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## Identification and Characterization of Functional Human Monoclonal Antibodies to *Plasmodium vivax* Duffy Binding Protein

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### Abstract

*Plasmodium vivax* (Pv) invasion of reticulocytes relies on distinct receptor-ligand interactions between the parasite and host erythrocytes. Engagement of the highly polymorphic Pv Duffy-binding protein (DBP<sub>II</sub>) with the erythrocyte's Duffy antigen receptor for chemokines (DARC) is essential. Some Pv-exposed individuals acquired antibodies (Abs) to DBP<sub>II</sub> that block DBP<sub>II</sub>-DARC interaction and inhibit Pv reticulocyte invasion, and Ab levels correlate with protection

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**Authors' contributions.** CLK, LC, JHA, NT and MUF conceived the project. LC, SD, VCN and CD performed experiments to generate mAbs. SS, SS, CA, SB and RMF provided access to subjects from Cambodia. EC and NT provided recombinant DBP<sub>II</sub> and DBP<sub>II</sub> with a BirA insert. VCN and MUF performed the Pv invasion experiments in Brazil, and BW, JP and CR conducted the Pv invasion experiments in Cambodia. CLK, LC, SD and MUF analyzed the data and CLK wrote initial manuscript drafts. LC, SD, JHA, NT, MUF, CA and RMF helped to revise the manuscript.

¶The authors contributed equally to the work.

Microarray data were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) under master accession number GSE127729. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE%20127729>

against Pv malaria. To better understand the functional characteristics and fine-specificity of protective human Abs to DBPII, we sorted single DBPII-specific IgG<sup>+</sup> memory B cells from three individuals with high blocking activity to DBPII. We identified 12 DBPII-specific human monoclonal antibodies (mAbs) from distinct lineages, which blocked DBPII-DARC binding. All mAbs were Pv strain-transcending and targeted known binding motifs of DBPII with DARC. Eleven mAbs competed with each other for binding, indicating recognition of the same or overlapping epitopes. Naturally-acquired blocking Abs to DBPII from individuals with high levels residing in different Pv endemic areas worldwide competed with mAbs, suggesting broadly shared recognition sites. We also found that mAbs inhibited Pv entry into reticulocytes *in vitro*. These findings suggest that IgG<sup>+</sup> memory B cell activity in individuals with Pv strain-transcending Abs to DBPII displays a limited clonal response, with inhibitory blocking directed against a distinct region of the molecule.

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## Introduction

*Plasmodium vivax* (Pv) malaria is the most widespread of human malarias, with a social and economic burden that is underappreciated (1). Standard measures against *P. falciparum* (Pf) malaria, such as bed nets, indoor residual spraying and antimalarial drugs, are less effective with Pv because of latent phases of the parasite and increased transmissibility, as sexually-committed parasites emerge directly from the liver (2, 3). Thus, a vaccine is likely to play a key role in controlling Pv malaria. Successful vaccine strategies may rely, in part, on targeting relatively conserved antigenic epitopes involved in erythrocyte invasion pathways (4).

For Pv invasion of reticulocytes to proceed, several multistep receptor-ligand interactions are required. Crucial to the process is engagement of the cysteine-rich domain II of the Pv Duffy-binding protein (DBPII) with the N-terminal region of the Duffy antigen receptor for chemokines (DARC) on the host erythrocyte surface (5–7). Amino acids 1–60 form the N-terminal ectodomain of DARC and are sufficient for DBPII binding (5). During invasion, DBPII initially binds to a single DARC molecule, then dimerizes to form a heterotrimer, which rapidly develops into a heterotetrameric complex of DBPII and DARC (8, 9). DBPII is composed of three subdomains (SD), with SD2 containing essential residues for initial DARC binding, and other SDs contributing to formation of the heterotetrameric complex (8, 9). In individuals identified as DARC-negative, clinical vivax malaria rarely occurs (10, 11). Thus, binding of DBPII to DARC on the reticulocyte surface is essential, making DBPII a leading vaccine candidate.

In highly endemic areas, young children exposed to Pv infection usually acquire clinical immunity by four to five years of age (12, 13). There are likely multiple antigen targets of naturally-acquired immunity to Pv, and one such target appears to be DBPII. A subset of Pv-infected individuals (8–15%) acquires binding inhibitory antibodies (BIAbs) which block DBPII binding to DARC (12, 14–16). Subjects with high levels of BIABs have a reduced risk of Pv infection and illness (12, 16), and anti-DBPII antibodies (Abs) purified from the serum of individuals with high BIAB activity inhibit Pv invasion of reticulocytes *in vitro* (17). However, in considering vaccine strategies, complicating factors include DBPII

polymorphisms and the presence of multiple Pv strains in endemic regions. Although total Ab responses to DBPII in a given Pv-infected individual are often strain-specific, a subset of individuals will develop high levels of BIABs which are strain-transcending (12, 17, 18), suggesting the importance of conserved epitopes which elicit broadly inhibitory Abs. For highly polymorphic viral pathogens such as HIV, respiratory syncytial virus and influenza, broadly neutralizing Abs are essential for protective immunity (19, 20), and the same may be true for Pv infection.

To examine the hypotheses that in Pv-exposed and/or immune individuals, functional Abs develop which i) interfere with DBPII-DARC interaction, ii) are strain-transcending and iii) target conserved region(s) of the DBPII molecule worldwide, we isolated DBPII-specific memory B cells from individuals with high levels of BIABs to generate a panel of human monoclonal Abs (mAbs) to DBPII. We found that mAbs derived from different B cell lineages recognized the same region in the DBPII-binding domain for DARC. These mAbs were broadly inhibitory for DARC binding to all DBPII variants tested and blocked Pv invasion of reticulocytes *in vitro*. Further characterization of the epitopes in the DBPII-binding region will assure these motifs are retained and amplified in a vaccine design for Pv malaria.

## Methods

### Study population

Blood samples were obtained from Cambodians with documented Pv malaria residing in Pursat Province (21, 22). Samples were screened for blocking Abs to PvDBPII (see below). Additional plasma samples were provided from cohort studies conducted in Brazil (16) and Papua New Guinea (12). Cambodian and Brazilian adults with high levels of blocking Abs to PvDBPII donated up to 200 ml of peripheral venous blood. Institutional review boards from the United States National Institutes of Health (NIAID protocol #08-N094, [Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00663546) NCT00663546), Cambodian Ministry of Health, University Hospital of the University of Sao Paulo (1025/10), National Human Research Ethics Committee of the Ministry of Health of Brazil (551/2010), Medical Research Advisory Counsel of Papua New Guinea (PNGIMR No 1409, PNG MRAC No. 1400, and UH IRB No. 04-14-19) and University Hospitals of Cleveland Medical Center approved the protocols. Written informed consent was obtained from all study participants or their parents/guardians.

### Protein expression and purification

Plasmids containing DNA coding for amino acids 211–525 of PvDBP (region 2 of PvDBP, RII-PvDBP) for DBP variants (Sal 1, 7.18, P, O and DEK null, sequences previously described (8, 9, 23–25)) were expressed as inclusion bodies in *E. coli* and recovered as previously described (9, 17). After stirring at 4°C for 36 h, soluble recombinant DBPII (rDBPII) was concentrated and purified by size-exclusion chromatography. To generate tetramers of rDBPII, BirA-tagged DBP was obtained as previously described (8, 9, 25). A construct containing an N-terminal BirA site (amino acids: GLNDIFEAQKIEWHE) was linked with a short, flexible six amino acid sequence. Following expression of rDBPII with a BirA site, biotin ligase was used for biotinylation (BirA, Genecopoeia, Rockville MD).

RDBPII with a BirA site bound erythrocytes in a manner identical to that observed for rDBPII lacking a BirA site. We also expressed a tetanus toxin C-terminal fragment (TTCF) containing an N-terminal thrombin-cleavable 6x histidine tag followed by a BirA site and a short, flexible linker sequence (26).

### **DBPII ELISAs and binding inhibition of DBPII to DARC fusion protein**

ELISAs were performed as previously described(27). Antigen units were defined using a pool of plasma from 20 Papua New Guinean individuals with high Ab titers to rDBPII (Sal I variant); a 1:50 dilution of this pool was defined as standard 1 with 500 units of activity. Standards involved a serial 2-fold dilution of this plasma pool and were run on every plate.

To assess Ab blocking activity, plasma from Pv-exposed individuals was incubated with rDBPII at specified concentrations. We then measured levels of binding inhibition of rDBPII to a fusion protein containing amino acids 1–60 from the DARC N-terminal region fused to the Fc region of human IgG (nDARCIg). Pooled plasma samples with high blocking activity from Papua New Guinea or Brazil served as positive controls. Diluent alone or pooled samples from North Americans not exposed to malaria were used for negative controls. Percent inhibition was calculated by  $(1 - (\text{optical density [OD] of test sample} / \text{OD of negative control})) \times 100$ .

### **Peripheral blood mononuclear cell preparation**

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood drawn from otherwise healthy Cambodian and Brazilian adults previously infected with Pv, all of whom had high titers of blocking Abs to DBPII (>80% binding inhibitory activity at a titer of 1:10 or higher) as previously described (12). Cells from twenty individuals were cryopreserved prior to use.

### **ELISPOT assay**

To identify antigen-specific B cells and measure their frequency, memory B cells were converted into immunoglobulin-secreting cells by culturing PBMCs at 37°C and 5% CO<sub>2</sub>, using cRPMI 1640 + L-glutamine with 10% heat inactivated fetal bovine serum (FBS), with addition of B cell agonists CpG 2006 at 2.5 µg/ml, *S. aureus* Cowan (SAC) suspension at 100 ng/ml, IL-10 at 25 ng/ml and pokeweed mitogen at 10 ng/ml for 6 days (28). Following activation, cells were washed twice and re-suspended to  $3-6 \times 10^5$ , then added at varied concentrations to Millipore Multiscreen HTS Immobilon-P membranes coated with rDBPII or rPvMSP1<sub>19</sub> (2.5 µg/ml) or tetanus toxoid (TT, 1 µg/ml) for 6 h at 37°C and 5% CO<sub>2</sub>, to detect antigen-specific B cell secretion (ELISPOT assay). To determine total number of IgG-secreting B cells,  $2-6 \times 10^3$  activated PBMCs from the same cultures were added to plates coated with 5 µg/ml of polyclonal anti-human IgG for 6 h. Cells were washed off and AP-conjugated anti-human IgG (H+L) added, and spots detected with BCIP/NBT as substrate. The number of spots in each well was counted using an ELISPOT Reader (Cellular Technology Limited, CTL).

### Tetramer preparation

To generate tetramers, premium-grade allophycocyanin-labeled streptavidin (Invitrogen Molecular Probes) was mixed on ice with mono-biotinylated rDBP11 with a BirA site (described above) using rDBP11:SA-APC ratio of 4:1 (26), modified with 5 additions of SA-APC at 20 min intervals and mixing at 10 min intervals. Afterward, any aggregates were removed with 17,000xg centrifugation for 20 min at 4°C. All tetramers were prepared fresh for each experiment.

