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Molecular engineering of Ghfp, the gonococcal orthologue of *Neisseria meningitidis* factor H binding protein

Running title: Molecular engineering of Ghfp

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Abstract

The knowledge of sequences and structures of proteins produced by microbial pathogens is continuously increasing. Besides offering the possibility to unravel the mechanisms of pathogenesis at molecular level, structural information provides new tools for vaccine development, such as the opportunity to improve viral and bacterial vaccine candidates by rational design. Structure-based rational design of antigens can optimize the epitope repertoire in terms of accessibility, stability and variability. In the present study, we used epitope mapping information on the well characterized antigen of *Neisseria meningitidis* factor H binding protein (fHbp) to engineer its gonococcal homologue Ghfp. Meningococcal fHbp is typically classified in three distinct antigenic variants. We introduced epitopes of fHbp variant 1 onto the surface of the Ghfp, which is naturally able to protect against meningococcal strains expressing fHbp of variants 2 and 3. Heterologous epitopes were successfully transplanted, as engineered Ghfp induced functional antibodies against all three fHbp variants. These results confirm that structural vaccinology represents a successful strategy to modulate immune responses as well as a powerful tool to investigate on extension and localization of immuno-dominant epitopes.

Introduction

Neisseria meningitidis is still responsible for fatal disease worldwide (1). Glyco-conjugate vaccines against serogroups A,C,W,Y are available since early 2000s (2), while prevention of infection by meningococcus serogroup B (MenB) strains has to be afforded to alternative antigens due to the poor immunogenicity of the serogroup B polysaccharide and its structural similarity to human neural antigens, which raised concerns about the risk to induce auto-reactive antibodies (3). The research of novel candidates culminated with the development of two protein-based vaccines approved for use in humans, one (Trumenba®) licensed in U.S. for use in individuals 10 through 25 years of age (4, 5), the second (Bexsero®) recommended in more than 30 countries for all the age groups including infants (6). Both vaccines contain factor H-binding protein (fHbp, alternatively named rLP2086 or GNA1870), a lipoprotein expressed by a large majority of circulating strains (7) and able to elicit a potent protective immune response against serogroup B (8-11). fHbp plays a fundamental role during the meningococcal infection, providing the bacterium with a way to evade the host serum surveillance. The protein, secreted across the outer membrane, is able to bind and sequester the human complement regulator factor H on the bacterial surface. This interaction prevents the activation of the alternative complement pathway and protects meningococci from killing (12, 13).

fHbp shows a high level of genetic diversity. So far, more than 700 diverse fHbp peptide sequences are known, with amino acid identity ranging from about 62 to 99% (http://pubmlst.org/neisseria/fHbp/). On the basis of such variability, fHbp sequences have been classified as belonging to variant 1, 2 and 3 (8) or to sub-families A and B (9). Serological studies indicate that the genetic variability can have a profound influence in determining the ability of antibodies to kill fHbp-expressing strains, as the immune response elicited by each variant ensures poor coverage against strains expressing heterologous alleles (8, 9). Inclusion of additional antigens (11) or combinations of distant fHbp sub-variants (9), are both strategies pursued to expand the vaccine coverage to virtually all circulating meningococcal strains. The fHbp sub-variant 1.1, included in the vaccine Bexsero® (11), represents the prototypic member of variant 1. In the past, we engineered this molecule in order to expand its coverage to variants 2 and 3. The resulting chimeric protein was able to protect mice against a panel of meningococcal strains expressing all the three variants (14). Recently, the gonococcal homologue of fHbp (Ghfp)

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has been characterized by Jongerius et al. and proposed as an alternative broad-coverage vaccine candidate against meningococcal disease (15). Ghfp shows 60-94% of sequence identity to fHbp and demonstrated the ability to induce in mice antibodies able to kill natural meningococcal strains expressing different fHbp variants, although the effective response against variant 1 was relatively low and limited to the sub-variant 1.10. Moreover, Ghfp was unable to bind human factor H (15, 16), a desirable feature that can prevent partial masking of the protein surface to the immune system (15).

In the present work we explored the possibility to increase the coverage of the immune response raised by Ghfp against meningococcal strains by inserting on its surface epitopes of fHbp sub-variant 1.1.

The knowledge of the fHbp structure (17-20) provides the unique opportunity to deeply analyze distribution and accessibility of conserved and variant-specific residues. Moreover, a considerable ensemble of epitope mapping studies has been reported on fHbp. Pioneering mutagenesis studies identified critical residues for binding to bactericidal antibodies (21, 22). Subsequently, nuclear magnetic resonance (NMR) (23), hydrogen-deuterium exchange mass spectroscopy (HDX-MS) (24) and x-ray crystallographic studies (25) allowed remarkable progresses in mapping protective epitopes.

The ensemble of this information makes members of the fHbp family ideal candidates for rational design studies attempting to modulate their immunogenicity by the introduction of heterologous epitopes from different variants.

In order to introduce fHbp variant 1-specific epitopes onto Ghfp, we modified the gonococcal protein surface according to the information deriving from the NMR epitope mapping on fHbp. We previously mapped by NMR the epitope recognized by the monoclonal antibody MAb502 specific for fHbp sub-variant 1.1 (23). Here, we used the same approach to map the epitope of a second fHbp 1.1-specific monoclonal antibody called JAR 5 (26). Both MAb502 and JAR5 have been reported to induce a complement-mediated killing of meningococcal cells in presence of rabbit complement (22, 26). We decided therefore to introduce onto Ghfp both mAb502 and JAR5 epitopes. Mice immunized with the resulting chimeric proteins elicited sera able to kill a wide panel

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meningococcal strains belonging to variants 1, 2 and 3. This work represents an epitope mapping-based rational design that increased the antigenicity of Ghfp and it is in principle applicable to any vaccine candidate whose potential coverage is limited by sequence variability.

Materials and methods

Bacterial strains

Escherichia coli DH5α and BL21 (DE3) were purchased from Invitrogen and used as cloning and expression strain, respectively. Ampicillin (Sigma) was used at concentration of 100 μg ml^{-1}.

Antibody generation

The hybridoma cell line expressing JAR5 (26) was kindly provided by D. M. Granoff (CHORI). The murine IgG2b isotype monoclonal antibody JAR5 and the corresponding Fab fragment were produced and purified by Areta International SrL (Gerenzano, Italy).

NMR sample preparation and interaction studies

To express recombinant $^{2}H/^{15}N$ –labeled fHbp sub-variant 1.1 for NMR measurements, *E. coli* BL21(DE3) (pET21b-fHbp) was grown on M9 minimal medium in 80% $^{2}H_2O$ with the addition of glucose and 3.0 g of $^{15}NH_4Cl$ (98% isotopic enrichment, Aldrich), as the sole carbon and nitrogen source, respectively. The culture was induced at A_{590} of 4.0 with 1.4 mM sterile filtered isopropyl 1-thio-β-D-galactopyranoside (Sigma) for 70 min. The protein lacking the N-terminal leader peptide and the lipobox motif and containing a C-terminal 6x-His tag was purified by two chromatographic steps: Ni2+ affinity (His-Trap HP, 5-mL column; GE Healthcare), and cation exchange (HiTrap SP HP). Analytical gel filtration analysis showed that the recombinant protein was eluted as a monomer. The protein sample used for NMR experiments was subsequently dialyzed against 20mM sodium phosphate buffer at pH7.0. NMR samples contained 10% (v/v) $^{2}H_2O$ for NMR spectrometer lock.

