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Enhanced protective antibody to a mutant meningococcal factor H-binding protein with low-factor H binding

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Meningococcal factor H-binding protein (FHbp) is an antigen in 2 serogroup B meningococcal vaccines. FHbp specifically binds human and some nonhuman primate complement FH. To investigate the effect of binding of FH to FHbp on protective antibody responses, we immunized infant rhesus macaques with either a control recombinant FHbp antigen that bound macaque FH or a mutant antigen with 2 amino acid substitutions and >250-fold lower affinity for FH. The mutant antigen elicited 3-fold higher serum IgG anti-FHbp titers and up to 15-fold higher serum bactericidal titers than the control FHbp vaccine. When comparing sera with similar IgG anti-FHbp titers, the antibodies elicited by the mutant antigen gave greater deposition of complement component C4b on live meningococci (classical complement pathway) and inhibited binding of FH, while the anti-FHbp antibodies elicited by the control vaccine enhanced FH binding. Thus, the mutant FHbp vaccine elicited an anti-FHbp antibody repertoire directed at FHbp epitopes within the FH binding site, which resulted in greater protective activity than the antibodies elicited by the control vaccine, which targeted FHbp epitopes outside of the FH combining site. Binding of a host protein to a vaccine antigen impairs protective antibody responses, which can be overcome with low-binding mutant antigens.

Introduction

Microbial virulence factors that interact with host complement regulators have been proposed to be attractive vaccine candidates (1). One example is factor H-binding protein (FHbp), which is an antigen in 2 recently licensed meningococcal serogroup B vaccines (2, 3). The protein was discovered independently by 2 groups of investigators based on its ability to elicit complement-mediated serum bactericidal activity in mice (4, 5). Subsequent studies found that the antigen bound a complement downregulator, FH (6). Binding of FH enhanced resistance of the bacteria to the alternative complement pathway (6, 7), which is an important mechanism by which meningococci evade innate host defenses (8, 9). To reflect this important virulence mechanism, the antigen was renamed FHbp (6).

Additional studies showed that binding of FH to FHbp was specific for human and some nonhuman primate FH (9–11). When humans are vaccinated, the FHbp antigen is thought to form a complex with FH, whereas when WT mice are vaccinated, there is no complex formation. In human FH transgenic mice (12–15) and infant rhesus macaques (16, 17), binding of FH to FHbp vaccines impaired protective serum anti-FHbp antibody responses. Further, mutant FHbp vaccines containing single amino acid substitutions that decreased FH binding elicited higher serum bactericidal antibody responses in human FH transgenic mice than control FHbp vaccines that bound human FH (12, 14, 18, 19).

The FHbp antigens in currently licensed meningococcal serogroup B vaccines bind human FH. Humans immunized with these vaccines develop complement-mediated serum bactericidal antibody responses (20–23). However, the activity is low against some strains (24, 25), and the possible negative effect of binding of human FH to the vaccine on impairing human anti-FHbp bactericidal antibody responses has not been investigated because, to date, all of the FHbp vaccines tested bound human FH.

In an infant macaque model, even low binding of FH to the FHbp antigen diverted the anti-FHbp antibody repertoire to epitopes outside of the FH binding site (16, 17) and lowered serum bactericidal titers (17). In the present study, we describe a mutant FHbp with 2 amino acid substitutions that resulted in even lower binding of human FH than our previously described low-FH binding mutant vaccine candidate containing a single amino acid substitution (12). The purpose of the present study was

Conflict of interest: DMG and PTB are inventors on applications for patents, or on issued patents, (US 2011-0256180 A1) (US2015/041616) in the area of meningococcal vaccines. Rights to these inventions have been assigned to UCSF Benioff Children's Hospital Oakland.

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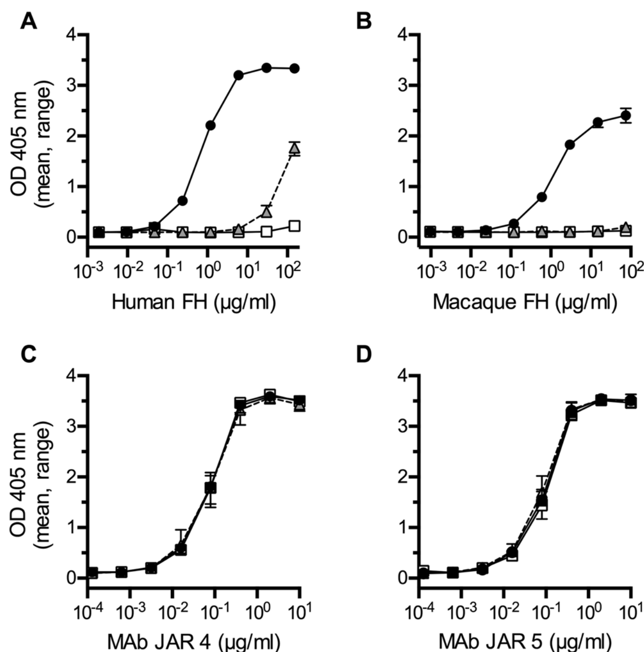

Figure 1. Binding of FH and anti-FHbp mAbs to FHbp mutants by ELISA. (**A**) Binding of purified human FH to the control FHbp WT (black circles with solid black line), R41S single mutant (gray triangles with dashed line), or R41S/H248L double mutant (white squares with solid line). (**B**) Binding of purified rhesus macaque FH to FHbp WT and mutants (symbols same as in **A**). For **A** and **B**, bound FH was detected with a polyclonal sheep anti-human FH antibody. (**C**) Binding of murine anti-FHbp mAb JAR 4 to each of the mutants and WT FHbp. (**D**) Binding of murine anti-FHbp mAb JAR 5. For **C** and **D**, bound mAbs were detected with a polyclonal goat anti-mouse IgG antibody, and symbols are the same as in **A**. For all 4 panels, the mean and range of 2–4 replicates are shown.

to determine whether this mutant FHbp antigen elicits superior protective antibodies in an infant primate model when compared with a control FHbp antigen that binds human and macaque FH. The results in the macaque model should be more relevant for predicting human anti-FHbp antibody repertoire and protective activity than the previously used transgenic mouse models.

Results

A mutant FHbp antigen with low FH binding. In a previous study, we found that binding of human FH to FHbp was markedly decreased when arginine at FHbp residue 41 was replaced by serine (R41S) (12). By ELISA, the R41S mutant had ~100-fold lower binding of human FH than the respective WT FHbp that lacked the serine substitution. In the present study, we replaced a second amino acid residue, histidine at position 248 with leucine (H248L) in FHbp peptide identification (ID) number 1. When compared with the R41S mutant, the addition of the second amino acid substitution lowered human FH binding further (Figure 1A). In parallel experiments, we confirmed that the substitution of the 2 amino acids in FHbp also decreased binding of macaque FH by > 100-fold (Figure 1B). However, the relative binding of the R41S mutant and double mutant to macaque FH were similar to each other. Control anti-FHbp bactericidal mAbs, JAR 4 (26, 27) and JAR 5 (27, 28), bound almost identically to the FHbp mutant and WT FHbp antigens (Figure 1, C and D). These results showed that comparable amounts of each of the proteins were adsorbed to the wells of the microtiter plate. Also, the mAb binding data showed that introduction of the amino acid substitutions did not affect either epitope (JAR 4 epitope includes residues 25–27 and 57–59 located in the N-terminal domain of FHbp, and JAR 5 includes residues 121 and 122; refs. 26, 28).

