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Characterization of the immune response and cross protection activity elicited by the Neisserial Heparin Binding Antigen (NHBA), a component of the 4CMenB vaccine

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A mio padre Leonidhë,

per i suoi insegnamenti

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DISCLAMER

Dr. Enea Ndoni was enrolled at the Life Sciences Graduate Program at University of Siena. The research project he worked on was in collaboration with and funded by Novartis Vaccines, now acquired by the GSK group of companies.

1. ABSTRACT

Invasive disease caused by capsular group B *Neisseria meningitidis* (MenB) is life threating disease causing hundred thousands of deaths every year, still remaining an unmet medical need in many countries. Although disease can be observed at all age groups, infants and adolescents are the most at risk populations showing the highest incidence in case numbers. Since the MenB capsule was not-immunogenic the development of a MenB vaccine which makes the use of other antigens becomes necessary. 4CMenB is a multicomponent vaccine against serogroup B *N. meningitidis* composed by three major protein antigens, factor H-binding protein (fHbp), Neisserial Heparin-Binding Antigen (NHBA) and Neisserial adhesin A (NadA), combined with outer membrane vesicles (OMVs) from the New-Zealand epidemic strain (NZ98/254). Neisserial Heparin Binding Antigen (NHBA) is a surface-exposed lipoprotein expressed by all *N. meningitidis* strains analyzed so far and is composed of two major domains, a highly variable amino-terminal (N-term) domain which anchors the protein on the bacterial outer membrane through the lipobox motif, and a highly conserved carboxyl-terminal (C-term) domain. These domains are separated by a short and quite conserved Arginine-rich (Arg-rich) motif which has been reported to be involved in different mechanisms that mediate meningococci adhesion, infection and survival within the host's blood stream. NHBA is susceptible to cleavage by NalP, a bacterial protease which has its cleavage site upstream of the arginine region. Moreover human proteases such as human lactoferrin (hLf) and kallikrein are able to process NHBA downstream the the Arg-rich region. Both bacterial and human proteases-mediated cleavage releases the C-term of NHBA in the supernatant, while the N-term of the protein remains anchored on the bacterial surface. NalP cleavage did not impact SBA titers elicited by anti-NHBA antibodies but little is known about the impact that host's proteases have on bactericidal titers. Based on sequence analysis it has been reported that NHBA has two major alleles, the so called "short" and "long" variants, which differentiate by the presence or absence of a 190 bp long fragment. Despite its sequence variability, NHBA is able to induce a robust and broad immune response against meningococcal strains expressing vaccine homologous and heterologous variants. Although anti-NHBA antibodies are able to induce bacterial killing when tested in serum bactericidal activity assay (SBA), the regions involved in eliciting cross protective immune response remain still unknown. Aims of this study were to use monoclonal antibodies (mAbs) raised against the NHBA vaccine variant peptide 2 (NHBAp2) to (i) map the NHBA regions involved in eliciting the functional response, (ii) test their ability to induce cross protection against strains expressing epidemiologically relevant homologous and heterologous NHBA variants, and (iii) investigate the molecular mechanism of NHBA-mediated bactericidal activity. To this end we used a panel of anti-NHBA mAbs selected to

recognize different regions of the protein. Our results showed that only anti-N-term mAbs were able to induce killing of bacterial strains expressing the homologous NHBAp2 and closely related heterologous NHBA variants. Synergy between monoclonal antibodies targeting the N-term and the C-term of NHBA resulted in a significant increase of bactericidal titers but cross protection remained restricted to closely phylogenetic NHBA variants. Anti C-term mAbs were not able to induce SBA activity when tested individually, but surprisingly they became bactericidal when tested in combination. Moreover they were able to induce full cross protection against a panel of strains expressing phylogenetically distant heterologous NHBA variants.

Our results suggest that the partial release of the NHBA C-terminal portion upon NalP and serum proteases could explain why anti-C-term mAbs are not able to induce complement mediated bactericidal killing when tested individually. However, the simultaneous binding of C-term mapping mAbs on the same NHBA molecule can induce the formation of a very stable ternary complex that probably allows a more efficient C1q engagement and C3 deposition, thus leading to the observed co-operative bactericidal activity. These results suggest that synergy between anti-NHBA antibodies is at the basis of the mechanism of NHBA-induced bactericidal activity, which could explain the robust and cross-protective immune response elicited by anti-NHBA polyclonal antibodies following immunization. Collectively, the body of experimental data suggests that both domains of NHBA are required to elicit complement mediated bactericidal activity against strains expressing the vaccine homologous and heterologous NHBA variants.

2. INTRODUCTION

2.1. *Neisseria meningitidis*

Commonly named meningococcus, *Neisseria meningitidis* is a Gram-negative, diplococcal-shaped aerobic bacterium that lives as an obligate pathogen colonizing exclusively humans (DeVoe 1982). Meningococci which are piliated and non-spore forming colonize the mucosal membranes primarily in the respiratory tract. It is estimated that up to 10-25% of the population is meningococcus asymptomatic carrier in the nasopharynx tract (Stephens 2009).

N. meningitidis is surrounded by a lipid membrane containing outer membrane proteins and lipopolysaccharides and it can be either capsulated or non-capsulated. The polysaccharide capsule is essential for pathogenicity as it confers resistance to phagocytosis and complement-mediated lysis and offers protection against environmental attacks. As a consequence, non-capsulated bacteria are attenuated *in vivo* since they cannot persist in the blood and only rarely may cause invasive disease (van Deuren, Brandtzaeg et al. 2000).

On the basis of the immunogenicity and structure of the polysaccharide capsule, *N. meningitidis* can be classified into 12 serogroups (Branham SE, 1953). Among them, only six serogroups (A, B, C, W, X, Y) have been associated with invasive meninogococcal disease in humans and are thus considered pathogenic (Rouphael and Stephens 2012). Based on class 1 outer membrane proteins (PorA), class 2 or 3 outer membrane proteins (PorB) and lipopoly[oligo]saccharide structure, a further classification into serosubtype, serotype and immunotype, respectively, has been reported (Rosenstein NE, 2001), (Stephens DS *et al*, 2007). Since classification based on a few genes variation that probably are under selective pressure is not anymore suitable, new methods of epidemiological classification have been developed. Multi-locus enzyme electrophoresis (MLEE) uses the electrophoretic mobility of various cytoplasmic enzymes to classify meningococci into clonal families with similar characteristics. Eventually, genetic typing system based upon polymorphisms in multiple house-keeping genes (Multi-locus Sequence Typing, MLST) has now become the gold standard for molecular epidemiological typing. This technique has shown that majority of disease-associated clinical isolates cluster into a minority of sequence type (ST) and thus allowed to define hyper-virulent invasive meningococcal lineages (Maiden M. *et al*, 1998).

Meningococcal outer membrane is composed of many proteins, some of which enable the organism to adhere to, and to invade, host cells. Pili, composed of pilin subunits, are filamentous structures essential in mediating the interaction between meningococci and both epithelial and endothelial cells, and are also associated with other phenotypes such as a high level of competence, bacterial auto-agglutination and twitching motility. Pilins are classified into class I and class II based on their differences in antigenicity (Nassif X., 1999). Opa and Opc proteins are capable of mediating adhesion to and invasion into eukaryotic cells, but this effect is visible only in the absence of a capsule (Virji M. *et al*, 1993, 1992). Outer membrane also encloses lipopolysaccharide (LPS), which helps in serum resistance and plays a key role in the pathogenesis of meningococcal disease (Vogel U. and Frosch M., 1999; McNeil G., Virji M. and Moxon ER, 1994).

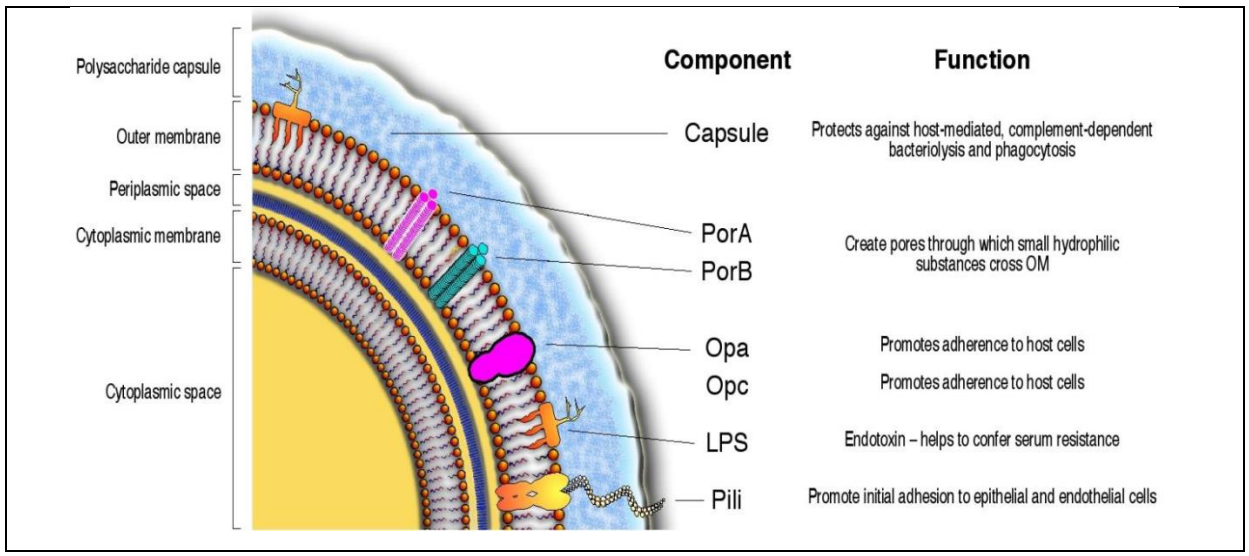


Figure 1. Schematic representation of the different bacterial compartments and of the main components of the outer membrane, together with their known function (image adapted from Rosenstein NE, 2001).

2.2. *Epidemiology of meningococcal disease*

Globally the annual number of meningococcal invasive disease cases is estimated to be 1.2 million with 135,000 deaths, although the incidence of the disease varies from <1 case/100 000 population, in Europe and North America, to 10-1,000 cases/100,000 population, in the "meningitis belt" of sub-Saharan Africa (Jafri et al., 2013). Direct contact

with respiratory droplets or secretions is the most common way of bacterial transmission from a healthy carrier or an infected person. It has been described that carriage can be an immunizing process that results in the elicitation of a specific and systemic protective antibody response (Yazdankhah and Caugant, 2004). Nevertheless, bacterial exposition is able to induce invasive diseases in a subset of individuals. Diagnosis of meningococcal disease is often challenging since initials symptoms mimic the symptoms of common viral infection, such coryza, pharyngitis, fever, loss of appetite, nausea, vomiting and headache. Without an appropriate and urgent treatment, the infection can progress rapidly and result in death or permanent disability in as little as 24 hours from the first symptoms. Even with appropriate antibiotic therapy, the case-fatality ratio of meningococcal infection disease is 10% to 15% rising up to 40% in septicemia cases (Pinkbook CDC 2015). Most severe clinical forms of invasive meningococcal diseases (IMD) are meningitis (30-60%) and septicaemia (20-30%) often complicated by with *Purpura Fulminans* (petechial or purpuric rash) manifestations (Pace and Pollard, 2012). Infants and adolescents are particularly susceptible to invasive disease but all age groups are at risk for meningococcal infections. Lack of maternal antibodies in newborns and infants and the high rate of nasopharyngeal colonization in adolescence, respectively, increase the risk of meningococcal invasive infections in these population groups (Kovarik and Siegrist, 1998; Christensen et al., 2010; Soriano-Gabarró et al., 2011). Figure 1 shows the results of an epidemiological study were are reported the number of hospitalization cases due to IMD divided per group age performed by the Italian national health agency (ISS, 2014). This study identifies three major peaks that correspond to three different age groups. The highest incidence of the disease is observed in infants followed toddlers. The third age group in which IMD occurs is composed by adolescents and young adults between 15 and 19 years-old. The predominant serogroup responsible for invasive meningococcal disease in new-born babies is serogroup B (ISS, 2014; ISS, 2016).

Although peak incidence occurs among infants and adolescents, more than one-third of sporadic cases are reported in adults older than 18 years of age. Early stages of the disease are very similar to viral infections such as influenza, but the disease onset may be fulminant. Thus, identification of symptoms might be difficult and this can delay appropriate therapeutic treatment. Rapid progression of the disease from bacteremia and/or meningitis to life-threatening septic shock syndrome or meningitis can occur within the first few hours

after initial symptoms appear. Because of the severity of clinical outcomes and the difficult management of meningococcal diseases, vaccination remains the only successful way for community prevention and disease control.

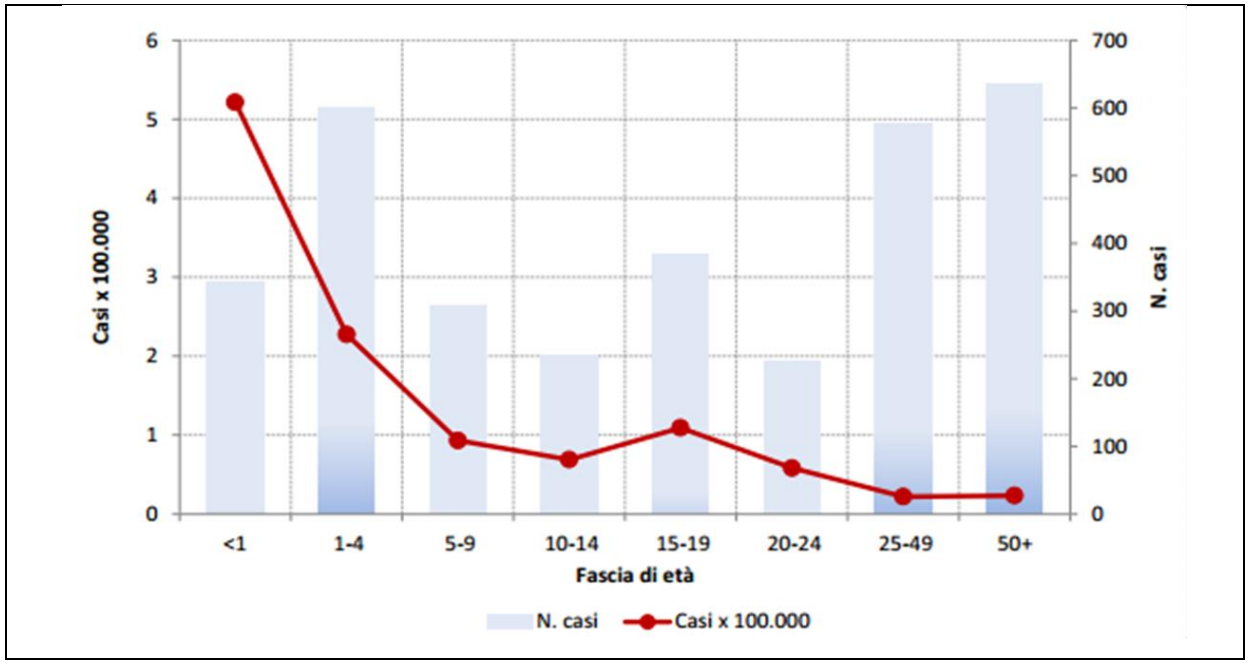


Figure 2. Incidence of meningococcal invasive diseases in Italy Figure adapted from ISS report (2014). Number of hospitalization cases per age groups due to invasive meningococcal disease in Italy from 2001 and 2012

2.3. *Anti-meningococcal vaccines*

Given its rapid progression and difficulties related to diagnosis (Rosenstein, Perkins et al. 2001, Thompson, Ninis et al. 2006), vaccination is the most effective option for meningococcal disease prevention. Currently, no broadly protective vaccine is available to provide protection against all serogroups of *Neisseria meningitidis* although different meningococcal vaccines have been developed against the distinct serogroups (Zahlanie, Hammadi et al. 2014). Several polysaccharide and conjugate meningococcal vaccines are available against serogroups A, C, Y and W. The tetravalent vaccine composed of only purified capsular polysaccharides, was seen to be efficacious in adolescent and adults but poorly immunogenic in infants and failed to induce immunological memory. However, when conjugated to a carrier protein, capsule polysaccharides show a greatly improved

immunogenicity in infants (Granoff and Pollard 2007, Nassif X. 2009, Virji M. 2009). Monovalent, bivalent, and tetravalent polysaccharide conjugative vaccines are available and effective against meningococcal serogroups A, C, Y and W (Zahlanie, Hammadi et al. 2014); http://www.who.int/ith/vaccines/meningococcal/en/]. When a massive vaccination campaign with MenC conjugated vaccine was implemented in UK, a dramatic decline in the incidence of serogroup C disease in all age groups that received the vaccine (Borrow R. et al. 2000; Miller E. et al. 2001) was observed, with a vaccine efficacy of 97 and 92 per cent for teenagers and toddlers, respectively (Ramsay M. et al. 2001).

Following the introduction of the MenC conjugated vaccine, serogroup B has remained the most critical target for anti-meningococcal vaccine development. It is responsible for 32 per cent of all cases of meningococcal disease in the United States and for 45–80 per cent or more of the cases in Europe (Scholten RJ. et al, 1993). The group B polysaccharide capsule is not suitable as vaccine antigen. It consists of a homolinear polymer of α(2→8)N-acetyl neuraminic acid, also known as polysialic acid that is structurally similar to the sialic acid found in human neural tissue, hence poorly immunogenic and potentially capable of eliciting auto-antibodies (Finne, Leinonen et al. 1983, Finne, Bitter-Suermann et al. 1987). Therefore the research of non-capsular antigens, such as surface exposed proteins or LOS became crucial for the development of a vaccine against meningococcus serogroup B (MenB). Outer membrane vesicles (OMVs) preparations have been used as an alternative strategy to MenB vaccine development. At the basis of this strategy is the use of surface-exposed proteins contained in the OMVs. The first OMV vaccines were developed in Norway and Cuba and showed efficacy in humans ranging from 50 to 80 per cent (Tappero JW. et al, 1999). Although both vaccines induced a good protection against the homologous strain, they failed to induce protection against heterologous strains (Rosenstein, Fischer and Tappero, 2001). The most abundant outer membrane protein and the major protective antigen in both vaccines is PorA, which is known to be highly variable across different isolates of serogroup B *N. meningitidis*. To overcome these limitations, a multicomponent recombinant protein-based vaccine, named 4CMenB was developed against serogroup B *N. meningitidis* strains,. This vaccine has been approved for human vaccination in Europe, Australia, Canada, US and many other countries worldwide.

4CMenB is composed of 4 different components, three are major protein antigens: factor H-binding protein (fHbp), Neisserial Heparin-Binding Antigen (NHBA) and Neisserial adhesin A (NadA) which are combined with OMVs from the New-Zealand epidemic strain (NZ98/254) (Pizza M and Scarlato V. 2000, Giuliani MM et al 2006) (fig.3). fHbp binds to human factor H, the major inhibitor of the alternative complement pathway, favouring the *N. meningitidis* survival in human blood (Madico et al., 2006). NadA is a surface protein that mediates the adhesion and the entrance of the bacterium in epithelial cells and also likely in endothelial cells (Capecchi et al., 2005; Scietti et al., 2016). NHBA is a surface lipoprotein able to bind heparin and heparan sulfate proteoglycans, and it is involved in different steps of meningococcal pathogenesis, including bacterial adhesion to epithelial cells, biofilm formation, bacterial survival in the blood and vascular leakage (Serruto et al., 2010; Arenas et al. 2013; Casellato et al., 2014; Vacca et al., 2016).

Another vaccine against serogroup B meningococcus (rLP2086) has been approved for adolescent vaccination and contains two alleles of the recombinant fHbp (Anderson AS et al 2012).

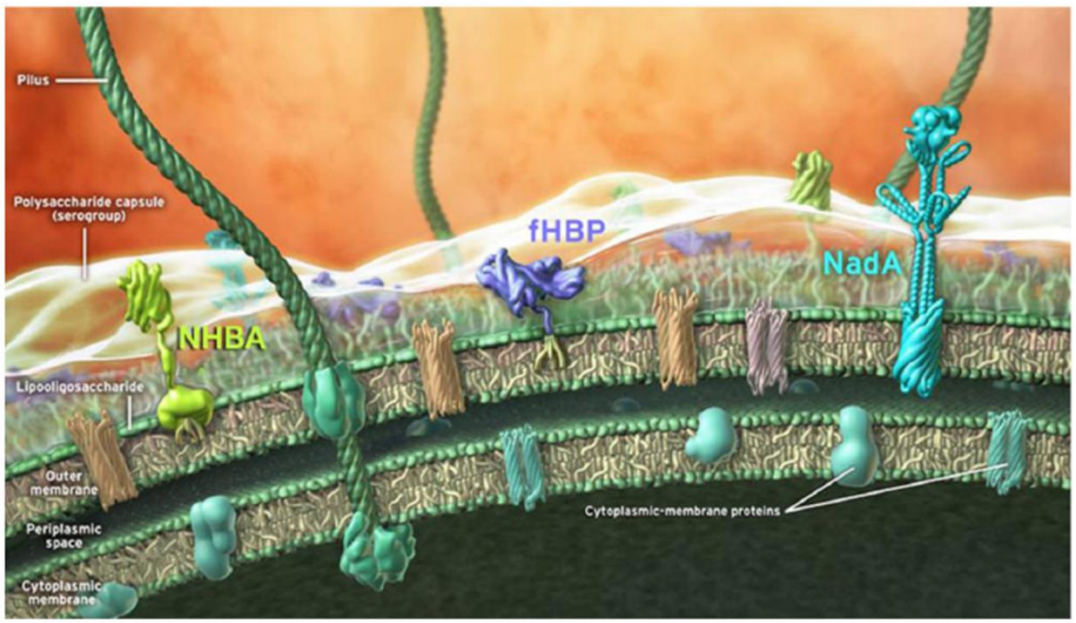


Figure 3. **Schematic representation of the Bexsero vaccine antigens on the surface of *N.meningitidis***. Figure adapted from Serruto et al. (2012). The different bacterial compartments (outer membrane, periplasmic space, cytoplasmic membrane) and the main antigens identified through reverse vaccinology approach (NHBA, fHbp and NadA) are depicted. Other components of the meningococcal membranes are also shown (pilus, polysaccharide capsule, lipooligosaccharide and integral inner and outer membrane proteins).

It has been demonstrated that antibodies induced by meningococcal polysaccharide, polysaccharide protein conjugates, outer membrane protein and recombinant protein based vaccines protect against meningococcal disease (Frasch C. et al 2009, Giuliani MM et al 2010) Effectiveness of anti-meningococcal vaccines is evaluated using the serum bactericidal activity (hSBA) assay using human serum as complement source, which is the only accepted surrogate for protection (Borrow et al 2005). This assay measures the concentration of vaccine induced antibodies able to induce bacteriolysis of meningococci via the classical pathway which is the primary mechanism of protection against invasive meningococcal disease applied by the natural immunity (Goldschneider et al., 1969). Due to difficulties in obtaining human serum as source of exogenous complement, very often in preclinical studies, the use of rabbit serum as complement source for SBA assay (rSBA) have become widely accepted.

2.4. *Neisserial Heparin Binding Antigen (NHBA)*

Originally annotated as GNA2132 (genome-derived neisserial antigen 2132), this vaccine component was later renamed by Serruto and colleagues as *Neisserial Heparin Binding Antigen* (NHBA) (Serruto D et al 2010) due to its ability to bind heparin *in vitro*. NHBA is an important virulence factor of *Neisseria meningitidis*, being involved in adhesion (Vacca et al., 2016), biofilm formation (Arenas et al., 2013) and possibly even in serum resistance through its ability to bind heparin (Serruto et al., 2010) (V. Esposito et al., 2011). NHBA was originally identified by reverse vaccinology approach (Pizza et al 2000, Rappuoli R 2000 and Masignani V et al 2002), and is one of the key components of the 4CMenB vaccine (Serruto et al 2012). NHBA encoding gene was reported to be present in all *N. meningitidis* strains, and it is also found in *N. gonorrhoeae* and the commensal species *N. lactamica*, *N. polysaccharea*, and *N. flavescens* (Pizza et al., 2000; Jacobsson et al., 2006; Bambini et al., 2009; Lucidarme et al., 2009; Lucidarme et al., 2010, Muzzi et al., 2013). Gene sequences from genetically diverse group B strains reveal the existence of more than 400 protein sub variants that have some association with clonal complexes and sequence types (Muzzi et al., 2013; Bambini et al., 2009). Expression of *nhba* has been recently described to be temperature dependent; *nhba*

gene is strongly upregulated at 30-32°C (Lappann et al. 2016, Borghi S. 2017), a temperature encountered by the bacterium during transmission and initial colonization of the nasopharynx (Keck et al., 2000). The upstream regulatory region of the *nhba* gene is characterized by the presence of the 150 base pair Contact Regulatory Element of Neisseria (CREN) in hyper virulent strains belonging to clonal complex ST-32. This regulatory element, specific to pathogenic *Neisseria* species, activates induction of the downstream associated genes upon contact with target eukaryotic cells (Deghmane et al., 2002). Transcription of *nhba* gene is induced upon contact with epithelial cells in MC58 strain witch harbors the CREN regulatory element in the promoter region of the gene (Deghmane et al., 2003).

NHBA is a surface exposed lipoprotein with a very well conserved lipobox motif (-LXXC-) located at the distal part of the N-terminal region and anchoring the protein on the bacterial surface (Serruto et al., 2010). Multiple sequence analysis revealed that NHBA consists of two principal domains: a highly variable amino-terminal region annotated as intrinsically unfolded by commonly used structure prediction algorithms, and a highly conserved C-terminal region whose structure has been resolved by NMR spectroscopy. NHBA C-terminal region (residues 246 to 428, in strain 2996) is an independently folded globular domain with high thermal stability, and its structure consists of a β-barrel with 8 anti-parallel strands. (Pizza et al., 2000, Esposito et al., 2011). A peculiar Arginine-rich motif (-RSARSRRS-) is present between the N-term and C-term domains and is present in all *Neisseria* species harboring *nhba* gene (Muzzi et al., 2013). The Arginine-rich (Arg-rich) motif is responsible for the protein binding *in vitro* to heparin and heparan sulfate proteoglycans (Serruto et al., 2010; Vacca et al., 2016). Structurally the C-terminal domain of NHBA shows similarities to transferrin binding protein B (TbpB) and to the C-terminal domain of fHbp, although these proteins share very low sequence similarity (Esposito et al., 2011).

