Title: An Adenoviral Vectored Vaccine Confers Sero-Protection Against Capsular Group B Meningococcal Disease

Authors: Christina Dold[1], Leanne Marsay[1], Nelson Wang[1], Laura Silva-Reyes[1], Elizabeth Clutterbuck[1], Gavin K. Paterson[2]‡, Kelsey Sharkey[3]‡, David Wyllie[2†], Peter T. Beernink[3], Adrian V. Hill[2], Andrew J. Pollard[1], Christine S. Rollier[1,4]*

Affiliations:

[1] Oxford Vaccine Group, Department of Paediatrics, University of Oxford and the NIHR Oxford Biomedical Research Centre; CCVTM, Churchill lane, Oxford OX37LE, United Kingdom.

[2] Jenner Institute, University of Oxford; Old Road Campus Research Building, Oxford OX3 7DQ, United Kingdom.

[3] Division of Infectious Diseases and Global Health, Department of Paediatrics, University of California San Francisco; 94143 San Francisco, USA.

[4] School of Biosciences and Medicine, University of Surrey; Guildford, GU2 7XH United Kingdom.

*Corresponding author. c.rollier@surrey.ac.uk

‡G.K. Paterson present address: Royal (Dick) School of Veterinary Studies and the Roslin Institute, University of Edinburgh; Edinburgh, EH25 9RG, United Kingdom.
‡K. Sharkey present address: Lawrence Berkeley National Laboratory; Berkeley CA 94720, USA.
† Deceased

One Sentence Summary: A single dose of a clinically-relevant adenovirus-based vaccine induces a strong and functional antibody response against group B meningococcus.

Abstract: Adenoviral-vectored vaccines are licensed for prevention of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Ebola virus, but, for bacterial proteins, expression in a eukaryotic cell may impact the antigen's localization, conformation, or lead to unwanted glycosylation. Here, we investigated the potential use of an adenoviral-vectored vaccine platform for capsular group B meningococcus (MenB). Vector-based candidate vaccines expressing MenB antigen factor H binding protein (fHbp) were generated, and immunogenicity was assessed in mouse models, including the functional antibody response by serum bactericidal assay (SBA) using human complement. All adenovirus-based vaccine candidates induced high antigen-specific antibody and T cell responses. A single dose induced functional serum bactericidal responses with titers superior or equal to those induced by two doses of protein-based comparators, as well as longer persistence and a similar breadth. The fHbp transgene was

further optimized for human use by incorporating a mutation abrogating binding to the human complement inhibitor factor H. The resulting vaccine candidate induced high and persistent SBA responses in transgenic mice expressing human factor H. The optimized transgene was inserted into the clinically-relevant ChAdOx1 backbone, and this vaccine has now progressed to clinical development. The results of this preclinical vaccine development study underline the potential of vaccines based on genetic material to induce functional antibody responses against bacterial outer membrane proteins.

Main Text:

INTRODUCTION

Neisseria meningitidis is a leading cause of childhood meningitis and septicemia in several countries, including the United Kingdom. Effective conjugate vaccines against the capsular groups A, C, W and Y, are licensed, For the serogroup B (MenB), the licensed vaccines 4-component MenB vaccine (4CMenB, Bexsero, GlaxoSmithKline) and recombinant lipoprotein 2086 (rLP2086, Trumenba, Pfizer) are based on subcapsular protein antigens *(1–5)*. 4CMenB also contains outer membrane vesicles (OMV), used to control a previous outbreak in New Zealand *(6)*. Both vaccines are licensed for adolescents and adults in a two-dose schedule *(4)*, but the persistence of the protective response appears limited*(7)*, and there is no evidence that 4CMenB can reduce bacterial colonization within an organism *(8)*. These factors negatively affect the cost-effectiveness of an adolescent program for MenB vaccines *(9)*. A low cost, single-dose MenB vaccine capable of inducing sustained protective immune responses would be well positioned *(10)*.

Viral-based vaccine platforms such as adenoviral and poxviral vectors induce both innate and adaptive immune responses in mammalian hosts *(11, 12)*. Although they were originally developed for their well-recognized ability to induce potent cellular immunity, a single dose of an adenovirus-based vaccine is able to induce potent neutralizing antibodies against some pathogens, as demonstrated with vaccines against rabies *(13)*, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) *(14, 15)*, malaria *(16)* and Ebola virus *(17)*. Their capacity to induce strong interferon (IFN)-γ producing T cell responses should provide the ideal conditions for class switching to complement-fixing bactericidal antibody against MenB *(18)*.

The use of viral vectors to induce antibody responses to bacterial outer membrane proteins is complicated by the differences between prokaryotic and eukaryotic expression systems, which may result in incorrect or sub-optimal expression of bacterial antigens in mammalian cells. If successfully expressed, there may still be a loss of protective epitopes due to misfolding or aberrant post-transcriptional modifications *(19)*. However, some successes at eliciting functional responses were described for bacterial antigens from *Streptococcus pneumoniae (20)* and *Yersinia pestis (21)*. In the current study, a series of vectors were constructed expressing the MenB protective antigen factor H binding protein (fHbp) *(22–25)*. The vectors were assessed for antigen expression in mammalian cells, and for immunogenicity and induction of protective bactericidal activity in mouse models. One candidate was selected and optimized for human use by incorporating a point mutation, which abrogates binding of fHbp to the human complement inhibitor factor H. The resulting vaccine candidate is now in clinical development.

RESULTS

Adenoviral vaccine vectors encoding fHbp induce bactericidal activity in mice.

Recombinant replication-deficient human adenovirus 5 (HuAd5) vectors encoding a full length (immature sequence including the signal sequence), or a truncated version (without the signal sequence) of fHbp were created (HuAd5 fHbp and HuAd5 fHbp-truncated, respectively). Both vectors induced antibody responses in BALB/c mice as soon as two weeks post a single intramuscular injection, as evidenced by antibodies detected against whole H44/76 bacteria containing a homologous fHbp variant (Fig. 1A). The antibody titers were of similar magnitude to those induced by a single dose of native H44/76 OMVs containing the same fHbp variant as well as many more antigens including the immunodominant PorA protein. Endpoint enzyme-linked immunosorbent assay (ELISA) titers reached 32,000 to 256,000 at week 6 post HuAd5 fHbp injection, and 16,000 to 128,000 with native OMVs (Fig. 1A). An analysis of the IgG subclasses at week 6 indicated that HuAd5 fHbp induced IgG1 and IgG2a mainly, whereas the native H44/76 OMVs induced responses that also included high IgG2b and IgG3 antibodies (Fig. 1B). The latter could be directed against any of the immunogenic antigens comprised within OMVs, in particular the immunodominant PorA. Both the full length and truncated fHbp-encoding adenoviruses elicited T cell responses (Fig 1C).

Importantly, both vectors were able to induce functional antibody responses, as evidenced by the bactericidal activity detected in pooled mouse serum samples at week 42 (Fig. 1D). Individual SBA titers and T-cell responses were similar when a tissue plasminogen activator (tPA) signal sequence was used instead or in addition of the wild type fHbp signal sequence (fig. S1A and S1B). The vector comprising both the tPA signal sequence followed by the full-length (immature) bacterial signal sequence (HuAd5 fHbp) was selected for further analysis and optimization. Induction of bactericidal response was confirmed in a second mouse inbred strain (NIH Swiss) against strain H44/76-SL as well as against another bacterial strain expressing a lower amount of fHbp variant 1.1 (BZ83, Fig. 1E), and also after intranasal and sublingual delivery (fig. S1C). A dose response experiment suggested that bactericidal responses appeared highest after vaccination with 10^9 infectious units (IU) per mouse (Fig. 1F), a relatively high dose in mice. Finally, a longitudinal study was performed. As a single dose OMV vaccines is poorly immunogenic, and OMVs are not used as single dose in humans, a single dose adenovirus was compared with two doses OMV in order to better represent real use (Fig. 1G). We observed that a single injection with HuAd5 fHbp elicited SBA titers that were consistently equivalent to or higher than OMV-induced responses, at different time points, included when compared with two doses of OMVs (Fig. 1H). The quantitative antibody responses, detected by ELISA, were similar between a single dose HuAd5 fHbp, expressing a single antigen, and two doses of OMVs, containing many more antigens (Fig. 1I). Moreover, the SBA titers remained stable up to 32 weeks post the single injection with HuAd5 fHbp (Fig. 1H). Altogether these results confirm the capacity of the adenoviral-vector platform to elicit functional antibody responses after a single dose in mice.

The adenovirus-induced SBA response is not increased with a prime-boost regimen.

Heterologous prime-boost regimens using a vectored vaccine construct have been shown to induce higher antibody responses than single dose or homologous prime-boost modalities *(26)*. Combinations of HuAd5 fHbp and native (n) OMVs from strain H44/76 were assessed in mice

(Fig. 2A and B). The SBA responses induced by two-dose approaches (homologous and heterologous) did not induce higher SBA titers than those observed at 6 weeks post a single injection (Fig. 2C). Remarkably, the SBA assay against strain H44/76-SL detects bactericidal antibodies directed to all antigens present in the OMVs, including the immunodominant and protective antigen PorA, and thus may advantage the OMV vaccine, due to this PorA-specific response, as well as potential bactericidal responses to other lesser known antigens. Therefore, strain BZ83 was used as target in the SBA assay as it contains low amounts of homologous fHbp variant 1.1, and a PorA (P1.5-2,10) heterologous to the OMVs used for immunizing mice (P1.7, 16), and thus allows a fairer comparison of the fHbp-specific bactericidal antibodies in this study. Only HuAd5 fHbp was able to elicit SBA responses against this strain, whereas nOMVs did not (Fig. 2D).