The mutant FHbp antigen elicits higher serum antibody responses. Beginning at approximately 3 months of age, we immunized macaques with either 25 μg of the mutant FHbp antigen with low FH binding (n = 11 macaques) or 25 μg of a control WT FHbp antigen that bound macaque FH (n = 11 macaques). Both vaccines were mixed with aluminum hydroxide and given i.m. The vaccine schedule was 3 doses given at 0, 1, and 5 months. All 22 macaques also received separate i.m. injections of a control, licensed polysaccharide-protein conjugate vaccine (*Haemophilus influenzae* type b–*Neisseria meningitidis* serogroups C and Y–tetanus toxoid, referred to herein as Hib-MenCY-TT) (29) in the other leg. Four additional infant macaques (2 with FH that bound strongly to FHbp and 2 with FH that bound weakly) served as negative controls and were immunized with aluminum hydroxide alone in one leg and saline in the other leg, which corresponded to the adjuvant in the FHbp vaccine and the diluent for the non-adjuvated Hib-MenCY-TT vaccine, respectively (see Methods).

The mean ages of the groups at the time of the first dose were 3.5, 3.4, and 3.3 months (Table 1). We tested the ability of serum FH in each animal to bind to WT FHbp by ELISA. Because we lacked a macaque FH standard of known concentration, we assigned serum FH titers based on the serum dilution that gave an optical density (OD) of 1.5 in the assay. As shown in Table 1, the reciprocal serum FH geometric mean titers (1/GMT) were similar in the 2 FHbp vaccine groups (3,327 in the mutant group vs. 3,698 in the control FHbp group; $P = 0.34$ by t test). Both FHbp vaccine groups also had similar respective serum IgG antibody responses to 2 antigens tested in the control conjugate vaccine, serogroup C polysaccharide and tetanus toxoid (Table 1). All 4 negative control animals injected with aluminum hydroxide and saline

Table 1. Characteristics of the macaque vaccine groups and antibody responses to control antigens[A]

	Mutant FHbp + Hib-MenCY-TT (*n* = 11)	Control WT FHbp + Hib-MenCY-TT (*n* = 11)	Aluminum Hydroxide + Saline (*n* = 4)
No. (%) male	6 (54)	6 (54)	1 (25)
Mean age (mo.) at dose 1 ± SD	3.5 ± 0.4	3.4 ± 0.6	3.3 ± 0.5
No. (%) caged outdoors	6 (55)	8 (73)	2 (50)
Serum FH, 1/GMT before vaccination (95% CI)[B]	3,327 (2,849–3,886)	3,698 (3,103–4,407)	2,397 (1,455–3,954)
Serum antitetanus toxoid postdose 3, 1/GMT (95% CI)[C]	134 (49–365)	111 (50–246)	<50
Serum antimeningococcal group C polysaccharide postdose 3, 1/GMT (95% CI)[C]	73 (40–132)	84 (44–161)	<50

[A]Animals were immunized i.m. at a 0-, 1-, and 5-month schedule. The vaccinated groups received FHbp in one leg, which was given with aluminum hydroxide and a control conjugate vaccine (Hib-MenCY-TT) in the other leg. The 4 negative control animals received aluminum hydroxide alone in one leg and saline in the other leg to correspond to the respective vaccines. [B]Titer of serum macaque FH that bound to FHbp by ELISA preimmunization. Data are shown for the 2 negative control macaques with high FH binding to FHbp. The 2 negative control animals with low FH binding to FHbp had a 1/GMT of 89. [C]IgG titers to tetanus toxoid and meningococcal group C polysaccharide from serum collected 3 weeks after dose 3. Data are from one assay in which all sera were tested in parallel.

had undetectable serum IgG antibody titers to these antigens (<1:50, Table 1). Thus, the 2 FHbp vaccine groups had similar serum titers of macaque FH that bound to WT FHbp and had similar serum antibody responses to 2 components of the control conjugate vaccine that did not bind FH.

The serum IgG anti-FHbp antibody responses of the individual macaques in each vaccine group are shown in Figure 2, A–C. The test antigen in the ELISA was recombinant WT FHbp ID 1, which was used as the control vaccine. One month after the second FHbp dose, the serum IgG anti-FHbp titers of the mutant FHbp vaccine group were ~3-fold higher than the control FHbp vaccine group (Figure 2A, 1/GMT of 9,575 vs. 3,548, $P = 0.004$). Four months later (immediately before vaccine dose 3), the serum IgG titers had declined in both groups but were still 3.5-fold higher in the mutant vaccine group (Figure 2B, 1/GMT of 743 vs. 212 in the control FHbp vaccine group, $P = 0.004$). Three weeks after the third dose, the serum IgG anti-FHbp titers increased in both FHbp vaccine groups and remained 3-fold higher in the mutant FHbp vaccine group than control (WT) FHbp group (Figure 2C, 1/GMT of 8,210 vs. 2,786, $P = 0.0003$). At all 3 time points, the negative control animals injected with aluminum hydroxide and saline had titers <1:100, which was the lowest dilution tested.

To assess anti-FHbp antibody protective activity, we first measured serum bactericidal titers against serogroup B strain H44/76, which naturally has high expression of FHbp ID 1 (8) with 100% amino acid identity to the control (WT) FHbp vaccine that bound FH. One month after the second dose, the reciprocal GMT was 3.7-fold higher in the mutant vaccine group (Figure 2D, 174 vs. 46 in the control FHbp vaccine group, $P = 0.002$). Four months later, at the time of dose 3, serum bactericidal antibody titers had declined in both FHbp vaccine groups (Figure 2E); the reciprocal GMT was 2-fold higher in the mutant vaccine group (32 vs. 17 in the control FHbp vaccine group, $P = 0.05$). One month after a third dose of an FHbp vaccine (Figure 2F), serum bactericidal titers increased in both vaccine groups and were 5.3-fold higher in the mutant group (1/GMT of 586 vs.110 in the control FHbp group, $P = 0.0007$).

