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Synergic complement-mediated bactericidal activity of monoclonal antibodies with distinct specificity

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Introduction

Definition of synergy in biology

In biology, synergy is defined as "*the interaction of biological structures or substances that produce an overall effect which is greater than the sum of the individual effects of any of them*" (0). Various examples of synergy exist in nature, including the emergence of biochemical properties in mixed microbial communities which are not found in clonal monocultures (2), bacterial metabolites that have a greater antibiotic activity against microbial competitors when used in combination compared to the sum of their individual antibiotic activites (3), and multiple transcription factors acting synergistically in the activation of mammalian genes transcription (4).
In pharmacology, the synergistic effect of combinations of different drugs has been exploited for instance to treat HIV infection with increased efficacy compared to monovalent therapies (5).

Synergic interactions between antibodies: state of art and proposed mechanisms

Synergic interactions between antibodies have been reported as early as the 1980s. Antibodies are able to interact and trigger a synergistic biological effect thanks to a number of different molecular mechanisms. In the first antibody synergy studies, it was observed that antibodies could synergize thanks either to mechanisms triggered by interactions with the antigen (6) or to mechanisms triggered by the interactions between the Fc tails of distinct antibody molecules (7).

Synergic antigen-binding

Synergistic antigen-binding interactions between murine Monoclonal antibodies (mAbs) were the first mechanisms observed. A number of scientific studies reported that antibodies could bind to their specific antigen and cause enhanced binding of second antibodies to the same antigen (6, 8). The mechanism that was proposed to explain this enhancement was that simultaneous binding by a single antibody to two antigen molecules could bring them in spatial proximity, increasing the probability that a second mAb could bind simultaneously both of the antigens forming a stable tetrameric complex. An

alternative mechanism proposed to explain this synergistic affinity enhancement postulates that a conformational change is induced in the antigen following the binding to the first antibody, resulting a better presentation of the epitope to the second antibody, which leads to an increase of affinity of the second antibody (9, 10, 11).

Synergic functional properties

Antibodies can act synergically also enhancing their functional properties. Synergistic neutralizing activity were observed in particular in the case combinations of antibodies directed against toxins including the tetanus neurotoxin (12), *Bacillus anthracis* Lethal Factor and Protective Antigen (13, 14), and Botulinum neurotoxins (15). Synergistic neutralizing mAb activity was also observed against viral antigens, including Human Immunodeficency Virus (HIV) envelope glycoproteins gp41 and gp120 (16; 17; 18), Severe Acute Respiratory Syndrome (SARS) Coronavirus antigens (19), Hepatitis C Virus (HCV) antigens (20), and Ebolavirus GP glycan cap (21). Furthermore, synergic mAb couples neutralizing a cytokine, Tumor Necrosis Factor (TNF), were reported (22). Recently, a synergic effect has been also reported between antibodies targeting tumor antigens. It has been observed that antibodies traditionally used as diagnostic tools to evaluate tumor progression were able to suppress tumor growth when used in combination (23; 24; 25).

Different mechanisms have been proposed as responsible for such neutralizing synergy (15). In particular:

1) Multiple antibodies bind simultaneously to different functional sites of the antigen, inhibiting strongly its functional activity
2) Binding of a first antibody to the antigen induces formation of antigen-antibody bridges that cause an increase in affinity of the mAb couple for the antigen, compared with each single mAb.

Antibodies have been reported to be able to cooperate thanks to interactions between the crystallizable fragment (Fc) regions, upon binding to multivalent antigens (7; 26; 27). The work of Diebolder et al. (28) provided insights about the correlation between Fc-Fc interactions and synergistic activation of effector functions, explaining that when multiple

antibodies bind to the respective antigens on the surface of a membrane Fc-Fc interactions promote the formation of ordered IgG hexamers providing optimal conditions for C1q engagement and complement activation.

Cooperative C1q recruitment and complement activation: a recently identified antibody synergy molecular mechanism

Our understanding of the mechanisms responsible for antibody synergy has been greatly improved thanks to recent studies on C1q recruitment. C1q is an hexameric glycoprotein of 460 kDa consisting of 18 peptide chains organized in 6 subunits of 3 chains. Each chain contains a collagen-like fiber near the N-terminus and a C-terminal globular domain which mediates the functional interaction with Fc portions of antibodies (29). Following Fc binding, a structural rearrangement of C1q leads to the activation of the C1 complex, the first step of the classical pathway (30, 31, 32, 33).

Several studies have reported that antibodies, provided that their Fc regions are in proximity, can interact with C1q in a cooperative manner that triggers a synergistic complement activation, which leads to synergistic deposition of the complement proteins C3 (34), and C4b (35) on the target cell, resulting in turn to a synergistic activation of MAC perforin complex and bacterial killing (36; 35; 37; 38; 39, 40). C1q is associated in the internal side of its stems with two serine proteases, C1r and C1s, forming a calcium-dependent tetramer called $C1r_2s_2$ or C1 complex (32). Association of C1q to its targets causes an activation of the C1 complex through C1r that cleaves C1s, initiating the downstream reactions of the complement cascade. The current hypothesis to explain cooperative antibody-mediated C1q recruitment postulates that interaction of C1q with one single antibody molecule is characterized by a very low affinity (28). In these conditions there is little or no outward movement of the C1q stems from the original antibody-free conformation. When C1q interacts with multiple Fcs closely clustered in the space (i.e. on a surface with high antigen density, or when multiple antibodies bind simultaneously to one single antigen with the proper spatial orientation) the stems interact with high affinity with the Fcs and move outward. Such conformational change results in the activation of C1rs subunits which in turn activate the whole C1 complex (Fig.1).

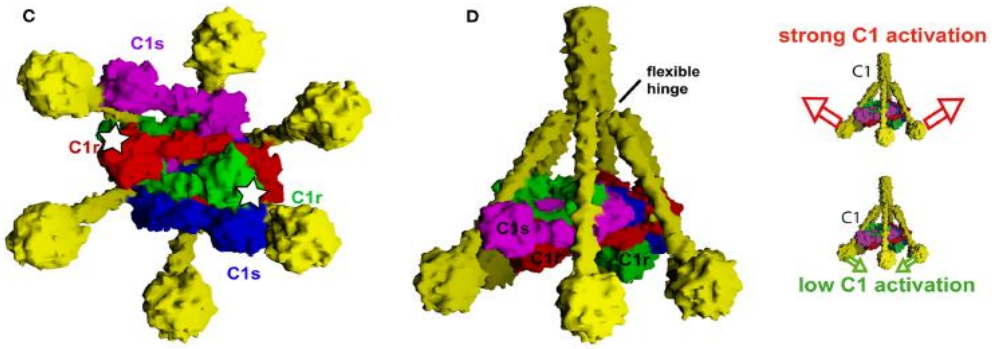

Fig.1: Bottom view (C) and Side view (D) of the C1 complex. Red arrows indicate an outward movement of the stems of C1q induced by binding to ligands (including immune complexes)

Variables playing a role in C1q recruitment and complement activation

Antibody-mediated C1q recruitment does not occur always at the same extent, and a number of factors have been reported to influence it, acting in different key points of the mechanism. These factors can critically determine whether the classical pathway is activated or not, and in some cases lead to a synergy between antibodies:

- Antigen expression and density on the surface of target cells: Antigen density has a critical influence on C1q engagement because it contributes to determine the spacing between the Fcs of antigen-bound antibodies, therefore higher antigen densities correspond to a better engagement of C1q and complement activation. C1q binds with high avidity to multivalent IgG complexes (41), and when the antigen density favors a situation in which two or more antibody Fc tails become adjacent, C1q is multivalently and stably engaged. This condition occurs either when a single antigen adopts a clustered disposition on a surface, as reported for the Neisserial Adhesin A (42); or when a single antigen sparsely distributed is expressd at sufficient density (as in the case of the meningococcal factor H binding protein, see Ref. (43).
- Antibody epitope: The position and conformation of the epitope on the antigen of a single antibody molecule can influence the efficency with which the antibody activates complement by imposing structural restraints to C1q recruitment. This has been observed particularly for antibodies recognizing different epitopes of the same tumor antigen (44; 45).
- IgG subclass: IgG antibodies belonging to different subclasses are characterized by different structural patterns in the constant regions, particularly in their hinges and upper CH2 domains (46), which affect the interaction of IgGs with C1q and therefore of complement activation. Human IgG1 and IgG3, thanks to favorable structural patterns, can recruit efficently C1q, while

IgG2 and IgG4 do so much less efficently (47) or only in certain conditions (48; 28). Different C1q recruitment efficency corresponds to different functionality in the activation of complement-mediated killing, as demonstrated by Giuntini et al. (49).

Aim of the study

The aim of the present study was to investigate whether individual monoclonal antibodies with specificity for distinct bacterial antigens could synergistically activate complement-mediated killing, and to explore the molecular mechanisms driving this synergy, understanding which variables play a role in it and modulate its potency. To this purpose, antibodies specific for two well characterized antigens sparsely distributed on the cell surface of *N. meningitidis* serogroup B (MenB) were selected as a model. The selected antigens were factor H binding protein (fHbp) and Neisserial Heparin binding Antigen (NHBA), both included in the multi-component meningococcal B vaccine Bexsero (50). Bexsero contains three meningococcal surface-exposed recombinant proteins: fHbp-GNA2091 (factor H binding protein variant 1.1 fused with GNA2091), NHBA-GNA1030 (Neisserial Heparin Binding Antigen variant p2, fused with GNA1030), and NadA (Neisserial adhesin A variant 3.8), plus Outer Membrane Vesicles (OMV) from a New Zealand strain (50). Each of these proteins is able to induce seroprotective human-complement mediated bactericidal activity. FHbp is a 27kDa lipoprotein that can be classified into three main variant groups named “group 1” (or “subfamily B”), “group 2” and “group 3” (collectively named “subfamily A”) and can bind to human complement factor H (FH), a downregulator of the alternative complement pathway helping *N.meningitidis* to survive in human blood, evading alternative-complement mediated clearance (51). The three-dimensional structure of fHbp alone (73, 74) and in complex with bactericidal mAbs (40, 52, and 70) as well as with FH (53) has been solved. NHBA is a 60kDa protein present in all meningococcal strains and it can be classified in many different peptidic variants, mainly cross-protective. Gene-sequence analysis from genetically diverse serogroup group B meningococcal strains revealed the existence of more than 600 NHBA peptidic variants (54; 55). It has been shown to bind heparin and heparan sulfate structures increasing bacterial serum resistance and contributes to epithelial cell binding (56). By nuclear magnetic resonance, the structure of a distal C-terminal eight-stranded beta barrel (57) was obtained. Both FHbp and NHBA have been reported to be sparsely distributed on the meningococcal surface (38).