One of the most immunogenic vaccine regimens with regard to induction of T cell responses is based on heterologous adenovirus prime-poxvirus boost regimen *(27)*. A Th1-biased T cell response may be associated with better functional responses to protein-based meningococcal vaccines *(18)*. Therefore, the heterologous vectored prime-boost approach was explored, using a modified vaccinia Ankara (MVA) vector encoding the same fHbp 1.1 sequence as in the adenovirus prime (Fig. 2E). MVA was a poorer primer of antibody responses as compared with HuAd5, as evidenced by the lower and slower induction of SBA response after a single dose (Fig. 2F). The prime-boost regimen, whether HuAd5-MVA or MVA-HuAd5, did not induce substantially higher SBA titers than HuAd5 alone, (Fig. 2F). The SBA responses persisted in all groups up to week 50 (Fig. 2F). Addition of an adjuvant previously shown to increase the immunogenicity of adenoviral vectors (AddaVax) also did not impact the SBA response induced by HuAd5 fHbp (Fig. 2F), nor the amount of binding antibodies as detected by ELISA at week 50 (Fig. 2G). At week 50 post the single injection or prime, bone marrow B cell responses were explored in a subset of groups as these are associated with longer persistence of circulating antibodies. The results did not suggest that the heterologous prime-boost elicited higher B cell responses (Fig. 2H). Similar results were observed in an independent experiment using bone marrow and spleens of mice immunized with each vector alone or immunized with a prime-boost regimen, where higher numbers of fHbp-specific B cells were detected in the mice that received at least one HuAd5 injection (HuAd5 alone, HuAd5-MVA or MVA-HuAd5, fig. S2A). Altogether, these results suggests that the SBA response induced in this model by a single HuAd5 fHbp injection may be at a plateau at that dose and time points.

A single dose of HuAd5 fHbp compares favorably with 4CMenB.

The adenovirus-based vaccine candidate was compared with one, two and three doses of 4CMenB, which contains detergent-extracted OMVs from strain NZ98/254 (PorA P1.7-2,4), and a recombinant fHbp protein variant 1.1 (Fig. 3A). 4CMenB was assessed at one-tenth and one-fifth of the human dose, the latter administered intraperitoneally because the volume exceeded the permitted volume for intramuscular injection. At week 20, antibody responses measured by ELISA were higher after two or three doses of the licensed vaccine as compared with a single dose of adenoviral vaccine (Fig. 3B). However, the SBA responses against a strain containing an homologous fHbp (H44/76-SL) were equivalent or higher with the adenovirus vaccine (Fig. 3C, top panels). 4CMenB administration schedule is two or three doses, and indeed the SBA response induced by a single dose of 4CMenB in mice was low (Fig. 3C, first time point a week 3). SBA responses against strain NZ98/254 (homologous to the OMV in 4CMenB) were also

explored. This strain expresses intermediate amounts of heterologous fHbp ID 14. Results showed that the adenoviral vaccine was able to induce SBA responses against that heterologous fHbp with titers in the same range as 4CMenB except for the latest time points (from 40 weeks post prime, Fig. 3C, bottom panels). At week 56, a terminal bleed was performed and allowed the assessment of individual SBA responses and their persistence over a year post vaccination. The results mirrored the SBA titers obtained with the pooled serum samples. A single dose of adenovirus vaccine elicited higher or similar SBA titers than two or three doses of 4CMenB, with titers comprised between 1:128 and 1:1,024 at 56 weeks after a single injection (Fig. 3D). Enumeration of antigen-specific B cells in the bone marrow and spleen suggested that a single adenoviral vaccine induced persistence of B cell responses in these organs as well as two or three doses of 4CMenB (Fig. 3E). An exploration of the CD45RA+ CD19+ B cells in lymph nodes and spleens two weeks after injection with a single dose of HuAd5 fHbp, 4CMenB or OMVs confirmed the capacity of the viral vector to induce early high B cell responses in mice, that may explain the persistence of protective SBA titers for up to a year in mice after a single dose (fig. S2B).

In an independent longitudinal experiment (Fig. 3F), assessment of the SBA response was compared with a higher dose of 4CMenB (two-fifths of a human dose). In this study, a single dose of HuAd5 fHbp induced similar SBA titers to those induced by three doses of 4CMenB against strain H44/76-SL (Fig. 3G), and also induced SBA responses against strain BZ198 (PorA P1.7-2,4, similar to the one in the 4CMenB vaccine, and fHbp 1.5), albeit lower than three doses of 4CMenB containing a homologous PorA to that strain (Fig. 3H).

Lastly, immunogenicity was confirmed in outbred mice (CD-1, Fig. 3I). The single dose adenovirus vaccine induced similar SBA titers to those elicited by three doses of 4CMenB, and induced better persistence from six months post first injection against strain H44/76-SL (Fig. 3J). Altogether, these results demonstrate that a single dose adenovirus-based vaccine is sufficient to induce immunity in mouse models.

HuAd5 fHbp induces bactericidal responses against different strains.

Many variants of fHbp circulate in invasive meningococcal strains *(28)*; therefore the capacity of the vaccine candidate to induce serological evidence of protection against strains expressing different fHbp variants in different quantities was measured by SBA using pooled serum samples at different time points post injection with a single dose of the adenovirus vaccine, or one, two or up to three doses of the licensed vaccines, 4CMenB or rLP2086. Twelve target strains were selected, varying either by the variant expressed or by the putative quantity of fHbp expressed on their surface (Table 1). The SBA responses induced by a single dose of the protein-based vaccines was absent, or very low (titer of 1:4) against a single strain out of the 12 tested (Table 1, week 2). None of the vaccines induced SBA against a strain expressing a low amount of fHbp 1.1 (Table 1, M08 0240375). However, a single dose of HuAd5 fHbp induced earlier SBA responses than those generated by the protein-based vaccines against strains expressing medium and high amount of homologous fHbp 1.1 (Table 1, M08 0240063 and M07 40800, respectively) despite the fact that 4CMenB contains other antigens able to induce SBA responses. Both HuAd5 fHbp and 4CMenB were able to induce SBA responses against strains expressing variants 1.13 and 1.15, but not against 1.14 (Table 1). Responses were induced against strains expressing low and medium amounts of variant 1.4. Only rLP2086 was able to induce SBA

responses against strains containing fHbp variant 3.187 and 3.45, as expected (Table 1). rLP2086 appeared to induce limited SBA against some of the variant 1 strains included in this panel, despite containing a variant 1 fHbp, but this may be an artefact of the small number of strain assessed in this study. Altogether, these results show that the strain coverage induced by a single dose of fHbp inserted in the adenovirus delivery platform was similar to that induced by three doses of 4CMenB when tested using this particular panel of strains.

Modifications of the vaccine candidate were designed to increase clinical potential.

Pre-existing immunity to human adenovirus serotypes such as the serotype 5 has the potential to neutralize the vaccine and thus dampen its immunogenicity. One solution is to use adenoviruses that do not circulate in humans, such as chimpanzee serotypes, including ChAdOx1 and ChAdOx2 *(29, 30)*. In addition, the influence of two different CMV promotors on antigen expression was explored, a long and a shorter version described previously *(31)*. The vectors were assessed at suboptimal doses in order to detect differences that may not be observed when using the higher dose of 10^9 IU/mouse (Fig. 4). There was no statistically significant difference ($p>0.05$) between the two clinically-relevant backbones at week 20 post vaccination and ChAdOx1 was selected (Fig. 4).

Mutations of the fHbp transgene to prevent binding to human factor H increases the bactericidal response.

In humans, *N. meningitidis* fHbp binds to the human complement inhibitor factor H (fH), thus decreasing the innate response to the invading bacteria and allowing its survival in the bloodstream *(32)*. This interaction may affect the anti-fHbp antibody repertoire when fHbp is used as vaccine antigen, and decrease SBA due to fH covering important epitopes when binding on fHbp in the vaccine *(33)*. Therefore, mutant fHbp proteins with lower binding to human fH have been generated *(34–36)*, and are associated with higher SBA titers in the presence of human fH *(34, 37)*. We thus explored if the same would occur when fHbp is expressed within the host cells by an adenoviral vector. Two vectors were constructed, HuAd5 fHbp-H248L and -S223R, containing previously described mutations that decrease fH binding *(38)*. The expression of the fHbp mutants in cells infected with the vectored vaccines was at least equivalent to the expression of the wild type antigen in infected HeLa cells (21 to 32% of infected cells, Fig. 5A, top panels). The fHbp mutants expressed in infected HeLa cells had reduced binding to human factor H present in human serum, as well as to recombinant human fH, and the reduction was independent of the adenoviral backbone used (Fig. 5A, middle and bottom panels, respectively). Mouse fH does not bind to fHbp, and we verified that both mutants induced SBA responses comparable with those elicited by the wild-type fHbp in BALB/c mice (Fig. 5B and C) and in outbred mice (Fig. 5D). SBA titers were assessed in transgenic mice expressing human fH and were at similar amounts to those found in healthy humans in the two experiments described previously *(34)* (fig. S3A and B). In this model, the vector expressing the mutant S223R induced superior SBA titers as compared with vectors containing the wild type sequence or the H248L mutation (Fig. 5E and 5F). In a longitudinal study using human fH-expressing transgenic mice, a single HuAd5 fHbp-S223R dose elicited comparable or higher titers than three injections of 4CMenB that persisted up to 17 weeks post-injection (Fig. 5G). The S223R mutation was therefore introduced in the ChAdOx1 backbone (ChAdOx1 fHbp-S223R). Induction of SBA

responses by a single dose of ChAdOx1 fHbp-S223R was confirmed in three strains of mice, including an outbred strain (Fig. 6A). Dose responses in BALB/c and CD-1 highlighted the higher variability observed in outbred mice, where a higher dose is required to obtain 100% seroconversion. SBA responses induced by a single dose ChAdOx1 fHbp-S223R were similar to those induced by 4CMenB administered 3 times in the presence of human fH, and persisted up to week 21 (Fig. 6B). Altogether, these results highlight the potential of an adenoviral-based vaccine expressing a mutated fHbp for use in humans.

DISCUSSION

In this study, we explored the immunogenicity of adenovirus-based vaccine candidates expressing fHbp. Screening of different transgene designs was performed using HuAd5 and allowed a comprehensive exploration of different signal sequence and mutations. The optimal transgene was inserted into the clinically-relevant ChadOx1 vector. We demonstrate that a single dose of ChAdOx1 fHbp-S223R induces higher SBA responses in mice than three doses of 4CMenB in the presence of human factor H. This MenB vaccine is now in phase I human clinical trials. Although the expression of CMV-driven transgenes in adenovirus vectors was shown to be dose-dependent, it is not known if the quantity of antigen expressed, the timing, or the pattern recognition or danger signals provided after infection with the adenovirus are responsible for the response after a single dose. The capacity of adenoviral vaccines to induce T cell responses may also support higher B cell responses and contribute to better persistence as compared with conventional adjuvants, such as aluminum.