NHBA protein has been described to be susceptible to proteolytic cleavage by both bacterial and human proteases, respectively NalP and lactoferrin (hLf). The cleavage sites of these proteases are upstream and downstream of the Arg-rich motif, respectively (Serruto et al., 2010) (Figure 4). NalP cleavage site is less conserved due to sequence variability while human proteases cleavage site is highly conserved among NHBA variants (Vacca I 2014).The cleavage results in the release of the C-term fragment into the supernatant, while the N-term fragment remains anchored to the bacterial surface. The C-term fragment generated

by NalP cleavage, named C2, was recently reported to alter endothelial permeability, contributing to extensive vascular leakage (Casellato et al., 2014). The cleavage operated by hLf generates the C1 fragment, which lacks the Arg-rich motif. Recently other human proteases, including kallikrein and the C3-convertase of the alternative complement pathway, was seen to cleave NHBA downstream of the Arg-rich motif generating a C-terminal fragment that does not contain the Arg-rich motif. Characterization of the released C-term fragment by mass spectrometry evidenced that the cleavage site for hLF, kallikrein and C3-convertase proteases is highly conserved and the C-term fragment generated is the same (Serruto et al, 2010, PantanoE., 2016, Di Fede M.,2017).

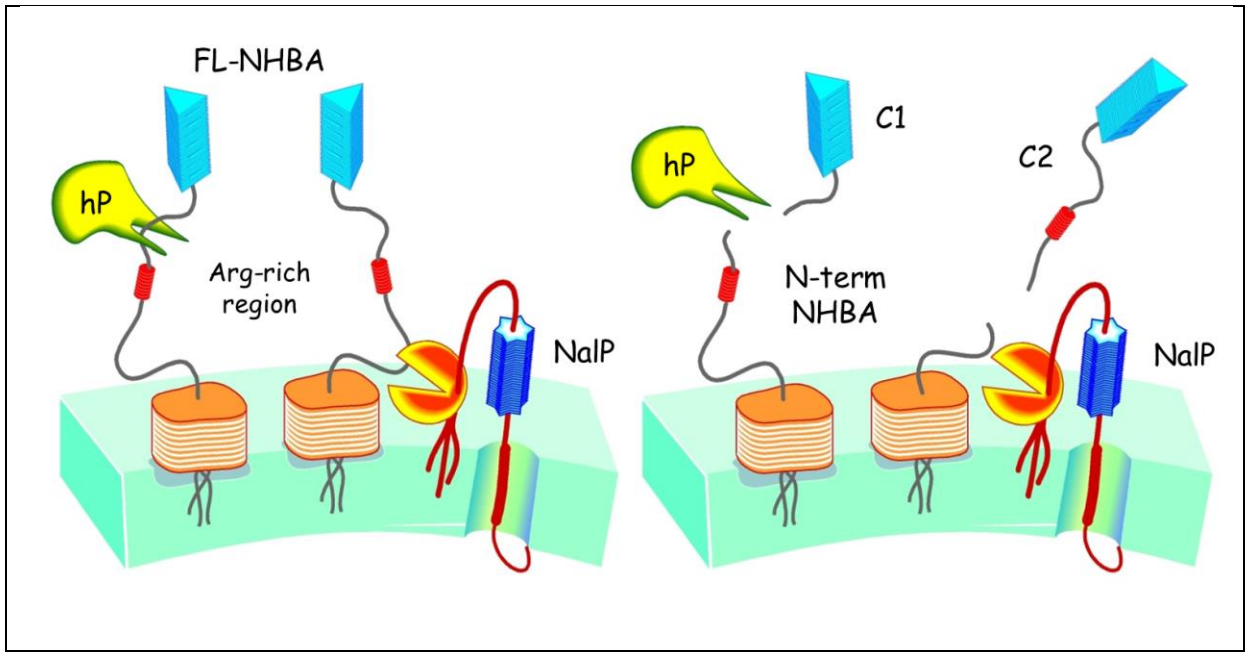


Figure 4. Schematic representation of NHBA cleavage by human and bacterial proteases. On the left panel are represented human proteases (hP), the full length NHBA (FL-NHBA) and NalP bacterial protease exposed on bacterial surface. On the right panel are represented NHBA fragments generated by human proteases and NalP cleavage: the N-term NHBA remains on bacterial surface while the digested C1 and C2 fragments generated by human proteases (lactofetrrin, kallikrein and C-convertase) and bacterial proteases, respectively, are released.

NHBA is one of the three major components of the 4CMenB vaccine and is a protective antigen able to induce a specific and robust immune response against strains expressing homologous and heterologous NHBA variants (Giuliani et al 2006, Serruto et al 2010 Giuliani et al 2010 and Vesikari et al., 2013). Moreover anti-NHBA antibodies were seen to passively protected infant rats against meningococcal bacteremia after challenge with serogroup B

meningococcal strains (Welsch et al., 2003) and were able to inhibit meningococcal adhesion to epithelial cells (Vacca et al., 2016).

Although anti-NHBA polyclonal antibodies are able to induce bacterial killing when tested in serum bactericidal activity (SBA), the regions involved in eliciting cross-protective immune response remain still unknown

3. AIMS OF THE PROJECT

In the present study we used monoclonal antibodies raised against NHBA with the aim to:

- Investigate complement mediated activity of monoclonal antibodies and map the regions of the antigen involved on eliciting the immune response
- Investigate cross protection of anti-NHBA monoclonal antibodies against a panel of MenB strains expressing epidemiologically relevant NHBA variants
- Investigate the mechanism of immune response elicited by NHBA

4. RESULTS AND DISCUSSION

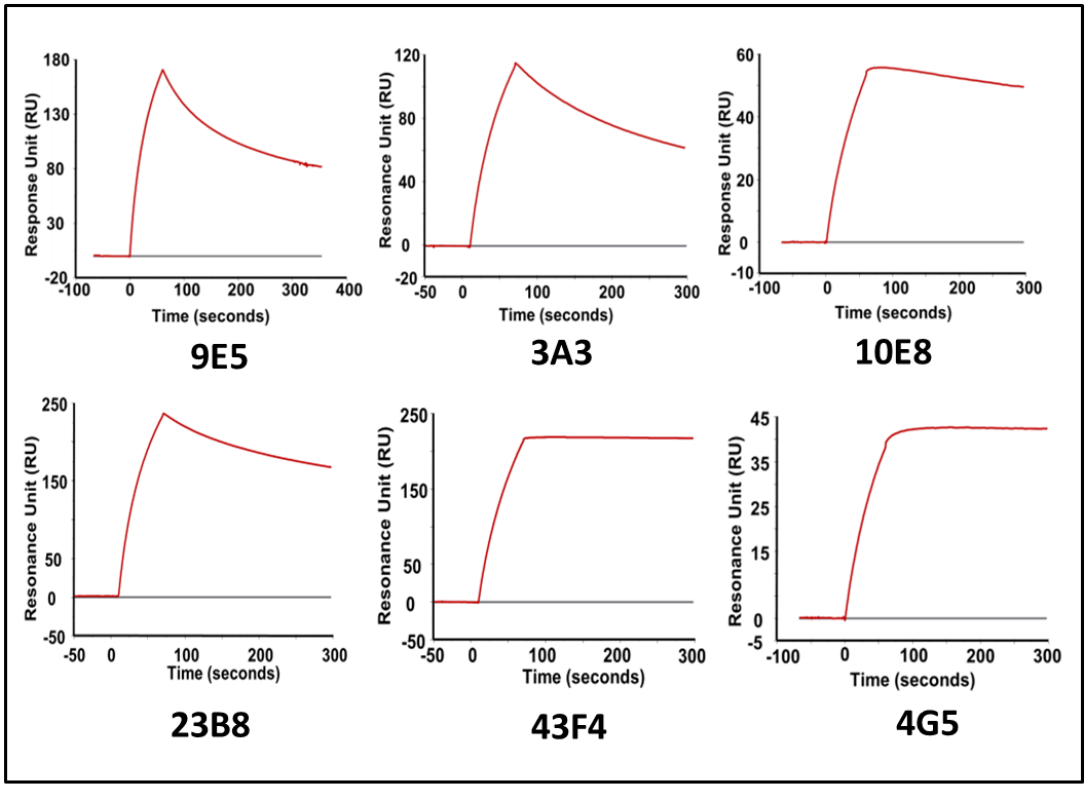
4.1. *Monoclonal antibody isolation and characterization*

4.1.1. Isolation of anti-NHBA murine mAbs and affinity characterization by SPR

Monoclonal antibodies were obtained by immunizing mice with different NHBA forms, including the full length molecule, and NHBA derived fragments, formulated with different adjuvants. Overall, this step led to the isolation of six monoclonal antibodies, belonging to the IgG 2a and 2b subtypes, that we characterized further for their ability to bind the recombinant NHBAp2 (NHBA peptide 2 corresponds to the vaccine variant) defined by Surface Plasmon Resonance (SPR) analysis. Binding profiles of anti-NHBA mAbs (Fig.5A) show that all mAbs were able to bind the recombinant NHBA p2 variant (rNHBAp2) with a high association rate (ascending tract of the sensogram). Monoclonal antibodies 43F4 and 4G5 showed a very stable binding since their dissociation rate is very low (the plateau tract of the sensogram). Conversely mAbs 9E5, 3A3, 10E8 and 23B8 manifest a higher dissociation rate as displayed by the descendent tract of the respective sensograms (Fig. 5A).

To better characterize mAb-rNHBAp2 interaction, SPR single-cycle kinetics (SCK) titrations of rNHBAp2 (0,39nM to 25nM) were performed to calculate constant affinities (KD) (Fig. 5B). Binding affinities of all the mAbs tested are within the nano molar range but affinity constants of mAbs 23B8, 43F4 and 4G5 were 1 to 2 log higher than affinity constants of mAbs 3A3, 9E5 and 10E8 (Fig. 5B). On the overall all mAbs tested are able to bind with high affinity rNHBAp2 variant and these data are in agreement with binding profile data.

A



B

Affinities of selected anti-NHBA mAbs				
mAb	**Protein**	**Kon (1/Ms)**	**Koff (1/s)**	**KD (M)**
3A3	rNHBAp2	$1,6 \times 10^{+5}$	$3,9 \times 10^{-3}$	$2,3 \times 10^{-9}$
9E5	rNHBAp2	$3,8 \times 10^{+5}$	$6,1 \times 10^{-3}$	$1,6 \times 10^{-8}$
10E8	rNHBAp2	$1,6 \times 10^{+5}$	$2,5 \times 10^{-4}$	$1,6 \times 10^{-9}$
23B8	rNHBAp2	$6,9 \times 10^{+5}$	$5,5 \times 10^{-4}$	$7,9 \times 10^{-10}$
4G5	rNHBAp2	$2,1 \times 10^{+5}$	$4,6 \times 10^{-5}$	$2,2 \times 10^{-10}$
43F4	rNHBAp2	$2,6 \times 10^{+6}$	$4,6x\ 10^{-5}$	$1,5 \times 10^{-10}$

Figure 5. Monoclonal antibodies binding profiles and affinities. A. Surface plasmon resonance sensograms show binding profiles of rNHBAp2 binding on each individual mAb. **B**. Binding affinities obtained by single cycle kinetics titrations. Kon: association constant; Koff: dissociation constant; KD: affinity constant

4.1.2. MAb recognition of the Native NHBA on live bacteria by flow cytometry analysis

The ability of murine mAbs to recognize the native surface exposed NHBA on live encapsulated bacteria was evaluated by flow cytometry analysis. We hence employed serogroup B meningococcal strain M4407, a natural strain isolated from patients with invasive meningococcal disease (IMD) (provided by CDC). Polyclonal antiserum raised against NHBAp2 was used as positive control (Fig. 6). Flow cytometry results showed that all tested mAbs were able to recognize the native NHBA protein surface exposed on live bacteria and this in agreement with SPR data. Polyclonal antibody was used as positive control.

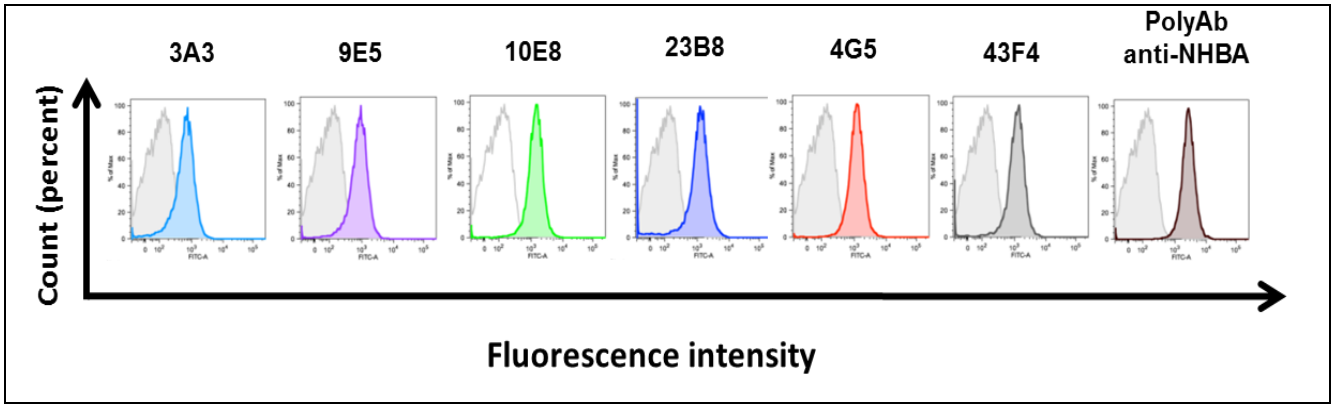


Figure 6. Flow cytometry analysis. Monoclonal antibodies recognition profiles of native and surface exposed NHBAp2 variant on M4407 MenB strain. Anti-NHBA murine polyclonal antibody was used as positive control.

4.1.3. Epitope mapping of anti-NHBA monoclonal antibodies

Epitope mapping of mAbs presented in this study was carried out with different approaches. Protein based microarray such as Protein-chip and peptide array scanning were used to identify the regions of the proteins targeted by each mAb. Furthermore, we used SPR and Hydrogen Deuterium Exchange Mass - Spectrometry (HDX) to better characterize the epitope targeted by mAbs 4G5, 23B8 and 43F4.

4.1.4. Protein microarray based epitope mapping

Protein based microarray epitope mapping relies on a protein fragment library designed to cover the whole NHBA sequence. For this approach two platforms were used.

Protein-chip microarray (Fig.7.A) consists in overlapping NHBA protein fragments ranging from 30 amino acids to the full-length NHBA protein spotted onto nitrocellulose-coated microscope slides. Most of the peptides be included in the array were selected among the most immuno-reactive NHBAp2 fragments identified by phage display. In addition, other fragments were designed to cover all potential epitopes and domains of the antigens, by cutting at random coils on the base of secondary structure prediction. All selected protein fragments were designed in house, cloned, expressed and purified in *E.coli* and finally spotted onto the chip. The overall folding of NHBA fragments was confirmed by analysing a large panel on mAbs previously characterized, targeting both linear and conformational epitopes both on the N-term and C-term of NHBA (Bruttini M., PhD thesis 2015). This approach is used to identify epitopes that can either be linear or conformational.

Peptide array (Fig.7.B) consists of 15-mer synthetic peptides (JPT) library, overlapping of 5 amino acids on both 5' and 3' and covering the entire NHBA sequence. Given the short length of peptides, this technique is mainly used to identify linear epitopes.

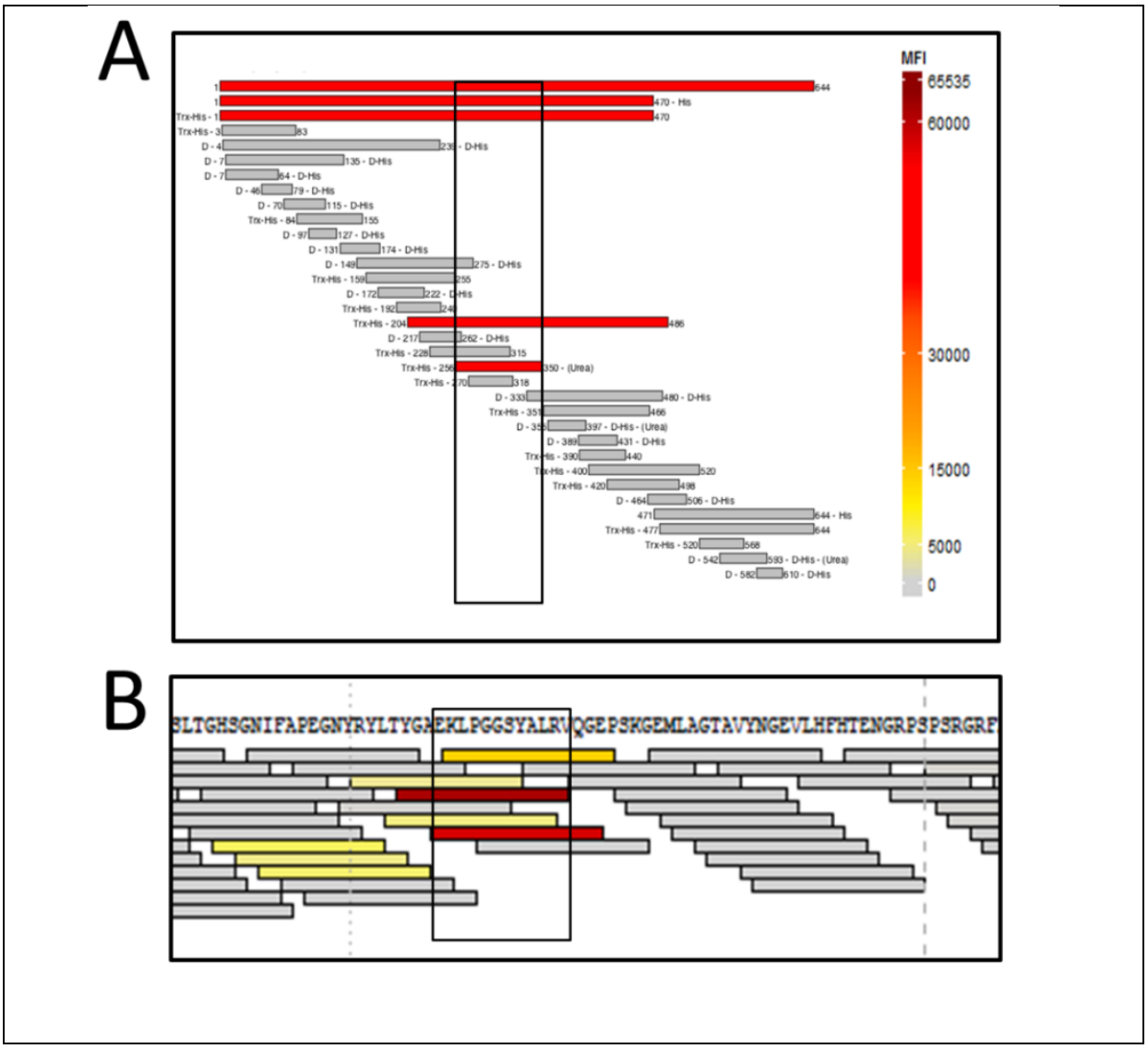


Figure 7. Lay out of mAb23B8 protein chip and peptide array analysis. A. Full length and fragments of NHBAp2 spotted onto the nitrocellulose-coated microscope slides. The color-range bar on the right indicates binding intensity. Grey: no binding, purple: high binding signal. The black square indicates the target region identified by protein chip. **B**. In red are shown 15-mer peptides that bind to mAb 23B8 in peptide array analysis. The epitope is defined as the minimum region identified between two or more positive overlapping peptides.

Regions targeted by each mAb in protein chip and peptide array mapping are reported in figure 9A. Based on protein chip analysis it was observed that mAbs 3A3, 10E8 and 9E5 targeted regions are located on the N-terminal domain of NHBA, while mAbs 23B8 and 43F4 recognized regions mapping on the C-term domain of the antigen (Fig. 9B). Differentially from the other mAb tested protein chip analysis revealed that MAb 4G5 targeted an extended region consisting on more than 200 amino acids mapping both on the N-term and on the C-term of NHBA (fig 9B).

Peptide scanning analysis showed that epitope of mAbs 3A3, 10E8, 9E5 and mAb 23B8 recognizes linear epitopes while mAbs 4G5 and 43F4 target most likely conformational epitopes since no specific binding on the 15-mer peptides spotted on the chip were observed (Fig. 9A). For mAb 23B8, although the region identified by peptide array (Y282-M376) overlaps with the one identified by protein chip (E357-V368), there is a difference of around fifty amino acids on the peptide length identified by the two epitope mapping approaches. This could potentially imply that other contact points might be involved on the interaction between mAb 23B8 and NHBA. These results are in agreement with protein chip results.

4.1.5. Investigating the epitope of mAb 4G5 by Surface Plasmon Resonance (SPR) analysis

The target region of mAb 4G5 identified by protein chip analysis, consists of a 262 amino acids peptide (K226-D492) comprising the terminal region of the N-term domain and the whole C-term domain of NHBA. To explore which domain of NHBA is mainly involved on the interaction with mAb 4G5 we performed SPR analysis using recombinant NHBA N-term, C-term fragments and the β-barrel fragment (Fig. 8A and 8B. The full length recombinant NHBA protein was used as positive control. Results showed that mAb 4G5 was able to bind the full length and C-term fragment of rNHBA while no binding were observed for the N-term domain and C-term β-barrel NHBA fragment (Fig. 8C). These results suggest that the core epitope of mAb 4G5 is probably located on the well conserved C-term domain of NHBA.

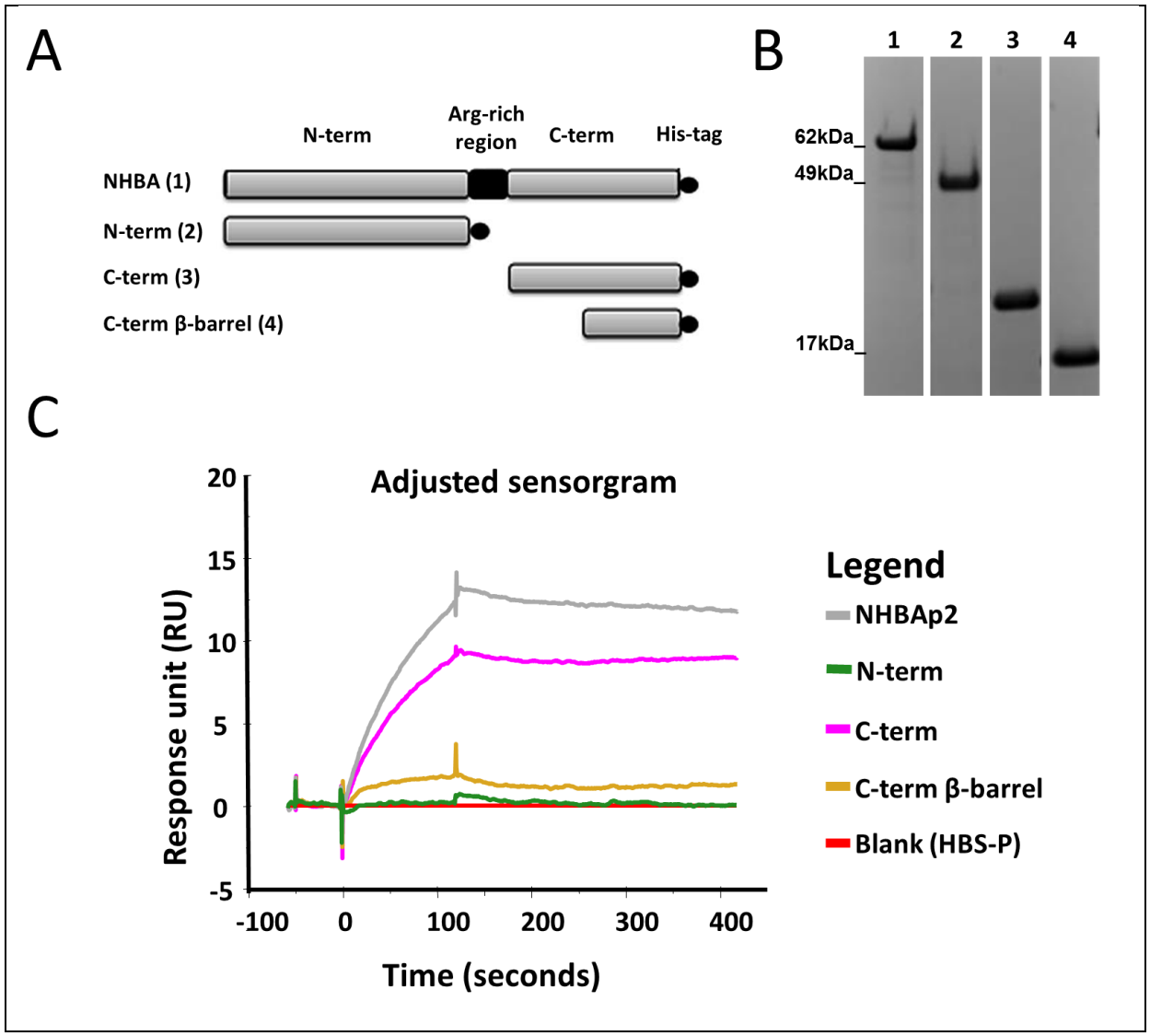


Figure 8. Analysis of mAb 4G5 epitope by SPR analysis. A. Schematic representation of NHBA fragments used for SPR analysis adapted from Vacca I et al 2016. **B**. 4 µg of each protein were analyzed by SDS-PAGE to investigate for protein degradation. **C**. SPR analysis: Sensograms of NHBA fragments and full length protein were normalized on the blank injection.

