD_{600} was monitored at 60 minutes timepoints, stopping the incubation when the OD remained with stable for 1,5 hours. Bacteria were then pelleted at 4000 x g for 60 minutes and supernatants were collected to recover nOMVs by ultracentrifugation (2 hours at 32.000 rpm in a Beckman SW32Ti rotor). nOMVs pellets were then resuspended in 30 ml of sterile PBS, pelletted again at the same conditions, resuspended in 200 µl of sterile PBS and stored at 4°C.

nOMVs quantification

To assess quantity of resuspended nOMV from each MenB strain, the BCA method was utilized. Briefly, a standard curve was made with a BSA solution, in PBS, from 2 mg/ml to 0,0625 mg/ml in 4 dilution points. nOMVs were diluted 1 in 5 and this dilution was utilized to have a 5 points curve in 2-fold dilution. Each point was made in duplicate. 5 µl of each solution were added in a 96-well flat bottom plate and 25 µl of DC protein assay reagent A (5000113; BIO-RAD) and 200 µl of protein assay reagent B (5000114; BIO-RAD) were added in each well. Samples were incubated for 15 minutes at room temperature and then signals were acquired with Infinite M Plex (TECAN). Concentration of nOMV of each MenB strain was extrapolated from the standard curve using Excel2007 software (Microsoft). 10 µg of each nOMVs diluted in LDS sample buffer 4X (NP0007;

ThermoFisher) with sample reducing agent 10X (NP0004; ThermoFisher) were then loaded in a 4-12% acrylamide gel and run in MES buffer (180 V for 30 minutes). Gel was then stained with ProBlue safe stain (G00PB002; GIOTTO BIOTECH). Gel images were acquired with GelDoc EZ System (BIO-RAD).

Electron microscopy Immunogold experiments

MenB strains were grown in the same conditions utilized to perform SBA experiments. Once OD_{600}=0,25 was reached, 5 ml of culture were pelleted at 2500 x g for 15 minutes, washed with sterile-filtered PBS, and pelleted again to eliminate the washing buffer. Bacteria were then resuspended in water - 4% paraformaldehyde (P6148, Sigma-Aldrich) for 5 minutes at room temperature to inactivate bacteria. Following this incubation, bacteria were pelleted again to eliminate fixation buffer and resuspended at a final OD_{600}=1.

Aliquot of 5 ul of the bacteria suspension were adsorbed to 300-mesh nickel grids, blocked in PBS - 1% bovine serum albumin and incubated for 1 h with mAbs, diluted 0.5 µg/ml in PBS,. Grids were then washed several times and incubated for 1 h with 12-nm gold-labeled anti-human secondary antibody (Jackson ImmunoResearch, 109-205-088) diluted 1:40 in PBS. After several washes with distilled water the grids were air-dried and analyzed using a TEM FEI Tecnai G2 spirit microscope operating at 120kV. The micrographs were acquired using a Tvips TemCam-F216 (EM-Menu software).

FACS experiments

MenB strains were grown in the same conditions utilized to perform SBA experiments. Once OD_{600}=0,25 was reached, 5 ml of culture were pelleted at 2500 x g for 15 minutes, washed with sterile-filtered PBS, pelleted again to eliminate the washing buffer and resuspended in 5 ml of PBS.

25 ul of bacterial suspension were put into wells of a 96-well plate containing 25 ul of purified mAbs, in PBS, at the concentration of 1,0 µg/ml and the plate was incubated at room temperature for 1 hour. After a 5 minutes centrifuge at 2500 x g liquid was removed and pelletted bacteria were resuspended in 50 ul of PBS 1% BSA containing the Goat anti-Human IgG H&L FITC conjugated (ab8854, Abcam) diluted 1:100. Plate was incubated for 1 hour at room temperature in the dark and, after a 5 minutes centrifuge at 2500 x g liquid was removed and pelletted bacteria were resuspended in

paraformaldehyde (47608, SIGMA), diluted in H20 at 1%, for 1 hour at room temperature. After a 5 minutes centrifuge at 2500 x g liquid was removed and pelletted bacteria were resuspended in sterile-filetered PBS and stored at 4°C until read of fluorescence was performed with BD FACSCantoII instrument (BD Biosciences). Data produced were analyzed by FlowJo v10 software (BD Biosciences).

4 Results

4.1 More than 1000 plasmablasts were isolated from PBMcs derived from human vaccinees with single-cell FACS sorting

To examine the immune response elicited by OMV contained in the 4C-MenB vaccine formulation, we single-cell sorted human plasmablasts from the blood of 3 adults vaccinees. These subjects were chosen on the basis of their high frequency of OMV-specific plasmablasts, previous analyzed via ELISPOT (data not shown). As extensively reported in literature [118, 119], plasmablasts represent one of the first response of the human adaptive immune system to non-self antigens, deriving from stimulated antigen-presenting B-cells [120]. Given the fact that their proliferation is determined by the activation mediated by Ags for which these cells are specific and that the peak in plasmablast production is from 6 to 8 days after immunization [121], we selected the blood of vaccinees after 7 days from their third dose of 4C-MenB immunization as the best source to obtain plasmablasts specific for OMV antigens (Figure 4.0).

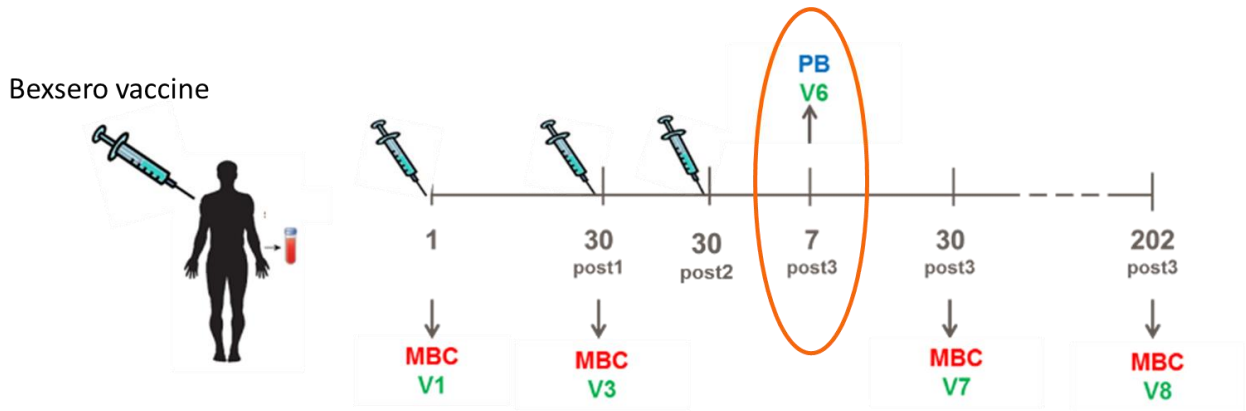


Figure 4.0 Clinical trial study design. Different blood withdrawals were collected from vaccinated subjects. PBMCs were collected from blood of vaccinees 7 days post third immunization (orange circle). PB is for plasmablast, MBC for memory B cells and Vn means visit number n.

From each subject, a mean of $5{,}0*10^6$ cells were utilized to perform the single-cell sorting of plasmablasts based on the exposure of specific markers (CD19, CD20, CD27 and CD38) on cell surface.

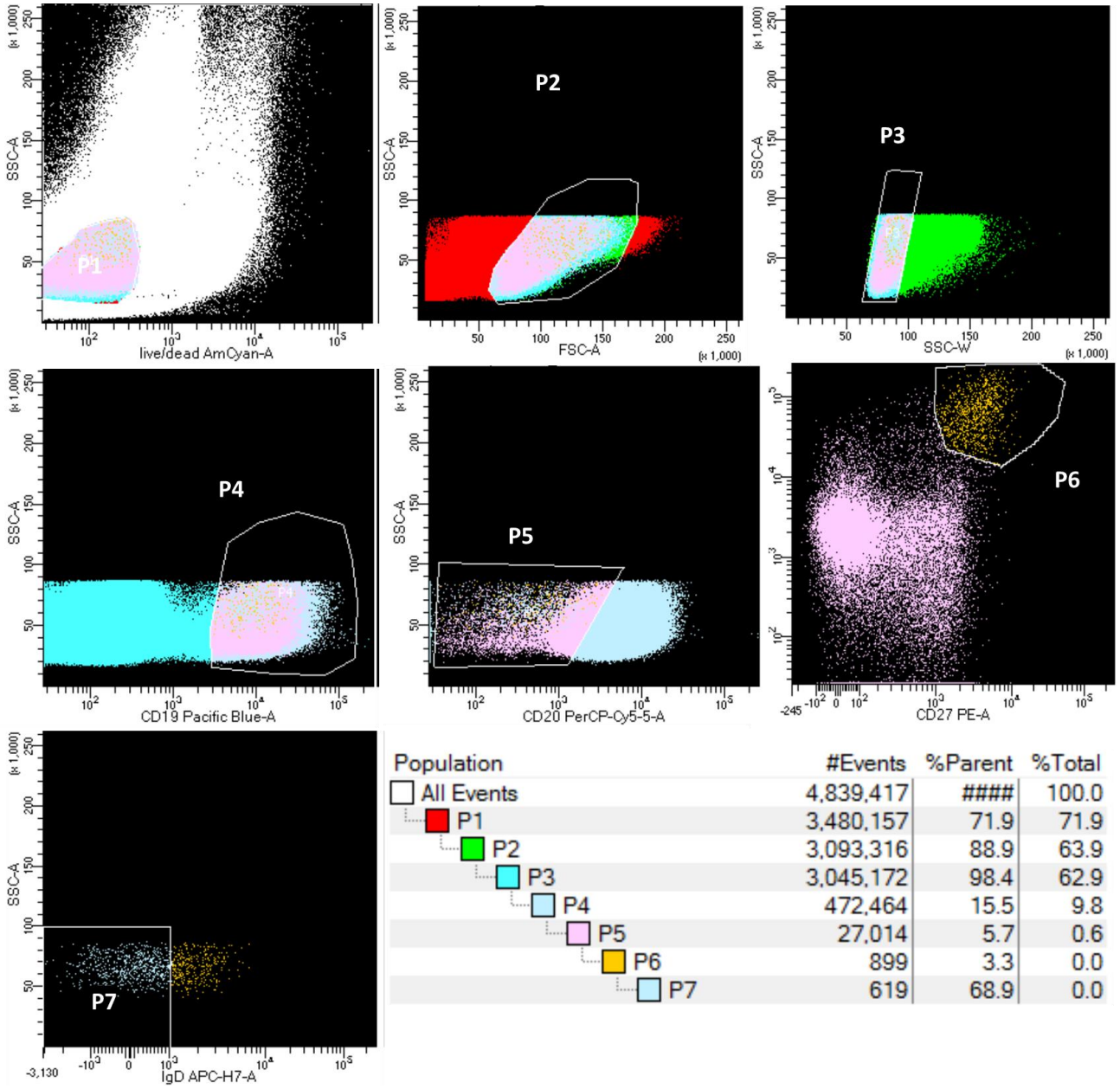


Figure 4.1 FACS Aria gating strategy. Live cells were selected with Live-Dead fluorophore (P1) and, based on their morphology (P2), single cells (P3) were examined for presence/absence of specific markers CD19 (P4), CD20 (P5), CD27 and CD39 (P6), to select the plasmablasts population negative to IgD (P7). Image is made from results of one representative subject of the three selected.

Despite the low frequency of plasmablasts in total PBMCs isolated from the blood (0,0127% of total cells, as depicted in Figure 4.1) we have been able to isolate 1024 cells that are actively producing antibodies. Since isolated PBs are not able to survive in culture and release natural mAbs in the culture

supernatant, we developed a PCR-based method to express the mAbs in a recombinant form, through the transfection of a mammalian cell line.

4.2 Amplification and cloning of variable regions encoded by sorted PBs

Single PBs were sorted into wells containing lysis buffer and reverse transcription was performed immediately after the sorting to prevent degradation of the mRNA. The cDNA was then used as template to amplify the variable regions of the antibody genes from the heavy and light chain loci, as they represent the unique feature of each mAb, determining its specific binding to Ags (Figure 4.2).

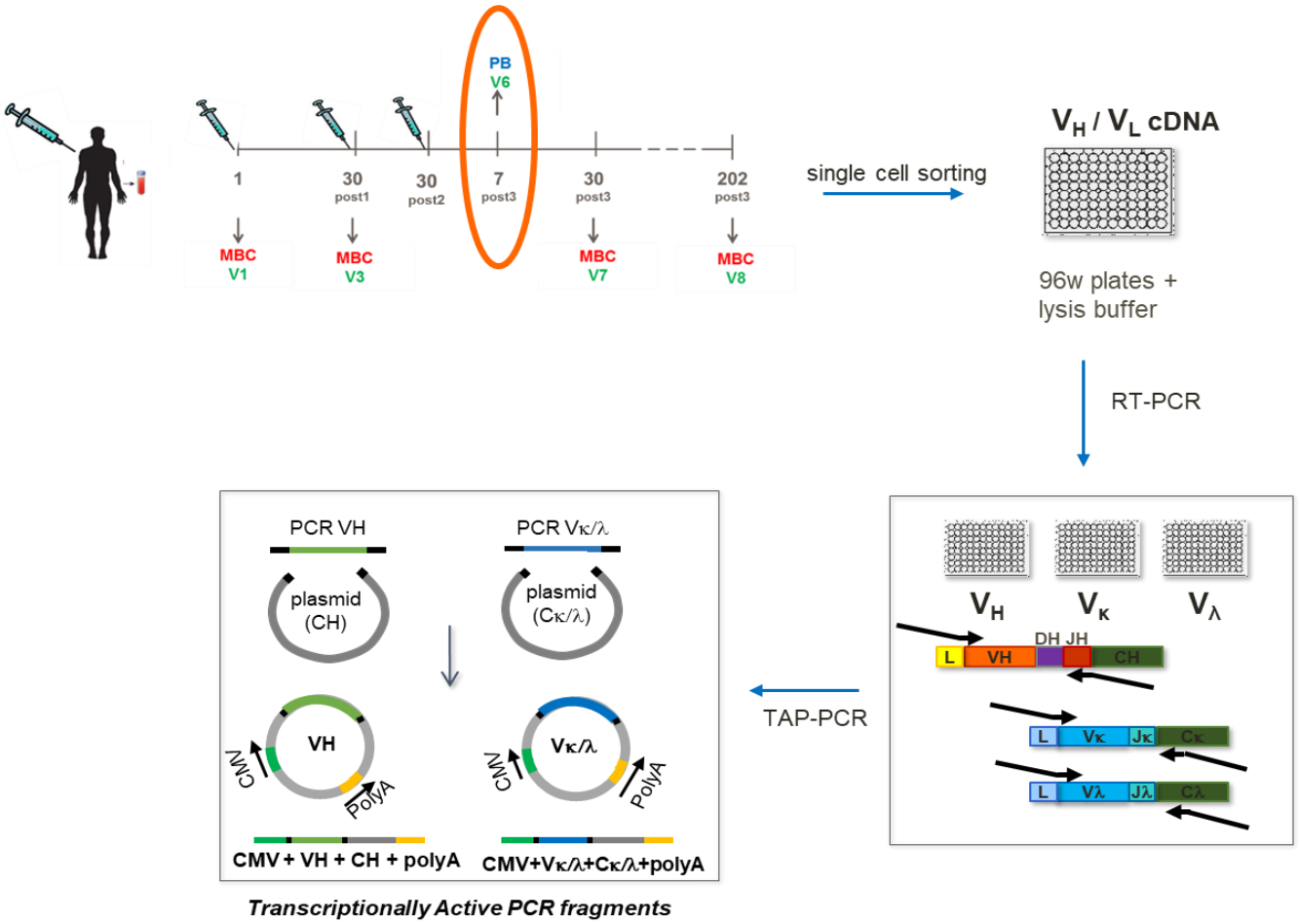


Figure 4.2 TAP generation pipeline from blood of immunized subjects. After the first RT-PCR step, 2 nested PCR were performed. The second PCR was performed to insert the tails flanking the VH and VL regions of the antibody genes and overlapping with the linearized plasmid flanking regions. These amplified regions were then inserted into 2 different scaffold plasmids which contained the constant regions of the antibody genes of IgG1 and ligated. Coding regions were eventually amplified from ligation products to transfect mammalian cells and produce the mAbs of interest.

