

# Immunogenicity of a Synthetic Vaccine Based on *Plasmodium vivax* Duffy Binding Protein Region II

Francis B. Ntumngia, Samantha J. Barnes, Amy M. McHenry,\* Miriam T. George, Jesse Schloegel,\* John H. Adams

Global Health Infectious Disease Research Program, Department of Global Health, University of South Florida, Tampa, Florida, USA

**Molecules that play a role in *Plasmodium* merozoite invasion of host red blood cells represent attractive targets for blood-stage vaccine development against malaria. In *Plasmodium vivax*, merozoite invasion of reticulocytes is mediated by the Duffy binding protein (DBP), which interacts with its cognate receptor, the Duffy antigen receptor for chemokines, on the surface of reticulocytes. The DBP ligand domain, known as region II (DBPII), contains the critical residues for receptor recognition, making it a prime target for vaccine development against blood-stage vivax malaria. In natural infections, DBP is weakly immunogenic and DBPII allelic variation is associated with strain-specific immunity, which may compromise vaccine efficacy. In a previous study, a synthetic vaccine termed DEKnull that lacked an immunodominant variant epitope in DBPII induced functional antibodies to shared neutralizing epitopes on the native Sal1 allele. Anti-DEKnull antibody titers were lower than anti-Sal1 titers but produced more consistent, strain-transcending anti-DBPII inhibitory responses. In this study, we further characterized the immunogenicity of DEKnull, finding that immunization with recombinant DEKnull produced an immune response comparable to that obtained with native recombinant DBP alleles. Further investigation of DEKnull is necessary to enhance its immunogenicity and broaden its specificity.**

The global control of malaria is threatened with the spread of drug-resistant parasites and insecticide-resistant mosquitoes. The impact of this on the public health infrastructure and economic stability of the countries most affected is a cause for concern. Vaccines are an attractive mode of control since they are cost-effective and easily administered. Despite years of research, effective malaria vaccines have remained elusive and none has been introduced as a commercial product. However, efforts to develop a vaccine remain optimistic for a number of reasons. First, individuals in regions where malaria is endemic develop naturally acquired immunity to clinical manifestation of the disease (1, 2). Second, passive transfer of IgG from immune individuals conveyed protection to naive individuals (3). Third, vaccination with attenuated sporozoites induced partial protection (4). Finally, naturally and artificially acquired antibodies to different *Plasmodium* antigens have been shown to inhibit parasite invasion of erythrocytes, as well as parasite growth and development, *in vitro* (5–8).

Successful host red blood cell invasion by *Plasmodium* merozoites depends on specific ligand-receptor interactions (9, 10). In *Plasmodium vivax*, a critical step in the invasion of reticulocytes during asexual blood-stage infection is the interaction between the merozoite Duffy binding protein (DBP) and its cognate receptor, the Duffy antigen receptor for chemokines (DARC), on the reticulocyte surface. It is believed that DBP plays an essential role during the irreversible process of junction formation just before invasion (11, 12). This is evident in the virtual absence of *P. vivax* malaria in populations lacking DARC expression on their erythrocyte surface (13, 14). This dependence of DBP on DARC for erythrocyte invasion makes DBP a promising candidate as a vaccine against the asexual stages of the parasite. Naturally occurring anti-DBP antibodies are prevalent in people living in regions where malaria is endemic (15, 16). There is evidence that these anti-DBP antibodies can block DBP-erythrocyte binding, as well as inhibit parasite invasion in short-term *in vitro* cultures (5, 17–21). These data further strengthen the case for a vaccine based on DBP. In *P. vivax*

DBP, a cysteine-rich domain, termed region II, consisting of 330 amino acid residues is considered the ligand domain for adherence to DARC during invasion (22, 23). Structural studies have revealed that DBPII can be divided into three subdomains (24, 25), and other studies have demonstrated that critical residues for receptor recognition are located within subdomain 2 (22, 26–28). Interestingly, this region is highly polymorphic, a pattern consistent with high selection pressure on DBPII (26, 29–32). This poses a great challenge to the development of a DBP-based vaccine that will be effective against diverse *P. vivax* strains.

We have identified B-cell epitopes within the ligand domain of DBP that are associated with protection (17). The immunodominant B-cell epitopes identified are polymorphic, surface-exposed motifs that a previous study determined are not important for receptor recognition but flank residues critical for receptor recognition (22, 25). As most naturally acquired infections with *P. vivax* tend to elicit weakly reactive and strain-specific antibodies (2, 17, 33), we hypothesize that the polymorphic dominant B-cell epitopes represent an evasion mechanism that misdirects the immune response away from the functional, more conserved Duffy recognition epitopes that are potential targets for broadly neutralizing immunity. Similar to what occurs in other microbial agents (34, 35), these variant immunodominant epitopes in DBPII tend

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Address correspondence to John H. Adams, usfmalaria@gmail.com.

\* Present address: Amy M. McHenry, Department of Biology & Geology, Southwestern Adventist University, Keene, Texas, USA; Jesse Schloegel, Sydney Medical Program, Sydney Medical School, University of Sydney, Sydney, Australia.

