Meningococcal Factor H Binding Protein Vaccine Antigens with Increased Thermal Stability and Decreased Binding of Human Factor H

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Neisseria meningitidis causes cases of bacterial meningitis and sepsis. Factor H binding protein (FHbp) is a component of two licensed meningococcal serogroup B vaccines. FHbp recruits the complement regulator factor H (FH) to the bacterial surface, which inhibits the complement alternative pathway and promotes immune evasion. Binding of human FH impairs the protective antibody responses to FHbp, and mutation of FHbp to decrease binding of FH can increase the protective responses. In a previous study, we identified two amino acid substitutions in FHbp variant group 2 that increased its thermal stability by 21°C and stabilized epitopes recognized by protective monoclonal antibodies (MAbs). Our hypothesis was that combining substitutions to increase stability and decrease FH binding would increase protective antibody responses in the presence of human FH. In the present study, we generated four new FHbp single mutants that decreased FH binding and retained binding of anti-FHbp MAbs and immunogenicity in wild-type mice. From these mutants, we selected two, K219N and G220S, to combine with the stabilized double-mutant FHbp antigen. The two triple mutants decreased FH binding >200-fold, increased the thermal stability of the N-terminal domain by 21°C, and bound better to an anti-FHbp MAb than the wild-type FHbp. In human–FH-transgenic mice, the FHbp triple mutants elicited 8- to 15-fold-higher protective antibody responses than the wild-type FHbp antigen. Collectively, the data suggest that mutations to eliminate binding of human FH and to promote conformational stability act synergistically to optimize FHbp immunogenicity.

Neisseria meningitidis serogroup B is one of the leading causes of bacterial meningitis and sepsis in North America and the European Union (1, 2). The disease burden is highest in infants (2), who have not yet developed natural immunity, and in young adults living under crowded housing conditions, such as dormitories and military barracks. Two protein-based vaccines were recently developed to protect against meningococcal serogroup B disease. One of the vaccines, MenB-FHbp (Pfizer), is licensed in the United States; the second, MenB-4C (Bexsero; GSK), is licensed in the United States, the European Union, Australia, and Canada. MenB-4C is now part of the routine immunization program in the United Kingdom. In the United States, both vaccines are recommended for persons at increased risk of acquiring meningococcal serogroup B disease, including those with persistent complement deficiencies, those potentially exposed during serogroup B outbreaks, and microbiologists with routine exposure to N. meningitidis (3).

Both of the licensed serogroup B vaccines include factor H binding protein (FHbp), which is a highly sequence-variable surface antigen; more than 930 amino acid sequence variants have been identified (http://pubmlst.org/neisseria/FHbp). Based on amino acid sequence identity, FHbp variants can be classified in two subfamilies (4), three variant groups (5), or 10 modular groups (6). The two licensed vaccines contain divergent FHbp sequence variants in variant group 1, which corresponds to subfamily B. In addition, the MenB-4C vaccine containing non-lipidated FHbp uses aluminum as an adjuvant, whereas the MenB-FHbp vaccine relies on aluminum and the adjuvant properties of the lipid moieties of the two FHbp variants. The MenB-FHbp vaccine includes an FHbp sequence variant from each of the two subfamilies (7), whereas the MenB-4C vaccine includes FHbp and three other protective antigens (8, 9). Thus, two different strategies were used to expand the cross-protection by antibodies elicited by the licensed vaccines against diverse meningococcal strains.