With this aim, given the low amount of starting material, we performed two nested PCR reactions, using the primers pairs for amplification across the conserved regions flanking the VH and VL loci including both K and Lamda primer mixes listed in M&M section. After the second PCR, samples were run on a 2% agarose precast gel to verify the presence and the expected length (around 400 bp) of the PCR products (Figure 4.3).

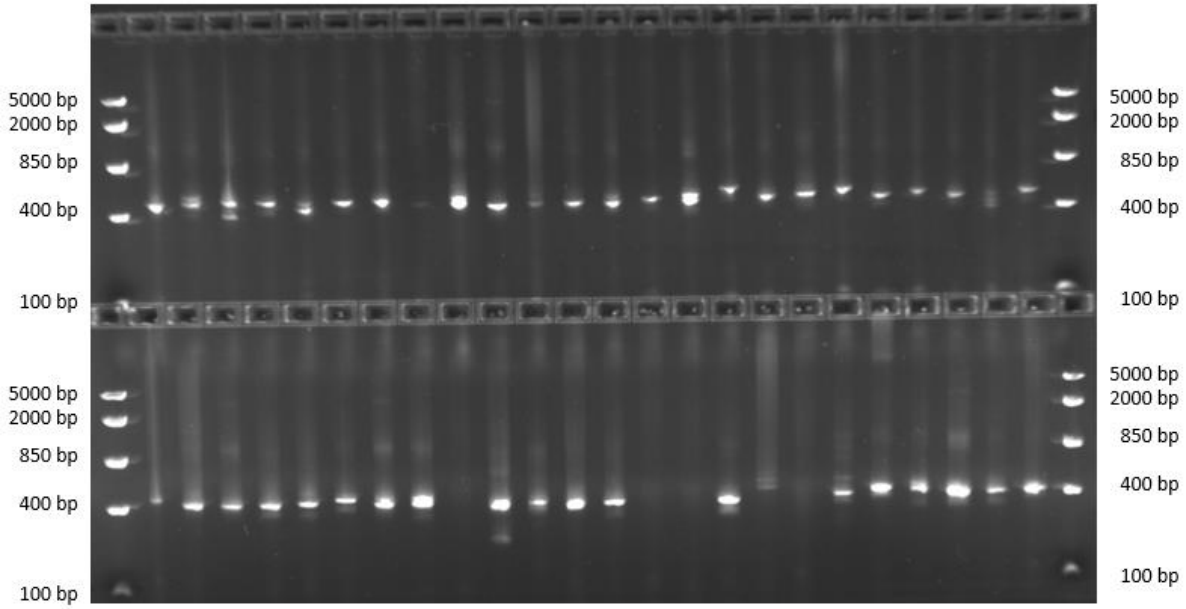


Figure 4.3 PCR1b products on gel. Three uL of PCR products corresponding to the variable regions of the heavy and light chain of mAbs were run for 25 minutes at 160V and detected by GelDoc instrument. Upper lanes: the VH fragments; lower lanes: the corresponding VL fragments from 2 lanes of a representative plate. Middle range Fastruler DNA marker was run on both sides of gel.

The correct amplification of the regions of interest was successful in 80% of wells, as shown in Figure 4.3. In some of them there are double bands, which is not surprising as we performed the PCR reactions with a mix of primers, that could end up producing some byproducts. Nonetheless, the main reaction products are of the size of interest, so we proceeded with the ligation into the two vectors selected (one for VH and the other for VL) followed by TAP reactions. To confirm the success of this last PCR another 2% agarose gel was run with the purified products.

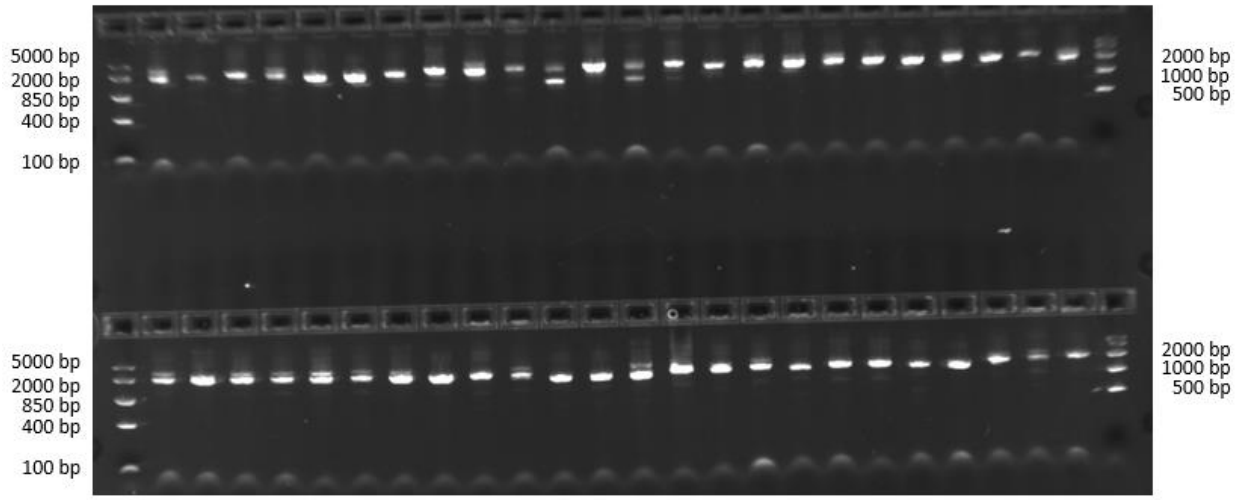


Figure 4.4 TAP products on gel. PCR products were run for 25 minutes at 160V and detected by GelDoc instrument. On the upper portion of the gel are run the VH and on the lower portion the corresponding VL from 2 lanes of a representative plate. Middle range fastruler DNA marker was run on left side of gel while High range fastruler DNA marker was run on the right side of the gel.

The TAP PCR step further increased the recovery of desired products, as shown in Figure 4.4. This data can be explained assuming that even PCR1b products that are present in low amounts are sufficient to perform a ligation with the corresponding plasmid and to be used as template in the subsequent. The TAP obtained for paired heavy and light chain of mAbs derived from each sorted cell have been used for co-transfection into human Expi293F cells for the expression of recombinant mAbs. The correct TAP reaction product is around 2000 bp and, mostly in VH reactions, some lower byproducts can be observed. These results are not alarming as the wrong band will generate an abortive reaction product.

4.3 Transfection of TAP products on mammalian cells was successfully performed

To express and test the mAbs of interest we used the human Expi293F cell line, which is broadly used for this scope [122] and allows mAbs to be decorated with a human glycosylation pattern, which is fundamental for the correct mAb functions [123]. The co-transfection of VH and VL inserted into the corresponding constant regions of mAb was performed in 96-well plates. After 6 days of cell culture, supernatants were screened for the presence of mAbs by SDS-PAGE followed by Coomassie proteins staining (Figure 4.5).

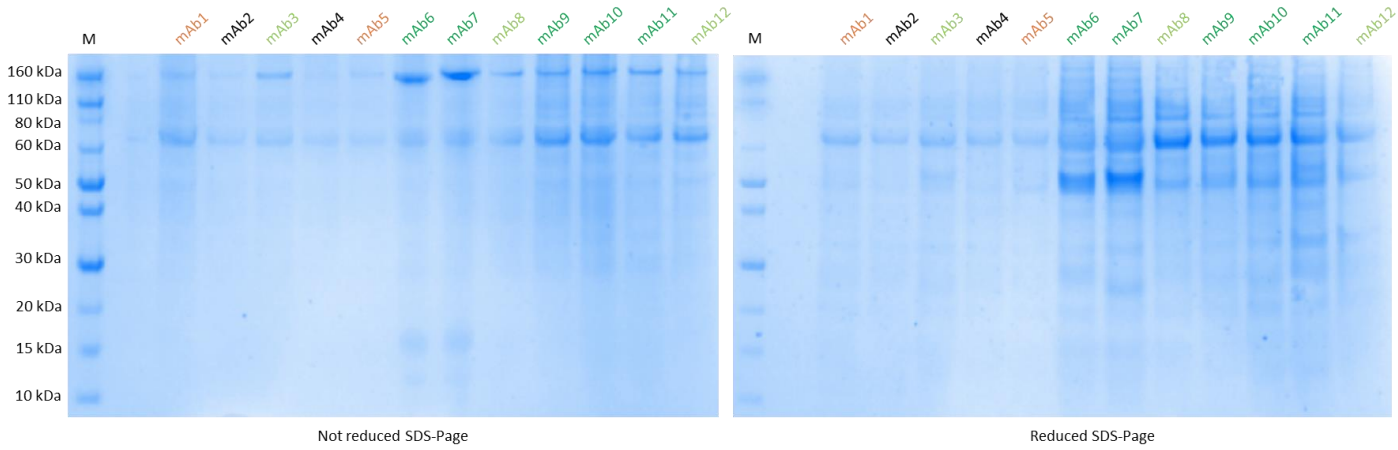


Figure 4.5 Analysis of TAP transfections products on gel. Raw supernatants from transfected Expi293F cells were run for 25 minutes at 180V in both reducing (right side of the image) and not reducing (left side of the image) conditions and detected by GelDoc instrument. In black are reported mAb with an expression level too low to be detected by this technique, in orange mAbs expressed at low yield but sufficient to be detected, while in green and bright green mAbs expressed at middle or high amount, respectively.

Even if the transfection conditions utilized were the same for all the transfected constructs, the quantity in the raw supernatants of mAbs is highly variable, depending likely on the monoclonal sequence. Nonetheless, as the mAbs were detectable in most of the observed wells as bands at the expected MW of 150KDa, we proceeded with the analysis of their binding capacity and their relative quantification by Luminex binding assays.

4.4 The majority of mAbs are expressed and 90% of 4C-MenB-positive monoclonal antibodies are not-PorA specific HumAbs

Due to the vast diversity of circulating Ab in the human bloodstream, a technique which allow to characterize them and discriminate the ones of interest is pivotal to determine the subset of Ab to be expressed. To screen Ab for binding to specific antigens different methods are reported in literature, that can be reconducted into 2 major categories [124]:

I) ELISA-based methods;
II) Luminex-based methods.

Since the Luminex-based methods have the advantages of: 1) being more sensitive, being able to detect very low amount of Abs, 2) being multiplexable, allowing the simultaneous testing of mAbs to

different Ag-coated beads, and 3) require very low amount of working volume [125-128], we decided to rely on this technique to perform the binding screening.

Luminex technology relies on the use of magnetic fluorophore-coded beads, each one coated with a different analyte that can be utilized to screen the mAbs of interest. Binding between antigens coated on beads and mAbs usually takes place in a well of a 96/384-well plate and then a phycoerythrinated (PE) Ab specific for human Fc fragment is added to the mixture. Signal from each bead is then acquired on a dual-laser flow-based instrument, such as Luminex 200 (96-well plates) or FlexMap (384-well plates), where one laser identifies the bead (i.e. the antigen) and the other the presence or absence of PE (i.e. the binding of the mAb). With this tool, the screening of a high number of different mAbs for binding to selected antigens becomes possible in a short timeframe, allowing the identification of the subset of mAbs of interest [129]. The same method also allows the quantification of the mAb, thanks to the capability of Staphylococcal protein A (SpA) to bind to the Fc regions of mAbs. SpA was coupled to luminex beads to capture the mAb in the raw supernatant and a standard curve (made with a purified human mAb diluted in expression media) was set to quantify the mAb (Figure 4.6).