Mouse IgG isotypes differ in their capacity to promote bactericidal activity *(39)*. We quantified the antigen-specific IgG1, IgG2a, IgG2b, and IgG3 induced by fHbp-expressing vectors. HuAd5 fHbp induced IgG responses dominated by IgG2a, whereas the nOMV vaccines also induced IgG2b and IgG3. Induction of IgG2a has been observed with adenoviral vectors encoding different antigens (viral, parasitic, and bacterial) in mouse models *(40–42)*, suggesting that this induction of this IgG2a subclass is not driven by the antigen itself, but by the adenovirus vector. IgG1 is not reported as a primary driver of bactericidal activity, but this observation was made for antibodies against the outer membrane PorA protein only, and only in mice *(39)*. It is not known if fHbp-specific subclass antibodies would behave similarly and which subclass is responsible for SBA responses after HuAd5 injection. The IgG1 and IgG2a induced by the nOMV vaccine may be against other antigens (as shown by the ELISA used whole cells), or may be against other epitopes in fHbp due to the different presentation (OMV versus mouse host cells). The titers of binding antibodies was lower after OMV injection than those elicited by HuAd5 fHbp by week 26, which supports the hypothesis that the lower SBA at later time points may in part be due to lower persistence of antibodies after injection with OMVs.

The fHbp gene was inserted as either the immature protein (bacterial signal sequence followed by the protein encoding gene) or the mature protein only (with the bacterial signal sequence removed) to manipulate the N-terminal sequence and the resulting folding of the proteins. In both cases, we elected to add a tPA signal sequence to target the protein to the secretion pathway and to promote antigen presentation on the plasma membrane. Therefore, two signal sequences were encoded for the immature construct. The tPA followed by the bacterial sequence is an original design compared with other adenoviral vaccines expressing bacterial antigens *(43–45)*. The design with the double signal sequence consistently induced higher antibody titers and SBA.

This may suggest that preserving the native fHbp signal sequence contributed to correct processing by signal peptidase and supported native folding for this antigen, as previously observed with the SARS-CoV-2 spike protein *(46)*. However structural data suggest the same folding for fHbp, regardless of presence or absence of leader peptide and regardless of variant type; thus, the exact mechanism for when two signal sequences are used is unknown *(46)*. Moreover, although it is expected that the expressed fHbp is glycosylated due to the presence of the tPA (which may cause issues for bacterial antigens in this type of vaccine platform), we do not know if it would be lapidated. The contribution of each element in the signal sequence has been explored separately *(47)*.

The lack of boosting of the SBA responses with heterologous prime-boost regimen was surprising given existing literature suggesting that such regimen leads to higher immune responses *(12, 26, 48, 49)*. The lack of boosting may be a dose effect, as a high dose of adenoviral vaccines was used in this study ($1x10^9$ IU per mouse). In this study, any regimen including an adenovirus injection, whether as a prime or a boost, induced high bactericidal antibody responses, and a remarkable persistence of antibody titers, linked with higher numbers of bone marrow antibody-secreting B cells as compared with protein-based vaccines. Whether a single dose will elicit similar high and long-lasting SBA responses in humans is currently being explored. Persistence of antibody responses with a single adenoviral vaccine injection in humans has been observed for an Ebola virus vaccine. After a single dose in children, the antibody titers decreased during the first six months and remained remarkably stable at 12 months *(50)*. However, higher total responses and better antibody persistence were observed after a second dose of ChAdOx1 nCoV-19 *(51)*, suggesting that there are differences in immunogenicity due either to the backbone vector in humans, or to the antigen itself. There is induction of neutralizing antibody responses against the vector, which increases with increasing numbers of doses. However, this induction of neutralizing activity does not seem to affect the antibody response to the expressed transgene protein *(51)*. Whether the administration of ChAdOx1 nCoV-19 interferes with another ChAdOx1-based vaccine will need to be addressed during clinical development.

A limitation is that a MenB vaccine based on a single antigen is unlikely to induce sufficiently broad protection *(52)*, as the prevalence of different fHbp variants differs across geographical regions. The absence of cross-reactivity across families was previously observed in fHbp protein-based vaccines *(53)*. The licensed vaccine based solely on fHbp (rLP2086) contains two variants *(52, 54)*. However, introducing two fHbp variants in one adenovirus vector is challenging, as the homology between the two variants is highly likely to lead to internal recombination depending on the position of the transgenes. In this context, a mRNA-based approach may be more amenable to mixing several antigens than the adenovirus platform. However, the challenges of preserving the correct expression and presentation of bacterial B cell epitopes are likely to be similar between adenovirus and mRNA platforms. Our attempts to induce bactericidal responses to other protective MenB antigens in adenoviral vectors were unsuccessful *(19)*. Therefore, in an attempt to improve the bactericidal antibody response induced by a single fHbp variant and produce a clinically-relevant vaccine, we elected to introduce a point mutation abrogating binding to human fH *(38)*. In multiple studies, it has been suggested that reduced fH-fHbp binding induces higher SBA responses in mouse models *(33, 36, 37, 55)*. We introduced two mutations described previously *(38)* and demonstrated that when expressed with the adenoviral vaccine platform, the S223R mutation induced the highest SBA titers in the presence of human fH in mice. It would be of interest to assess if the mutation

resulted in higher cross-reactivity as shown for the original insert. This question is being addressed in humans as part of the phase I clinical trial, as another limitation of this study is that it relies on mouse models using the accepted correlate of protection (SBA).

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In conclusion, our results demonstrate that outer membrane bacterial antigen targets can be expressed in eukaryotic cells from viral vectors and retain a relevant conformation, so that a functional antibody response is elicited. Here, fHbp is presented in a relevant conformation when expressed by a viral vector, and the resulting vaccine is able to induce a rapid, strong, long-lasting and functional antibody response. This vaccine is now being tested in a first-in-human phase I clinical trial in healthy adults, and has the potential to address the lingering need for a more cost-effective vaccine against serogroup B *Neisseria meningitidis* which has low manufacturing costs *(56)*, and only requires a single injection to provide sustained protection in adolescents *(57)*. The results of these preclinical studies have the potential to be transferable to other gene-based vaccine delivery platforms, such as mRNA, and further highlight the potential of such vaccines to be used for other bacterial diseases.

MATERIALS AND METHODS

Study design

The overall objective of this study was to investigate the potential of a viral-vectored vaccine platform to induce functional protective antibody responses against the bacterial disease caused by group B meningococcus. The outer membrane protein target selected was known to contain protective epitopes. Several vaccine candidates based on replication-deficient adenoviruses were constructed and preclinical batches produced. Groups of mice were immunized with defined doses of vaccines, and the individual mouse experiments within this study were designed to address different questions: murine experiments were performed to explore the strength, longevity and cross-reactivity of the responses, as described in the corresponding figure. The treatments included vaccine comparators and/or naïve animals. The measurement of the immune responses included quantitative (ELISA) and qualitative (serum bactericidal activity) antibody assays. All data were included in the analysis. Sample size determination was performed based on previous experience with immunization with MenB fHbp mutants and number of available transgenic animals, the number of animals per group is indicated in the figure legends. Each animal was allocated randomly to a treatment group by the animal caretaker. The experimenter was not involved in the randomization. Assays included either two (SBA) or three (ELISA) technical replicates.

Vaccine candidates

The nucleotide sequence for the antigen fHbp, variant 1.1 was obtained from the GenBank sequence database (https://www.ncbi.nlm.nih.gov/genbank/, NMB_1870). The sequences were codon-optimized for expression in mammalian cells. Recombinant adenoviruses (Ad5, ChAdOx1 and ChAdOx2) were generated as described previously using a Gateway-compatible entry vector *(51, 58, 59)*, using a CMV promotor and a tissue plasminogen activator signal

sequence (tPA). The antigen was inserted as 'full length' using the immature fHbp sequence, including the signal sequence that is cleaved in the mature protein, or truncated (labelled t) where the bacterial signal sequence was omitted (mature protein). Empty or irrelevant adenoviral vectors were used as controls. Although vectors are dosed as viral particles (VP, quantified by OD280) in humans, the antigen-specific immunogenicity is due to infectious virus (IU, quantified by titration) that leads to transgene expression as opposed to viral particles which also measures non-infectious virus. Therefore, dosing as IUs was selected for these preclinical studies aiming at comparing different transgene designs. The P:I ratios (particles:infectivity) were measured for all batches. All HuAd5 expressing the various designs had P:I ratios below 39. The ChAdOx1 and ChAdOx2 preclinical batches had P:I ratios ranging from 195 to 545. The modified vaccinia Ankara (MVA) vectors encoding the same antigen were generated as described previously *(60)*. Outer membrane vesicles (OMVs) were generated and purified as described previously *(61, 62)*.

Immunogenicity experiments in mice

Procedures were performed according to the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Oxford Animal Care and Ethical Review Committee or the Institutional Animal Care and Use Committee at UCSF Benioff Children's Hospital Oakland. Experimental design followed ARRIVE guidelines. Randomized healthy 6- to 8-week-old female BALB/c-OlaHsd and NIH-OlaHsd, Hsd:ICR (CD-1)outbred mice (Harlan, UK), or 8 to 16-week old human factor H transgenic (hfH Tg BALB/c mice of both sexes (Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, USA)*(35)*, were housed in specific pathogen-free conditions. Sex, age and human fH concentration for the hfH Tg mice were randomized in each vaccine group. Injections were performed by intramuscular route unless otherwise indicated. Blood was collected from tail bleeds or terminal cardiac bleeds at various time points and allowed to clot, then centrifuged at 15000 x g for 10 minutes. Sera were aliquoted and stored at -20^{o}C until use. Spleen, lymph nodes and bone marrow were harvested following cervical dislocation under terminal sedation. Mouse serum samples collected in the USA were shipped and assayed in the UK.

Detection of antibodies by ELISA against whole cells or recombinant proteins

Immulon 2HB plates (Thermo Fisher Scientific) were coated with heat-killed whole-cell preparations of *N. meningitidis* in phosphate-buffered saline (PBS) (optical density (O.D.) 600nm = 0.1), or with recombinant fHbp protein expressed in *E.coli* using an fHbp expression construct as previously described *(63)*, at 2.5 µg/ml in carbonate-bicarbonate buffer (Sigma Aldrich). Serum samples were serially diluted in PBS containing 0.5% (v/v)Tween-20 and 1% (w/v) bovine serum albumin (BSA). High, medium, and low positive quality controls were used in each plate (anti-PorA monoclonal antibody P1.7 or anti-fHbp monoclonal antibody JAR4, National Institute of Biological Standards and Controls). Serum from naïve BALB/c mice was used as negative control along with buffer only. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc) and visualized with 3,3',5,5'-Tetramethylbensidine substrate (TMB, Sigma Aldrich). The reaction was stopped with 50 µl H_2SO_4, and O.D. were measured at 450 nm with a reduction at 600 nm. Endpoint titers

were defined as the serum dilution corresponding to the O.D. reading above two times the average of naïve negative control serum.