It was previously shown that couples of monoclonal antibodies recognizing non-overlapping epitopes of fHbp (35; 37; 40; 64) can synergistically trigger complement-mediated killing, and the proposed molecular mechanism for this cooperation was that the adjacency of the Fcs of the pairs of mAbs bound to the antigen led to an enhanced recruitment of C1q. Additional studies (38; 65) demonstrated that polyclonal sera containing antibodies recognizing individually fHbp or NHBA can trigger augmented complement-mediated bactericidal activity of MenB strains when combined together, supporting the hypothesis that a critical density of antibodies directed at more than one surface antigen can enhance the antibody-mediated engagement of C1q and augment classical complement pathway killing. The aim of this study was to provide direct evidences that monoclonal antibodies recognizing different antigens can cooperate to activate the complement cascade. Mabs specific for fHbp or NHBA inducing weak or no complement-mediated killing of meningococcal cells in vitro were tested in vitro to evaluate their ability to become effective when used in combination. Previous studies (28) showed that the nature of the recognized epitope can have a critical influence on the capability of one antibody to trigger complement mediated killing, determining the orientation of it when it is bound to the antigen and imposing structural constraints to C1q recruitment. The panel of mAbs tested in this study for single and combined bactericidal activity included mAbs specific for a range of different epitopes of fHbp and NHBA, in order to evaluate their influence on complement-mediated killing.

As aforementioned above, antigen density is a variable that can considerably influence the activation of complement-mediated killing. Previous studies have shown that regular distribution of epitopes in clustered patterns (42) or high expression level of antigen sparsely distributed on the bacterial surface (43), are key variables to induce complement-mediated killing. In this study, functional assays of antibody cooperativity were performed with strains expressing fHbp and NHBA at different levels, in order to investigate the reciprocal effects of antigen expression and density on mAb cooperativity.

We considered mAbs belonging to the same subclass and therefore expressing the same constant regions (for the human mAbs IgG1, for the murine ones IgG2b) in order to prevent variability of bactericidal activity due to different Fc affinity for C1q. Both IgG sublcasses are 150kDa antibodies (Janeway et al., 2001) made of two identical heavy chains of 50 kDa and two light chains of about 25kDa (See figure 2). The heavy chains consist of three constant heavy regions (CH1, CH2, CH3) and one variable heavy region (VH) and are linked to each other and to one single light chain each

by disulfide bonds, resulting in a Y-shaped quaternary structure. The light chains consist only of one constant region named Cl and one variable region called Vl. The structure can be ideally divided in two portions, called respectively Fab (Fragment-antigen binding portion) and Fc (crystallyzable fragment). The Fab contains variable regions which are responsible for binding to the antigen, while the Fc, which is often glycosilated, contains the constant regions, that mediate the functional activity of the antibody. Each Fab consists of one light chain plus part of one heavy chain, while the Fc consists of the latter of the heavy chains. The Fab and Fc regions are connected together by a region called "hinge" which is responsible for the flexibility of the molecules and varies in length among the four human subclasses.

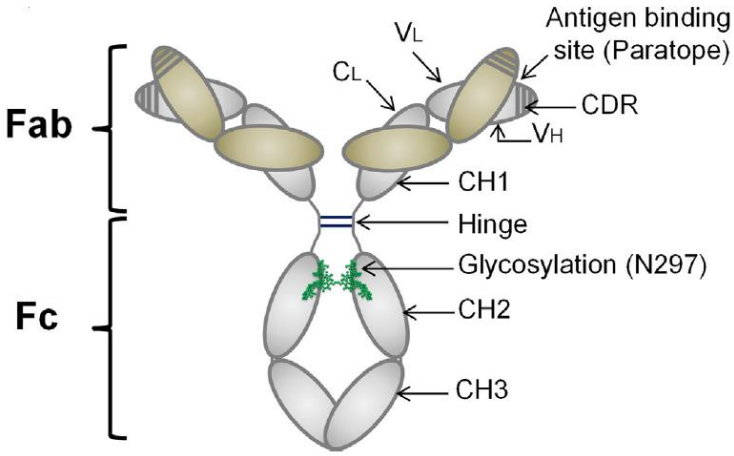

Fig.2: Structure of an IgG antibody.

Human IgG1, together with IgG3, are IgG subclasses that can efficiently recruit C1q and trigger C4b deposition (48). Both human IgG1 and murine IgG2b can bind to C1q and activate the classical pathway cascade. The binding site on IgG1 and IgG2b for C1q is located on the CH2 domain, and the residues involved in the interaction are in human IgG1 D270, K322, P329, and P331 (58), while in the murine IgG2b they are residues E318, K320, and K322 (59). Each IgG has two C1q binding sites, on the opposite sides of the Fc, but only one needs to be involved in C1q binding for complement activation (28). Human IgG1 have been reported to be able to form hexameric structures that interact optimally with C1q (28). They have lower flexibility than IgG3, but greater flexibility than IgG4 and IgG2, with flexibility well correlating with the hinge length (60). Thanks to the hinge region, the Fab regions can reorient, allowing the IgG1 antibodies to bind multiple antigens in different positions (61). Furthermore, the relatively long hinge region of IgG1 (15 aminoacids) and the remarkably long one of IgG3 (62 aminoacids) confers great flexibility and makes in these subclasses the C1q binding site more accessible to C1q than the one of IgG2 (12

aminoacids) and IgG4 (12 aminoacids) (62), leading to a more efficient functional activity in terms of C1q recruitment and complement activation (49). Different capability of complement activation has been observed also abong murine IgG subclasses, showing a hierarchy of IgG3 >> IgG2b > IgG2a >> IgG1 (63).

The present PhD project intends to provide the proof of principle that antibodies recognizing different antigens can cooperate in events like C1q recruitment and C3b deposition resulting in enhancement of bactericidal titers, as well as to clarify the contribute of antibody epitope, antigen density and conformation to such cooperativity. Compared to previous studies that investigated synergic bactericidal activity between antibodies with different specificity by using polyclonal sera (36,38), this work exploits the specificity of mAbs to detect any difference in the contribute provided by single epitopes and to remove potential interferences caused by the heterogenicity typical of a polyclonal population.

Materials and Methods

Human monoclonal antibody expression and purification

A panel of human monoclonal antibodies isolated from patients immunized with Bexsero was expressed in mammalian cells. For the expression of human mAbs, the Expi293 Expression System (Gibco, Life Technologies) was used. The expression system is designed to allow large-scale transfection of suspension 293-line human embryonic kidney cells in a defined, serum-free medium. Transfection and expression experiments were performed directly in Expi293 Expression Medium.

Frozen cells were supplied by Life Technologies in frozen vials containing 1mL of cells at $1x10^7$ viable cells/mL in 90% Expi293 Expression Medium and 10% DMSO and were thawed directly into Expi293 Expression medium in 125-ml polycarbonate, disposable, sterile, vent-cap Erlenmeyer shaker flasks containing pre-warmed Expi293 Expression medium. Cell cultures were established incubating cells in a 37°C incubator with humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 125rpm. Cell viability was monitored determining the viable and total cell counts using a hemocytometer with trypan blue and an automated cell counter. Maintenance cell cultures were passed when they reached a density of 3-5 x 10^6 viable cells/ml, typically every

3-4 days, diluting them 1:10 (3ml of cells in 27ml of expression medium). When cells were subcultured, they were transferred into pre-warmed growth medium.

For the transient transfection of high-density Expi293 cells, a cationic lipid-based transfection reagent, ExpiFectamine293 reagent, included in the kit, was used. Plasmid DNA for transfection was clean, sterile and in DNA-ase free water. Transfections were performed in a total 30 ml volume. On day 0 (pre-transfection) cell viability was determined and cells were seeded at a density of 2 x 10^6 viable cells/ml with viability over 95%. On day 1 (transfection day), 7,5 x 10^7 cells were suspended in a final volume of 25,5ml of expression medium in a 125-ml polycarbonate, disposable, sterile, vent-cap Erlenmeyer shaker flask. Plasmid DNA and Expi293 reagent were initially incubated separately with Opti-MEM I medium (a reduced serum medium that facilitates optimal formation of DNA-expifectamine 293 reagent complexes) and then incubated together for 20-30 minutes. DNA-Expifectamine293 reagent complexes were then added to each shaker flask bringing volume up to 28,5ml. Approximately 16-18 hours post-transfection, two transfection enhancers (Enhancer 1 and Enhancer 2) were added to the culture bringing up the volume to 30ml. Expression medium (containing the secreted mAb) was harvested at 72 and 144 hours post-transfection and stored at -20°C until purification. From the expression medium, mAbs were purified by affinity chromatography with protein G columns (*GE Healthcare*) using an Akta Purifier instrument (*GE Healthcare*). Binding and washing buffer contained 50mM NaH2PO4, 300mM NaCl pH 7,2, mAbs were eluted with 0,1M Gly HCl pH 2,7 and Tris 2M pH 9,0 was used to compensate the pH variation.

Murine mAbs production and purification

The murine mAbs were produced and purified by Areta International Srl (Gerenzano, Italy). For details see the “Materials and Methods” section of Ref. (70).

Detection of antibody binding by Fluorescence Activated Cell Sorting.

The ability of murine and human mAbs to bind the antigens exposed on the surface of *N. meningitidis* bacteria expressing different variants of the proteins was determined using an FACS flow cytometer (FACScanto II, BD Biosciences). Antibody binding was detected using an anti-mouse or anti-human (whole-molecule) FITC-conjugated antibodies (Sigma-Aldrich) at a 1:100

dilution. Assay controls included a polyclonal serum raised against fHbp/NHBA proteins and as a negative control, bacteria plus PBS+ 1% bovine serum albumin (BSA) and secondary antibody.

Starting from glycerol stocks, *N. menigitidis* stains were grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Mueller hinton broth containing 0,25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C with shaking until the OD_{600} reached 0,25. 5ml of bacterial culture were centrifuged at 3500rpm x 10 minutes. The surnatant was discarded, and the pellet was resuspended in 5ml of PBS+ 1% bovine serum albumin. 50 µl of the previously resuspended bacteria were added in each well of a 96-well plate (Costar, 3799) to a mix containing mAbs (or polyclonal serum for the positive control and PBS+ 1%BSA for the negative control) at a final concentration of 10 µg/ml in PBS + 1%BSA and incubated with the mAbs at room temperature for 1 hour. Two washing steps with 150 µl of PBS + 1%BSA were performed. Bacteria were resuspended in 100 µl of secondary antibody 1:100 in PBS + 1%BSA and incubated for 30 minutes at room temperature. Two additional washing steps were performed with 150 µl of PBS + 1%BSA. Bacteria were resuspended in 150 µl of freshly-prepared PBS + 0,5% paraformaldehyde and incubated for 30 minutes at room temperature. Bacterial inactivation was checked seeding 15 µl of bacteria on Agar chocolate plates. On the following day, bacteria were diluted 1:40 in PBS 1x and fluorescence data was acquired with FACScanto II and analyzed with FlowJo software.

Complement-mediated bactericidal activity.