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to create an inherent bias toward the induction of a nonprotective, strain-specific humoral immune response. Recently, we designed a novel synthetic DBP-II immunogen, termed DEKnull, that lacks a strain-specific immunodominant variant epitope normally present on DBP-II (36). We demonstrated that removal of this dominant variant epitope lowered DBP immunogenicity, but importantly, inhibitory anti-DBP-II antibodies were elicited against conserved neutralizing epitopes on the native Sal1 strain, which was used as the template and shared with other DBP allelic variants. Therefore, recombinant DEKnull (rDEKnull) was able to produce inhibitory anti-DBP antibodies against diverse DBP-II alleles (37). Previous studies have demonstrated that naturally acquired immunity to the erythrocytic stages of malaria parasites is strongly dependent on antibodies (38–40). In areas where malaria is endemic, immunity to vivax malaria is gradually acquired with age as a result of a boosting effect due to repeated exposure to infection (2, 19, 41, 42). Acquired immunity, in addition to being biased toward strain specificity, is relatively slow to develop, never sterile, weak, short-lived (43), and usually unstable (17). There is no long-lasting protective immunologic memory in the absence of continued exposure to infection (17, 43–46) and a failure to consistently boost upon reinfection (47). The present study was designed to further our understanding of the immunogenicity of synthetic DEKnull as a vaccine candidate and to determine whether immunization with rDEKnull can induce an immune response that is relevant to diverse naturally occurring DBP-II alleles of *P. vivax*.

## MATERIALS AND METHODS

**Production of recombinant proteins.** Recombinant DBP-II antigens from three naturally occurring alleles (Sal1, 7.18, and P), the synthetic DBP-II allele (DEKnull) (36), and rPvMSP1-19 were expressed and purified as described previously (37, 48). Briefly, the genes coding for the various antigens were synthesized, codon optimized for expression in *Escherichia coli*, and cloned into expression vector pET21a+ (Novagen) with a C-terminal 6×His tag to facilitate purification by affinity chromatography. The resulting plasmid was transformed into *E. coli* BL21(DE3) LysE (Invitrogen). Recombinant proteins were expressed, purified under denaturing conditions, and refolded by rapid dilution as previously described (20, 49, 50). The refolded antigens were evaluated for native conformation and further analyzed for function by a standard erythrocyte-binding assay (36, 48). Endotoxins were removed from the antigens with the GenScript ToxinEraser endotoxin removal kit, and endotoxin levels in the final products were determined with the ToxinSensor Chromogenic LAL Endotoxin Assay kit. All antigens had endotoxin levels of  $\leq 40$  EU/ml.

**Immunization schedule.** Female BALB/c mice (6 to 8 weeks old) were purchased from Harlan Animal Research Laboratories. Immunizations were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of South Florida. Mice were randomly assigned to six groups of 15 mice each, and preimmune serum was collected from each mouse. The first five groups were primed twice 3 weeks apart with rDEKnull at 25  $\mu$ g/dose subcutaneously, and the sixth group (control) was primed with rSal1 at 25  $\mu$ g/dose. All antigens were emulsified in TiterMax Gold as an adjuvant according to the manufacturer's specifications. Three weeks after the second dose (day 42), test sera were collected and total serum anti-DBP-II IgG titers (baseline titers) were quantified by enzyme-linked immunosorbent assay (ELISA) against the priming antigen. Serum IgG titers were then monitored until an approximately 50% decline in titer was observed (day 217). On day 224 (32 weeks after the first immunization), mice in each group received a booster immunization (anamnestic boost) of 25  $\mu$ g of rDBP-II each from one of three naturally occurring alleles (Sal1, 7.18, and P), rDEKnull, or rPvMSP1-19 and the control group received rSal1 (Table 1). Three weeks

TABLE 1 Immunization schedule

Immunization group	Priming antigen (days 0 <sup>a</sup> and 21 <sup>b</sup> )	Anamnestic boost antigen (day 224 <sup>b</sup> )
1	DEKnull	DEKnull
2	DEKnull	Sal1
3	DEKnull	7.18
4	DEKnull	P
5	DEKnull	MSP1-19
6	Sal1	Sal1

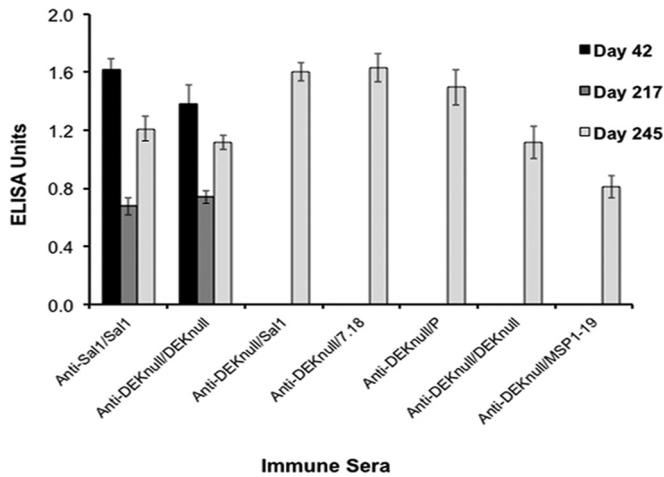
<sup>a</sup> Antigen emulsified 1:1 (vol/vol).