Serum bactericidal assay (SBA)

SBAs were performed as described previously using 25% (vol/vol) human serum as a complement source, from donors screened for no intrinsic SBA *(64)*. Heat-inactivated murine serum samples were serially diluted in Hanks Balanced Salt Solution supplemented with 0.5% BSA. SBA titer was defined as the reciprocal of the highest dilution of serum that yielded ≥50% decrease in colony forming units relative to that of control wells within 60 minutes at 37ºC without CO_2. Meningococcal target strains were provided by the Manchester Meningococcal reference Unit, UK).

Enumeration of antigen-specific antibody-secreting B cells by enzyme-linked immune-spot assay (ELISPOT)

Ninety-six well filtration ELISPOT plates (Millipore) were coated with recombinant fHbp at 2.5 µg/ml or 1:1000 dilution of goat-anti-mouse IgG (BioLegend, positive controls), or PBS (blank wells). Splenocytes or bone marrow cells (acquired by flushing the bones with PBS through a needle) were incubated in duplicates at a concentration of $4x10^5$, $2x10^5$ and $1x10^5$ cells per well. Detection of spots was performed with alkaline phosphatase conjugated goat-anti-mouse IgG (Invitrogen) followed by alkaline phosphatase substrate (Bio-RAD). Spot counts were performed using an AID ELISpot Reader ELR03 and ELISpot software as described previously *(65)*. Results were expressed as the number of antigen-specific spots detected per million cells, minus the number of spots counted in the absence of antigen (medium only). A negative result was recorded as 1.

Detection of fHbp expression and hfH binding by flow cytometry

Human epithelial HeLa cells (CCL-2, the American Type Culture Collection) were infected with 5×10^8 IU of adenovirus constructs and incubated overnight at 37°C. Infected cells were stained with anti-fHbp monoclonal antibody JAR5 (National Institute of Biological Standards and Controls) followed by anti-IgG AlexaFluor-488 (Invitrogen, 1:10000 dilution), for 30 minutes at 4°C. The cells were washed with AutoMacs running buffer (Miltenyi) pre- and post-antibody staining, fixed and permeabilized with a Fixation/Permeabilization kit (BD Biosciences). The antibody incubation steps with JAR5/anti-IgG AlexaFluor-488 were repeated for intracellular staining. The stained cells were then ran on a FACSCalibur flow cytometer (BD Biosciences). The percentage of fHbp expressing cells was measured using FlowJo software (BD Biosciences).

Statistics

Antibody titers as measured by ELISA are presented as median +/- 95% confidence intervals, SBA titers are presented as geometric mean titers +/- 95% confidence intervals. Statistical analysis of differences between antibody titers were performed using either Kruskal-Wallis test, Mann-Whitney test, two-way ANOVA with Bonferroni post-tests, or one-way ANOVA with

Dunn's multiple comparisons test when appropriate and as stated, using Prism 5 (Graphpad Inc.). The experimental units are single animals. No data exclusion was done. Potential confounders were minimized by changing orders of treatments and measurements and random cage location.

List of Supplementary Materials

Fig. S1 to S3

MDAR Reproducibility Checklist

Data file S1

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CSR, AVH and AJP conceptualized the study. AJP and CSR generated the funding. DW, PTB, AJP and CSR led the investigations. CSR, CD, LM, DW and PTB developed the methodologies. The experiments were performed by CD, LM, NW, LSR, EC, GKP for the cloning, generation of vaccine candidates, immunogenicity in

wild type mice including all readouts, KS and PTB performed the immunogenicity studies in transgenic mice. Visualization of the results was performed by CD. CD, LM, LC, DW, PTB, AJP and CSR supervised the laboratory work. CSR and CD wrote the paper. CD, LM, KS, PTB, AJP and CSR edited the paper. All authors reviewed the paper.

*Competing interests***:** AJP is Chair of U.K. Dept. Health and Social Care's (DHSC) Joint Committee on Vaccination & Immunisation (JCVI). The views expressed in this article do not necessarily represent the views of DHSC, JCVI. CD, LM, DW, CSR, AJP and AVH are named inventors on a patent in the field of meningococcal vaccines (PCT/GB2021/052692, Compositions And Methods For Inducing An Immune Response). AJP waives his rights under any patent. PTB is a named inventor on patents related to meningococcal vaccines (Issued U.S. Patent No. 10,995,122 B2). AVH is named as an inventor on a patent covering use of simian adenoviral vectored vaccines. PB has performed paid consultancy work for Pfizer. CSR has performed paid consultancy for Guidepoint. NW, LSR, LC, JPD, KS, and GKP declare that they have no competing interests.

*Data and materials availability***:** All data are available in the main text or the supplementary materials. Materials transfer agreements (MTAs) will be required for access to the materials. All MTA request should be directed to Andrew J. Pollard at Andrew.pollard@paediatrics.ox.ac.uk

Figures

Fig. 1. **HuAd5 vectors expressing different versions of fHbp are immunogenic in mice.** Groups of mice (n=8 to 16) were immunized with HuAd5 expressing fHbp full length or truncated, or nOMVs as indicated. **(A)** Serum IgG antibody responses were detected in serum samples by ELISA against heat inactivated H44/76 bacteria, 2 and 6 weeks post a single injection. **(B)** IgG subclass titers were measured at week 6. In (A and B), the titers for each individual mouse, the median and 95% confidence interval of the group, are presented. **(C)** Individual (n=6) fHbp-specific T cell responses were assessed in spleens two weeks post a single injection of 10^8 or 10^9 infectious units per mouse. **(D)** Mice (n=4 to 6) were immunized as per (A), and SBA responses were measured in pooled serum samples at week 42. **(E)** SBA titers were measured in pooled serum samples from BALB/c and NIH Swiss mice (n=4 to 6 per group and strain) two weeks post injection with 10^9 infectious units of HuAd5 fHbp (blue); white bars are titers observed in pooled naïve mouse serum samples. **(F)** Shown is a dose response in BALB/c mice. Individual IgG and SBA titers were measured 6 weeks after a single injection with HuAd5 fHbp at the doses indicated on the x-axis (n=4 to 8). **(G)** Shown is a schematic of the longitudinal study (n=10) assessing the persistence of antibody responses after a single dose of HuAd5 fHbp (blue) as compared with two doses of native (n) OMVs (pink). **(H and I)** SBA titers in pooled serum **(H)** and individual IgG titers **(I)** at the different time points are shown. In (E, F, and H), the horizontal dotted red line denotes the putative threshold associated with protection (titer of 1:4). Individual data in (A, B, F left panel and I) are presented as median +/- 95% confidence intervals, and data in (C and F right panel) are presented as geometric mean titers +/- 95% confidence intervals . Data were analyzed by Kruskal Wallis with Dunns multiple comparison test.

Fig. 2. A single adenovirus is sufficient to induce high SBA response in mice. Shown are the effects of prime-boost regimen using HuAd5 and nOMV combinations **(A, B, C and D)**, or HuAd5 and MVA combinations **(E, F, G and H)**. Mice (n=5 to 6 per group) were immunized with the regimen indicated (10^9 infectious units HuAd5, 5 μg nOMV or 10^7 infectious units MVA). **(A)** Shown is the timeline for the HuAd5 and nOMV immunization regimen**. (B and C)** Individual serum IgG titers are shown. **(C)** SBA titers in pooled serum were measured against strain H44/76-SL expressing the homologous fHbp (variant 1.1) **(C)** or strain BZ83 expressing an homologous fHbp but heterologous to the PorA in the nOMV **(D)**. The horizontal dotted red line denotes the putative threshold associated with protection (titer of 1:4). **(E)** Shown is the timeline for the HuAd5 and MVA immunization regimen. **(F)** SBA responses measured in pooled serum samples at different time points. (**G and H**) At week 50, individual serum IgG titers **(G)** and bone marrow B cell responses **(H)** were measured in each group. Individual data in (B and G) are presented as median +/- 95% confidence intervals, and data in (D, F and H) are presented as the geometric mean +/- 95% confidence interval of the group as

indicated. Data in (B , G and H) were analyzed by Kruskal Wallis with Dunns multiple comparison test.

Fig. 3. A single dose of adenovirus vaccine induces a persistent humoral response in mice. Kinetics of SBA responses against different strains and comparison with 4CMenB are shown**. (A)** Groups of mice (n=4 to 8) were immunized as indicated and blood samples were collected at different time points. At the termination of experiment, spleens and bone marrow were collected. HD, human dose. **(B)** Individual anti-fHbp endpoint titers were measured by ELISA at weeks 6 and 20; data show individual titers, the median and 95% confidence intervals for each group. **(C)** SBA using human complement was performed using pooled serum samples at the different time points against strain H44/76-SL expressing the homologous fHbp and strain NZ98/254 expressing a heterologous fHbp but homologous for the OMV component in 4CMenB. The titer obtained for each pooled sample is indicated. **(D)** At week 56, individual SBA titers were measured; geometric means and 95% confidence intervals are indicated. **(E)** At week 56, individual antibody-secreting cell numbers were calculated in spleens and bone marrow samples; geometric means and confidence intervals are indicated for each organ. **(F)** A second longitudinal study compared HuAd5 fHbp vaccination with a higher dose of 4CMenB. (**G and H**) SBA titers were measured at different time points in pooled serum samples against strain H44/76 **(G)** and BZ198 **(H). (I and J)** In an independent experiment (**I**), sufficient blood volumes were collected at four time points to measure individual SBA titers (**J**). Individual SBA titers, geometric means, and confidence intervals are reported. The horizontal dotted red line in (D, E, G, H, and J) denotes the putative threshold associated with protection (titer of 1:4). Data in (B, D, E and J) were analyzed by Kruskal Wallis with Dunns multiple comparison test.

Fig. 4. The clinically relevant ChAdOx1 vector encoding the selected antigen design induces SBA in mice. The mpact of clinically-relevant modifications to the vaccine on the SBA response in mice is shown. Groups of BALB/c mice (n= 4 or 6) were immunized with a single dose of adenovirus vaccine using different backbones and either a short or a longer version of the CMV promoter as indicated. Individual SBA titers, geometric mean and 95% CI are shown. The horizontal dotted red line denotes the putative threshold associated with protection (titer of 1:4).