We also measured bactericidal antibody titers in postdose 3 immunization sera against 3 additional serogroup B strains (Figure 3). Two of these isolates were previously described mutants of strain H44/76, one with ~2-fold lower expression of FHbp ID 1 than in the WT H44/76 parental strain (30) and the second with high expression of FHbp ID 13 instead of ID 1, the antigen in the vaccine. FHbp ID 13 has 93% amino acid identity to ID 1. The third test strain, CH860, was a WT isolate from an outbreak in Quebec, Canada (31), with high expression of FHbp ID 15, which shares 87% amino acid identity with the control WT FHbp ID 1 vaccine. A maximum-likelihood tree (32) showing the sequence divergence between FHbp ID 1, –13, and –15 (all in subfamily B) is shown in relation to several representative subfamily A sequence variants (Figure 3A). The macaques immunized with the mutant FHbp vaccine had a 4.6-fold higher serum bactericidal GMT ($P = 0.001$) than the control FHbp

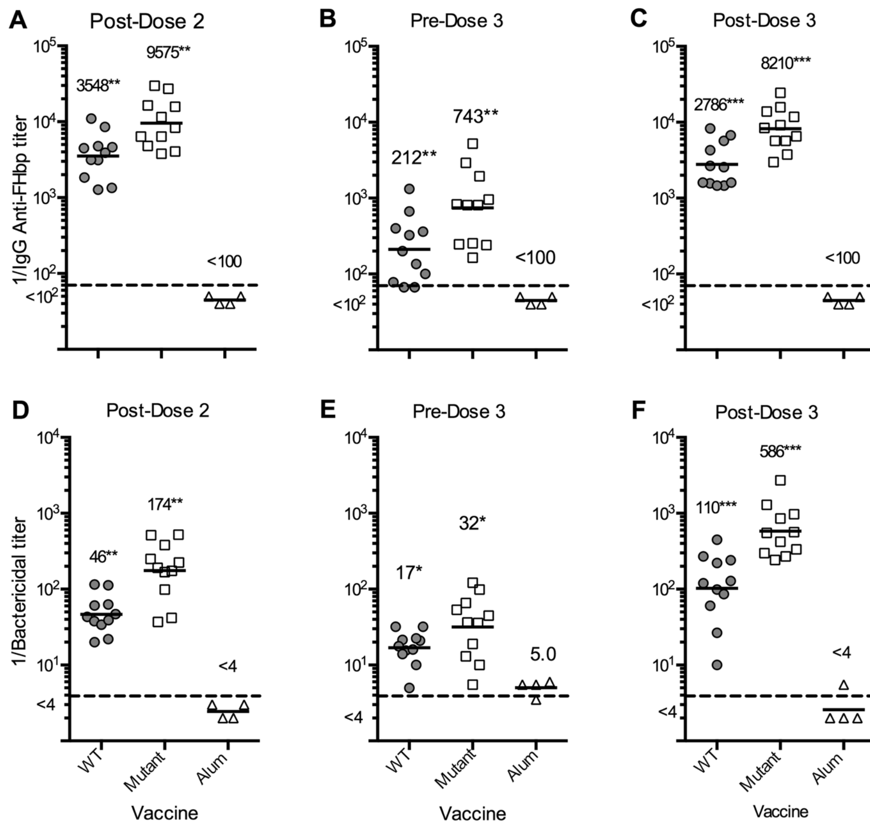

Figure 2. Serum antibody responses to FHbp vaccines given at a 0-, 1-, and 5-month schedule. (**A–C**) IgG anti-FHbp antibody titers as measured by ELISA using immobilized WT FHbp. Each symbol represents the titer of an individual animal, and the horizontal lines indicate the GMTs. The numbers above each group indicate reciprocal GMT for the respective group (WT, n = 11; mutant, n = 11, alum, n = 4). (**A**) One month after dose 2; (**B**) before dose 3 (4 months after dose 2); (**C**) 3 weeks after dose 3. (**D–F**) Serum bactericidal antibody responses against strain H44/76 (FHbp ID 1, exact match to FHbp sequence in control WT vaccine). *P < 0.05; **P < 0.01; and ***P < 0.001 (2-tailed), comparing respective control WT and mutant FHbp vaccine groups by t test of $\log_{10}$-transformed titers. For exact probabilities, see text. Data represent mean bactericidal titers for each animal based on 2 or 3 replicate assays.

vaccine group when tested against the H44/76 mutant strain, with 2-fold lower expression of FHbp ID 1 than the parental WT strain (Figure 3B) and 6- to 15-fold higher reciprocal GMTs (P < 0.0001) against the 2 strains with divergent FHbp sequence variants (Figure 3, C and D).

Postimmunization sera from the mutant FHbp group inhibit binding of FH to meningococci. In previous studies, serum anti-FHbp antibodies, which were elicited in WT mice whose mouse FH didn't bind to the FHbp vaccine antigen, inhibited binding of human FH to FHbp on the bacterial surface (12, 15). With less bound FH, the bacteria were more susceptible to complement-mediated anti-FHbp bactericidal activity (33). In contrast, anti-FHbp antibodies elicited in humans or macaques immunized with the MenB-4C vaccine, which contains an FHbp antigen that binds FH, didn't inhibit binding of FH to meningococci (16, 17, 34). In the present study, we used flow cytometry to investigate the ability of postimmunization serum antibodies of the macaques to inhibit binding of FH to live meningococci (WT strain H44/76). In this experiment, the respective pre- and postimmunization macaque sera were tested in parallel at a dilution of 1:150. The pre- and postimmunization sera served as sources of macaque FH, and the postimmunization sera served as sources of anti-FHbp antibody. Figure 4A shows binding of serum FH to meningococci from a representative macaque immunized with the control WT FHbp. There was greater binding of FH with the postimmunization serum (solid line) than with the respective preimmunization serum (dashed line). Thus, the anti-FHbp antibodies in the postimmunization serum did not inhibit FH binding to the bacteria and slightly enhanced FH binding when compared with the respective preimmunization serum that lacked anti-FHbp antibodies. In contrast, in a macaque immunized with the mutant FHbp antigen (Figure 4B), there was lower FH binding to meningococci with the postimmunization serum than the respective preimmunization serum (i.e., inhibition of FH binding). Histograms showing binding of macaque FH to meningococci when incubated with predose 1 or postdose 3 sera from each pair of macaques in the study are shown in Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/jci.insight.88907DS1). The median fluorescence intensity (MFI) values are summarized in Figure 4D (WT FHbp group) and Figure 4E (mutant FHbp group). After immunization, there was a significant increase in serum FH binding in the WT FHbp group (MFI before vaccination of 487 increasing to 766 one month after dose 3; P = 0.003). In contrast, in the mutant FHbp group, there was a significant decrease in FH binding (MFI of 493 before immunization, which decreased to 294 after immunization; P = 0.005).