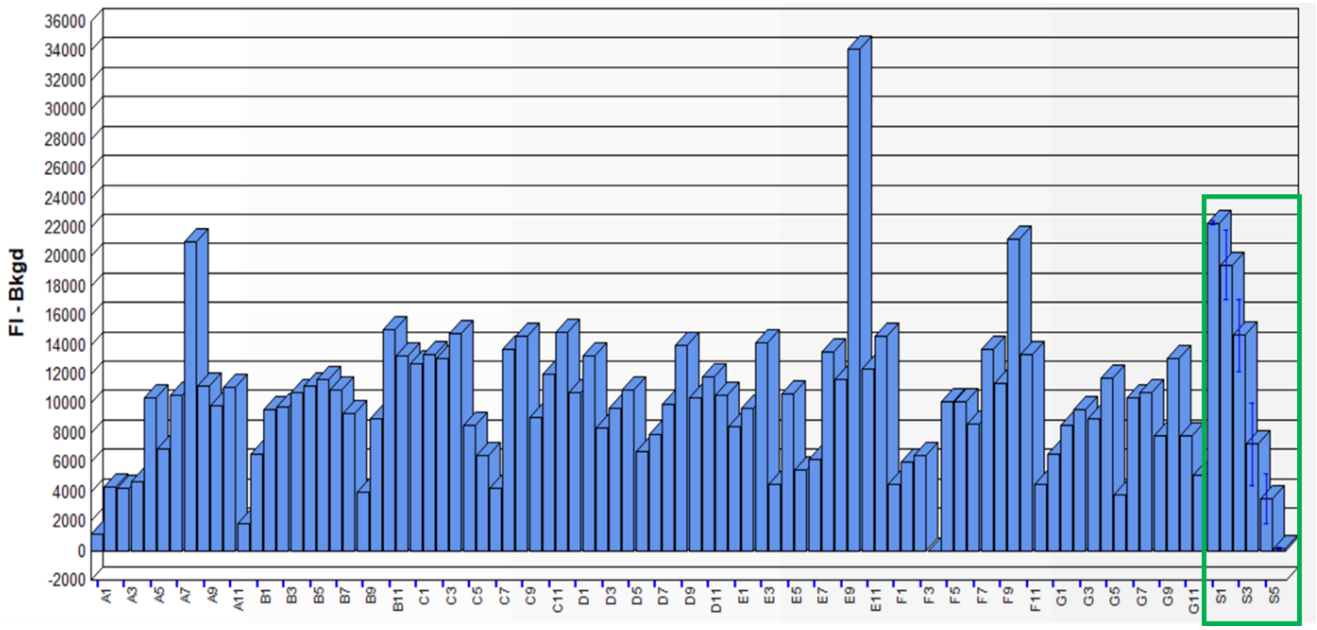
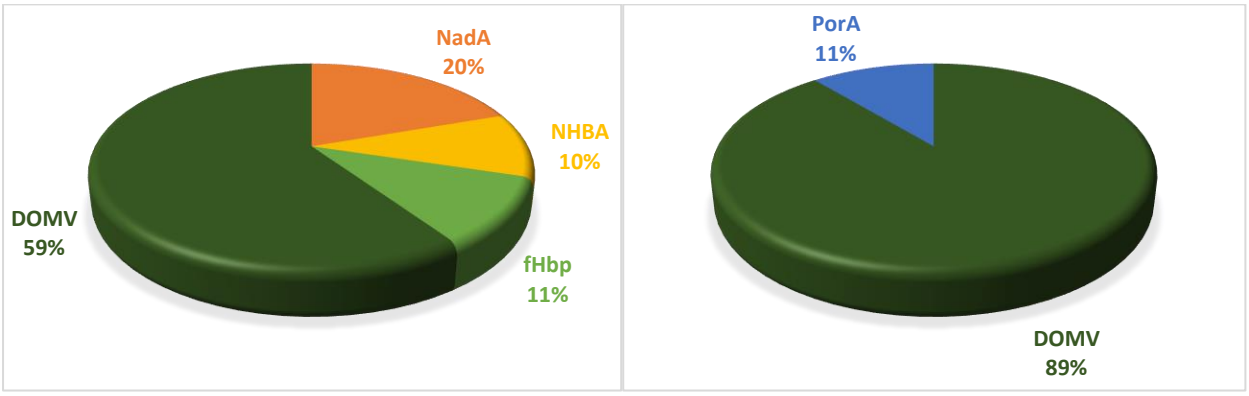


Figure 4.6 Results of the Luminex IgG binding assay. Graph shows the quantification of the mAbs contained in the raw supernatant and is representative of 1 of the 4 expressed plates from subject 2. On the X axis is reported the well position related to the transfection plate and in the Y axis is reported the mean fluorescence intensity (FI) measured with the Luminex, subtracted of the background. On the right side of the slide (green box) is reported the standard curve, performed with 5 duplicate points of a purified mAbs in 2-fold dilution (starting from S1 at 20 ng/ml) in expression medium.

The IgG binding assay confirmed the high transfection efficiency already observed with the SDS-PAGE. Considering the standard curve utilized, we fixed a measure of 1000 MFI as the threshold to consider as positive the presence of mAb in the transfection's supernatant. The results confirmed the expression of mAb in the 97% of the transfected wells. We coupled to Luminex beads all the 4C-MenB components and the recombinant PorA of NZ98/254, in order to interrogate our mAbs in raw supernatant of transfected Expi293F cells also for their binding properties (Figure 4.7).

We observed that around 16% of the 1024 mAbs screened with the Luminex binding assay were positive for one of the 4C-MenB antigens. More in detail, 41% of mAbs were specific for the protein antigens included in the vaccine composition (10% were NHBA-specific, 11% fHbp-specific and 20% NadA-specific). As for the remaining 60%, these were OMV-specific mAbs, of which quite surprisingly only 11% recognize the recombinant PorA utilized in our binding assay (Figure 4.5).



N = 168 N = 100

Figure 4.7 Results of the Luminex-based 4C-MenB binding assay. Ags coupled to Luminex beads were: NadA, NHBA, fHbp, PorA and OMV derived from NZ98/254 strain. The pie chart on the left represents the distribution of mAbs that were found specific for the 4C-MenB Ags. The pie chart on the right represents the percentage of the 100 OMV-positive mAbs that were PorA specific or that recognize other OMV component.

It is reported in literature that the main Ag eliciting protective response in 4C-MenB OMV is PorA. However from our 3 subjects we isolated 100 OMV positive mAbs of which 11 only recognised PorA. Therefore, we concluded that most mAbs (89) that recognize MenB NZ98/254 strain's OMVs were targeting unknown antigens.

4.5 Results from functional rSBA initial screening

To look at the functionality of the OMV-positive HumAbs found, we performed rSBA with the supernatants from transfected cells. The assay has been performed in parallel on two different MenB strains: the NZ98/254 and the M07576 MenB strains: NZ98/254 carries a PorA VR1 7-2 and VR2 4, while M07576 carries a PorA VR1 22-1 and VR2 14. The NZ98/254 is the reference bacterial strain from which the OMV component of 4C-MenB vaccine is produced and therefore represents the homologous strain, while the M07576 strain is mismatched for the major outer membrane protein PorA and belongs to a different clonal complex (ST-35).

As we can see in the graph reported below (Figure 4.8), we could identify 3 populations of mAbs with different features:

- Active against NZ strain (5 mAbs, PorA-specific from Luminex binding data);
- Active against M07576 strain (14 mAbs);
- Active on both MenB strains (2 mAbs).

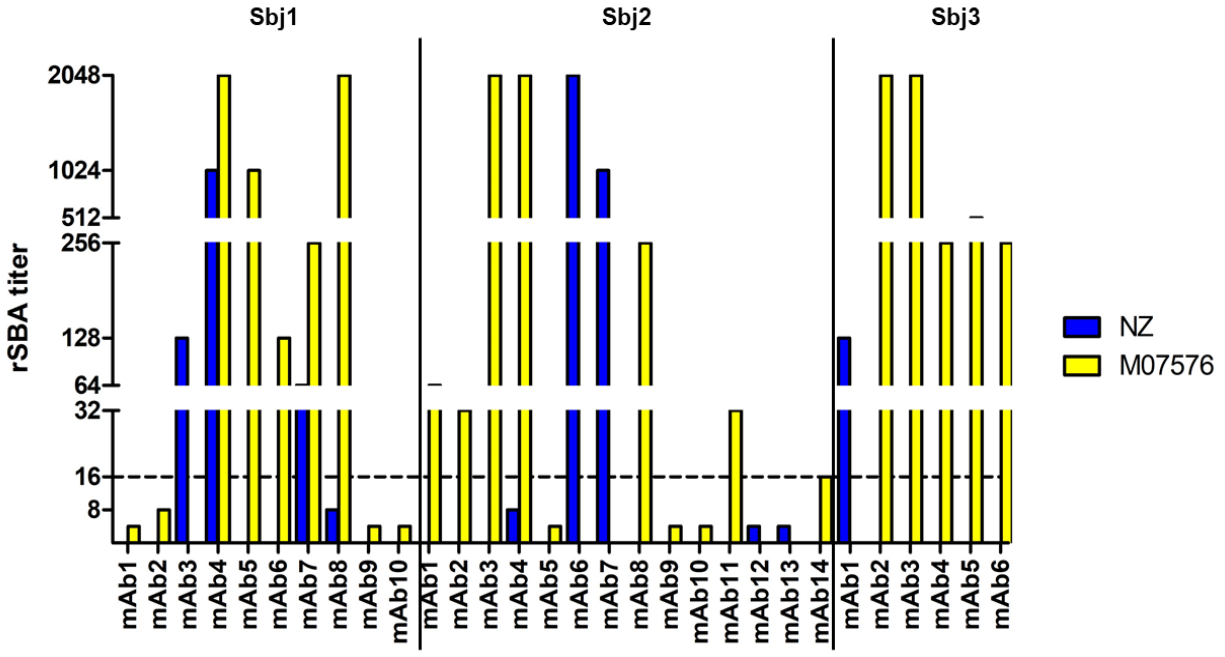


Figure 4.8 Results of the rSBA screening assay. Raw supernatants of transfected cells were tested in 2-fold dilutions in a 10 points curve, made in duplicate. Titers reported (Y axis) are the reciprocal of the highest dilution at which mAbs exerted bactericidal activity, calculated as a reduction of more than 50% of colonies retrieved in the control conditions, as described in M&M section. mAbs were grouped per subject (X axis). Only mAbs that gave a positive result are reported in the graph.

21 of the 100 mAbs tested in rSBA resulted positive for bactericidal activity towards at least one of the MenB test strains. Surprisingly only 5 out of 21 bactericidal mABs were able to kill the NZ98/254 strain, while, the majority (14/21) of mAbs identified as bactericidal in this screeningwere able to kill the PorA-mismatched strain M07576. The 5 mABs that are bactericidal against NZ were all identified as PorA specific mAbs at Luminex, and this is in agreement with the use of NZ as the reference MenB strain utilized to detect killing of PorA-specific Abs elicited by 4CmenB vaccine in clinical trials. Instead the identification of 14 mAbs bactericidal only against the heterologous strains suggests that they recognize non-PorA antigens that may be abundant in the M07576 strain and suggests the importance of other Ags contained in the OMV component in eliciting protection. This result is in line with the data reported from Invasive meningococcal Disease (IMD) patients, where cross-bactericidal mAbs not PorA-specific were found [130]. Ultimately, 2 mAbs were able to kill both MenB strains, highlighting the presence of common Ags responsible for cross-killing other than NadA, fHbp and NHBA. We cannot exclude that the mAbs from the supernatants that are not bactericidal have not functional activity as their concentrations may be too low under these experimental conditions.

4.6 Sequences from prioritized mAbs highlighted a prevalence of mAbs of the IgG isotype and an enrichment in specific gene families

To better investigate these OMV-specific mAbs, we decided to produce a subset of 70 OMV-specific mAbs of interest in a larger scale, selected on the basis of their Luminex signal on NZ98/254 OMVs and of their concentration according to the following criteria. mAbs with a high signal on OMVs were prioritized not taking into account their concentration in the raw supernatant. mAbs showing a weak signal on OMVs were prioritized only if their concentration was below 10 ng/ul, suggesting that their low recognition signal was due to poor expression. This decision was made to exclude from the following analysis mAbs that weakly recognized the OMVs despite their high concentration (Table 4.0, mAbs in red). These criteria were applied to Sbj2 and Sbj1, while all OMV-positive mAbs from Sbj3 were expressed, as control. The list of 100 tested mAbs is reported in Table 4.0, which shows the mean fluorescence intensity (MFI) obtained at Luminex for each mAb on the OMVs.

mAb ID	OMV_NZ98/254	Relartive mAb Concentration (ng/ul)	
Sbj1_mAb1	895	7,2	Selected
Sbj1_mAb2	9552	4,1	Selected
Sbj1_mAb3	29739	11	Selected
Sbj1_mAb4	27921	17	Selected
Sbj1_mAb5	12863	3,8	Selected
Sbj1_mAb6	880	2,4	Selected
Sbj1_mAb7	27261	19	Selected
Sbj1_mAb8	7183	13,2	Selected
Sbj1_mAb9	1766	25	Excluded
Sbj1_mAb10	28864	28,3	Selected
Sbj1_mAb11	28677	17,5	Selected
Sbj1_mAb12	28567	22	Selected
Sbj1_mAb13	1906	17,1	Selected
Sbj1_mAb14	852	16,9	Selected
Sbj1_mAb15	31292	23	Selected
Sbj1_mAb16	1273	2,6	Selected
Sbj1_mAb17	23171	35	Selected
Sbj1_mAb18	35804	17,3	Selected
Sbj1_mAb19	42030	16,2	Selected
Sbj1_mAb20	31294	28,4	Selected
Sbj1_mAb21	2167	13	Selected
Sbj2_mAb1	15894	8,4	Selected
Sbj2_mAb2	32638	16	Selected
Sbj2_mAb3	7530	4,2	Selected
Sbj2_mAb4	31929	21,7	Selected
Sbj2_mAb5	32214	12	Selected
Sbj2_mAb6	27695	16,4	Selected
Sbj2_mAb7	32637	14	Selected
Sbj2_mAb8	639	21	Excluded
Sbj2_mAb9	874	17,5	Excluded
Sbj2_mAb10	1479	5,3	Selected
Sbj2_mAb11	1627	8	Selected
Sbj2_mAb12	704	28,3	Excluded
Sbj2_mAb13	33149	17	Selected
Sbj2_mAb14	11647	12	Selected
Sbj2_mAb15	153731	30,9	Excluded
Sbj2_mAb16	44356	18,5	Excluded
Sbj2_mAb17	31055	27,9	Selected
Sbj2_mAb18	1234	18,6	Excluded
Sbj2_mAb19	1219	29	Excluded
Sbj2_mAb20	1341	8,4	Selected
Sbj2_mAb21	628	17	Excluded
Sbj2_mAb22	1345	3,3	Selected
Sbj2_mAb23	94934	28,6	Selected
Sbj2_mAb24	1114	18,2	Selected
Sbj2_mAb25	122587	33,7	Selected