Meningococci recruit the complement regulator factor H (FH), using FHbp (10) and several alternative ligands, including neisserial surface protein A (NspA) (11) and porin B2 (PorB2) (12). By binding FH using one or more of these ligands, meningococci downregulate complement alternative pathway amplification, which renders the bacteria more resistant to complement-mediated killing. Antibodies to FHbp elicit complement-mediated bactericidal activity and can inhibit binding of FH to FHbp, which defeats this bacterial evasion mechanism. However, in human–FH-transgenic mice, binding of FH to the FHbp vaccine antigen decreases protective antibody responses, possibly by interfering with antigen uptake, processing, or presentation. To overcome this limitation of the FHbp antigen, considerable effort has been devoted to identifying mutant FHbp antigens with decreased binding of FH, including structure-based (13–16) and mutant library (17) approaches. Candidate mutants have been identified in variant groups 1 (13, 14, 16), 2 (15, 16, 18), and 3 (16), and a subset of these mutants have been evaluated in human–FH-transgenic-mouse immunogenicity models.
In previous studies, we investigated the vaccine potential of FHbp ID 22 in variant group 2, since this sequence variant is prevalent in serogroup W strains in sub-Saharan Africa (19, 20) and the same or similar sequence variants are present in serogroup B strains in the United States and the European Union (1, 21). Additional studies showed that FHbp antigens in variant group 2 were less thermally stable than those in variant group 1 or 3 (16, 18). We recently stabilized an FHbp variant group 2 protein by replacement of two amino acid residues, L130 and G133, with their counterparts, R130 and D133, from a variant group 1 protein (22). The L130R G133D double mutant had 21°C-higher thermal stability of the N-terminal domain and 7-fold-decreased binding of human FH compared to the wild-type FHbp ID 22 (22).

In the present study, we combined the amino acid substitutions previously reported to increase FHbp stability (22) with new, additional single-amino-acid substitutions to decrease further binding of human FH to FHbp antigens. Collectively the data show the vaccine potential of several novel FHbp mutants in variant group 2, which could be combined with other FHbp sequence variants or other meningococcal antigens to increase the protective antibody responses to maximize the breadth of protection against meningococci.

MATERIALS AND METHODS

Identification and purification of FHbp mutants. An error-prone PCR mutant FHbp library was displayed on the surface of Escherichia coli as previously described (17). Mutant clones with low binding of purified human FH and high binding to a control murine anti-FHbp monoclonal antibody (MAb) (JAR 41) were isolated by fluorescence-activated cell sorting (FACSArta; BD Biosciences), and mutants were identified by DNA sequencing of the FHbp gene. Single FHbp mutants were generated from plasmid pET21b-FHbp ID 22 by inverse PCR using Phusion polymerase and the protocol in the Phusion site-directed mutagenesis kit (Thermo-Fisher Scientific). The oligonucleotide primers used for specific mutagenesis (5′-3′) were as follows: F129S_r, GCTCTTGTACGCCTTTGG; F129S_r, TGAGACGGTTGTATCAGCT; V131D_f, ACAGCGTTGATGAGG; V131D_r, TTACCGAGGCTGC; K219N_f, TGGCACTTACCACCTCGCCCT; K219N_r, TTCTCTTGTCC; CGCGCTGAGG; G220S_r, AGCACTTACCACCTCGCC; G220S_r, TTACCGAGGCTGC; L130R/G133D_r, AGGATTTGTCGGAGAA; L130R/G133D_r, GACACACAGGAGGCTGTTATAT. The oligonucleotide primers had first been phosphorylated with polynucleotide kinase (New England BioLabs). The L130R G133D double mutant, which was engineered to increase thermal stability, was described previously (22). Triple mutants were constructed using the pET21b-FHbp ID 22 L130R G133D mutant as the plasmid template and the appropriate primers.

Soluble FHbp mutants lacking the N-terminal signal sequence were expressed in E. coli BL21(DE3) from pET21-based plasmids. FHbp containing a C-terminal hexahistidine tag was purified by Ni²⁺ affinity and ion-exchange chromatography as previously described (17). Purified FHbp was diazylated against phosphate-buffered saline (PBS) containing 3% sucrose and stored at −30°C prior to use. The FHbp concentration was determined from the absorbance at 280 nm (Nanodrop 1000; Thermo-Fisher Scientific) using a molar extinction coefficient of 10,430 M⁻¹ cm⁻¹, which was calculated with ProtParam (23; http://web.expasy.org/protparam/).