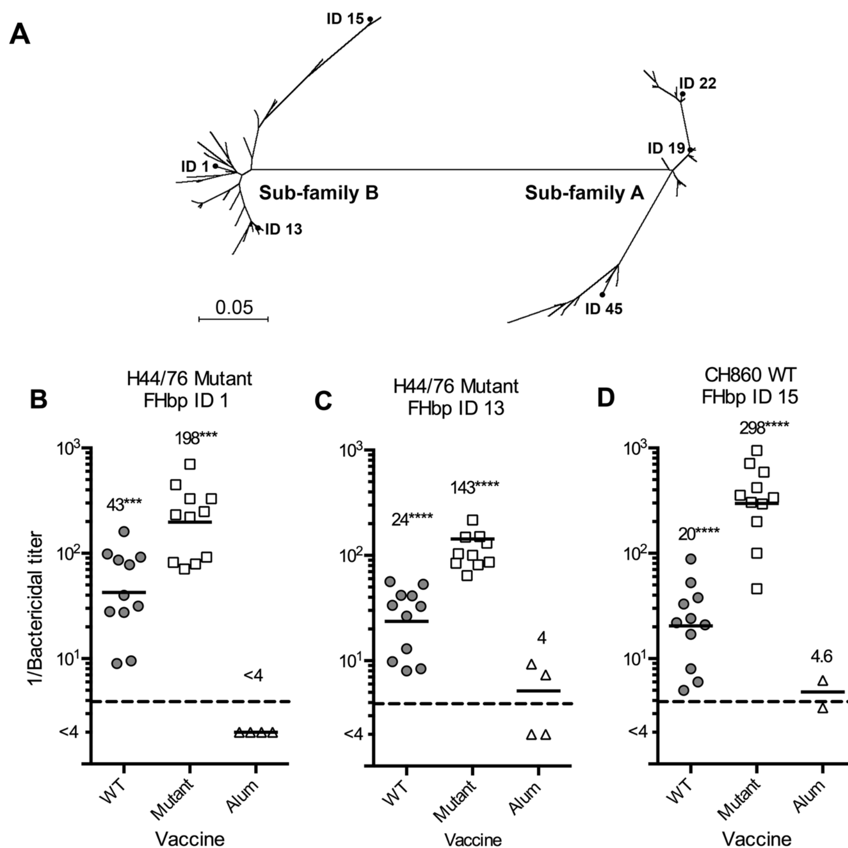

Figure 3. Serum bactericidal antibody responses against meningococcal strains with divergent FHbp sequences or lower FHbp expression. (**A**) Unrooted, maximum-likelihood phylogram of 70 representative FHbp amino acid sequence variants (57) computed with MEGA 7 (32). The subfamily B sequence variants expressed by the 3 test strains in **B**–**D** are indicated with labels; ID 1 represents the sequence of the control WT FHbp vaccine. Three representative subfamily A sequence variants also are shown for comparison. The scale bar indicates 5% amino acid sequence divergence. (**B–D**) Serum bactericidal antibody responses 3 weeks after 3 doses of a recombinant WT or mutant FHbp vaccine. Each symbol represents the titer of an individual animal; the number of animals per groups is the same as in Figure 2. The horizontal lines and numbers in the panel indicate the reciprocal GMTs. (**B**) Mutant of strain H44/76 with 50% expression of FHbp ID 1 compared with parental WT strain (30). (**C**) Mutant of strain H44/76 with high (100%) expression of FHbp ID 13 compared with parental WT strain. (**D**) WT strain CH860 with high expression of FHbp ID 15. ***$P < 0.001$; ****$P < 0.0001$ (2-tailed), comparing respective WT and mutant FHbp vaccines by t test of $\log_{10}$-transformed titers. For exact probabilities, see text. Data in **B** are titers measured in one assay in which all sera from pairs of control FHbp and mutant FHbp immunized animals were tested in parallel. Data in **C** and **D** represent mean titers for each individual animal from 2 or 3 replicate assays.

As a human control, we tested predose 1 and postdose 3 sera from an adult immunized with a meningococcal serogroup B vaccine (MenB-FHbp, see Methods) containing FHbp antigens that bind FH (Figure 4C). At a 1:600 dilution, the postdose 3 serum (solid line) gave approximately 10-fold more FH binding to meningococci than the preimmunization serum (dashed line). Collectively, the human data validate the observations in the infant macaques immunized with a WT FHbp antigen that binds FH, in which the postimmunization serum antibodies enhanced binding of FH.

Figure 4F shows the ratios of the MFI in individual postdose 3 serum versus preimmunization serum. Ratios ≥ 1.0 represent no inhibition or enhancement of FH binding, and ratios < 1 represent inhibition of FH binding. The postimmunization sera from the macaques immunized with the control (WT) FHbp vaccine (gray circles) enhanced FH binding (median ratio of 1.54 after dose 3, which was higher than the theoretical ratio of 1.0; $P = 0.003$ by a Wilcoxon matched-pairs signed-rank test). In contrast, the postimmunization sera from the animals immunized with the mutant FHbp vaccine inhibited FH binding (white squares, median ratio of 0.68 after dose 3 [lower than 1.0]; $P = 0.005$). Similar respective enhancement or inhibition results in the 2 vaccine groups were observed in sera obtained 1 month after dose 2 (Supplemental Figure 2).

One possible explanation for the greater inhibition of FH binding in postimmunization sera from the macaques immunized with the mutant vaccine is that there are higher serum IgG anti-FHbp antibody titers than in the macaques immunized with the control (WT) FHbp vaccine (Figure 2, A and C). To control for higher serum antibody titers, we performed a subanalysis of the data for the 6 macaques in the WT FHbp vaccine group with the highest serum IgG anti-FHbp titers and the 6 macaques in the mutant FHbp group with the lowest IgG anti-FHbp titers after 3 doses of vaccine (Figure 5). The resulting groups had similar IgG anti-FHbp titers (reciprocal GMT of 4,573 and 5,204, $P > 0.5$, panel A). Despite similar serum IgG anti-FHbp antibody titers, binding of FH to meningococci was enhanced in the animals in the control (WT) FHbp group, with a median ratio of 1.68, which was significantly > 1.0 ($P = 0.03$ by Wilcoxon signed-rank test); further, there was a trend for inhibition in the animals in the mutant FHbp vaccine group, with a median ratio of 0.78 ($P = 0.16$, 2-tailed, compared with a theoretical