4.1.6. HDX epitope mapping of mAbs 4G5, 43F4 and 23B8

The HDX approach is based on the differential rate of deuterium incorporation by a protein when it is bound or not by its ligand. The rate at which backbone amide hydrogens exchange in solution is directly dependent on the dynamics and structure of the protein. Therefore, regions embedded within the structure or occluded by the presence of a ligand will exchange more slowly than regions fully exposed to the solvent (Hager-Braun C and

Tomer KB 2005, Malito et al 2013). To better characterize the conformational epitopes targeted by mAbs 4G5 and 43F4 and to confirm that no additional regions are targeted by mAb 23B8 we performed hydrogen deuterium exchange mass-spectrometry (HDX) analysis. We did not perform the HDx analysis on mAbs targeting the N-term of NHBA (3A3, 10E8 and 9E5) since the peptides identified by protein chip and peptide array showed good overlapping. The first step consists in generation of a peptidic map in order to monitor the deuterium uptake of the peptides generated during the analysis. The peptidic map was generated performing five online pepsin digestions. The resulting peptidic map was seen to have 79.4% coverage of the entire protein (Fig. 9).

Hydrogen-deuterium exchange (HDX) experiments, with and without the mAbs, were performed in the same experimental session. For each mAb the epitope was confirmed by at least two independent experiments. HDX analysis revealed that for mAb 4G5difference in deuterium uptake was observed in two discontinuous regions identified by peptides 243-KSEFEKLSDADKISNYKKDGKNDKFVGL-272 located upstream the Arginine-rich region on the N-terminal domain of NHBA and 333-VSLTGHSGNIFAPEGNYRYLTYGAEKLPGGSYALRVQGEPSKGEML-378 located on the C-terminal domain of the protein. The region targeted by mAb 43F4 identified by HDx analysis corresponds to the C-term peptide 356-AGTAVYNGEVLHFHTENGRPYPTRGRFAAKVDFGSKSVD-395. Finally the epitope targeted by mAb 23B8 located as well on the C-term domain was identified on 327-YRYLTYGAEKLPGGSYA-343 peptide. HDX epitope mapping results are in agreement with peptides identified by protein-chip and pep-scan confirming that mAb 4G5 and 43F4 target conformational epitopes while mAb 23B8 recognize a linear epitope. Epitopes of mAbs 4G5, 23B8 and 43F4 are reported in figure 10A and displayed on the schematic representation of NHBA on figure 10B upper panel. Moreover epitopes targeted by mAbs 23B8, 43F4 and part of the epitope of mAb 4G5 are displayed on the 3D NMR structure of the C-term domain of NHBA previously described by Esposito et al 2011 (fig.10B lower pannel)

Figure 9. Peptidic map of NHBA-GNA1030. The NHBA-GNA 1030 fusion protein was used to performed online pepsine digestions and peptides. Peptides generated were separated by 18C reverse phase column high performance liquid chromatography (HPLC and then analyzed for their time of flight (TOF) by Synapt G2 mass-spectrometer.

A

mAb	Protein chip	Peptide array	HDX
	Target region	Linear epitope	Epitope
3A3	K29 - A86	44-54 (KETEAKEDAPQ)	ND
10E8	A106 - S137	105-115 (NAADTDSLTPN)	ND
9E5	M153 - T173	165-171 (AGNTAAQ)	ND
4G5	K226 - D492	no binding	K243-L274 (N-term) V333-L378 (C-term)
23B8	Y282 - M376	357-368 (EKLPGGSYALRV)	Y349-A365
43F4	L377-D492	no binding	A378-D417

B

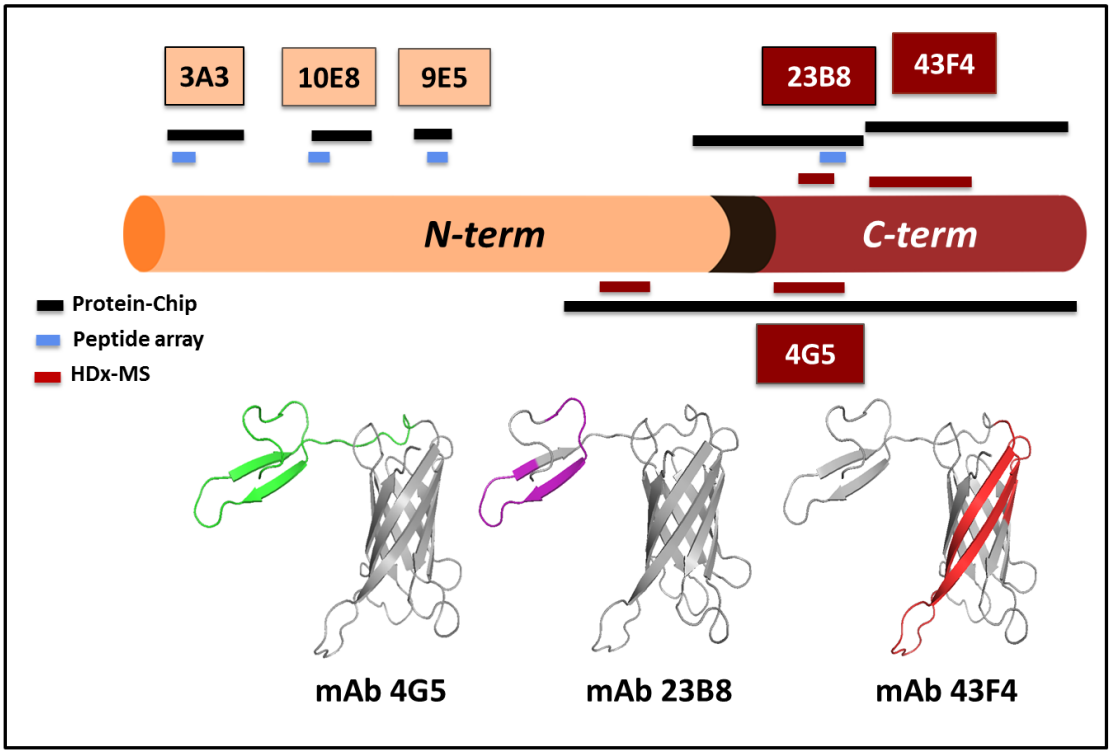


Figure 10. Epitope mapping of anti-NHBA monoclonal antibodies using protein chip, peptide array and HDX. A. Target region recognized by each mAb with the different techniques is reported on the table. MAb 4G5 and 43F4 did not bind any probe spotted on the chip on peptide array because they target conformational epitopes. ND: not done. **B.** Upper panel: regions targeted by each mAb identified by protein chip, peptide array and HDx on the schematic representation of NHBA. Lower panel: epitope of mAbs 23B8, 43F4 and part of the epitope of mAb 4G5 displayed on the 3D NMR structure of the C-term domain of NHBA.

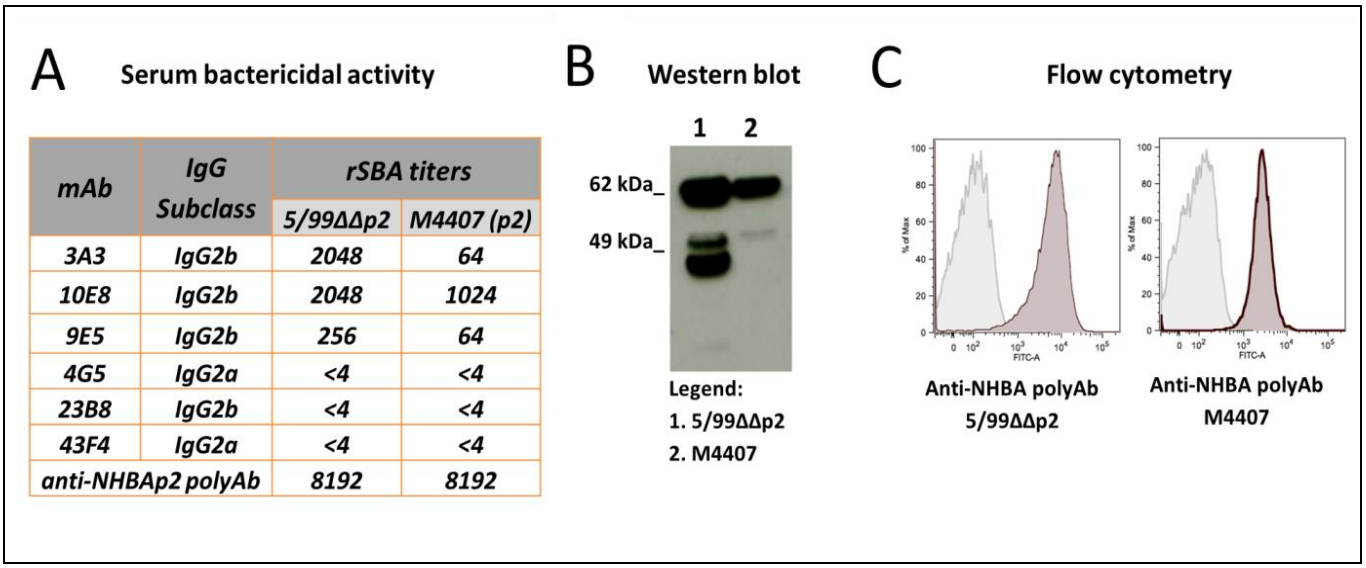
4.1.7. Functional characterization by Serum Bactericidal Activity assay

Bactericidal antibodies, i.e. able to kill the bacteria in the presence of complement, are the major component of protective immune response against MenB (Goldschneider, Gotschlich et al. 1969). Measurement of serum bactericidal antibodies (SBA) is widely used to analyse antibody response to meningococcal vaccines. SBA titre has been described to correlate with protection against invasive meningococcal disease (Borrow, Balmer et al. 2005, van Alphen and van den Dobbelsteen 2008)

To evaluate the ability of monoclonal antibodies to induce complement mediated killing of live bacteria Serum Bactericidal Activity Assay (SBA) was performed as described in Giuliani et al 2005 using pooled baby rabbit serum (BRS) (Cedarline) as complement source. To this aim two strains were used: *5/99ΔnadAΔnhba:npbap2* (5/99ΔΔp2) and M4407. 5/99ΔΔp2 is a recombinant MenB strain where the NHBA gene is under an IPTG inducible promoter which allows high antigen expression levels (Serruto D. et al 2010). M4407 is a natural strain (provided by CDC) that expresses a heterologous fHbp in the variant 2 group, a relatively high expresser of NHBA with 100% amino acid identity to the antigen used in the vaccine, and lacked a gene for NadA.

SBA results showed that mAbs 3A3, 10E8 and 9E5 tested individually were able to induce bactericidal killing on 5/99ΔΔp2 MenB strain. Conversely mAbs 4G5, 23B8 and 43F4 were SBA negative. Similarly to what observed for 5/99ΔΔp2 strain, only mAbs 3A3, 10E8 and 9E5 were able to induce bactericidal killing against M4407 strain but lower SBA titers were observed (Fig. 11A). These data indicate that only mAbs targeting the N-term of NHBA are able to induce bactericidal killing.

To investigate the difference observed in SBA titers between 5/99ΔΔp2 and M4407 we analyzed NHBA expression by western blot and flow cytometry on both strains (Fig. 11B and 11C). A marked difference in total NHBA expression was noticed between 5/99ΔΔp2 and M4407 strains by western blot analysis. Flow cytometry analysis evidenced as well higher surface exposed NHBA expression on strain 5/99ΔΔp2 respect to M4407 strain, but the difference was less marked respect to western blot analysis.



mAb	IgG Subclass	rSBA titers	
		5/99ΔΔp2	M4407 (p2)
3A3	IgG2b	2048	64
10E8	IgG2b	2048	1024
9E5	IgG2b	256	64
4G5	IgG2a	<4	<4
23B8	IgG2b	<4	<4
43F4	IgG2a	<4	<4
anti-NHBAp2 polyAb		8192	8192

Figure 11. Complement mediated bactericidal activity of anti-NHBA murine mAbs against 5/99ΔΔp2 and M4407 MenB strains A. Bactericidal titers of mAbs tested against 5/99ΔΔp2 and M4407 MenB strains using pooled baby rabbit serum (BRS) as complement source. SBA titers are expressed as reciprocal serum dilution. SBA titers <4 indicate negative SBA titers. Polyclonal antibody (polyAb) was used as positive control. NHBA expression analysis on 5/99ΔΔp2 and M4407 MenB strains performed by western blot and by flow cytometry are showed on panel **B** and **C**, respectively.

4.1.8. Cooperative bactericidal activity of anti-NHBA monoclonal antibodies

It has been described elsewhere that combination of mAbs targeting epitopes mapping on different antigenic regions are able to induce cooperative bactericidal activity (Welsh et al 2008 Beernink et al, 2008). To fully investigate the contribution of the two major domains of NHBA, SBA was performed testing combination of mAbs targeting (i) different epitopes on the N-term, (ii) epitopes on the C-term and N-term and (iii) epitopes on different regions of the C-term of NHBA.

Each experiment was performed twice, in two different days, in order to take into account the assay variability. Figure 12 shows the frequency distribution of the difference between SBA titers obtained within the duplicate experiment per each condition tested (mAbs tested alone or in combination). The difference in SBA titers was calculated as $|Log_2$SBA titersExp1/ Log_2SBA titersExp1$|$).

Twenty-one out of 23 of tested conditions showed a difference lower than 2 dilutions in SBA titers and only 2 out of 23 show a difference between 2 and 3 dilutions in SBA titers. The

overall range @95% of the difference between SBA titers is lower than 3 dilutions. No difference higher than 3 dilution observed in our dataset.

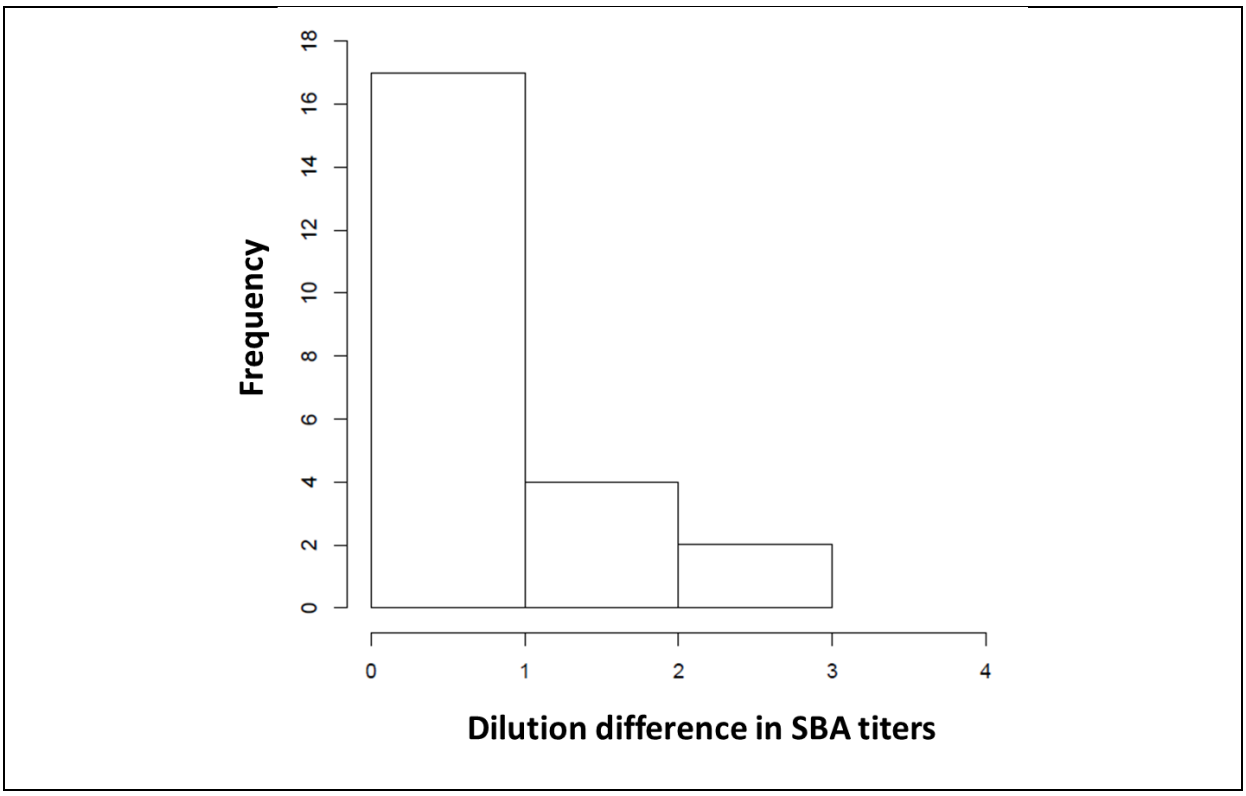


Figure 12. Frequency distribution of the difference between SBA titers obtained within the duplicate experiment per each condition tested. Condition tested**:** mab tested alone and mAbs tested in combination. On the X axis is reported the dilution difference in SBA titers while on the Y axis is reported its frequency distribution for all duplicate experiments**.**

According to assay variability analysis and to criteria assigned to the SBA dataset (see material and methods) results indicate that no increase in SBA titers was observed from combination of mAbs mapping on the N-term of NHBA (fig. 13A). MAb combinations, 43F4+9E5, 23B8+3A3, 23B8+10E8 and 23B8+9E5 targeting the C-term and N-term of the protein, show an increase in SBA titers (Fig. 13B). These data suggests that SBA negative mAbs, mapping on the C-term can contribute on increasing SBA titers when combined to SBA positive mapping on the N-term. Surprisingly all mAb combination targeting the C-term of NHBA were able to induce cooperative bactericidal activity with 23B8+43F4 mAb combination showing the highest SBA titer among all the combination tested (Fig. 13C).

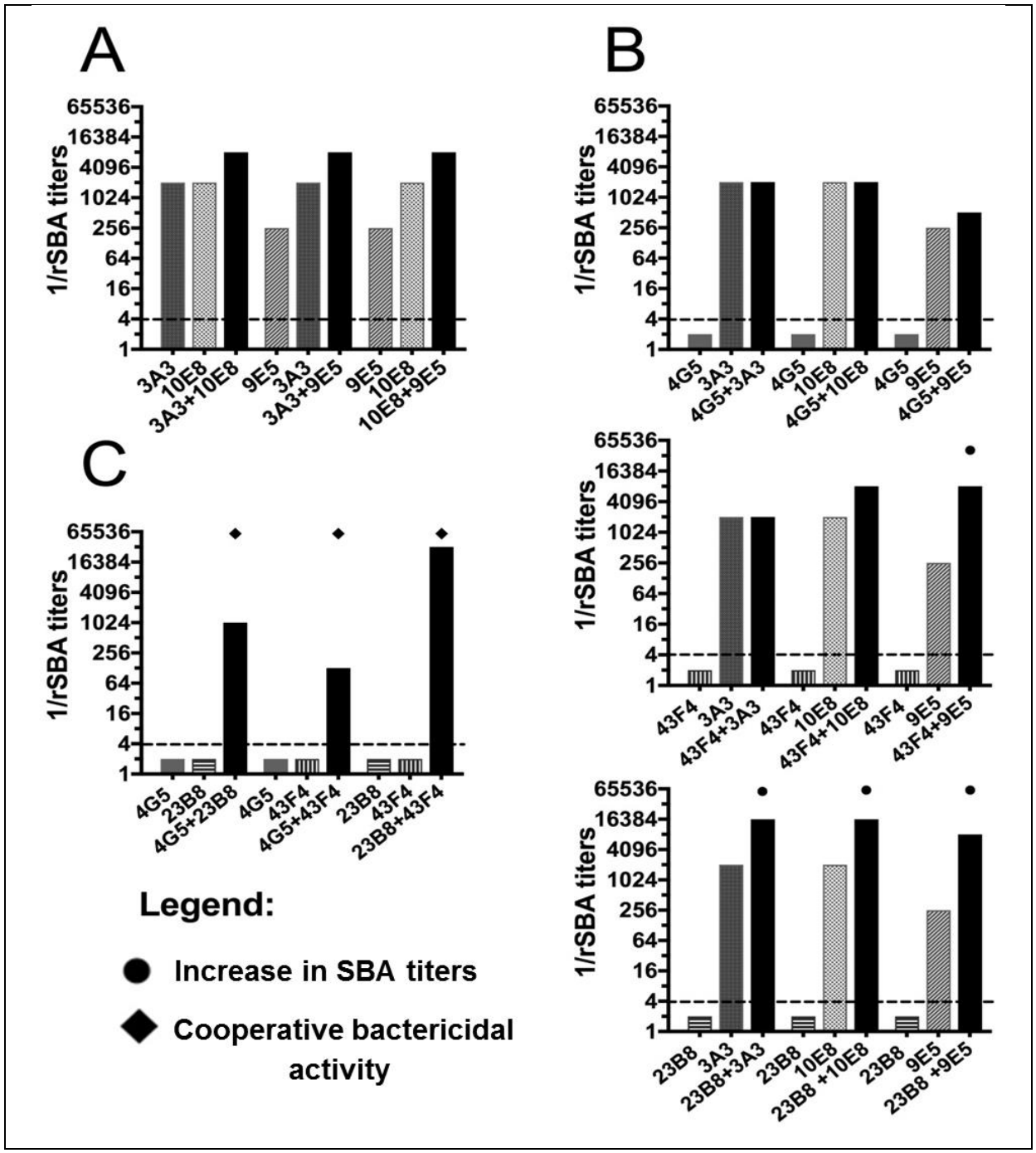


Figure 13. Serum bactericidal activities of mAbs tested alone and in combination against strain 5/99ΔΔp2. SBA titers of mAbs targeting (**A**) only N-term domain, (**B**) both C-term and N-term domains and (**C**) only the well conserved C-term of NHBA. On Y axis are reported reciprocal SBA titers, while on X axis mAbs tested alone and in combination. The SBA titers showed in this graph are the lower SBA titer measured in the duplicate experiments

4.1.9. Discussion and conclusions

The first step of the study was to investigate the ability of monoclonal antibodies to recognize the recombinant and the native form of NHBA. SPR analysis showed that mAbs were able to bind the vaccine rNHBA p2 variant, within nano molar range and mAbs 4G5, 23B8 and 43F4 showed higher affinity with respect to mAbs 3A3, 10E8 and 9E5. Moreover, by flow cytometry analysis it was shown that they were able to recognize the native form of the protein surface exposed on M440 MenB natural strain.

From protein chip and peptide array analysis it emerged that mAbs 3A3, 10E8 and 9E5 target specific linear epitopes on the N-term domain of NHBA; for this reason, these Epitopes were not further characterized. The region targeted by mAb 4G5 which comprised a 226 amino acid peptide (K226-D492), spanning on both major domains of NHBA on protein chip, was further characterized by SPR analysis. Results of SPR experiment showed that mAb 4G5 recognized the longer C-term fragment of NHBA but not the shorter C-term β barrel fragment indicating that apparently the epitope localized only on the C-term region of NHBA. Interestingly the HDx analysis revealed that mAb 4G5 target a conformational epitope composed of two discontinuous peptides, K243- L274 on the N-term and V333-L378 mapping upstream the β barrel on the C-term. These data suggests that of the two peptides identified, peptide V333-L378 could be the core epitope of mAb 4G5.

Peptide array and protein chip analysis showed that mAb 23B8 targets a linear epitope on the C-term domain of NHBA. A more detailed investigation with HDx analysis revealed that mAb 23B8 epitope comprised a slightly longer peptide that the one identified by peptide array analysis (Y349-A365).

The target region of mAb 43F4, identified by protein chip, was seen to map entirely on the β-barrel region of the C-term domain (L377-D492). HDx analysis allowed a better characterization of the epitope targeted by 43F4 confirming its conformational nature within the C-term β barrel fragment. Interestingly the epitope of mAb 43F4, identified by HDx analysis, is very similar to the anti-NHBA human mAb 5H2 epitope isolated by plasma blasts of a 4CMenB immunized donor (Maritan M., 2017). These evidences suggest that murine and human immune systems induce monoclonal antibodies able to target similar epitopes on the well conserved C-term of NHBA highlighting the importance of this domain.

Protein microarray, SPR and the HDx analysis provide concordant results, but the latter allowed a better characterization of the contact regions between mAbs under investigation and the antigen. Overall these results provided an explanation of the SPR data where mAbs targeting conformational epitopes show higher affinity respect mAbs targeting linear epitopes. These results are in agreement with data previously reported by Malito and collaborators (Malito et al 2013).

The use of Serum Bactericidal Antibody Assay (SBA) described by Goldschneider and colleagues is still considered as the "gold standard" to assess functionality of anti-meningococcal antibodies (Goldschneider et al 1969; Frasch et al 2009). We tested anti-NHBA monoclonal antibodies in the SBA assay using rabbit serum as source of complement (rSBA). We showed that monoclonal antibodies of 3A3, 10E8 and 9E5 targeting the N-term of NHBA induced individually *in vitro* killing of bacteria against both strains. Unexpectedly C-term mapping mAbs 4G5, 23B8 and 43F4 which showed higher affinity for NHBA were unable to induce complement mediated bactericidal killing. Anti-PorA IgG2a and IgG2b mouse mAbs have been described to have similar avidity and ability in inducing bactericidal activity against epidemic MenB strain H44/76 (Michaelsen TS., et al 2003). This report suggests that the different rSBA results observed between mAbs targeting the N-term and the C-term of NHBA is not probably due to different IgG subtype of mAbs 4G5 and 43F4.
MAbs 3A3 and 9E5 showed the same rSBA titer of 64 when tested individually against strain M4407 but different titers were observed when assayed against strain 5/99ΔΔp2. As previously described 5/99ΔΔp2 strain express higher levels of NHBA and an overall increase in rSBA titers is observed for both mAbs. Interestingly mAb 3A3 showed a 3 fold higher rSBA titer respect to the 9E5 rSBA titer, 2048 and 256 respectively. Even though flow cytometry analysis showed similar recognition on surface exposed NHBA by both mAbs, the difference in rSBA titers might be the result of cumulative effects. One possible explanation might reflect the combination of two effects (i) higher NHBA surface exposure on 5/99ΔΔp2 strain and (ii) the different affinity that mAbs 3A3 and 9E5 displayed for NHBA. The higher NHBA expression increase the avidity which reflects the increase of rSBA titers for both mAbs, but the higher affinity of 3A3 might result in a more prolonged complement activation resulting in higher bactericidal activity.