<sup>b</sup> Antigen emulsified 1:0.5 (vol/vol) in adjuvant.

following the anamnestic boost (day 245), the mice were bled for serum and individual mouse serum was stored at  $-20^{\circ}\text{C}$  in barcoded tubes until needed. The final IgG titers (day 245) for each group were determined against the booster antigen, with the exception of the group boosted with rMSP1-19, whose IgG titer was determined against rDEKnull.

**Quantitation of anti-DBP-II antibody titers.** Immunized mice were evaluated for allele-specific anti-DBP-II antibody titers by ELISA as previously reported (37, 48). Briefly, ELISA plates (Nunc) were precoated with 0.2  $\mu$ g of rDEKnull, Sal1, P, 7.18, or PvMSP1-19 per well, and unbound surfaces were blocked with 5% (wt/vol) skim milk in phosphate-buffered saline–0.05% Tween 20 for 2 h at room temperature. Serial tripling dilutions of individual mouse sera (starting at 1:4,000) in blocking solution was added to triplicate wells and again incubated for 2 h on a shaker at room temperature. After washing, wells were incubated with a goat anti-mouse alkaline phosphatase-conjugated secondary antibody (KPL Inc.) for 90 min as described above, and bound antibody was detected with alkaline phosphatase conjugate (BluPhos Microwell Substrate kit; KPL Inc.). Absorbance at 630 nm was determined on a microplate reader (BioTek Instruments Inc.). DBP-II-specific monoclonal antibody 3D10 (48) was used as the standard calibrator on each plate. All optical density (OD) values were normalized at a point on the standard curve where the OD at 630 nm ( $\text{OD}_{630}$ ) was 1.0, and antibody values were expressed in ELISA units (EU), which were determined as the ratio of the  $\text{OD}_{630}$  generated by the test antibody to the  $\text{OD}_{630}$  of the standard (37). Antibody titers were determined as the serum dilution required to achieve 1.5 EU (51). Anti-MSP1-19 antibody served as a negative control, while preimmune serum was used as a background control.

**Lymphocyte proliferative assay.** Splenocytes from immunized mice were prepared as previously reported (52, 53), with some modifications. Briefly, spleens were harvested and washed twice in Iscove's modified Dulbecco's medium (IMDM; Invitrogen). Single-cell suspensions were prepared by teasing spleen tissues into tiny pieces in a petri dish containing IMDM. Red blood cells were lysed with ACK lysis solution (Invitrogen), and membrane debris was removed by passing the cell suspension through a cell strainer (Fisher Scientific). The cells were washed with IMDM, resuspended in IMDM–10% fetal bovine serum (FBS), and layered over Ficol1 Paque Plus. The splenocytes were recovered by centrifugation and washed in IMDM–10% FBS. Cell viability was determined by trypan blue staining. The cells were resuspended in IMDM–10% FBS–0.5 mM 2-mercaptoethanol–1× streptomycin-penicillin (Invitrogen). Splenocytes were seeded into 96-well flat-bottom plates at a concentration of  $2 \times 10^5$ /ml in 100  $\mu$ l of complete medium and stimulated with 1  $\mu$ g/ml rDEKnull, Sal1, 7.18, P, or PvMSP1-19 in triplicate wells. Additional wells stimulated with concanavalin A (ConA; Sigma-Aldrich) at a final concentration of 1  $\mu$ g/ml or left unstimulated (with culture medium alone) served as positive and negative controls, respectively. Plates were incubated for 72 h at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified incubator. A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used to determine the number of proliferative cells with the CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation Assay reagent (Promega). A 20- $\mu$ l volume of the reagent was added directly to 100  $\mu$ l of culture in the wells and incubated for 4 h at



**FIG 1** Anamnestic response to rDEKnull. Mice were primed twice 3 weeks apart with either rDEKnull or rSal1 (control), and their serum anti-DEKnull IgG titers were determined (day 42). Antibody titers were allowed to decline by ~50% (day 217). Mice from each group were boosted on day 224 with a naturally occurring rDBP<sub>II</sub> allele (Sal1, 7.18, or P), rDEKnull, or rMSP1-19, and their serum anti-DBP<sub>II</sub> IgG titers were again determined on day 245 (final blood collection). Bars represent the mean antibody titers (EU) for reactivity of immune sera from each group at a  $1 \times 10^5$  dilution against the boosting antigen for that group. Error bars represent standard errors.

37°C, and absorbance at 490 nm was recorded with a 96-well Synergy 2 ELISA plate reader (BioTek Instruments). Results were expressed as a stimulation index (SI), which is the ratio of the absorbance of the stimulated culture to that of the unstimulated culture.