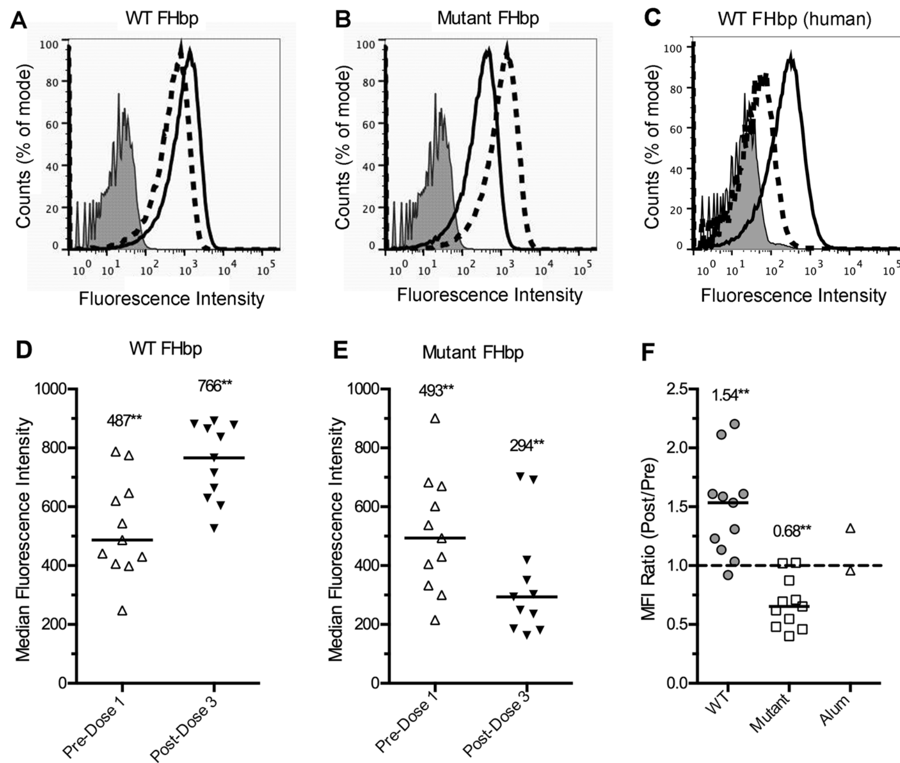

Figure 4. Effect of FHbp vaccination on binding of serum FH to live meningococci as measured by flow cytometry. (**A** and **B**) Histograms showing binding of macaque serum FH to live meningococci of group B strain H44/76 when incubated in 1:150 dilutions of predose 1 (dashed line) or postdose 3 macaque sera (solid line). Gray-filled histogram shows bacteria that were not incubated with macaque serum. (**A**) Representative macaque immunized with the control (WT) FHbp antigen showing higher FH binding in postimmunization than preimmunization serum (i.e., no inhibition and slight enhancement of FH binding). (**B**) Representative macaque immunized with the mutant FHbp vaccine showing lower FH binding in postimmunization than preimmunization serum (i.e., inhibition). (**C**) Representative sera from an adult human immunized with 3 doses of a meningococcal serogroup B vaccine (MenB-FHbp) containing FHbp that binds FH. The postdose 3 (solid line) shows approximately 10-fold more binding of FH to meningococci than the preimmunization serum (dashed line). The human sera were tested at a 1:600 dilution. (**D** and **E**) Binding of macaque serum FH to strain H44/76 before dose 1 and 3 weeks after 3 doses of WT (**D**) or mutant FHbp antigen (**E**). All sera were tested at a 1:150 dilution. After immunization, there was a significant increase in serum FH binding in the WT FHbp group (MFI before vaccination of 487 increasing to 766 one month after dose 3, $P = 0.003$). In contrast, in the mutant FHbp group, there was a significant decrease in FH binding (MFI of 493 before immunization, which decreased to 294 after immunization, $P = 0.005$). The number of animals per groups is the same as in Figure 2. (**F**) Ratios of MFI of serum FH binding (postdose 3/predose 1) for each individual animal. Each symbol represents median ratio determined in 2–3 independent assays. Ratios ≥ 1 signify no inhibition or enhanced FH binding. Ratios < 1 indicate inhibition of FH binding. $^{**}P < 0.01$, comparing median ratio of mutant or control FHbp vaccine group to a theoretical ratio of 1.0 by a one sample Wilcoxon signed rank test (2-tailed).

mean of 1.0). The difference in the median ratios of FH binding in the 2 vaccine groups was significant ($P = 0.002$). This difference in anti-FHbp antibody repertoire did not translate into a significantly higher serum bactericidal activity of the mutant vaccine group against WT strain H44/76 with FHbp ID 1 that exactly matched the vaccine antigen (1/GMT of 339 in the mutant vaccine group vs. 214 in the control vaccine group, $P = 0.10$, Figure 5C). However, for strain CH860 with a divergent FHbp sequence ID 15, despite similar IgG anti-FHbp titers, the bactericidal GMT of the mutant vaccine group was 5.8-fold higher than the WT vaccine group (Figure 5D, $P = 0.002$).

Antibodies elicited by the mutant FHbp antigen elicit greater classical pathway activation. The ability of serum antibody to activate complement component C4b deposition on meningococci can be used as a marker of classical complement pathway activation (16). For strain CH860, despite similar IgG anti-FHbp titers, postdose 3 immunization sera from the mutant FHbp vaccine group elicited a 5.3-fold higher median C4b deposition on live meningococci as measured by flow cytometry than postimmunization sera from the WT FHbp vaccine group (Figure 5E, $P = 0.009$, by a 2-tailed nonparametric Mann-Whitney *U* test). Further, there was a direct correlation between the amount of C4b deposition on the bacteria and the respective serum bactericidal titers ($r^2 = 0.92$, $P < 0.0001$, Figure 5F). Thus, the anti-FHbp repertoire elicited by the mutant vaccine, which was directed in part at epitopes in the FH