The interaction between the Fab fragment of JAR5 with $^{2}H/^{15}N$-labeled fHbp sub-variant 1.1 was investigated with $^{1}H–^{15}N$ TROSY (Transverse Relaxation-optimized Spectroscopy)-HSQC experiments. All the NMR measurements were performed at 298 °K on a Bruker Avance 900 spectrometer, working at a 900.13-MHz

frequency and equipped with a cryogenically cooled probe. Titrations were performed on 0.4 mM $^{2}H/^{15}N$ – labeled fHbp 1.1 protein samples with the unlabeled JAR5 up to an fHbp–JAR5 molar ratio of 1:1.5. ^{1}H and ^{15}N resonance assignments for the fHbp sub-variant 1.1 protein were already available (27).

Cloning and expression of Ghfp mutants

The DNA sequence of *N. gonorrhoeae* strain FA1090 *ghfp* devoid of the region encoding the leader peptide and the N-terminal glycine stretch was used as starting point to generate the three chimeric proteins. The amino acid substitutions were introduced avoiding the use of rare codons for arginine. The 3 synthetic genes were purchased from GeneArt (Invitrogen) to include *Nde*I and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. Each gene was digested with *Nde*I/ *Xho*I and cloned into the corresponding sites of the pET21b(+) vector (Novagen). The expression vectors were transformed into *E. coli* BL21 (DE3). The recombinant cells were grown at 37°C to an optical density at 600 nm of ~0.5, at which time 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added and the cultures were allowed to grow for 3 hours. Cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C.

Protein Purification

Bacterial pellets were re-suspended in 10 ml of Buffer A IMAC (50 mM NaH_2PO_4 [Sigma], 300 mM NaCl [Fluka], 30 mM imidazole [Merck]; pH 8.0), sonicated and then centrifuged at 35,000 × *g* for 30 min. The supernatant was collected and subjected to two serial purification steps using metal affinity chromatography (IMAC) and ionic exchange chromatography with a desalting step in between. All purification steps were performed using an AKTAXpress chromatographic system, and the OD_{280} was monitored. For the IMAC purification step, filtered supernatants were automatically injected into 1-ml Ni^{2+}-HisTrap HP columns with a flow rate of 1 ml/min, and columns were washed with 20 column volumes (CV) of washing buffer (50 mM NaH_2PO_4 [Sigma], 300 mM NaCl [Fluka], 30 mM imidazole [Merck]; pH 8.0). Then, the His tag fusion proteins were eluted with 5 CV of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole; pH 8.0), and automatically loaded on three 5-ml HiTrap (GE) desalting columns connected in series and eluted with a flow rate of 5 ml/min in 50 mM Tris-HCl pH8.0. For ionic exchange chromatography, the eluted proteins were

automatically loaded on 1-ml HiTrap Q HP columns with a flow rate of 1 ml/min. Subsequently, the column was washed with 10 CV of 50 mM Tris-HCl, pH 8.0. The elution was set up in a linear gradient, between 50 mM Tris-HCl (pH 8.0) and 50 mM Tris-HCl, 1.0 M NaCl (pH 8.0) buffer in 10 CV, and 1-ml fractions were collected. Protein purity resulted more than 95% for all the samples, as by densitometry analyses of SDS-Page 12% gel. Protein aggregation and apparent Molecular weight were checked by analytical size-exclusion chromatography (Waters Acquity UPLC system equipped with BEH200 1.7 mm column 4,6x300mm (waters), 150 mM NaP buffer pH 7.0 at a flow rate of 0.4 ml/min). All the protein samples resulted more that 95% in the monomeric form. A table summarizing the features of the purified recombinant proteins is reported as Table S1 in Supplementary Material.

Surface Plasmon Resonance (SPR) analysis

SPR was used to analyze the binding of fHbp and chimeric proteins to MAb502 and JAR5. All SPR experiments were performed using a Biacore T200 instrument at 25 °C (GE Healthcare). In brief a carboxymethylated dextran sensor chip (CM-5; GE Healthcare) was prepared where high densities (~10,000 response units (RUs)) of anti-mouse antibodies from a commercially available Mouse Antibody Capture Kit (GE Healthcare) were immobilized by amine coupling. The anti-mouse IgG chip was used then to capture ~1000-1500RU of MAb 502 and JAR5. Proteins, purified as described before, and diluted in buffer contained 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.05% (vol/vol) P20 surfactant, pH 7.4 (HBS-EP), to a final concentration of 200 nM for the single injection experiments and to a range of five consecutive injections of increasing analyte concentration (2.5 nM to 40 nM) for the Single Cycle Kinetics (SCK) experiments (28) were injected over the captured antibodies. Surfaces were then regenerated with 10 mM glycine pH 1.7. Anti-mouse antibody-coated surfaces without captured monoclonal antibody were used as the reference channel. A blank injection of buffer only was subtracted from each curve, and reference sensorgrams were subtracted from experimental sensorgrams to yield curves representing specific binding. The data shown are representative of at least two independent experiments. SPR data were analyzed using the Biacore T200 Evaluation software (GE Healthcare). For the SCK experiments each sensorgram was fitted with the 1:1 Langmuir binding model, including a term to account

for potential mass transfer, to obtain the individual k_{on} and k_{off} kinetic constants; the individual values were then combined to derive the single averaged K_D values reported.

Binding to human factor H was also analyzed in two experimental setups. First, purified full length factor H (Calbiochem) was covalently immobilized by amine coupling on a CM5 chip to reach a density of ~2500 RU. Proteins at a concentration of 200nM in PBS were injected and binding levels were compared. Regeneration between injections was achieved by a single injection of 10mM Glycine pH 3, 3M NaCl solution. In order to assess the effect of several factor H concentrations on the binding, proteins were covalently immobilized by amine coupling on a CM5 chip on different flow cells to reach a density of ~300-400 RU. Full length factor H was then injected at increasing concentrations (0.07-2µM) and binding to the different surfaces was compared. Following each injection, sensor chip surfaces were regenerated with a 30-second injection of 50mM NaOH.

Differential Scanning Calorimetry (DSC)

Thermal stability of the mutants was checked by DSC experiments performed with a MicroCal VP-Capillary instrument (GE Healthcare) with integrated auto-sampler. Samples were dialyzed in PBS to a final protein concentration of 0.5 mg/ml. DSC scans were recorded in the temperature range of 10-110°C with a thermal ramping of 200°C per hour and a 4 second filter period. Data were analyzed by subtraction of the reference data for a sample containing only buffer, using the Origin 7 software (OriginLab).

Antigen formulation

All formulations were performed in sterile conditions under a flow hood. Each recombinant protein was adsorbed onto aluminum hydroxide at protein, aluminum (alum), and NaCl concentrations of 100 µg/ml, 3 mg/ml, and 9 mg/ml, respectively, in 10 mM histidine (pH 6.5). Water for injection and histidine buffer were premixed. Sodium chloride was added to result in a final formulation osmolality of 0.300 mosmol/kg. Alum addition was calculated on the basis of the concentration of the alum stock to obtain a final concentration of 3 mg/ml. Antigens at respective concentrations were added to the mix and left for 15 min under stirring at room temperature and then stored overnight at 4°C before the immunization. Final formulations were isotonic and at

physiological pH. All alum formulations were characterized soon after immunization, antigen adsorption was >90%, and adsorption profile was similar for all antigens and adjuvants tested.

Bactericidal activity assay

To prepare antisera, 20 µg of protein were used to immunize 6-week-old CD1 female mice (Charles River). Eight mice per group were used. The antigens were administered intra-peritoneally (i.p.), together with aluminum hydroxide (3 mg/ml), on days 0, 21, and 35. Two weeks after the third immunization, the sera were collected and pooled. Serum bactericidal antibody activity of mice immune sera was evaluated as previously described by Borrow et al (29) against the *N. meningitidis* strains listed in Table 1. Pooled baby rabbit serum was used as the complement source. Bactericidal titers in presence of rabbit complement (rSBA) were expressed as the reciprocal of the final serum dilution step giving ⩾50% killing at 60 min compared to the number of CFU at time zero.