Fig. 5. A point mutation in the transgene abrogates binding to human fH and increases SBA responses in the presence of human fH. Point-mutations were introduced in the fHbp transgene (H248L) and (S223R). **(A)** In vitro expression of the resulting protein, and capacity to bind human factor H was verified in HeLa cells infected with the adenoviruses as mentioned. In the top row, expression of the antigen was measured by flow cytometry using an anti-fHbp monoclonal antibody (JAR5), and expressed as percentage of positive cells. Middle and bottom rows: HeLa cells were infected with the adenoviruses as mentioned, followed by incubation with human serum fH (middle row) or with recombinant human fH (bottom row). Detection of bound human fH was performed using a commercial anti-human fH antibody by flow cytometry. Representative panels from an individual experiment are shown. **(B)** Immunogenicity of the mutant-expressing vectors was measured in the absence or presence of human (h) fH. Groups of BALB/c, CD-1 or hfH transgenic (Tg) mice (n=5 for BALB/c and CD-1, n=12 for hfH Tg mice) were immunized once with the adenovirus as mentioned. **(C to E)** individual serum SBA titers against strain H44/76-SL were measured at weeks 2, 6 or 14 in BALB/c (**C**), CD-1 (**D**), and fH Tg BALB/c (**E**) mice. **(F)** Individual SBA titers were measured in fH Tg mice vaccinated in an independent experiment repeating the assessment of HuAd5 fHbp S223R. Individual human fH amounts and correlation with SBA titers for both experiments are shown in fig. S3. **(G)** Shown is a comparison of immunogenicity between HuAd5 fHbp-S223R and 4CMenB in transgenic mice expressing human fH (n=12). Tg mice were immunized once with the vectors expressing the S223R mutant, or three times with 4CMenB, and individual SBA titers were measured at several time points post injection against strain H44/76-SL. For (C to G), geometric means and 95% confidence intervals are indicated. Data were analyzed by Kruskal Wallis with Dunns multiple comparison test, except for (D), data were analyzed by Mann-Whitney test. The horizontal dotted red line denotes the putative threshold associated with protection (titer of 1:4).

Fig. 6. SBA responses are induced by the clinical vaccine composition ChAdOx1 fHbp-S223R in mice. (A) Immunogenicity and dose response experiment results are shown for ChAdOx1 fHbp-S223R vaccination in three strains of mice (two inbred and one outbred). Individual SBA titers at week 6 are indicated with geometric means and 95% confidence intervals. **(B)** Longitudinal analysis of SBA responses in the presence of hfH are shown. hfH Tg mice (n=10) were immunized as indicated. Individual SBA titers geometric means and 95% confidence intervals are shown for mice followed up to 21 weeks post prime. Data were analyzed by Kruskal Wallis with Dunns multiple comparison test. The horizontal dotted red line denotes the putative threshold associated with protection (titer of 1:4).

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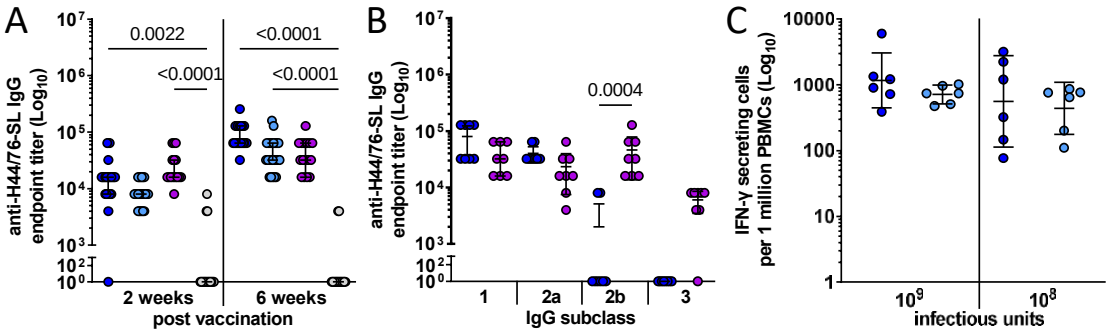
Figure 1



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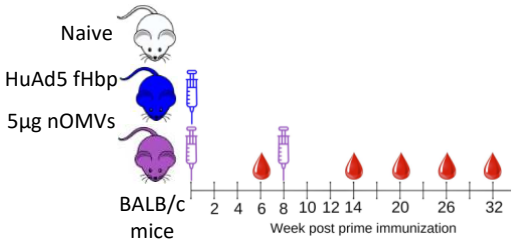
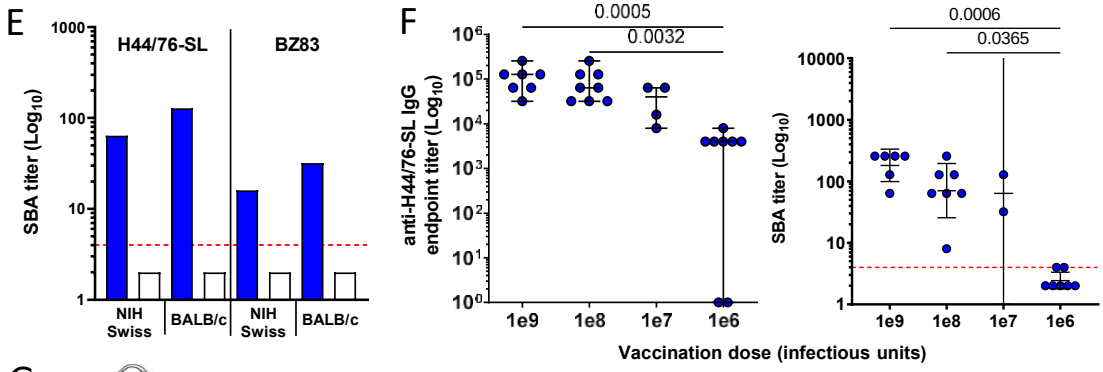
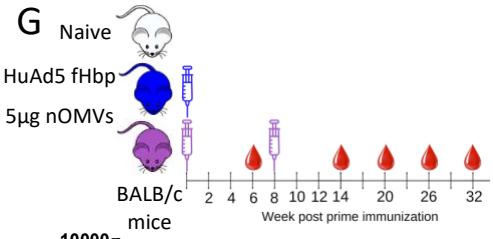
Vaccine	Week 42 SBA titers	
	H44/76-SL	BZ83
HuAd5 fHbp	256	128
HuAd5 fHbp truncated	128	32
lgtBLpxL1 nOMVs	<4	<4

Numbers are the serum bactericidal activity titer in pooled sera from mice immunized with the vaccines as indicated, against strains H44/76-SL or BZ83 at week 42







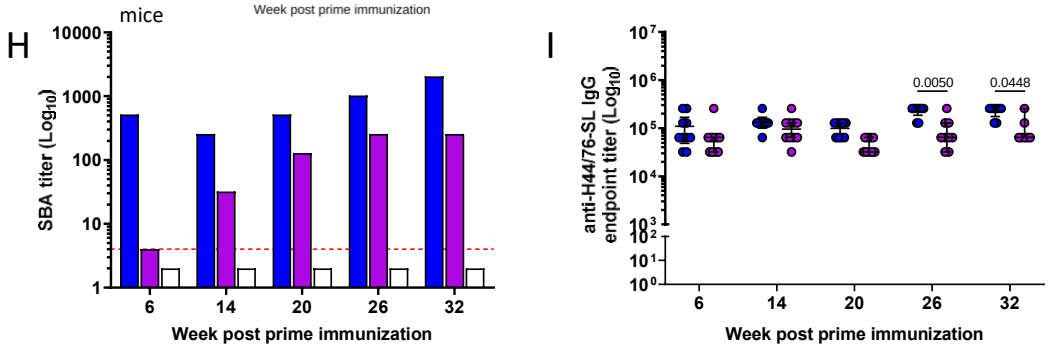


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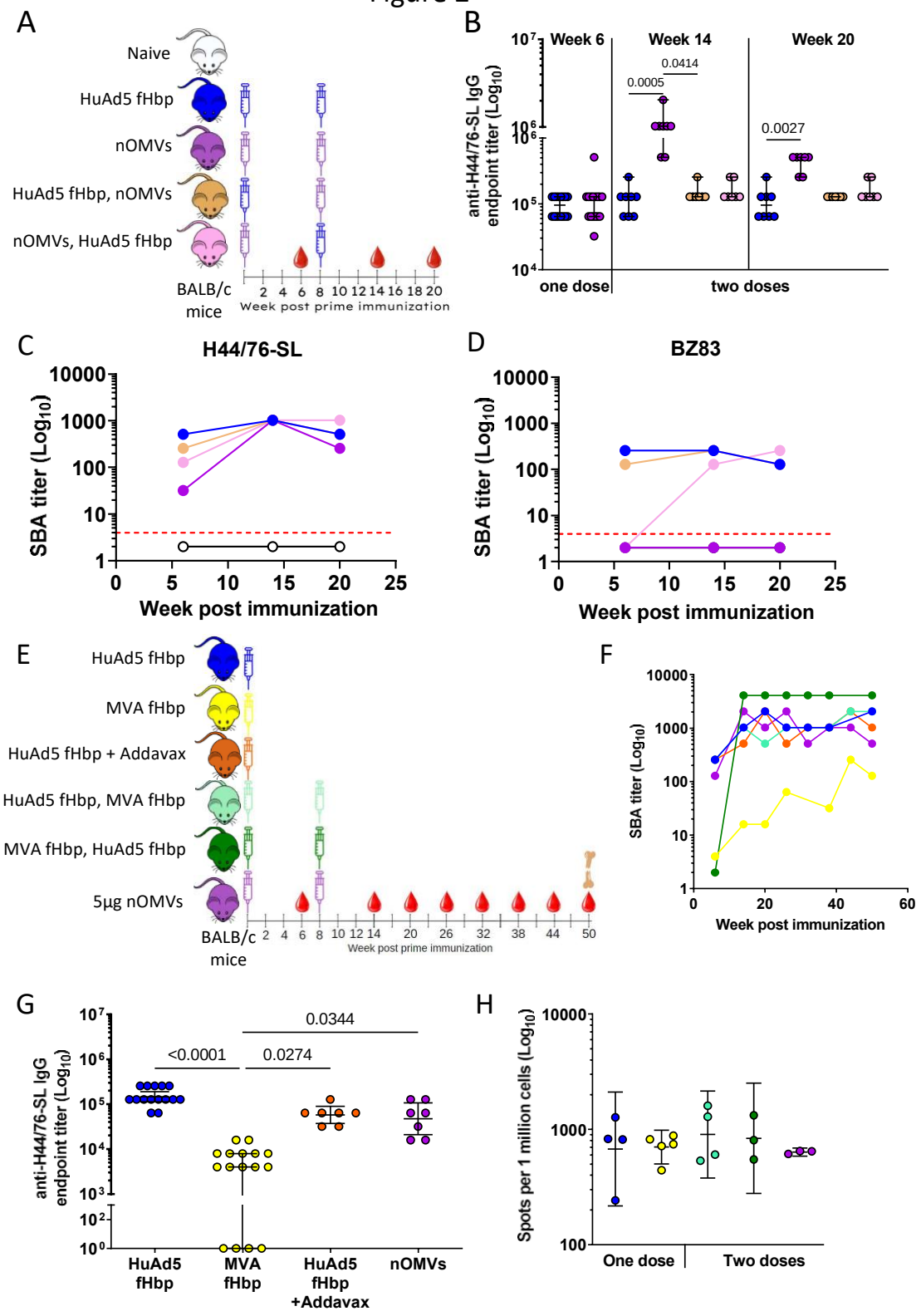
Figure 2