**Inhibition of DBP-erythrocyte binding by COS-7 assay.** Expression plasmid constructs were engineered to target different variants of DBP<sub>II</sub> alleles on the surface of transiently transfected COS-7 cells as fusion proteins to the N terminus of enhanced green fluorescent protein (EGFP). DBP<sub>II</sub>-erythrocyte binding inhibition assays were performed as previously reported (21, 54, 55). Briefly, wells of transfected COS-7 cells were preincubated with different concentrations of purified total serum IgG

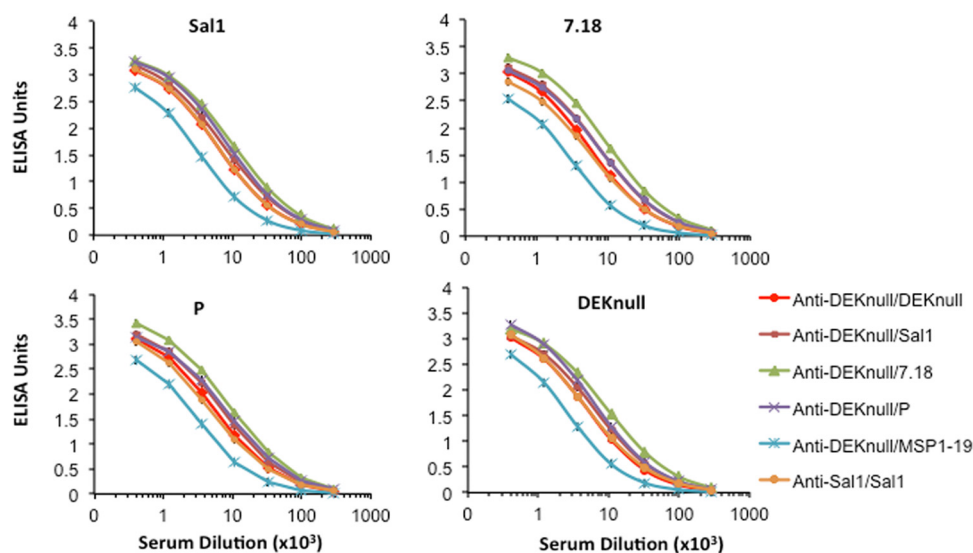
from pooled mouse serum from the different immunization groups prior to the addition of human erythrocytes. Purified total serum IgG from mice immunized with rMSP1-19 served as a negative-control antibody. Binding inhibition was determined by assessing the percentage of rosettes in wells of transfected COS-7 cells in the presence of test IgG relative to that in wells of transfected cells in the presence of purified IgG from preimmune sera.

**Statistical analyses.** The distributions of antibody titers and inhibition concentrations for each antiserum were compared between the different alleles tested for any statistically significant differences in antibody reactivity and inhibitory responses by one-way analysis of variance and multiple-comparison analysis by the Bonferroni test with SAS software.

## RESULTS

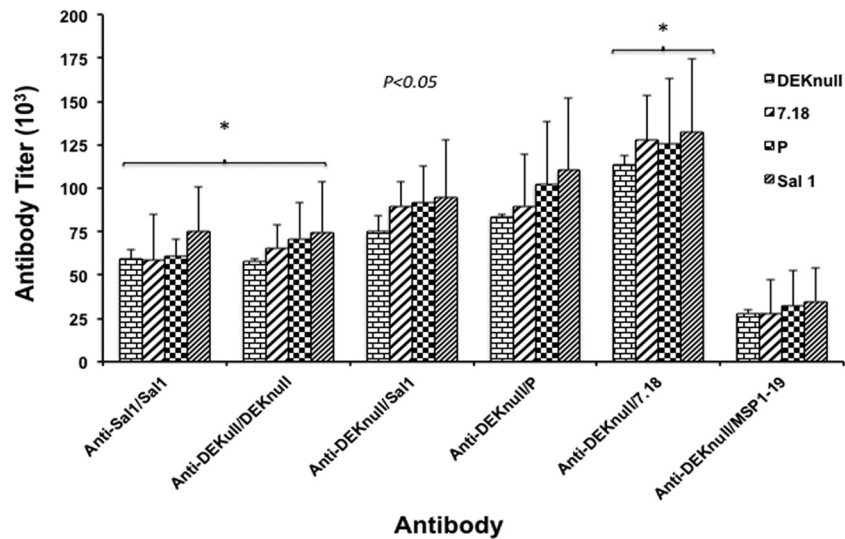
**Anamnestic response to rDEKnull.** To determine if rDEKnull vaccine could induce secondary immune responses against native alleles of DBP<sub>II</sub>, BALB/c mice were primed with two consecutive doses of rDEKnull 3 weeks apart. ELISA was used to determine baseline serum anti-DEKnull IgG antibody titers 3 weeks after the second immunization (day 42). The serum anti-DEKnull IgG titers were monitored until a 50% decline in the IgG titer was observed (day 217), and then a third injection of one of three naturally occurring rDBP<sub>II</sub> alleles, Sal1, 7.18, or P; rDEKnull; or a control antigen, rPvMSP1-19, was administered (Table 1). Three weeks after the final immunization (day 245), serum anti-DBP<sub>II</sub> IgG antibody titers were again determined against the boosting antigen. Mice in all of the immunization groups showed strong immune responses to the anamnestic boost (Fig. 1). This was evident by a significant increase in antibody titers between days 217 and 245 ( $P = 0.0007$ ) in all of the groups, with the exception of the DEKnull/MSP1-19 prime-boost group. No significant differences in antibody titers were observed between days 42 and 245 ( $P = 0.4$ ).