### Cell staining and sorting of antigen-specific memory B cells

Single cells were identified and sorted according to previously described techniques (29) from cryopreserved PBMC without activation. Staining and single cell sorting of DBP11- or TTCF-specific IgG<sup>+</sup> memory B cells were performed as follows. B cells from cryopreserved PBMC were enriched using immunomagnetic positive selection with anti-CD19 magnetic MACS beads (MiltenyiBiotec). Cells were washed at least twice with 5 ml FACS buffer with 3 mM EDTA and adjusted to a cell density of 1–2×10<sup>6</sup> cells/ml. Cells were stained with mouse anti-human CD20 (PE-Cy5.5, Invitrogen) and anti-human IgG Abs (PE-Cy7 clone G18–145; Becton, Dickinson and Co., BD) along with DBP11- or TTCF-prepared tetramers using streptavidin coupled with allophycocyanin (BD) and SYTOX Green Dead Cell Stain (Invitrogen) to gate out dead cells. Stained CD19<sup>+</sup> cells were sorted on a BD FACSAria II equipped with chilled stage sorting based on size and complexity. Doublet discrimination was performed to exclude aggregated cells. Individual DBP11- or TTCF-specific CD20<sup>+</sup>, IgG<sup>+</sup> memory B cells were single cell sorted using a 100 µm nozzle at 20 psi with the following parameters: yield mask 0/32, purity mask 32/32 and phase mask 0/32. Individual cells were sorted directly into 4µl mRNA extraction buffer on a cooled 96-well metal block. After cells were collected, plates were frozen immediately on dry ice and stored at –80°C until further processing.

### cDNA synthesis

The 96-well plates with single cells were thawed on ice; a cold volume of 7 µl containing 300ng random hexamers (Qiagen Operon), 12U Rnasin (Promega) and 0.9% NP-40 (Thermo Scientific Pierce) was added to each well. After thorough pipetting and rinsing, wells were capped, centrifuged at 4°C, heated to 68°C in a thermal cycler for 5 min and placed on ice for at least 1 min. Reverse transcription was performed with the addition of 7 µl containing 3.6 µl 5X reverse transcriptase buffer, 10 U RNAsin (Promega), 62 U Superscript III RT (Invitrogen), 0.62 µl dNTPs 25 mM each (Omega Bio-Tek) and 1.25 µl 0.1 M DTT (Sigma). All wells were capped, the plate placed in a cold rack and vortexed for 10 sec before centrifugation at 300xg. Thermal cycler conditions for reverse transcription were as follows: 42°C 5 min, 25°C 10 min, 50°C 60 min, 94°C 5 min and 4°C hold. When completed, 10 µl of nuclease-free PCR water was added to each well.

### Ig gene amplification

Immediately following cDNA synthesis, IgG genes (Igg) were amplified in a total of 20 µl per well for the first round of nested PCR for IgG heavy chain (Igh), IgG kappa (Iggκ) and IgG lambda (Iggλ), utilizing primers (Supplemental Table 1) as previously described (30).

In brief, a master mix was prepared consisting of 15.58  $\mu$ l water, 2  $\mu$ l 10X HotStar PCR buffer (Qiagen), 0.065  $\mu$ l 5' primer mix, 0.065  $\mu$ l 3' primer, 0.2  $\mu$ l dNTP solution and 0.09  $\mu$ l HotStarTaq per well, to which 2  $\mu$ l cDNA from individual sorted B cells were added and IgG amplified under the following conditions: thermal cycle PCR at 94°C for 15 min; 50 cycles at 94°C for 30 sec, then 58°C (Iggh and Igg $\kappa$ ) or 60°C (Igg $\lambda$ ) for 30 sec, then 72°C for 55 sec; then one cycle at 72°C for 10 min. Second round of nested PCR for Iggh, Igg $\kappa$  and Igg $\lambda$  utilized 2  $\mu$ l of first-round PCR product with second-round primers (30) and the same master mix protocol, with the following conditions: thermal cycle PCR at 94°C for 15 min; 50 cycles at 94°C for 30 sec, then 58°C (Iggh and Igg $\kappa$ ) or 60°C (Igg $\lambda$ ) for 30 sec, then 72°C for 45 sec; then one cycle at 72°C for 10 min. The PCR product generated was purified and sequenced, with V(D)J genes determined using IMGT/V-Quest (31).

### Specific V(D)J region amplification and cloning

Primers specific with restriction enzyme sites for V and J regions were used to amplify the first-round PCR product to generate a fragment for cloning based on previously described primers (30) (Supplementary Table 1). PCR product was purified, restriction enzyme digested, cloned into *Iggh*, *Igg $\kappa$*  or *Igg $\lambda$*  expression vectors and chemically transformed into 5  $\mu$ l aliquots of TOP10 *E. coli* cells (Thermo Fisher Scientific). Successful transformants were screened by PCR amplification utilizing a vector-specific primer paired with an insert-specific primer, sequenced and compared to the second-round PCR product sequence.

### Definition of clonal groups

Clonal groups were based on heavy chain nucleotide sequences. Any PCR product with >0.8% nucleotide sequences with a Phred score <20 was excluded. From PCR-amplified sequences, we determined heavy chain alleles using IMGT/V-QUEST (<http://www.imgt.org>, international ImMunoGeneTic information system). Due to primer mixture ambiguities, the first 20–22 nucleotides of IgGH variable regions were designated as germline, thus this region was not evaluated for somatic hypermutations. IMGT/V-QUEST was used to assign V(D)J organization, and sequences were grouped by shared IgHV genes and CDR3 length. Clonal grouping was determined using Sequence Manipulation Suite: Ident and Sim (32) using Ab-specific clusters as previously defined (33). A clonal group is defined by the same IGHV gene and CDR3 length, along with >72% similarity of each CDR3 amino acid sequence.

### Protein microarray platform

Microarrays PfPv500.1 (Antigen Discovery, Inc.) were 1054 Plasmodium ORFs spotted as in vitro rapid translation/transcription (IVTT) reactions printed and probed (34, 35). array results deposited in Gene Expression Omnibus, reference GSE127729.

### MAB expression and purification

Two plasmids that included coding sequences for full-length IgG1 heavy and light chains were transfected into HEK293-H cells using polyethyleneimine (PEI). Five hundred micrograms PEI were incubated for 25 min at room temperature with 250 $\mu$ g of each plasmid and then added to the HEK293-H at a density of  $1 \times 10^6$  cells/ml in total volume of 500ml

(29, 30). Transfected cells were adapted for growth in Freestyle 293 serum-free expression medium (Gibco, Thermo Fisher Scientific) under suspension conditions of 37°C and 8.5% CO<sub>2</sub>. Cells were centrifuged 96 h post transfection and culture medium was harvested, filtered through a 0.22 µm filter and supernatants concentrated 20 times using a 50 kDa cut-off Vivaflow 50 System (Vivasciences). One volume of IgG binding buffer (Thermo Scientific Pierce) was added and IgG was purified on a Protein A HP HiTrap column (GE Healthcare) eluted with IgG elution buffer (Thermo Scientific Pierce) and neutralized with 1M Tris pH 9.0. Proteins were concentrated and buffer exchanged with PBS using Amicon Ultra4 10kDa. Protein concentrations were determined on Nanodrop (Thermo Fisher Scientific) and sample purity was analyzed by SDS-PAGE.

### **DBPII western blot**

One microgram rDBPII (Sal I variant) was loaded per lane, either as reduced (using 5% β-mercaptoethanol) or non-reduced protein, and was subsequently separated on 4–20% SDS-PAGE gels (Bio-Rad), then transferred electrophoretically onto PVDF membranes (MilliporeSigma) and probed by western blot with different mAbs at 0.2 µg/ml, followed by addition of HRP-conjugated goat anti-human Ab (Jackson ImmunoResearch).

### **Native PvDBP extraction and immunoblot**

Native proteins were extracted from a cryopreserved Pv Malagasy isolate after the culturing achieved a majority of late schizonts. Approximately 50 µl of packed infected erythrocytes were lysed by 3 cycles of freeze/thaw in 15 ml of 5 mM Tris·HCl pH 8.0, then centrifugation for 20 min at 16,000xg at 4°C without brake. All subsequent centrifugations were performed in the same manner. The pellet was re-suspended in 30 ml of 0.1 M Na<sub>2</sub>CO<sub>3</sub> pH 11.5 and centrifuged. After re-suspension in 15 ml of 0.1 M Na<sub>2</sub>CO<sub>3</sub> pH 11.5, protease inhibitors were added and the mixture was incubated 30 min on ice. After 20 min centrifugation, the pellet was solubilized in 100 µl sodium dodecyl sulfate (SDS) at 2.5% and boiled at 100°C for 10 min. Afterward 25 µl of the protein were separated on 4–20% SDS-PAGE gels (Bio-Rad) under non-reducing conditions, then transferred electrophoretically onto PVDF membranes (MilliporeSigma). Membranes containing Pv proteins were probed with 0.2µg/ml of biotinylated mAbs, followed by HRP-conjugated Streptavidin (Thermo Fisher Scientific).

### **Measurement of mAb binding inhibition of DBPII to Duffy-positive erythrocytes**

To assess ability of mAbs to block binding of rDBPII to Duffy positive erythrocytes, a fingerstick blood sample from a donor with homozygous Duffy antigen (FyB/FyB) was collected using EDTA microtainer tubes. Whole blood was washed twice in PBS, re-suspended in 100µl PBS-1% BSA for 30 min at 37°C, then washed twice with PBS-0.2%BSA and counted. A total of 1×10<sup>6</sup> erythrocytes were re-suspended to 100µl. Sixty µl of rDBPII at 1µg/ml diluted in PBS-0.2% BSA were added to mAbs and incubated 20 min under gentle agitation. MAb final concentrations of 16, 4, 1, 0.25 and 0.0625 µg/ml were used, corresponding respectively to mAb/rDBPII molar ratios of 4, 1, 0.25, 0.0625 and 0.015. The mix was added to 1×10<sup>6</sup> erythrocytes in PBS-0.2%BSA and incubated 30 min at 37°C. After 2 washes with PBS-0.2%BSA, rabbit polyclonal anti-DBPII serum at dilution 1/500 with PBS-0.2%BSA was added and incubated 30 min at 37°C. Cells were washed

again and incubated at 37°C in the dark for 30 min with 40 µl anti-rabbit IgG phycoerythrin (PE)-tagged Ab (Sigma), at a final dilution of 1/32 in PBS-0.2% BSA. After 2 washes in PBS-0.2% BSA and a final wash in PBS, erythrocytes were analyzed by flow cytometry. The positive control was binding inhibition by rabbit polyclonal anti-DBPII, while the negative control was binding inhibition by North American plasma samples or mAb specific to TTCF. Data were acquired by BD FACS Diva software and analyzed using FlowJo software v.10 (Tree Star, Inc).

### Measurement of avidity of mAbs to DBPII

ELISA plates coated with rDBPII were prepared as described above. MAbs at 2, 1 and 0.5 µg/ml were diluted in PBS–1% BSA; 100 µl of each dilution were added in duplicate and incubated 1 h at 37°C. After 3 washes with PBST, each series was incubated with PBS or 0.5M or 1M of the chaotropic reagent Ammonium Thiocyanate (NH<sub>4</sub>SCN) for 20 min at room temperature. After 3 washes with PBST, anti-human IgG (Fcγ specific, HRP-conjugated, Jackson ImmunoResearch) diluted at 1/1,000 in PBS–1% BSA was added to each well and incubated 1 h at 37°C. Following 3 washes with PBST, the HRP substrate tetramethylbenzidine (Bio-Rad) was added to wells and OD measured at 405 nm. The experiment was performed twice and the average of the two experiments was used to calculate percentages of high- and low-avidity Abs. For each mAb dilution, percentages were calculated using the following formula:

$$\text{Percentage of high avidity Ab} = [\text{OD with 1M NH}_4\text{SCN}]/[\text{OD with PBS}] \times 100.$$

Percentage of medium avidity Ab was calculated by  $[(\text{OD with 0.5M NH}_4\text{SCN})/(\text{OD with PBS}) \times 100] - \text{percentage of high avidity Ab}$ .