Serum complement-mediated bactericidal activity against *Neisseria meningitidis* was evaluated with pooled baby rabbit serum (Cedarlane) or human serum as a source of complement. The assay was performed in 96-well plates (Thermo Scientific).

Starting from glycerol stocks (in which 20-30 colonies had been inoculated in Mueller Hinton broth + 0,25 glycerol) conserved at -80°C, *Neisseria menigitidis* stains were grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Mueller hinton broth containing 0,25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C with shaking until the OD_{600} reached 0,25. The bacteria were diluted 10.000-fold in Dulbecco's phosphate buffered saline (DPBS) and 1% (wt/vol) Bovine Serum Albumin (BSA). The total volume in each well was 50 µl, consisting of 25 µl of serial twofold dilution of mAb, 12.5 µl of bacteria at the working dilution, and 12.5 µl of source of complement (baby rabbit serum or human serum). MAbs were tested starting from a concentration of 0,5 mg/ml. In mixing experiments, equal quantities of

antibodies were combined together. Lyophilized baby rabbit serum was reconstituted adding Milli-Q ultrapure water (Merck Millipore). Positive serum samples were included in each assay. Controls included bacteria incubated with heat-inactivated complement (with complement being inactivated by heating at 56°C for 30 minutes) and bacteria incubated with mAbs and without a source of complement. Immediately after adding the source of complement, 7 µl of the controls was plated on Mueller-Hinton agar square plates (time zero). The plate was incubated for 1 hour at 37°C on shaker; 7 µl of each mix was spotted from the 96-well plate on Mueller-Hinton agar plates (time 1). The agar plates were incubated overnight for 18 hours at 37°C, and the colonies corresponding to time zero and time 1 were counted.

Serum bactericidal titers were defined as the mAb dilution resulting in 50% decrease in CFU after a 60-min incubation of bacteria with the reaction mixture compared to the control CFU at time zero.

Detection of C3b deposition by Fluorescence Activated Cell Sorting.

Rabbit C3b deposition triggered on the bacterial surface by single mAbs and cooperative mAb couples was assessed by flow cytometry with a primary goat anti-rabbit C3b antibody (*Meridian Life Sciences*) acquiring fluorescence data the flow cytometer FACScanto II (BD Biosciences). Starting from glycerol stocks, *N. menigitidis* stains were grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Mueller hinton broth containing 0,25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C with shaking until the OD_{600} reached 0,25. 6ml of culture were centrifuged at 3500rpm x 5 minutes, the surnatant was discarded and the pellet was resuspended in 1 ml of DPBS 1%BSA 0,1%glucose. In each reaction well, 12,5 µl of bacteria + 12,5 µl of baby rabbit complement were added to the antibodies concentrated 50 µg/ml and incubated for 15 minutes at 37°C shaking. The 96 well-plate was then frozen in order to block the complement-mediated killing reaction. Bacteria were then washed with PBS 1%BSA, incubated with the primary anti-C3b antibody 1:500 for 1 hour, washed again, incubated with a secondary donkey anti-goat antibody conjugated with FITC fluorophore, washed again with PBS 1%BSA, incubated for 1 hour with PFA (paraformaldehyde) 2% and finally washed with PBS 1x. On the following day, bacteria were diluted 1:40 in PBS 1x and fluorescence data was acquired with FACScanto II and analyzed with FlowJo software.

Surface Plasmon Resonance.

A Biacore T200 instrument (Biacore, Uppsala, Sweden) was used to obtain the affinuty constants of the mAbs for the respective antigens; as running buffer we used HBS-EP$^+$. In these experiments, 10 kRU anti-mouse or anti-human antibodies (GE Healthcare) were immobilized on a CM-5 sensorchip by amine coupling. Subsequently, we captured 1 kRU of mAbs, and performed 5 injections, each with 1 min contact time, of fHbp/NHBA variants at increasing concentrations and followed by a 500 s dissociation period (30 µl/min). Kinetic parameters were determined using a 1:1 model of the Biacore T200 Evaluation 1.0 software. fHbp var1 wild-type and D85A mutant proteins were amine coupled in 50 mM NaOAc at pH 4.5 to the surface of a ProteOn GLM sensor chip, activated with 25% EDC/NHS. Sensorgrams were analyzed using ProteOn Manager Software.

Results

Selection of monoclonal antibodies and meningococcal strains.

In the first part of the work, a panel of previously characterized murine and human mAbs targeting fHbp or NHBA were selected following a number of criteria:

1) Affinity constant for the antigen: previous Surface Plasmon Resonance (SPR) data of the antibodiy affinity for the recombinant antigens was available. Only mAbs with high affinity for the antigen ($K_d < 10^{-8}$M) were kept in consideration, in order to select antibodies capable of binding efficiently to the antigens on the bacterial surface (able 1).
2) Bactericidal titer: the selected mAbs were assessed for their capability of eliciting complement-mediated killing with baby rabbit serum as a source of complement, against meningococcal strain NGH38. Most of the mAbs could not elicit singly bactericidal activity, and this was demonstrated by a negative bactericidal titer (rSBA <4); one murine mAb (9E5) could elicit singly bactericidal activity (rSBA = 128). Negative or low single bactericidal titer was a preferential selection criterium, as as our aim was to show that for strains in which the contribute of single antigens (fHbp or NHBA) is not sufficent to trigger complement-mediated killing of the bacterium, the combined effect of the two antigens provides a synergistical recruitment of C1q that allows activation of bacterial killing, providing a better coverage of the vaccine.
3) Variant specificity: the mAbs selected were predicted by protein chip to be able to bind to the variants of fHbp and NHBA expressed by strain NGH38, the strain used for the experiments.
4) Epitope: data of epitope mapping via different techniques (including X-ray crystallography, HDX-MS, protein chip) was available from previous studies of antibody characterization (see table 1). Only mAbs with non-overlapping epitopes were selected. The mAbs targeting fHbp were characterized by epitopes disposed in different regions of the protein, whereas the mAbs anti-NHBA were preferentially selected for capability of binding to the N-term domain of NHBA, which remains anchored to the membrane even after proteolytic cut (that occurs in physiological conditions and is mediated by host and bacterial proteases, as mentioned in (56)), in order to prevent epitope loss during the course of the bactericidal assays

5) Isotype: all the human mAbs were initially expressed as IgG1 with mammalian cells, while all murine mAbs were expressed as IgG2b with hybridoma cells. The reason for selecting mAbs belonging to the same subclass lies in avoiding variability of bactericidal activity due to different Fc affinity for C1q.

Constant regions	**mAb name**	**Antigen**	**Binding variants (Pepscan)**	**Affinity constants (K_D), M (SPR)**	**Epitope mapping**
Human (IgG1)	1A12	fHbp	1,2, 3 (B)	$1{,}78\times10^{-11}$ (var.1); $2{,}2\times10^{-10}$ (var.2); $2{,}10\times10^{-10}$ (var.3) (A)	161, 163, 180-186, 188, 190-192, 213-216 (B)
	4B3	fHbp	1,2, 3	N.a.	N.a.
	1G3	fHbp	1, 2, 3 (A)	$1{,}72\times10^{-10}$ (var.1); $1{,}25\times10^{-10}$ (var.2); $1{,}28\times10^{-10}$ (var.3) (A)	V2-L30, A43-F70 (A)
Murine (IgG2b)	14B3	fHbp	N.a.	N.a.	60-90 (C)
	11F10	fHbp	N.a.	N.a.	K257, K268 (C)
Human (IgG1)	10C3	NHBA	p2 (A)	$2{,}12\times10^{-8}$ (p2) (A)	149-275 (N-term) (A)
	12E1	NHBA	N.a.	$9{,}5\times10^{-11}$ (p2) (A)	46-64 (N-term) (A)
	5H2	NHBA	p2 (A)	$1{,}01\times10^{-9}$ (p2) (A)	351-466 (C-Term) (A)
Murine (IgG2b)	3A3	NHBA	P1,p2	$5{,}2\times10^{-9}$	22-32 (N-Term) (NAADTDSLTPN)
	9E5	NHBA	P1,p2,p3, p5,p29	$1{,}68\times10^{-8}$	143-149 (N-term) (AGNTAAQ)
	22A4	NHBA	P1,p2,p5, p29	$1{,}89\times10^{-9}$	167-173 (N-Term) (SSTNPSA)

Table 1. Main features of selected mAbs anti-fHbp and anti-NHBA already available in literature. (A)=from Ref. 64); (B)=from Ref.52); " (C)= data from Ref. 71; N.a."=not available; *α* =peptide classification according to PubMLST Neisseria database (https://pubmlst.org/neisseria/).

The epitope of the human crossreactive mAb 1A12, which is capable of binding to all the three variants of fHbp (52), was mapped by X-ray crystallography. The mAb interacts with several aminoacids, most of which are conserved across the three different fHbp variants. The epitopes of human mAb 1G3 and murine mAbs 11F10 and 14B3 were mapped my HDx-MS (71). 14B3 is capable of interacting with sparse aminoacids comprised in the region 60-90 of fHbp, while 11F10 interacts with two lysines (K257, K268). 1G3 interacts with two extended regions of approximately 30 aminoacids each (V2-L30, A43-F70). Both 1A12 (52) and 1G3 (64) can bind to all the three variants of fHbp with good affinity, with affinity constants ranging from 10^{-10} M to 10^{-11} M.
A summary of the epitopes recognized by each mAb is provided in Figure 3.