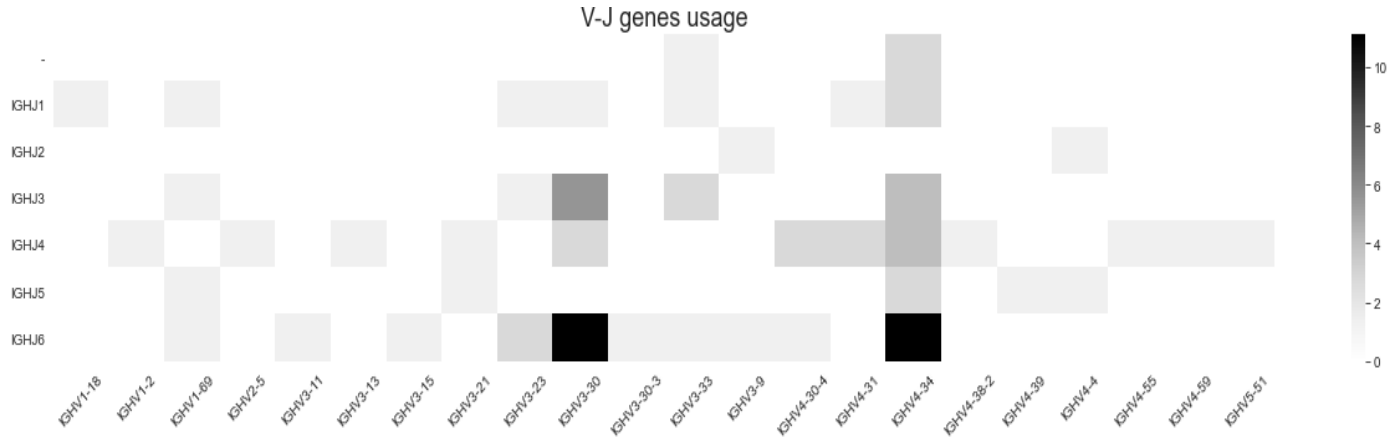
Sbj2_mAb26	36935	12,6	Selected
Sbj2_mAb27	121158	20,1	Selected
Sbj2_mAb28	2214	17	Excluded
Sbj2_mAb29	820	15,7	Excluded
Sbj2_mAb30	107748	22	Selected
Sbj2_mAb31	107936	38,5	Selected
Sbj2_mAb32	1669	19,1	Excluded
Sbj2_mAb33	107741	25,7	Selected
Sbj2_mAb34	530	19,4	Excluded
Sbj2_mAb35	8363	7,3	Selected
Sbj2_mAb36	690	15	Excluded
Sbj2_mAb37	102427	17,2	Selected
Sbj2_mAb38	36849	14	Selected
Sbj3_mAb1	1833	12,2	Excluded
Sbj3_mAb2	3280	16	Selected
Sbj3_mAb3	76440	21,7	Selected
Sbj3_mAb4	3265	2,1	Selected
Sbj3_mAb5	580	11,9	Excluded
Sbj3_mAb6	1268	17	Excluded
Sbj3_mAb7	4576	1,9	Selected
Sbj3_mAb8	515	13	Excluded
Sbj3_mAb9	2321	17,3	Excluded
Sbj3_mAb10	103888	27,2	Selected
Sbj3_mAb11	1164	21	Excluded
Sbj3_mAb12	613	11,7	Excluded
Sbj3_mAb13	110142	37,4	Selected
Sbj3_mAb14	4146	9	Selected
Sbj3_mAb15	89804	16,9	Selected
Sbj3_mAb16	3654	3,8	Selected
Sbj3_mAb17	6211	13,3	Excluded
Sbj3_mAb18	77009	25	Selected
Sbj3_mAb19	76176	19,6	Selected
Sbj3_mAb20	581	15	Excluded
Sbj3_mAb21	13488	13,6	Selected
Sbj3_mAb22	1466	12,2	Excluded
Sbj3_mAb23	6185	8	Selected
Sbj3_mAb24	6623	6,4	Selected
Sbj3_mAb25	12534	17,3	Selected
Sbj3_mAb26	1104	17	Excluded
Sbj3_mAb27	33104	22,4	Selected
Sbj3_mAb28	643	13	Excluded
Sbj3_mAb29	500	16,1	Excluded
Sbj3_mAb30	33349	27	Selected
Sbj3_mAb31	18272	8,4	Selected
Sbj3_mAb32	4386	3	Selected
Sbj3_mAb33	1953	2,8	Selected
Sbj3_mAb34	3972	5,4	Selected
Sbj3_mAb35	1383	19	Excluded

Sbj3_mAb36	67483	28,6	Selected
Sbj3_mAb37	1299	11	Excluded
Sbj3_mAb48	3616	17	Excluded
Sbj3_mAb39	11653	18,9	Selected
Sbj3_mAb40	4209	10,1	Excluded

Table 4.0 mAbs expression in raw supernatant and binding. For each mAbs, the mean MFI of three indipendent Luminex binding experiments on the beads coupled with OMV of NZ98/254 strain is reported. The color code of these cells goes from blank (low signal) to orange (high signal) depending on the binding intensity observed. mAbs information are reported in red when the concentration of mAb was higher than 10 ng/ul and binding to OMV was low, as previous mAbs with this behaviour were expressed and did not shown improved target's binding (data not shown).

Since the variable heavy and light chains (VH and VL, respectively) are the ones that determine mAbs specificity, we sequenced these regions either from PCR1b or TAP purified reactions. Sequencing was performed both with Sanger-based and Illumina-based techniques and the VH and VL of each mAb were retrieved.

VH and VL sequences were also compared, in order to exclude mAbs duplicates between the three subjects analyzed, and a characterization on their V and J genes usage was performed to highlight a possible preferential usage of the alleles of these genes (Figure 4.9).



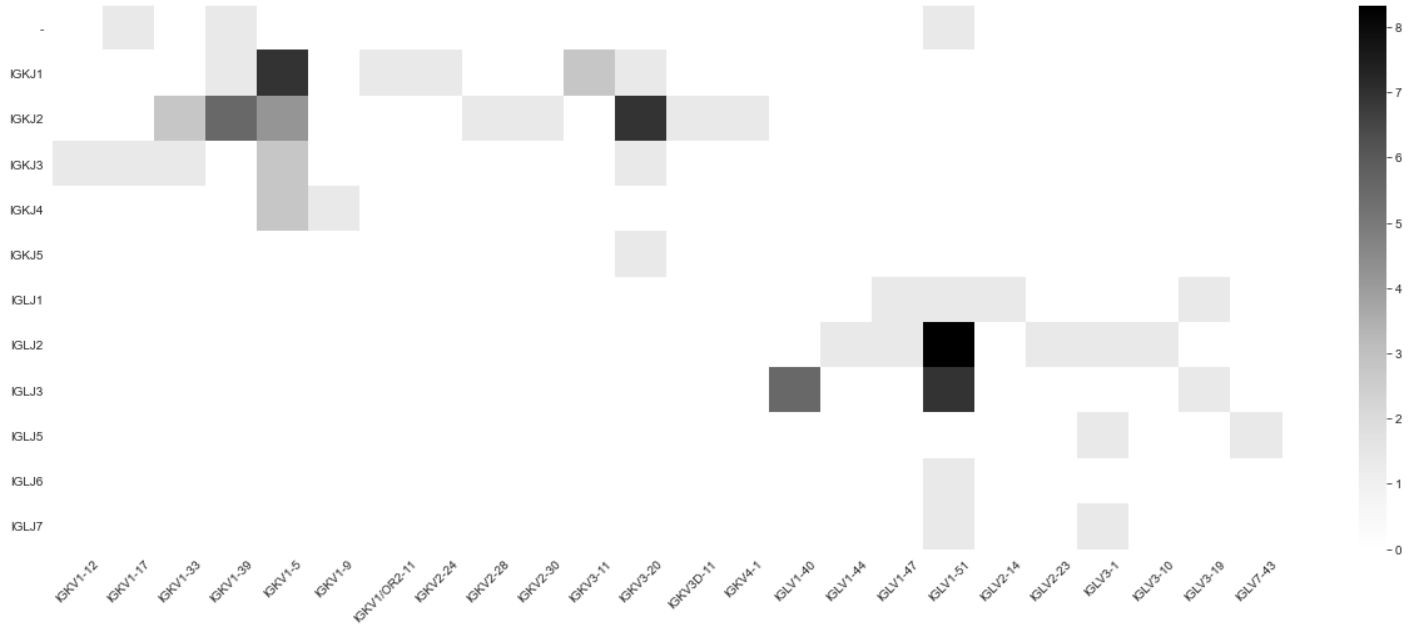


Figure 4.9 V and J gene usage in VH and VL. On top is reported the V-J usage of VH, while on bottom is reported the usage of VL. On the X axis of the graphs is reported the different V alleles founded in each mAb and on the Y axis is reported the paired J allele utilized. The darkness of the color used increases when more mAbs use that specific couple of alleles, as represented on the bar on the right of each graph. Numbers on this bar represent the number of mAbs that use that specific couple of genes.

We found an enrichment in IGHV3-30 and IGHV4-34 coupled with a IGHJ6 gene usage for the VH of sequenced mAbs, while for the VL we found a prevalence of IgKV1-5 with IGKJ1 and IGKV3-20 with IGKJ2 for the Vk of the obtained sequences and IGL1-51 with both IGJ2 and IGJ3 for the Vλ. Therefore, we concluded that OMVs from NZ98/254 MenB strain elicited mainly antibodies made up with these combinations of V and J genes.

From the sequences we could also retrieve information related to the isotype of the native mAb encoded by PBs: as expected, the most represented isotype of OMV-elicited mAbs was IgG. Interestingly, we could also identify some IgA and one IgM. The induction of IgG>IgA>IgM is in line with what already reported [131, 132] and reflects the stimulation of more blood-circulating mAbs (IgG) than secreted ones (IgA) (Figure 4.10).

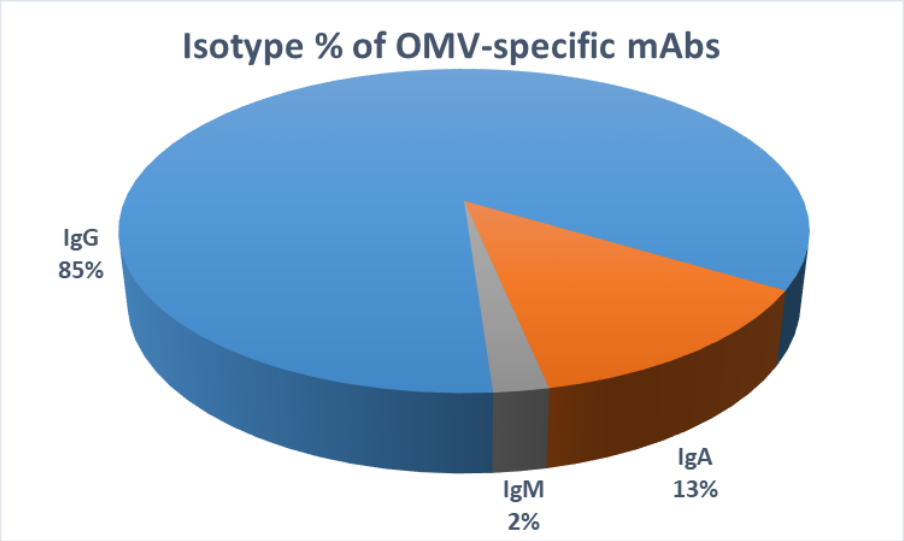


Figure 4.10 Isotype distribution across sequenced mAbs. The pie chart represents the relative percentage of each isotype across half of the different OMV-specific mAbs that were sequenced.

4.7 Expression and purification of 63 mAbs

For the expression of the subset of selected recombinant mAbs, we ordered at synthetic DNA strings codon optimized for expression,and cloned these in frame with the constant region of human IgG1 in in an expression plasmid for mammalian cell lines. For the expression and purification of each mAb from transient transfection with the plasmids generated we used the same cell line (Expi293F) utilized to perform the transfection with TAP fragments, to have the same glycosylation pattern. Cells were co-transfected with plasmids encoding for the 2 paired chains in Erlenmeyer flasks and 6 days after transfection the culture supernatant was collected for further purification. A small aliquot of each raw supernatant was loaded on SDS-PAGE to verify the mAb expression and its correct molecular weight. We noticed that not all mAbs were expressed at the same level from transfected cells. This result was in line with what we observed from previous small-scale expression with TAP fragments, and suggests that the expression level highly depends on the sequence of the different VH and VL chain. Seven out of 70 mAbs derived from the selected sequences were not visible in the gel. To exclude that the low sensitivity of the technique utilized to analyze the mAb was underestimating the expression, we used WBs on the supernatant from transfected cells (Figure 4.11).

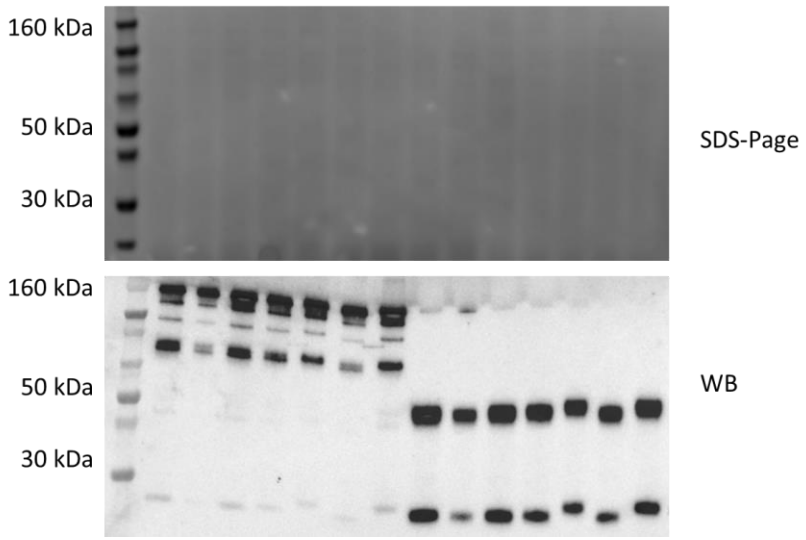


Figure 4.11 mAbs in raw supernatant run in reduced and not reduced condition. Seven mAbs in supernatant run under not-reduced and reduced conditions in SDS-PAGE at 180V for 30 minutes (top) and revealed in WB (bottom). Ten ul of mAbs in raw supernatant were loaded into each well.

Each time we detected bands at the expected position for the heavy and light chains (50 and 25KDa, respectively) in reducing conditions and at the position of the correctly assembled mAb in non-reducing condition (150KDa), we concluded that the mAb was correctly produced and assembled, but at a yield too low to be detected by SDS-PAGE. Therefore all mAb constructs were expressed albeit at variable levels and, we proceeded with purifications of all transfected supernatants with Protein-G sepharose resin (Figure 4.12).