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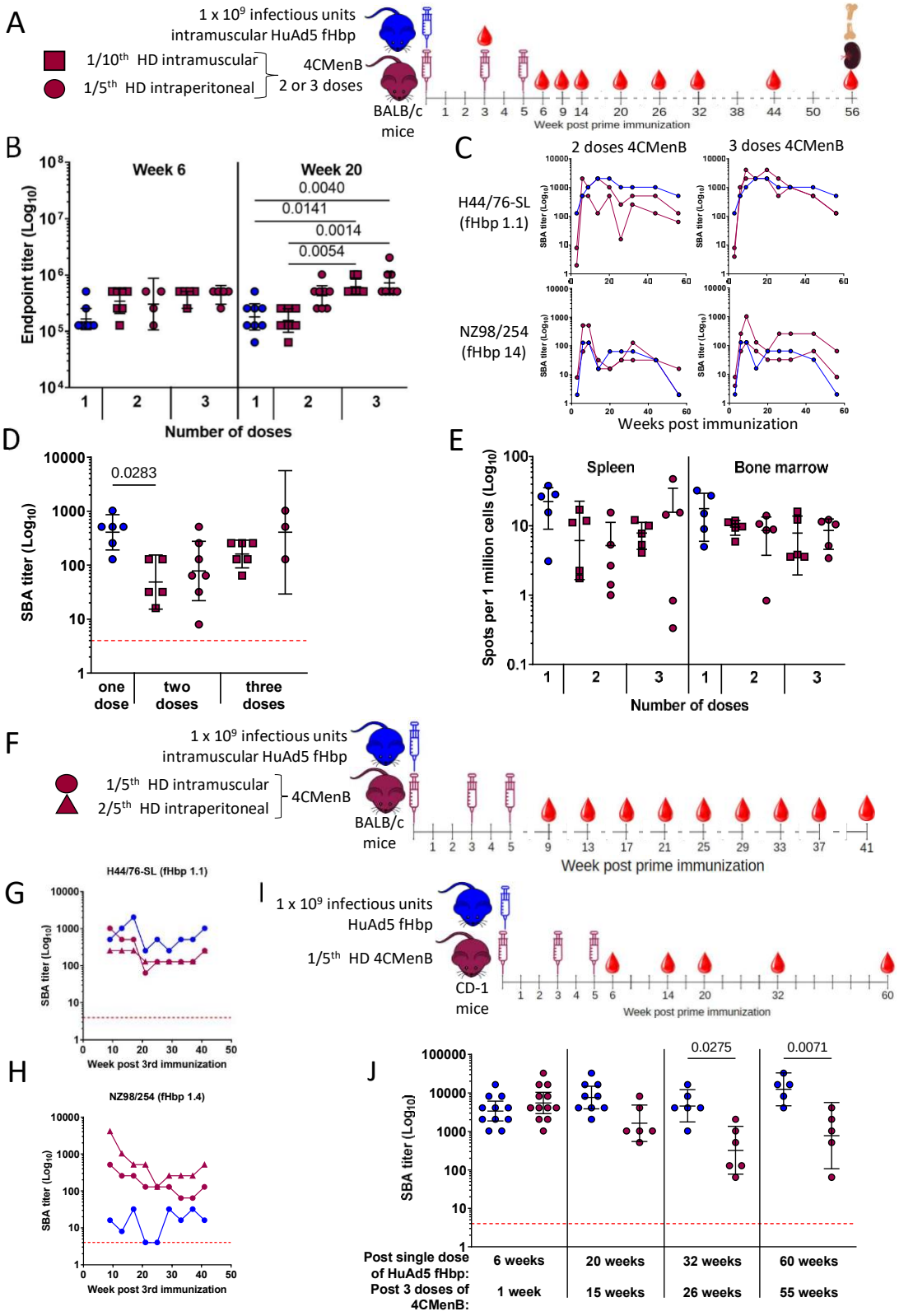