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fHbp-1.1      VNRTAFCCLSLTTALILTACSSGGGG-----VAADIGAGLADALTAPLDHKDKGLQSLTL
fHbp-2.16     VNRTAFCCLSLTAALILTACSSGGGG-----VAADIGAGLADALTAPLDHKDKSLQSLTL
fHbp-3.28     VNRTAFCCLSLTTALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTL
              ************:*************     ******:***************.*:****

                          ◊◊◊◊◊◊◊◊◊◊◊◊
fHbp-1.1      DQSVRKNEKLKLAAQGAEKTYGNGD---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLES
fHbp-2.16     DQSVRKNEKLKLAAQGAEKTYGNGD---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLES
fHbp-3.28     EDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLAS
              ::*: :* .*.*:*******:  **   ***********:*****:::****** *** *


fHbp-1.1      GEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYR
fHbp-2.16     GEFQIYKQDHSAVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLP-DGKAEYH
fHbp-3.28     GEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSGLGGEHTAFNQLP-GGKAEYH
              ****:***.***:.*:* *:*::.:: ..:: :*.* :..:.****:*::**..*:* *:

fHbp-1.1      GTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVL
fHbp-2.16     GKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTR
fHbp-3.28     GKAFSSDDPNGRLHYSIDFTKKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTR
              *.**.***..*:* *:***: *** *:*****: * **:****::*.* * **** *..

fHbp-1.1      YNQAEKGSYSLGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ
fHbp-2.16     YGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ
fHbp-3.28     YGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ
              *.. ***:* *.:**.:***:**** **  : ::.**:*.**
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Amino acid sequence alignment of the three prottypic fHbp variants. Mapped epitopes are colored according to:

1A12

1G3

◊ 14B3

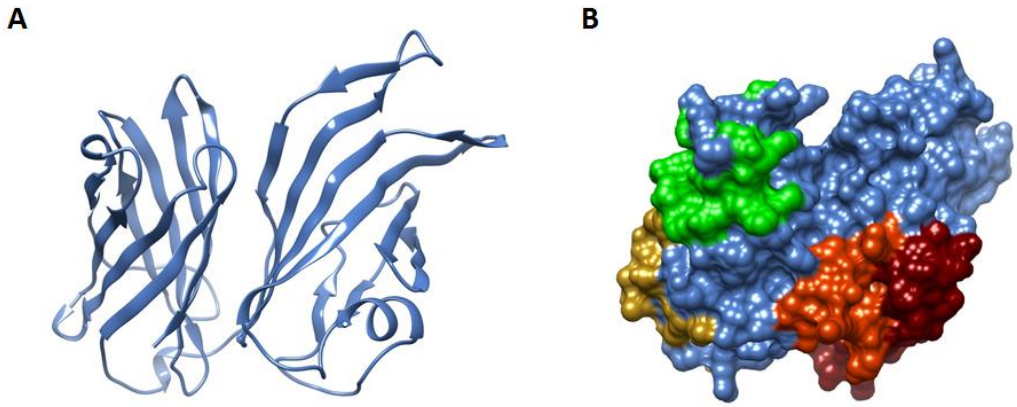

Fig. 3 Distribution of fHbp mAbs epitopes. A Schematic ribbon representation of fHbp. N- and C-terminal domains are indicated. B Space fill representation of the protein where atoms forming the different epitopes are colored as following: green =1A12; orange= specifically recognized by 1G3; dark red= residues shared by the 1G3 and 14B3 epitopes; yellow = epitope recognized by 4B3 (72)

The affinity for recombinant NHBA was previously assessed by SPR using recombinant NHBA p2, the homologous variant of NGH38 and the one contained in Bexsero. MAb affinities for NHBA ranged from 1,68 x 10^{-8} M (for mAb 9E5) to 9,5 x 10^{-11} M (for mAb 12E1). The epitope of the mAbs was estimated either by protein chip or HDx-MS, for the human mAbs published in (64). Epitopes of most of the mAbs were located in the N-term domain of NHBA: 9E5 and 22A4 recognized regions consisting of seven peptides, located respectively in positions 143-149 and 167-173; 3A3 recognized a decapeptide at the extremity of the N-term domain (22-32); the human mAbs 10C3 and 12E1 recognized both the N-term region of NHBA (with epitopes located in positions 181-275 and 46-64). However, one human mAb (5H2) had an epitope located on the C-term domain of the protein (351-466). The murine mAbs were obtained immunizing mice with two different variants of NHBA: 9E5 was obtained immunizing with NHBA dRR, a variant deleted from the arginine-rich region (RR); 22A4 was obtained immunizing with NHBA NZ (a wild type variant expressed by a New Zealand strain); 3A3 immunizing with NHBA AB3, a variant deleted from a 116 AA C-terminal region.

The strain that was mainly used in this study to investigate on the cooperativity between mAbs recognizing fHbp and NHBA is *N. meningitidis* strain NGH38, expressing fHbp variant 2.24 and NHBA peptidic variant 2. The reasons for this choice are the following:

1) It is a high-level NHBA expressor, considered in the MATS (Meningococcal Antigen Typing System) coverage system (66) as the reference strain for NHBA. Therefore, it was predicted to ensure a sufficient level of expression of NHBA allowing cooperative bactericidal activity.

2) It expresses fHbp at low levels in comparison to other strains, as observed in our flow cytometry experiments, therefore we expected low or negative anti-fHbp mAb titers against this strain.

NGH38 provides therefore a good balance between expression levels of NHBA and the naturally more abundant fHbp antigen. For such reason we considered this strain suitable to appreciate any contribute by anti-NHBA antibodies to an eventual cooperative protective synergy, which could be underestimated in the presence of high density of fHbp. A strain called M13520, expressing high levels of fHbp, was selected to investigate on the synergy between anti-fHbp and anti-NHBA mAbs in the presence of human complement, under the hypothesis that using an anti-fHbp mAb capable of inhibiting FH binding to fHbp could help to achieve bactericidal synergy in the presence of high levels of expression of fHbp.

All anti-fHbp antibodies except for 11F10 can bind to fHbp on NGH38 surface.

The capability of anti-fHbp mAbs to bind to fHbp on the surface of strain NGH38 was evaluated by Fluorescence Activated Cell Sorting (FACS). Briefly, bacteria 0,25 $O.D._{600}$ were incubated with anti fHbp human/murine mAbs and then incubated with FITC-conjugated (Fluorescein-Isothiocyanate) conjugated secondary antibodies. Results are showed in Figure 4. All the mAbs except for 11F10 were able to bind to fHbp v.2.24 presented on the surface of NGH38 suggesting that in the variant expressed by this strain, the epitope of 11F10 is not expressed or presented. 11F10 was therefore excluded by the following functional assays.

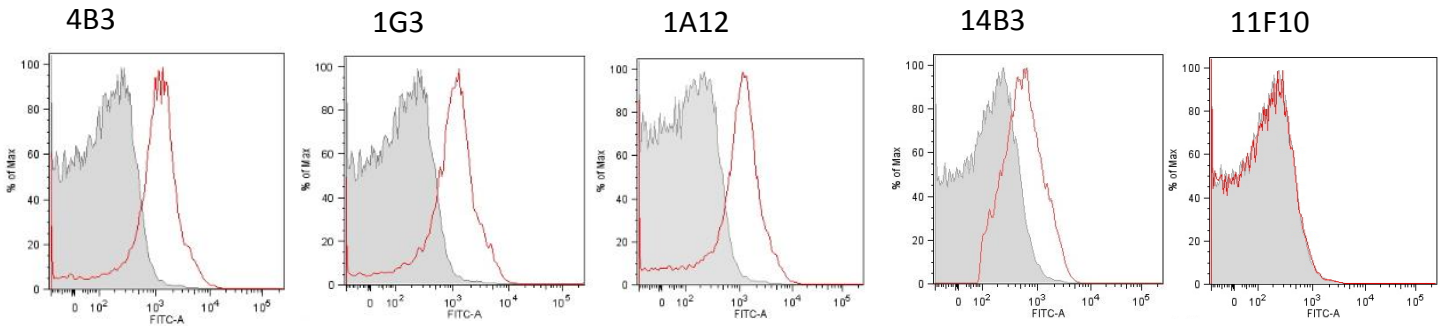

Figure 4. Binding of anti-fHbp mAbs to fHbp expressed on the surface of NGH38. The negative controls, consisting of bacteria+PBS, are represented by grey tinted lines. The FACS binding profile of the mAbs is represented by red lines.

All anti-NHBA antibodies can bind to NHBA on NGH38 surface.

Binding of the NHBA mAbs was investigated via FACS following the same experimental protocol for the anti-fHbp mAbs, with bacteria $O.D._{600}$ 0,25 incubated initially with the primary anti-NHBA antibody and then with a FITC-conjugated secondary antibody. Figure 5 shows the FACS binding profiles. All the mAbs targeting NHBA were able to recognize the antigen presented on the bacterial surface despite a less pronounced shift can be observed in the case of 3A3 and 12E1. Epitopes of both the antbodies have been mapped at the very beginning of the protein sequence (region spanning 22-64 residues, see Table 1). This could suggest a limited accessibility of the N-terminus of NHBA in native conditions. MAb 12E1 showed a higher affinity than the other anti-NHBA mAbs, apparently in contrast with what was observed in FACS. One possible explanation could be that the native antigen expressed on the bacterial surface and the recombinant antigen used for SPR have different folding patterns, resulting in an apparent higher binding efficiency in SPR rather than in FACS. Interestingly, we observed that mAb 3A3 anti-NHBA could recognize this NHBA variant (p2) on this strain, but it was not able to recognize the homologous variant p2, expressed by another strain (M13520), suggesting that in M13520 the epitope was somehow masked or lost.

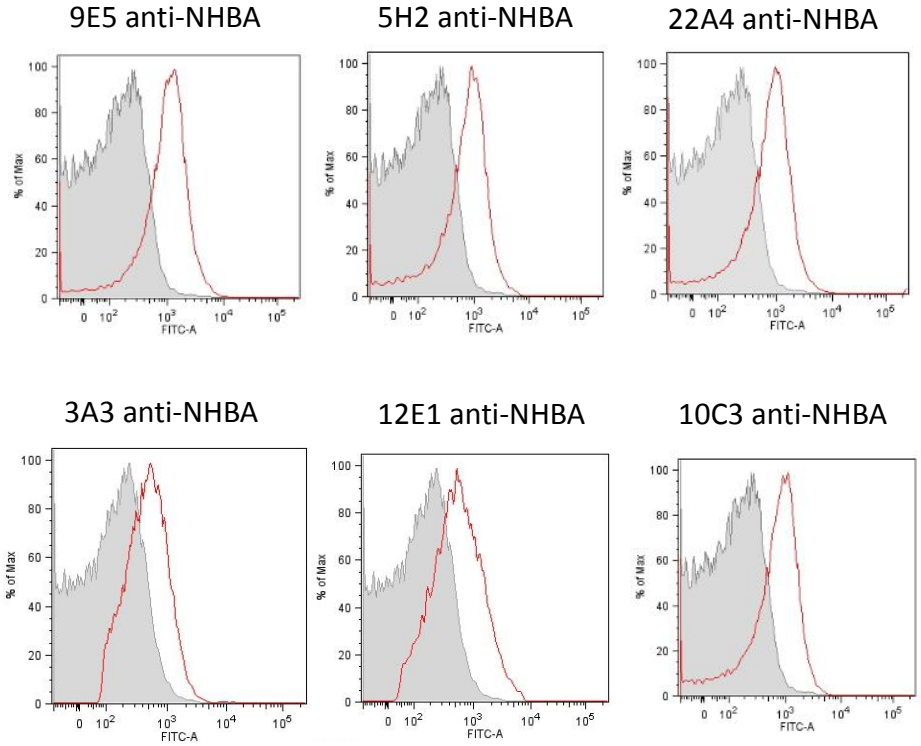

Figure 5. Binding of anti-NHBA mAbs to NHBA expressed on the surface of NGH38. The negative controls, consisting of bacteria+PBS, are represented by grey tinted lines. The FACS binding profile of the mAbs is represented by red lines.

Anti-fHbp and anti-NHBA mAbs can cooperate with rabbit complement.