### Competition experiments

For assays measuring competition between mAbs, plates were coated overnight at 4°C with mAbs at 0.5 µg/ml per well, then washed with PBST and blocked with 3% BSA in PBST for 2 h at room temperature. Plates were washed again with PBST, incubated with 50 µl mixtures of 0.04 µg/ml biotinylated rDBPII Sal I and either 1.6, 0.16 or 0.016 µg/ml of competitor mAb for 30 min at room temperature. Plates were washed 6 times with PBST and incubated with 50 µl of a 1:9,000 dilution of High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific) in PBS–1% BSA for 1 h at room temperature. Afterward 100 µl of 3,3',5,5'-tetramethylbenzidine (Bio-Rad) was added, and the reaction quenched with 100 µl of 2 M H<sub>2</sub>SO<sub>4</sub>. Absorbance at 450 nm was measured using a VersaMax plate reader (Molecular Devices).

For assays measuring competition between mAbs and naturally-acquired Abs, plates were coated with mAbs at 0.5 µg/ml per well and remained overnight at 4 °C, then washed with PBST and blocked with 3% BSA in PBST for 2 h at room temperature. Serum from naturally immune subjects, at a dilution of 1/50, was pre-incubated with biotinylated rDBPII Sal I (20 ng/ml) for 20 min at room temperature. The serum mixture was added to plates and incubated 1 h at room temperature. Detection of rDBPII was achieved using High Sensitivity

Streptavidin-HRP (Thermo Fisher Scientific) as described for competition assays between individual mAbs.

### Phagemid expression system

Preparation of phagemids was adapted from previously described protocols for the expression of other malarial antigens (36). Briefly, portions of the genes encoding for the four PvDBPII subdomain (SD) constructs (SD1, SD1+2+3, SD2+3, and SD3) were cloned into the phagemid pHEN-H6 via PstI and NotI for display on the surface of M13 bacteriophage. After purification, the ligation products were used to transform electrocompetent *E. coli* TG1, then cultured overnight on 2 yeast-extract tryptone (YT) agar containing 50 mg/ml ampicillin. The resulting colonies were screened by PCR for the presence of an insert of the correct size. PCR products were sequenced to confirm correct sequence integrity, orientation and frame. TG1 clones containing the pHENH6/DBPII SD were grown to an optical density (OD) of 2.0 in 10 ml of 2 YT broth with 50 mg/ml ampicillin. M13K07 helper phage (11,012 PFU) was added and allowed to infect cells. Ten ml of the culture was added to 200 ml of broth containing 70 mg/ml kanamycin + 50 mg/ml ampicillin and incubated at 37°C for 16 h with shaking. Constructs were expressed as chimeras, with fusion to the phage pIII minor coat protein. Phage particles were purified by centrifugation at 8,000xg for 15 min and 50 ml of 5 polyethylene glycol-NaCl solution was added to the supernatant containing the phage. The phage-polyethylene glycol was incubated on ice for 4 h. Following phage precipitation, the preparation was centrifuged at 8,000xg for 15 min. Supernatant was discarded and phage re-suspended in 1 ml PBS and stored at -80°C. Phage clones were normalized for insert concentrations using the c-Myc epitope tag immediately downstream from the NotI site in the pHENH6 vector. ELISA was performed using mAb 9E10 (anti-Myc, Life Technologies) on serial dilutions of individual phage preparations displaying the various PvDBPII SD to be analyzed. Phage preparations were then diluted such that similar titration curves were obtained when the ELISA was repeated at those dilutions. At that point, all preparations had similar levels of c-Myc epitope tag reactivity and therefore equivalent levels of the respective PvDBPII SD.

### Pv invasion assays

For the Brazilian experiments, cryopreserved Pv-infected erythrocytes (iRBCs) obtained from Brazilians with acute vivax malaria were thawed, enriched using Percoll gradients as described (37) and immediately re-suspended in 100 µl IMDM plus 10% human AB serum with GlutaMax (1:100) to a hematocrit of 6%, then cultured with a gas mix of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub> at 37°C. When cultures were first established, mAbs to be tested were added at indicated concentrations. Cultures of iRBCs were incubated for 20 h, at which point a thin blood smear was prepared and stained with Giemsa to monitor parasite viability and maturation. Between 0.3 and 0.5% of erythrocytes were infected with mostly early trophozoites. Cultures were incubated an additional 18–20 h and re-examined. Maturation to late trophozoite or schizont stage was noted in ~70% of iRBCs. Additional fresh blood cells (donor cells) were enriched for reticulocytes by differential centrifugation without the use of Ficoll, to achieve a reticulocyte count of ~5–6%, and added to cultures at a 1:1 (donor:iRBC) ratio. Cultures were incubated an additional 24 h to allow for new erythrocyte invasion. Cells were gently spun and two thin smear slides per culture well were prepared

and stained with Giemsa. The number of rings and early trophozoites per 50,000 erythrocytes (experiment 1) or 20,000 erythrocytes (experiments 2 and 3) were counted blindly three times using a Miller reticule as described (38). All experiments were performed with duplicate wells. Each experiment corresponded to a different Pv isolate.

For the Cambodian experiments, cryopreserved iRBCs obtained from Cambodians with acute vivax malaria were thawed and cultured in RPMI 1640 medium (Gibco) supplemented with 0.5% Albumax II (Gibco), 2.5% heat-inactivated human serum, 25 mM HEPES (Gibco), 20 µg/ml gentamicin (Sigma) and 0.2 mM hypoxanthine (C-C Pro) for ~24 or ~48h until a majority of schizont stage parasites were noted. The schizont-infected erythrocytes were enriched using magnetic selection, then cultured at a ratio of 1 erythrocyte to 5 reticulocytes that were enriched from cord blood and labeled with CellTrace Far Red dye (Thermo Fisher Scientific). The cultures were incubated for ~8h, stained with DNA stain Hoechst 33342 and examined by flow cytometry. Reticulocytes that were Hoechst 33342 and Far Red positive were scored as new invasion events (Supplemental Figure 1). Invasion of reticulocytes ranged from 0.11% to 1.83% (media alone) for the four experiments, each of which corresponded to one of four Pv isolates.

### Statistical analysis

Analysis was performed using STATA v13 or Graphpad Prism v6. Differences in parasite invasion of erythrocytes were determined using Mann-Whitney U test.

## Results

### Identification of individuals with high blocking activity and determination of frequency of DBPII-specific memory B cells

Three hundred and seventy-one Cambodians with a history of acute vivax malaria were screened for the presence of Abs reactive to rDBPII; 23 individuals (6.2%) had high binding inhibitory activity Abs (BIAbs; high activity defined as >80% binding inhibition at a serum dilution of 1:10 or higher as previously described (12)) to the rDBPII Sal I variant (Figure 1A). Twenty adults with high BIAbs were selected to determine the frequency of DBPII-specific memory B cells (MBCs) in adults by ELISPOT (Figure 1B and C). A mean of 0.80% ( $\pm 1.27\%$  SD, range 0.1–6.4%) of total IgG-secreting B cells were DBPII-specific, and the frequencies correlated with levels of DBPII Abs in plasma ( $r^2=0.43$ ,  $p=0.002$ ). The mean frequency of TT-specific MBCs was 0.82% ( $\pm 0.59\%$  SD, range 0.06–2.72%) and for PvMSP1<sub>19</sub>-specific MBCs, it was 1.01% ( $\pm 0.98\%$  SD, range 0.16–4.36%). The frequencies of DBPII-specific MBCs were highly correlated with frequencies of PvMSP1<sub>19</sub>-specific B cells ( $r^2=0.86$ ,  $p<0.0001$ ), but weakly correlated with frequencies of TT-specific MBCs ( $r^2=0.20$ ,  $P=0.045$ ).

### Cell sorting, sequence analysis and initial identification of clones

Lymphocytes from three donors with high titers of DBPII-blocking Abs underwent single cell sorting using an APC-labeled rDBPII tetramer (Sal I variant). The Sal I variant of DBPII occurs in Cambodia but is not common (39), thus using this variant should preferentially select for strain-transcending DBPII-specific B cells. From one Cambodian donor, *Igh* and

*Ig* PCR products from 98 individual B cells were sequenced (Figure 2A), and 16 B cell clonal groups were identified based on different VDJ sequences (Supplemental Table 2). One or two clones were selected from each group and expressed as full-length IgG1, thereby creating monoclonal antibodies (mAbs). MAb from 11 clones recognized rDBP II (Figure 3). Most of the mAbs reacted to all three variants, as exemplified by 092096 and 053054, with greatest reactivity to Sal I, followed by P and 7.18. In contrast, mAb 087085 appeared to be strain-specific, recognizing the Sal I variant well, but showing comparatively little reactivity to others. MAb 094083 recognized all three variants, but in a manner distinct from other mAbs, with equivalent reactivity to Sal I and P but less for 7.18.

From a second Cambodian donor, 72 DBP II-specific B cells were sorted, from which 21 IgGH were successfully amplified, representing 21 clonal groups. From two of these clonal groups, mAbs were generated, one of which recognized rDBP II (099100). From one Brazilian donor, 64 DBP II-specific B cells were simultaneously sorted. Twenty mAbs were expressed, two of which recognized rDBP II, representing two clonal groups. However, one of the mAbs was cross-reactive to non-malarial antigens, and one specifically recognized rDBP II (134135).

All clonal groups were somatically mutated from germline IgG, however the number of mutations varied greatly among the different clonal groups (Figure 2B). Related mutations in each group indicated common ancestor cells, which underwent clonal expansion and substantial diversification, presumably during germinal center reactions. The VDJ and CD3 region sequences of 13 anti-DBP II mAbs that were further characterized are shown in Figure 2C (see below). A similar approach was used to isolate T TCF-specific B cells. The anti-T TCF mAb 043038 was used as a negative control for subsequent experiments.

### **MAb polyreactivity, reduction dependence and ability to recognize native *P. vivax* proteins**

Anti-DBP II mAbs were not polyreactive, showing little or no recognition of a panel of Pv and Pf antigens by microarray analysis (Figure 4). The polyreactive mAb 110111 was obtained from a sort for another malarial antigen (PvCelTOS). Epitope reactivity of all anti-DBP II Abs was reduction dependent, except for 094083 (Figure 5) suggesting that most mAbs potentially recognize conformational epitopes. MAb 092096 and 099100 recognized native DBP from an extract of thawed Malagasy Pv-infected erythrocytes run on non-reducing gel (Figure 5B). Notable is recognition of DBP at about 225 kDa, twice the size of the predicted 120 kDa of full-length DBP.

### **DBP II-specific mAbs show strain-transcending inhibition of DBP II binding to erythrocytes**

All but one of the anti-DBP II mAbs blocked binding of rDBP II to Duffy-positive human erythrocytes (Figure 6), with molar ratios as low as 0.25:1 of mAb:rDBP II. The ability of mAbs to block binding of the three divergent rDBP II variants is noteworthy, indicating strain-transcending binding inhibition. By contrast, the anti-T TCF mAb 043038 and anti-DBP II mAb 087085 failed to block rDBP II binding. Although there was considerable variation in avidity among the mAbs (Figure 7), mAb avidity was not associated with blocking activity.