Synergy between anti-factor H binding protein (fHbp) mAbs assayed in pairs has been described elsewhere to augment bactericidal titers and to elicit cooperative bactericidal activity against serogroup B meningocicccal strains (Welsh et al, 2003, Welsh et al, 2004 and Beernink et al 2008). Here we tested in rSBA assay anti-NHBA mAbs in combination against 5/99ΔΔp2 strain in order to deeply investigate the contribution of the major domains of NHBA on complement mediated bactericidal activity. We found that combination of mAbs targeting the N-term of NHBA did not show a significant increase in SBA titers. Conversely combination of rSBA positive mAbs targeting the N-term with rSBA negative mAbs targeting the C-term resulted in ≥3 fold increase of bactericidal titers. Surprisingly all combinations of C-term mapping mAbs were seen to induce cooperative bactericidal activity. The pair 23B8+43F4 showed the highest rSBA titer than any other mAbs combination

Overall these results suggest that mAbs targeting the N-term of NHBA are individually able to induce bacterial killing when tested against 5/99ΔΔp2 MenB strain. mAbs targeting the C-term of NHBA even though individually are rSBA negative, when combined with N-term mapping mAbs act in synergy increasing bactericidal titers. Finally mAbs targeting the well conserved C-term of NHBA are able to induce bactericidal activity only when tested in combination. Moreover these results highlight the importance of both the N-term and C-term domains of NHBA in eliciting the observed protective immune response.

4.2. *Cross protection of monoclonal antibodies against a panel of epidemiologically relevant heterologous NHBA variants*

NHBA gene sequence analysis performed on a panel of group B meningococcal strains highlighted the presence of more than 400 variant peptides. The distribution of these diverse variant peptide sequences were seen to have some association with clonal complexes and sequence types of virulent strains (Bambini *et al* 2009, Muzzi *et al* 2013). Despite NHBA sequence variability, polyclonal antibodies to NHBAp2 were able to induce cross-protection against different strains expressing heterologous NHBA variants. We therefore wanted to investigate which regions of this antigen are important for its observed cross protection. To this aim we decided to investigate the ability of mAbs elicited against the homologous NHBAp2 vaccine variant to induce bactericidal killing against a panel of strains expressing epidemiological relevant NHBA heterologous variants.

4.2.1. Selection of NHBA peptides for the cross-protection studies

To evaluate the cross-protective activity of monoclonal antibodies raised against NHBA p2 variant, seven NHBA variant peptides were selected based on their prevalence in European and American clinical isolates from IMD patients. From this analysis variant peptides p2, p3, p5, p10, p18, p21 and p29 were selected. Generation of UPGMA (Fig.14A) showed the genetic distance existing among the different selected NHBA variants. Moreover, from the un-rooted phylogenetic tree (splits tree) (Fig.14B) it can be better appreciated that NHBA variants are distributed in three distinct clusters. Finally to better appreciate amino acid sequence conservation/variability, multiple sequence alignment of the selected NHBA variants were performed (Fig.15). As expected, sequence analysis showed that C-terminus domain of NHBA was highly conserved while the N-terminal domain showed high variability in the primary amino acid sequence. NHBA variants p2, p3, p5 and p29 have a 62 amino acid insertion/deletion (ins/del) sequence (A117 – T179), which allows classification of NHBA in long and short variants. Interestingly NHBA p29 variant an 11 amino acids ins/del sequence (V46 – A56) which is not observed in any of the other NHBA variants

in the current study (Fig. 15). Overall, the selected panel of the epidemiologically relevant selected variants is representative of NHBA amino-acidic diversity and cluster distribution.

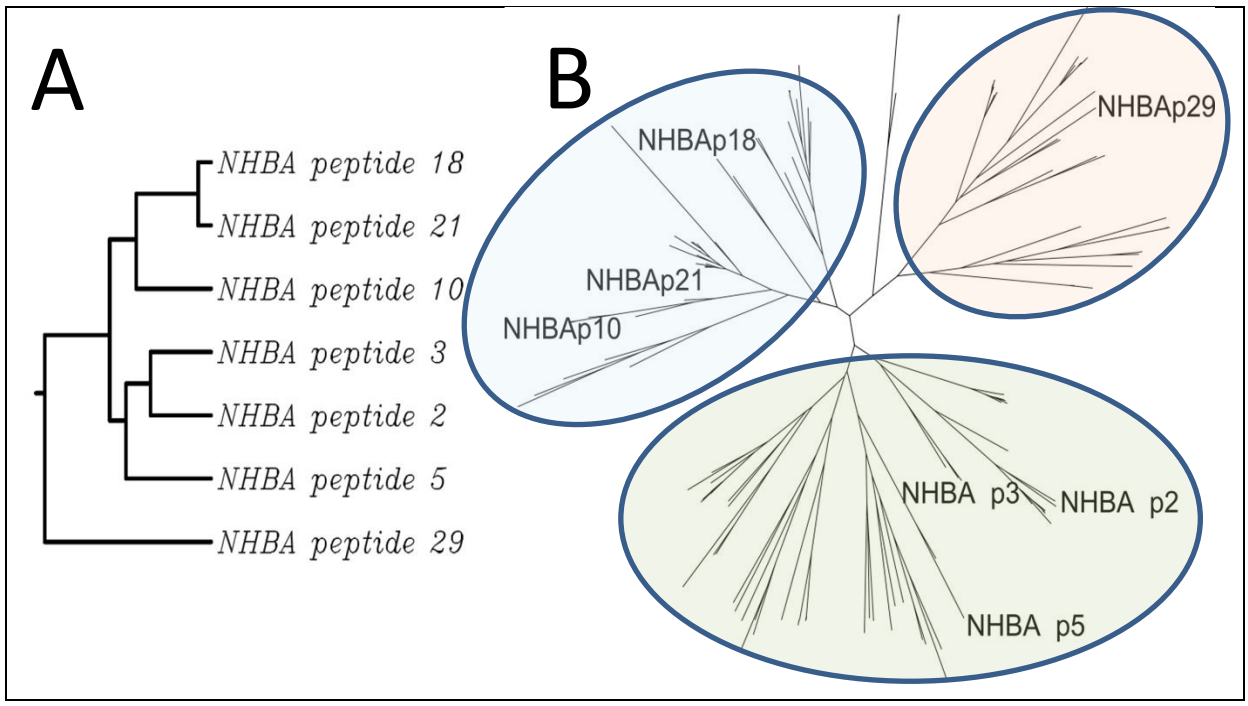


Figure 14. Phylogenetic analysis of NHBA variants. A. UPGMA phylogeneic tree between NHBA variants reported in this study. **B**. Unrooted phylogenetic tree obtained from the analysis of 150 NHBA variants. The highlighted areas shows that the diverse NHBA variants selected in this study are distributed in three different clusters.

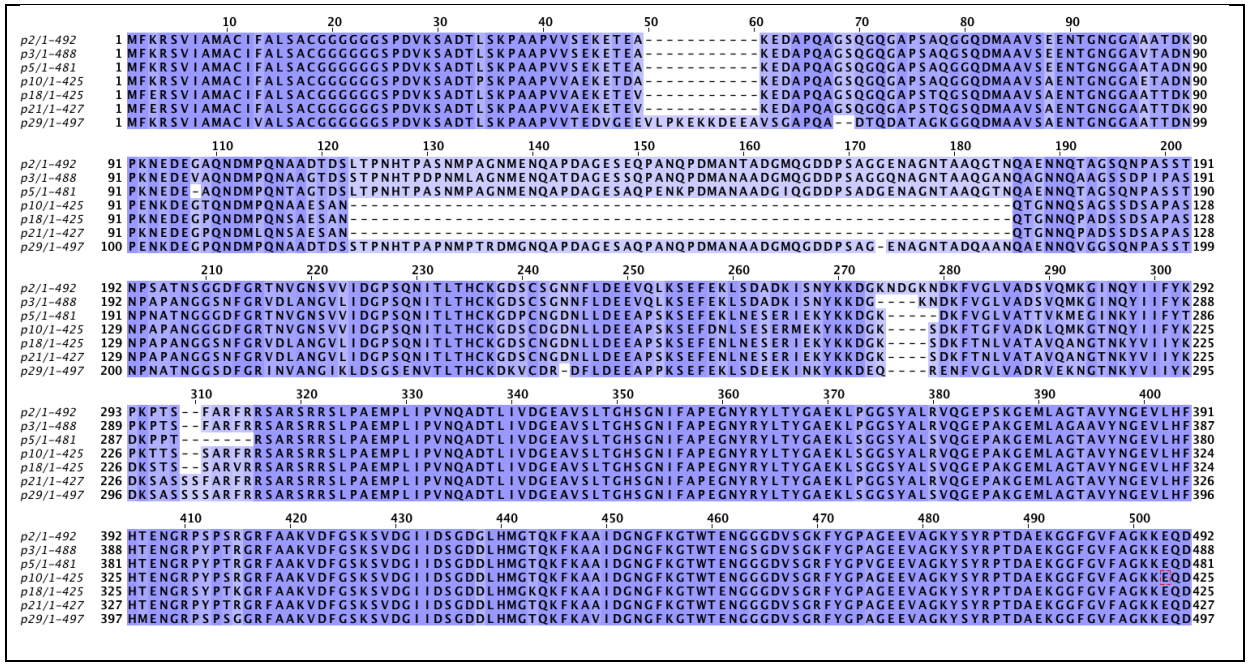


Figure 15. **Multiple sequence alignment analysis of different NHBA variants.** On the left are reported both NHBA variant peptide number and amino acid sequence length.

4.2.2. Cross reactivity of anti-NHBA mAbs

To explore the ability of mAbs raised against the vaccine variant to cross-react with heterologous NHBA variant peptides we used two approaches. The first was to investigate the ability of monoclonal antibodies to recognize native surface exposed NHBA on live bacteria by flow cytometry analysis and on total protein extracts by western blot analysis. For the second approach we used protein chip to validate the result observed by flow cytometry and western blot analysis on the full length rNHBA variants and peptide array analysis to investigate more in detail the peptides recognized by mAbs targeting linear epitopes.

4.2.3. Cross reactivity studies of anti-NHBA mAbs by flow cytometry and western blot analysis

For these studies we used recombinant isogenic strains expressing diverse NHBA variants (Vacca I. 2014). Differently from clinical isolates, where NHBA show variable expression levels, in 5/99ΔΔ:nhba strains, NHBA expression is under an IPTG inducible promoter, and therefore it can be controlled. The same genetic background and the inducible promoter allow similar levels of expression for each of the heterologous NHBA peptides under investigation. These are optimal conditions which –by excluding the bias of expression level- will allow assessing the contribution of NHBA sequence variability for cross reactivity and cross protection analysis.

Flow cytometry results (fig 16A) showed that mAbs targeting epitopes on the variable N-term region of NHBA were able to recognize only NHBA variants closely related to the vaccine variant. In fact mAb 3A3 cross reacted with the homologous NHBA p2 and heterologous p3 and p5 variants; mAb 10E8 was specific only to NHBA p2 homologous variant while mAb 9E5 cross reacted with variants p2, p3, p5 and p29. On the other hand mAbs 4G5 and 43F4, that target the well conserved C-term domain of NHBA, showed as expected cross reactivity with all NHBA variants. Flow cytometry results for mAbs 3A3, 10E8, 9E5, 4G5 and 43F4 were concordant with results obtained from western blot analysis. Flow cytometry and western blot analysis results for mAb 23B8 were almost overlapping. By flow cytometry analysis it was observed that mAb 23B8 was able to recognize NHBA variants p2,

p3, p5, p18 and p21. No binding was observed on strains expressing NHBA p10 and p29 variants. Similar results were observed on western blot analysis with the difference that mAb 23B8 showed no binding for the strain expressing NHBAp18 which was seen to be positive on flow cytometry analysis (fig. 16B). This result could be due to higher sensibility that flow cytometry has respect to WB analysis but overall these results suggest that, based on NHBA sequence variability, N-term mAbs cross-react only with closely related NHBA variants, while C-term mAbs are able to cross react with both distant and closely related NHBA variants.

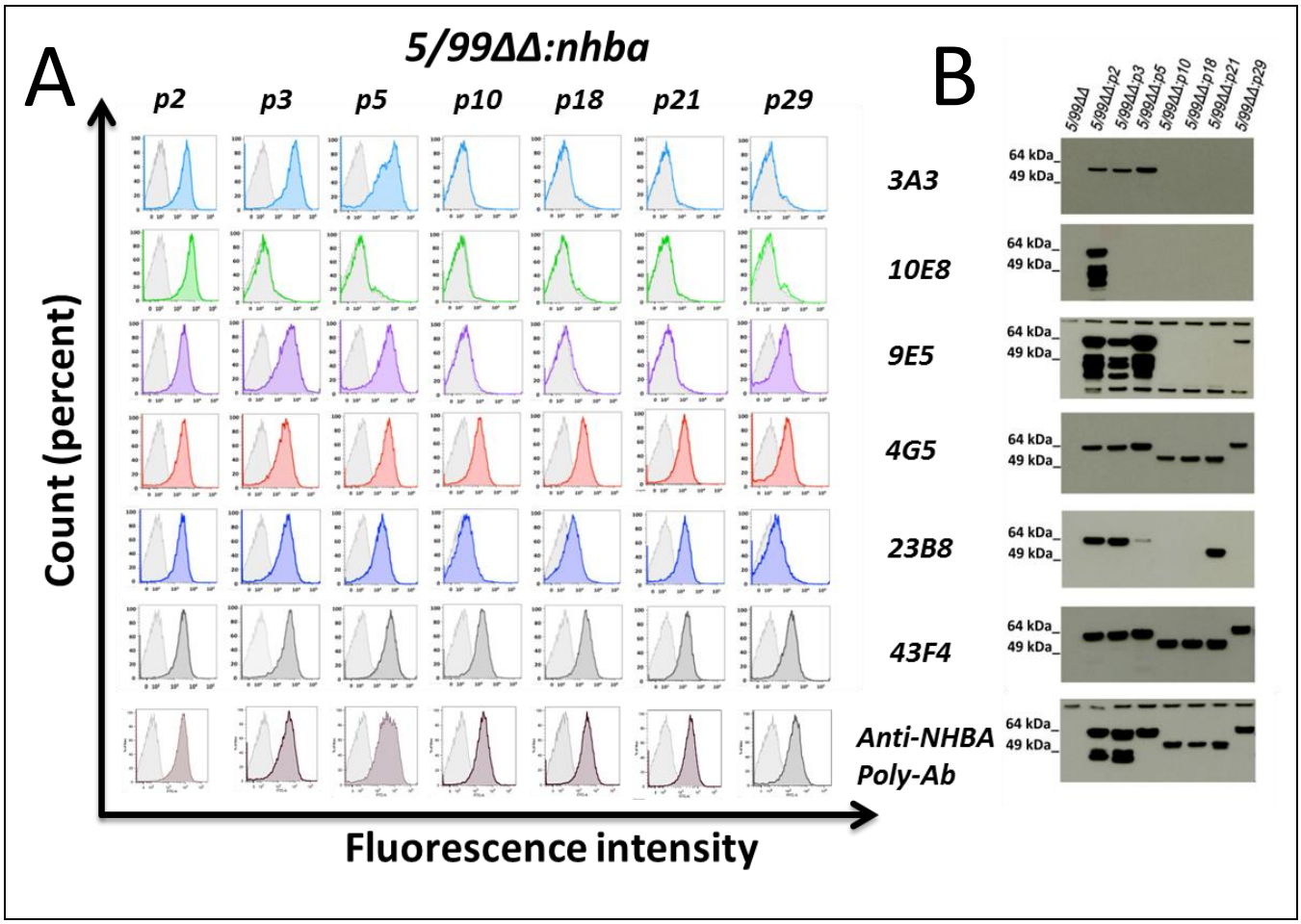


Figure 16. Cross reactivity of anti NHBA murine mAbs investigated by flow cytometry (A) and western blot (B) analysis on 5/99ΔΔ:nhba MenB recombinant strains. Anti-NHBAp2 polyclonal antibody was used as positive control both for flow cytometry and western analysis. 5/99ΔΔ was used as negative control (data not shown for flow cytometry analysis).

4.2.4. Cross reactivity studies of anti-NHBA mAbs by protein chip and peptide array analysis

Protein chip analysis. To validate flow cytometry and western blot results mAb recognition on full length rNHBA variants p2, p3, p5, p10, p18 p21 and p29 were analyzed by protein chip. Protein chip data (Tab. 1) showed that mAb 3A3 cross reacts with rNHBA variants p2, p3 and p5, mAb 10E8 was able to bind only the homologous NHBA p2 variant while mAb 9E5 was able to cross react with rNHBA variants p2, p3, p5 and p29. NHBA N-term targeting mAbs were not able to recognize short rNHBA variants p10, p18 and p21. MAbs 4G5 and 43F4 were able to cross react with both long and short NHBA variants. Multiple sequence analysis of mAb 4G5 epitope identified by HDx revealed that peptide K243-L274 (N-term) is highly polymorphic, while the peptide V349-L378 is highly conserved among the NHBA variants under analysis (fig 17B). The same analysis was performed on the HDx epitope of mAb 43F4 located on the C-term of NHBA which showed as well a high sequence identity of mAb 43F4 target region among the different NHBA variants (fig.17C). MAb 23B8 was seen to cross react with all NHBA variants tested as well, but its binding signal was lower on variants p3, p5, p10, p18 and p29 with respect to variants p2 and p21. Protein chip results show that mAbs targeting the variable N-term of NHBA cross react with closely related NHBA variants, while mAbs targeting the well conserved C-term cross react with distant and closely related NHBA variants.

Table 1. Cross reactivity of anti-NHBA mAbs by protein chip analysis. Plus sign (+) indicates mAb binding; minus sign (-) indicates no binding.

	Full length recombinant NHBA variants						
mAb	**p2**	**p3**	**p5**	**p10**	**p18**	**p21**	**p29**
3A3	+	+	+	-	-	-	-
10E8	+	-	-	-	-	-	-
9E5	+	+	+	-	-	-	+
4G5	+	+	+	+	+	+	+
23B8	+	+	+	+	+	+	+
43F4	+	+	+	+	+	+	+

Peptide array analysis. Since this method can detect only mAbs targeting linear epitopes, peptide array was performed only for mAbs 3A3, 10E8, 9E5 and 23B8 and not for mAbs 4G5 and 43F4 that target conformational epitopes (fig. 17). Results showed that mAb 3A3 was able to cross react with NHBA variants p2, p3, p5 and p10 but not with variants p18, p21 and p29.

Although the peptide recognized by **mAb 3A3** (Lys44-Gln54) is highly conserved among NHBA variants p2, p3, p5, p10, p18 and p21, a few polymorphisms are present on NHBA variants p10, p18 and p21. More in details Glu 47 is substituted in Asp in variant p10 and Ala 48 is substituted in Val in variants p18 and p21 (fig 17A). Since mAb 3A3 did not recognize variants p18 and p21, polymorphism 48:Ala->Val is probably responsible for abolishing the binding. NHBA variant p29 was not recognizes by mAb 3A3, since shows high sequence variability with variant p2 and moreover it has an insertion of ten amino acids (V49 and A59). These differences can certainly explain why mAb 3A3 did not bind NHBA variant p29. Overall peptide array analysis suggests that the epitope targeted by mAb 3A3 is present in NHBA variants p2, p3, p5 and p10. Since NHBAp10 variant was not recognize by mAb 3A3 in flow cytometry, western blot and protein chip analysis suggests that probably long and short NHBA variants might have a different protein folding. This could be due to amino acid sequence differences between long and short NHBA variants that might result on hiding the epitope or it's altered exposure.

Peptide array analysis showed that **MAb 10E8** specifically binds only to rNHBAp2 variant. Sequence analysis showed that the epitope have two polymorphisms, D108 substituted in Gly on NHBA p3 and p5 variants and L112 substituted in Ser on variants p3 and p29 (fig. 17A). Both polymorphisms seem to be involved on abolishing 10E8 binding to long NHBA variants p3 and p5. Multiple sequence alignment shows that short NHBA variants p10, p18 and p21 have multiple polymorphisms on 10E8 mAb epitope and moreover three terminal amino acids (113Thr-Pro-Asn115) of the epitope are missing since they belong to the 62 amino acid insertion/deletion (fig. 17A). Overall peptide array data identified amino acidic polymorphisms and sequence variability features between the long and the short NHBA variants that are supportive of mAb 10E8 NHBAp2 variant specificity. These results are concordant with previously showed flow cytometry, western blot and protein chip analysis.

MAb 9E5 was seen to cross reacts with all NHBA long variants (p2, p3, p5 and p29) but not with the short ones (p10, p18 and p21) on peptide array analysis. From multiple sequence alignment it can be observed that the epitope targeted by mAb pE5 (A165 – Gln 171) is highly conserved within p2, p3, p5 and p29 long NHBA variants. On the other hand this epitope is not present in short NHBA variants, p10, p18 and p21, due to the 62 amino acid ins/del sequence which distinguish the long from the short NHBA variants (fig. 17A).

Peptide array analysis showed that **mAb 23B8** cross reacted with NHBA variants p2, p3 and p21 and no binding was observed on NHBA variants p5, p10, p18 and p29. Multiple sequence analysis of the epitope identified by peptide array shows that 23B8 mAb targets a well conserved peptide (Lys 358 – Val 368) on the C-term of NHBA which have two polymorphisms, 360 Pro->Ser in variants p5, p10, p18 and 29 and 367 Arg->Ser in variants p5, p18 and p29 (fig. 17C). Although both substitutions might be responsible for binding abolishment, the polymorphism 360 Pro->Ser present in NHBA variant p10 seems to have a major impact on mAb 23B8 recognition since on this variant the Arg367 is conserved. In table 2 are reported cross reactivity results for mAb 23B8 among the panel of NHBA variants under investigation, obtained by flow cytometry, western blot, protein chip and peptide array analysis. These data suggests that mAb 23B8 cross react with NHBAp2, as previously showed, with NHBAp3 (long) and NHBAp21 (short) variants. Most probably mAb 23B8 is able to cross react as well with NHBA p5 since it showed a positive result in flow cytometry, western blot and protein chip but not in peptide array analysis. The latter result could be due to a combination of events which combines the presence of both polymorphisms (360 Pro->Ser and 367 Arg->Ser) to the shorter peptide recognized in peptide array, respect to the longer peptide identified by HDx. Similar results were observed for NHBAp18 variant where on flow cytometry and protein chip analysis mAb 23B8 was able to bind the native and the recombinant forms of the protein but negative results were obtained from western blot and peptide array analysis. A possible explanation of the negative results could be the lower sensibility of western blot analysis and the shorter peptide recognized on peptide array. MAb 23B8 was seen to recognize in protein chip the full-length NHBA variants p10 and p29 variants but no recognition was observed on flow cytometry, western blot and peptide array analysis. These results suggest that mAb 23B8 probably do not cross-react with NHBA variants p10 and p29. From the 3D structure of NHBA it can be observed that the HDx epitope of mAb 23B8 is comprised between two antiparallel beta sheets and their linking

loop. Overall these data suggest that the observed variability on cross reactivity of mAb 23B8 is probably due to the presence of the polymorphisms at the level of primary amino acidic sequence but also differences in protein folding could play an important role.

Table 2. Cross reactivity profile of mAb 23B8 among the panel of NHBA variants selected for this study obtained by flow cytometry, western blot, protein chip and peptide array analysis. On the last column of the table is reported the overlay of the epitopes identified by HDx (in red) and peptide array (highlighted in gray). In green are highlighted the amino acid polymorphisms +: recognition, -: no recognition.