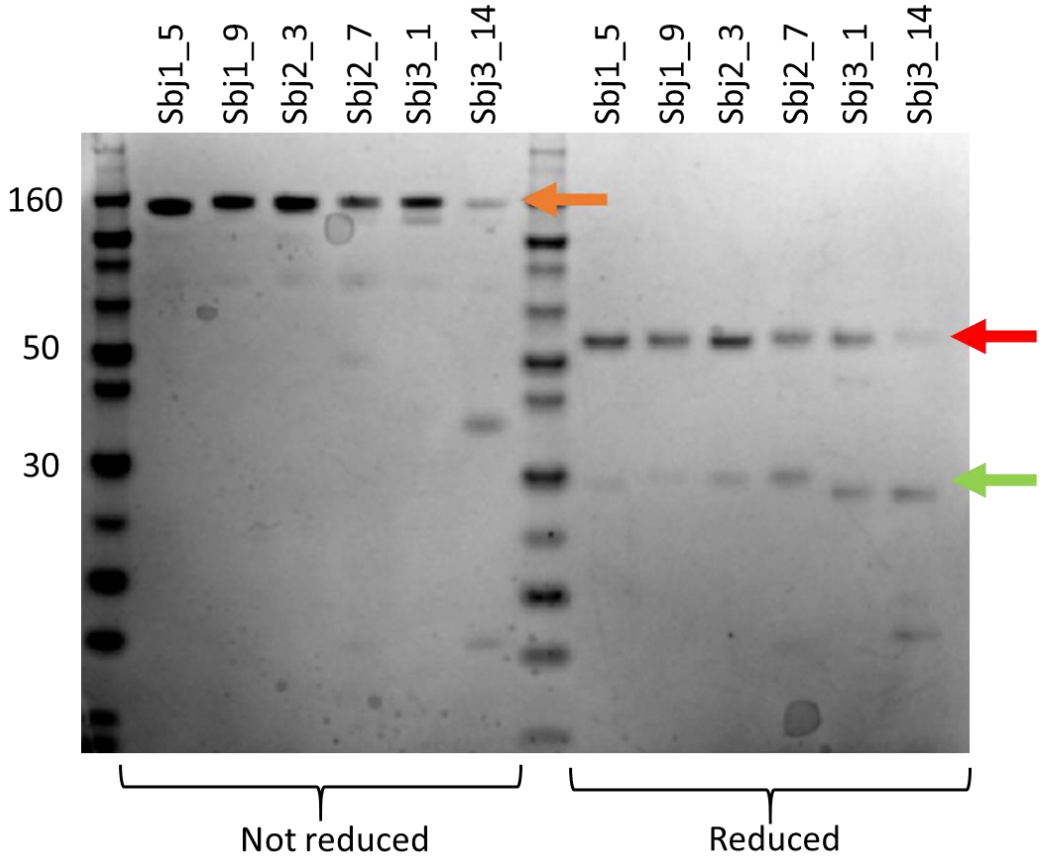


Figure 4.12 Purified mAbs run in reduced and not reduced condition. Two representative mAbs from each subject were subjected to SDS-PAGE at 180V for 30 minutes. Ten ul of purified mAbs were loaded into each well, to underline the different rate of expression of each mAb. On the left side of the image mAbd were loaded in non-reducing condition, while on the right side of the image same mAbs were boiled for 10 minutes with reducing agent and then loaded into the wells of the gel. Red arrow indicates the heavy chains, green arrow indicate light chains, orange arrow indicates assembled mAbs.

Succesfull purification were obtained for 63 out of 70 selected mAbs and was confirmed by both SDS-PAGE performed both in non-reducing conditions (Figure 4.12, orange arrow) and reducing conditions (Figure 4.12, red arrow for heavy and green arrow for light chains), we tried to identify their specific targets by protein array experiments and WBs.

4.8 Protein array to characterize mAbs targets

To identify the specific targets of the mAbs a technique that allows a high throughput screening on the targets was needed. Protein microarray satisfies the requirements of such screening, requiring a very low quantity of Ab to perform a screen on hundreds of different antigens. Indeed, this technique has already been used to characterize the immune response against bacterial infections [133] and characterize the immunosignatures associated with vaccinations [134]. In particular, in the context of 4C-MenB, efforts to elucidate epitopes of fHbp, NadA and NHBA upon vaccination were conducted with a vast panel of both human mAbs [135] and human sera [136] on a dedicated protein array. We utilized an ad-hoc, in-house produced protein microarray which displayed 61 different recombinant proteins of *Neisseria meningitidis* B and 14 additional MenB membrane proteins. These proteins were selected on the basis of the previous data (Viviani at al. under submission) reporting their abundance on the OMV component of 4C-MenB OMVs. Some of them were purified as his-tagged recombinant proteins, others were complex transmembrane proteins and were displayed on *Escherichia coli* derived OMV's surface. Each single purified recombinant mAb was probed on this protein microarray in the attempt to detect the target Ag.

From the results obtained, mAbs were divided into 2 different categories (Figure 4.13):

- mAbs with a specific target (left side of the pie);
- mAbs with an unknow target (right side of the pie).

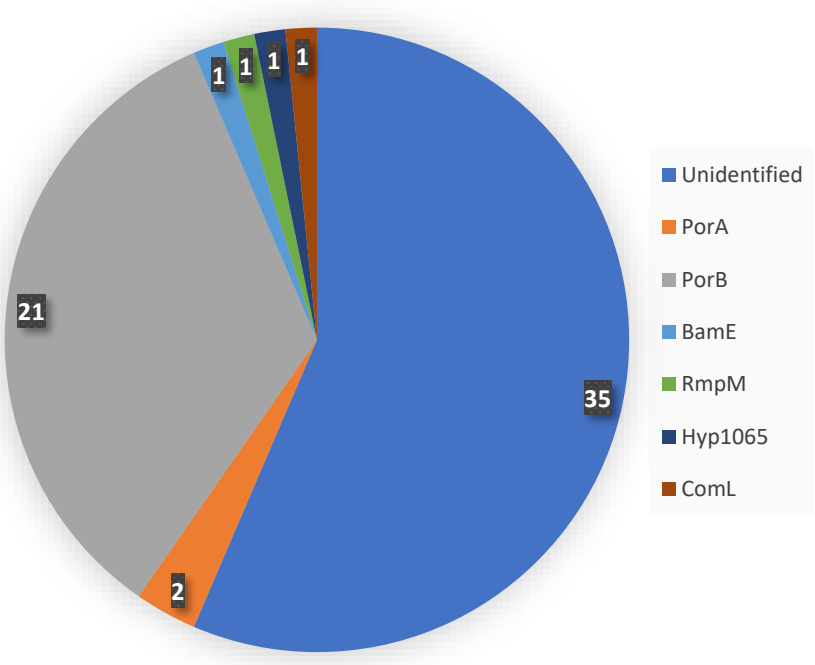


Figure 4.13 mAbs-specificity results from protein microarray experiments. The pie chart represents the number of mAbs for which a target was identified (on the left side of the pie chart) vs the mAbs for which the target has not been found yet (on the right side of the pie chart).

From this analysis we identified a target for half of the OMV-positive mAbs recombinantly expressed, among which some new immunogenic components of OMV from *Neisseria meningitidis* NZ98/254 strain including PorB, BamE, RmpM, Hyp1065 and the fimbrial biogenesis and twitching motility protein. PorB is one of the most abundant component of the OMV NZ98/254, with a relative abundance of up to 35 % of the total proteins, so we expected to find mAbs against this Ag. The relative abundance of the other identified proteins spans from not assessed quantity to 3,08 % of total OMV proteins [137]. This result highlighted the importance of proteins less represented on the OMV from 4C-MenB vaccine (defined as minor antigens) to elicit immune response in the vaccinated subjects.

Of notice, all the mAbs that were bactericidal against the M07576 strain, when tested in raw supernatant of transfected cells (Figure 4.8) resulted PorB-specific. In contrast, none of the other mAbs targeting the new immunogenic component of OMV were bactericidal per se against NZ98/254 and M07576 strains in the screening.

The rSBA screening performed with raw supernatant could anyway have been biased by the variable expression level of the mAbs on one side and by the expression level of the target antigen on the strains selected for the test on the other. To overcome the first point, all the mAbs have been expressed and purified, so that they can be tested at the same concentration. To overcome the strain selection issue, we decided to screen all the purified mAbs on a panel of natural OMVs derived from different MenB strains, to verify their binding profile. The results of this analysis will guide the subsequent rSBA analysis, that would be performed for each mAb only on the recognized strains. This approach would allow us to understand if PorB-mediated killing is shared by different MenB strains or is specific of M07576 strain.

4.9 mAbs binding to nOMV highlights differences in OMV composition between selected strains

To produce nOMV to be tested for specific mAb recognition, we selected 18 different MenB strains, on the basis of their genetic differences with respect to the NZ98/254 strain and of their sensitivity to

human sera from people immunized with 4C-MenB vaccine, as reported in the Table 4.1. These 18 strains were selected with the following criteria:

- 8 strains amongst our internal collection of MenB strains because of their sensitivity to sera from mice and people immunized with 4C-MenB but not from sera derived from adults vaccinated only with the recombinant protein components of 4C-MenB (first group), for which the Ag driving the SBA activity is contained in the OMVcomponent and unknown (Viviani et al., manuscript under submission);
- 10 strains showing susceptibility to sera from 4C-MenB vaccinees for which the main Ags driving SBA activity are unknown, as mismatched for 4C-MenB recombinant components (second group).

One additional condition that we considered as mandatory on the strains selection was the mismatched VR1 and VR2 region of PorA with the reference strain of the vaccine (NZ98/254), to further exclude that the bactericidal activity on the strain was guided by PorA.

Strain alias	*Clonal complex*	*PorA VR1*	*PorA VR2*	*PorB allele*	*NadA*	*fHbp*	*NHBA*
NZ98/254	ST-41/44 complex/Lineage 3	7-2	4	42 (3)	no	1	2
M07576	ST-35 complex	22-1	14	63 (3)	no	2	5
M08389	ST-162 complex	22	14	1781 (3)	no	2	20
M14569	ST-35 complex	22-1	14	63 (3)	no	2	21
M12898	ST-35 complex	5-1	2-2	63 (3)	no	2	143
M09929	ST-35 complex	12-1	16	42 (3)	no	2	19
LNP24651	ST-32 complex/ET-5 complex	7	16-26	63 (3)	yes	2	47
M07 241084	ST-41/44 complex/Lineage 3	19	15	37 (3)	no	2	31
M18711	ST-35 complex	22-1	14	63 (3)	no	2	339
M13547	ST-41/44 complex/Lineage 3	17	16-3	49 (3)	no	2	11
M08129	ST-41/44 complex/Lineage 3	7-4	1	1463 (3)	no	2	144
M07463	ST-41/44 complex/Lineage 3	17	16-3	266 (3)	no	2	10
M13520	ST-41/44 complex/Lineage 3	19	15-1	40 (3)	no	2	2
ARG-3753	ST-865 complex	21	16-36	350 (3)	no	2	24
ARG-3191	ST-865 complex	21	16-36	350 (3)	no	2	24
ARG-3175	ST-865 complex	21	16-36	350 (3)	no	3	24
ARG-3222	ST-865 complex	21	16-36	1405 (3)	no	2	24
ARG-3054	ST-865 complex	21	16-36	350 (3)	no	2	24

Table 4.1 Genotyping of the 18 selected MenB strains. The table report the main genetic information on the 4C-MenB Ags (PorA, NadA, fHbp and NHBA) and PorB variants in each one of the 18 selected strains. On the left side of the table, information also on the clonal complex and ST are reported for each strain.

For each strain, a growth curve was made (data not shown) to ensure that the collection of nOMV was done from 1 to 2 hours after bacteria entered the stationary phase of growth, depending on the strain, as not all of them had the same duplication time. The incubation was not protracted more than two hours in the stationary phase because after this time all MenB strains started to die. This compromises the quality of the nOMV, since dying bacteria release cytosolic proteins in the growth medium and degradation of the OMV components can be observed (Figure 4.14, panel A).

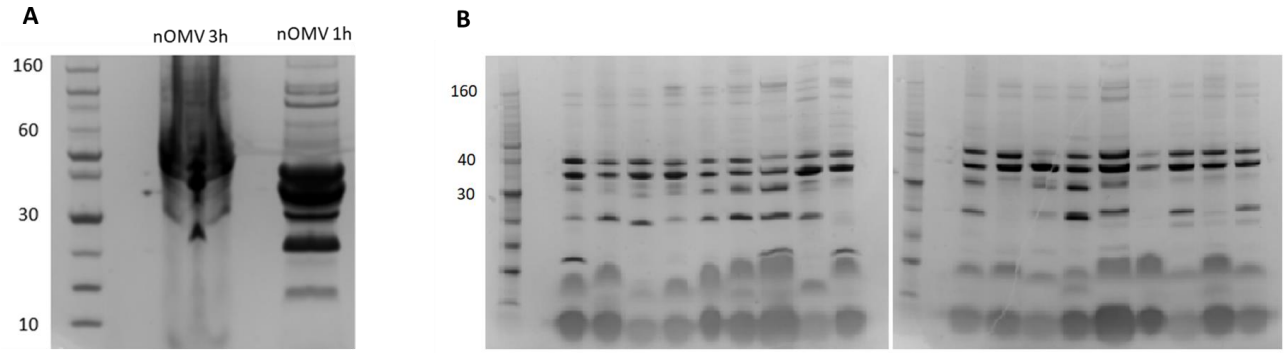
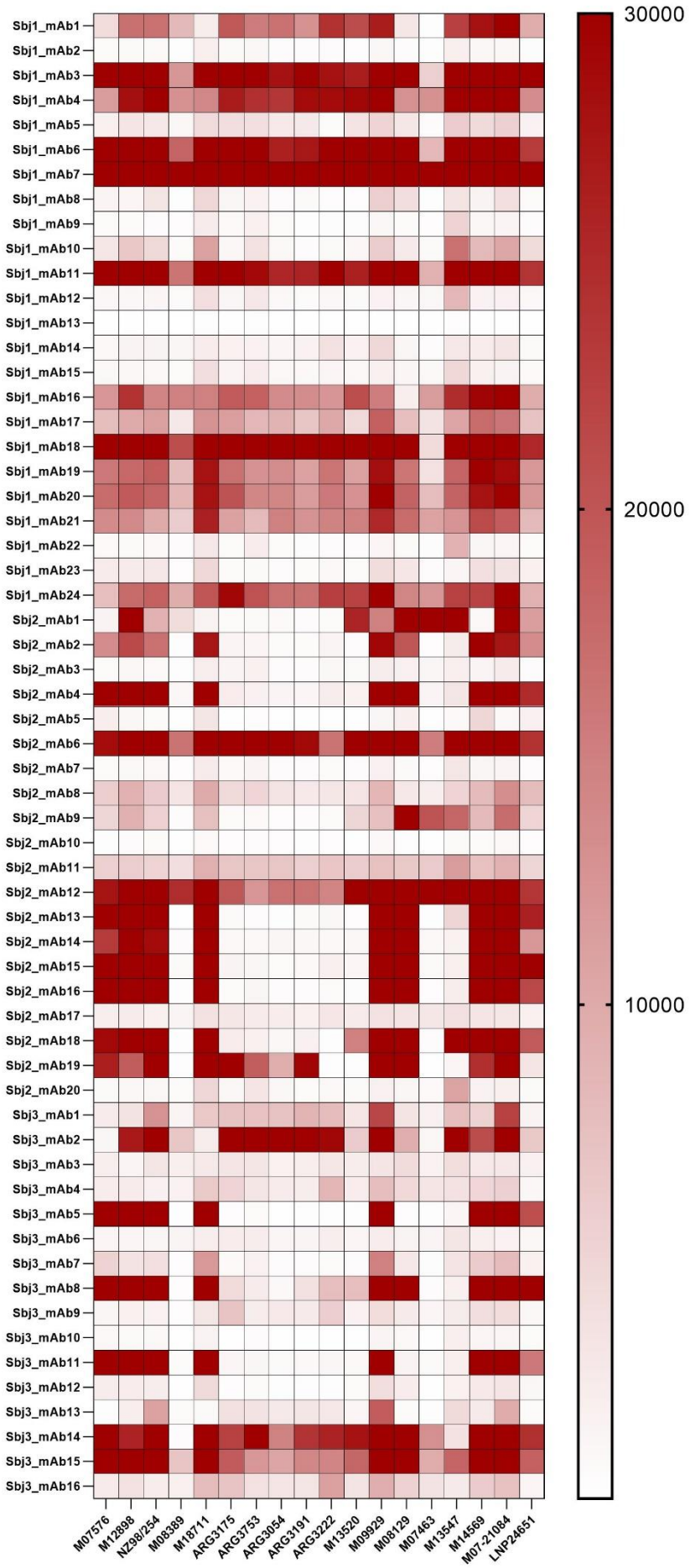