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Figure 4

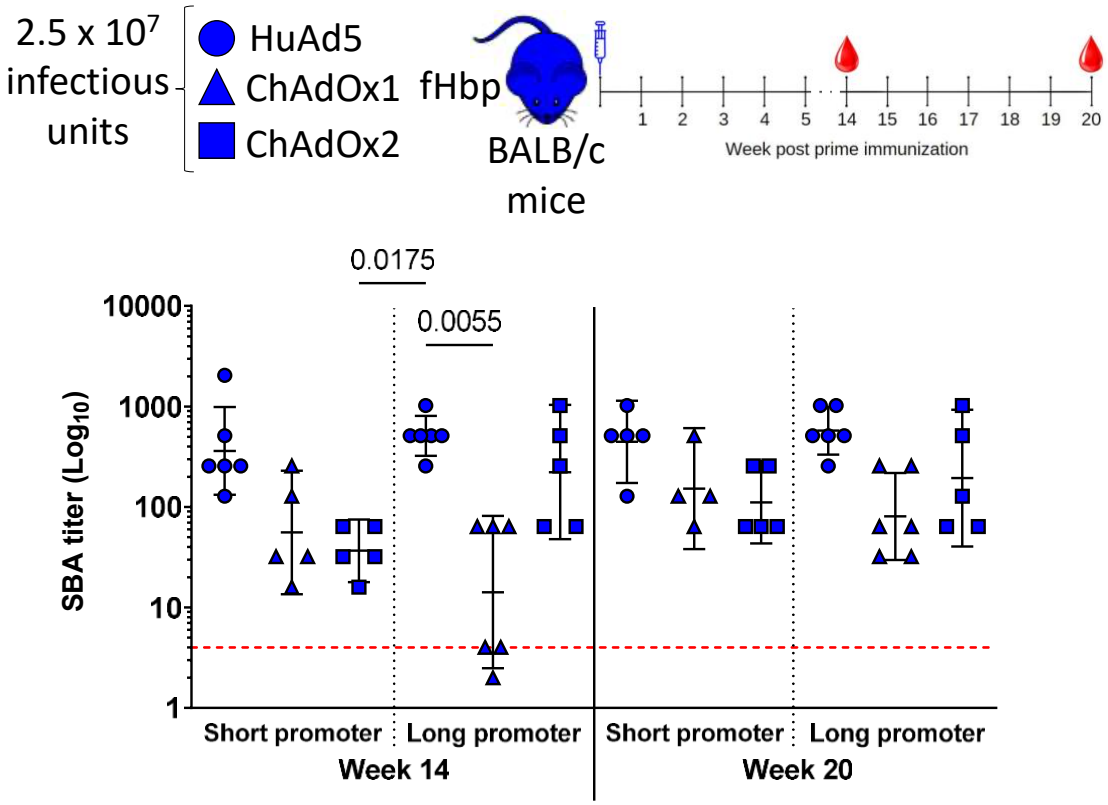

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Figure 5

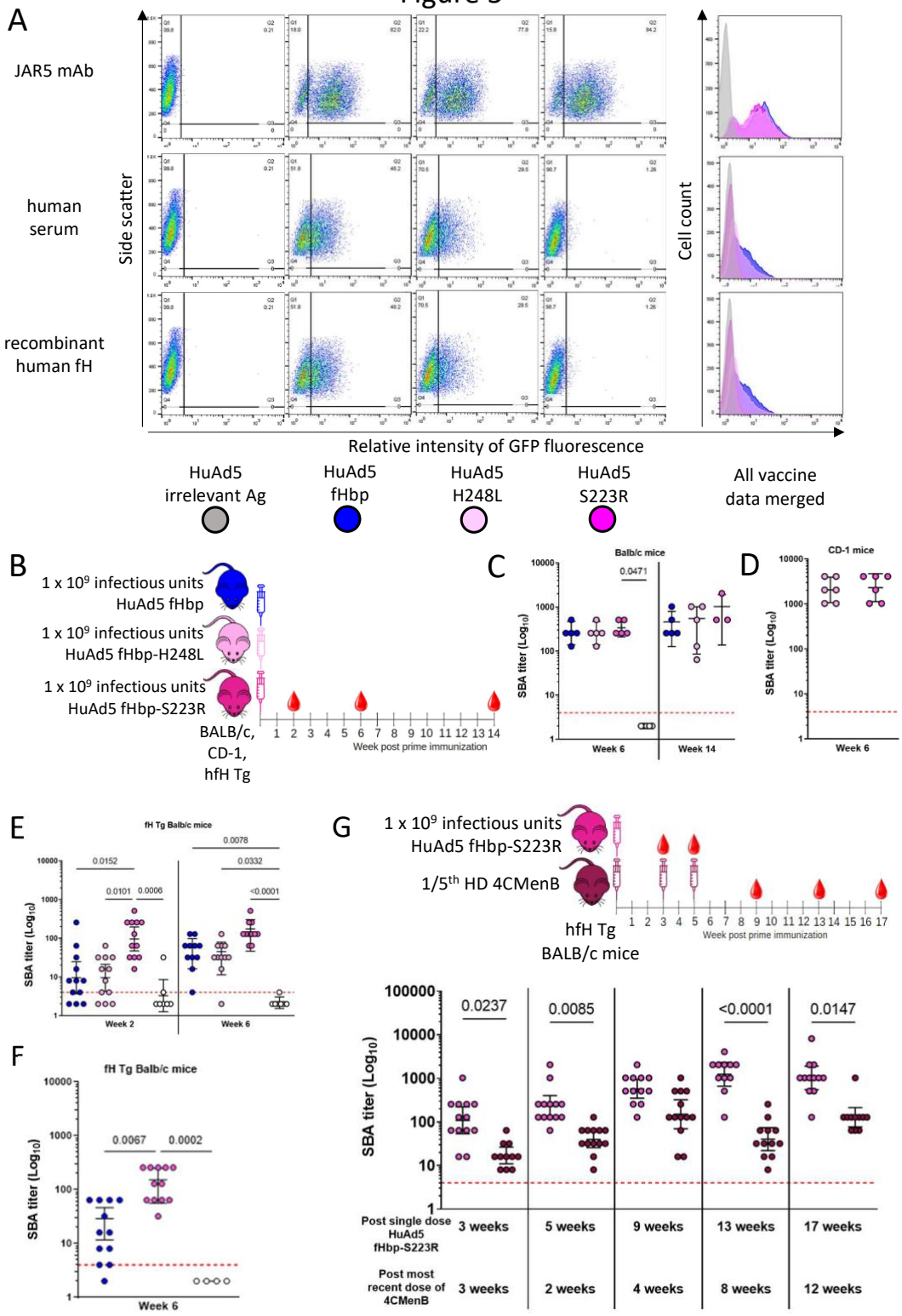

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Figure 6

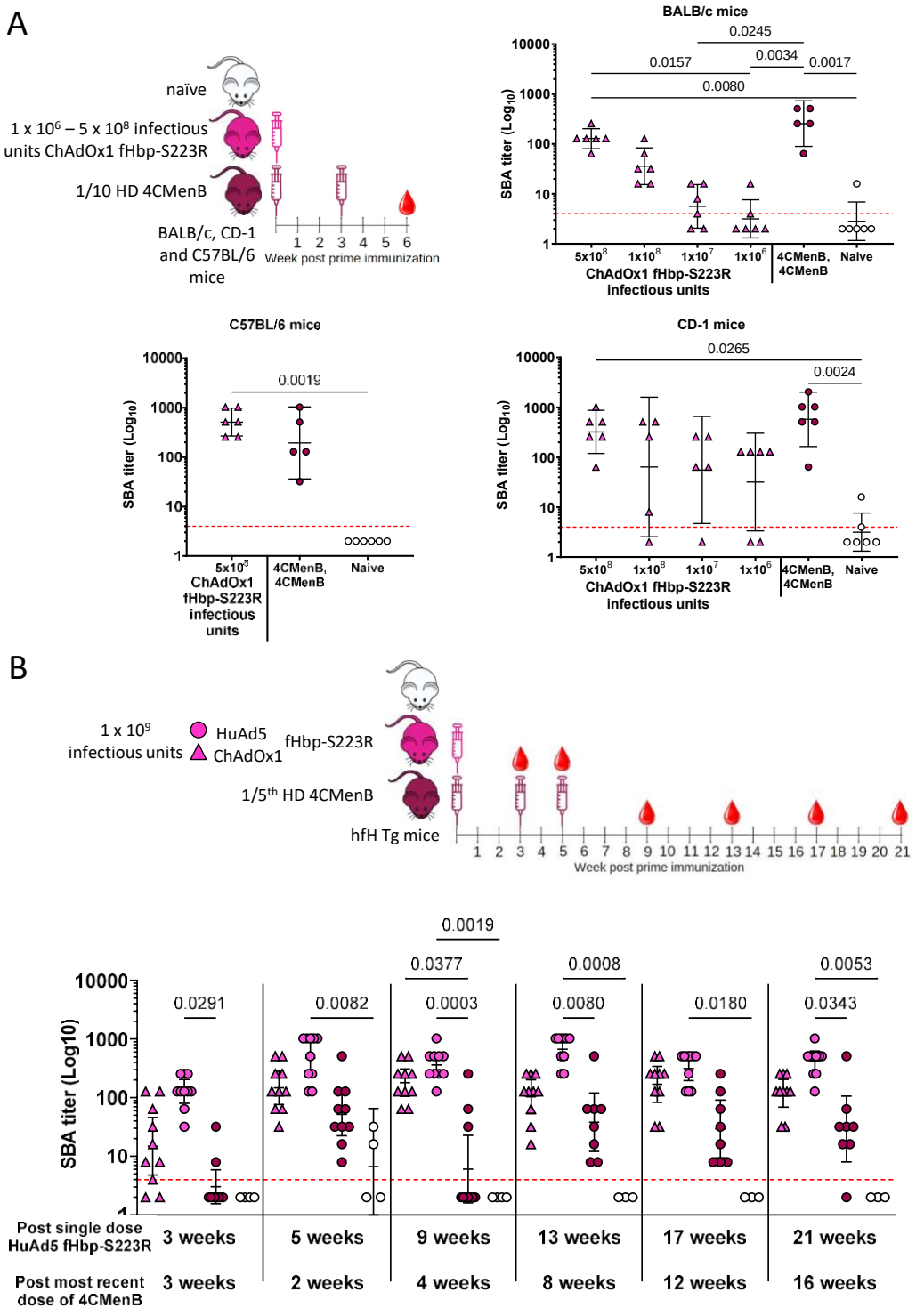

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Table 1. Bactericidal activity was measured against a panel of MenB strains. The target strains are indicated along with their fHbp variant, relative potency and clonal complex. The table shows the SBA titers elicited in groups of mice immunized with either rLP2086 (after 1, 2 or 3 doses), 4CMenB (after 1, 2 or 3 doses) or HuAd5 fHbp (single dose), at the time points indicated. The assays were performed using human complement and pooled serum samples from each group against each of the strain. * The relative potency for fHbp is reported from the meningococcal antigen typing system (MATS) (Plikaytis *et al.*, 2012). ND, not determined.

Target strain	fHbp variant	Relative potency *	Clonal complex	2 weeks post vaccination			9 weeks post single HuAd5 dose			11 weeks post single HuAd5 dose		
							6 weeks post protein dose 2			6 weeks post protein dose 3		
				rLP2086	4CMenB	HuAd5 fHbp	rLP2086	4CMenB	HuAd5 fHbp	rLP2086	4CMenB	HuAd5 fHbp
M08 0240375	1.1	0.218	ST-32 /ET-5	<4	<4	<4	<4	<4	<4	<4	<4	<4
M08 0240063	1.1	0.746	ST-32 /ET-5	<4	<4	32	<4	32	2048	8	512	1024
M07 0240800	1.1	1.243	ST-162	<4	<4	16	<4	64	1024	<4	512	1024
M07 0240639	1.13	0.034	ST-41/44 /Lineage 3	<4	4	16	<4	256	128	<4	1024	512
M07 0241016	1.14	0.07	ST-41/44 /Lineage 3	<4	<4	<4	<4	<4	<4	<4	<4	<4
M07 0240871	1.15	0.053	ST-269	<4	<4	<4	<4	<4	<4	<4	256	512
M08 0240103	1.4	0.032	ST-41/44 /Lineage 3	<4	<4	<4	<4	64	32	<4	512	128
M08 0240102	1.4	0.074	ST-41/44 /Lineage 3	<4	<4	<4	<4	<4	1024	<4	256	512
M11 240 181	3.187	ND	ST-213	<4	<4	<4	<4	<4	<4	64	<4	<4
M11 240 183	2.49	ND	ST-32	<4	<4	<4	<4	<4	<4	<4	<4	<4
M11 240 976	3.45	ND	ST-213	<4	<4	<4	<4	<4	<4	32	<4	<4
M13 240 519	2.19	ND	ST-213	<4	<4	<4	<4	<4	<4	<4	<4	<4