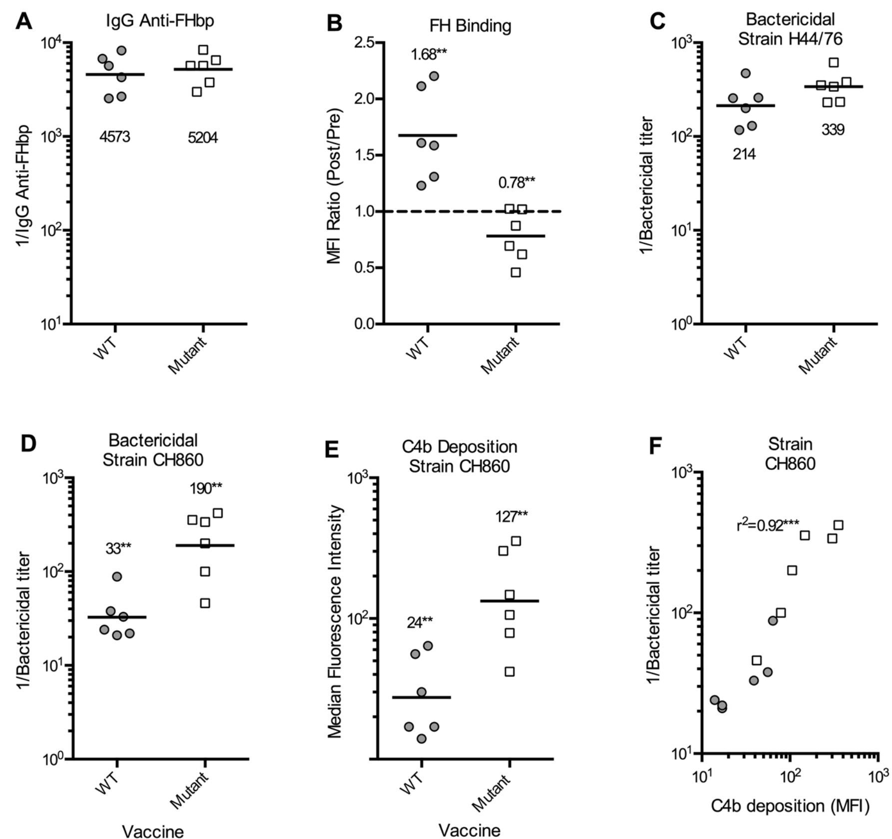

Figure 5. Effect of FHbp vaccination on binding of serum FH to meningococci, bactericidal activity, and C4b deposition. In a subanalysis to control for group differences in IgG titers, 6 macaques in the control WT FHbp group were selected based on the highest postdose 3 IgG anti-FHbp titers and 6 macaques in the mutant vaccine group with the lowest IgG anti-FHbp titers (see Figure 2C). (**A**) Serum IgG anti-FHbp GMTs of the vaccine groups were not significantly different, P = 0.6. (**B**) Ratios of FH binding to meningococci (WT strain H44/76) in postdose 3 sera to respective preimmunization sera from individual animals. All sera were tested at a dilution of 1:150. For the WT FHbp vaccine group, the MFI ratio of 1.68 was significantly > 1.0 (P = 0.03 by Wilcoxon signed-rank test compared with a theoretical ratio of 1.0.). For the mutant FHbp vaccine group, there was a trend for inhibition of FH binding by postimmunization serum (median ratio of 0.78, P = 0.16 [2-tailed]). The difference in the median ratios of FH binding in the 2 vaccine groups was significant (P = 0.002). (**C** and **D**) Bactericidal titers in postdose 3 sera against WT strains H44/76 with FHbp ID 1 (100% amino acid identity with vaccine) (**C**), and strain CH860 with FHbp ID 15 (89% identity) (**D**), for the subset of animals with similar IgG anti-FHbp titers. **P < 0.01, comparing GMT of the 2 vaccine groups against strain CH860 by t test of $\log_{10}$-transformed titers. Respective GMTs against H44/76 were not significantly different (P = 0.10). (**E**) C4b deposition on live meningococci of strain CH860 activated by macaque serum anti-FHbp antibodies in postdose 3 sera diluted 1:40. The MFI values for each individual animal are shown; the horizontal lines and numbers indicate the median of the individual values for each vaccine group. (**P < 0.01 by Mann-Whitney nonparametric test). (**F**) Relationship between serum bactericidal titers measured against strain CH860 and C4b deposition (data from **D** and **E**). r^2 = 0.92, P < 0.0001. Data in **A**–**D** for the subanalyses were recalculated from data shown in Figures 2–4. Data in **E** (C4b deposition) represent the MFI values for each animal based on one assay in which all sera were tested in parallel.

combining site, elicited higher complement-mediated serum bactericidal activity against a strain with an FHbp sequence divergent from the vaccine antigen, and the mechanism involved greater classical pathway activation.

Serum autoantibodies to FH. In a previous study, 2 macaques immunized with the licensed MenB-4C vaccine, which contains a FHbp antigen that binds macaque FH as one of its principal antigens, developed serum IgG antibodies to FH in sera obtained 1 month after the second dose (17). However, the serum anti-FH antibodies were transient and were not present after dose 3. In the present study, none of the animals immunized with either FHbp vaccine developed serum anti-FH antibodies after doses 2 or 3.

Discussion

FH is an important alternative complement pathway regulator and is one of the most abundant plasma

proteins (~200–600 μg/ml in humans) (12, 35, 36). In our previous study in infant macaques, even low binding of FH to a WT FHbp antigen skewed the serum anti-FHbp repertoire to FHbp epitopes outside of the FH binding site, which was associated with impaired booster serum bactericidal responses (17). In the present study, we used macaques with high FH binding to the WT FHbp antigen to investigate the immunogenicity of a mutant FHbp antigen with 2 amino acid substitutions and even lower binding to human FH than our previously investigated FHbp R41S mutant vaccine candidate, which had been tested in human FH transgenic mice (12). Our most important findings in the infant macaques were that the mutant antigen elicited higher serum IgG anti-FHbp titers and higher serum bactericidal responses than the control WT FHbp antigen that bound FH. Also, the mutant FHbp elicited an antibody repertoire directed, in part, at FHbp epitopes located in the FH binding site, since the postimmunization sera inhibited binding of FH to meningococci. In contrast, the antibodies elicited by the control FHbp antigen did not inhibit FH binding and, for reasons that are unknown, enhanced FH binding.

One possible factor contributing to the lower-serum IgG antibody titers of the macaques immunized with the control FHbp antigen could have been the binding of macaque FH to the vaccine antigen and the downregulation of the inflammatory potential of complement to enhance serum antibody responses (37). However, this mechanism would be expected mainly to decrease the magnitude of the serum antibody response to the WT vaccine antigen. Even when controlling for higher levels of serum IgG anti-FHbp antibody in the mutant FHbp vaccine group through a subanalysis (Figure 5), the macaques immunized with the mutant FHbp vaccine had higher serum bactericidal titers than the WT FHbp vaccine group against a strain with a divergent FHbp sequence. The greater bactericidal activity appeared to result from a different antibody repertoire.

As described above, some macaques have FH that binds weakly to FHbp (10, 11); these animals were excluded from our present study. However, in our previous study, even this low affinity binding of FH to a WT FHbp antigen resulted in an anti-FHbp antibody repertoire that did not inhibit binding of macaque FH to meningococci (16, 17). A big difference, therefore, in the present study is that the serum anti-FHbp repertoire to the mutant FHbp antigen partially inhibited binding of FH to meningococci. Further, the anti-FHbp antibodies elicited greater C4b deposition (classical pathway) than the antibodies elicited by the control FHbp antigen, which were directed largely at epitopes outside of the FH combining site.

Activation of the classical complement pathway requires binding of at least 2 IgG molecules to epitopes located in sufficient proximity to engage complement component C1q. Conceivably, by targeting the FH binding site of FHbp, the IgG antibodies elicited by the mutant FHbp vaccine were able to bind in closer proximity to each other and better engage C1q than antibodies elicited by the WT antigen, which were largely directed at epitopes outside of the FH binding site. While the enhanced FH binding to meningococci by postimmunization sera from the WT FHbp vaccine group also might be expected to diminish bactericidal activity by downregulating the alternative complement pathway, in our previous studies in macaques or humans immunized with the MenB-4C vaccine, postimmunization serum antibodies that enhanced FH binding could still recruit the alternative pathway and contribute to anti-FHbp bacteriolysis (16, 34). Collectively, our data indicate that the higher bactericidal antibody responses to the mutant FHbp vaccine were a result of greater activation of the classical pathway by antibodies directed, in part, at FHbp epitopes located near or within the FH binding site. We have no direct evidence that the enhanced binding of FH to meningococci incubated with sera from the animals immunized with the WT FHbp vaccine contributed to downregulating complement activation and lower bactericidal activity.