	Long/ Short	Flow cytometry	Western blot	Protein chip	Peptide array	HDx/Peptide array epitope
NHBAp2	**Long**	**+**	**+**	**+**	**+**	**YLTYGAEKLPGGSYALRV**
NHBAp3	**Long**	**+**	**+**	**+**	**+**	**YLTYGAEKLPGGSYALRV**
NHBAp5	**Long**	**+**	**+**	**+**	**-**	**YLTYGAEKLSGGSYALSV**
NHBAp10	**Short**	**-**	**-**	**+**	**-**	**YLTYGAEKLSGGSYALRV**
NHBAp18	**Short**	**+**	**-**	**+**	**-**	**YLTYGAEKLSGGSYALSV**
NHBAp21	**Short**	**+**	**+**	**+**	**+**	**YLTYGAEKLPGGSYALRV**
NHBAp29	**Long**	**-**	**-**	**+**	**-**	**YLTYGAEKLSGGSYALSV**

4.2.5. Summary of anti-NHBA mAbs cross reactivity studies

From the analysis of the cross reactivity results obtained by several methodologies, including data generated from flow cytometry, western blot analysis and protein microarray data, we conclude that:

1. mAbs 3A3 and 9E5, targeting different epitopes on the variable N-term domain of NHBA, cross react with heterologous NHBA variants phylogenetically closely related to the homologous NHBA p2
2. mAb 10E8 (N-term) was seen to bind specifically only the homologous NHBAp2 variant
3. mAbs 4G5, 23B8 and 43F4, targeting different epitopes on the well conserved C-term of NHBA cross react with both distant and closely related NHBA variants

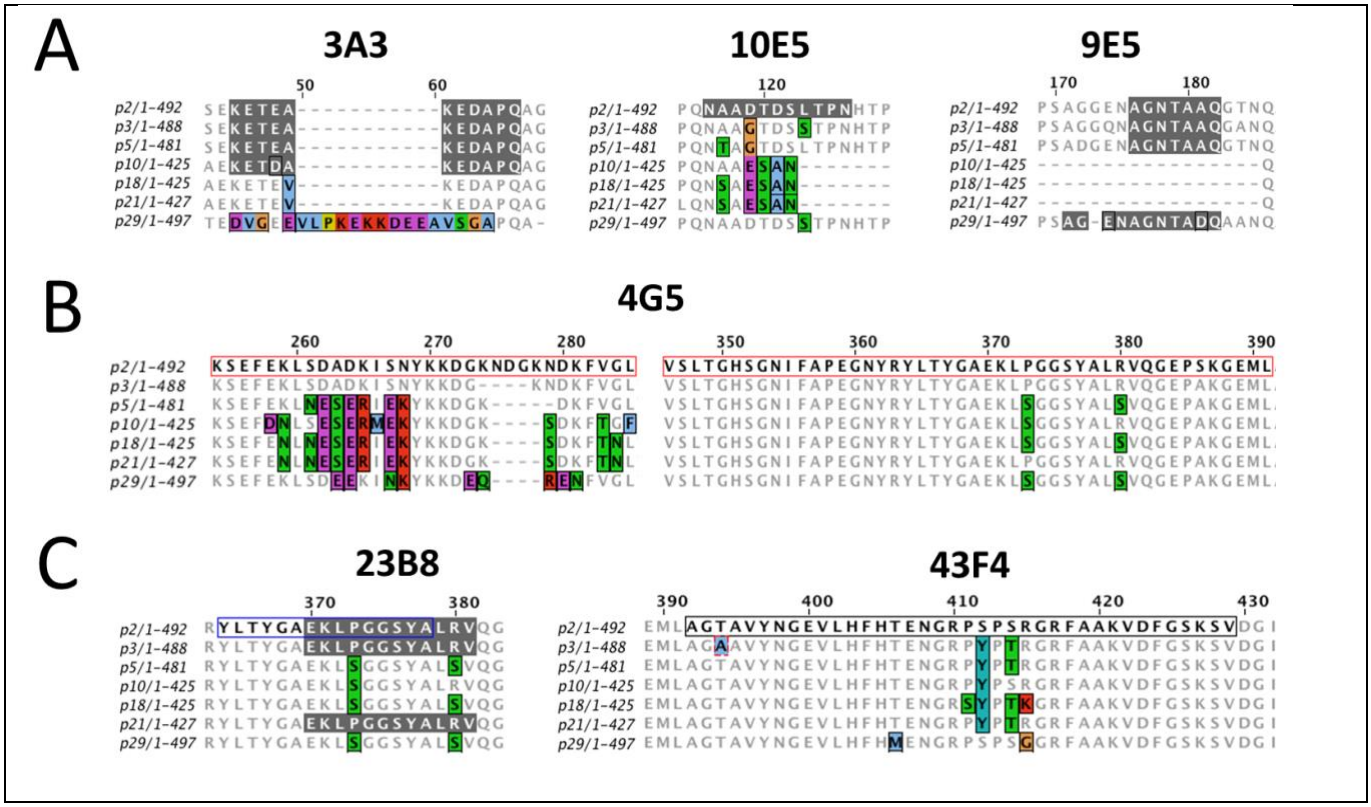


Figure 17. Multiple alignment of the epitopes recognized by murine mAbs in cross reaction studies by protein chip and peptide array. In the multiple alignment highlighted in grey are shown peptides recognized by mAbs 3A3, 10E8, 9E5 (**A**) and 23B8 (**C**, left panel) among different NHBA variants. Amino acid variability of peptides, belonging to heterologous NHBA variants not recognized by mAbs are highlighted in different colors. HDx epitopes of mAbs 4G5, 23B8 and 43F4 are displayed in red

(**B**), blue and black rectangles (**C**), respectively, on NHBAp2 variant. Despite epitope sequence variability mAbs 4G5, 23B8 and 43F4 were positive in protein chip analysis

4.2.6. rSBA analysis of mAbs tested alone and in combination against a panel of recombinant MenB strains expressing the selected panel of NHBA variants

Based on their cross reactive profile, cross protection of anti-NHBAp2 monoclonal antibodies were assayed against MenB strains expressing heterologous NHBA variants. To this aim monoclonal 3A3, 9E5, 4G5, 23B8 and 43F4 antibodies were tested alone and in combination in SBA experiments against MenB recombinant strains 5/99ΔΔ:nhba, complemented with different NHBA variants, previously used in flow cytometry cross reactivity analysis. These strains allow evaluating the impact of NHBA sequence variability on complement mediated killing since, differently from clinical isolates, they have the same genetic background and similar expression levels of the antigen. Monoclonal antibody 10E8 was excluded from this study because it was seen to bind specifically only the NHBAp2 vaccine variant (Fig. 16).

rSBA assay was performed as previously described on paragraph 1.4 and mAbs were tested alone and in combination to investigate their ability of to induce bactericidal killing. For the latter approach mAbs were combined based on their mapping region as follows: targeting only the N-term, both N-term and C-term and mAbs targeting only the C-term of NHBA.

Results showed in figure 18 are representative of a duplicate experiment performed in different days per each condition (mAbs tested alone and in combination). rSBA data against MenB strain 5/99ΔΔp2 is reported on the graph in order to facilitate interpretation of SBA titers among the different strains tested.

Monoclonal antibodies 3A3 and 9E5 both targeting the N-term of NHBA are able to induce bacterial killing against recombinant strains expressing the long NHBA variant p2, p3 and p5 (Fig. 18A). MAb 3A3 had the same SBA titer (2048), while mAb 9E5 showed an increase in rSBA titers among the three strains tested (SBA(p2)=256, SBA(p3)=1024 and SBA(p5)= 2048). The 9E5+3A3 mAb combination did not have a significant increase in SBA titers, when assayed against recombinant MenB strains expressing long NHBA variants (p2, p3 and p5).

Bactericidal titers of C-term mapping mAbs 4G5, 43F4 and 23B8 tested alone and in combination with N-term mapping mAbs 3A3 and 9E5 are reported in figure 18B and 18C respectively. When tested individually against MenB strains expressing long NHBA variants, C-term mAbs 4G5 and 43F4 showed negative rSBA titer. Monoclonal antibody 23B8 showed negative SBA titers against strains expressing NHBA variant p2, p3, and p29 but was able to induce bactericidal activity against the recombinant MenB strain expressing NHBA variant p5 (SBA titer=256). No significant increase in SBA titers was observed when mAb 3A3 was tested in combination with mAbs 4G5, 43F4 and 23B8 against strains expressing long NHBA variants (Fig 18B). When these mAbs were tested in combination, only the couple 3A3+23B8 showed a significant increase in rSBA titers against 5/99ΔΔcp2 MenB strain as already described (fig. 13B lower panel). C-term mapping mAbs 4G5, 23B8 and 43F4 were as well tested in combination with the other N-term mapping mAb 9E5 in rSBA assay. Results showed that a significant increase in rSBA titers was observed for mAb combinations 43F4+9E5 and 23B8+9E5 against strains expressing p2 (as previously described) and p5 NHBA variants (Fig 18C middle and lower panel). Moreover, mAb combinations 4G4+9E5, 43F4+9E5and 23B8+9E5 were able to induce cooperative bactericidal activity against 5/99ΔΔp29 (Fig. 13C upper, middle and lower panel).

C-term mapping mAbs, 4G5 and 43F4 were not able to induce bacterial killing when individually tested against MenB recombinant strains expressing both long and short NHBA variant peptides. Almost same scenario was observed for mAb 23B8 except for 5/99ΔΔcp5 against which was SBA positive. Combination of mAbs 4G5+43F4 induced killing of bacteria only against strains expressing long NHBA variants p2, p3 and p5 (fig 18D upper panel). 4G5+23B8 mAb combination was able to extend cross protection against strains expressing NHBA variants, p29 and p18 (fig 18.D middle panel). Only 23B8+43F4 mAbs combination was able to induce a full cross protective bactericidal activity against all the panel of recombinant MenB strains (fig. 18.D lower panel).

In summary based on these data we can conclude that:

1. mAbs 3A3 and 9E5, targeting the N-term, cross protect against recombinant strains expressing NHBA variants phylogenetically closely related to NHBAp2 variant.
2. 23B8+9E5 and 43F4+9E5 mAb combinations targeting both N-term and C-term of NHBA are able to significantly increase bactericidal titers but protection remains restricted to closely related NHBA variants.
3. Combination of mAbs 4G4+23B8 targeting the well conserved C-term of NHBA is able to extend cross protection to strains expressing more distant NHBA variants
4. Only synergy between 23B8+43F4 C-term mAbs induces full cross protection against the entire panel of recombinant MenB strains tested.

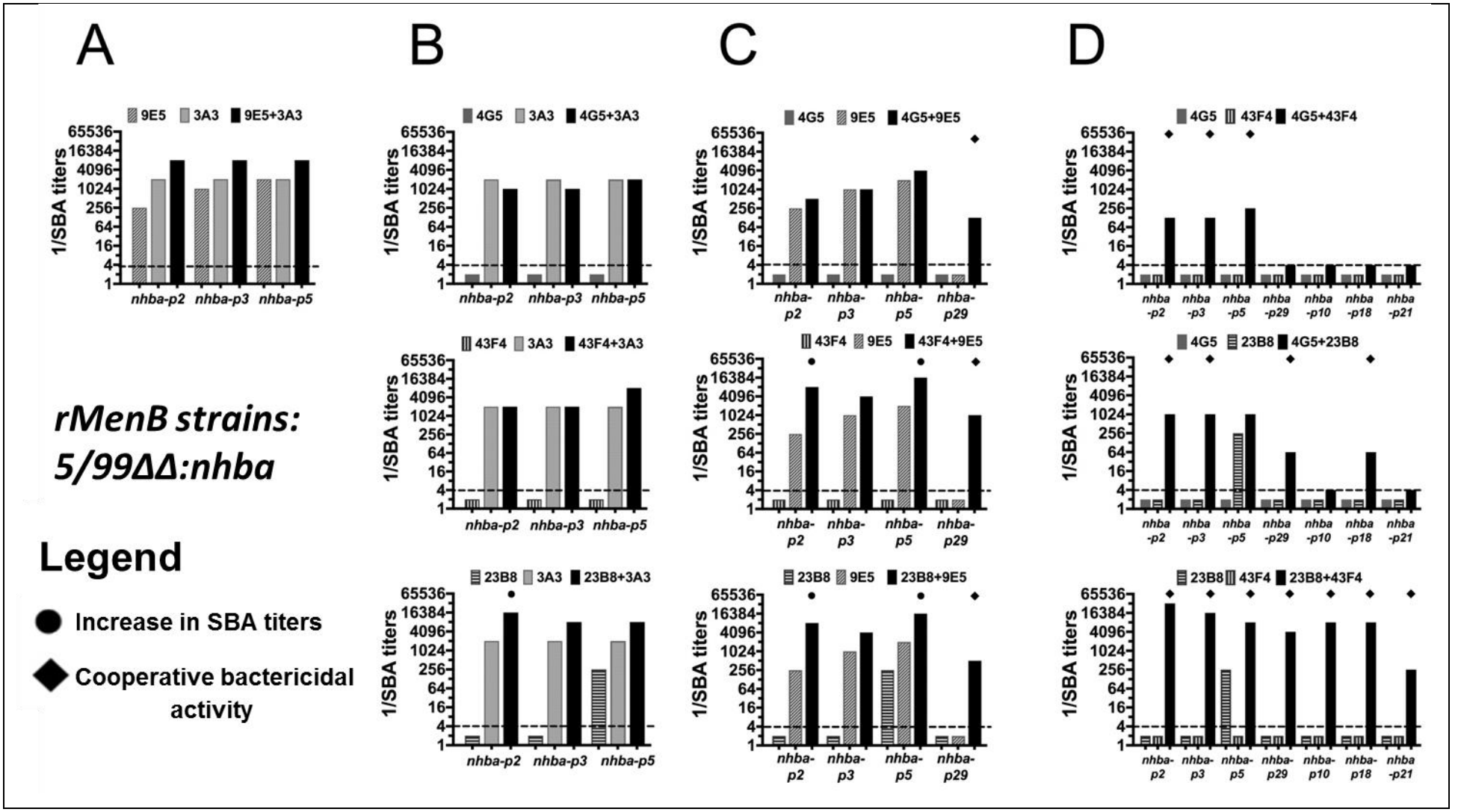


Figure 18 Cross protection of monoclonal antibodies against a panel of recombinant MenB (rMenB) strains expressing phylogenetic closely related and distant NHBA variants. Monoclonal antibodies 3A3 and 9E5 targeting the N-term of NHBA tested alone and in combination (**A**). C-term mapping mAbs 4G5, 43F4 and 23B8 tested alone and in combination with N-term mAbs 3A3 (**B**) and 9E5 (**C**). MAbs targeting the C-term of NHBA tested alone and in combination (**D**). Criteria for increase in SBA titers and cooperative bactericidal activity is reported in materials and methods

4.2.7. 23B8+43F4 mAb combination induce cross-protection against a panel of natural MenB strains which have variable NHBA expression

Monoclonal antibodies combination, 23B8+43F4, targeting the C-term of NHBA was seen to induce cross protection against recombinant strains expressing all NHBA homologous and heterologous variants selected for this study. In these strains *nhba* gene is under an IPTG inducible promoter that allows high and comparable NHBA expression levels among all strains. Differently from recombinant strains, clinical isolates are natural MenB strains isolated from infected patients. These strains have been described to have variable NHBA expression levels (Giuliani M. et al 2006). We therefore tested the ability of 23B8+43F4 mAb couple to induce bacterial killing against natural MenB strains. To this aim we selected a panel of MenB clinical isolates expressing the different NHBA variants used for the cross protection study. In particular we selected M4407 (NHBAp2), MC58 (NHBAp3), M2934 (NHBAp5), M12923 (NHBAp10), GB355 (NHBAp18), NM117 (NHBA p21) and M16686 (NHBAp29) natural MenB strains. We performed western blot analysis from total MenB protein extracts cultured in SBA growing conditions to investigate NHBA expression level on the selected natural strains. Total protein extracts from 5/99ΔΔp3 strain was used to evaluate differences in total NHBA expression between natural and recombinant strains. For western blot analysis we used polyclonal antibody raised against the vaccine variant NHBAp2.

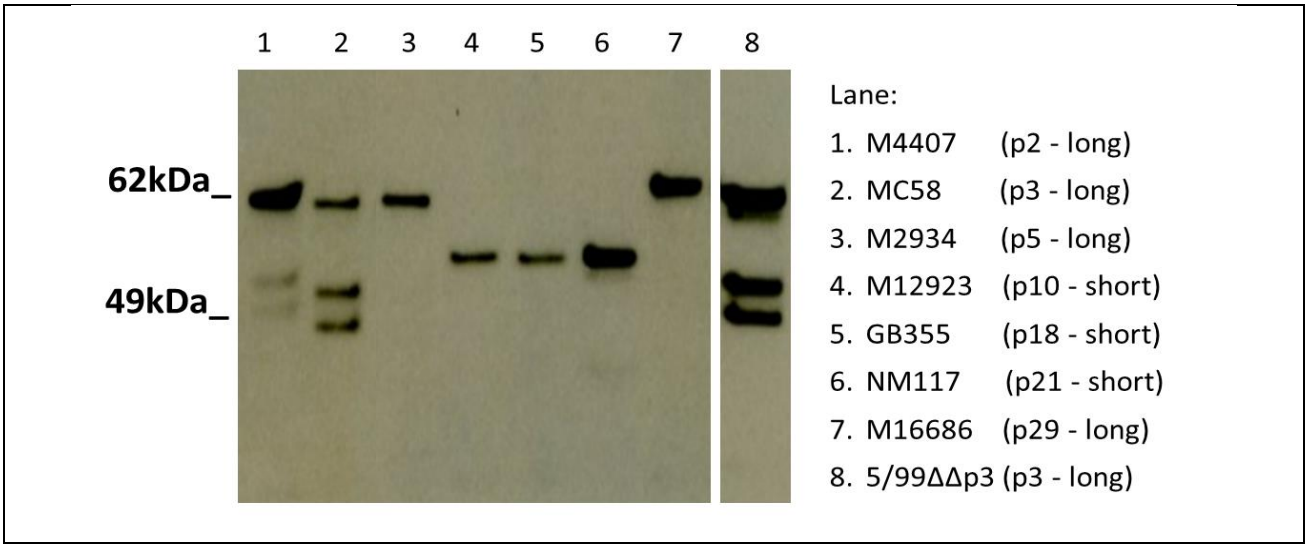


Figure 19. Analysis of different NHBA peptides expression on MenB clinical isolates. Western blot analysis of total protein extracts using polyclonal sera raised against NHBAp2 show a different migration pattern of long *versus* short NHBA variants. Full length NHBA variants, p2, p3 p5 and p29 (long variants) migrate around 62kDa, while short NHBA variants p10, p18 and p21 migrate 55kDa. Lower bands of variants p2 and p3 derive from NHBA cleavage probably due to NalP or other bacterial proteases. NHBA expression on 5/99ΔΔp3 recombinant strain is higher than expression of NHBA peptides on natural strains.

Results reported in figure 19 showed that NHBA total protein expression is variable within natural strains. Higher level of total NHBA expression was observed on the recombinant strain 5/99ΔΔp3 respect to the natural strains. The differences observed between different NHBA peptides (Fig. 19) are due to diverse sequence length between the long (p2, p3, p5 and p29) and the short NHBA variants (p10, p18 and p21).

Same conditions as for the recombinant strains previously reported were used to perform serum bactericidal activity assay against natural MenB strains. As positive control we used antisera raised against NHBAp2 vaccine variant. Results showed that 23B8+43F4 mAb combination is able to induce complement mediated killing of bacteria against all but one tested strains thus confirming its ability to induce cross protection against natural MenB strains (fig 20). Except for MC58 strain, 23B8+43F4 mAbs combination induced significantly higher bactericidal titers compared to the anti-NHBAp2 polyclonal antibody. Particularly a six fold increase in rSBA titers was observed when this mAb combination was tested against strain GB355 (p18), followed by fivefold increase against strain M2934 (p5). When testing the mAb combination against strains M16686 (p29), M12923 (p10) and NM117 (p21), three to four fold increase in rSBA titers was observed. As expected, no significant difference in SBA titers was observed when mAb combination was tested against M4407 expressing the homologous NHBAp2 variant. The difference in rSBA elicited by the mAb combination respect to titers induced by NHBAp2-antisera probably reflects the different functional antibody composition present in the two mixtures. The number of antibodies that binds with high affinity to heterologous NHBA variants and able to induce bactericidal killing is probably lower in the NHBAp2-antisera respect to the 23B8+43F4 mAb mixture. Moreover competition between mAbs targeting overlapping epitopes could take place, therefore not allowing an efficient complement deposition and bacteriolysis. The only strain in which mAb combination was not able to induce bactericidal activity was MC58. This strain has been described to express NalP, a bacterial protease capable to process NHBA cleaving away the C-term domain. Both 23B8 and 43F4 target the C-term of NHBA so its detachment from the bacterial surface due to NalP cleavage could be a plausible explanation of the observed negative rSBA result of the mAb combination. In fact the anti-NHBAp2 polyclonal antibody targeting different regions of the NHBAp3 variant was able to induce bactericidal killing.

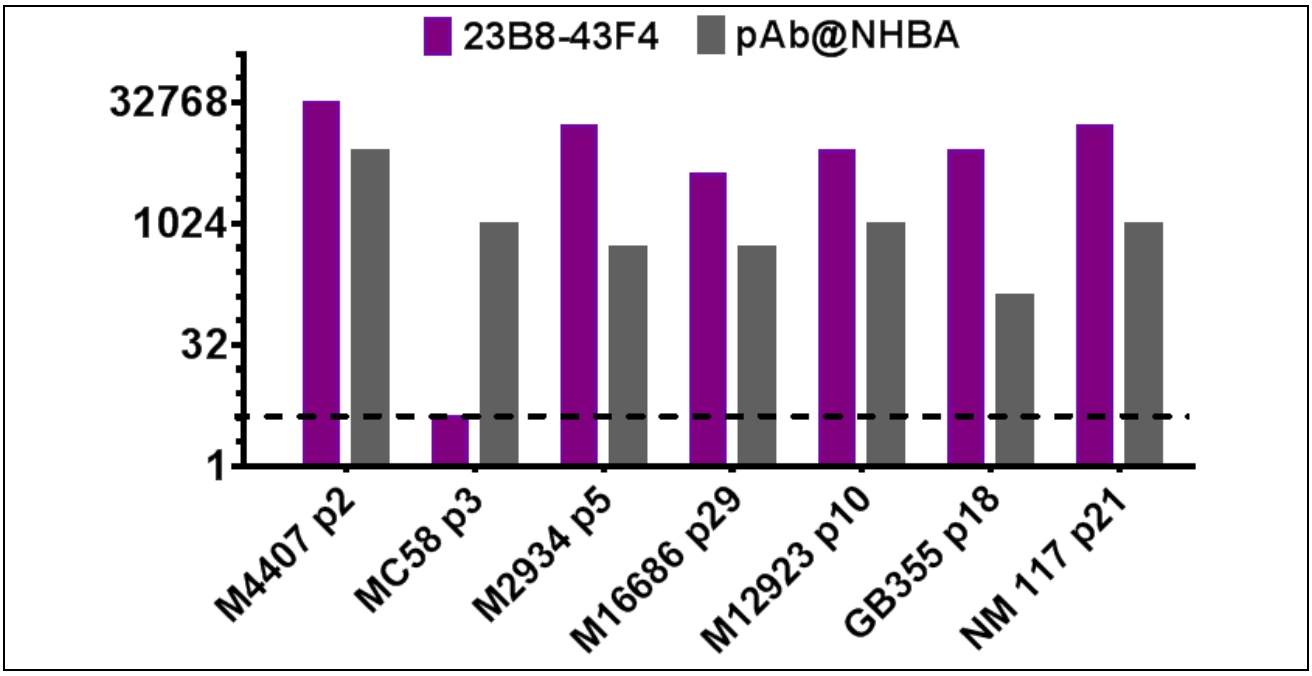


Figure 20. 23B8+43F4 mAb combination induce bactericidal activity against all MenB natural strains assayed except MC58. Anti-NHBA mouse polyclonal antibody was used as positive control

4.2.8. Investigating bacterial protease NalP impact on SBA titers of mAb combination 23B8+43F4

It has been previously shown that NHBA can be processed by the meningococcal protease NalP and human lactoferrin (hLf). Cleavage occurs either upstream and downstream of the NHBA Arg-rich region resulting in one of two possible cleavage fragments termed C2 and C1, respectively (Serruto et al., 2010). As previously shown, MC58 was the only MenB strain resistant to complement mediated killing to 23B8+43F4 mAb combination and moreover it has previously been described to express high levels of NalP. Therefore we evaluated NalP expression on natural and recombinant strains tested in rSBA to investigate its impact on bactericidal killing mediated by 23B8+43F4 mAbs combination. Since the recombinant strains have the same genetic background, NalP expression was evaluated only on one representative strain. To this aim, 5/99ΔΔp3 recombinant strain was selected.

Western blot analysis was performed on total protein extracts obtained from natural and recombinant strains grown on SBA conditions and NalP was detected using a polyclonal antibody raised the recombinant NalP protein. MC58*Δnalp* strain, where *nalp* gene is knocked out, was used as negative control (fig 21).

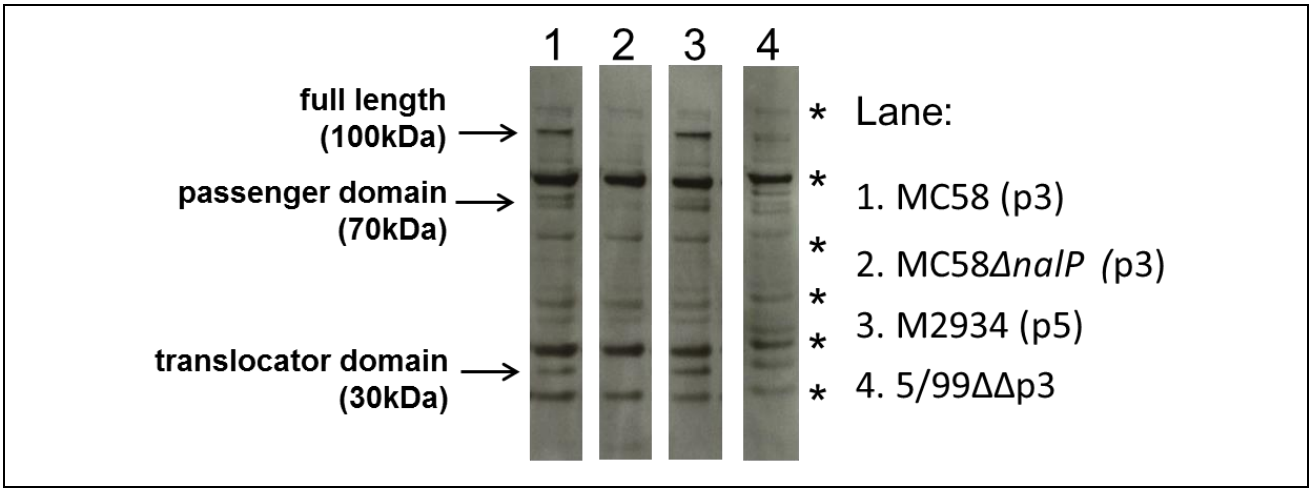


Figure 21 NalP expression on MenB natural and recombinant strains analysis. NalP expression detected on MC58 , M2934 and 5/99ΔΔp3. Full length NalP and its components passenger domain and translocator domain are indicated by the arrows. * indicates non-specific bands

Among all the strains tested, western blot analysis revealed that NalP was highly expressed on MC58, M2934 strains and lower expression was detected on 5/99ΔΔp3 recombinant strain (fig 22). These results are concordant with NHBA cleavage pattern previously observed by western blot (Fig.20) where NHBAp3 variant resulted cleaved in MC58 and 5/99ΔΔp3. A peculiar situation was observed on strain M2934 that express NHBA peptide p5 , which lacks the residues which are usually recognized by NalP (fig. 23) and therefore is not subject of NalP cleavage as shown in fig. 20. These results are in agreement with data previously reported by Vacca I. (Vacca I. 2014)

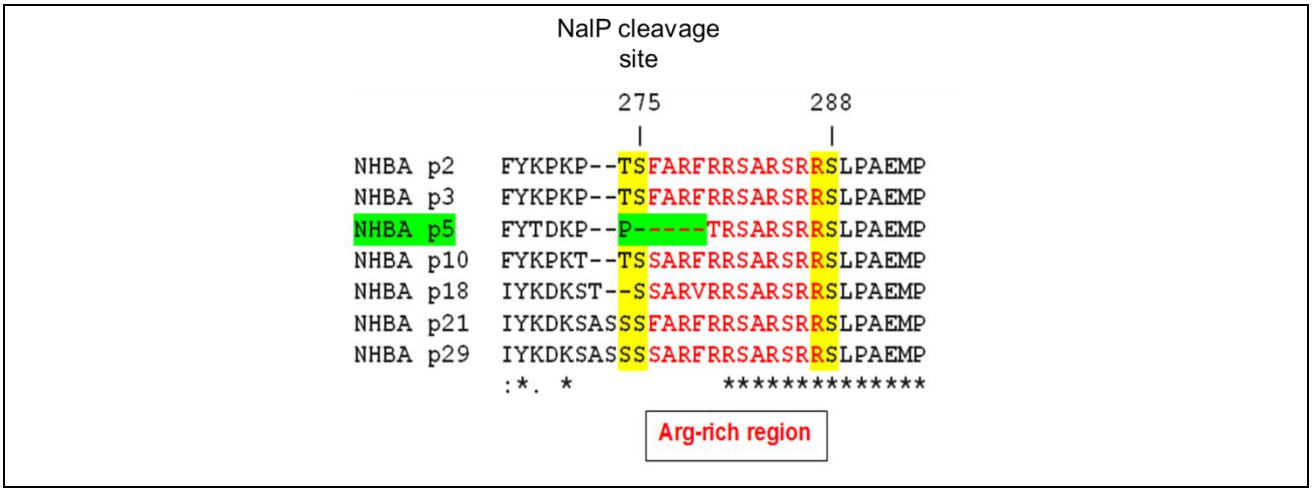


Figure 22 Multiple alignment of the Arginine rich region of NHBA variants p2, p3, p5, p10, p18, p21 and p29. In yellow are highlighted NalP and human Lf cleavage sites located respectively upstream and downstream of the Arginine rich region (highlighted in red). In green is highlighted NalP cleavage site deletion on NHBA p5 variant**.**

Western blot result suggests that NalP processing of NHBA might be responsible for MC58 resistance to bacterial killing mediated by 23B8+43F4. On the other hand NalP expression seems not to impact rSBA titers of the mAb couple against 5/99ΔΔp3 strain. To further investigate these experimental observations we evaluated monoclonal antibodies capability to bind the surface exposed NHBAp3. To this aim flow cytometry analysis was performed staining with mAbs alone and in combination on bacterial strains MC58 and 5/99ΔΔp3. MC58*Δnalp* strain was used as negative control for NalP cleavage (Fig. 23A), while anti-NHBAp2-polyclonal antibody was used as positive control for NHBA detection. Flow cytometry analysis showed that monoclonal antibodies neither alone nor in combination were able to recognize NHBA on the surface of MC58 strain (fig 23A). Conversely, mAbs both individually and in combination were able to recognize surface exposed NHBA on 5/99ΔΔcp3 and MC58*Δnalp* strains. The detection signal of mAbs binding NHBA on strain 5/99ΔΔcp3 was about 1 log higher than detection signal of the same mAbs tested on MC58*Δnalp* strain (fig 23A). This difference in mAb recognition reflects the difference of the surface exposed NHBA between the two strains. The same trend in surface exposed NHBA recognition, between 5/99ΔΔp3 respect to MC58 and MC58*Δnalp* strains was observed by anti-NHBAp2 polyclonal antibody, thus confirming the results observed with mAbs (data not shown). These results suggest that NalP cleaves away the C-term of NHBA impacting binding capability of mAbs 23B8+43F4 combination on NHBA surface exposed on MC58 strain but not in 5/99ΔΔp3. Finally we tested mAbs combination 23B8+43F4 against MC58*Δnalp* strain in order to test their ability to induce bactericidal killing when NalP cleavage is absent and using the same anti-NHBAp2 polyclonal antibody as positive control. RSBA results showed that 23B8+43F4 mAbs combination are able to induce bactericidal activity against MC58*Δnalp.* This confirms our hypothesis that in MC58 strain, 23B8+43F4 mAb combination does not induce bacterial killing due to NalP cleavage. In figure 23B are plotted rSBA results of 23B8+43F4 mAb combination assayed against MC58, MC58*Δnalp* and 5/99ΔΔcp3 strains.