Serum Bactericidal Assay (SBA), which can measure complement mediated killing via vaccine-induced antibodies in vitro (67), was exploited as a tool to demonstrate that mAbs targeting fHbp and NHBA can cooperate in the activation of complement-mediated killing. SBA was performed testing bactericidal activity triggered by single mAbs and mAb couples consisting of one mAb targeting fHbp and the other one targeting NHBA against strain NGH38, with baby rabbit serum as a source of exogenous complement.

According to (64), we considered a SBA titer 4-fold greater than the sum of titers induced by individual mAbs as a threshold to define cooperative the bactericidal activity induced by a mAb pair. Results are shown in table 2.

NGH38 strain						
			Anti-fHbp			
			Human			Murine
			1A12* (<4)	1G3 (<4)	4B3* (<4)	14B3 (<4)
Anti-NHBA	Human	12E1 (<4)	64**	<4	<4	<4
		10C3 (<4)	128**	<4	<4	<4
		5H2 (<4)	<4	<4	<4	<4
	Murine	9E5* (128)	**8192***	128	**8192***	128
		22A4* (<4)	<4*	<4	<4	<4
		3A3* (<4)	<4	64**	**2048***	<4

Table 2. Results of SBA against strain NGH38. Each antibody is characterized by a single titer (represented in the same box of the name of the mAb) and a combined titer with an antibody recognizing a different antigen (represented in the center of the table). Combinations between human mAbs are colored in green, combinations between murine mAbs are colored in blue, while human-murine combinations are colored in white. **=bacteriostatic titer;. Experiments were replicated at least 3 times. Bactericidal titers are consistent.

The results showed that most of the mAbs were not individually capable of eliciting rabbit-complement mediated killing of strain NGH38 (resulting in a titer <4) .For this strain, a negative titer for most of the single mAbs was expected, due to the low level of expression of single antigens. In the case of antigens sparsely distributed on the bacterial surface, low expression levels inded imply that the Fc tails of the antibodies are too far to engage efficiently C1q and trigger bactericidal activity.

The only exception to this paradigm was represented by MumAb 9E5, that could trigger killing of NGH38 with a rSBA titer of 128. This suggests that NHBA is expressed in NGH38 at sufficient levels

to trigger some bacterial killing even if not all NHBA-specific mabs can show bactericial activity, alone or in combination.

After performing SBA with the single mAbs, anti-fHbp and anti-NHBA mAbs were tested for combined bactericidal activity. Most of the mAb combinations had a negative titer. MAb 9E5 anti-NHBA, when combined with mAbs 1G3 anti-fHbp and 14B3 anti-fHbp, elicited a titer = 128, a titer which reasonably derives from the bactericidal activity of the single anti-NHBA mAb, since the titer did not vary when one anti-fHbp mAb was added.

Combination of 1A12 anti-fHbp with 12E1 anti-NHBA and 10C3 anti-NHBA triggered positive but bacteriostatic titers (respectively, rSBA = 64** and rSBA = 128**). A similar result was obtained when 3A3 anti-NHBA was combined with 1G3 anti-fHbp (rSBA = 64**).

Among the humab combinations, 1A12 was the most effective anti-fHbp partner, being able to trigger killing when combined with with two NHBA-specific Humabs. 4B3, on the contrary, was unable to trigger complement mediated killing in combination with the same NHBA specific mabs, despite showing comparable affinity to fHbp either in SPR and FACS. 4B3 and 1A12 recognize well distinct and non overlapping regions of fHbp, suggesting that epitope localization plays a role in inducing functional cooperativity among human-human mAb couples.

While none of the mumab pairs of the panel showed any cooperative effect, a striking synergy was observed in the case of three humAb-mumAb combinations: the bactericidal anti-NHBA mumAb 9E5 combined with two individually non-bactericidal anti-fHbp 1A12 (rSBA = 8192) and 4B3 (rSBA = 8192), as well as the couple 4B3 anti-fHbp + 3A3 anti-NHBA (rSBA = 2048). Noteworthy, both synergic couples 1A12+9E5 and 4B3+9E5 included 9E5, the only mAb already bactericidal *per se*. Instead, the synergic couple 4B3 + 3A3 included two non-bactericidal mAbs that, when combined together, elicited a synergic titer (rSBA = 2048). Overall these results demonstrate that anti-fHbp and anti-NHBA mAbs can cooperate in the activation of complement-mediated killing. The observation that different monoclonal antibodies show a different degree of synergic activity suggests that antigen density is not the only variable determining the cooperativity but multiple factors can influence synergy. In particular, nature of epitopes and IgG subclass play a major role in determining the capacity by single mabs to originate cooperative bactericidal couples.

It is interesting to notice that all the fHbp specific mAbs able to efficiently cooperate with NHBA-specific partners recognize the carboxyl-terminal domain of fHbp. Differently from the N-terminal

domain, wich is characterized by a less ordered conformation and low thermal stability (51), the C-terminal part of the molecule showed a remarkably stable fold. These results seem to suggest that well defined conformational epitopes can be more efficient to promote inter- antigenic cooperativity, likely by limiting the overall antigen-antibody flexibility and imposing conformational restraints to spatial reorientation of Fc tails.

Cooperative mAb couples elicit more efficient rabbit C3b deposition than single mAbs and non-cooperative mAb couples.

The classical pathway of complement activation is initiated when Fc regions proximity allow the antibodies to bind C1q. C1q engagement in turn activates C4, which ultimately leads to C3b deposition, membrane attack complex formation, and lysis of the bacterium. In order to investigate on the molecular mechanisms driving the synergy between anti-fHbp and anti-NHBA mAbs we used flow cytometry to measure C3b binding to the surface of live NGH38 cells as a surrogate marker for C1q binding and complement activation.

C3b deposition triggered by two single non-bactericidal mAbs (1A12 anti-fHbp, 22A4 anti-NHBA, rSBA <4), one single bactericidal mAb (9E5 anti-NHBA, rSBA =128), one non-cooperative non-bactericidal couple (1A12 + 22A4, rSBA <4) and one cooperative mAb couple (1A12 + 9E5, rSBA = 8192) triggered on the surface of NGH38 was evaluated and compared. Results are summarized in Fig.6.

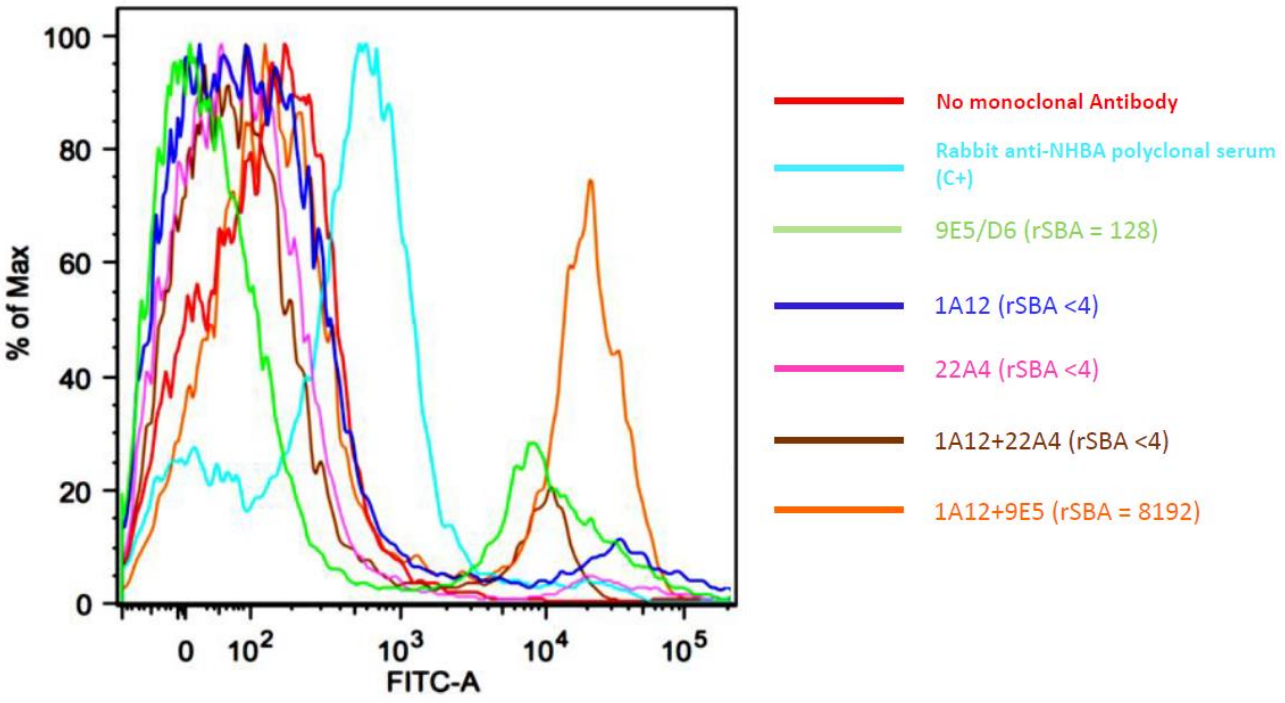

Figure 6. FACS analysis of rabbit C3b deposition triggered on the surface of live NGH38 cells triggered in the presence of single mAbs and mAb couples. Binding profiles are diversely colored: red = no mAb; light blue = rabbit anti-NHBA polyclonal serum (positive control); green = 9E5; brown = 1A12 + 22A4; dark blue = 1A12; pink = 22A4; orange = 1A12 + 9E5.

For all the C3b deposition FACS profiles, a double peak was observed, with the first corresponding to an intensity of 10^2 FITC-A and the second one to 10^4-10^5 FITC-A.

When NGH38 was incubated with complement in PBS (profile in red), no C3b deposition was observed, confirming that antibodies are required to trigger deposition of C3b on the bacterial surface. Incubation with single non-bactericidal mAbs 1A12 and 22A4 (dark blue, pink), triggered a poor C3b deposition. The non-bactericidal couple 1A12 + 22A4 triggered a more efficient C3b deposition (brown) compared to the one elicited by single mAbs, which is still not sufficient to trigger killing. A more efficient C3b deposition was triggered by the bactericidal mAb 9E5 (green), and the cooperative couple 1A12 + 9E5 (orange). It worthy to notice that synergy between mAbs leads to deposition of C3b on a greater number of cells compared to the C3b deposition triggered by single mAbs, and this result well fits with the fact that cooperative mAb couples are able to trigger killing of a greater number of cells in the bactericidal assay.

These results help to elucidate the mechanism of antibody cooperativity. The increased C3b deposition induced by the cooperative mab pair indirectly suggests that synergic antibodies trigger increased C1q recruitment, potentiating the downstream events of the classical complement pathway, which ultimately lead to bacterial killing. The role of the alternative pathway in this synergy remains to be elucidated yet.

Density of fHbp and NHBA on the bacterial surface can influence bactericidal activity of cooperative mAbs.