Binding of human FH and anti-FHbp MAbs to FHbp. Binding of human FH to FHbp was measured in an enzyme-linked immunosorbent assay (ELISA) (14) with up to 200 µg/ml of purified human FH, which was purified from human serum using a HiTrap NHS column (5 ml; GE Life Sciences) that had been coupled with 5 mg purified recombinant FHbp ID 1 using the manufacturer’s protocol. Binding of control murine anti-FHbp MAbs, JAR 4 (24), JAR 13 (25), and JAR 31 (26), to FHbp was measured by ELISA (27). Data are shown as the mean and standard error (SE) of four replicates obtained in two independent experiments.

Differential scanning calorimetry. Protein thermal stability was measured using a VP-DSC instrument (MicroCal). FHbp was dialyzed against PBS overnight, and the concentration was adjusted to 0.5 mg/ml. The reference solution was PBS that had been used as the dialysis buffer. Data were collected at a scan rate of 60°C/h using passive feedback mode. Reference data were subtracted, and the data were normalized with Origin 5 software (MicroCal); a non-2-state unfolding model was used to calculate transition midpoint (T_m) and enthalpy change (ΔH) values. Representative data from one of two or three independent experiments are shown.

Immunogenicity in mice. Groups of 4-week-old, female, wild-type CD-1 mice (n = 10 to 21 per group) were immunized with recombinant FHbp vaccines containing 10 µg of protein and 600 µg of aluminum hydroxide (Alhydrogel; Brenntag Biosector) as the adjuvant. Two doses were administered intraperitoneally 3 weeks apart, and blood was collected 3 weeks after the second dose. Human-FH-transgenic BALB/c mice were immunized using similar procedures, except that 2- to 4-month-old mice of both genders were used and three doses of vaccine were given. Prior to immunization, the mice were screened by ELISA to identify those with serum human FH concentrations of >240 µg/ml. The groups were randomized to ensure that the mean ages, human FH concentrations, and gender distributions of the groups were similar. The experiments in mice were performed in strict accordance with the recommendations in the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Children’s Hospital Oakland Research Institute.

Measurement of serum antibody responses. IgG anti-FHbp titers of sera from individual mice were measured by ELISA as described previously (28) but using purified recombinant FHbp ID 22 as the target antigen. The titers were assigned as the interpolated serum dilution needed to obtain an optical density at 405 nm (OD₄₀₅) of 1.5. The complement-mediated bactericidal antibody activities of individual mouse sera were measured using exogenous human serum, which had been depleted of IgG using a HiTrap Protein G column (5 ml; GE Life Sciences), as the complement source (19). The meningococcal serogroup B test strains had identical or closely matched FHbp sequence variants; the strains were 03S-0658 and 03S-0673 (both with FHbp ID 23 with 99.6% identity to the FHbp ID 22 wild-type vaccine antigen) (29) and a mutant of strain H44/76 that expresses FHbp ID 22 (100% identity) (30). Human serum used to purify FH for ELISA or as a source of complement in bacterial assays was obtained with written informed consent under a protocol approved by the Institutional Review Board of UCSF Benioff Children’s Hospital Oakland.

Statistical analysis. Statistical tests were performed on the mean log₁₀ transformed antibody titers. Unpaired, nonparametric t tests (Mann-Whitney tests) using two-tailed hypotheses were used for all comparisons. P values of <0.05 were considered to be significant.

RESULTS

Immunogenicity of an FHbp mutant with increased stability. As described in the introduction, we previously engineered a double mutant, L130R G133D, of FHbp ID 22 in variant group 2 that had 21°C higher thermal stability of the N-terminal structural domain (22). In the present study, to evaluate whether the mutations that increased the thermal stability of FHbp also affected its immunogenicity in the absence of binding of human FH, we immunized groups of wild-type CD-1 mice with two doses of the recombinant FHbp ID 22 wild-type or double-mutant protein. The two FHbp vaccines elicited similar serum IgG anti-FHbp antibody geometric mean titers (GMTs) (P = 0.30) and similar serum bactericidal antibody GMTs (P = 0.83 by Mann-Whitney test) (Fig. 1). The

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locations of the two amino acid substitutions are shown in the crystal structure of the double-mutant FHbp (Fig. 2A, yellow). The factor H binding, thermal stability, and immunogenicity of the double mutant, as well as the new mutants described below, are summarized in Table 1.