Overall these results suggest that high expression levels of NalP which results in active NHBA cleavage, have an impact on bacterial killing mediated by mAb combination 23B8+43F4 against natural strains with low NHBA expression as observed for MC58. On the other hand in strains expressing lower NalP levels combined to high expression levels of surface exposed NHBA, NalP cleavage does not seems to impact bacteriolysis mediated by the mAb combination. Moreover we showed that in strains where NalP expression level is high but NHBA lacks the residues usually recognized by this bacterial protease preventing NalP cleavage bactericidal killing mediated by

23B8+43F4 mAb combination was not impacted by NalP expression. We conclude that NalP expression might partially impact bactericidal killing mediated by monoclonal antibodies in strains with high NalP and low NHBA expression.

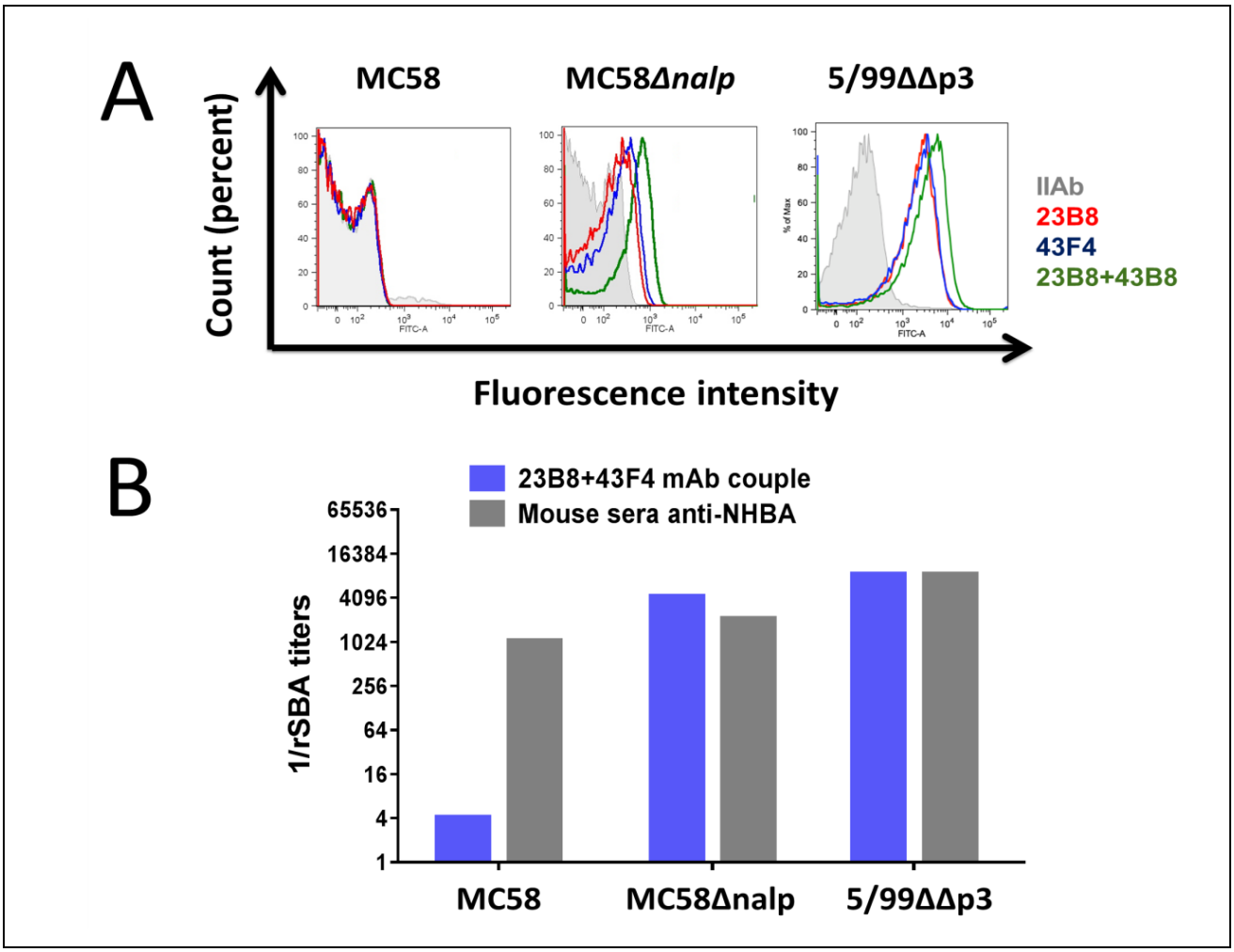


Figure 23 Flow cytometry and SBA analysis on MC58, MC58ΔnalP and 5/99ΔΔcp3. A. Surface exposed NHBA recognition of mAbs 23B8 (red line), 43F4 (blue line) individually and in combination (green line). **B**. SBA titers of mAb 23B8+43F4 combination (blue bars) against, MC58, MC58*Δnalp* and **5/99ΔΔcp3.** MC58 was the only strain resistant to bactericidal killing of the mAb combination. This strain has been previously described to express high NalP levels which cleaves NHBA upstream the Arginine-rich region (as shown on the WB analysis in fig. 22. NalP is a meningococcal phase variable auto-transporter with proteolityc activity and its cleavage site shows sequence variability among NHBA variant peptides. Grey bars show rSBA titers obtained by anti-NHBA mouse polyclonal antibody that was used as positive control.

4.2.9. Discussion and conclusions

NHBA sequence variability in circulating strains has been previously reported (Muzzi A. *et al* 2013, Vogel et al 2013 Bambini S. *et al* 2009). Unlike other main antigens of 4CMenB (fHBP, NadA and PorA), NHBA has not been classified into a limited number of distinct sequence variants, alleles or subtypes, since the variability is very broad and no specific group of sequences can be identified. To date more than 400 distinct amino acid sequences are reported in public databases, and different level of expression has been described for NHBA in natural strains (Vacca I., 2014). To investigate the capability of the mAbs presented in this study to confer cross protection against different representative strains, a limited number of NHBA peptide variants were identified by molecular epidemiology surveys performed on different panels of strains (Bambini S. *et al* 2009, Wang *et al,* 2011, Vogel et al 2013).

The six selected NHBA variants were phylogenetically diverse and could be divided in two main groups: p3, p5 and p29 the so called “long” NHBA variants which are more closely related to the homologous NHBAp2 vaccine variant and p10, p18 and p21 so called “short” NHBA variants, which are mode distant. Beside sequence variability the main difference between the two groups is the presence of a 62 amino acid insertion present on the long NHBA variant peptides.

We used the recombinant to investigate cross reactivity and cross protection of the murine mAbs, isogenic 5/99ΔΔ:nhba strain engineered to express the diverse NHBA variants p2, p3, p5, p10, p18, p21 and p29 under an IPTG inducible promoter (Vacca I. 2014). The same genetic background and the inducible promoter allow similar levels of expression for each of the heterologous NHBA peptides under investigation. This feature allows dissecting and evaluating specifically the contribution of amino acid sequence variability to NHBA immunogenicity. These strains were previously reported to be susceptible only to anti-NHBA antibodies mediated bactericidal killing, differing only in the amino acid sequence of the NHBA protein expressed (Vacca I. 2014).

We showed that mAbs 3A3 and 9E5 (mapping the N-term) were able to cross react with the homologous NHBA p2 and variants p3, p5 and p29, (the latter recognized only by mAb 9E5) phylogenetically closely related to the vaccine variant. MAb 10E8 was specific only to NHBA homologous p2 variant. On the other hand the C-term mAbs 4G5 and 43F4 cross reacted with both distant and closely related NHBA variants.

As previously showed, by HDx epitope mapping we identified the conformational epitope of mAb 4G5. This epitope is composed by two discontinuous peptides, K243- L274 and V333-L378

mapping respectively on the N-term and on the C-term of NHBA. Using multiple alignment sequences analysis, we showed the N-term peptide of the 4G5 epitope shows high sequence variability while the C-term peptide shows high sequence conservation (fig.17). This result is concordant with our previous hypotheses that the core epitope of mAb 4G5 might be located on the C-term of NHBA. Overall these data are concordant with the full cross reactivity profile of mAb 4G5.

By protein chip analysis we showed that mAb 23B8 recognized all the NHBA variants, while by flow cytometry we observed that it cross reacts only with p2, p3, p5, p18 and p21 surface exposed NHBA variants. By peptide array and multiple sequence analysis we identified two polymorphisms 360 Pro->Ser and 367 Arg->Ser that seems to be responsible abolishment of 23B8. We believe that the variability on cross reactivity of mAb 23B8 is probably due to the presence of the two polymorphisms within the target regions identified by HDx and peptide array analysis. Neverthe less differences at the level of primary amino acidic sequence of NHBA variants could play an important role in protein folding end epitope exposure.

The body of the experimental data here reported sugests that monoclonal antibodies targeting the variable N-term domain of NHBA cross-react with homologous and closely related heterologous NHBA variants, while mAbs targeting the highly conserved C-terminal domain are able to cross react with more distant NHBA variants.

Cross protection studies were conducted testing mAbs individually and in combination against a panel of recombinant MenB strains expressing the selected NHBA variants. We showed that mAbs 3A3 and 9E5, targeting the N-term of NHBA, were able to induce bactericidal activity against recombinant strains expressing the homologous NHBAp2 variant and closely related NHBA variants p3 and p5. Moreover combination of mAbs targeting the two major domains of the antigen (N-term and C-term respectively) were able to significantly increase SBA titters against strains expressing variant p2 and p5 and induce functional cooperativity against the strain expressing variant p29 variant. These results are concordant with cross reactivity data suggesting that even though synergy between mAbs targeting the major NHBA domains can contribute to increase bactericidal titers, protection remains restricted to closely related NHBA variants

MAbs 4G5 and 43F4 were not able to induce bactericidal killing against any of the strains tested. Combination of mAbs 4G5+23B8 targeting the well conserved C-term of NHBA were able to extend cross protection to strains expressing more distant NHBA variants. Only combination 23B8+43F4

was able to induce full cross protection against the entire panel of strains tested. This result was further validated testing 23B8+43F4 mAb combination against a panel of natural MenB strains (clinical isolates) expressing same NHBA variants expressed by the recombinant panel of MenB strains. We showed that although natural strains show lower NHBA expression level, respect to recombinant strains, similar bactericidal titers were observed by 23B8+43F4 mAb combination when tested against the panel of natural strains. With these results we confirm the ability on anti-C-term mAbs to induce cross protection against a panel of natural MenB strains expressing epidemiologically relevant NHBA heterologous peptides.

Furthermore we showed that NalP mediated NHBA cleavage has a limited impact on bactericidal activity. NalP cleavage generates the C2 fragment, which is released from bacterial surface. We showed that C2 fragment release from the surface of MC58 strain impacts bactericidal activity mediated by 23B8+43F4 mAb combination. Therefore, the susceptibility to cleavage might indicate a bacterial strategy to evade the immune system by displacing cooperative antibodies that would render the killing process even more efficient. The release of the C2 fragment, upon NalP proteolysis, has been recently reported to also alter endothelial permeability, contributing to extensive vascular leakage (Casellato et al., 2014).

On the other hand we demonstrated that high levels of surface exposed NHBA combined with low NalP expression did not impact bactericidal titers, as observed testing 23B8+43F4 mAb combination against 5/99ΔΔp3 strain. Moreover we showed that SBA titers were not impacted by NalP expression when tested against M2934 strain that express NHBAp5 variant that misses NalP cleavage site. Due to NHBA sequence variability NalP is not able to exert its proteolytic activity on NHBAp5. High levels of NHBA expression and absence of NalP cleavage retain the C-term of NHBA on bacterial surface. The increased availability of the C-term of NHBA might be at the basis of the bactericidal activity induced by mAb 23B8 when tested alone against 5/99ΔΔp5 recombinant strain. These results require further investigation since it was the only case when a C-term mapping mAb was individually able to induce bactericidal activity.

4.3. *Investigating the molecular mechanism of NHBA mediated bactericidal activity*

In the previous section we described that the release of the C-term fragment upon NalP cleavage , termed C2, might have an impact on bactericidal activity of 23B8+43F4 mAb combination, but little is known on the cleavage operated by host proteases. The cleavage operated by hLf generates the C1 fragment which lacks the Arg-rich motif. Recently other two human proteases, the C3-convertase and kallikrein have been reported to cleave NHBA. Similarly to hLf, the cleavage site for both proteases is located downstream the Arg-rich motif generating a C-terminal fragment that does not contain the Arg-rich motif. Characterization of the released C-term fragment by mass spectrometry evidenced that the cleavage site for hLF, kallilrein and C3-convertase proteases is highly conserved and the C-term fragment generated is the same (Serruto et al, 2010, Pantano 2016, Di Fede 2017). More recently it has been described that incubation of bacteria with different serum sources (human, mouse and rabbit) has two different effects on NHBA. The first is that incubation of bacteria with 25% (vol/vol) with human, mouse and rabbit serum, as used during SBA assay, was reported to actively induce cleavage of NHBA with a pattern similar to that observed for human proteases. The second effect refers of the increase of NHBA RNA and protein expression levels during incubation with serum (Borghi S. 2017).

We previously showed in this study that monoclonal antibodies (Fig. 11A) targeting the N-term domain of this antigen were individually able to induce bacterial killing when tested in rSBA against natural and recombinant strains expressing the NHBAp2 vaccine variant. Furthermore when combined with C-term domain mapping mAbs a significant increase in bactericidal titers was observed against recombinant strains expressing homologous and closely related heterologous NHBA variants.

On the other hand mAbs targeting the well conserved NHBA's C-term domain did not induce bacterial killing individually but their synergy was seen to induce bactericidal activity against strains expressing both distant and closely related phylogenetic NHBA variants.

Therefore, understanding the NHBA exposure pattern on bacterial surface and the mechanisms that drive antibody mediated bactericidal activity is an important goal to better understand the vaccine induced response and *N. meningitidis* pathogenesis.

To this aim we used mAbs 9E5, 23B8 and 43F4 to investigate different aspects important for the NHBA mediated bactericidal activities which are:

1. How is NHBA distributed on the surface of serogroup B meningococci during SBA growth conditions?
2. Does NHBA cleavage mediated by serum proteases have an impact on mAb recognition and their bactericidal activity?
3. Why mAbs targeting the N-term domain individually induce bacterial killing while mAbs mapping on the C-term domain do not?
4. Why combination of mAbs targeting the two major domains of NHBA result in increase of bactericidal titers?
5. Which is the mechanism that mediates cooperative bactericidal activity of mAbs 23B8 and 43F4?

4.3.1. NHBA shows sparsely surface exposure and is distributed in clusters on the bacterial surface

Surface exposure and distribution of vaccine antigens are correlated with their ability to elicit antibody mediated bactericidal activity. It has been previously reported that NHBA and fHbp show a different pattern of expression and antigen density on the bacterial surface (Welsch JA. et al.,2003 ;Giuliani et al 2006). To better understand how NHBA is distributed on the bacterial surface we performed confocal microscopy analysis on M4407 and 5/99ΔΔp2 strains grown in the same conditions as for the rSBA assay (final OD_{600}=0,25). We used these strains because both of them express the vaccine NHBAp2 variant but at different levels, as previously showed in figure 11B and 11C. Confocal microscopy experiments where performed using monoclonal antibodies alone and in combination while polyclonal antiserum raised against NHBAp2 variant were used as positive control. Results showed that in both strains NHBA is organized in clusters on bacterial surface even though the signal detected for strain 5/99ΔΔp2 was higher respect to that detected on M4407strain. These evidences are in agreement with the NHBA surface exposure analysis performed by flow cytometry, as previously shown (Fig. 11C). Moreover high local density of NHBA is observed within clusters but their overall distribution is not uniform for both strains (Fig.24).

The overall NHBA surface exposure detected by flow cytometry and surface distribution detected by confocal microscopy cannot explain the different ability of anti-NHBA mAbs to induce bactericidal activity. As previously showed only mAbs targeting the N-term of NHBA were bactericidal against both strains.

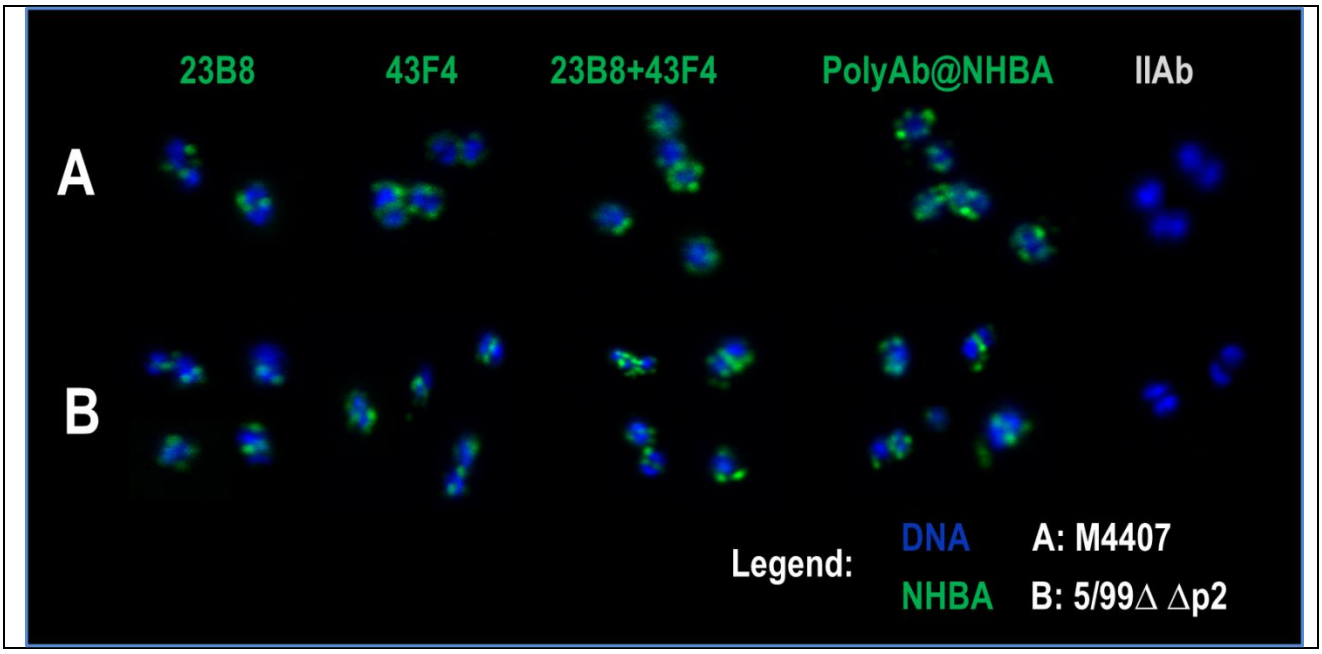


Figure 24 NHBA is exposed in clusters on bacterial surface of M4407 (A) and 5/99ΔΔp2 (B) MenB strains. Overlay image where in blue are shown nuclei DNA, and in green are shown NHBA clusters exposed on serogroup B meningococcal surface.

4.3.2. Characterization of NHBA cleavage by rabbit serum proteases

We previously showed by flow cytometry analysis, that recognition of surface exposed NHBA by monoclonal antibodies targeting the N-term and the C-term domains of the protein was similar. Moreover even though in the same non molar range, affinities of mAbs targeting the C-term was higher respect to mAbs targeting the N-term of the protein. It has been reported that NHBA is cleaved upstream and downstream of the Arg-rich motif both by bacterial (NalP) and human proteases (hLf, kallikrein and Complement C-3 convertase proteases). More recently it was reported that NHBA is also subject to serum proteases cleavage that shows a similar cleavage pattern as the one observed by human proteases (Borghi S. 2017). This cleavage pattern was seen to be similar within different serum sources (human, mouse and rabbit) suggesting that the cleavage site might be downstream the Arg-rich region which is supported by the fact that proteases cleavage site is highly conserved among NHBA variants. During the rSBA assay, bacteria, mAbs and baby rabbit serum (BRS) used as complement source, are mixed together and incubated for 1 hour, 5%CO_2 at 37°C. We wanted to investigate NHBA behavior in the presence of BRS during rSBA assay conditions. We incubated 1ug of the recombinant NHBAp2 protein (rNHBAp2) with different BRS

concentrations (1%, 5%, 10% and 25% vol/vol) and reaction mixture was incubated for 1 hour at 37°C, 5% CO_2. After 1 hour of incubation the reaction was blocked and samples separated on SDS-PAGE gel. Western blot analysis showed that incubation with 1%, 5% and 10% BRS generates three fragments of different molecular weight which migrate at 62 kDa, 49 kDa and around 20kDa corresponding to the full length, N-term and C-term of NHBA respectively. Incubation of rNHBAp2 with 25% BRS generates only two fragments corresponding to the N-term and the C-term of NHBA (Fig 25). This data suggest that a partial cleavage of rNHBA is observed when the protein is incubated with 1%, 5% and 10% BRS while incubation with 25% BRS totally cleaved the recombinant protein.

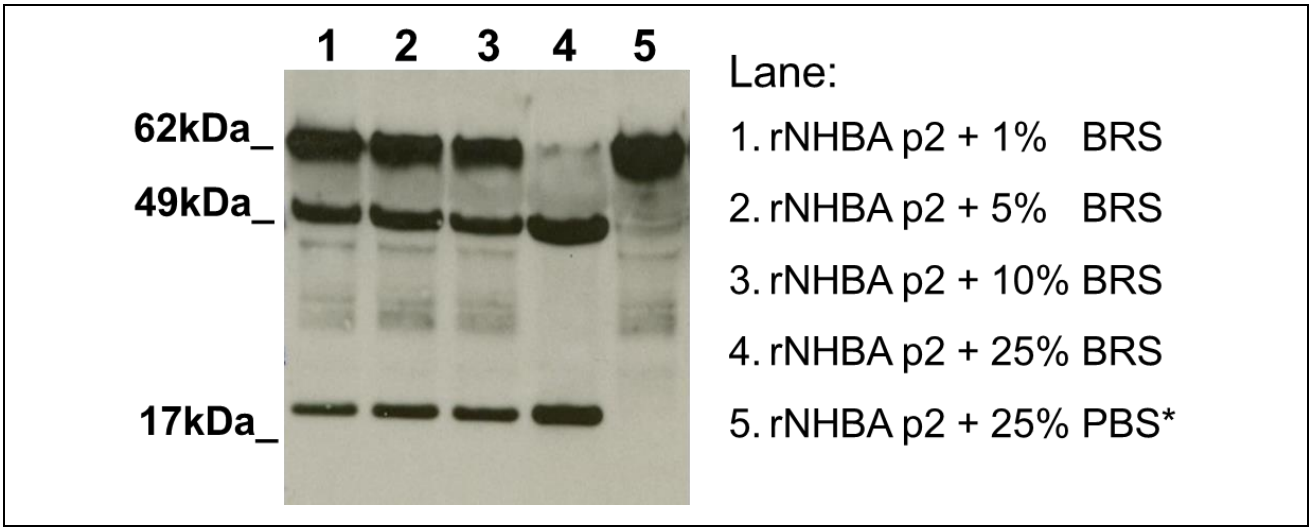


Figure 25. Western blot analysis of rNHBA incubated with a dose range of BRS. The band migrated at 62kDa corresponds to the full length NHBA. The bands that migrate at 49kDa and approximately 20kDa correspond to the N-term and the C-term of NHBA, respectively. rNHBA incubated in 25% PBS 1% BSA (*) was used to control specificity of BRS cleavage.