Fluorescence-Activated Cell Sorter (FACS) analysis of fHbp expression

The ability by mouse polyclonal anti-fHbp sera to bind the surface of meningococci was measured using a 1:100 dilution of mouse polyclonal antiserum raised by the same fHbp variant when available, or by closely related alleles (25). Primary antibody binding was detected by using an anti-mouse (whole-molecule) FITC-conjugated antibody (Sigma) at a 1:100 dilution.

Results

NMR epitope mapping

fHbp 1.1 is one of the protein sub-variants more frequently found during epidemiological surveys worldwide (30, 31). For this reason, several studies have been reported describing the epitopes of this protein recognized by bactericidal monoclonal antibodies. In a previous study, we mapped the fHbp site recognized by MAb502 in one of edges of the carboxyl-terminal beta barrel domain (23). Previous mutagenesis work reported by Beernink et al. indicated that JAR5 targets the N-terminal domain of fHbp, in a region overlapping the binding site to factor H

(21). Such preliminary evidences suggested that MAb502 and JAR5 recognized well distinct regions of the fHbp surface and were able to cooperate in inducing a protective immune response against fHbp sub-variant 1.1 (21). To identify the residues forming the JAR5 epitope we applied the same NMR-based approach that we previously used to map MAb502 (23). Briefly, ^{1}H, ^{15}N HSQC NMR spectra were acquired on $^{2}H/^{15}N$ –labeled fHbp in presence and absence of the JAR5 Fab fragment. Changes in the chemical environment caused by Fab binding were expected to change the chemical shift of backbone NH groups. Residues of fHbp experiencing chemical shift changes upon the addition of JAR5 are listed in Table S2. With the exception of Gln38, Ser39 and Asn43, all the affected residues were localized on four adjacent beta strands of the N-terminal domain (Fig.1A). Gln38, Ser39 and Asn43 were not considered as a part of the JAR5 epitope, due to the distance from all the other perturbed residues. Remarkably, the epitope defined by NMR includes both Gly121 and Lys122, previously identified by Beernink and colleagues as essential for binding to JAR5 (21).

The ensemble of perturbed residues formed a solvent accessible surface of 2860 $Å^2$, a value exceeding the range of 900-2000 $Å^2$ typical of conformational epitopes characterized so far (32-34). We cannot however exclude that some perturbation effects could be ascribed to local conformational rearrangements occurring after the interaction with the antibody rather than direct contact with JAR5, leading to an over-estimation of the epitope extension.

No overlap was observed between JAR5 and MAb502 epitopes (Table S2 and Fig.1B) while, in line with the observation that JAR5 can inhibit the fHbp interaction with factor H (21), JAR5 epitope resulted largely overlapping to the factor H binding site (Table S2)

Design of chimeric Ghfps

The aim of this work was to design a broad coverage antigen against *Neisseria meningitidis* by engineering the Ghfp surface. We selected as a scaffold the Ghfp from *Neisseria gonorrhoeae* strain FA1090, which encodes a fHbp homologue unable to bind human factor H and closely related to members of fHbp variant 3 (Fig.2). The substitution of some surface-accessible residues of Ghfp with amino acids specifically present in fHbp variant 1 was expected to result in the creation of a chimeric molecule containing epitopes of both variants. Extension and

235 localization of the fHbp sub-variant 1.1 epitopes recognized by MAb502 and JAR5 were used to identify the
236 portions of the Ghfp surface to be modified.

237 In order to produce chimeric proteins able to elicit antibodies cross-protective across all sub-variants 1, we
238 aligned the amino acid sequences of fHbp 1.1. to 1.3, 1.14 and 1.15, the most divergent sub-variants among the
239 highly common isolates belonging to variant 1 (30) (Fig.S3). Then, selected groups of concurrent substitutions
240 deduced from the multiple sequence alignment were introduced in each chimeric protein. In Fig.3 the amino acid
241 sequences of the wild-type gonococcal scaffold and the meningococcal allele MC58 used to elicit in mice both
242 JAR5 and MAb502 are compared to the mutants. Overall, 29, 30 and 31 amino acid substitutions were made on
243 FA1090 to generate NG_5.2, NG_5.6 and NG_5.8 respectively. The resulting three mutants, as well as Ghfp and
244 the fHbp sub-variants 1.1 and 3.28 were expressed in *E. coli* as hexa-histidine-tagged proteins.

245 The effects of the substitutions on thermal stability of the proteins were investigated by DSC. In DSC
246 experiments a melting temperature (Tm) value is given by the peak maximum in the scanned curve. Differently
247 from the meningococcal fHbp, typically showing two very well distinct transitions (Tm1 at 70°C and Tm2 at 80-
248 90°C), corresponding to N- and C-terminal domains respectively (17), the thermal unfolding of Ghfp appeared to
249 be much more cooperative. The DSC profile of the gonococcal protein could be de-convoluted in two nearly
250 overlapping peaks with very similar melting temperatures (Tm1 at 58°C and Tm2 at 67°C) and a sensibly low
251 enthalpy in the case of the second transition (Fig.4A).

252 All the mutants showed values of Tm1 similar to the gonococcal wild type, while Tm2 sensibly increased in
253 NG_5.2 and NG_5.6, reaching values more in line with those observed for the C-terminal domain of
254 meningococcal fHbp (Fig.4B). We concluded therefore that mutations introduce to mimic the MAb502 epitope
255 stabilized the C-terminal domain of NG_5.2 and NG_5.6, while the JAR5-related mutations left the N-terminal
256 domain substantially unaffected.

257 **Functional analysis of the mutants**

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The interaction of each mutant with the full length human factor H was tested by SPR, in order to evaluate whether substitutions had any impact on such interaction. Differently from the strong concentration-dependent interaction observed between fHbp variant 1.1. and human fH, no binding was detectable in the case of Ghfp and all the immobilized mutants to increasing concentrations of human factor H up to 2μM (Fig.5). These results led to exclude that any residue necessary to re-establish the interaction was introduced by the JAR5 epitope grafting.

In order to check the ability by the mutants to properly present the MAb502 and JAR5 epitopes, the interaction with each monoclonal antibody was also investigated by SPR (Table 2 and Fig. 6). As expected, substitutions introduced in NG_5.2 conferred to the molecule the ability to bind MAb502 with affinity comparable to fHbp sub-variant 1.1. Binding to MAb502 was instead compromised in NG_5.6 and NG_5.8. In both these mutants the gonococcal serine 204 was replaced by histidine (Fig. 3), the residue naturally occurring in sub-variants 1.14 and 1.15 (Fig. S3). We hypothesize that the absence of arginine 204, previously identified as critical for the interaction of fHbp sub-variant 1.1 with MAb502 (22), prevented the binding to NG_5.6 and NG_5.8.

All the three mutants were able to bind JAR5 with comparable affinity, although only NG_5.2 showed the slow dissociation rate characteristic of fHbp sub-variant 1.1.

Overall the SPR analysis provided a preliminary indication that surface regions corresponding to MAb502 and JAR5 epitopes were successfully introduced on the gonococcal protein and sufficiently well exposed on the protein surface to be recognized by respective monoclonal antibodies.