**Anti-DBP<sub>II</sub> reactivity profiles.** Sera from the anamnestic boosts were evaluated for cross-reactivity with heterologous rDBP<sub>II</sub> alleles, as well as rPvMSP1-19. All the immunization groups elicited high levels of cross-reactive anti-DBP<sub>II</sub> antibody responses against heterologous DBP<sub>II</sub> alleles (Fig. 2). With the



**FIG 2** Anti-DBP<sub>II</sub> reactivity profiles. Antisera from the different immunization groups were evaluated in an ELISA by endpoint dilution for cross-reactivity with variant recombinant DBP<sub>II</sub> alleles. Antigen preparations (2  $\mu$ g/ml) were allowed to adsorb to the wells of microtiter plates and then allowed to react with different dilutions of antiserum from individual mice. Each curve is a four-parameter logistic regression curve for antisera from each group ( $n = 14$ ) against the different alleles, and error bars represent standard deviations.





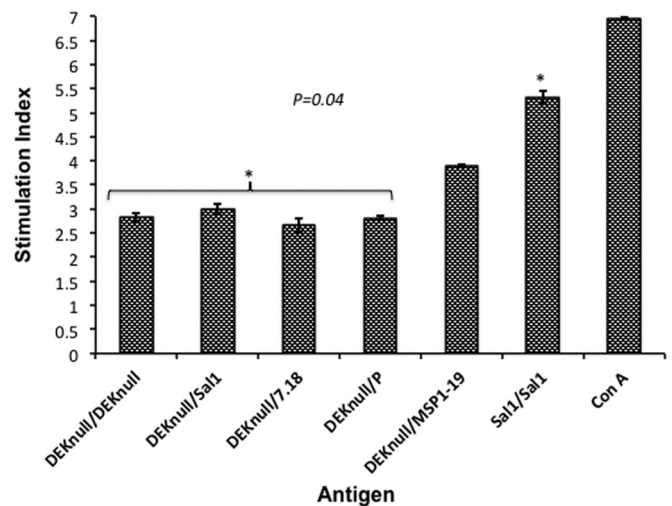
**FIG 3** Quantitative analysis of anti-DBPII antiserum binding specificity. The binding specificity of antiserum from each immunization group was compared against that of recombinant Sal1, 7.18, P, and DEKnull by ELISA. Antibody titers were calculated as the serum dilution required to achieve 1.5 EU. Each bar represents the titer for each antiserum against a specific recombinant DBPII allele, and error bars indicate standard deviations. Asterisks indicate significant differences in antibody titers between the two groups.

exception of the anti-DEKnull/MSP1-19 group, there was no response to rPvMSP1-19 (not shown). The DEKnull-primed and MSP1-19-boost mice showed lower levels of reactivity to rDBPII. We used 1.5 EU as a basis to determine potential differences in antigen-specific anti-DBPII antibody responses. Similar anti-DBPII reactivity profiles were observed for each immune serum against the respective rDBPII alleles (Fig. 3). Only one prime-boost combination (DEKnull/7.18) produced an anamnestic response with antibody titers greater than the homologous prime-boost vaccinations of Sal1/Sal1 and DEKnull/DEKnull (Bonferroni multiple-comparison adjustment,  $P < 0.05$ ). Interestingly, the DEKnull-primed-heterologous-DBPII-boosted mice showed anamnestic responses to all of the native alleles relatively higher than those of the Sal1/Sal1 homologous prime-boost mice. No anti-DBPII boosting response was observed with the rDEKnull-primed rPvMSP1-19 heterologous-boost mice; however, this group did produce a high anti-MSP1-19 response (not shown).