### Most anti-DBP2 mAbs recognize similar/overlapping epitopes

To determine whether binding inhibitory mAbs recognize shared epitopes of DBP2, competition experiments were performed using ELISA plates coated with different mAbs. To identify blocking activity, biotinylated rDBP2 was pre-incubated with the same panel of mAbs in solution, with molar ratios of mAb:rDBP2 at 10:1, 1:1 and 0.1:1 (Figure 8, 0.1:1 data not shown). MAbs 053054, 055056, 065098, 066067, 071063, 080064, 092096 and 099100 competed with each other, indicating shared or overlapping epitopes. MAbs 081082, 088084 and 134135 also competed with other mAbs, but to a more limited extent. This is likely a consequence of overall lower avidity and/or steric inhibition by nearby, but not totally overlapping, epitopes. By contrast, the blocking mAb 094083 showed no competition with other mAbs, indicating recognition of a different epitope. Of note, 094083 was the only mAb tested that was not reduction sensitive with respect to rDBP2 (Figure 5).

### Blocking Abs recognize subdomain 2 of PvDBP2

To define the region of PvDBP2 interacting with mAbs, blocking activity was examined using an assay in which rDBP2 binds to the N-terminal 60 amino acids of the Duffy antigen receptor for chemokines (nDARC), which is expressed as a fusion protein with human FcG1. Binding in this assay involves interaction of subdomain 2 (SD2) of rDBP2 with the Duffy antigen, as part of the initial step in heterotetramer formation (8, 9). Testing was done with a subset of four human anti-DBP2 mAbs representing the observed breadth of mAb responses to PvDBP2. MAbs 096092 and 053054 represented mAbs that blocked binding of rDBP2 to nDARC, and they also competed with most of the other mAbs, indicating they targeted the same or overlapping epitope(s). MAb 094083 also blocked binding of rDBP2 to nDARC, but it recognized a different epitope, while mAb 087085 was a non-blocking mAb (Figure 9A). By contrast, murine anti-DBP2 mAbs reported to inhibit DBP2 binding to erythrocytes *in vitro* showed no blocking activity in this assay (25). This is consistent with the demonstration that murine anti-DBP2 mAbs recognize epitopes in SD3 of PvDBP2. To support the observation that SD2 is the recognition site for the human mAbs, we performed a phage display analysis. Seven of 8 human mAbs tested recognized phage displays of SD 1+2+3 and SD 2+3, but not phage displays of SD 1 or 3 (Figure 9B). As positive controls, pooled human Pv immune sera recognized all phage display constructs, and the murine anti-DBP2 mAbs 3D10 (epitope in SD1) and 2H2 (epitope in SD3, (25)) recognized constructs expressing SD1 or SD3, respectively. There was no recognition of the phage display constructs by pooled non-immune North American human sera. Pooled human immune sera reactive to rDBP2 recognized all constructs shown in Figure 9 except the SD2-only phage, suggesting improper folding (data not shown).

### Blocking mAbs do not target a highly polymorphic epitope of DBP2 in SD2

To better define the binding region of mAbs to SD2, we determined whether mAbs would block a modified rDBP2 molecule where the DEK epitope, the most polymorphic and immunogenic portion of SD2 (DEKAQRRKQ), has been replaced with ASTAATSRTS in a construct referred to as DEKnull(24, 40). Despite this alteration, binding of DEKnull to reticulocytes and the nDARCIg fusion protein is preserved (24, 41). All blocking mAbs

inhibited binding of DEKnull to nDARCIg (Figure 10), indicating the DEK substitution in SD2 was not critical in determining the binding reactivity of blocking mAbs.

### **Anti-DBP<sub>II</sub> mAbs share epitopes associated with blocking Abs from naturally immune subjects**

To evaluate whether naturally-acquired blocking Abs to DBP<sub>II</sub> recognize the same epitopes as isolated mAbs, we examined serum samples from residents of Pv-endemic areas in Papua New Guinea, Cambodia and Brazil (Figure 11). The overall level of binding inhibition was determined by the degree to which a serum sample inhibited rDBP<sub>II</sub> binding to its ligand using the nDARCIg assay (y-axis, assay described in Figure 9A). In a separate assay, we then evaluated whether the same serum sample could significantly block binding of rDBP<sub>II</sub> to the test mAb coated on a plate (x-axis). If so, this would suggest that a mAb derived from a given individual is competing for the same rDBP<sub>II</sub> epitope as DBP<sub>II</sub>-specific Abs from other naturally immune residents of their community or a geographically distinct malaria-endemic region. The amount of binding inhibitory activity in serum samples from different Pv endemic areas of the world was associated with the ability of the same sample to block binding of rDBP<sub>II</sub> to the blocking mAb 099100 (Figure 11). A similar association was observed for other anti-DBP<sub>II</sub> blocking mAbs. The competitive relationship was significant for serum samples from individuals with the highest blocking activity (> 80%), with mAbs competing for a median of 82% to 95% of blocking activity. By contrast, the blocking mAbs showed comparatively little competition with serum from individuals with lesser blocking activity (40–79%). For comparison, results with the non-blocking mAb 087085 are also shown. Thus, mAbs identified in the current study target similar epitopes as naturally-acquired blocking Abs from diverse Pv-endemic populations.

### **MAbs inhibit Pv invasion of reticulocytes**

*In vitro* culturing of *P. vivax* is inherently difficult. Thus far, we have been able to test three anti-DBP<sub>II</sub> mAbs for the ability to inhibit Pv invasion of human reticulocytes, and all three inhibited invasion in a short-term Pv *ex vivo* assay using Brazilian and Cambodian clinical isolates (Figure 12). As controls, we used cytochalasin D (Brazilian experiments), a cell-permeable fungal toxin that inhibits actin polymerization needed for merozoite invasion of erythrocytes, or heparin (Cambodian experiments). For three Brazilian Pv isolates, the addition of mAb 134135 derived from a Brazilian subject produced 57%, 69% and 41% inhibition, respectively, of Pv invasion at 100 µg/ml, relative to no added Ab (Figure 12). MAb 099100, derived from a Cambodian resident, showed a similar level of inhibition using Brazilian Pv isolates (~50%). For both mAbs 134135 and 099100, inhibition was dose-dependent, with less inhibition at concentrations of 25 and 50 µg/ml (data not shown). Concentrations higher than 100 µg/ml were not tested. MAb 099100 and 053054, the latter derived from a second Cambodian resident, showed similar levels of Pv invasion inhibition (40–50%) using four separate Pv isolates from Cambodia (Figure 12). There was little or no inhibition with anti-TTCTF mAb 043038. Thus, the addition of anti-DBP<sub>II</sub> mAbs 134135, 99100 and 053054 resulted in similar reduction of Pv invasion of reticulocytes, irrespective of the geographic origins of Pv isolates.

## Discussion

Successful vaccines often model effective immune responses acquired by natural infection. In the case of Pv malaria, naturally-acquired Abs to DBP inhibit DBP-DARC binding and parasite invasion (17), and levels correlate with protection against clinical disease (12, 16). The DBPII molecule is highly polymorphic, but only at certain residues, indicating the different domains of the ligand are under diversifying or purifying selective pressures aimed at retaining function while evading the host immune response (42). We have previously identified individuals from diverse Pv-endemic areas who have high levels of blocking Abs capable of inhibiting DBP-DARC binding (12, 16). These Abs are strain-transcending, suggesting that critical and conserved DBP-binding sites are important for protective Ab responses. To identify the critical sites, we generated panels of mAbs to DBPII by isolating and sequencing *Igg* from individual DBPII-specific MBCs derived from subjects with high titers of blocking Abs to DBPII. These mAbs, which arose from diverse B cell lineages acquired by three of our study participants, were further characterized with respect to reactivity with DBPII. We identified 12 binding inhibitory mAbs. All were strain-transcending, and most of the mAbs had multiple somatic mutations, indicating they had undergone a period of affinity maturation consistent with gradual acquisition of natural immunity to blood-stage *Plasmodium* infection. Eleven of the mAbs recognized the same or closely overlapping regions of subdomain 2 (SD2) of PvDBP region II, which contains the binding motif for DARC. The Ab repertoire associated with these conserved epitope(s) contains diverse paratopes, indicating multiple B cell lineages cluster around one primary solution for antigen recognition, dominated by CDR3 ontogenies.

Most importantly, the epitope(s) recognized by mAbs reacted with Pv immune sera from residents of diverse geographic endemic regions, suggesting a globally conserved protective epitope(s). We examined almost 1600 serum samples from 971 residents of Pv-endemic areas of Papua New Guinea, Cambodia and Brazil, all of whom had varying levels of blocking Abs to recombinant DBPII (rDBPII). Sera from immune individuals with high blocking activity competed for rDBPII binding to mAbs, indicating the sera contains Abs that recognize the same conserved regions as the mAbs (although the possibility of steric hindrance, due to some Abs binding to adjacent epitopes, should be considered). For many of these immune individuals, the predominant blocking activity was directed to portions of rDBPII recognized by mAbs, suggesting that strain-transcending naturally-acquired Abs recognize one or a few binding sites on rDBPII. These observations are consistent with those noted in studies of highly variable viral surface proteins (43, 44). For example, a panel of 14 broadly reactive mAbs capable of neutralizing 90% of HIV-1 strains targeted a common “supersite” on gp120 involved in binding to CD4 (43).

The epitopes recognized by mAbs appear to be located in SD2 of DBPII, a region containing the initial contact residues required for DBPII binding to DARC (8). By contrast, blocking murine mAbs specific for DBPII-DARC binding are known to target epitopes in SD3 (25). Compared to murine mAbs, the human mAbs are more potent inhibitors of rDBPII binding to DARC, emphasizing the importance of the DBPII-SD2–DARC interaction in the initial reticulocyte invasion process. In addition, these blocking mAbs derived from elite responders do not recognize the dominant B cell epitope in SD2, the polymorphic DEK

epitope (41), supporting the notion that their principal recognition sites are conserved motifs. Such a pattern for the host immune response to DBP<sub>II</sub> is similar to that described for critical viral invasion ligands, e.g., HIV, RSV and influenza (43–45). In those models, strain-transcending blocking Abs target conserved critical binding domains necessary for receptor engagement of host cells, with polymorphic and immunogenic residues bordering conserved regions. The immunogenic residues divert Ab responses away from critical binding sites. Since Ab responses to DBP<sub>II</sub> in a majority of individuals are often strain-specific (23) and non-blocking, a similar model emerges in which anti-DBP<sub>II</sub> blocking Abs derived from elite responders preferentially recognize a conserved SD2 critical binding epitope(s), which is surrounded by highly immunogenic and polymorphic residues such as DEK.

The explanation as to why only a subset of individuals acquires strain-transcending DBP blocking Abs after Pv infection may be related to several factors. One aspect relates to genetic restriction by certain HLA class II alleles (16, 46). In one study, however, all individuals not previously exposed to malaria who were immunized with rDBP<sub>II</sub> produced binding inhibitory Abs (47), suggesting genetic restriction may not be a significant issue. Another factor is the restricted V<sub>H</sub> gene origin of the Ab heavy chain, which favors specific epitope recognition. Our data show that in anti-DBP<sub>II</sub> blocking mAbs, the V1–24 allele predominates. A third possibility is that frequent antigenic exposure is required to induce repeated somatic mutations and affinity maturation to achieve recognition of the critical binding domain. This is supported by the general observation that individuals with more exposure to Pv are more likely to develop strain-transcending blocking Abs (16). In our studies, one mAb was non-blocking and had a median of six somatic hypermutations, compared to a median of 20 for strain-transcending blocking mAbs. There also may be variation in parasite strains affecting the acquisition of naturally-acquired immunity, such as polymorphisms in the non-binding domain which affect DBP conformational presentation and Ab access, as well as an increase in the number of genes encoding DBP (22). Despite these factors, our studies showed that strain-transcending blocking mAbs to DBP<sub>II</sub> arose from multiple B cell lineages derived from residents of geographically distinct areas, and this suggests multiple pathways are involved in the generation of high-activity binding inhibitory Abs. This observation suggests a DBP<sub>II</sub>-based vaccine may be able to produce strain-transcending blocking Abs in most individuals.