The immunogenicity of NG_5.2, NG_5.6 and NG_5.8 was then evaluated by a serum bactericidal assay (SBA) on the strains reported in Table 1. To confirm the fHbp accessibility to antibodies, we first probed the meningococcal strains by FACS (Fig. 7). Mouse polyclonal sera elicited by homologous or closely related fHbp sub-variants were used to detect fHbp on the bacterial surface. FACS profiles revealed that fHbp was well accessible to antibodies in all the strains tested. Moreover, strains with higher (MC58, M01-02400660, M08-02400104), intermediate (M12566, M01-0240988, M01-02400355,) or lower (M14879, NZ98/254, M1239,

LNP024551) fHbp accessibility could be distinguished, suggesting that sequence diversity and protein exposure could both have influence on the bactericidal titers.

Groups of eight mice were immunized with NG_5.2, NG_5.6 or NG_5.8. Controls included animals vaccinated with Ghfp, fHbp sub-variants 1.1 and 3.28. The ability of the chimeric proteins to elicit functional antibodies was evaluated by measuring the complement-mediated killing induced by the immune sera *in vitro*. Values of rSBA <16 were considered negative as this is the starting dilution for the experiments.

A summary of the rSBA analysis is reported in Table 3. Ghfp induced a bactericidal immune response against meningococcal strains expressing fHbp variants 2 and 3 but failed to protect mice against variant 1. Conversely, bactericidal activity against variant 1 was observed, at different levels, after vaccination with each of the three mutants. Complement mediated killing of all the variant 1 isolates was induced by sera of mice immunized with NG_5.6 and NG_5.8. Despite that fact that NG_5.2 was the only mutant able to bind MAb502 (Fig. 5B), the NG_5.2 immune sera exhibited moderate bactericidal titers all the variant 1 strains. The loss of bactericidal activity against NZ98/254 and the low titer against M14879 could be due to the limited amount of fHbp detected on their surface (Figure 7). To explain the relatively low titers observed against MC58 we speculated that few mismatches of surface-exposed residues like aspartate 163 (glycine in MC58) and histidine 178 (asparagine in MC58) (Fig.3) might have counteracted the positive effects of the epitope grafting. Alternatively, changes in the conformational equilibrium induced by the NG_5.2-specific substitutions (Fig.4) could have indirectly influenced the variant 1 epitope presentation.

NG_5.6 and NG_5.8 retained also the ability to kill all the strains of variant 2 and variant 3, although a sensible decrease in bactericidal activity compared to the gonococcal wild type was observed, particularly against the low fHbp expressing strains of variant 3 M1239 and LNP24551. Bactericidal titers against variants 2 strains were also elicited by NG_5.2. This mutant resulted unable to promote the complement-mediated killing against M1239 and LNP24551 (Table 3), likely due to the combined effect of low fHbp abundance and sequence diversity.

Discussion

Molecular grafting of functional epitopes is a promising way to improve variable antigens or realize novel proteins with pre-specified functionalities. Side chain and backbone remodeling have been recently proposed as protein design strategies to stabilize and optimize protein antigens for presentation of contiguous conformational epitopes (35-37).

In a previous study, we engineered the C-terminal domain of fHbp 1.1 by introducing residues specific of variants 2 and 3 within patches of about 1000 A^2 (14). The mutagenesis was applied to the entire immuno-dominant carboxyl-terminal domain of the protein, whose surface was systematically explored in order to identify the region(s) able to well tolerate the epitope grafting in terms of folding and immunogenicity. In the present work we decided to explore the possibility to modulate the immunogenicity of the gonococcal fHbp orthologue Ghfp by selectively grafting pre-defined meningococcal epitopes from the distantly related fHbp sub-variant 1.1.

A deep structural knowledge of sub-variant 1.1-specific protective epitopes allowed limiting the mutagenesis on the regions of protein surface specifically recognized by anti-sub-variant 1.1 antibodies. We previously reported the characterization by NMR of the epitope recognized by the murine monoclonal antibody MAb502. The antibody binding site covered a surface of 1992 A^2 entirely located on one apex of the carboxyl-terminal domain of the protein and distant from the site of interaction with factor H (23). In the present study, we mapped the epitope of a second monoclonal antibody, JAR5, previously reported to target Gly121 and Lys122 on fHbp sub-variant 1.1 and able to inhibit the binding to factor H (21). Such observations suggested that the region recognized by JAR5 was well distinct from the MAb502 epitope. The present results confirmed this prediction. The JAR5 epitope identified by NMR resulted entirely located within the N-terminal domain of fHbp excluding any overlap with the region recognized by MAb502. Remarkably, the JAR5 epitope resulted localized in the

same region where the epitopes of two murine IgG1 monoclonal antibodies (17C1 and 30G4) were previously mapped by hydrogen-deuterium exchange mass spectrometry (24). Both 17C1 and 30G4 displayed, although to different extents, synergistic bactericidal activity against strains of variant 1 when used in combination with MAb502. These results suggested that co-transplantation of the JAR5 and MAb502 epitopes in a fHbp variant 3-like environment could potentially result in a molecule able to induce a potent protective immunity against variant 1 strains.

Serum bactericidal activity assay measures the ability of immune sera to mediate killing of meningococci *in vitro,* in presence of an exogenous source of complement. In a previous study, Jongerius and colleagues evaluated the ability by Ghfp to induce bactericidal antibodies against meningococcal strains expressing variants 1, 2 or 3 (15). They tested a panel of seven isolates and observed comparable bactericidal activity across the three variants. A remarkable exception was represented by MC58 strain (fHbp sub-variant 1.1), which was resistant to killing by anti-Ghfp antibodies. In the present work, we analyzed a different set of meningococcal isolates, which included four strains expressing different sub-variants 1. All the fHbp variant 1-expressing isolates tested were not killed by anti-Ghfp serum according to the observation that molecules of variant 3 do not induce bactericidal antibodies against variant 1 (8, 38).

Ghfp induced bactericidal antibodies against M12566 and M1239, expressing fHbp 2.19 and 3.28 sub-variants respectively, with SBA titers comparable to those reported by Jongerius et al. for the same sub-variants (15). M08-240104 and M01-240355 strains, expressing fHbp 2.16 and 3.4 sub-variants respectively, showed a more pronounced sensitivity to the bactericidal activity of the anti-Ghfp immune sera. In the case of M01-240355 this could be due to the highest sequence similarity to Ghfp of the fHbp 3.4 sub-variant (93.51 % of identity at amino acid level) compared to all the other meningococcal strains of the panel (Table S3). The M08-240104 sequence 87% identical to Ghfp cannot be however invoked to explain the high sensitivity of this strain to the Ghfp immune serum. It is possible that the high fHbp expression level, together with the conservation of a small number of specific residues residing within crucial epitopes, render M08-240104 more susceptible to killing by anti-Ghfp antibodies.

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Overall, the results of the bactericidal activity assay showed that the mutagenesis was able to introduce a local molecular mimicry of fHbp variant 1 sufficient to elicit antibodies bactericidal against a panel of natural meningococcal strains expressing different sub-variants. In particular, NG5.6 and NG5.8 were both able to elicit a protective immune response against all variant 1 strains tested, included isolates expressing some of the most prevalent alleles like 1.1 and 1.13 (7).

A general decrease of bactericidal titers against variant 3 was observed in sera elicited by the mutants compared to those obtained by the immunization with the wild type gonococcal protein. The total area including MAb502 and JAR5 epitopes accounts for about 13% of the fHbp surface and both epitopes resulted localized on the predicted accessible side of the molecule. The changes introduced in the gonococcal protein reduced the surface area available to elicit variants 3 specific antibodies. This could has been particular critical in the case of variant 3 strains, were fHbp expression levels were generally lower compared to variant 1. Alternatively, the modifications could have specifically altered epitopes critical for variant 3. Finally, we cannot exclude that modifications in the amino acid sequence could have introduced some local conformational change of the molecule that altered the original epitope repertoire. The DSC profile of the mutants indicated that substitutions increased the thermal stability of the proteins, presumably stabilizing the overall fold. However, how this could reflect changes in the immunogenicity remains unclear.