In previous studies of human-mouse chimeric anti-FHbp mAbs, IgG3 was more bactericidal than IgG1 (38, 39), and IgG2 had the least activity (39). Therefore, it is possible that different IgG subclass responses of the macaques to the 2 FHbp vaccines contributed to the higher serum bactericidal antibody bactericidal activity of the mutant vaccine group. To our knowledge, there are no published data on the effector function of rhesus IgG subclasses. Rhesus macaque IgG3 lacks the long hinge region present in human IgG3 (40), and whether macaque IgG3 has greater ability to activate the classical complement pathway than IgG1 is not known. Further, measurement of rhesus macaque IgG subclass responses is not straightforward, since antibodies that specifically bind to rhesus IgG subclasses are not readily available. In preliminary studies, we used mouse mAbs specific for rhesus macaque IgG1, IgG2, and IgG3. The predominant IgG subclass response in both FHbp vaccine groups was IgG1 with relatively low IgG2 and IgG3 responses (Supplemental Figure 3). However, while the anti-rhesus IgG

subclass mAbs were specific for each subclass (panel C), the relative reactivity of the anti-IgG1 mAb was higher than the IgG2 or IgG3 mAbs, which limited interpretation of the low responses to these subclasses. Nevertheless, our preliminary data are consistent with the predominant IgG1 subclass response of humans immunized with other protein antigens such as tetanus toxoid (41) or a meningococcal outer membrane vesicle vaccine that does not bind to a host antigen (42).

In human infants immunized with the MenB-4C vaccine, serum bactericidal titers wane rapidly, and a booster dose at 12 months is required to maintain immunity (43). In the infant macaques immunized with the FHbp vaccines, peak serum bactericidal titers present 1 month after dose 2 decreased after 4 months but remained significantly greater in both FHbp vaccine groups than in the control macaques that did not receive a FHbp vaccine (Figure 3E). One limitation of the present study is that we do not have comparable serum antibody persistence data after dose 3. However, at 1 month after dose 3, there was evidence of an increase in antibody quality (functional activity) compared with 1 month after dose 2. For example, a serum IgG anti-FHbp GMT of 1:3,548 in the control FHbp vaccine group 1 month after dose 2 was associated with a bactericidal GMT of 1:46 against WT strain H44/76, whereas a similar IgG GMT of 1:2,786 one month after dose 3 was associated with a 2-fold higher bactericidal GMT of 1:110. In the mutant vaccine group, a serum IgG GMT of 1:9,575 after dose 2 was associated with a bactericidal GMT of 1:174, whereas a similar IgG GMT of 1:8,210 after dose 3 was associated with ~3-fold higher bactericidal GMT of 1:586. Because of the higher anti-FHbp antibody quality after dose 3 in both vaccine groups, it is possible that bactericidal titers will persist longer and also remain higher after dose 3 in the mutant vaccine group. However, further studies are needed to resolve this question.

In the present study, neither FHbp vaccine elicited serum autoantibodies to macaque FH. However, serum autoantibodies to FH have been observed in previous studies in animal models (15, 17) and in humans immunized with each of the licensed serogroup B vaccines (DMG and SG, unpublished observations). Thus, the use of a mutant FHbp vaccine with low FH binding has the added potential benefit of diminishing the theoretical risk of eliciting autoimmune responses that may decrease human FH function.

In summary, the present data indicate that a mutant FHbp antigen with very low FH binding gives higher serum bactericidal antibody responses than a control WT FHbp antigen in infant macaques, which indicates that the antibody repertoire to the mutant antigen inhibits FH binding. These results imply that the recombinant FHbp antigen in the MenB-4C vaccine, which binds human FH, can be improved by using a low FH binding mutant. Similarly, in human FH transgenic mice, a meningococcal native outer membrane vesicle (NOMV) vaccine with overexpressed lipidated mutant FHbp R41S with low FH binding elicited higher serum anti-FHbp bactericidal titers than a control NOMV vaccine with overexpressed WT FHbp that bound human FH (14). Thus replacing the recombinant lipidated FHbp antigen in the licensed MenB-FHbp vaccine with a mutant low-FH binding FHbp antigen also should enhance protective antibody responses to that vaccine.

The vaccine potential of other microbial proteins that bind host proteins also is an area of active investigation. These vaccine antigens include M-protein (*Streptococcus pyogenes*) (44, 45), PspC (*Streptococcus pneumoniae*) (46, 47), OspE (*Borrelia burgdorferi*) (48, 49), NspA (50), and TbpB (*Neisseria meningitidis*) (51). Our results with a mutant FHbp antigen in a nonhuman primate model increase our understanding of the deleterious effect of binding of a host protein to a vaccine antigen on immunogenicity and have the potential to lead to the design of FHbp antigens and other microbial vaccine antigens with decreased binding to host antigens in order to achieve greater protective antibody responses in humans.

Methods

***Rhesus macaques*.** The animals were housed at the California National Primate Research Center (Davis, California, USA) as previously described (16). Polymorphisms in macaque FH are associated with high- or low-FH binding to FHbp (10, 11). At ages 2–3 months, we screened sera from ~100 animals for binding of macaque FH to FHbp ID 1 by ELISA, as previously described (11). The macaque sera with the high FH binding phenotype were confirmed by flow cytometry with live meningococci and by DNA sequencing of FH exon encoding domain 6. Twenty-two high FH-binding macaques (11 pairs, each matched by age and sex) were selected for immunization with 1 of the 2 recombinant FHbp vaccines. Four additional monkeys, 2 pairs each with either high FH binding or low FH binding, served as negative controls.

Vaccines. We prepared 2 recombinant FHbp vaccines using methods previously described (12). The control FHbp vaccine consisted of the WT protein (subfamily B, peptide identification number 1), which bound strongly with both human and macaque FH (Figure 1). The mutant vaccine had an identical amino acid sequence to the control FHbp, except for 2 amino acid substitutions, R41S and H248L, which decreased FH binding by >250-fold. Each 0.5-ml dose of the WT or mutant recombinant FHbp vaccine contained 25 μg of protein, which was adsorbed with aluminum hydroxide (Alhydrogel, Brenntag Biosector; 0.5 mg Al^{3+}). All of the FHbp-vaccinated animals were also immunized with a human dose of a US-licensed polysaccharide-protein conjugate vaccine (Hib-MenCY-TT; MenHibrix, GlaxoSmithKline Biologicals).