New FHbp mutants with low binding of human FH. The two substitutions in FHbp ID 22 that increased the thermal stability in our previous study also decreased binding of human FH by 7-fold compared with the wild-type FHbp (22). Since previous studies of FHbp mutants suggested that at least 30- to 100-fold decreases in FH binding were needed to achieve optimal immunogenicity in the presence of human FH (17,18), in the present study, we sought additional FHbp substitutions to decrease FH binding further. Using a random mutant FHbp library displayed on the surface of E. coli (see Materials and Methods), we identified two mutants, F129S and V131D, that resided in the N-terminal domain and two mutants, K219N and G220S, in the C-terminal domain (Fig. 2A, red). The amino acid substitutions were located in the FH binding site, which spans both domains of FHbp, as seen in the structure of FHbp in variant group 2 (22) modeled as a complex with human FH (Fig. 2B).

The purified recombinant wild-type FHbp, which lacked the N-terminal signal sequence and lipidation site, had high FH binding (Fig. 3A), as we had observed previously (31). Based on the FH concentrations needed to obtain an optical density of 1.0 (Fig. 3A, horizontal dotted line), the K219N and G220S mutants decreased FH binding by approximately 30- to 200-fold, respectively, and the F129S and V131D mutants decreased FH binding by >200-fold compared with the wild-type protein (Fig. 3A). We next measured the binding of three murine anti-FHbp MAbs to the FHbp mutants to probe their conformational integrity. MAb 4, which recognizes a conformational epitope in the N-terminal domain of FHbp (32), bound to the mutants in the C-terminal domain, K219N and G220S, but not to the mutants in the N-terminal domain, F129S and V131D (Fig. 3B). MAb 13, which recognizes an epitope in the C-terminal domain of FHbp (25), bound to all of the mutants except the G220S mutant (Fig. 3C). MAb 31, which also recognizes an epitope in the C-terminal domain (our unpublished observations), bound similarly to the wild-type protein and all four mutants (Fig. 3D).

Immunogenicity of FHbp mutants with low FH binding. Since mouse FH does not bind to meningococcal FHbp, mutant FHbp antigens with low binding of human FH are not expected to impact the antibody responses of wild-type mice unless the mutations disrupt important epitopes. As a test of vaccine potency, therefore, we immunized groups of wild-type mice with two doses of the new recombinant FHbp mutants. As a positive control, we immunized a group of mice with the wild-type FHbp, and as a negative control, we immunized another group of mice with the aluminum hydroxide (Alhydrogel) adjuvant alone. Because the
mutants were identified at different times, two new FHbp mutants were tested in each of two independent experiments. After two vaccine doses, we measured the serum IgG anti-FHbp titers of individual mice by ELISA (Fig. 4A and B). The IgG GMTs for the mice that were immunized with each of the four new mutants were not significantly different from those for the wild-type FHbp (P > 0.56 by Mann-Whitney test).

As a test of antibody functional activity, we measured human complement-mediated bactericidal activity in the sera of individ-

### TABLE 1 Summary of properties of FHbp mutants

<table>
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<tr>
<th>FHbp variant</th>
<th>FH binding (fold decrease)</th>
<th>Thermal stability T&lt;sub&gt;m&lt;/sub&gt; (°C)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bactericidal antibody titer (ratio to WT FHbp)</th>
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</thead>
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<td>C terminal</td>
<td>WT mice</td>
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<td>38.5/82.3</td>
<td>1.0</td>
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<td>59.4/81.2</td>
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<td>ND</td>
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</tr>
<tr>
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<td>ND</td>
<td>0.3</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>L130R G133D</td>
<td>&gt;200</td>
<td>59.6/66.3</td>
<td>ND</td>
</tr>
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</table>

*<sup>a</sup> T<sub>m</sub>, transition midpoint from differential scanning calorimetry experiments.

*<sup>b</sup> ND, not determined.

*<sup>c</sup> Tg, transgenic.