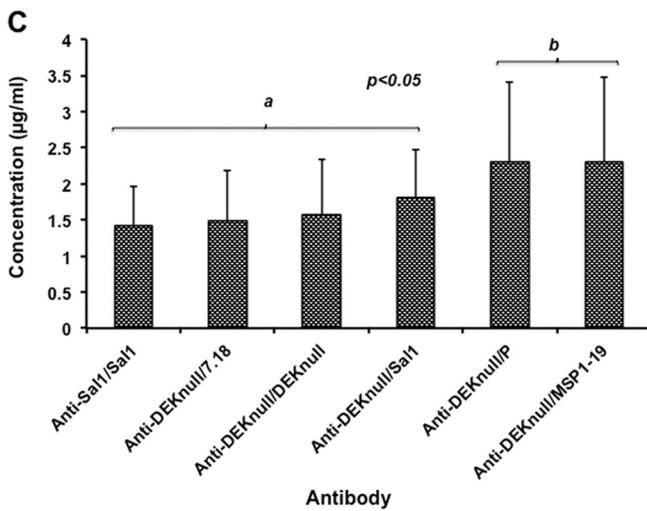
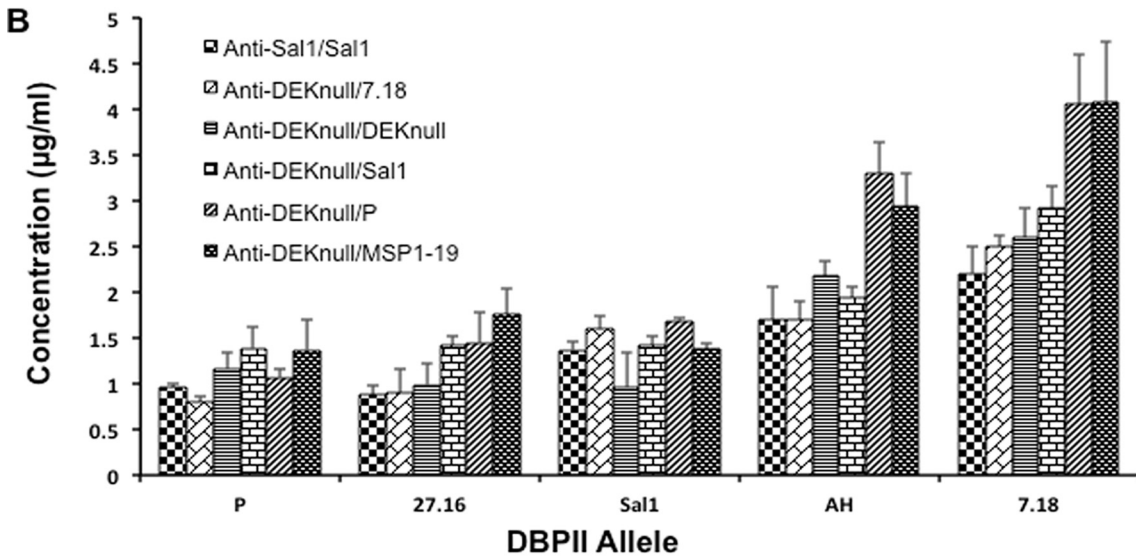
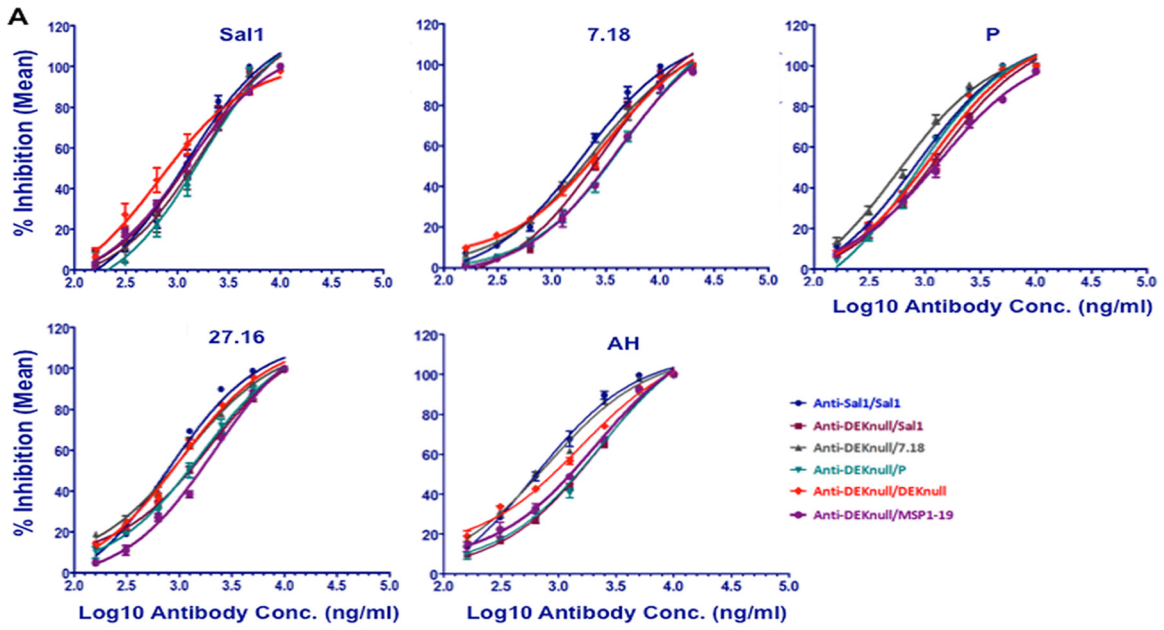
**Lymphocyte proliferative response.** The immunogenicity of the different antigens was further assessed for a T-cell response. Cultured splenocytes from mice primed with rDEKnull and boosted with the different native rDBPII alleles were stimulated with the same antigens used in the boosting immunization, and antigen-specific T-cell proliferation was determined. The number of proliferating cells was determined as the ratio of cells stimulated with antigens to cells stimulated with culture medium alone. Irrespective of the stimulating antigen, all the splenocytes from the DEKnull-primed mice showed a 3- to 4-fold increase in proliferation (SI, 2.9 to 3.9) compared to that of adjuvant controls (SI, 0.9), but this was significantly smaller than that of the Sal1/Sal1 homologous prime-boost group (SI, 5.3) ( $P = 0.04$ ). Splenocytes from the Sal1 homologous prime-boost mice stimulated with ConA (control) produced the highest level of proliferation, as expected (Fig. 4).