Following the results obtained with the recombinant protein we investigated the behavior of NHBA expressed in live bacteria in presence of baby rabbit serum. We incubated bacteria from M4407 strain (grown in SBA assay conditions) in 25% of BRS (vol/vol). In order to better understand the kinetics behind the proteolytic cleavage of NHBA, the reaction was incubated over a 1 hour time course experiment.

Western blot analysis showed that NHBA expressed on live bacteria was subject to processing by baby rabbit serum (Fig. 26). Cleavage kinetics is time dependent, but conversely from the recombinant protein, bacterial expressed NHBA was not totally cleaved when incubated with 25%

BRS. No NHBA cleavage was observed when bacteria or the recombinant protein were incubated with heat inactivated BRS (Hi BRS) at 56°C for 30 minutes (data not shown) or with PBS. This indicates that NHBA cleavage is probably mediated by serum proteases which are generally inactivated upon heat treatment at 56°C for 30 minutes.

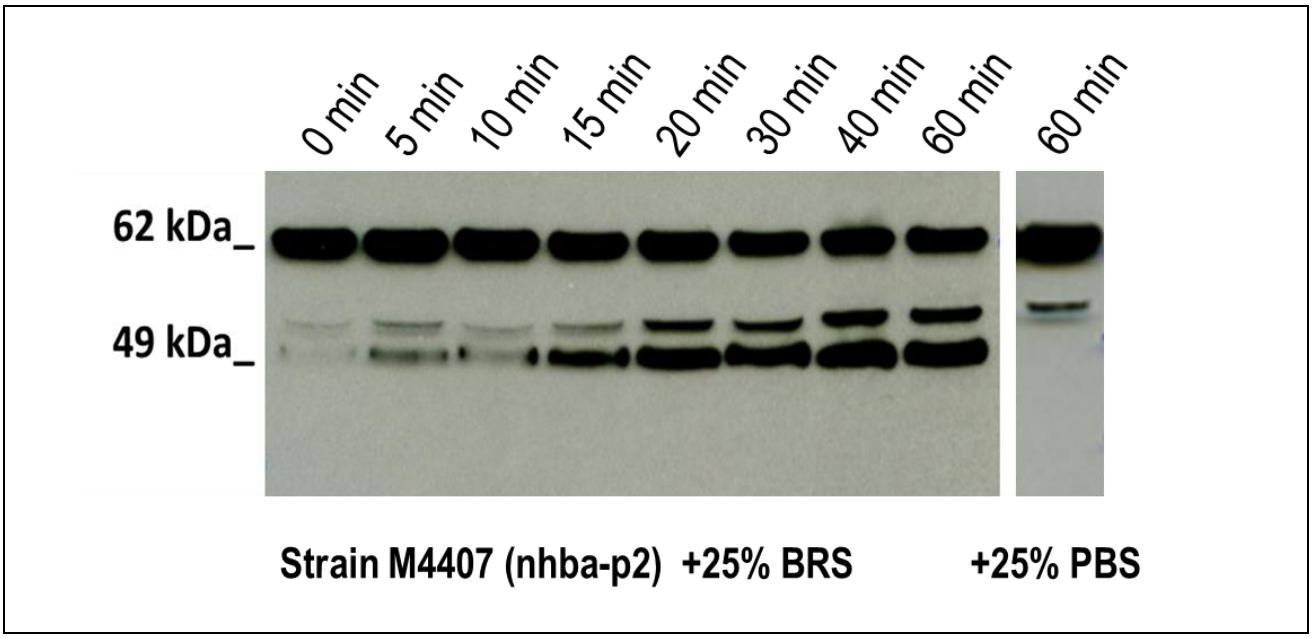
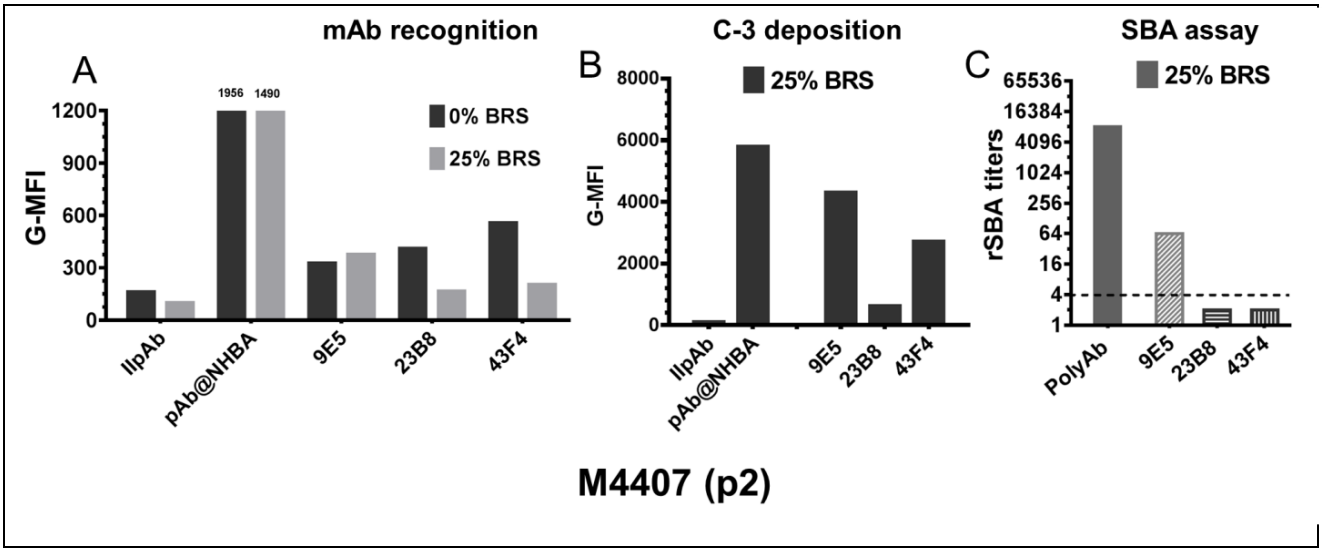


Figure 26 Characterization of NHBA cleavage by baby rabbit serum on live bacteria by western blot analysis. M4407 strain expressing NHBA p2 vaccine variant was incubated with 25% BRS in different time points. Incubation of M4407 with 25% PBS was used as control. For NHBA detection mouse anti-NHBA polyclonal antibody was used. NHBA full length protein corresponds to the 62 kDa band while the N-term to the 49kDa band.

4.3.3. Investigating the impact of serum proteases on NHBA recognition by monoclonal antibodies

From the western blot analysis performed on total protein extracts of M4407 strain we showed that NHBA is partially cleaved by serum proteases. We than investigated if this cleavage might have an impact on NHBA surface exposed recognition by mAbs targeting both the N-term and the C-term of NHBA. We used mAbs 9E5, 23B8 and 43F4, which were selected based on their epitope mapping and behavior in the rSBA assay. MAb 9E5 target the N-term of NHBA and was able to induce bacterial killing, while mAbs 23B8 and 43F4 target the C-term of NHBA and were rSBA negative. MAbs, individually, were incubated with bacteria in the presence and in the absence of 25% BRS for 20 minutes at 37°C and NHBA recognition in both experimental conditions was analyzed by flow cytometry. Results showed that no difference was observed in surface exposed NHBA recognition by mAb 9E5 independently from BRS presence. Conversely mAbs 23B8 and 43F4 showed more than 50% of signal reduction on NHBA recognition in presence of BRS (fig 27A).This result was expected since as previously observed by western blot analysis the C-term of NHBA was actively cleaved by serum proteases.

Figure 27. NHBA recognition by N-term mapping MAbs is not impacted by serum proteases cleavage and



correlates with their ability to individually induce comp*l*ement deposition and *bactericidal* activity. **A.** Individual mAb recognition of surface exposed NHBA in *the* presence of 25% BRS. **B.** Complement C-3 *(C3/C3b)* deposition mediated by anti NHBA monoclonal antibodies. **C.** SBA titers of monoclonal antibodies 9E5, 23B8 and 43F4 tested individually.G-MFI: geometric mean fluorescence intensity.

4.3.4. Investigating C3/C3b deposition of mAbs targeting the N-term and the C-term of NHBA

We previously showed that NHBA recognition of C-term mapping mAbs is impacted by NHBA cleavage mediated by serum proteases and this could impact the ability of mAbs to induce bacterial killing. To test this hypothesis we measured C3/C3b-deposition on M4407 bacterial surface mediated by each mAb. Deposition of the C3/C3b component is a crucial step of the classical complement cascade which leads to the membrane attack complex (MAC) formation that induces bacterial lysis. Flow cytometry results showed that the level of C3/C3b deposition is higher for mAb 9E5 with respect to levels induced by mAbs 23B8 and 43F4 individually (Fig.27.B). These data suggests that due to the C-term cleavage from serum proteases mAbs 23B8 and 43F4 are not able individually to induce an efficient C3/C3b deposition. The lack of effective C3/C3b deposition probably does not allow an efficient activation of the MAC complex and thus not able to induce bactericidal activity. C3/C3b deposition data are in agreement with rSBA of mAbs 23B8 and 43F4 previously reported as not able to induce bactericidal killing (Fig. 27C). NHBA cleavage by serum proteases does not impact 9E5 recognition which targets the N-term of the protein. This probably allows a higher C-3 deposition, leading to an efficient MAC formation that induces bacterial killing as previously observed (Fig. 27C)

4.3.5. The cooperative bactericidal activity induced by mAbs 9E5+43F4 combination is the result of higher C3/C3b deposition

In this study we previously showed that combination of mAb 9E5 and 43F4, targeting respectively the N-term and the C-term domains of NHBA, were able to increase rSBA titers when tested in combination. Moreover we showed that the positive rSBA titer of mAb 9E5 is mediated by higher C3/C3b deposition respect to SBA negative mAb 43F4. We used the same approach to investigate mAb recognition of NHBA exposed on bacterial surface but this time we tested mAbs alone and in combination. Moreover we tested in the same conditions their ability to induce C3/C3b deposition. In both experiments we used the M4407 natural MenB strain, which express NHBAp2 variant. Flow cytometry analysis revealed that a higher C3/C3b

deposition is observed when mAbs 9E5 and 43F4 are combined on the reaction mixture respect to mAbs tested individually (Fig 28B). rSBA titers of mAbs tested in combination against M4407 resulted in 6 fold higher (4096) respect to mAbs tested alone. In fact mAb 9E5 elicited low SBA titers (64) while mAb 43F4 had no bactericidal activity (Fig 28C). The significant 6 fold increase in rSBA titers indicates that mAbs 9E5 and 43F4 are able to induce a cooperative bactericidal activity when tested against M4407.

When we looked at the mAb recognition on the surface exposed NHBA in the presence and in the absence of 25% BRS we noted that recognition of 9E5+43F4 mAb combination was within the same range of mAb 9E5 tested individually (Fig 28A). Overall C3/C3b deposition and rSBA results of 9E5+43F4 mAb combination are concordant, but functional data are not supported by the recognition pattern of the mAb combination in the presence of 25% BRS

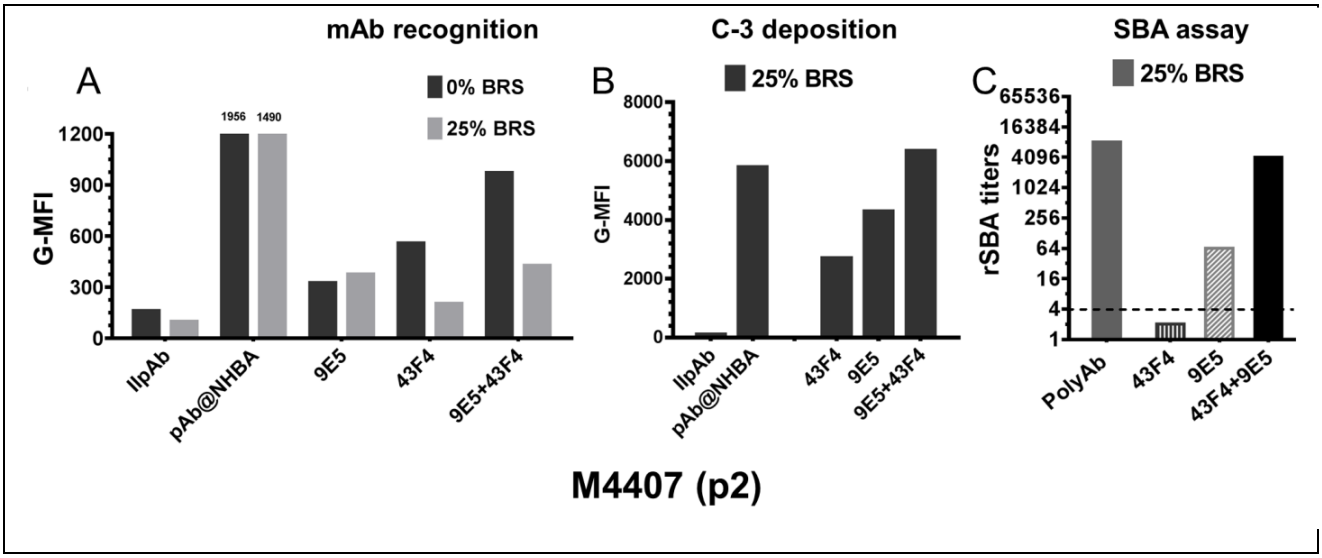


Figure 28 Combination of mAbs 9E5+43F4 induces a very efficient C-3 deposition leading to cooperative bactericidal activity. A. Recognition of surface exposed NHBA by flow cytometry of mAbs 9E5, 43F3 individyally and in combination in presence and absence of 25%BRS. B. Combination of mAbs induces a more efficient C-3 deposition. C. SBA titers of mAbs tested individually and in combination

4.3.6. Constitution of a stable ternary complex between 9E5, 43F4 and NHBA probably drives their higher cooperative bactericidal activity when tested in combination

Functional data were not supported by the recognition pattern of NHBA by 9E5+43F4 mAb combination in presence of 25% BRS. Recently Malito and collaborators reported that simultaneous binding to fHbp by two different antibodies targeting non overlapping epitopes form a very stable ternary complex, which promotes a multivalent high-avidity binding to C1q (Malito et al 2016). Complement component C1q is the first step of the initiation of the classical pathway cascade, which mediates the formation of the C3-convertase leading to C3/C3b deposition. These conditions are favorable to enable synergistic bactericidal activity (Malito et al 2016) Here we used a Surface Plasmon Resonance, similarly to the approach reported by Malito and colleagues, to test the hypothesis of a ternary complex formation between mAbs 9E5, 43F4 and rNHBAp2. Our experimental design was slightly different from the one described by Malito and collaborators. We decided to immobilize monoclonal antibodies on the chip and not the rNHBAp2 in order not to alter epitope exposition or to increase avidity of mAbs for the antigen. In this way rNHBA will be captured by the mAb immobilized on the chip and the second mAb will be injected on the detection cell. We used an anti-Fhbp mAb to control rNHBAp2 capture specificity on the chip. The experiment was performed in both orders, with mAb 43F4 immobilized on the chip that captures injected rNHBA, followed by successive injection of mAb 9E5 (Fig. 29A) and vice versa (Fig. 29B). The formation of a highly stable ternary complex was observed when rNHBA was captured on mAb 43F4. When NHBA was captured on mAb 9E5 immobilized on the chip, low efficiency of the ternary complex formation was observed due to the high dissociation rate of mAb 9E5 towards rNHBA. These results suggests that binding of mAb 43F4, which binds rNHBA with high affinity and has a very low dissociation rete, increases the binding stability of mAb 9E5 allowing the formation of a very stable ternary complex. We can though hypothesize that binding of mAb 43F4 can induce allosteric changes on rNHBA that potentiates binding capability of mAb 9E5 and the formation of a very stable ternary complex. Additional studies are required to test this hypothesis. Overall these results bring a comprehensive explanation of the cooperative bactericidal activity elicited by 9E5+43F4 mAb combination.

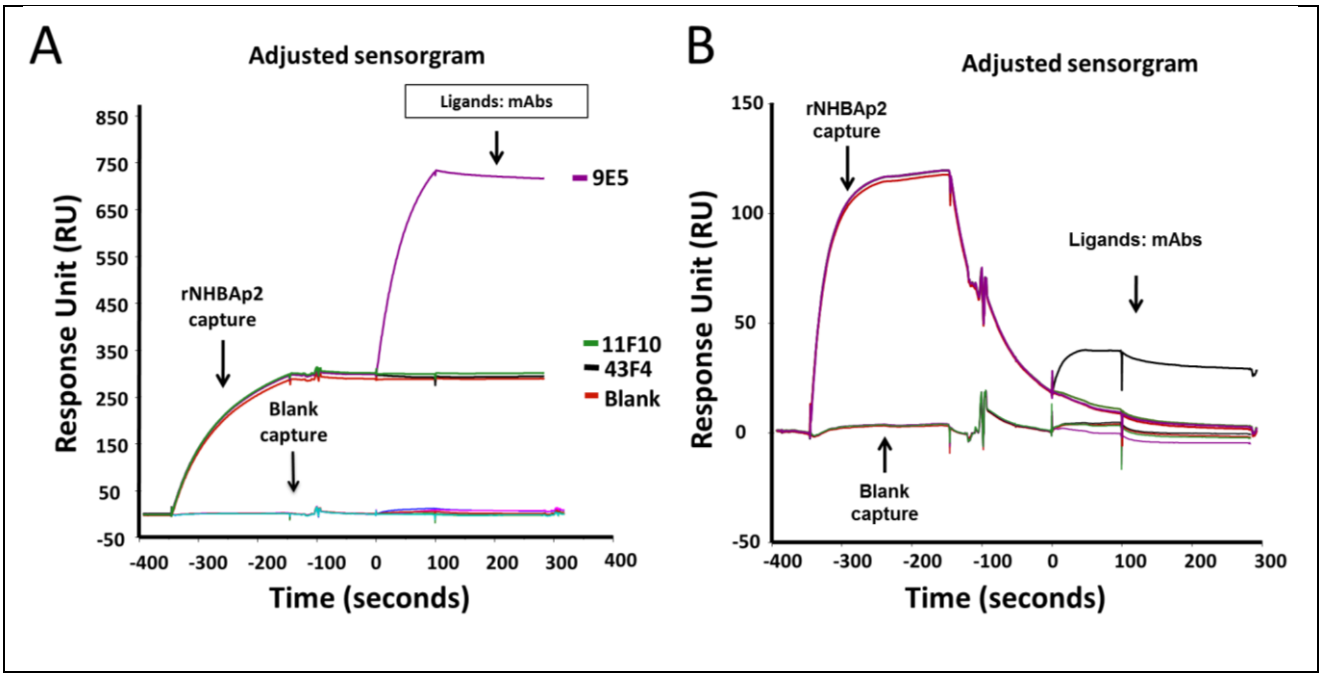


Figure 29 . MAb 43F4 and mAb 9E5 are capable to bind the same NHBA molecule forming a stable ternary complex . **A.** rNHBA p2 is captured by mAb 43F4 (black line) that is immobilized on the chip. mAb 9E5, successively injected is able to bind the same rNHBA p2 molecule. two negative controls were used: (i) a second injection of mAb 43F4 revealed that binding of rNHBA p2 is specific to mAb43F4 and no a-specific binding of the protein, on the chip, was observe; (ii) injection of an fHbp specific mAb, 11F10 (green line), revealed that binding of mAb 23B8 is specific only to NHBAp2 that is captured by mAb 43F4. **B.** the same experimental setting was used on a second experiment but in this case mAb 9E5 was immobilized on the chip to capture the rNHBA p2 protein. Successively mAb 43F4 was injected.

4.3.7. Ternary complex formation between C-term mapping mAbs and NHBA drives efficient complement deposition and results in cooperative bactericidal activity

MAbs 23B8 and 43F4, which target different epitopes on the well conserved C-term domain of NHBA, were not able to induce bactericidal killing when tested individually both against natural (M4407) and recombinant (5/99ΔΔp2) strains. When these mAbs were tested in combination very high rSBA titers were observed and moreover these mAbs were able to induce cross protection among natural and recombinant isogenic strains expressing a panel of epidemiologic relevant NHBA variants. Since NalP is phase off in M4407, our hypothesis is that serum proteases cleavage of the C-term by bacterial surface is probably responsible for their lack of bactericidal killing when tested individually. To test this hypothesis we investigated by flow cytometry their recognition pattern on surface exposed NHBA in the presence and in the absence of active BRS. Flow cytometry analysis showed that both mAbs individually and in combination show more than 50% of signal reduction in presence of BRS (fig 31 A). Moreover we performed C3/C3b deposition analysis of mAbs individually and in combination. These experiment showed that a tremendously high C3/C3b deposition signal where observed when mAbs 23B8+43F4 were tested in combination (fig.31 B), whereas for mAbs tested individually low C3/C3b deposition was observed. 23B8+43F4 mAb combination shows that high levels of C3/C3b deposition correspond to high bactericidal titers (fig 31C). Nevertheless complement deposition data as well as rSBA data are not supported by NHBA recognition pattern of mAbs in combination on M4407 MenB strain. Since HDx analysis, previously reported, showed that mAbs 23B8 and 43F recognize two different epitopes on the well conserved C-terminal domain of NHBA (fig 32.A) we used the SPR approach to investigate if mAbs 23B8 and 43F4 are able to bind simultaneously rNHBA. Results show that mAb 23B8 (blue line) binds rNHBAp2 captured by mAb 43F4 immobilized on the CM5 chip (fig. 32 C). These results indicate that mAbs 23B8 and 43F4 can simultaneously bind rNHBAp2 forming a ternary complex. The *plateau* tract of mAb 23B8 binding signal indicates that the complex is very stable. The experiment was repeated but this time rNHBAp2 was captured by mAb 23B8 immobilized on the chip and injection of mAb 43F4 was performed to test ternary complex formation. Similar results were obtained even though complex stability was lower respect to the previous experiment (fig 32D).

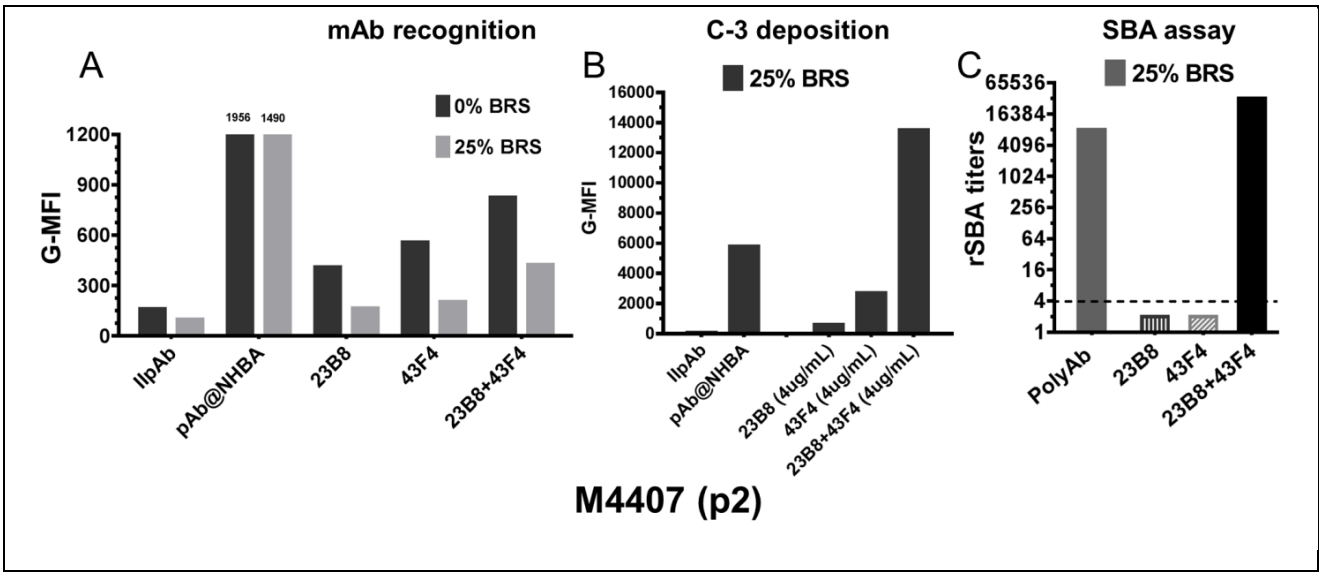


Figure 30. Combination of mAbs 9E5+43F4 induces a very efficient C-3 deposition leading to cooperative bactericidal activity. A. Recognition of surface exposed NHBA by flow cytometry of mAbs 9E5, 43F3 individyally and in combination in presence and absence of 25%BRS. B. Combination of mAbs induces a more efficient C-3 deposition. C. SBA titers of mAbs tested individually and in combination

Ability of mAbs to bind simultaneously on the same NHBA molecule was tested as well using the recombinant C2 NHBA fragment (Serruto et, al 2010, Casellato et al 2013 and Vacca et al 2016). Results were very similar respect to the data obtained with the full length rNHBAp2 variant (data not showed).

These results indicate that the ability to form a stable ternary complex drives the complement mediated cooperative bactericidal activity induced by 23B8+43F4 mAb combination.