FIG 3 Binding of human FH and anti-FHbp MAb to FHbp mutants. (A) Binding of purified human FH to wild-type FHbp or site-specific mutants by ELISA. Bound FH was detected using a sheep anti-human FH antibody. (B) Binding of murine anti-FHbp MAb JAR 4, which binds to an epitope in the N-terminal domain of the protein (32). (C) Binding of anti-FHbp MAb JAR 13, which binds to an epitope in the C-terminal domain of the protein (25). (D) Binding of anti-FHbp MAb JAR 31. In panels B through D, bound murine MAb were detected with goat anti-mouse IgG. All the data are from two independent experiments, each performed in duplicate.

FIG 4 Serum antibody responses of mice immunized with FHbp mutants. (A and C) Experiment 1. (B and D) Experiment 2. (A) Serum IgG anti-FHbp titers in individual mice immunized with the WT or the V131D or K219N mutant measured by ELISA (n = 17 to 21 mice per group). The horizontal lines represent the geometric mean titers (GMTs). Alum, negative-control mice immunized with Alhydrogel adjuvant alone. Mice with titers less than the lowest dilution tested (1:100) were assigned a titer of half that value. (B) In an independent immunization experiment, the serum IgG anti-FHbp titers elicited by two additional mutants, F29S and G220S (n = 10 mice per group), were measured as described for panel A. (C) Serum bactericidal antibody titers measured against serogroup B strain 035-0673, which expresses a closely matched FHbp in variant group 2 (99.6% identity to the WT vaccine antigen). Mice with titers less than the lowest dilution tested (1:10) were assigned a titer of half that value. (D) Serum bactericidal antibody titers measured as described for panel C. The horizontal lines represent the GMTs.
ual mice. The bactericidal GMTs elicited by the new mutants K219N, F129S, and G220S, were not significantly different from that elicited by the respective wild-type FHbp (P = 0.62 by Mann-Whitney test) (Fig. 4C). In contrast, the V131D mutant elicited a 6-fold-lower serum bactericidal GMT than the wild-type FHbp (244 versus 1,569; P = 0.0001 by Mann-Whitney test) (Fig. 4C).

Characterization of FHbp triple mutants. Binding of human FH to the K219N and G220S triple mutants was much lower than to the wild-type protein and also lower than to the respective single mutants (Fig. 5A and B). MAb JAR 4 bound to the K219N triple mutant slightly more than to either the wild-type protein or the single mutant (Fig. 5C). Whereas MAb JAR 4 bound to the G220S single mutant much less than to the wild-type protein, the triple mutant containing G220S bound the MAb more than the wild type (Fig. 5D). In contrast, binding levels of JAR 31 to the wild-type and single- and triple-mutant proteins were indistinguishable (data not shown).

For comparison with the triple mutants, the thermal stabilities of the FHbp ID 22 wild-type and double-mutant proteins are shown (Fig. 6A). The thermal stability of the K219N triple mutant was similar to that of the L130R G133D double mutant, with a slightly higher Tm for the C-terminal domain of the triple mutant (Fig. 6B). In contrast to the other FHbp mutants, the G220S triple-mutant protein exhibited two overlapping transitions (Fig. 6C). The first transition, which corresponded to the unfolding of the N-terminal domain, was similar in temperature to the L130R G133 double mutant. The C-terminal domain of the G220S mutant unfolded at 66°C, which was about 16°C lower than the C-terminal domain of either the wild-type or the L130R G133 double-mutant protein.

Immunogenicities of FHbp triple mutants in human-FH-transgenic mice. We tested the immunogenicities of the FHbp triple mutants containing L130R G133D to increase stability and K219N or G220S to further decrease human FH binding in groups of human-FH-transgenic BALB/c mice. The transgenic mice received three doses of recombinant FHbp vaccines at 3-week intervals. There were no significant differences between the IgG anti-FHbp GMT of either mutant and that of the control wild-type FHbp (P = 0.0001) (Fig. 7B). In contrast, the serum bactericidal GMTs elicited by the K219N and G220S triple mutants were 8- and 15-fold higher, respectively, than that elicited by the wild-type protein (P = 0.0001) (Fig. 7B).