**Assessment of anti-DBPII functional activity.** The purified total IgG fraction of pooled sera from final bleeds (day 245 sera) of each immunization group was evaluated for inhibition of COS-7

cell surface-expressed DBPII alleles from binding to human erythrocytes in a standard COS-7 cell assay. Inhibition of DBPII-erythrocyte binding was determined by assessing the number of COS-7 cells with adherent erythrocytes (rosettes) in cultures of transfected COS-7 cells incubated in the presence of different concentrations of purified total serum IgG from the immunized mouse groups relative to IgG from preimmune serum. A dose-dependent anti-DBP binding-inhibitory response was observed with anti-



**FIG 4** Lymphocyte proliferation. Groups of BALB/c mice were primed with rDEKnull and boosted with recombinant DEKnull, Sal1, 7.18, P, or MSP1-19. An rSal1 prime-boost group served as a control. Cultured splenocytes harvested 3 weeks after boosting were stimulated with the booster antigen. The T-cell proliferative response was quantified by an MTS assay, and the SI was determined as the ratio of the absorbance at 490 nm of stimulated cells to that of unstimulated cells. Bars represent mean SI values  $\pm$  standard deviations of triplicate wells. The asterisks indicate significant SI differences between the control group (Sal1-Sal1 prime-boost) and the DEKnull prime-heterologous-boost groups.



bodies from each immunization group (Fig. 5A). No inhibition was observed with total IgG from control mice immunized with MSP1-19 alone (not shown). The 50% inhibitory concentration ( $IC_{50}$ ) was used as a basis for determining the anti-DBP-II functional ability of each antibody group to inhibit DBP-II-erythrocyte binding (Fig. 5B). Each antibody group showed variations in anti-DBP-II inhibitory responses against the different alleles, with the 7.18 allele being the most resistant to inhibition, followed by the AH allele. Statistical analysis with the Bonferroni multiple-comparison adjustment grouped the different antibodies into two distinct groups (*a* and *b*;  $P < 0.05$ ). The anti-DEKnull-primed recombinant P-boost antibodies showed no boosting effect since the inhibitory effect was similar to that of the control anti-DEKnull/MSP1-19 antibodies (Fig. 5C).

## DISCUSSION

It has been established that immunodominant variant epitopes tend to create an inherent bias toward a strain-specific immune response (34, 35) and limit the induction of an immune response toward more conserved protective epitopes. Correspondingly, immunization with a single *P. vivax* DBP-II allele results in strain-specific protective immunity (56). This is also true for the *P. falciparum* vaccine candidate PfAMA1 (57, 58). On the other hand, it has been demonstrated that antibodies to epitopes common to different DBP-II alleles (37) or PfAMA1 alleles are relevant for the induction of broader parasite strain inhibition (59, 60). An effective DBP vaccine therefore needs to focus the immune response towards conserved epitopes that are targets of neutralizing inhibitory antibodies. Recently, we demonstrated that a synthetic DBP-II vaccine, DEKnull, lacking a dominant variant B-cell epitope induced inhibitory antibodies to shared neutralizing epitopes on native Sal1 from which it was derived (36). The results demonstrated that removal of the dominant variant epitope from the DEKnull vaccine lowered the immunogenicity of DBP-II, but DEKnull immunization produced a more consistent anti-DBP-II response against diverse DBP-II alleles in an *in vitro* erythrocyte binding inhibition assay than three single native alleles, confirming that anti-DBP antibodies target shared neutralizing epitopes on DBP-II (37).

The development of immunological memory even after a long period of nonexposure forms the basis of a successful vaccine in inducing long-term protective immunity against a given pathogen (61). Therefore, for an immunogen to serve as an effective vaccine, it should be able to produce an enhanced memory response to all of the native variants of the pathogen that either completely prevents reinfection or greatly reduces the severity of disease. In regions where malaria is endemic, individuals develop anti-DBP inhibitory antibodies following natural exposure (17, 21); however, there is no long-lasting protective immunologic memory in

the absence of continued exposure to infection (17, 43–46) and sometimes there is failure to consistently boost upon reinfection (47). To further characterize the immunogenicity and vaccine potential of synthetic DEKnull, we conducted a prime-boost immunization study to evaluate the potential of rDEKnull vaccine to induce a B-cell memory response, as well as a T-cell response to naturally occurring DBP-II alleles following primary DEKnull vaccination. Mice were immunized twice with rDEKnull, and serum IgG antibody titers were allowed to decline to ~50% before a boost with a natural DBP-II allele. Our data demonstrate that DEKnull prime–heterologous-boost mice generally produced an anamnestic response to native DBP-II alleles better than that of native Sal1/Sal1 homologous prime-boost mice (Fig. 3). However, this was not reflected in the anti-DBP-II inhibitory responses, as multiple-comparison analysis showed no differences in inhibitory responses between antibodies from the different immunization groups, with the exception of the DEKnull/P prime-boost group (Fig. 5C). This observation supports previous data that showed no correlation between the antibody response to DBP-II and inhibition of DBP-II-erythrocyte binding (17, 48). Antibodies from all of the vaccination groups showed similar anti-DBP-II-specific responses to each native allele, suggesting that the antibodies bind to similar epitopes on DBP-II. Even though the DEKnull prime–heterologous-boost groups induced a relatively stronger antibody response to all DBP-II alleles than the Sal1/Sal1 homologous prime-boost control group, only the DEKnull/7.18 prime-boost immunization induced an anamnestic response with antibody titers significantly higher ( $P < 0.05$ ) than those of the Sal1/Sal1 and DEKnull/DEKnull homologous prime-boost groups (Fig. 3).