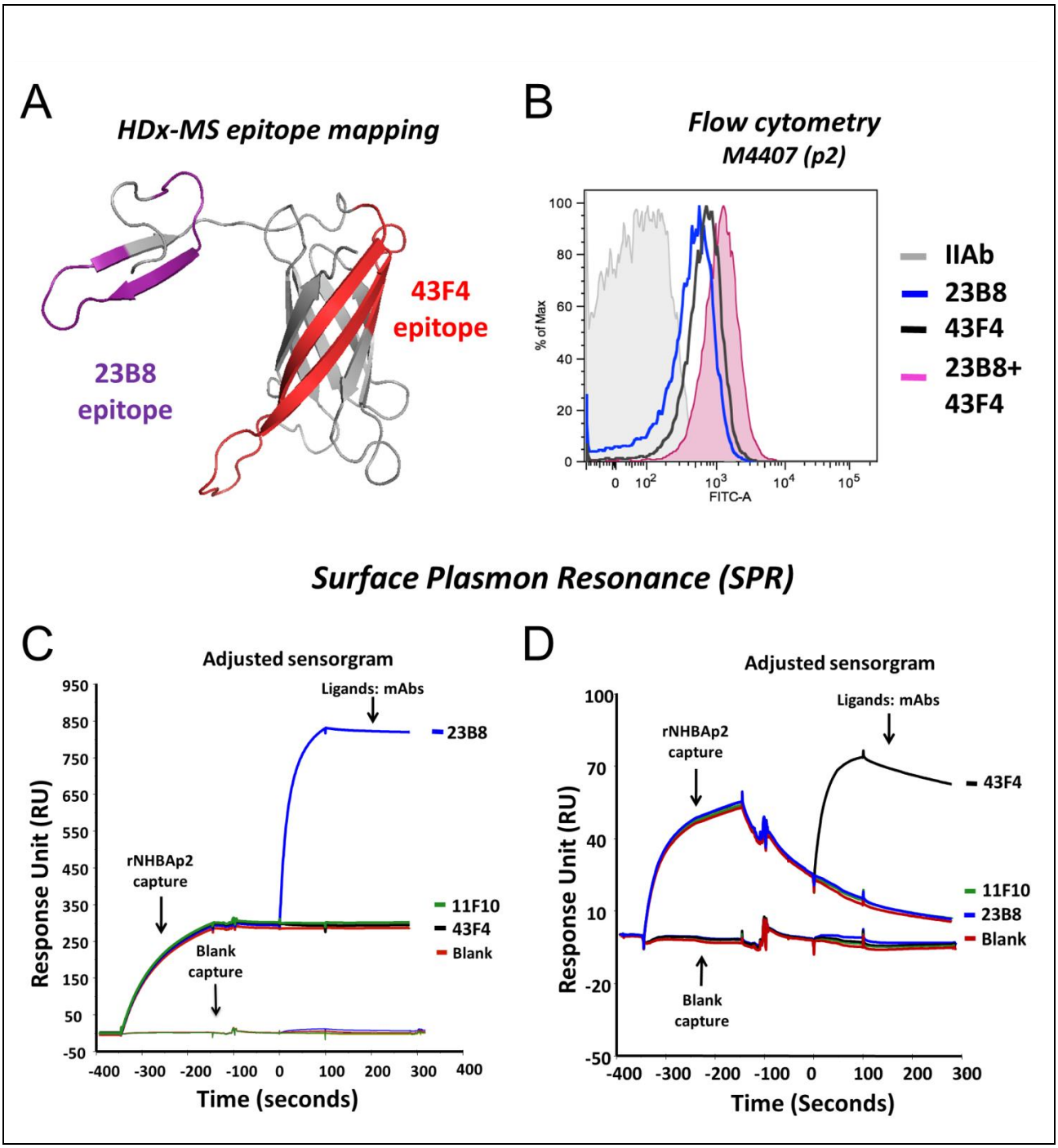


Figure 31. **MAbs 23B8 and 43F4 are able to bind on the same NHBA molecule generating a stable trimeric complex**. A. Epitopes targeted by 23B8 and 43F4 displayed together on the 3D structure of the C-term domain of NHBA. B. Recognition of surface exposed NHBA by mAbs alone and in combination. In C and D are reported sensograms of SPR experiments showing the simultaneous binding of rNHBAp2 by both mAbs. Blue line shows binding of mAb 23B8, black line binding of mAb 43F4. Green line reports that mAb 11F10 that is specific to fHbp used as negative control, did not bind on the captured NHBA. Red line illustrates that no binding signal is observed when blank solution (HBS-EP buffer) is injected on the captured NHBA. SPR data are representative of a triplicate experiment.

4.3.8. Discussion and conclusions

The spatial orientation and antigen density on the surface of a bacterium can be important in determining whether IgG directed against that antigen can support C1q binding and complement activation (Michaelsen TE, et al 1991). Failure of an individual mAb to activate complement-mediated bacteriolysis could result if the distance between most of the surface exposed antigen molecules on the bacterial surface exceeded that required for optimal C1q engagement by antibody binding. In contrast, binding of 2 antibodies could be sufficient for adjacent Fc domains to engage C1q and activate bactericidal activity by the classical complement pathway, as long as the 2 antibodies recognized appropriately spaced non-overlapping epitopes (Welsch et al 2008).

In this study we showed that mAbs targeting the N-term of NHBA were able to individually induce bactericidal killing against serogroup B strains expressing different levels of NHBA. On the contrary mAbs targeting the C-term of the protein were not able to induce bactericidal killing individually but only paired with other N-term or C-term mapping mAbs.

By confocal microscopy we showed that in both M4407 and 5/99ΔΔp2 strains during early-log phase, NHBA is sparsely distributed and organized in clusters on bacterial surface. Overall a good signal was obtained using C-term mAbs and polyclonal antibodies for confocal analysis and these results are concordant with flow cytometry previously showed. It has been described elsewhere that NHBA is processed by different proteases (Serruto, et al 2010, Pantano. E., 2016, Di Fede M.,2017), so we investigated the behavior of NHBA in presence of rabbit serum (BRS). The full length recombinant protein was seen to be totally cleaved when incubated with 25% BRS generating a unique intense lower band around 49kDa. Successively we showed that NHBA cleavage on live bacteria has a different behavior. On M4407 strain, NHBA cleavage occurs since the first minutes after incubation and at 15 minutes the 49kDa band indicating the N-term of the protein becomes more evident. NHBA cleavage becomes more pronounced in the successive time points but it isn't fully cleaved after 1 hour incubation. Since our aim is to understand NHBA behavior during the SBA assay we didn't investigate NHBA cleavage at longer time points.

Our hypothesis is that serum incubation of bacteria induces a strong proteolytic activity of NHBA, but the protein is continuously produced by bacteria since the full length protein is always detectable on western blot analysis. This hypothesis is in accordance with very recent data where is reported that incubation of meningococci with different serum sources (human, mouse and

rabbit) induces strong NHBA cleavage and simultaneously *nhba* transcription activation which results in increased NHBA protein level (Borghi S.,2017). Serum proteases such as C3B-convertase or kallikrein have been reported to cleave NHBA (Pantano E., 2016, Di Fede M.,2017) so it's not surprising to observe an increase in C-term cleavage. However the actors involved in NHBA cleavage and up-regulation remains still to be identified (Borghi S.,2017). These results could partially explain why N-term mAbs are able to induce NHBA complement bactericidal activity when tested individually and C-term mAbs aren't.

We showed that C-term mAbs, 23B8 and 43F4 show a 50% decrease in NHBA surface recognition in presence of 25% BRS, while N-term mAb 9E5 recognition is not impacted by BRS presence. This reflects the higher C3/C3b complement component deposition mediated by mAb 9E5 and its bactericidal activity. Our results suggest that the removal of the C-term domain from the bacterial surface does not allow effective C3/C3b deposition mediated by mAbs 23B8 and 43F4 leading to inefficient activation of the terminal complement cascade. Therefore, the susceptibility to cleavage by serum components/proteases might belong to a meningococcal strategy of immune evasion.

We identified by HDx that mAbs 23B8 and 43F4 target two closely but not overlapping epitopes located on the C-term domain of NHBA. Moreover we demonstrated through SPR approach that mAbs 23B8 and 43F4 are able to bind simultaneously the same NHBA molecule. The resultant ternary complex shows a high stability. Furthermore C3/C3b deposition of mAbs tested in combination was much higher respect to mAbs tested individually. No aspecific complement deposition was observed in control experiments.

These observations led us to formulate the hypothesis that although NHBA is partially cleaved on bacterial surface during SBA assay, simultaneous binding of 23B8 and 43F4 to the same NHBA molecule, generates a highly stable ternary complex which allows efficient C3/C3b deposition and terminal complement cascade activation leading to bacteriolysis via pore formation. These data are in accordance with the cooperative bactericidal activity induced by 23B8+43F4 mAbs previously reported in this study.

Finally we showed that also mAbs 9E5 and 43F4, targeting the N-term and the C-term respectively, can simultaneously bind the NHBA protein. Interestingly 9E5+43F4 mAb combination showed similar NHBA recognition as mAb 9E5 tested alone, in the presence of BRS by flow cytometry analysis. On the other hand C-3 deposition of mAb combination was much higher respect to mAbs 9E5 and 43F4 tested individually. These results are in agreement with the cooperative bactericidal

activity observed when 9E5 and 43F4 mAbs are tested in the rSBA assay in combination against M4407 strain. Similar results has been recently reported by Malito and collaborators, where two mAbs anti fHbp were able to bind on two non-overlapping epitopes generating a stable ternary complex and inducing co-operative complement mediated bacteriolysis (Malito et al 2016).

From a structural point of view it is interesting that, from the SPR experiments was showed that binding of mAb 43F4 is probably responsible of inducing allosteric modifications that allows a more efficient binding of the second member of the mAb combinations. This was observed in the combination with mAb 23B8 and was even more evident in the combination with mAb 9E5 but further investigation is needed to fully characterize this observation.

The data presented in this study provide a detailed characterization of the anti-NHBA panel of monoclonal antibodies and helps to elucidate the functional and structural basis for the synergistic complement-mediated bactericidal activity induced by co-operative mAbs targeting the same NHBA molecule. Collectively this work suggests that in the context of a polyclonal response (such as the one elicited following vaccination) we expect to have antibodies such as 9E5, 23B8 and 43F4, so generally we don’t expect that cleavage could have a negative impact on NHBA-mediated bactericidal activity

5. MATERIALS AND METHODS

Monoclonal antibodies isolation

The mAbs used in this work were produced by Takis S.r.l. (Rome, Italy) by conventional hybridoma techniques from the spleen cells of immunized mice with full length molecule, and NHBA derived fragments in different formulations. Procedures involving mice were conducted in accordance to the guidelines of the European Union for the handling of laboratory animals. mAbs were purified from culture supernatants by Protein G affinity columns (Thermo). The mAb IgG subtype was determined using a mouse mAb isotyping kit (Thermo). An overview of antibody production is reported in below.

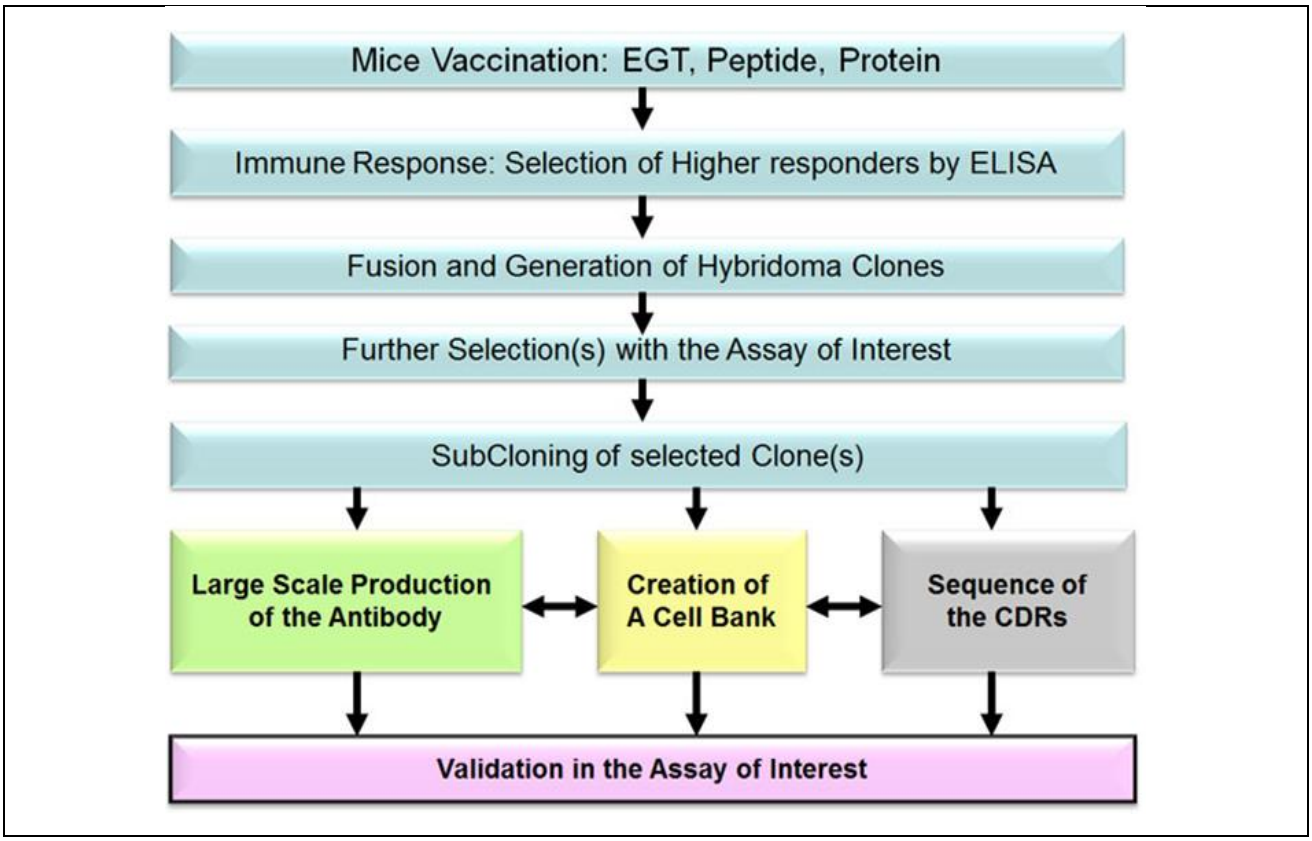


Figure 32. Murine monoclonal antibodies production workflow via standard hybridoma technology.

Bacterial strains used in this study

Natural MenB strains	Recombinant MenB strains	Knck-out strains
M4407	5/99ΔnadaΔnhba:p2	MC58Δnalp
MC58	5/99ΔnadaΔnhba:p3	5/99ΔnadaΔnhba
M2934	5/99ΔnadaΔnhba:p5	
M12923	5/99ΔnadaΔnhba:p10	
UK 355	5/99ΔnadaΔnhba:p18	
NM117	5/99ΔnadaΔnhba:p21	
M16686	5/99ΔnadaΔnhba:p29	

Surface plasmon resonance (SPR)

Binding of purified mAbs to the vaccine recombinant NHBA peptide 2 (rNHBAp2) variant was assessed by surface plasmon resonance (SPR) analysis. Briefly anti-mouse IgG Fc-specific antibodies are covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip (GE Healthcare Life Sciences). Following mAb (ligand) capture by anti-mouse IgG on the sensor chip surface, rNHBAp2 (analyte) was injected on the system. The ligand-analyte association (K_{on}) is described by ascendant tract of the curve (60 seconds, 30uL/min). On the other hand ligand-analyte dissociation (K_{off}) is described by the descendant tract of the curve during buffer injection on the system (240-300 seconds of injection time).

Single-cycle kinetics (SCK) experiments were performed using a commercially available Capture Kit (GE Healthcare) used to immobilize antibodies directed against FC regions of mouse IgG molecules, by amine coupling on a carboxymethylated dextran sensor chip (CM5; GE Healthcare). K_D and kinetic parameters were calculated by performing a titration series of five consecutive injections of purified rNHBAp2 diluted in HBS-EP at increasing concentration followed by a single final surface regeneration step with buffer containing 10 mM glycine pH 2.1 (180 s; 10 µL/min). All sensorgrams were corrected for nonspecific binding with readings from a protein-free reference flow cell. Data were analyzed using the standard SCK method (Karlsson, Katsamba et al. 2006) implemented by the Biacore T200 evaluation software (GE Healthcare). K_D values reported in this study are representative of a duplicate experiment.

We used Biacore T200 instrument to assess monoclonal antibodies ability to bind simultaneously on the recombinant full length or C-terminal doamain of NHBAp2 vaccine antigen. In this way we wanted to investigate biochemically the cooperativity of murine mAbs couple 23B8+43F4 that were seen to induce bacterial killing only when tester in combination (Abdiche, Miles et al. 2014). Moreover we used the same approach to investigate synergy of mAbs 9E5+43F4 in which an increase in rSBA titers were observed. In this experimental setting one of the mAbs is immobilized on the chip in order to capture NHBA, then the second member of the couple is injected in a sandwich based approach.

Flow cytometry analysis

Bacteria from liquid cultures grown until OD_{600} of0,25 or 0,5 were pelleted, washed with PBS 1% BSA and sequentially incubated with monoclonal antibodies alone or in combination (4ug/mL final concentration) or with mouse anti-NHBA polyclonal antibody (1:512- 1:1000 final dilution) and incubated for 1 hour at room temperature (RT). After two wash steps rabbit anti-mouse IgG Alexa Fluor 488 conjugated (Life Tecnologies) secondary antibody (1:1000) was added to bacteria and incubated for 45 minutes RT . After the secondary antibody incubation bacteria were washed fixed with paraformaldehyde and plated to check bacterial inactivation. Samples were analyzed wirth Canto II cell counter and histograms generated using Flowjo 8.60 software.

Protein chip and Peptide array assay

The protocol used is similar for both protein chip and peptide array, and described hereafter: Following 1h slide blocking at RT with microarray blocking buffer each mAb (diluted in blocking 1:1000) was incubated for 1 h at RT in dark humid chambers. After three PBST (0.1% Tween20 in PBS) washes, slides were incubated with goat-anti-mouse AlexaFluor®647-conjugated secondary antibody (Jackson Immunoresearch,) for 1 h and then washed. Image acquisition was performed using a Powerscanner (Tecan).

Peptidic map generation and Hydrogen Deuterium Exchange Mass Spectrometry (HDx-MS) analysis

NHBA peptidic map generation. To generate and separate peptides covering the full length protein in order to monitor the deuterium uptake in the entire sequence when the antigen is coupled with mAb. The NHBA p2 variant (54 pmol) was treated with 2 M guanidinium chloride for 1 hour at 60°C and subsequently injected in the nanoACQUITY ultra-performance liquid chromatography system for the digestion, that was performed for 2.5 minutes at 20°C online with a pepsin column (Poroszyme® Immobilized Pepsin Cartridge). The peptides generated were trapped, concentrated and desalted using a pre-column (VanGuard BEH 1.7 µm, 2.1x5 mm) and separated using a AQUITY UPLC BEH C18 reverse phase column, 1.7 µm, 1.0x100mm (Waters) with a linear gradient from 15 to 45% of acetonitrile/water, 0.1% formic acid over 6.8 min at 40 µl/min. The mass spectra were acquired in a resolution mode (m/z 100-2000) on a Waters SynaptG2 mass spectrometer equipped with a standard ESI source. Mass accuracy was ensured by continuously infusing a GFP solution (600 fmol/µL in 50% acetonitrile, 0.1% formic acid) through the reference probe of the ESI source. The identity of each peptide was confirmed by MS^E analyses. MS^E was directly performed by a succession of low (6 V) and high collision (25 V) energies in the transfer region of the mass spectrometer. All fragmentations were performed using argon as collision gas. Data were processed using Protein Lynx Global Server 3.0 (Waters) and each fragmentation spectrum was manually inspected to confirm the assignment. The DynamX 3.0 software (Waters) was used to select the peptides considered. Only the peptic peptides present in at least four out of five repeated digestions of NHBA were included for the analysis

HDx epitope mapping. The antigen/mAb complex was formed by adding 324 pmol rNHBAp2 vaccine antigen to the mAb, using a molar ratio of 1:1 and incubated for 30 min at room temperature. For the labeling deuterated buffer (PBS with a pH of 7.4) was added to the mAb/antigen complex in order to reach around 80% deuterium excess. 30 uL of the sample was removed and quenched with 30 uL of 200 mM sodium phosphate buffer added with 4 M guanidinium chloride (PBS-GnCl) ice-cold. Incubation with PBS-GnCl dissociate the antigen/mAb complex and to lower the pH to 2.4. In parallel, a control experiment without mAb was prepared using same conditions previously described (PBS was used instead of the antibody preparation). All quenched samples were immediately flash frozen in liquid nitrogen and stored for <24h at −80°C. The DynamX 3.0 software (Waters) was used to select peptides considered for the analysis

and to extract the centroid mass of each of them, and for each charge state, as a function of the labelling time. For each mAb the epitope was confirmed by at least two independent experiments.

Serum bactericidal activity (rSBA)

Serum bactericidal activity against *N. meningitidis* strains was evaluated as reported elsewhere (Giuliani, Santini et al. 2005). Bacteria grown until early log phase (OD_{600} of ~0.25) were diluted in Dulbecco's Phosphate Buffered Saline (DPBS) containing 1% bovine serum albumin (BSA) and 0.1% glucose at the working dilution of 10^4-10^5 and incubated with serial two fold dilutions of monoclonal antibodies tested alone or in combination starting from a concentration of 250µg/ml or mouse anti-NHBA polyclonal antibody diluted 1:250. Serum bactericidal titers were defined as the serum dilution resulting in 50% decrease in colony-forming units per milliliter after a 60 min incubation of bacteria with the reaction mixture compared to the control CFU/ml at time zero. Pooled baby rabbit sera (Cedarlane) have been used as a complement source (rSBA).
Criteria for evaluation of bactericidal titers of mAbs alone and in combination:

1. We assigned the lower SBA titer measured in the duplicate experiments when the result between the two experiments was different.(i.e mAb 10E8 Exp.1= 4096 Exp2= 2048, titer assigned 2048)
2. since range @95% of the ratio between SBA titers is <3 dilutions, we define a significant increase in bactericidal activity (or synergistic effect) when SBA titer of the mAb combination is ≥3 dilutions than SBA titers of mAbs tested individually. (i.e mAb 23B8 SBA titer ≤4; mAb 3A3 SBA titer 2048; 23B8+3A3 SBA titer 16384)
3. cooperative bactericidal activity is considered when when mAbs that individually show low or no bactericidal activity become bactericidal when coupled together as described by Beernink et al 2008.

Western blot

In the case of liquid cultures, strains were grown until OD_{600} of ~0.25 or 0,5 and one milliliter of the culture was pelleted and resuspended in 50 µL or 100 µL, respectively, of SDS loading buffer. Recombinant proteins or total protein extracts were separated by SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels in MES 1X (Life Technologies) and then transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C with PBS + 0.05% Tween 20 (Sigma) and

10% powdered milk (Sigma). After blocking membranes were incubated in with monoclonal antibody individually (0,8ug/mL), in combination (1:1000) and rabbit or mouse anti-NHBAp2 polyclonal antibody (1:2000) diluted in PBS + 0.05% Tween 20 (Sigma) and 3% powdered milk (Sigma) and incubated for 1 hour at room temperature. An anti-mouse or anti-rabbit antiserum conjugated to horseradish peroxidase (Dako) was used as secondary antibody. Bands were visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's instructions.

Recombinant and M4407 surface exposed NHBA cleavage assay

To investigate BRS mediated cleavage, 1 µg of rNHBAp2 were incubated in 1%, 5%, 12,5% and 25% BRS (vol/vol) and in 25% PBS1X 1%BSA as negative control in 100uL reaction volume. After 1 hour incubatio at 37°C, 55uL of LDS buffer and reducing agent mix was added and all samples incubated at 100°C for 10 minutes to inactivate the reaction.

BRS cleavage on surface exposed NHBA on live bacteria (strain M4407) was performed resuspending 1mL washed bacterial pellet (OD_{600} of ~0.25) in 25% BRS diluted in PBS 1% BSA (vol/vol) and incubated in a time course experiment for a maximum of 1 hour of incubation. For each time point a control sample incubated with 25% PBS 1% BSA was prepared. After the incubation bacteria were pelleted and resuspeneder in 55uL SDS buffer and inactivated incubating at 100°C for 10 minutes.

Both recombinant protein and bacteria derived samples were than analyzed by western blot analysis.

To investigate NHBA cleavage in presence of 25% BRS by flow cytometry 3mL of bacteria (OD_{600} of ~0.25-0,3) were pelleted and resuspended in 512 uL PBS 1% BSA. Than 12.5uL of BRS were added to final volume of 50uL containing mAbs alone and in combination at a final concentration of 4ug/mL. For each sample tested a negative control with no BRS or with25% of heat inactivated BRS was used. Samples were incubated foe 20 minutes at 37°C 5%CO2 in mild shaking. After washing rabbit anti-mouse IgG Alexa Fluor 488 conjugated (Life Tecnologies) secondary antibody (1:1000) was added to bacteria and incubated for 45 minutes RT . After the secondary antibody incubation bacteria were washed fixed with paraformaldehyde and plated to check bacterial

inactivation. Samples were analyzed wirth Canto II cell counter and histograms generated using Flowjo 8.60 software

Confocal microscopy analysis

2 mL of bacterial growth at OD_{600} of ~0.25 were washed and resuspended on 200uLof PBS 1% BSA. 20uL of bacterial suspension, were used per each staining condition. Staining was performed on the slide with 150uL of monoclonal antibodies alone and in combination diluted 4ug/uL or mouse polyclonal anti-287-953 diluted 1:512 and incubated for 45min at RT. After several wash steps slides were incubated in 150ul of rabbit anti-mouse antibody Alexa fluor 488 conjugated (Life technologies), diluted 1:1000 for 30 minutes at RT. Samples were dried with vacuum, mounted using ProLong gold antifade reagent with diamidino-2-phenylindole (DAPI; Invitrogen) and analyzed by confocal microscopy using a Zeiss LSM 710 confocal microscope

Antibody mediated complement C-3 deposition assay

C3 complement component deposition was performed as previously described in Ercoli G. et al 2015. Bacteria in exponential phase were incubated with mAbs alone and in combination (4 ug/mL) and 25% baby rabbit serum, as a source of complement for 20 minutes at 37°C in agitation. The capacity of specific sera to promote C3 deposition was revealed using anti-human C3 polyclonal antibody and goat anti-Rabbit Secondary Antibody, Alexa Fluor® 488 conjugate. Stained bacteria were analyzed with a Canto II flow cytometer (Beckton-Dickinson) by using Flowjo software. Results are displayed in histograms reporting geometric mean fluorescence intensity (G-MFI).

Phylogenetic analysis

For phylogenetic and multiple alignment analysis Splits tree, Clustal and JalView softwares were used.

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