*Immunogenicity***.** The immunizations and blood draws on the predesignated pairs were performed on the same day. At ages 3–4 months, the animals were vaccinated i.m. in one leg with a FHbp vaccine and in the other leg with the Hib-MenCY-TT conjugate vaccine. Vaccination was repeated 1 and 5 months later (i.e., 0-, 1-, and 5-month schedule). Blood samples were obtained immediately before dose 1, four weeks after dose 2, immediately before dose 3, and 3 weeks after dose 3. Negative control animals were immunized with aluminum hydroxide alone in one leg and saline in the other leg to correspond to the constituents of the respective FHbp and conjugate vaccines.

ELISA. Serum IgG titers to FHbp ID 1, meningococcal group C polysaccharide, and tetanus toxoid were measured by ELISA as previously described (52). IgG subclass-specific anti-FHbp titers were measured by ELISA using mouse mAbs specific for rhesus macaque IgG1, IgG2, and IgG3 (clones 7H11, 3C10, and 2G11, respectively; provided by the NIH Nonhuman Primate Reagent Resource Program at MassBiologics, http://www.nhpreagents.org). The bound mouse mAbs were detected by affinity-purified goat anti-mouse IgG (whole molecule) conjugated with alkaline phosphatase (1:7,000; Sigma-Aldrich); incubation lasted for 1 hour at room temperature.

Binding of macaque serum FH to FHbp was measured by ELISA using purified, recombinant FHbp ID 1 to capture FH as previously described (11, 12). After overnight incubation at 4°C, bound FH was detected with goat anti-human FH antibody (Complement Technology Inc.). The bound goat IgG was detected with AP-conjugated donkey anti-goat IgG (1:5,000; Sigma-Aldrich); incubation lasted for 1 hour at room temperature. The results were expressed as serum FH titers based on the serum dilution giving an OD at 405 nm ($OD_{405\ nm}$) of 1.5 after 30 minutes incubation with phosphatase substrate (para-nitrophenylphosphate; Sigma-Aldrich).

Binding of serum IgG antibodies to purified macaque FH was tested by ELISA (15, 16). For measuring autoantibody to FH, we only considered the serum positive if binding of the macaque IgG antibody to the solid -phase FH was inhibited by the addition of 50 μg/ml of soluble macaque FH to reaction, as previously described (15).

*Neisseria meningitidis***.** We measured serum bactericidal antibody responses against 4 serogroup B strains. Two were WT case isolates, H44/76 (from an epidemic in Norway in the 1970s, ref. 53), and CH860, isolated in 2009 during a period of hyperendemic serogroup B meningococcal disease in Quebec, Canada (31). Both strains had high surface expression of subfamily B FHbp. H44/76 has FHbp ID 1 (100% identical to the control WT FHbp vaccine). CH860 has FHbp ID 15 (87% identical to FHbp ID 1). We also tested serum anti-FHbp bactericidal antibody responses against 2 previously described mutants of strain H44/76, one in which the gene encoding the native FHbp ID 1 had been replaced by subfamily B FHbp ID 13 (93% amino acid identity with ID 1) (54) and one in which was engineered to have ~50% lower expression of FHbp ID 1 than in the parental WT strain (30).

*Bactericidal assay***.** Bacteria were grown to mid-log phase in Frantz media supplemented with 4 mM D,L-lactate (Sigma-Aldrich) and 2 mM cytidine 5'-monophospho-N-acetyl-neuraminic acid (CMP-NANA; Carbosynth) to enhance sialylation of lipooligosaccharide (55). Test sera were heated for 30 minutes at 56°C to inactivate intrinsic complement. The exogenous source of human complement was human serum depleted of IgG with a protein G column (HiTrap Protein G HP, 5 ml; GE Healthcare) (12). Serum bactericidal antibody titers were assigned as the interpolated dilution resulting in 50% survival of the bacteria, compared with CFU/ml of bacteria incubated at 37°C for 60 minutes with negative control sera and complement.

Flow cytometric assays to measure inhibition of binding of macaque FH to live meningococci by postimmunization sera and C4b deposition. The FH binding assay was performed as previously described, using approximately 1×10^7 CFU/ml of bacteria incubated for 1 hour at room temperature with a 1:150 dilution of either preimmunization macaque serum (as a source of macaque FH) or postimmunization macaque serum (as a source of macaque FH and anti-FHbp antibody). As a control we also tested a 1:600 dilution

of a pre-dose 1 and post-dose 3 serum from a human adult immunized with a FHbp vaccine (MenB-FHbp, Pfizer) that bound FH. Bound macaque or human FH was detected with a sheep polyclonal antiserum to human FH (Abcam) followed by washing and the addition of donkey anti-sheep IgG antibody conjugated with AlexaFluor 488 (Sigma-Aldrich). After washing and fixing with 0.5% (v/v) formaldehyde in PBS, binding was analyzed by flow cytometry (LSRFortessa, BD Biosciences) and data were analyzed using FlowJo version 10. For measuring C4b deposition, approximately 1×10^8 CFU/ml of bacteria were suspended in a reaction containing a 1:40 dilution of postdose 3 macaque serum, 9% IgG-depleted human serum as a complement source and Dulbecco's phosphate–buffered saline containing 0.1 g/liter $CaCl_2$ and 0.1 g/liter $MgCl_2 \cdot 6H_2O$ (Mediatech) (pH 7.4) with 1% (wt/vol) bovine serum albumin (Equitech-Bio) (D-PBS-BSA). After 10 minutes of incubation at room temperature, the bacteria were washed twice with D-PBS-BSA, and bound C4b was detected with fluorescein isothiocyanate–conjugated anti-human C4b (Meridian Life Science) by flow cytometry.

Statistics. For calculation of GMT, titers below the limit of the detection were assigned half the value of the lowest dilution tested. Since the original study design included pairs of macaques assigned to the control (WT) or mutant vaccine group, which were matched for age, sex, and dates of immunization, we used a paired *t* test to determine significant differences in antibody responses by vaccine group. For the subanalyses presented in Figure 5, we used unpaired *t* tests to determine significant differences in antibody responses (panels A, C, and D), and a one-sample Wilcoxon signed-rank test for comparing the MFI ratio for FH binding (panel B), or a nonparametric (Mann-Whitney *U*) test (panel E) as specified in the text. All statistical tests were 2-tailed; *P* values less than or equal to 0.05 were considered statistically significant.

Study approval. The animals were born and housed at the California National Primate Research Center (Davis, California, USA) in accordance with American Association for Accreditation of Laboratory Animal Care Standards, and "Guide for the Care and Use of Laboratory Animals" (56). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of California Davis (protocol 18217).

Author contributions

PTB and DMG designed study and analyzed and interpreted results; PTB identified mutant FHbp; KS prepared and characterized WT and mutant FHbp antigens; SG, EL, and FAG performed all assays of the macaque sera and analyzed results; all authors contributed to writing the manuscript. Primary data and materials are available from the authors.

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