We have shown that three mAbs partially block Pv invasion into reticulocytes, with a range of 40 to 69% inhibition. This is comparable to levels of inhibition (up to 64%) we previously observed using polyclonal rabbit IgG to DBP<sub>II</sub> (17), and similar to that found in a recently reported study showing murine mAbs specific to PvRBP2b, at concentrations ranging from 25 to 125 µg/ml, blocked Pv invasion of reticulocytes *in vitro* by 42 to 52% (48). For comparison, murine Abs elicited by PvAMA1 immunization inhibited Pv re-invasion *in vitro* by only a mean of 25% (range 11% to 43%) (49). Variable or incomplete blocking activity by the mAbs may result from polymorphisms and/or copy number variation of DBP in parasite lines used in assays, as mentioned above. Additionally, *in vivo* IgG subclass or post-translational modification by glycosylation of the IgG heavy chain may further enhance Ab flexibility, or enable recruitment of effector cells to facilitate blocking and/or merozoite clearance (50), and those effects and mechanisms may not recapitulated *in vitro*.

Until recently, few anti-*Plasmodium* mAbs have been generated from humans (almost exclusively to Pf molecules (51–54)), and prior studies have mostly used a phage display of single-chain fragments of *Iggh* and *Iggl* (scFv) (55–59). The scFv approach mixes *Iggh* and *Iggl* from different B cells, so it may not adequately represent a B cell repertoire. Recently, there have been multiple human mAbs isolated that recognize Pf circumsporozoite protein (PfCSP), generated from individuals either vaccinated with radiation-attenuated sporozoites, or fed upon by infected mosquitos, using chloroquine treatment to prevent blood stage infection (60–63). Most of the mAbs to PfCSP were isolated by the same method used in the current study, i.e., by single cell sorting of antigen-specific B cells. These mAbs conferred protection against sporozoite challenge *in vivo* using murine transgenic models. Some mAbs recognized conserved linear repeat NANP epitopes, previously known to be associated with protection (64, 65). However, other protective mAbs identified unique epitopes on PfCSP(62). This finding will help inform second-generation pre-erythrocytic subunit vaccines, which by virtue of an increased breadth of Ab responses, aim to prevent sporozoites from reaching the liver. With *P. vivax*, successful production of mAbs has been reported to date for only one Pv protein (66), in a study using the scFv approach to generate three anti-DBPII mAbs from Pv-exposed Koreans. These mAbs partially blocked binding of DBPII to COS cells transfected with a plasmid coding for DARC, but they were not further characterized, nor was it determined whether they could inhibit Pv invasion of reticulocytes *in vitro*.

There were limitations of the study. The mAbs were generated from a small number of individuals, thereby limiting the generalizability of the targets of highly inhibitory Abs. However, blocking mAbs from all three subjects (two from Cambodia, one from Brazil) recognized the same region of PvDBPII. The possibility that there might be a globally conserved recognition site was reinforced by the ability of blocking mAbs to compete with the vast majority of naturally- acquired blocking Abs from diverse individuals and locations worldwide. A second limitation of the study is that, to date, the precise epitopes of the mAbs have not been identified. Such studies are currently underway.

Ultimately, an assessment of the functional importance of these mAbs will require passive transfer to non-human primates susceptible to Pv, with a possible goal of developing a regimen for treatment or prevention of human Pv infection. Such passive immunization could provide short-term protection of up to 6 months, and bypass many of the immunoregulatory effects of malaria infection and other obstacles to vaccination, by directly providing the desired immune response. Given the relatively high concentration of mAbs required to inhibit Pv invasion *in vitro*, it might be difficult to achieve these levels *in vivo* following immunization or passive transfer of mAbs. However, mAbs *in vivo* may work by other mechanisms to reduce blood stage parasitemia, such as complement activation and opsonic phagocytosis/killing of parasites, thus requiring lower levels than that predicted *in vitro*. Humanized mAbs have been used to protect/treat premature infants in respiratory syncytial virus infections (45) and have been proposed as therapy for West Nile virus (67), Ebola virus (68) or HIV infections (69, 70). Treatment of *Plasmodium* infection with mAbs might be used in cases of seasonal malaria, when subjects are critically ill or when multidrug-resistant clones are present. With respect to continued progress toward effective malaria vaccines, further studies with mAbs can help identify which Ab targets confer

protection, the levels of Abs required, the optimal Ab isotypes and subclasses, and the potential synergies among Abs targeting different proteins or epitopes. Thus, these results provide a framework for the improved design of malaria vaccines in an effort to elicit a highly effective immune response.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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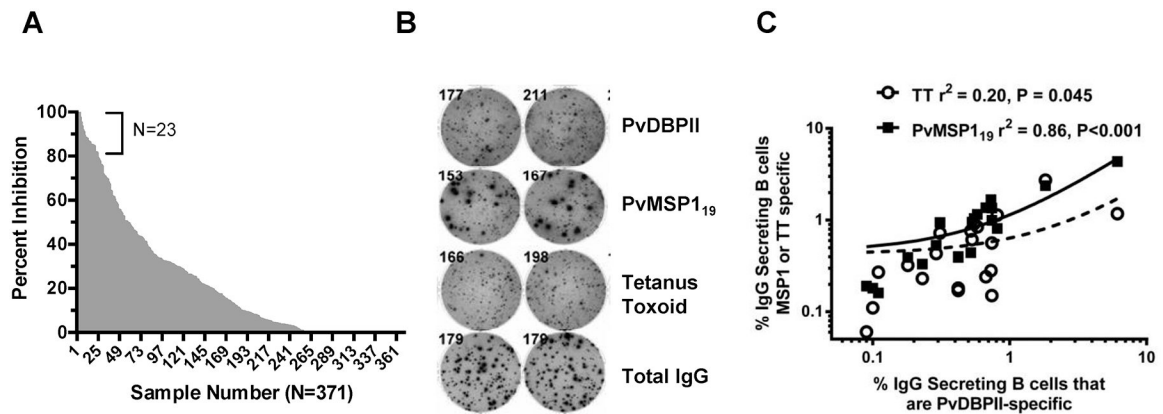
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**Key points**

*P. vivax* Duffy binding protein:DARC interaction is essential for red cell invasion

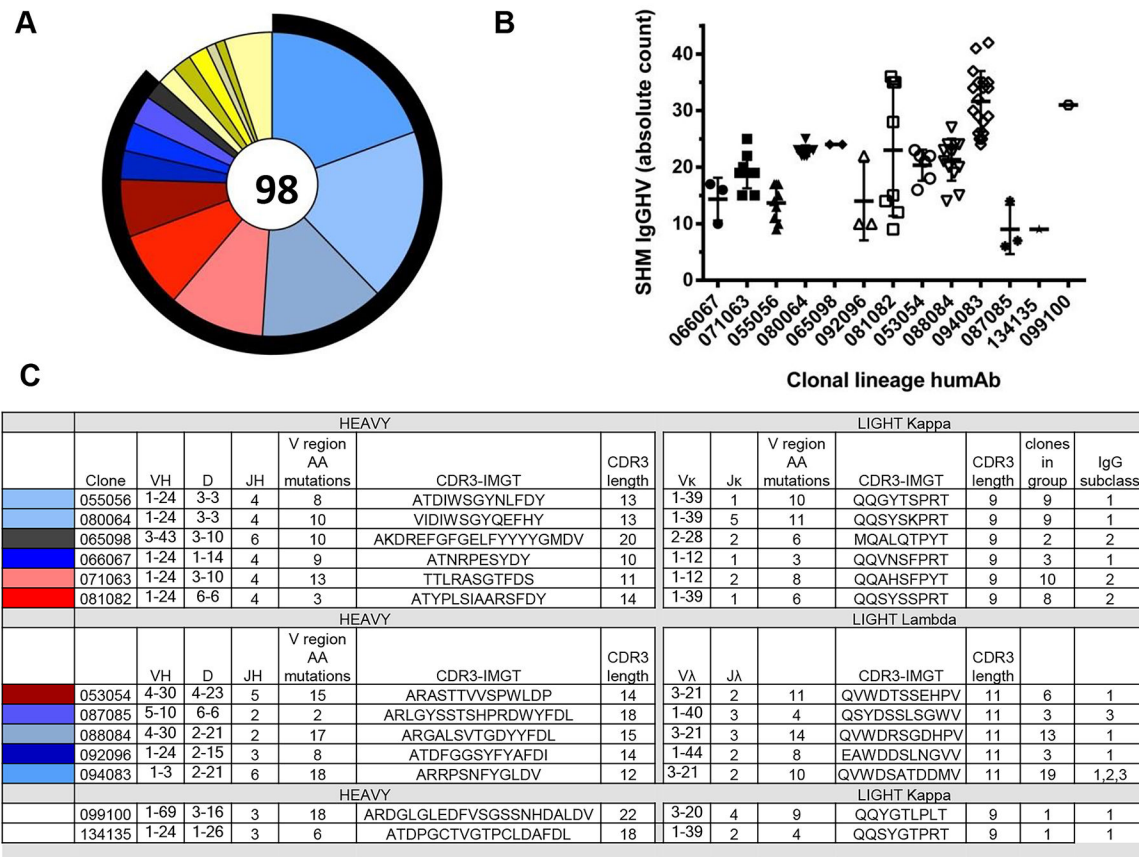
*P. vivax* exposed individuals acquire antibodies that block this interaction