Figure 4.14 Difference between nOMV collected after 1 and 3 hours and MenB lysates.
Panel A) reports the difference in SDS-PAGE performed in reducing condition between nOMV from M09929 strain collected after 1 or 3 hour after the start of the stationary phase. The strain behaviour is representative of all the 18 strains utilized to obtain nOMVs. Panel B) SDS-PAGE profile of each MenB strain nOMV. Ten µg of each OMVs lysates were loaded in each well.

Differences in nOMV composition were detectable already from the SDS-PAGE made with each purified nOMV (Figure 4.14, panel B). We proceeded with the generation of the nOMV-dedicated microarray to screen the purified mAbs for binding to the selected MenB strains.

4.10 Binding profile of purified mAbs revealed differences between mAbs and, intriguingly, also among PorB-specific mAbs

To assess on which MenB strain bactericidal activity should be analysed for the different mAbs, we tested the nOMV-array with all the purified anti-OMV mAbs (Figure 4.15).


Sbj1_mAb1
Sbj1_mAb2
Sbj1_mAb3
Sbj1_mAb4
Sbj1_mAb5
Sbj1_mAb6
Sbj1_mAb7
Sbj1_mAb8
Sbj1_mAb9
Sbj1_mAb10
Sbj1_mAb11
Sbj1_mAb12
Sbj1_mAb13
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Sbj1_mAb19
Sbj1_mAb20
Sbj1_mAb21
Sbj1_mAb22
Sbj1_mAb23
Sbj1_mAb24
Sbj2_mAb1
Sbj2_mAb2
Sbj2_mAb3
Sbj2_mAb4
Sbj2_mAb5
Sbj2_mAb6
Sbj2_mAb7
Sbj2_mAb8
Sbj2_mAb9
Sbj2_mAb10
Sbj2_mAb11
Sbj2_mAb12
Sbj2_mAb13
Sbj2_mAb14
Sbj2_mAb15
Sbj2_mAb16
Sbj2_mAb17
Sbj2_mAb18
Sbj2_mAb19
Sbj2_mAb20
Sbj3_mAb1
Sbj3_mAb2
Sbj3_mAb3
Sbj3_mAb4
Sbj3_mAb5
Sbj3_mAb6
Sbj3_mAb7
Sbj3_mAb8
Sbj3_mAb9
Sbj3_mAb10
Sbj3_mAb11
Sbj3_mAb12
Sbj3_mAb13
Sbj3_mAb14
Sbj3_mAb15
Sbj3_mAb16
M07576
M12898
NZ98/254
M08389
M18711
ARG3175
ARG3753
ARG3054
ARG3191
ARG3222
M13520
M09929
M08129
M07463
M13547
M14569
M07-21084
LNP24651
30000
20000
10000

Figure 4.15 Binding patterns differ for the different mAbs on selected MenB strains nOMVs.

In the figure is reported the mean signal acquired from 8 replicate of each nOMV spotted onto the array at the concentration of 0,5 µg/ml. Blank cells represent a signal below the chosen cutoff and the more red the cell is the more the signal is saturated.

We observed that 17 mAbs showed a signal below the selected cutoff on NZ98/254 strain. This could be due to the lower sensitivity of the microarray technology when compared to the Luminex-based binding assay in which the same mAbs resulted specific for the NZ98/254 OMVs.

As none of this 17 mAbs was bactericidal when tested in the raw supernatant of transfected cells, we speculated that their binding on bacterial surface was not sufficient not only to give a positive signal on the nOMV-array, but also to have functional activity on the related strain. To confirm this hypothesis, 2 of these 17 mAbs were tested in rSBA on all the 18 MenB strain selected and, none of them resulted bactericidal, confirming our hypothesis.

Therefore, we proceeded to test by rSBA the bactericidal activity of each mAb giving a positive signal on the array on the corresponding MenB strain.

4.11 Fourteen out of the 18 selected MenB strains are killed by PorB-specific mAbs, but with different killing patterns

The 45 mAbs showing a positive signal on any of the 18 nOMVs spotted on the array have been tested on the recognized MenB strains in rSBA. The killing pattern shown by all tested mAbs indicated that they can be clustered in 3 different categories (Figure 4.16):

- PorB-specific and bactericidal mAbs;
- Not bactericidal mAbs with non-PorB target Ags;
- Bactericidal mAbs with non-PorB target.

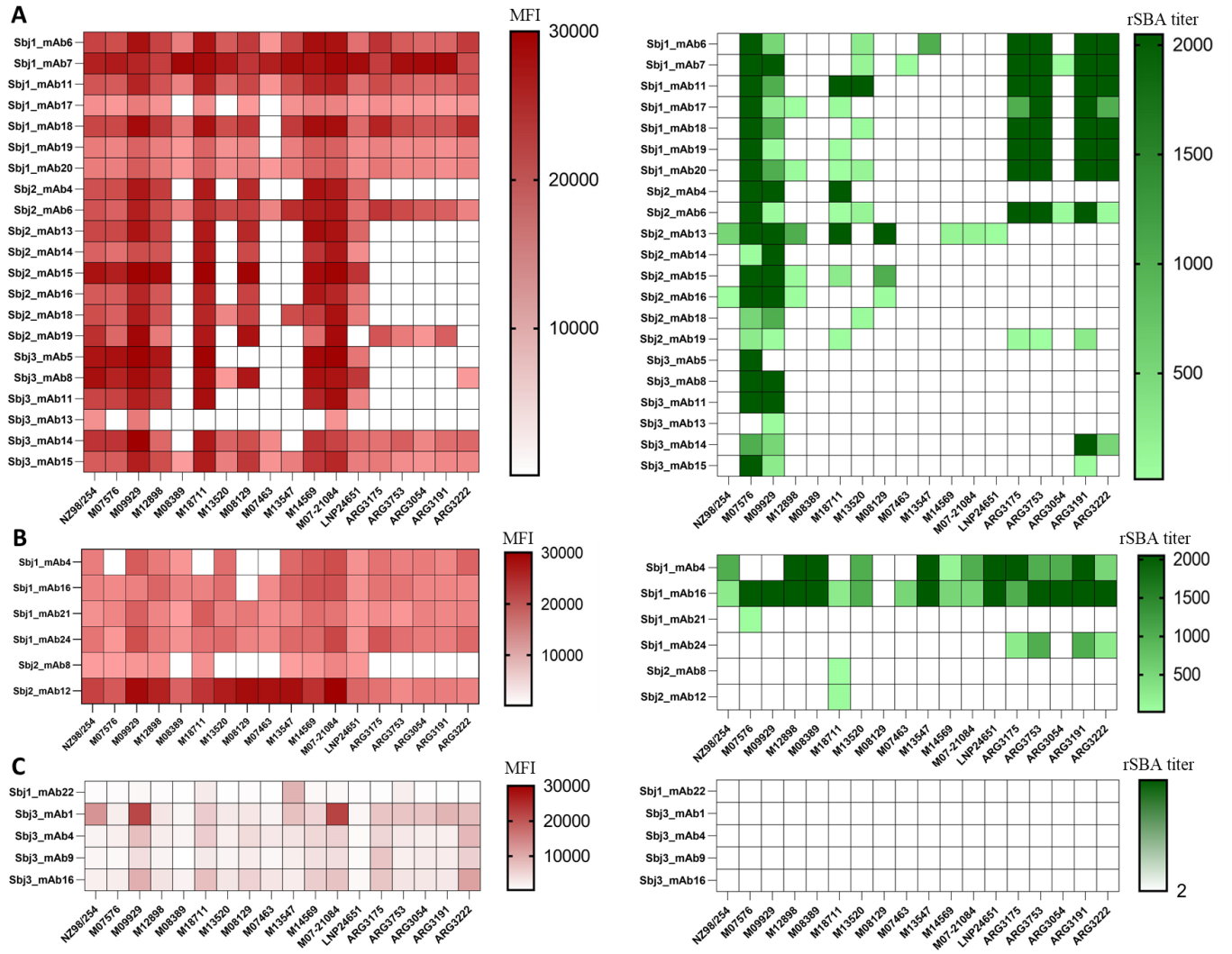

Figure 4.16 heatmaps of binding and killing of mAbs with an identified target.

In the figure are reported heatmaps for binding (red) or killing (green) of the tested mAbs. Cells are colored on the bases of the signals obtained with the nOMVarray (red) or the reciprocal of the dilution were mAb shown bactericidal activity (green). From top to bottom of the figure are shown patterns of: mAbs PorB-specific (A), mAbs with unknown target (B) and mAbs targeting minor Ags of the OMV component of 4C-MenB vaccine (C).

None of the mAbs recognizing minor OMV Ags resulted bactericidal per se on any of the tested strains, despite their cross-recognition on different MenB strains. We cannot exclude that these mAbs could exert bactericidal activity in combination with other mAbs, as would naturally happen in the context of a physiological immune response.

Regarding the group of mAbs with unknown target, we found that 3 of them were able to kill different MenB strains while other 4 were able to kill only 1 MenB strain. To understand which Ags are targeted

by these mAbs we performed immunoprecipitation coupled to mass spectrometry. Results that we obtained from these experiments are preliminar and, for this reason, not shown in this work as they need to be corroborated by more experimental evidences.

We finally analyzed the bactericidal activity of PorB-specific mAbs on the 2 MenB strains (M07576 and M09929) that were previously identified as reference strains for killing mediated by PorB (Viviani et al., under submission). All the PorB-specific mAbs tested were able to kill the M07576 and the M09929 strains, with the exception of mAb Sbj3_5, that did not show activity on M09929. This result could imply either the lack of the epitope recognized by mAb Sbj3_5 on the PorB of M09929 strain, or its lower accessibility. Overall, the results obtained with these 2 strains confirmed the specificity of our PorB-specific mAbs. Looking further in the activity of this subset of PorB-specific mAbs, we observed that other 14 strains from the selected panel were killed by these mAbs. Intriguingly, killing capability on these strains was not as homogeneous as observed with the reference strains for PorB. Indeed, we could identify different classes of monoclonal antibodies: mAbs binding and killing, mAbs binding but not killing and mAbs not binding a specific strain. In addition, the sensitivity of a specific strain to bactericidal killing was depending on the mAb tested. We made the following hypothesis to explain the different subclasses of monoclonals:

- mAbs with different SBA activity on the same strain are targeting different PorB epitopes, whose presence or absence due to sequence diversity between the 18 porins determines the outcome of SBA;
- mAbs binding without killing strains that are however killed by other PorB-specific mAbs could:adopt a different geometry on bacteria due to the different exposition of the targeted epitope, impairing the recruitment of C1q at the site of binding on the bacterial membrane; ot they could bind an epitope that is masked on the membrane of live bacteria but is accessible on the membrane of the nOMV that were utilized in the array.

To verify the impact of sequence variability on mAb recognition, we aligned the protein sequences of the PorB derived from all the 18 selected MenB strains. This analysis revealed that variability can be found mainly in the loops regions, as we expected from literature [138], which is in line with the concept that transmembrane regions are fundamental for the correct protein structure and conformation in the outer membrane of the bacteria and as a consequence they are highly conserved. In particular, 3 loops were subject to the higher variability observed, generating an exposed variable region on the porin (Figure 4.17, loops 4, 5 and 6 highlighted in green red and dark gray, respectively).