Previous studies have demonstrated that DBP-II contains a cluster of T-cell epitopes that are recognized by individuals in areas where malaria is endemic, with a correlation between cellular immunity and protection and the prevalence and intensity of *P. vivax* infection (30, 62). This implies that cellular immune responses to DBP-II play a role in the development of acquired immunity. To examine the cellular immune response to DEKnull, T-cell responses were determined by lymphocyte proliferation assays following restimulation of splenocytes from each immunization group with the different naturally occurring rDBP-II antigens. The Sal1/Sal1 homologous prime-boost group induced a significantly stronger proliferative response than the DEKnull homologous and heterologous prime-boost groups, suggesting that the “DEK” epitope in DBP-II represents an important T-cell epitope on DBP-II.

We also evaluated the potential biological and functional anti-DBP-II activity of the antibodies from the various groups in an *in vitro* erythrocyte binding inhibition assay. Differences were observed in the anti-DBP-II binding-inhibitory responses consistent with anti-DBP-II antibodies binding different functional epitopes

**FIG 5** Inhibition of erythrocyte binding to rDBP-II expressed on COS-7 cells. Purified total serum IgG from the different prime-boost groups was tested for inhibition of DBP-II-erythrocyte binding against a panel of COS-7 cell-expressed DBP-II alleles by endpoint dilution. A monolayer of transfected COS-7 cells expressing rDBP-II from five different alleles was incubated with the purified serum IgG at different concentrations prior to the addition of human erythrocytes. Binding was scored by counting rosettes in 30 microscopic fields at a magnification of  $\times 200$ . Percent binding inhibition was determined relative to that of the purified IgG from preimmune sera used as a control. (A) Charts show nonlinear regression curves for the inhibitory activities of the different antibodies against each DBP-II allele. Each antibody concentration was tested in triplicate for two independent experiments. (B) Quantitative analysis of anti-DBP-II binding inhibition. Bars represent the  $IC_{50}$ s of each antibody against individual alleles. (C) Multiple comparisons of anti-DBP-II binding-inhibitory responses. The overall inhibitory responses of serum anti-DBP-II IgG from each group against all five COS-7-expressed alleles were compared by Bonferroni multiple-comparison adjustment. Bars represent the mean  $IC_{50}$ s of each antibody against all the natural alleles. Antibodies were classified into two inhibitory groups (*a* and *b*), with a statistically significant difference in inhibitory responses between the groups. Error bars represent standard deviations.



TABLE 2 Panel of DBPII alleles used for protein expression and COS 7 assay

DBPII allele	Accession no.	Residue <sup>a</sup> at position:																			
		308	333	371	375	384	385	386	387	388	389	390	391	392	393	417	424	437	447	992	503
DBPII-Sal1	P22290.2	R	L	K	N	D	E	K	A	Q	Q	R	R	K	Q	N	L	W	S	K	I
DEKnull	NA <sup>b</sup>	R	.	.	.	A	A	T	A	A	T	S	R	T	S	.	.	.	.	.	.
DBPII-27.16 <sup>c</sup>	AAL79076	S	.	.	.	G	.	.	.	.	.	H	.	.	.	.	I	.	K	E	.
DBPII-7.18	AAL79051.1	S	.	.	.	G	.	Q	.	.	.	.	.	.	.	K	I	R	.	.	K
DBPII-AH <sup>c</sup>	AA34130.1	S	.	E	.	G	.	Q	.	.	.	.	.	.	.	K	I	R	.	.	K
DBPII-P	AAL79073.1	R	F	.	D	G	K	N	.	.	.	H	.	.	.	K	I	.	.	.	K

<sup>a</sup> Differences in amino acid residues between the different alleles are shown. A dot indicates that the residue is the same as that in DBPII-Sal1.

<sup>b</sup> NA, accession number is not available.

<sup>c</sup> Allele used for COS-7 assay only.

within the different alleles. No significant difference in inhibition was observed for any antibody group against the Sal1, 27.16, and P alleles. The 7.18 allele was the most refractory to inhibition, followed by the AH allele. These two alleles differ by just one amino acid and so cluster more closely to each other than the other natural alleles (Table 2). These data are in line with other studies that demonstrate that variation in DBPII plays a critical role in altering its antigenic properties and confers significant differences in sensitivity to inhibition by serum antibodies, resulting in a bias toward strain-specific immunity (56). Pairwise comparisons with a Bonferroni multiple-comparison adjustment classified the different antibodies into two statistically significantly different groups (Fig. 5C), with group *a* antibodies showing stronger inhibitory activity than group *b* antibodies ( $P < 0.05$ ). Together, the data are consistent with our hypothetical framework that variation is an evasion mechanism responsible for strain-specific immunity and that stable broadly neutralizing immunity is achieved when antibodies target functionally conserved epitopes, thereby blocking erythrocyte binding and invasion.

In summary, we have demonstrated that synthetic DEKnull is able to elicit immunological memory responses similar to those elicited by naturally occurring DBPII alleles. While the anti-DBPII inhibitory response induced by rDEKnull is lower than that to DBPII Sal1, the functional activity of these antibodies demonstrated a broader coverage of diverse DBPII alleles. Further investigation is necessary to enhance the immunogenicity of DEKnull and broaden its specificity.

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