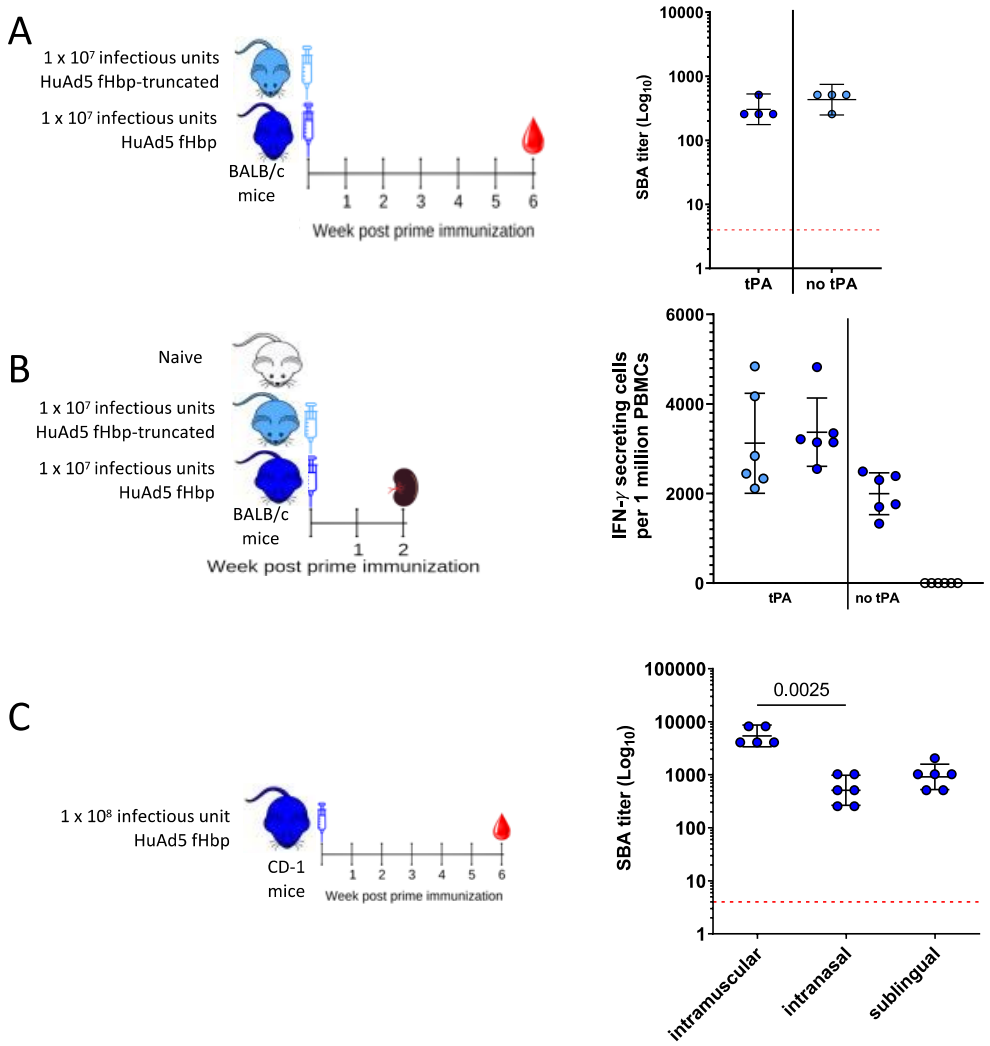

Fig. S1. Immunogenicity of adenoviral vectored vaccine candidates in mice models

(**A**) Individual serum bactericidal assay (SBA) titers in mice immunized with a single dose of human adenovirus 5 (HuAd5) containing a tissue plasminogen activator (tPA) signal sequence followed by the mature form of the group B meningococcus (MenB) antigen factor H binding protein (fHbp) (referred to as truncated in this study), as compared with the full length fHbp with its own signal sequence, and no tPA (n=4 mice per group) (**B**) Individual T cell responses in mice immunized with a single dose of HuAd5 containing a tPA signal sequence followed by the mature form of fHbp (referred to as truncated in this study, light blue), as compared with the immature, full length fHbp with its own signal sequence, with or without tPA (both dark blue) (n=6 mice per group). IFN- γ, interferon-γ; PBMC, peripheral blood mononuclear cells. (**C**) Outbred CD-1 mice were immunized by the routes indicated on the X-axis. IU, infectious units. Individual SBA titers were measured at week 6 (n=5 to 6 mice per group). Data in (C) were analyzed by Kruskal Wallis with Dunns multiple comparison test. Geometric mean and 95% confidence intervals (CI) are indicated for all panels. The red dashed lines indicate the protective SBA titer of 4.

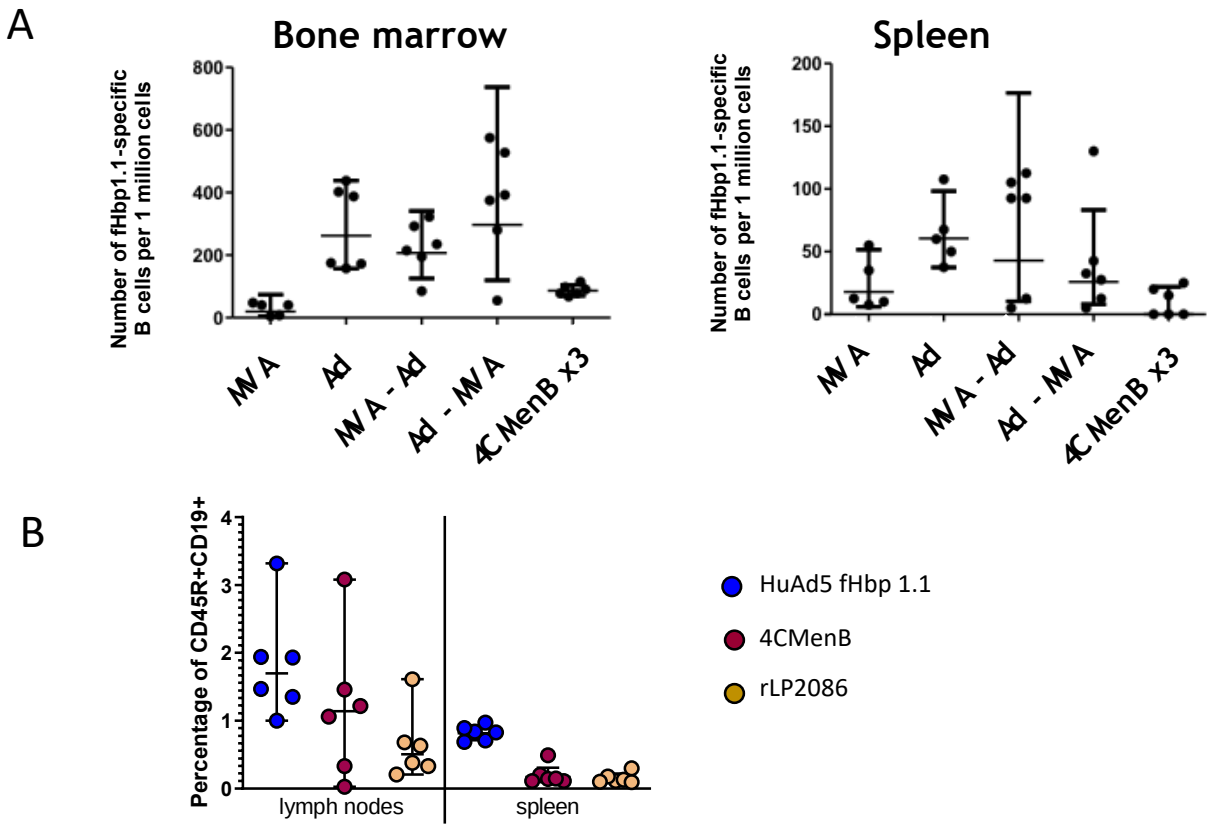

Fig. S2. B cell responses induced by the different vaccine regimen in mice.
(**A**) Number of fHbp-specific antibody-producing B cells in bone marrow and spleens after immunization with a single dose of HuAd5 or modified vaccinia Ankara (MVA) expressing fHbp variant 1.1, or a prime-boost regimen with HuAd5 followed by MVA, or MVA followed by HuAd5 (eight weeks apart), or three injections of one-tenth of a human dose of 4CMenB, as assessed by a B cell assay. Data are presented as geometric mean with 95% confidence intervals. (**B**) Percentage of CD45RA+ CD19+ B cells of total cells in lymph nodes and spleens two weeks after immunization with a single dose of HuAd5 fHbp (blue), 4CMenB (red), or rLP2086 (orange), as measured by flow cytometry. Data are presented as median±95% confidence intervals, and analyzed by Kruskal-Wallis test.

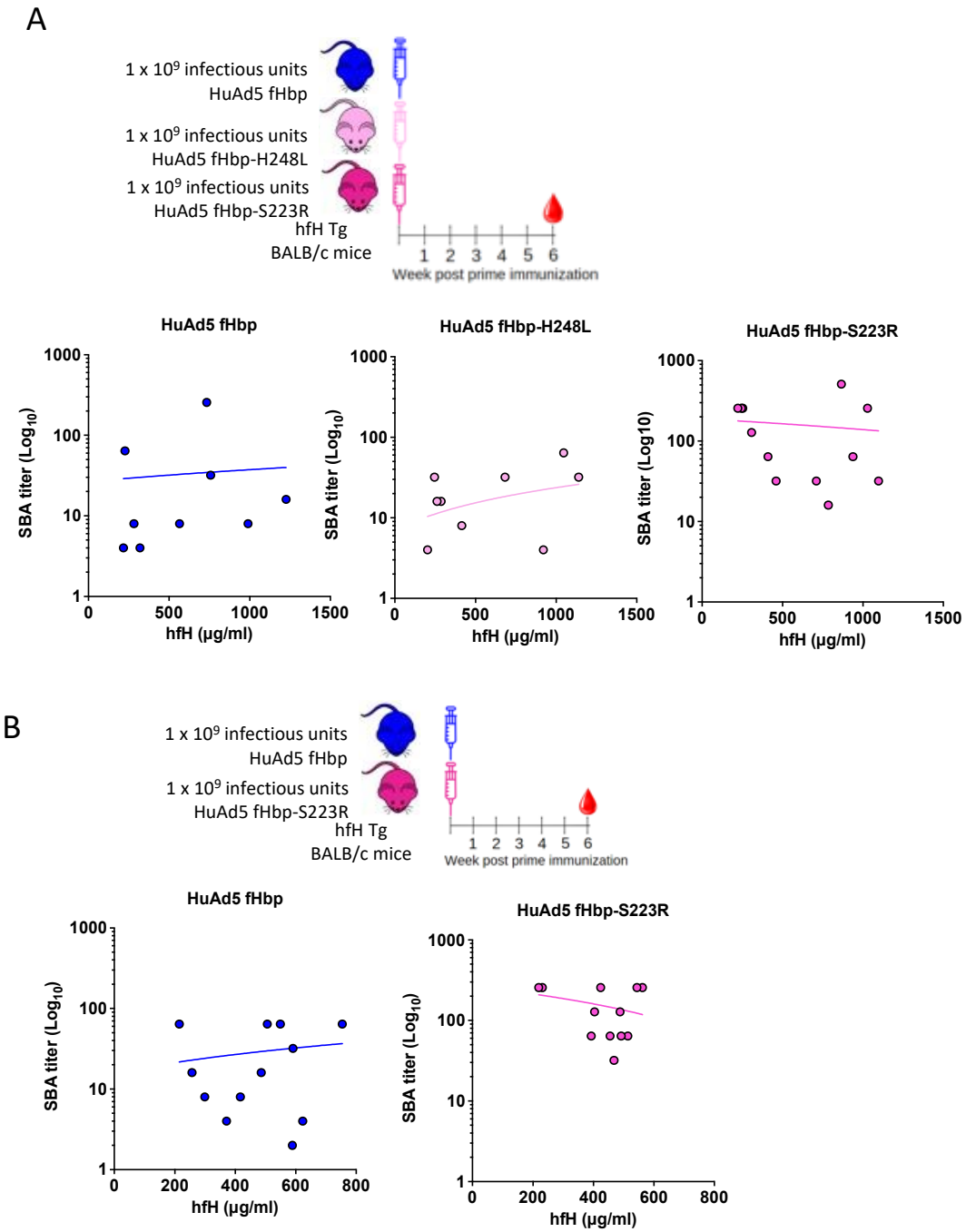

Fig. S3. Level of human factor H in mice and relation with the SBA titer after immunization with the wild type and mutant vaccine designs.
(**A** and **B**) Human factor H concentrations were measured in the immunogenicity experiments in human fH transgenic mice (x-axis), in relation with the SBA titers (y-axis). (**A**) and (**B**) show two independent experiments.

Data file S1. Raw, individual-level data for experiments where n<20.