Human derived Monoclonal Abs recognize a globally conserved blocking epitope

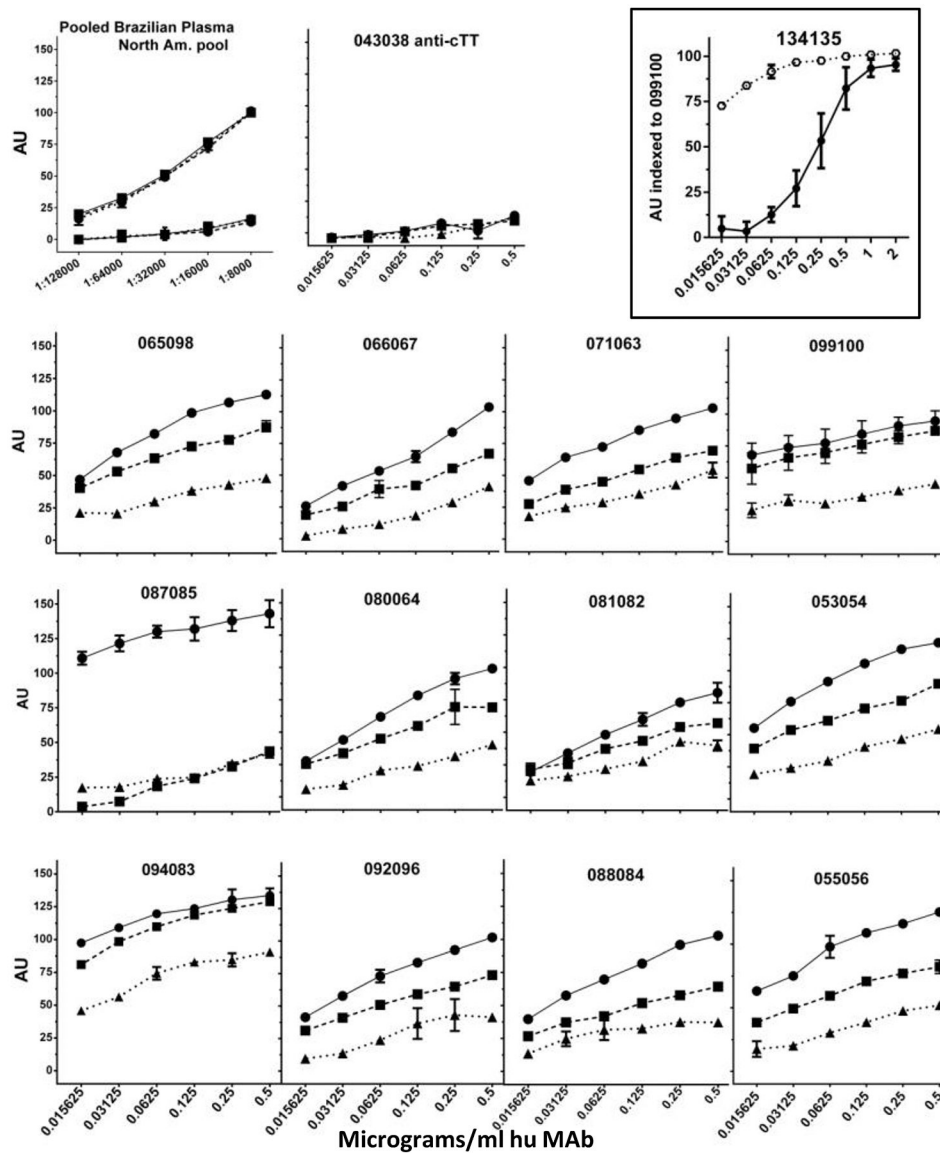


**Figure 1.**

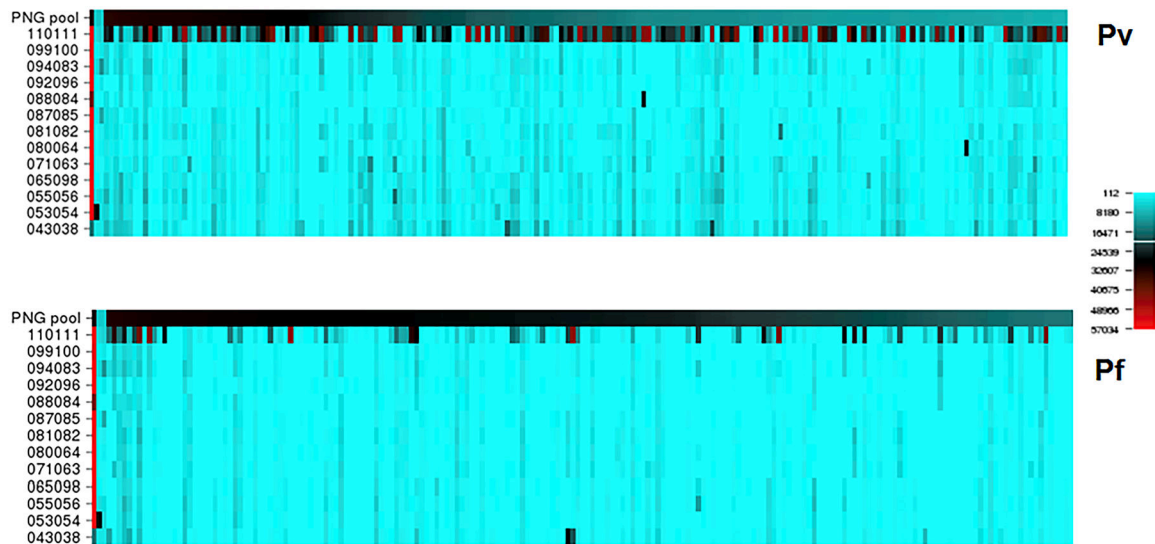
(A) Blocking Abs to PvDBP II from Cambodians with prior *P. vivax* infection. Panel (B) shows ELISPOT of antigen-specific MBCs following 6 days of stimulation performed in duplicate. Cells from PBMC-stimulated cultures were added as follows: 300,000 to wells coated with specific antigens; 3,000 to wells coated with anti-human IgG. Values by each well indicate the number of spots detected by an ELISPOT reader. (C) The frequency of IgG secreting B cells that are PvDBP II-specific (x-axis) or PvMSP1<sub>19</sub> or tetanus toxoid (TT)-specific (Y-axis). The correlation of the frequency of PvDBP II-specific IgG secreting B cells with frequency of PvMSP1<sub>19</sub>- or TT-specific B cells is shown. Identical activation of PBMCs from North Americans not exposed to malaria failed to show any malaria antigen-specific spots (data not shown).

**Figure 2.**

(A) The relative proportion of different clonal groups of 98 PvdBPII-specific B cells, which were sorted and sequenced for *Iggh* from one individual (color-coded). A clonal group is fully defined in Methods, but has the same VDJ rearrangement, the same CDR3 length and at least 72% similarity of each CDR3 amino acid sequence. Eighty-four percent of *Iggh* sequenced is in clonal groups that have blocking Abs (black ring) and is more likely to come from clonal groups with an expanded B cell population (see color coding). MAbs 134135 and 099100 are derived from sorts of a Brazilian and another Cambodian donor, respectively. (B) Somatic hypermutation of IgGH variable region base pair counts (absolute number) for each clonal group with anti-DBPII mAbs from the sort shown in (A). (C) The table shows antigen-specific B cells whose *Iggh* and *Igghw* were cloned into IgG1 expression vectors; mAb products recognized rDBPII but no other antigens. The number of non-synonymous mutations in the B cell clones differing from germline (somatic mutations); sequences of CDR3 *Iggh* and corresponding *Iggl* or *Iggk* and IgG subclasses of individual B cells are shown.

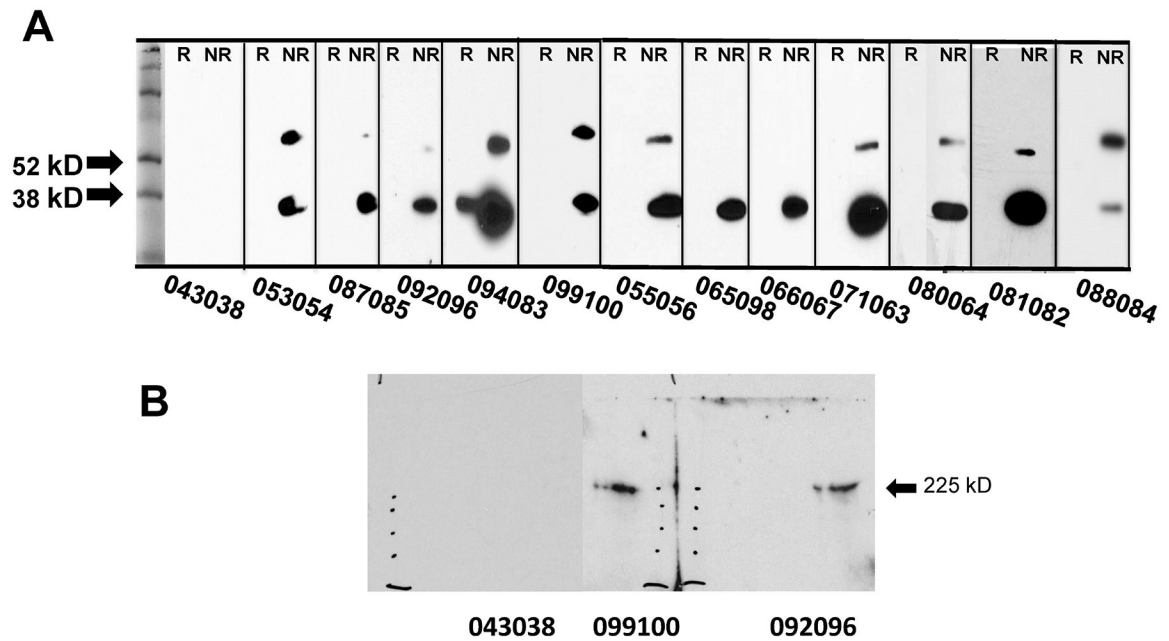


**Figure 3.** Human mAb recognition of 3 diverse variants of rDBPII: Sal I (circle, solid line), P (square, dashed line) and 7.18 (triangle, dotted line). The positive control is pooled sera from Brazilian subjects previously exposed to Pv. Negative controls are pooled sera from North American individuals without any history of exposure to malaria (upper left panel) and the mAb 043038, which reacts to the C-terminal fragment of tetanus toxoid (TTCF). Samples were run in duplicate and mean $\pm$ SD arbitrary units (AU) are shown. Reactivity is in AU, defined as 100 of the pooled Brazilian serum at 1:8000 dilution (see positive control). The positive control was run on all plates examining mAbs. The mAb 134135 was assayed on a different date using an ELISA with Sal I rDBPII in solution and indexed to 099100.



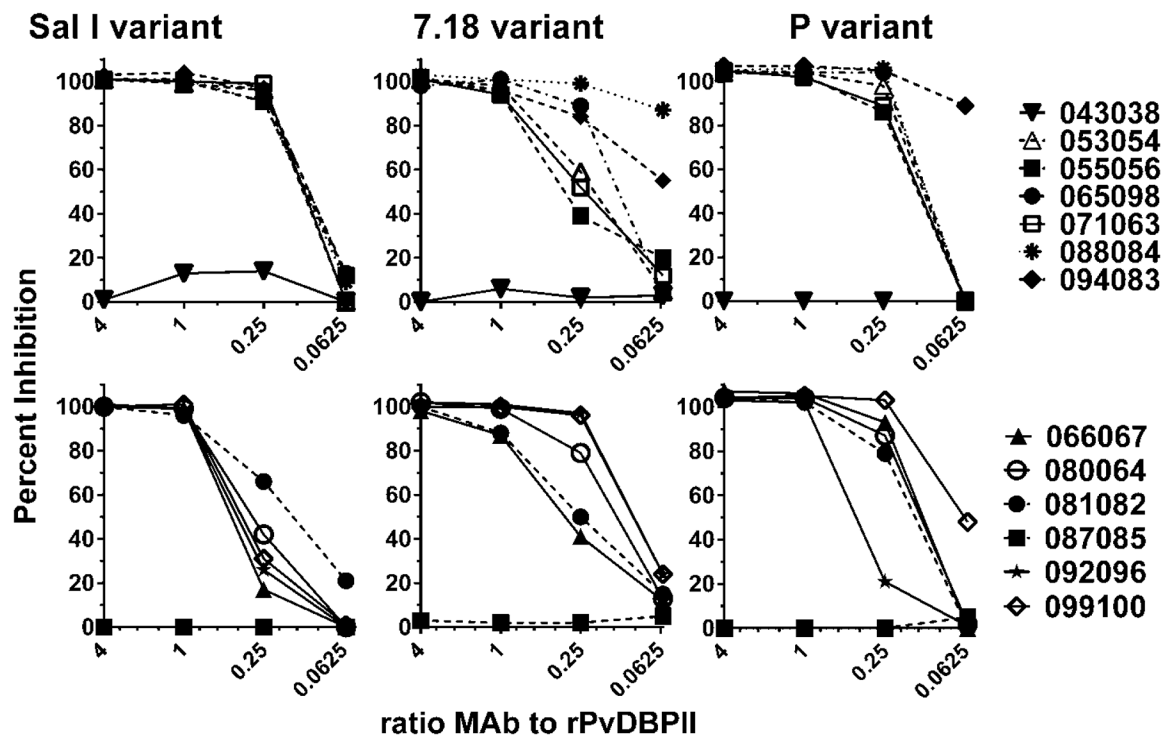
**Figure 4.**

Microarray of Pv and Pf proteins probed with human mAbs, then detected with PE-conjugated anti-human IgG Ab and measured by a protein microarray reader with signal ranging from 112 to 57,043 fluorescent units. Each column represents a single expressed protein. The rows indicate recognition by individual mAbs. The first column is 1.0 mg/ml of rDBP<sub>II</sub> spotted onto the chip. The top row shows pooled serum from PNG individuals exposed to malaria and samples arranged in order of the strength of reactivity to this pool. The second row shows a polyreactive human mAb generated to PvCEL<sub>TOS</sub>. The bottom rows show reactivity of a mAb that recognizes TT and not rDBP<sub>II</sub> (see Figure 3).