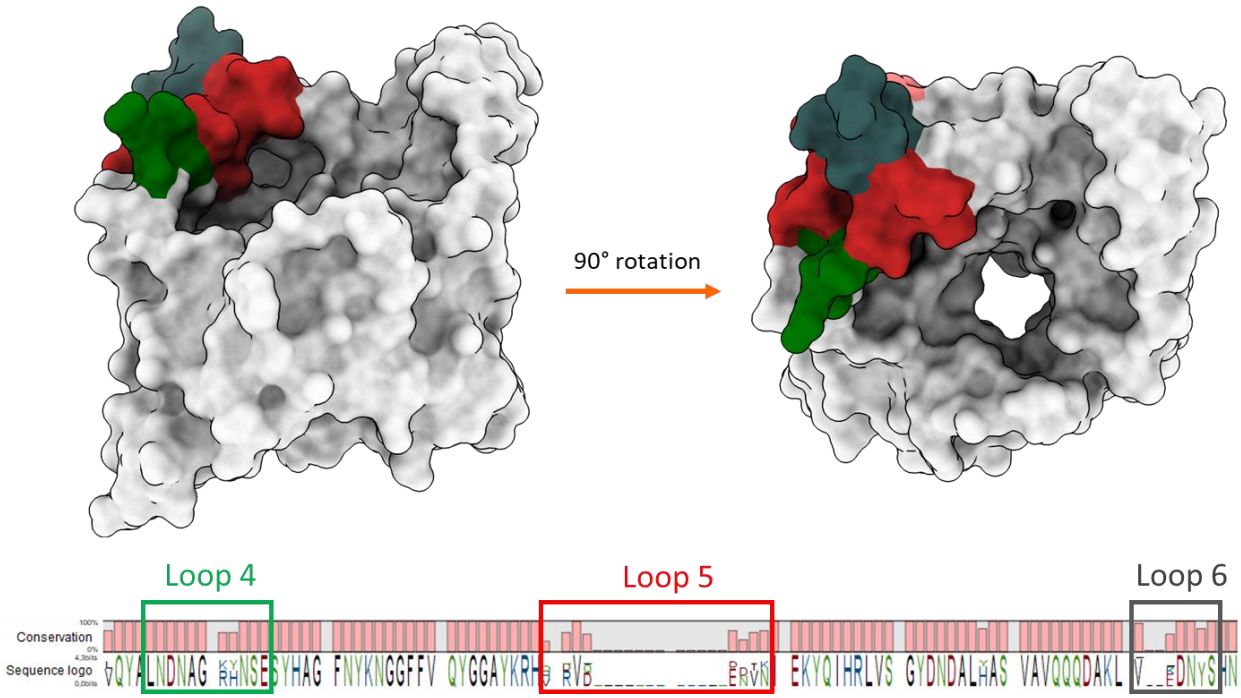

Figure 4.17 Alignment and structure design of the 18 PorB from the selected MenB strains.

On the top of the figure is shown the ribbon representation of the NZ98/254 PorB3 with the most variable loops colored in green (loop 4), red (loop 5) and dark gray (loop 6). On the bottom of the figure is reported the amminoacidic sequence alignment of the region which contains the 3 loops of interest. Image was done with ChimeraX and alignments with CLC Genomic software.

Since we found PorB with identical sequences in menB strains that gave different killing result with the same monoclonal, in order to understand the meaning of the observed phenotype we performed Electron Microscopy (EM) Immunogold experiments (to visualize the distribution of the mAbs on the surface of bacteria) coupled with FACS experiments (to evaluate the homogeneity of the bacterial populations observed by EM).

4.12 PorB-specific mAbs bind with different distributions on strains

We performed Immunogold experiments by EM on MenB strains incubated with mAbs showing one of the 3 different patterns described above as representative condition. When we analysed a strain that was bound by a specific mAb in the nOMV array and not killed in rSBA, the EM experiment confirmed an absent or very low binding of the mAb on the selected strain. On the other hand, when we analysed a strain that was bound by a specific mAb in the nOMV array and killed in rSBA, the EM experiment confirmed a very high binding of the mAb on the selected strain. We found an interesting pattern of binding on MenB strains that were recognized in the nOMV array but not killed by PorB-specific

mAbs: indeed, when a MenB strain is recognized in the nOMV array but not killed in rSBA we can observe a mixed population of bacteria in the EM preparation: there are bacteria fully covered by the anti-PorB mAb, as expected for an antigen that represents one of the main components of the bacterial surface, and bacteria with no o few antibodies bound on the surface (Figure 4.18).

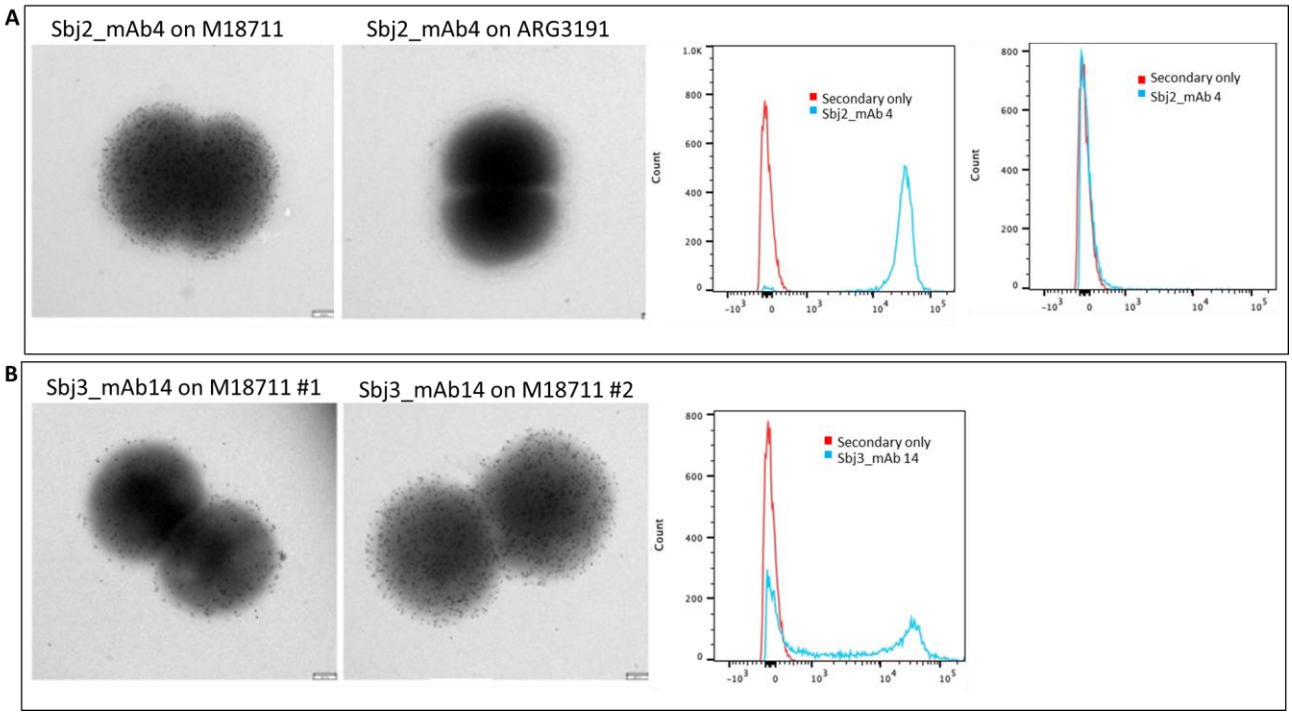

Figure 4.18 EM and FACS results from 2 MenB strains stained with 2 different mAbs.

The figure shown the EM image derived from Immunogold of mAbs reported on top of the image on the 2 mentioned MenB strains. Strains were chosen as M18711 is recognized and killed by Sbj2_2 mAb (positive control) while is recognized but not killed by Sbj3_13 mAb. ARG3191 was chosen as negative control, as it is not recognized in the nOMV array neither is killed in rSBA. On the right of each image is reported the FACS graph obtained by the staining of live bacteria of the corresponding MenB strain stained with the same mAb utilized to perform EM experiments. On panel A is reported the staining on the two different strains M18711 and ARG3191 of Sbj2_mAb2. On panel B is reported binding of Sbj3_mAb13 on only M18711 MenB strain.

We then repeated the same binding analysis using the FACS and what we observed is that while the first two classes of strains show an homogeneous population, with a peak completely overlapped or completely shifted with respect to the negative control (i.e. unstained bacteria), for the class of bacteria with a mixed binding in EM we observed a spread population, with bacteria showing different fluorescence levels. This data suggests that, in this specific case, the mAb binds the surface of bacteria

in the sample at different extents, from no or very low binding to high binding. Bacteria that are not bound (or bound to an extent too low to trigger bactericidal activity by rabbit complement) are then able to survive the killing mediated by complement in rSBA experiments. The phenomenon described has been observed with other 4 mAbs tested on M18711, M14569, M09929, and ARG3054 MenB strains (data not shown), suggesting a common explaination for the opbserved discrepancy between OMV-array and SBA. To confirm this hypothesis, FACS experiments were performed with all PorB-specific mAbs on NZ98/254, M14569, M18711, and M09929 strains, while for the subset of 4 PorB-specific mAbs utilized in the EM experiments we expanded the FACS analysis to all the 18 selected MenB strains (data not shown). Results recapitulated the behaviour observed on the 4 initial strains on all the tested MenB strains. Therefore, we can speculate that what we described here is not typical of mAb Sbj3_13 on M18711 strain, but is shared between all the PorB-specific mAbs that have the same behavior on the MenB strains, which are positive in OMV-array but not killed.

Since PorB is not a phase-variable gene, we hypothesized that this behavior could be due to specificity of the mAb for an epitope of PorB which is masked by some structure of another OMV component which is subject to phase-variation and is expressed at different extents during bacterial growth. To assess if PorB-specific mAbs sharing this particular binding pattern could recognize similar epitopes, we utilized a bioinformatic tool to compare their aminoacidic sequence and predict the epitope recognized.

4.13 PorB-specific mAbs characterization with bioinformatic tools

We firstly verified if PorB-specific mAbs share common characteristics in terms of clonotypes and V-J junctions utilized with the same IgBLAST-type protocol previously applied to the sequence of all our mAbs [139].

We noticed that anti-PorB mAbs were enriched in IGHV1-18, IGHV1-69 and IGHJ2, in the variable heavy chains, and in IGKV1-39, IGKV3D-11, IGLV3-19, IGKJ2, and IGLJ7 in the variable light chains. In addition, we also noticed that while the non-PorB specific mAbs were spread across all gene families, not all the V and J genes were utilized in the PorB-specific mAbs. Specifically, only IGHV1-18, IGHV1-60, IGHV3-21, IGHV3-23, IGHV3-30, IGHV30-4, and IGHV4-34 were utilized for the V junction on the heavy chain and IGHJ1, IGHJ3, IGHJ4, and IGHJ6 for the J junction. Regarding the light chains IGKV1-5, IGKV1-39, IGKV3-20, IGKV3D-11, IGLV1-51, and IGLV3-19 were utilized for the V junction and IGKJ1, IGKJ2, IGLJ3, and IGLJ7 for the J junction (Figure 4.19).

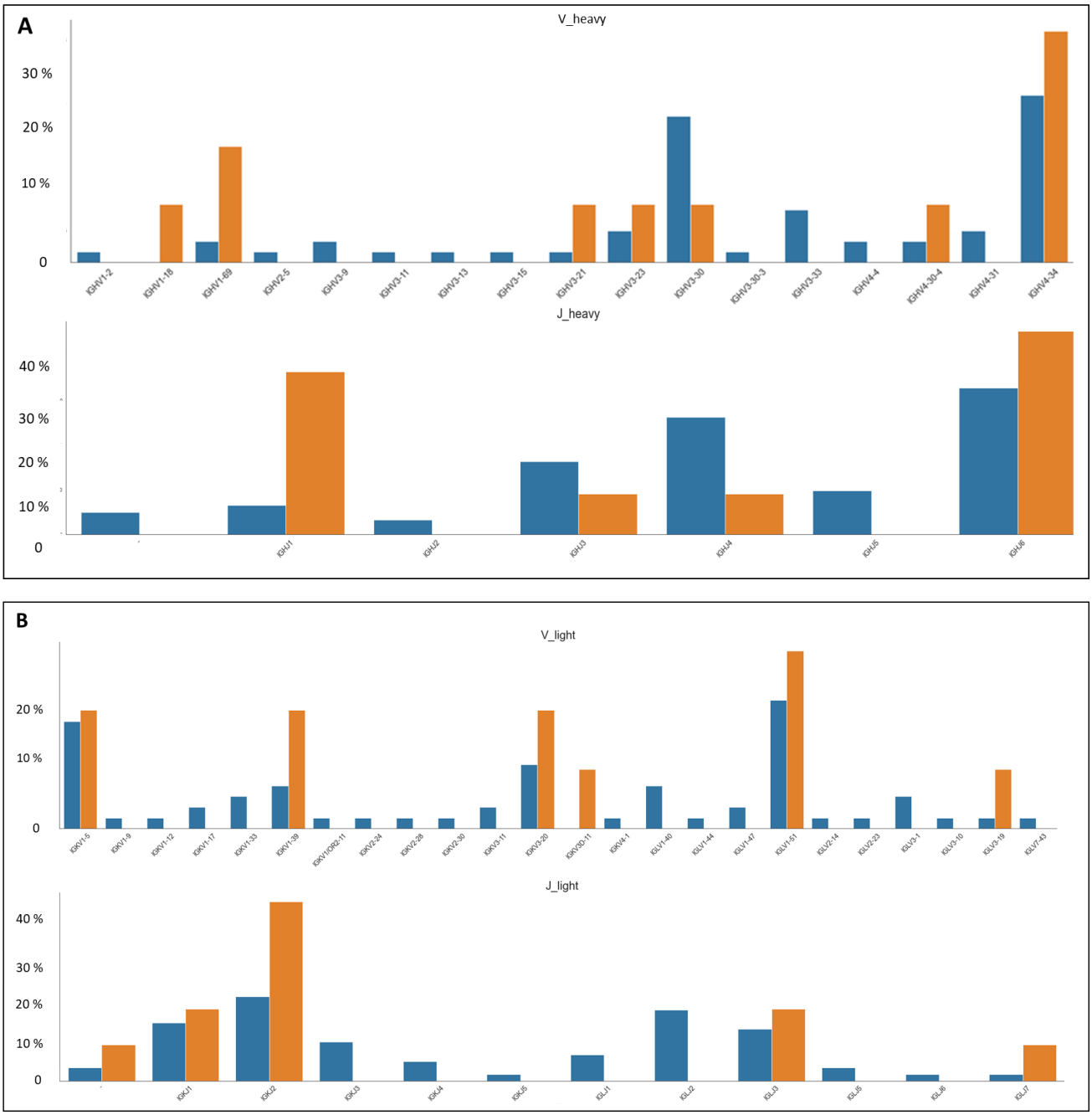

Figure 4.19 V and J gene usage of PorB specific mAbs. On panel A is reported the V-J usage of VH, while on panel B is reported the usage of VL. On the X axis of the graphs is reported the different V and J alleles found in each mAb, and on the Y axis is reported the percentage of mAbs. The color used is orange for PorB-specific mAbs and blue for all the other mAbs.