It is reported in literature that antigen density on the bacterial surface can influence complement-mediated killing triggered by antibodies raised against one antigen (43). According to the previous studies, higher antigen density induces proximity of the Fcs of antigen-bound antibodies, leading to a more efficient C1q recruitment and complement activation. In order to understand whether this factor can influence the synergy between antibodies recognizing different antigens on the

bacterial surface, a set of experiments including bactericidal assays and FACS experiments was performed.

Three different meningococcal strains were cosidered to study the effect of variable antigen densities on complement mediated killing: M07576, M13520 and NGH38. An initial FACS experiment with polyclonal sera anti-fHbp and anti-NHBA was performed in order to evaluate the density of both antigens on the bacterial surface (Figure 7).

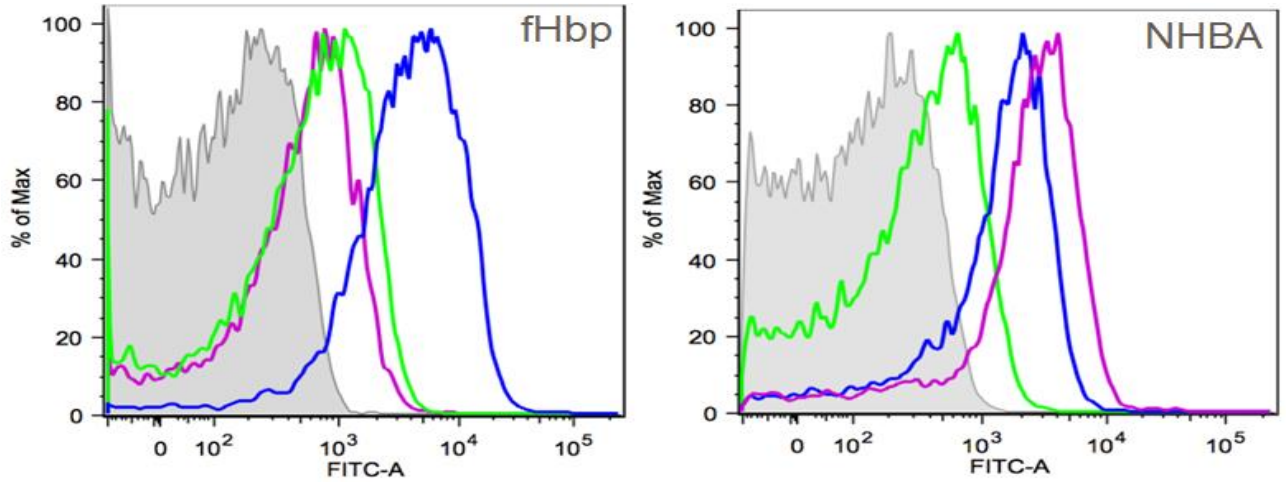

Figure 7. FACS expression comparison of fHbp and NHBA on the surface of M07576 (green), M13520 (blue), and NGH38 (pink) with polyclonal sera.

The three strains considered expressed fHbp and NHBA at different levels: M07576 (green) was a low expressor for both of the antigens; M13520 (blue) expressed fHbp at high levels and NHBA at an intermediate level; NGH38 (pink) expressed fHbp at a low level and NHBA at the highest level.

Subsequently, a FACS experiment was performed in order to confirm that the cooperative antibodies 4B3 anti-fHbp and 9E5 anti-NHBA could bind the three strains considered as a model for antigen density, and to compare the antigen density detected by these antibodies in the three strains (Figure 8).

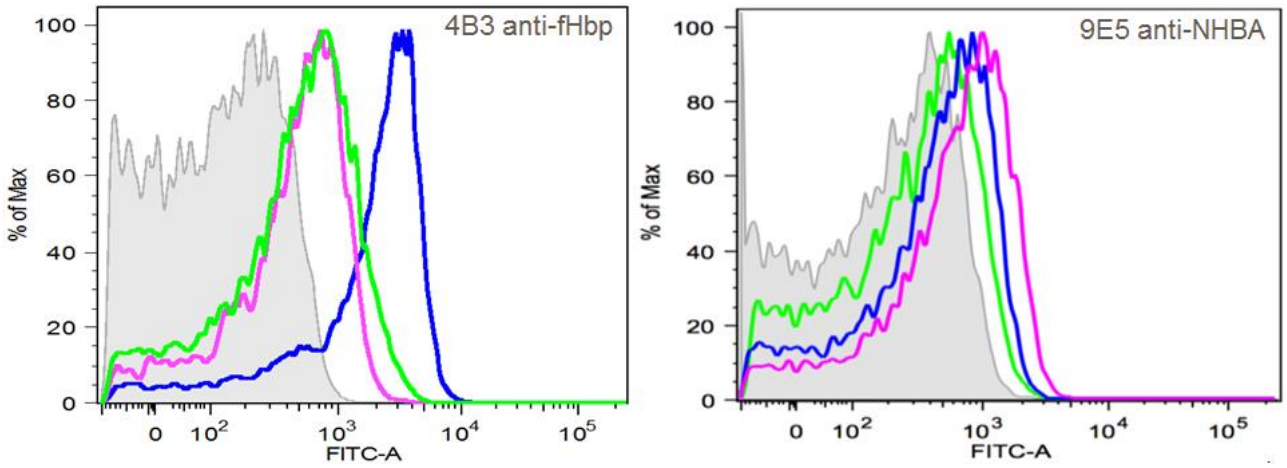

Figure 8. FACS binding of 4B3 and 9E5 to the respective antigens on the surface of M07576 (green), M13520 (blue), and NGH38 (pink).

Both of the antibodies could bind to the respective antigens on the surface of the three strains, and the density detected in the three strains was variable and the reciprocal level of expression was comparable with the one observed with polyclonal sera: as for fHbp, 4B3 detected a low level of density in M07576 and NGH38 and a high level in M13520. Instead, the density of NHBA was M07576 < M13520 < NGH38.

After characterizing the density of the antigens on the surface of these strains, bactericidal assays were performed with two of the previously identified synergic mAb couples: 4B3 + 9E5 and 1A12 + 9E5 (Table 3), using baby rabbit serum as a source of complement.

	M07576	M13520	NGH38
1A12 α-fHbp	<4	128*	<4
4B3 α-fHbp	<4	1024	<4
9E5 α-NHBA	<4	<4	128
4B3+9E5	<4	4096	8192
1A12+9E5	<4	1024*	8192

Table 3. Bactericidal assays performed with M07576, M13520 and NGH38, three strains expressing fHbp at different levels, with single mAbs and cooperative mAb couples.

The results showed that against a strain expressing both fHbp and NHBA at low levels (M07576), neither the single anti-fHbp (1A12, 4B3)/anti-NHBA (9E5) mAbs, nor the cooperative mAb couples could elicit complement-mediated bacterial killing. Considering a strain with increased fHbp

density (M13520), the single-anti-fHbp mAb titer turned positive, as well as the titer of the anti-fHbp mAbs combined with 9E5 anti-NHBA, which was 3-fold increased compared to the titer of the single mAbs. The titer of the single mAbs anti-fHbp were negative when they were tested for bactericidal activity against the fHbp low-expressor NGH38, against which, however, mAb 9E5 anti-NHBA could elicit individually bactericidal activity and synergistic complement activation when combined with anti-fHbp mAbs with a <5 fold increase in bactericidal titer.

These data clearly shows a correlation between antigen density on the bacterial surface and complement-mediated killing activation either by single mAbs (a result which was expected) and cooperative mAb couples targeting different antigens: when the density of one antigen is too low, as in the case of fHbp in M07576/NGH38 and NHBA in the case of M07576/M13520, the molecules of the antigen on the bacterial surface are too far from eachother, impeding Fc-mediated C1q recruitment and complement activation. The activation of complement-mediated killing becomes possible when the density of the antigen reaches a level that allows adequate proximity of the Fcs of antigen-bound antibodies (as in the case of fHbp for the high fHbp-expressor M13520 or NHBA for NGH38). When mAbs targeting fHbp and NHBA are simultaneously bound to the respective antigens on the bacterial surface and at least one antigen is bactericidal singly, the ensuing increased Fc density allows a synergistic recruitment of C1q, as demonstrated by the synergistic bactericidal titer of the 1A12+9E5 and 4B3+9E5 couples against M13520 and NGH38. It is possible to speculate that despite the increased Fc density for the mAb couples, it is still not sufficient to trigger bactericidal activity against strain M07576. The bactericidal titer of the two cooperative couples against a strain expressing at high levels NHBA (rSBA = 8192), NGH38, when compared to the titer elicited by the cooperative couples against M13520 (rSBA = 4096 for 4B3 + 9E5 and rSBA = 1024 for 1A12 + 9E5), showed that NHBA, when expressed at sufficient levels, can enhance the bactericidal activity of anti-fHbp mAbs more efficiently than anti-fHbp mAbs can with anti-NHBA ones, underlining the relevance of NHBA as a protective antigen.

Anti-fHbp and anti-NHBA mAbs can cooperate in the presence of human complement.

In order to further characterize the synergy between mAbs anti-fHbp and anti-NHBA, we investigated the possible cooperativity of these mAbs in the presence of human complement. In literature it has been widely reported that the binding of human FH to fHbp is one of the major

mechanisms allowing *N. meningitidis* to evade complement-mediated killing (51). Therefore, the presence of one mAb inhibiting FH binding to fHbp can impair FH-mediated immune evasion and therefore contribute to a more efficient activation of bacterial killing.

To test this hypothesis we selected mAb couples 9E5 + 1A12 and 9E5 + 4B3, which demonstrated potent synergy in the presence of rabbit complement.Mabs 1A12 and 4B3 were initially tested for their ability to bind to fHbp on the surface of strain M13520, and to inhibit hFH binding to meningococcal cells. We observed that both of the mAbs can bind to fHbp on the bacterial surface, but only 4B3 could inhibit FH binding to fHbp (Fig.9).Functional synergy between 4B3 and 1A12 with 9E5 was then investigated by SBA. Bactericidal assays were performed with M13520 and results are displayed in Figure 10.

None of the single mAbs were bactericidal including 9E5 that gave positive SBA with NGH38 and rabbit complement. This could be in part explained by a less efficient presentation of NHBA in M13520 compared to NGH38 as suggested by the differences in FACS profile (Fig.11). Moreover, the abundance of fHbp observed in the case of M13520 (Fig.8) could favor a higher recruitment of human fH on the surface of meningococcal cells during the bactericidal assay that interferes with the 9E5-mediated bactericidal killing. In line with this hypothesis a low bactericidal activity could be observed only in the case of the couple formed by 9E5 and the fH-inhibiting 4B3, whereas in the case of the couple including a non-FH inhibiting mAb (1A12) no synergy occurred. These results underlined the crucial role of FH inhibition and the contribute of the alternative pathway in the triggering killing of meningococcal cells.