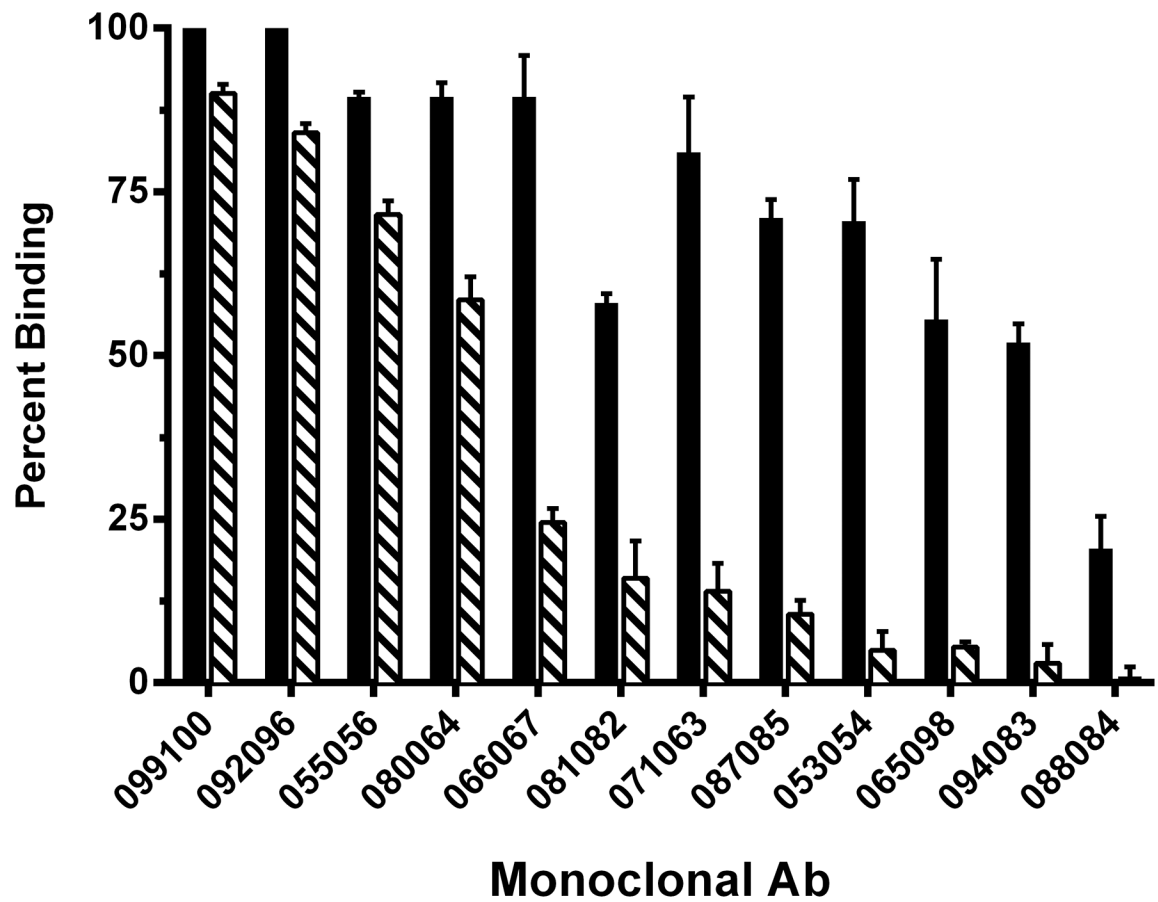
**Figure 5.**

Presence or absence of reduction sensitive mAbs. **(A)** Recombinant DBP II (Sal 1) was run on gels under reducing (R) and/or non-reducing (NR) conditions and the protein transferred to nitrocellulose membranes and probed with mAbs. The larger band on some blots is dimerized DBP II. **(B)** Anti-DBP II mAbs recognize partially purified lysates from cultures of Pv-infected erythrocytes matured to schizont stage by western blot analysis under non-reducing conditions.



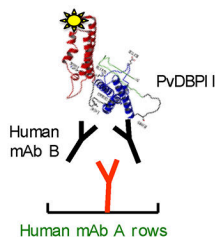
**Figure 6.**

DBP II-specific mAbs block binding of rPvDBP II to human erythrocytes. Three different rPvDBP II were used, corresponding to 3 distinct DBP variants: Sal I, P and 7.18. MAbs were mixed with rDBP II at the molar ratio indicated prior to addition to DARC (FyB/FyB)-positive erythrocytes, and levels of binding measured by flow cytometry. Percent inhibition was compared to rDBP II binding in absence of mAbs. Samples were measured in triplicate. Coefficient of variation was <10% for all samples.



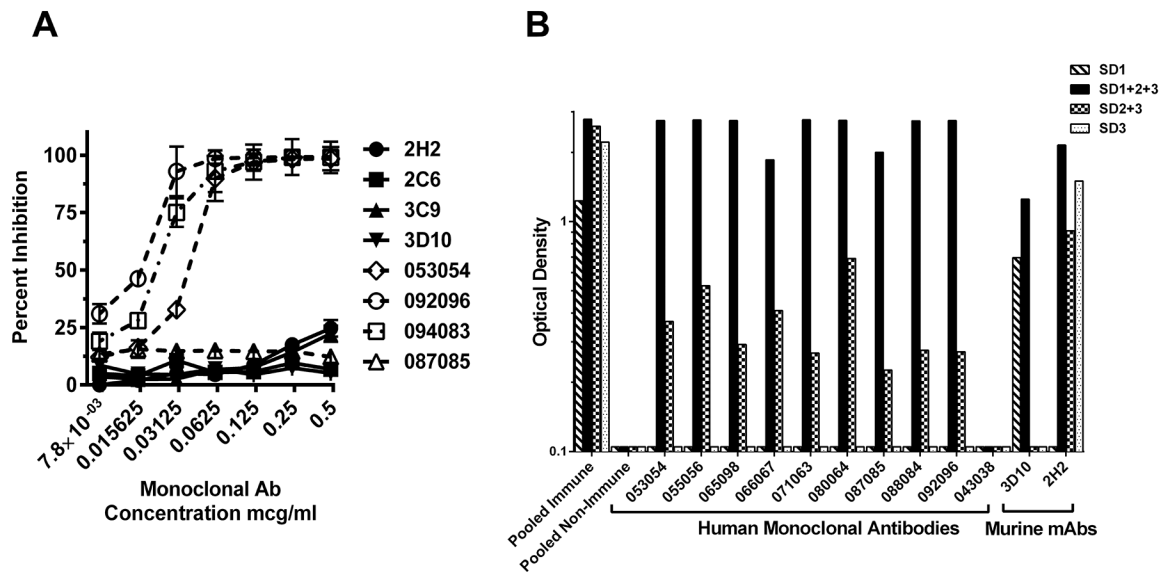
**Figure 7.**

Avidity of mAbs for rDBPII (Sal I) by ELISA. Percent of mAb binding  $\pm$  SD to rDBPII after 20 min incubation with 1 M (hatched bars) or 0.5 M (black bars) of NH<sub>4</sub>SCN as compared to incubation with PBS. The assay is described in detail in Methods.



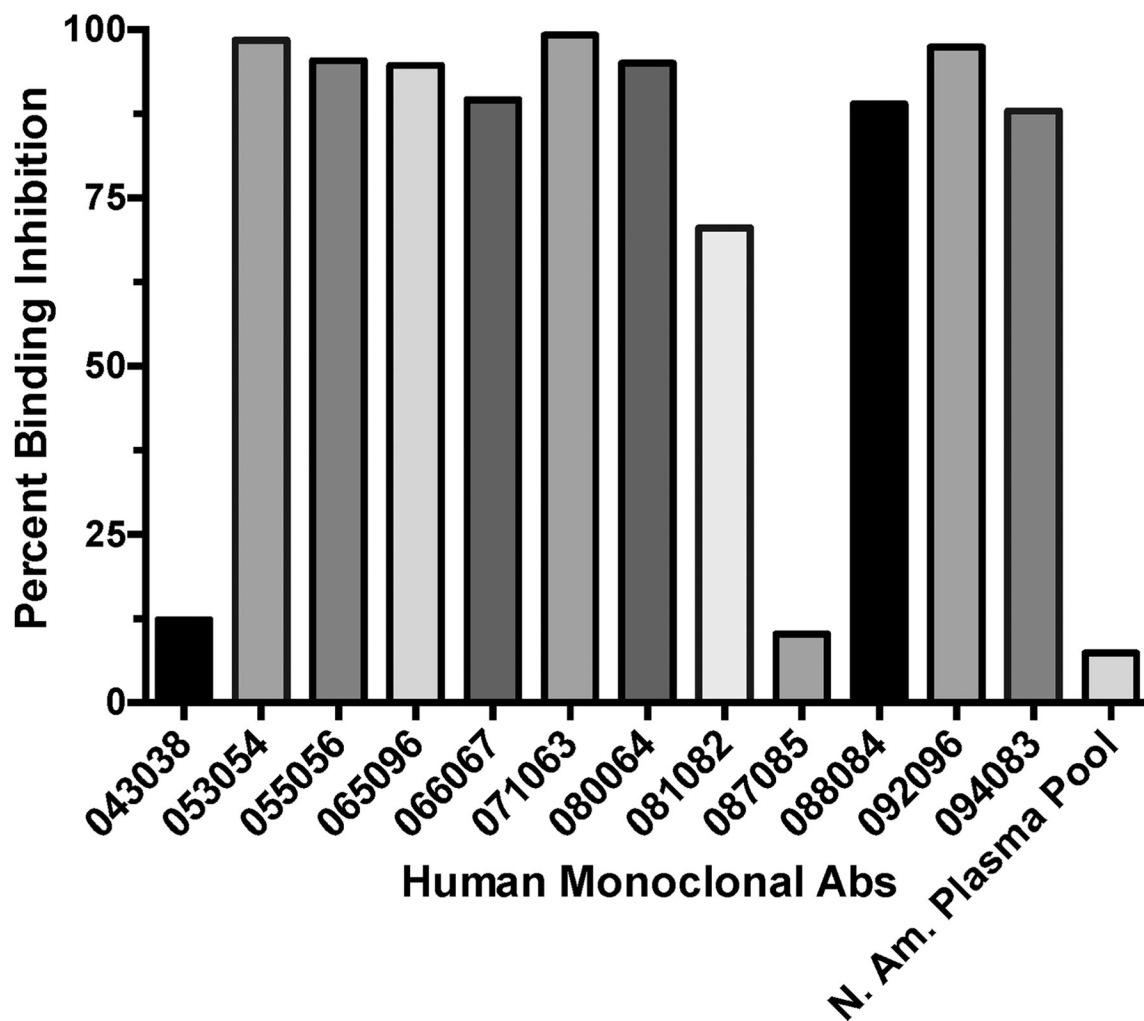
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coating	053054	91	85	96	84	86	56	86	67	95	78	93	77	38	12	50	19	95	85	0	0	85	70	6	2	
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	065098	96	91	96	88	88	69	87	71	96	89	96	88	56	27	70	37	99	96	0	0	88	80	13	4	
	066067	98	93	93	87	94	82	99	87	100	100	100	93	78	42	95	70	100	98	3	0	94	81	45	16	
	071063	98	90	99	87	91	65	96	69	100	91	95	83	51	21	67	31	97	90	0	0	94	81	18	5	
	080064	95	88	97	89	94	59	90	64	95	81	99	84	45	15	62	24	100	94	1	0	93	82	8	2	
	081082	100	96	100	96	96	75	93	73	100	92	100	80	62	17	78	33	100	97	0	0	95	84	15	3	
	088084	94	86	82	71	65	54	88	65	97	80	95	65	28	7	58	16	100	92	0	0	95	84	10	1	
	092096	89	75	92	79	76	44	75	53	89	69	89	56	29	6	43	12	100	95	0	0	94	73	6	1	
	094083	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	69	32	83	65	1	0
	099100	98	65	99	70	88	55	86	58	89	56	92	57	38	11	49	20	96	63	1	1	100	76	5	1	
134135	96	73	91	66	96	91	89	62	99	68	93	65	86	47	100	86	100	66	18	0	100	83	65	26		