In conclusion, we enhanced the potential of Ghfp as a vaccine candidate by threading in defined portions of its surface two well characterized heterologous functional epitopes. Although a clear correlation between the bactericidal titers obtained in mice with rabbit complement and bactericidal response in humans has not been yet defined, the positive titers reported in the present study indicate that the chimeric proteins have the potential to raise protective immunity against a wider panel of meningococcal strains compared to native Ghfp. The detailed epitope characterization obtained by NMR provided valuable information for antigen optimization, permitting to limit the mutagenesis within restricted regions of the protein surface and minimize the changes of naturally occurring sequences. This aspect assumes particular relevance for the optimization of large proteins where molecular dimensions and sequence variability could require the screening of a massive number of mutants.

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Acknowledgments

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We declare that we have not conflicts of interest.

Figure Legends

Figure 1 A - NMR mapping of the epitopes recognized by JAR5 and MAb502. Residues involved in the interaction with JAR5 are depicted in green (A). The epitope of mAb502 is colored in red and reported in panel B according to Ref. (23).

Figure 2 - Classification tree of the different fHbp alleles used in this study. Ranges of amino acid sequence identity of fHbp variants 1, 2 and 3 to Ghfp are reported in brackets. Multiple sequence alignment has been carried out with ClustalW (39) available at NPS@ server. Dendrogram was obtained at Phylogeny.fr server with TreeDyn (40).

Figure 3 - Multiple sequence alignment of the engineered proteins (NG_5.2, NG_5.6 and NG_5.8) to the wild type Ghfp and the fHbp sub-variant 1.1. The asterisk marks positions 163, 178 and 204, critical for mAb502 binding to fHbp sub-variant 1.1.

Figure 4 - DSC analysis of engineered Ghfp proteins. The overlapping peaks in the melting curve of Ghfp (Panel A, grey line) have been calculated by applying a non-2 state fitting model according to the Levenberg-Marquardt non-linear least-square method using Origin 7 software. All the mutants generated two well distinct peaks (Panel B), consistent with two unfolding events.

Figure 5 - Interaction of immobilized engineered proteins with factor H analyzed by SPR. BIAcore sensorgrams show the dose dependent response over time (resonance units [RU]) during the binding of increasing concentrations of factor H (up to 2µM) on immobilized recombinant fHbp while no binding is observed with the immobilized Ghbp proteins.

Figure 6 - Interaction of engineered Ghfp proteins with JAR5 (A) and mAb502 (B) analyzed by SPR. Representative BIAcore sensorgrams show the response over time (resonance units [RU]) during the binding of purified recombinant proteins to immobilized mAbs.

Figure 7 - FACS analysis of fHbp surface expression and factor H binding of *N. meningitidis* strains used in this study. The presence of fHbp on the meningococcal cell surface was detected by binding of mice polyclonal sera

411 elicited the same fHbp sub-variant when available, or by closely related alleles. In each panel, amino acid
412 identity between fHbp used to immunized mice and the genetic variant expressed by the strain tested is reported
413 in brackets. Shaded and white profiles show reaction with pre-immune and immune serum, respectively.

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TABLES

Strain	Clonal complex	ST	Year	Country	Serogroup:serotype:serosubtype	fHbp sub-variant
MC58	32	74	1985	UK	B:15:P1.7,16b	1.1
M14879	1157	1157	2006	USA	B:NA:P1.22,14-6	1.13
NZ98/254	41/44	42	1998	NZ	B:4:P1.4	1.14
M01-0240660	269	269	2001	UK	B:NA:P1.19,15	1.15
M08-240104	35	35	2008	UK	B:4:P1.14	2.16
M12566	41/44	5111	2004	USA	B:4,7:P1.4	2.19
M1239	41/44	437	1995	USA	B:14:P1.23, 14	3.28
M01-240988	213	213	2001	UK	B:1:NA	3.30
M01-240355	213	213	2001	UK	B:1:NA	3.31
LNP24551	32	34	2008	FR	B:4:P1.5,2	3.116

Table 1 – Meningococcal strains used in this study. The fHbps are named in terms of the translated (protein) sequence, as variant class.protein ID, in accordance with the public fHbp database (http://pubmlst.org/neisseria/fHbp/), in which new protein variants are assigned a sequential numerical identifier, alongside a prefix corresponding to the Novartis variant designation (variant 1, 2, or 3). For example, fHbp 1.1 refers to Novartis sub-variant 1, neisseria.org protein subvariant 1. ST, sequence type as determined by MLST; NA, not assigned

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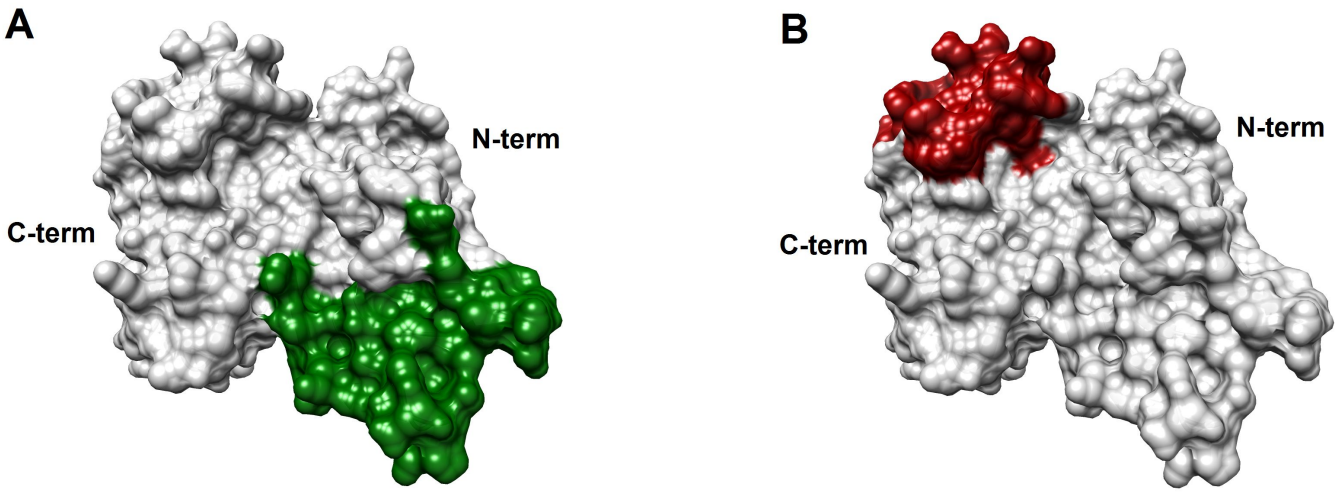
mAb	protein	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
MAb502	fHbp	1,93 E+06	0.84 E-02	4.34 ± 0.03 E-09
MAb502	NG 5.2	0.63 E+06	2.85 E-03	4.53 ± 0.01 E-09
JAR5	fHbp	0.81 E+06	2.15 E-04	2.63 ± 0.001 E-10
JAR5	NG 5.2	0.58 E+06	2.15 E-04	3.71 ± 0.02 E-10
JAR5	NG 5.6	0.82 E+06	3.71 E-03	4.53 ± 0.09 E-09
JAR5	NG 5.8	0.88 E+06	3.86 E-03	4.39 ± 0.15 E-09