DISCUSSION

In previous studies, FHbp antigens that were engineered to decrease binding of FH had increased immunogenicity in the pres-
eficacy of human FH (14, 18, 33). In a recent study, we constructed a random mutant FHbp library displayed on the surface of E. coli to identify additional substitutions that decreased binding of human FH to FHbp ID 1, which is a component of a licensed meningococcal serogroup B vaccine (MenB-4C) (17). In that study, we adapted an ELISA (28) to use purified human FH concentrations as high as 200 μg/ml (17), which approaches the concentrations in human serum (approximately 280 μg/ml) (14).

In the present study, we applied a method to display a random mutant FHbp library on the surface of E. coli (17) to a divergent FHbp sequence variant, ID 22, in variant group 2. We identified four new FHbp mutants with decreases in FH binding similar to or larger than those of variant group 2 mutants that previously had been identified by structure-based approaches (15, 16, 18). The K219N mutant had an ~30-fold decrease in FH binding, which was similar to that of the previously described D211A mutant (15, 18). The other three mutants, F129S, V131D, and G220S, had at least 200-fold decreases in FH binding. Interestingly, amino acid residues F129 and V131 are hydrophobic residues that are near the FH epitope recognized by JAR 4, which is in the N-terminal domain (32). The triple mutant containing G220S restored binding to MAb JAR 4, and its binding to the MAb was better than that of the wild-type FHbp ID 22 antigen. The K219N triple mutant also bound JAR 4 better than the wild-type antigen, which confirms our previous observation that epitopes in the N-terminal domain are stabilized by increasing the thermal stability of the domain.

Previously, we observed inverse correlations between the amount of FH binding and FHbp immunogenicity. In one study, FHbp ID 1 mutants with the largest decreases in FH binding elicited the highest protective antibody responses (17). In another study using a different FHbp sequence variant, ID 22, the D211A mutant with a 30-fold decrease in FH binding elicited higher protective antibody responses in human-FH-transgenic mice than the T221A mutant with a 15-fold decrease in FH binding (18). In the present study, we observed a similar pattern in the ID 22 triple mutant containing G220S, which had a >200-fold decrease in FH binding (Fig. 5B) and elicited higher protective antibody responses than the K219N triple mutant (Fig. 7B). Taken together, these studies suggest that the most effective FHbp antigens have the least possible FH binding.

Despite the prevalence of FHbp in variant group 2 in epidemic
strains in Africa (20), the United States (1), and the European Union (21), neither of the currently licensed meningococcal serogroup B vaccines includes an FHbp antigen from this variant group. Several preclinical studies have been conducted using FHbp variant group 2 antigens (27, 34, 35). Since FHbp variant group 2 is more prevalent than variant group 3 (1, 21) and there is a high degree of cross-protection between them (26, 36), these antigens have the potential to provide greater coverage against the majority of prevalent disease-producing strains. The two licensed vaccines contain FHbp as a key antigen, and invasive meningococci lacking an fhbp gene have been identified (37). Since meningococci have redundant mechanisms to bind FH and other complement downregulators (38), inclusion of additional virulence factors that mediate complement evasion may delay the emergence of escape mutants.

Meri and coworkers recognized that binding of a host protein to a microbial vaccine antigen might decrease its immunogenicity and proposed that modification of the vaccine antigen so that it no longer binds the host protein would increase its immunogenicity (39). Based on our previous studies that confirmed these predictions (14), we combined amino acid substitutions to decrease binding of human FH and to increase thermal stability. The engineered antigens elicited as much as 15-fold higher protective antibody responses in the presence of FH that binds FHbp, as evaluated in human–FH-transgenic mice. Future studies of such FHbp mutants in nonhuman primates, such as rhesus macaques, in which FH binds FHbp (40), and/or humans will be needed to demonstrate further the ability of engineered FHbp antigens to increase the efficacy of FHbp vaccine antigens while decreasing their potential to elicit autoantibody anti-FH antibodies (41, 42).

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