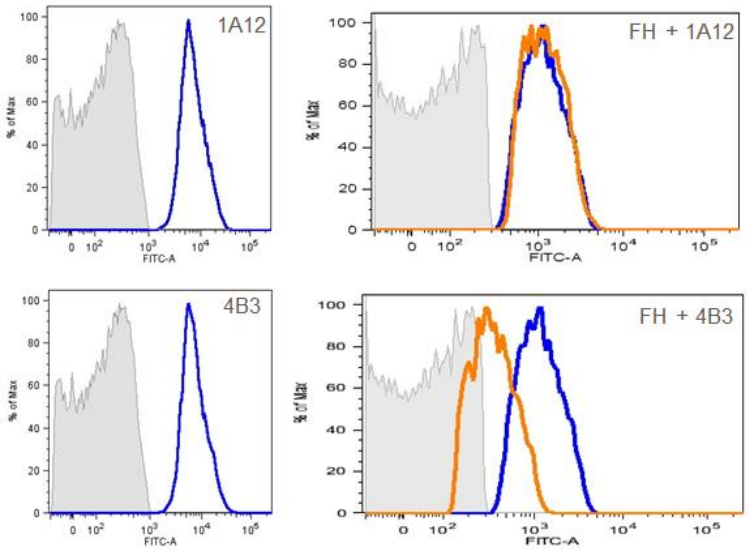

Figure 9. On the left: binding of 1A12 and 4B3 to fHbp on the surface of M13520. On the right, hFH binding inhibition. Orange: binding of FH in the presence of 4B3 Blue: binding of FH without 4B3

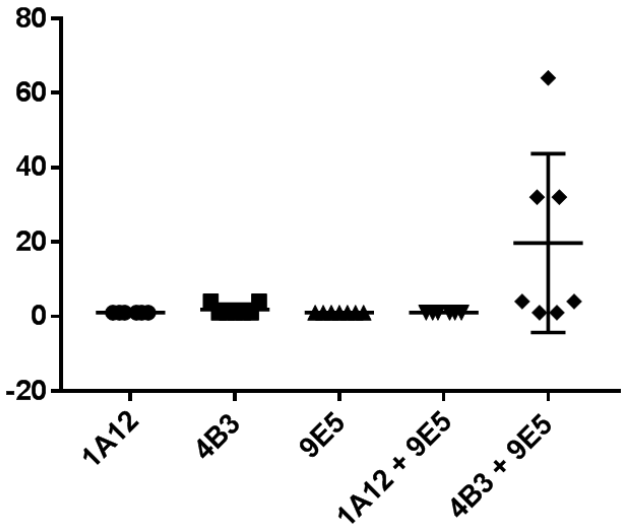

Figure 10. Results of the bactericidal assays performed against strain M13520 with human complement, of single mAbs 1A12, 4B3 and 9E5, and couples 1A12+9E5, 4B3+9E5.

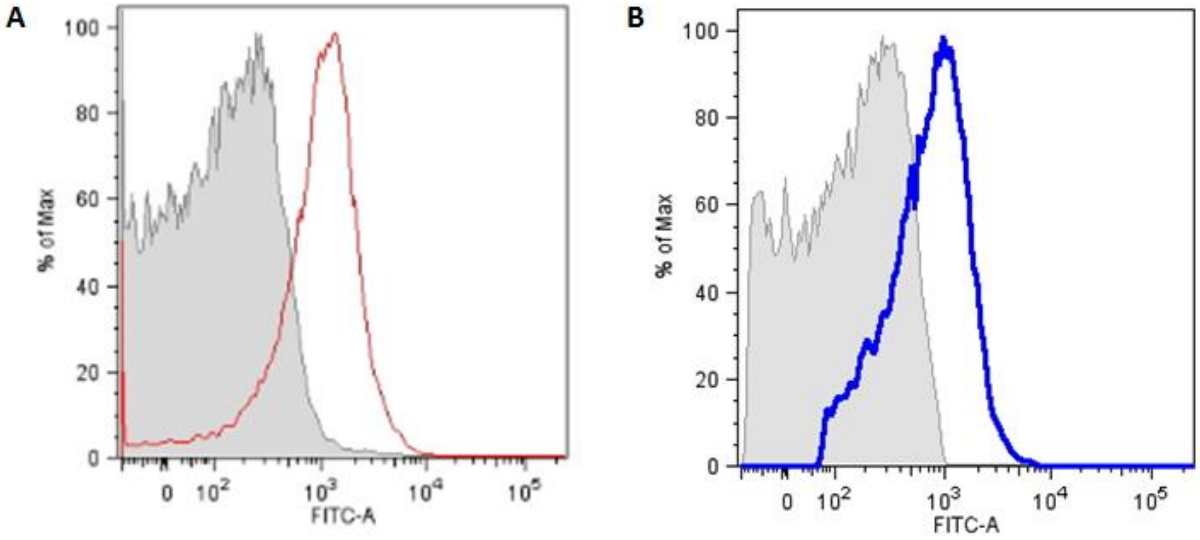

Fig. 11 Comparison of FACS profiles obtained by probing NGH38 (A) and M13520 (B) strains with the NHBA-specific mAb 9E5

Discussion

The complement system plays a major role in immune defense against meningococcal infections (68), and can be activated via different pathways: classical, alternative and lectin. The classical pathway is initiated upon recognition of pathogens by the immune system, when antibodies bind to their target antigens on the pathogen's surface and interact via the Fc tails with the protein C1q. This interaction activates the C1 complex, which triggers a series of downstream reactions that lead to opsonization of the pathogen with C3b and formation of a membrane attack complex (MAC) and ultimately result in lysis of the pathogen.

Antibody-mediated recognition of specific microbial antigens is responsible for the selectivity of the classical pathway, which is in phisiological conditions activated against non-self cells (pathogens). This mechanism is the key of the functionality of certain vaccines, including a vaccine for meningitis B called 4CmenB (Bexsero), containing three surface-exposed recombinant meningococcal proteins: fHbp-GNA2091 (factor H binding protein variant 1.1 fused with GNA2091), NHBA-GNA1030 (Neisserial Heparin Binding Antigen variant p2, fused with GNA1030), and NadA (Neisserial adhesin A variant 3.8), plus Outer Membrane Vesicles (OMV) from a New Zealand strain. Each of the recombinant antigens of Bexsero can be recognized by the immune system and elicits the production of antibodies that can efficiently promote killing of *Neisseria meningitidis* in the presence of complement via the classical pathway, conferring immune protection against the pathogen.

The molecular mechanisms by which antibodies can trigger the classical pathway have been widely studied, often using the antigens of 4CmenB as a model. Several studies have shown that antibodies do not activate complement always to the same extent, and that several variables play a role in antibody-mediated complement activation, mostly modulating the activation of complement at the level of C1q recruitment: antigen density can influence the spacing between the Fcs in a way that higher levels of total surface antigen density (43) or clusters of high density of surface antigens (42) can provide Fc adjacency that leads to an optimal engagement of C1q; a study demonstratd that C1q is favorably engaged even when two antigens are sparsely disposed with high density (Outer Membrane Vesicle Antigens) (36). In the aforementioned study (36), however, the antigens used to elicit bactericidal polyclonal antibodies were overexpressed by genetically engineered strains by promoter replacement, whereas in our study we focused on

monoclonal antibodies elicited by the antigen variants expressed by wild-type strains on order to achieve a more realistic model. Antibody epitope can influence the structural 3-dimensional disposition of antigen-bound antibodies favoring variably C1q engagement (44; 45; 28); the structure of the IgG constant regions depends on the IgG subclass and can influence the electrostatic interactions between Fcs and C1q (47, 48; 28, 49) having consequences on the functionality of antibodies. Furthermore, it has been demonstrated that antibodies can, in certain conditions, trigger a synergistic activation of complement thanks to mechanisms based on a cooperative recruitment of C1q. In particular, it was shown that when couples of antibodies bind simultaneously to one antigen, the proximity of their Fcs causes an enhanced C1q recruitment and complement activation, with consequent enhanced C3b deposition on the surface of the target cell. This has been demonstrated for monoclonal antibodies targeting fHbp (36; 35; 37; 38, 39, 49), provided that they have non-overlapping epitopes, so that they can simultaneously bind to the antigen. Moreover, it was observed that polyclonal sera, containing antibodies targeting multiple epitopes of different antigens (in particolar, fHbp and NHBA), can trigger synergistically complement when combined together (38; 65). However, the mechanisms responsible for synergistic complement activation mediated by antibodies targeting different antigens are still to be elucidated.

The aim of this project was to demonstrate that monoclonal antibodies targeting different antigens can synergistically activate complement-mediated killing, and to perform investigatory experiments to elucidate the factors that can modulate this synergy. To this purpose, we exploited two antigens well known and characterized of 4CmenB, fHbp and NHBA, as a model to explore cooperativity between monoclonal antibodies targeting distinct antigens. Using monoclonal antibodies, rather than polyclonal sera, allowed us to investigate singly on the functional contribution of different variables on this synergy: epitope (each mAb has only one epitope), and subclass (mAbs have only one subclass, while polyclonal sera contain antibodies belonging to different subclasses). Furthermore, it was possible to investigate on the influence of antigen density on this synergy performing experiments with strains expressing fHbp and NHBA at different levels.

Firstly, we selected a panel of mAbs targeting fHbp/NHBA which were suitable for our investigation. The selection of the mAbs was based on a number of criteria: good affinity for the antigen (we wanted antibodies capable of binding efficiently to the antigens on the bacterial

surface), low or negative bactericidal titer (in order to demonstrate that synergy between non-bactericidal or weakly-bactericidal mAbs can lead to strong complement activation), variant specificity (the antibodies selected were predicted to be able to bind to the antigen variants expressed by the meningococcal strain that was used for the functional assays, NGH38). Furthermore, targeting the N-term domain of NHBA was a preferential feature for the anti-NHBA mAbs, since it has been reported in literature that the C-term domain of this antigen can be cleaved by serum and bacterial proteases (56) causing a loss of the epitope during the functional assays performed in the presence of serum. The panel of mAbs included human mAbs and murine mAbs and all the human antibodies belonging to the same species were expressed with the same subclass: human mAbs were expressed as IgG1 and murine mAbs as IgG2b. The reason for this choice lies in the fact that we wanted to prevent the variability in complement activation due to different constant regions in our experiments. The antibodies were expressed in two different expression systems: the human ones in mammalian cells, and the murine ones in hybridoma cells.

The strain that we used as a model to study cooperativity between anti-fHbp and anti-NHBA mAbs was NGH38, a meningococcal strain characterized by a high level of NHBA expression, which we predicted to ensure sufficient antigen density for complement activation against this strain, and low level of fHbp expression, as demonstrated by our experiments, in order to demonstrate that when the density of fHbp is not sufficient to trigger complement-mediated killing, the combination of antibodies targeting fHbp and NHBA can lead to complement activation.

We firstly verified that all the mAbs included in our mAb panel are capable of binding to the respective antigens expressed on the surface of bacterial strain NGH38 by flow cytometry. All the anti-NHBA mAbs could bind to NHBA, whereas all the anti-fHbp mAbs except for one mumAb, 11F10, could bind to fHbp. This experiment allowed an additional selection of the mAbs that we wanted to use for the functional assays, excluding 11F10 from the following experiments.