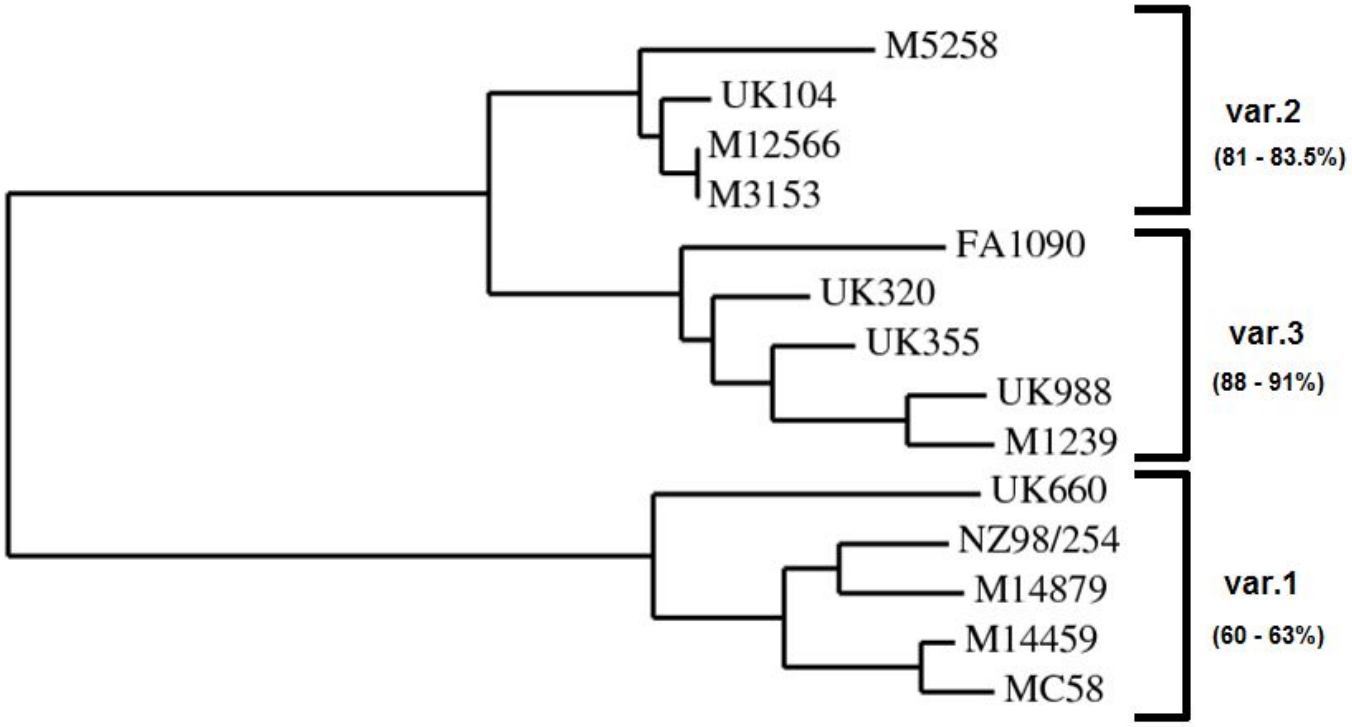
578

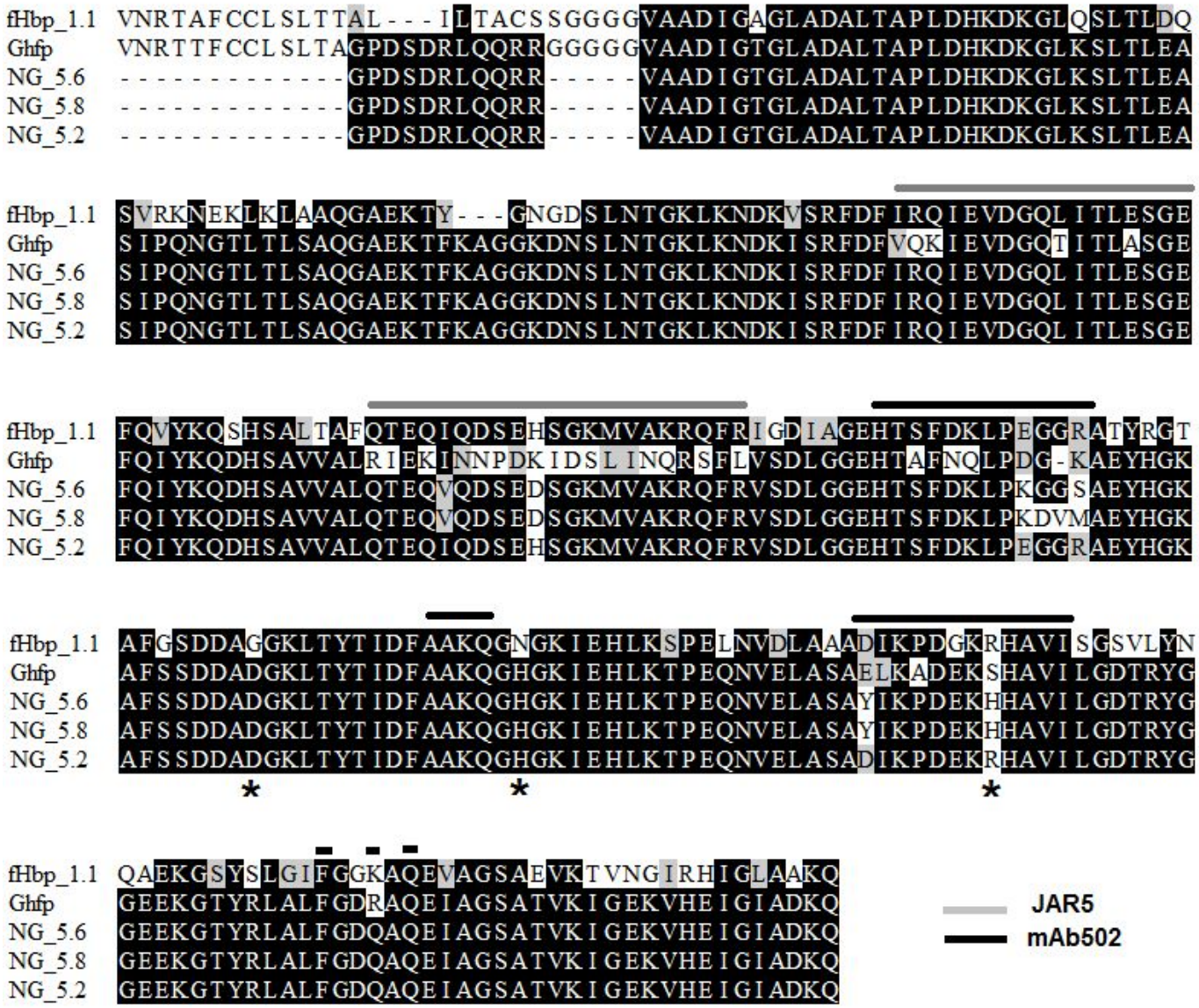
579 Table 2. Summary table of SCK experiments of the monoclonal antibodies binding to the Ghfp proteins with k_{on},
580 k_{off} and K_D measurements. Examples of sensorgrams are reported in the Supplementary Material section (Fig. S5)