These are the first reported data on the characterization of the gene usage of PorB-specific mAbs and, even if it is derived from a subset of only 20 mAbs, set the basis for the characterization of these kind of mAbs in a population of different 4C-MenB vaccine elicited mAbs.

We used the Parapred software [140] to predict and compare paratopes in the PorB-specific mAbs that bound MenB strains without exerting killing and we managed to find a similar paratope for 2 out of the 10 mAbs of interest. Specifically, we found that mAbs Sbj2_18 and mAbs Sbj2_20 have a paratope score higher than the cut off (0.95), predicting binding on the same epitope (Table 4.3).

mAb ID	Paratope predicted	Score
Sbj1_18	_____F__YY_E_N_S_R_K____VKAERF_F_____Y_____	0,954
Sbj1_20	_____F__YY_E_NYS_R_K____VKAERF_F_____Y_____	
Sbj1_18	_____F__YY_E_NYS_R_K____IKTERF_F_____Y_____	0,909
Sbj1_17	_____F__YY_E_N_S_R_K____VKAERF_F_____Y_____	
Sbj2_4	_____RSY____SDD__K_Y___LSARRGYYYF_____	0,833
Sbj2_16	_____AY_____R_____LS_RRGFYYF_____	

Table 4.3 Paratope scores made with Parapred tool. In the table are reported the paratopes predicted for each mAb couple listed and the score of similarity given by the software, on the right column. Scores above 0.95 (green) are considerate significant, while scores under this value are (red) are considerate probable. Table reports only mAbs with a score higher than 0.8.

This result is in line with the rSBA data, as Sbj1_18 and Sbj1_20 have the same binding and killing profiles on the 18 tested MenB strains. Nonetheless, the software did not manage to identify other similar paratopes for the other PorB-specific mAbs, highlighting the need for more experiments, like docking, to elucidate the different behavior of these mAbs on different MenB strains.

5 Discussion

The 4C-MenB vaccine, which is currently utilized to prevent invasive meningococcal disease caused by *Neisseria meningitidis* serogroup B, is composed of three recombinant proteins (fHbp, NHBA, and NadA) and the outer membrane vesicles (OMV) from the NZ98/254 strain. While the contribution of each recombinant component of this vaccine has been well characterized, thanks to studies both pre- and post-vaccine licensure, the contribution of the OMV component to vaccine protection has not been completely clarified yet. PorA on OMV surface has been pinpointed as the major driver for protective Abs in the vaccinees, as it is one of the most abundant and immunodominant Ags on these vesicles. Nonetheless, the importance of other OMV components on bacterial clearance has been highlighted in early phase II clinical trials, where protection was elicited also on mismatched PorA MenB strains. Ags responsible for the induction of protective mAbs are not know yet [141, 142].

The aim of this work was the characterization of the immunological response of vaccinees after 3 doses of 4C-MenB vaccine, to shed light on the OMV Ags responsible for cross-strains protection.

To do so, we single-cell sorted 1024 PBs from 3 vaccinated subjects, to retrieve the VH and VL regions of the immunoglobulins encoded by the isolated cells and expressed the corresponding recombinant mAbs in raw supernatants of transfected mammalian cells. Looking at binding properties of mAbs produced we found a similar frequency of 4C-MenB-specific mAbs between the 3 different subjects (14%, 18% and 15% for subjects 1, 2 and 3, respectively). Intriguingly, more than half of the identified 4C-MenB-specific mAbs were OMV-specific (59%). This data are in line with previous works that highlighted the importance of OMV in the formulation of this vaccine [143, 144] and is concordant with high ELISA titers of sera of the three selected subjects on NZ98/254 OMV (data not shown). Whether these mAbs mediate cross-protection on different MenB strains is the topic of this study.

To answer this question, we looked at the functionality of this subset of mAbs on different MenB strains. Therefore, we performed rSBA with the reference strain of the vaccine (NZ98/254, whose one of the main antigen involved in bactericidal killing is PorA) and a strain mismatched for PorA (M07576), in order to discriminate the antibodies mediating non-PorA related killing. What we observed was that the majority of bactericidal mAbs were killing the M07576 strain but not the NZ98/254 strain. This was a preliminary result, as we looked at the activity of these mAbs in raw supernatant and at different concentrations (as each mAb has a different expression level), that needed to be confirmed with purified and normalized mAbs. Nonetheless, it pointed out the importance of other OMV antigens in eliciting cross-protection in addition to PorA.

When we sequenced the Heavy and Light chain variable regions of these mAbs, needed to express them as recombinant mAbs, the data we obtained not only confirmed the unicity of their VH and VL,

but also showed an enrichment in some V and J alleles utilized to assemble OMV-specific mAbs in different 4C-MenB vaccinated subjects. Despite the intrinsic limitation of our analysis, due to the small size of our sample (n=70), a trend in the usage of IGHV3-30, IGHV4-34, IGHJ4, and IGHJ6 is visible for the VH of the antibodies and of IGKV1-5, IGKV3-20, IGKJ1, IGLV1-51, IGLJ2, and IGLJ3 is visible for the VL. The preferential use of these genes to assemble OMV-specific mAbs is not subject-specific, since this phenomenon is shared between the 3 analysed subjects. This is, to our knowledge, the first evidence of a preferential gene usage to assemble OMV-specific mAbs from the human immune system after 3 doses of 4C-MenB vaccine.

Once we obtained the purified mAbs, we proceeded with the analysis of their functionality testing them in rSBA on a selected panel of MenB strains mismatched for PorA. Eighteen different strains (NZ98/254 as vaccine reference strains + 17 mismatched PorA strains) were utilized in this analysis and the selection was based on previous data (unpublished data) showing that their killing was exclusively mediated by Abs targeting the OMV component of 4C-MenB vaccine after immunization in infants (5 strains) or adults (12 strains). To rationalize the assessment of bactericidal activity of produced mAbs on these 18 strains, we developed a native OMV (nOMV) array to test in parallel the binding of each purified mAbs to nOMV derived by all the strain. Data obtained from the array showed different binding profiles between the mAbs, as we expected from their monoclonality and the fact that the specific target of each mAb can be differentially expressed/exposed between the 18 selected strains. Thus, we proceeded testing in rSBA each mAb on all the strains recognized on the nOMV array.

Twenty-nine out of 63 tested mAbs had bactericidal activity on at least one of the selected strains and only 6 of them were bactericidal against NZ98/254 strain. This data was not entirely unexpected, as we already observed low percentages of PorA-specific mAbs in the subset of OMV-specific mAbs in the Luminex binding assay. Two of these 6 mAbs were the only PorA-specific mAb that we obtained as recombinant purified mAb. The other 4 bactericidal mAbs on NZ98/254 strain showed activity also on other MenB strains, suggesting a shared and protective target between these strains. With the aim to unravel these targets, as well as the ones of the other 19 cross-protective mAbs, we utilized a protein array displaying 39 *Neisseria meningitidis* proteins previously identified as main OMV component [145]: 13 were express as recombinant proteins, while the remaining 26 were outer membrane proteins (OMP) presented on the surface of *E. coli*-derived vesicles (called Generalized Modules for Membrane Antigens, or "GMMA". We prioritized OMP above other Ags because they account for the 90% of the OMV content as shown in a recent paper where different 4C-MenB lots undergo quantitative and qualitative proteomic analysis [145]. The exposition of 26 of these proteins on GMMA was preferred as their hydrophobic nature posed a challenge on their expression and correct folding outside the

membrane context. As proper folding of these proteins is necessary to conserve the exposed epitopes in their native conformation (a mandatory quality to mediate mAbs recognition of conformational epitopes), the GMMA approach was selected to present these protein, as it has already been proved successful on presenting bacterial Ags [146]. From these protein array experiments, we identified the following targets: PorB, PorA, RmpM, Hyp1065, ComL and BamE. This result is partially in line with the work of Awayne [147], where immunoprofiling of sera from adults vaccinated with OMV from H44/76 strain was done with protein microarrays and the most reactive proteins found were: PorB, PorA, RmpM, OpcA, PilQ, BamC, and GNA1162. The last 2 proteins were not included into our array due to their absence (BamC) or very low abundance (GNA1162) in the OMV component of 4C-MenB vaccine [148]. We can exclude the role of BamC in eliciting mAbs after vaccination (it is absent on 4C-MenB OMV), while the effect of GNA1162 cannot be ruled out, as some of the tested mAbs did not recognize any target on the array. Moreover, we did observe 1 mAb elicited by BamE, which has a percentage lower than 0,1% in the OMV relative abundance (similar to the abundance of GNA1162), suggesting that even such low amount on the OMVs can be sufficient to induce an immune response in human. What was interesting was the lack of mAbs against OpcA and PilQ, reported in literature as highly reactive proteins in the OMV component. Possible explanations could be linked to the small number of subject analysed, with the hypothesis that in this specific subset of vaccinated individuals these protein were not immunogenic, or the fact that Awayne [147] analysed at sera response while we looked at specific mAbs derived from PBs and the proportions can be different within these 2 samples.

In addition, we found 3 new immunogenic components of the NZ98/254 OMV: ComL, BamE and Hyp1065. Both ComL and BamE are fundamental for the correct assembly of Omp85 (a conserved outer membrane protein) and associate with it on the outer membrane alongside BamA, BamC and RmpM [149]. Unfortunately, bactericidal activity was not observed for mAbs specific for these targets, suggesting a secondary role in the protection of 4C-MenB vaccine. Nonetheless, we founded only 2 mAbs targeting these proteins, so we can not rule out the possibility of elicit protection of these component. What we can affirm is the exclusion of the specific epitopes targeted by these 2 mAbs from the portion of protein which could elicit protection.

Intriguingly, looking at the activity of mAbs expressed in supernatant of transfected cells, we found that all the mAbs able to kill the M07576 MenB strain were PorB-specific and the mAbs able to kill NZ98/254 strain were PorA-specific. While the relevance of PorA-specific mAbs was known [8], to our knowledge these are the first data supporting the functional activity of PorB-specific mAbs. The main reasons behind the focus on PorA and not PorB during the years of development and study of MenB vaccines resides in the fact that while the former porin has been demonstrated immunodominant

and protective, the latter was considered highly immunogenic [150] but poorly protective due to its hypervariability in the external loops [151]. Nonetheless, looking at the activity of the purified PorB-specific mAbs on the 18 selected MenB strains, we found that most mAbs exerted killing on more than one strain.

The availability of these strains gave us the possibility to test also the other purified mAbs, expanding the knowledge on the importance of minor Ags on the OMV component of 4C-MenB vaccine. As a result of these experiments, we assessed that PorB and PorA are able to elicit mAbs with bactericidal activity per se. This data do not exclude that mAbs specific for less represented Ags on the OMV surface can impact the immune response against MenB strains in vaccinees. There are indeed examples in the literature of mAbs elicited by 4C-MenB components that gain bactericidal activity only when combined [152, 153]. However, the lack of bactericidal activity of the only RmpM-specific mAb we found is in line with what reported in literature [154]. Indeed, RmpM interact with PorA/B anchoring them to the peptidoglycan layer via its N-terminal region, excluding its exposition on the outer membrane of live meningococci [155].

As we found 6 bactericidal mAbs without a specific target, nor on the protein array nor in WBs, we concluded that their targets are probably proteins which were not included into the protein array or non-proteic components of the NZ98/254 OMVs.

As 14 out of the 18 selected MenB strains were killed by PorB-specific mAbs we focused on this subset to unravel the driver of this bactericidal activity. What we observed was that, in addition to the reference strains M07576 and M09929, some mAbs were able to kill other MenB strains and, in two cases, the NZ98/254 as well. Indeed, all the selected strains share the PorB3 serotype, but not all of them are killed by all anti-PorB mAbs. As the external loops of PorB possess high variability, we compared their sequence trying to explain the different binding and killing patterns shown by our PorB-specific mAbs. We observed diversity amongst strains (as all the 18 strain possess the PorB3 serotype, but not the same serosubtype) that could partially but not entirely explain the different sensitivity to PorB-mediated killing. Indeed, PorB sequences are identical between some strain that are always killed by PorB-specific mAbs (as M07576) and some that are not (as M14569). For this reason, we looked further on the binding of these mAbs on the bacterial surface with FACS and EM.

We observed with EM that MenB strains that were bound but not killed by PorB-specific mAbs had at least 2 different staining profile: bacteria fully coated by mAbs and bacteria partially coated by mAbs. This data was confirmed by FACS, which gave us also information on the distribution of each population with the different mAbs. Therefore, we concluded that PorB-mediated killing is depending on both, epitope sequence and epitope accessibility and the latter can vary in the bacterial population during its grown. Thus, we hypothesized a phase-variable binding of PorB-specific mAbs to different

MenB strains, suggesting that epitopes recognized by PorB-specific mAbs were masked during some phases of MenB growth. As PorB is not a phase-variable gene, we hypothesised that this phenomenon could be due to LPS shielding effect. Indeed, masking of PorB3 serotype by LPS was already reported in literature by Michaelsen and colleagues [156]. In their work they demonstrate that PorB3 accessibility highly increase after ethanol treatment of bacteria, which disrupt the LPS, and conclude that the porin is not a good vaccine candidate due to its poor accessibility. This conclusion however was drawn after analysing the binding of whole human sera derived from vaccination in only 3 different MenB strains, while we observe single mAbs on 18 different strains. On the contrary, their result strengthen the fact that PorB3 is masked in some strain (as the ones that they utilize in their analysis, which were not included in our panel of strains, and the M14569 in ours) but in others it is fully accessible during all the growth phases (as in M07576 and M09929, which are always killed by PorB-specific mAbs) or accessible only in some phases of growth. Nonetheless, there are PorB3 epitopes that are always accessible, one example is Sbj2_mAb13 of our study which is bactericidal on MenB strains that are not stained by other PorB-specific mAbs. It will be interesting to better investigate in the future the nature of this epitope and use this information to find a way to better present it in the OMV of future 4C-MenB formulations, to exploit PorB-mediated killing.

In conclusion, we showed that OMV are a fundamental component of 4C-MenB vaccine to enhance the breadth of coverage against a panel of 18 genetically different strains. Specifically, we were able to identify RmpM, BamE, ComL and Hyp1065 as immunological component of OMV and to assess the protective role of PorB. Since the latter is an OMP, as its more famous counterpart PorA, the best way to present it to our immune system is through vesicles, where it maintains its native conformation. This are additional data which confirm the importance of OMV in the immunogenicity of the 4C-MenB vaccine formulation and in the broad protection across MenB strains it induces.

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