In order to demonstrate that anti-fHbp and anti-NHBA mAbs can cooperate, the capability of triggering complement of single mAbs and inter-antigenic mAb couples was tested with bactericidal assays against strain NGH38. SBA was initially performed with single mAbs. All single mAbs, except for 9E5 anti-NHBA, were not bactericidal; 9E5 anti-NHBA elicited a bactericidal titer = 128. Considering that when an antigen is sparsely expressed at low levels on the bacterial surface, the Fc tails of the antibodies are too far to engage efficiently C1q and trigger bactericidal activity, a non-bactericidal titer for the anti-fHbp mAbs was an expected result, given the low level

of expression of fHbp in NGH38; furthermore, the level of expression of NHBA, despite being relatively high in this strain, is quantitatively lower than the one of fHbp, and this could explain why also most of the anti-NHBA mAbs were not bactericidal singly. One mAb anti-NHBA, 9E5, was bactericidal singly (SBA titer= 128), demonstrating that, in the presence of favorable epitopes, the density of NHBA in NGH38 can be sufficient to ensure activation of killing by monoclonal antibodies.

The mAbs were then tested for combined bactericidal activity, considering an at least 4-fold increase in bactericidal titer as a parameter defining mAb cooperativity (64). All the possible anti-fHbp/anti-NHBA combinations were tested. Most of the mAb combinations were not bactericidal, and three mAb combinations elicited a bacteriostatic titer (1A12 + 12E1, 1A12 + 10C3, 1G3 + 3A3). It is possible to speculate that these bacteriostatic mAb combinations can recruit C1q only on the surface of a part of the cell population in the reaction mixture, generating a bacteriostatic activity rather than an efficient killing of bacterial cells. A positive bactericidal titer (128) was obtained when the singly-bactericidal mAb 9E5 was combined with 1G3 and 14B3. However, it is possible to assume that this bactericidal titer is generated by the activity of mAb 9E5 *per se*, since the titer does not vary when compared with the one of the single mAb. Out of all the possible combinations, three were cooperative and consisted always in one human mAb combined with one murine mAb: 1A12+9E5, 4B3+9E5, 4B3+3A3. Two of these couples included one mAb already bactericidal *per se,* 9E5 anti-NHBA (1A12+9E5, 4B3+9E5), and one included non-bactericidal mAbs (4B3 + 3A3). The couples including one mAb which was singly bactericidal activated complement 2-fold more potently than the couples including singly non-bactericidal mAbs, triggering a 2-fold greater bactericidal titer. These results underline the efficiency of the epitope of 9E5 in triggering synergistic complement-mediated killing.

Moreover, it was observed that most of the couples could not elicit bactericidal activity, while three couples were synergic and elicited striking bactericidal activity. One of the key factors of this gap in combined bactericidal activity could be the epitope localization of the mAbs: it is possible that in the couples that are not bactericidal, the epitopes of the mAbs are located in reciprocal positions of the antigens that do not favor Fc-mediated C1q recruitment, and that in the cooperative couples the epitopes of the mAbs of the pairs are responsible for a reciprocal orientation of the Fcs that favors C1q recruitment when both of the antibodies are bound to the antigens on the cell surface. Overall, these results demonstrate that anti-fHbp and anti-NHBA

mAbs can cooperate in the activation of complement-mediated killing, but that this synergistic bactericidal activity can occur only in certain conditions and is not triggered always to the same extent. Another variable that probably influences the activation of this synergy, is the Fc region: while some human/human couples were able just to trigger a bacteriostatic activity, bactericidal synergy was observed only in human/murine couples. It is possible to hypothesize that the murine constant regions (in particular, the IgG ones) may play a role in potentiating the interaction of the Fcs with C1q, enhancing its recruitment and increasing complement activation.

Following the observation that anti-fHbp and anti-NHBA mAbs can cooperate, our experiments focused on elucidating the molecular mechanisms involved in synergistic antibody-mediated complement activation. Previous studies (35) reported that synergistic bactericidal activity of mAbs targeting fHbp leads to an enhanced deposition of the C4b on the bacterial surface, which ultimately leads in C3b deposition, membrane attack complex formation, and bacteriolysis. Our experiments used C3b deposition as a surrogate marker for complement activation, and focused on elucidating the molecular mechanisms on which inter-antigenic cooperativity is based, understanding whether synergistic C3b deposition occurs also in the synergy between mAbs recognizing different antigens or not. Therefore, we characterized by flow cytometry the levels of C3b deposition that is promoted when the strain NGH38 is incubated with complement and single mAbs, cooperative mAb couples, and non-cooperative mAb couples, under the hypothesis that mAb synergy leads to enhanced C3b deposition. We observed that a part of the cell population did not undergo to C3b deposition, while the other part showed a C3b deposition characterized by efficiency which was increasing with mAb bactericidal activity: single non-bactericidal mAbs (1A12, 22A4) and non-bactericidal mAb couples (1A12+22A4) elicited C3b deposition only in a limited part of the cell population, single bactericidal mAbs (9E5) elicited C3b deposition in a greater part of the population, and synergistic mAb couples (1A12+9E5) could trigger C3b deposition on the greatest portion of the cell population. These results show that synergistic mAb bactericidal activity, which is likely due to potentiated C1q engagment by the cooperative mAb couples, leads to greater activation of the processes involved in the production and deposition on the bacterial surface of the opsonin C3b, which is therefore deposited on a greater number of cells, that are subjected to complement-mediated lysis. Noteworthy, these results well fit with what we observed in SBA: when NGH38 was incubated with synergistic mAb couples in the bactericidal

assays, we observed an increased killing of meningococcal cells compared to what we observed for single mAbs and non-cooperative mAb couples, and this is likely the consequence of enhanced C3b deposition and activation of the downstream events that lead to lysis. Overall, these results showed that the enhancement of C3b deposition is a critical step for synergistic complement activation and leads to the more efficient killing of the target cells.

Additional experiments were performed to investigate on the possible influence of antigen density on mAb synergy. Two cooperative mAb couples (1A12+9E5, 4B3+9E5) were tested for capability of triggering complement-mediated killing of three strains expressing fHbp and NHBA at different levels (M07576, M13520, NGH38). The strain expressing both of the antigens at low levels (M07576) was killied neither by the single mAbs nor by the mAb couples; a strain expressing fHbp at a high level and NHBA at an intermediate level (M13520) was killed by single anti-fHbp mAbs and mAb couples, but not by single anti-NHBA mAbs; finally, a high-level expressor of NHBA and low-level expressor of fHbp (NGH38) was killed by single anti-NHBA mAbs and cooperative couples, but not by single anti-fHbp mAbs. A clear interplay between antigen density and susceptibility to complement-mediated killing can be observed in these results: when the antigen density is not sufficient to ensure C1q recruitment, *i.e.* when the molecules of one single antigen or of two antigens are sparsely disposed on the bacterial surface and they are too far from eachother to allow the engagement of C1q mediated by the Fcs of antigen-bound antibodies, complement-mediated killing is not triggered, whereas, when at least one antigen is expressed at sufficiently high levels, or a pair of antigens provides a sufficient total Fc density to ensure C1q recruitment, complement is activated and bacteria are killed. Furthermore, the most potent synergistic complement activation occurred when NHBA is highly expressed (rSBA titer of cooperative couples against NGH38 reached 8192), demonstrating the efficacy of this antigen in triggering bactericidal activity and therefore conferring protective immunity. These results showed that antigen density can critically influence complement-mediated killing triggered by mAbs targeting different antigens.

Finally, we investigated on the possible cooperativity between anti-fHbp and anti-NHBA mAbs with human complement. For our experiments, we used a high level fHbp expressor strain, M13520, and we tested a combination including a mAb capable of displacing FH from fHbp (4B3 anti-fHbp), under the hypothesis that displacement of FH from fHbp, when expressed at high levels, would lead to a downregulation of the fHbp-mediated inhibition of the classical pathway

and therefore lead to killing. We obtained results which showed that the overall potency in complement activation mediated by the mAb couple (4B3 anti-fHbp + 9E5 anti-NHBA) was weak, but greater than the one of single mAbs, demonstrating that anti-fHbp and anti-NHBA mAbs can cooperate in complement activation even with human complement. Bactericidal assays performed with the same strain and human complement, but with a mAb couple including a mAb that could not inhibit FH binding (1A12) resulted always in negative titers, underlining the crucial influence of FH binding inhibition in the activation of synergistic human complement-mediated killing.

Conclusions

This study provides advances in the understanding of the mechanisms involved in complement-mediated killing, focusing in particular on the initial mechanisms involved in the activation of complement against pathogenic bacteria. Previous studies demonstrated that mAbs targeting the same antigen and polyclonal sera targeting different antigens can synergistically activate complement-mediated killing, and that complement activation can be influenced by several factors, including antigen density, epitope and Fc regions. Our study aimed to understanding whether mAbs with distinct specificity (targeting different antigens) can synergistically activate complement, the molecular mechanisms playing a role in this synergy, and which variables can influence it. We successfully demonstrated that mAbs targeting distinct antigens can cooperate in the activation of complement-mediated killing both with rabbit and human complement. Furthermore, we reconfirmed that previously reported variables modulating complement activation such as epitope presentation, Fc regions and antigen density play a role also in this type of cooperativity, acting mainly on the step of C1q recruitment. Moreover, we demonstrated that enhanced C3b deposition is one of the main molecular mechanisms involved in promoting synergistic complement activation. Our study advances the scientific knowledge in the field of vaccinology: previous studies had shown that vaccines containing several antigens confer better protection than those containing only one antigen (69). Our study provides evidence supporting this theory and overall, it helps to elucidate the molecular mechanisms by which the presence of multiple antigens acting in synergy in one vaccine triggers a more protective immune response and therefore represents a remarkable added value of multicomponent protein-based vaccines. Furthermore, we contributed to establishing the rationale of implementing immunotherapies against infections and tumors based on a treatment with monoclonal antibody combinations, showing that when used in certain combinations, mAbs can potently activate complement effector

functions against target cells. Using cooperative antibody couples in immunotherapy could provide the advantage of eliciting a strong and consistent immune response against tumor or microbial pathogen cells, rather than using single mAbs, which could activate poorly complement, or polyclonal sera, which contain antibodies that activate complement with variable efficiency.

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The purpose/aim of this Phase 3b study conducted in Poland was to assess the safety of a Meningococcal Group B Vaccine and to collect blood donations to be used in furthering the development of vaccines against N. meningitidis. Healthy Adults from 18 to 50 Years of Age received 2 doses of legacy Novartis Meningococcal Group B Vaccine given 2 month apart. Informed consent form was signed by all subjects.
Bexsero™ is a trademark of the GSK group of companies.

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