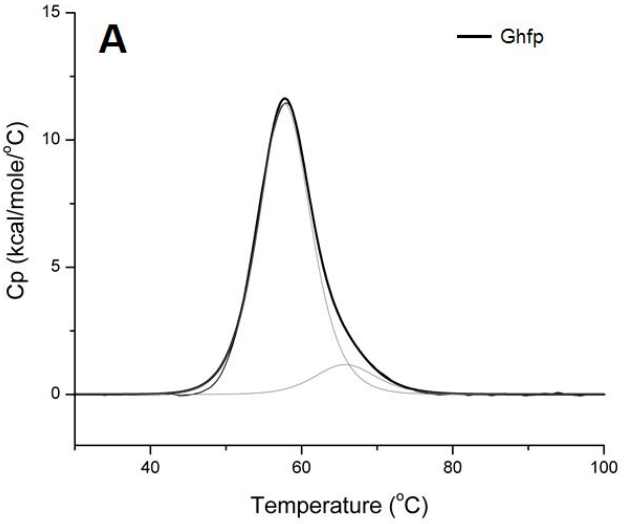
rSBA							
		antigen					
STRAIN	fHbp variant	NG5.6	NG5.8	NG5.2	NGFA1090	fHbp 3.28 (M1239)	fHbp 1.1 (MC58)
MC58	v1.1	**512**	**128**	**256**	<16	<16	>8192*
M14879	v1.13	**1024**	**1024**	64	<16	<16	1024
NZ98/254	v1.14	**256**	**512**	<16	<16	<16	128*
M01-240660	v1.15	**4096**	**4096**	**512**	<16	<16	2048
M08-240104	v2.16	**2048**	**128**	**256**	**≥8192**	**2048**	16
M12566	v2.19	**128**	**128**	**1024**	**512**	**256**	<16
M1239	v3.28	64	16	<16	**256**	**2048**	<16*
M01-240988	v3.30	**512**	**128**	32	**512**	32	<16
M01-240355	v3.31	**256**	**256**	**128**	**2048**	**512**	<16
LNP24551	v3.116	64	64	<16	**512**	<16	<16

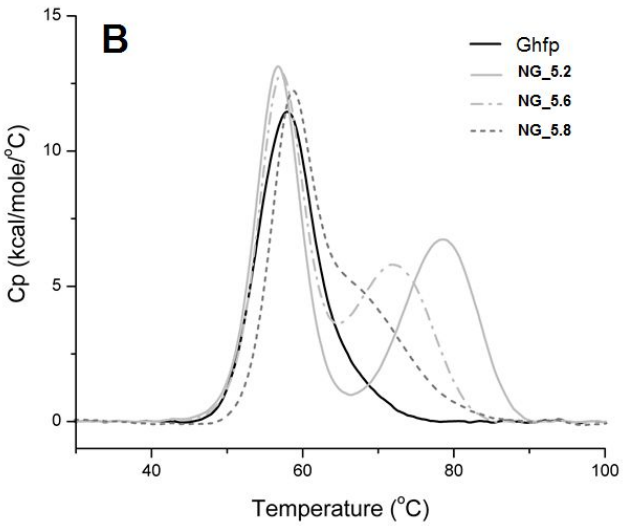
Table 3 - Serum bactericidal titers elicited in mice by engineered antigens against the panel of strains described in Table 1. Wild type Ghfp, as well as fHbp sub-variant 1.1 and 3.28 have been also included as control.
* from Ref. 37

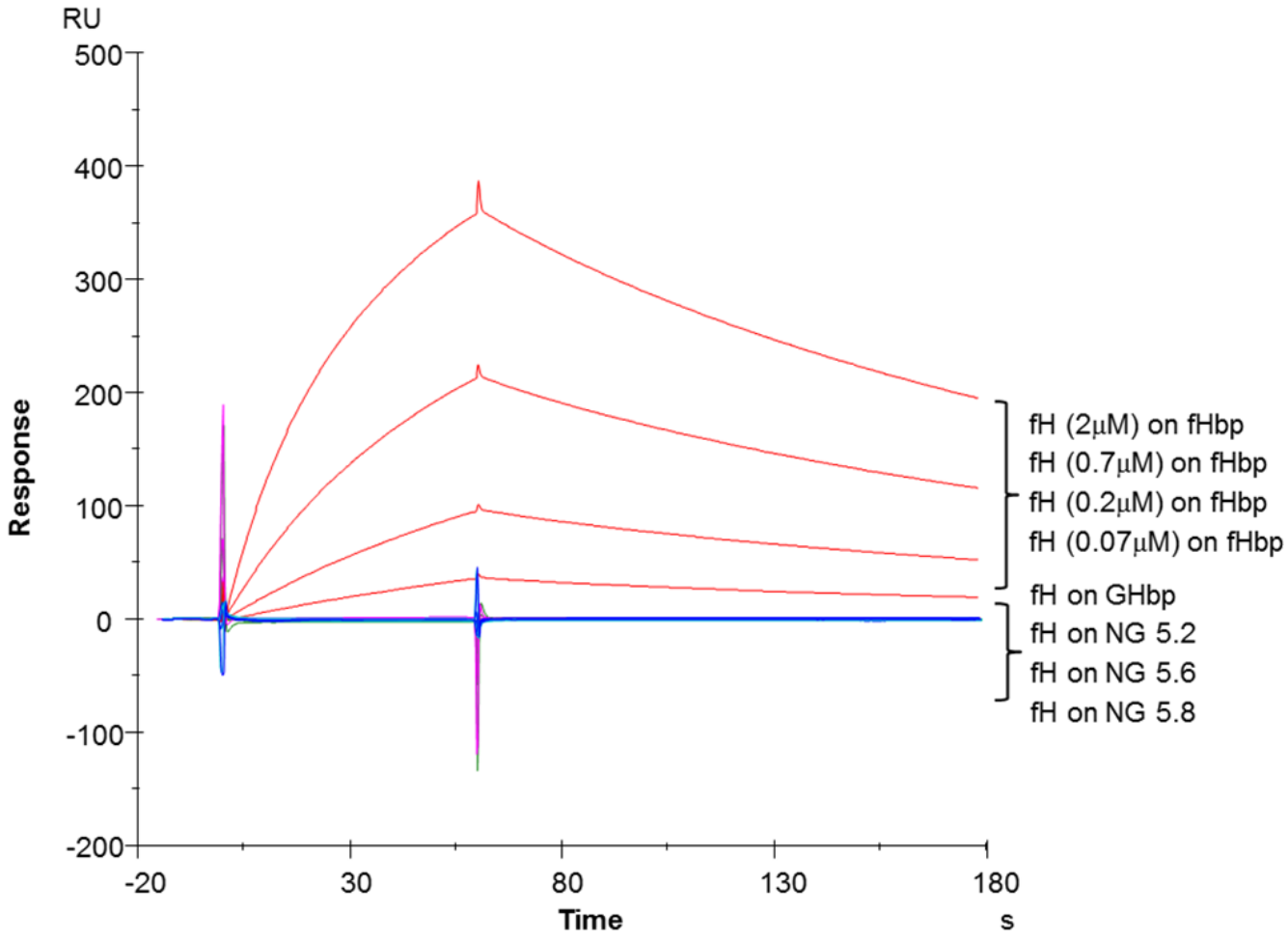


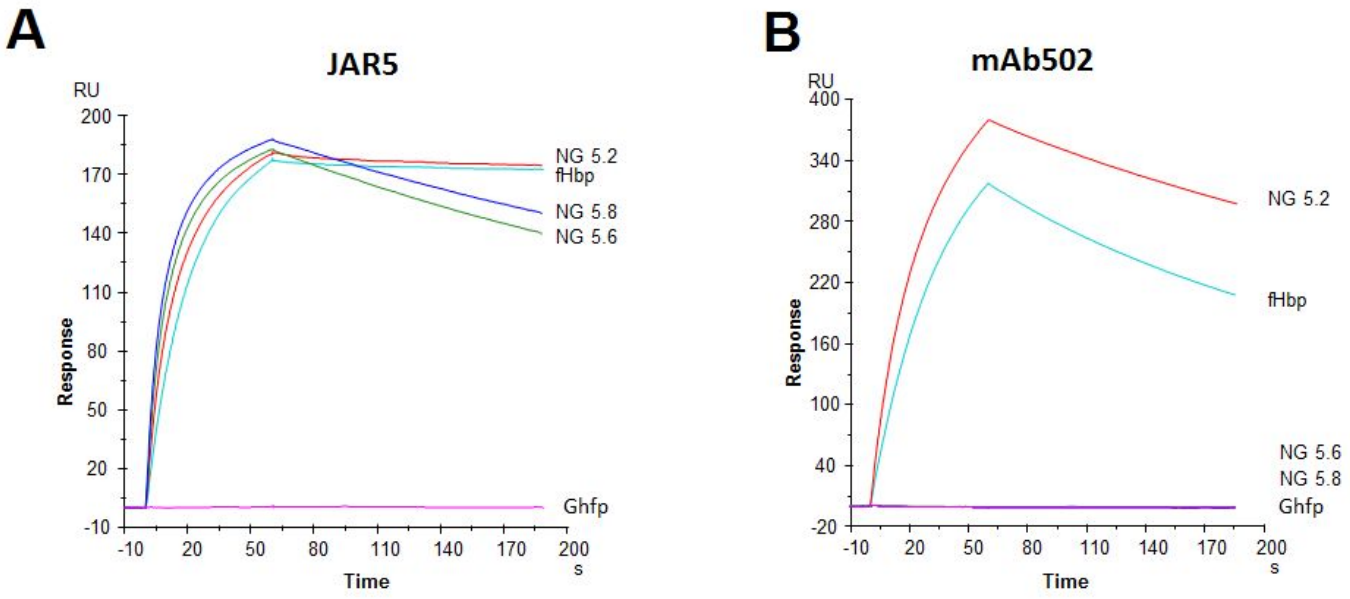


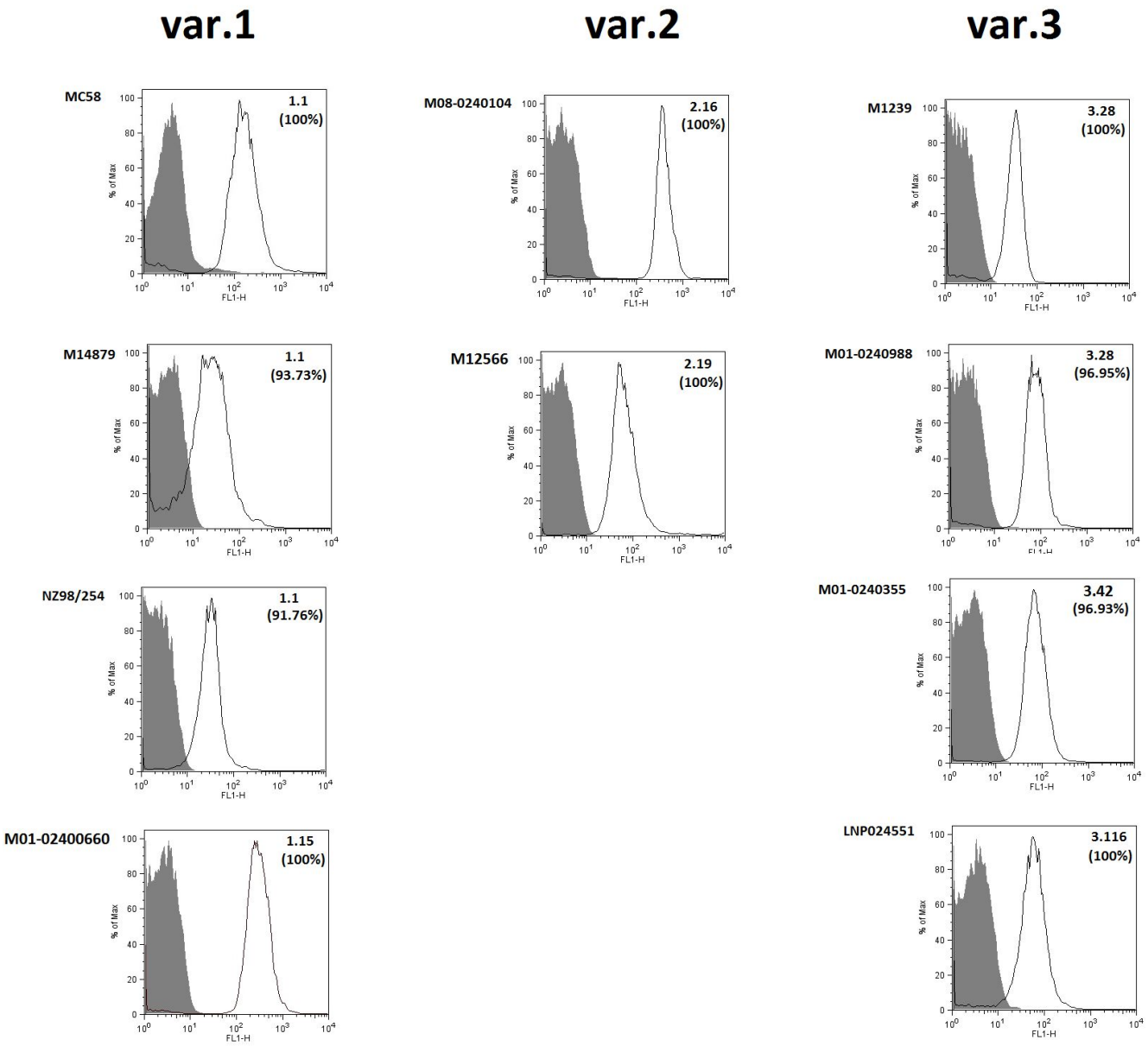












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M08-240104	35	35	2008	UK	B:4:P1.14	2.16
M12566	41/44	5111	2004	USA	B:4,7:P1.4	2.19
M1239	41/44	437	1995	USA	B:14:P1.23, 14	3.28
M01-240988	213	213	2001	UK	B:1:NA	3.30
M01-240355	213	213	2001	UK	B:1:NA	3.31
LNP24551	32	34	2008	FR	B:4:P1.5,2	3.116

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JAR5	NG_5.2	0.58 E+06	2.15 E-04	3.71 ± 0.02 E-10
JAR5	NG_5.6	0.82 E+06	3.71 E-03	4.53 ± 0.09 E-09
JAR5	NG_5.8	0.88 E+06	3.86 E-03	4.39 ± 0.15 E-09

rSBA							
		antigen					
STRAIN	fHbp variant	NG_5.6	NG_5.8	NG_5.2	Ghfp	fHbp 3.28 (M1239)	fHbp 1.1 (MC58)
MC58	v1.1	**512**	**128**	**256**	<16	<16	>8192*
M14879	v1.13	**1024**	**1024**	64	<16	<16	1024
NZ98/254	v1.14	**256**	**512**	<16	<16	<16	128*
M01-240660	v1.15	**4096**	**4096**	**512**	<16	<16	2048
M08-240104	v2.16	**2048**	**128**	**256**	**≥8192**	**2048**	16
M12566	v2.19	**128**	**128**	**1024**	**512**	**256**	<16
M1239	v3.28	64	16	<16	**256**	**2048**	<16*
M01-240988	v3.30	**512**	**128**	32	**512**	32	<16
M01-240355	v3.31	**256**	**256**	**128**	**2048**	**512**	<16
LNP24551	v3.116	64	64	<16	**512**	<16	<16