**Figure 8.** Competition experiments between mAbs to determine if they share the same or overlapping epitopes. ELISA plates were first coated with one of the mAbs shown in the left column in green (see cartoon, left panel). Next, biotinylated rDBP II (Sal I variant) was incubated with one of the mAbs shown in the top row at molar ratios 10:1 or 1:1 of mAb:rDBP II in solution for 1 h, prior to adding to a plate coated with mAb. Values shown are mean percent inhibition of mAb in solution relative to rDBP II in the absence of mAb. Each competition experiment was performed in duplicate. Replicates showed <10% coefficient of variation. Red indicates high blocking activity, whereas blue comparatively lower blocking activity. Biotinylated rDBP II was detected with streptavidin HRP.



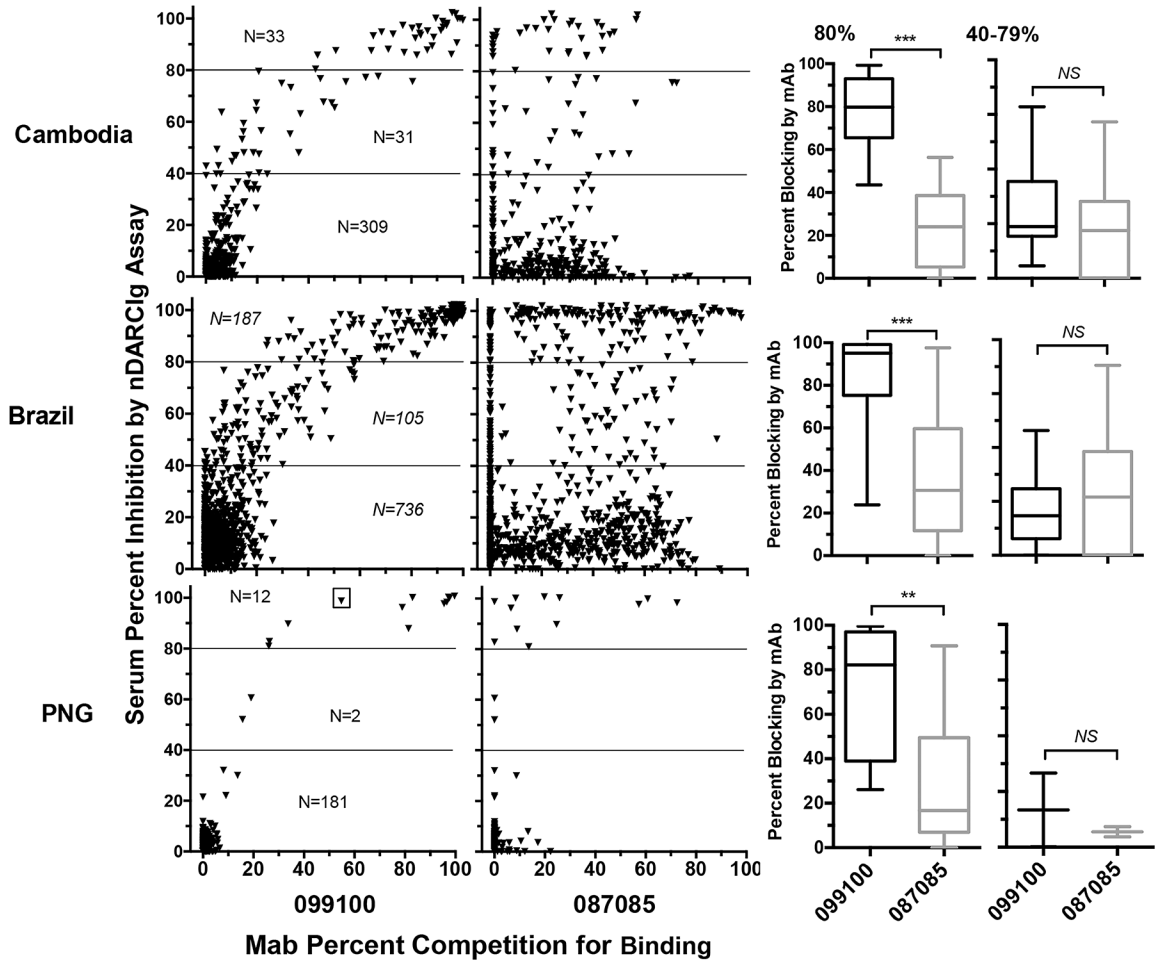
**Figure 9.**

(A) Comparison of binding inhibitory activity of 3 blocking human mAbs (053054, 092096, 094083), a non-blocking DBP II-specific human mAb (087085), 3 blocking murine anti-DBP mAbs (2C6, 2H2, 3C9) and one non-blocking murine mAb 3D10. Binding inhibition was measured using an assay that detects binding of rDBP II (Sal I variant) to the nDARCIg fusion protein (N-terminal 60 aa of DARC fused to Fc portion of human IgG1). Concentrations of mAbs are shown in  $\mu\text{g/ml}$ . Percent inhibition (y-axis) was calculated relative to rDBP II (11 ng/ml) binding to nDARCIg in the absence of Abs. Binding inhibition was performed in duplicate and percent mean blocking activity  $\pm$  SD is shown. The experiment was repeated once. (B) Human mAb recognition of DBP II (Sal I) subdomains (SD) expressed by phage display. Controls were murine mAb 3D10, which recognizes an epitope on SD1 (3D10 used for recognition of phages displaying for SD1 and SD1+2+3) and murine mAb 2H2, which recognizes an epitope on SD3 (2H2 used for recognition of SD2+3 and SD3 phages). The assay was performed in triplicate with co-efficient of variation  $<20\%$  for all the results.

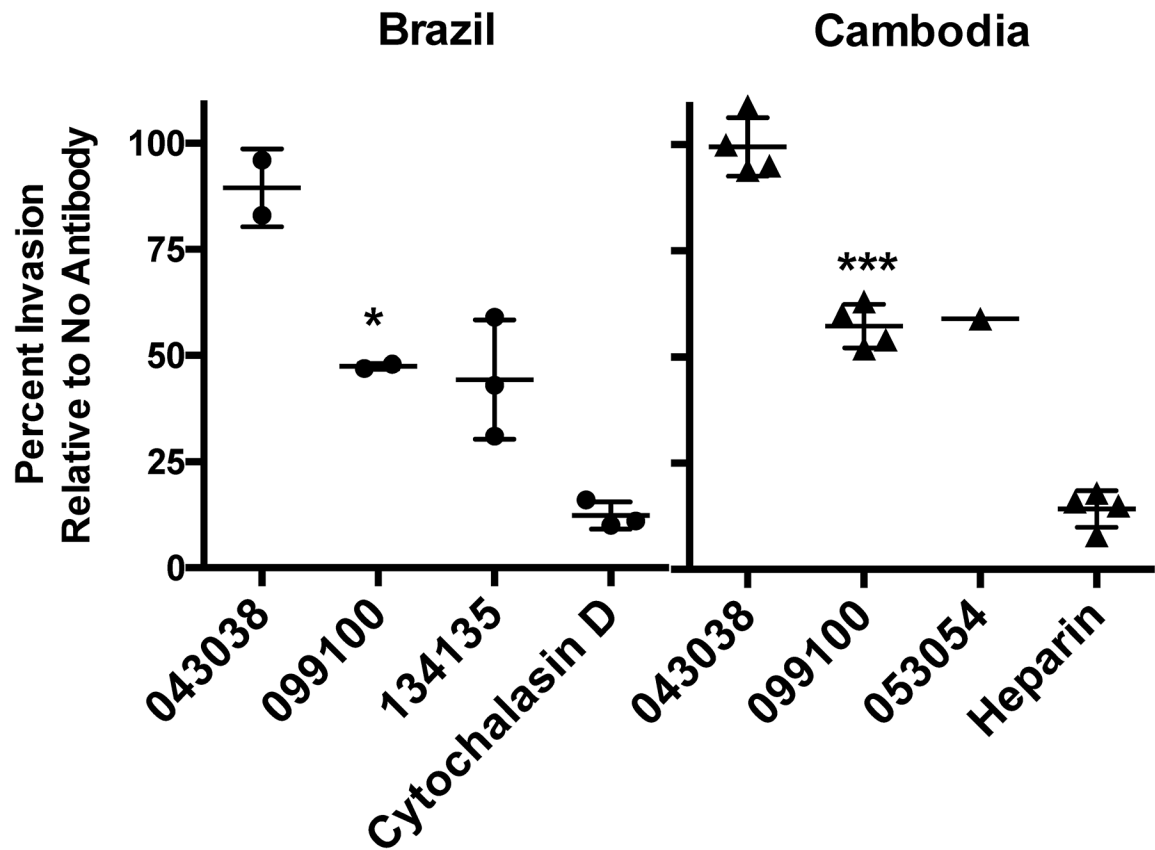


**Figure 10.**

Alanine replacement of the highly polymorphic and immunogenic DEK epitope did not affect binding of the mAbs. The assay was performed as described in Figure 9A, with the exception that the concentration of DEKnull was 22 ng/ml. All mAbs were used at a concentration of 30 ng/ml, and pooled North American plasma was used at a dilution of 1:2,000. The assay was performed in duplicate, with replicates <20% coefficient of variation. The experiment was repeated once with similar results.



**Figure 11.** Human mAbs compete with epitopes recognized by serum from individuals with high levels of DBPII-specific blocking activity ( 80%) residing in diverse Pv-endemic populations. The scatter plots show the relationship of the degree of blocking activity of serum assessed by rDBPII:nDARCIg assay (y-axis, and see Fig. 9 legend) to the extent to which the blocking mAb 099100 and the non-blocking mAb 087085 compete with naturally-acquired inhibitory Ab (x-axis). For example, the serum sample from PNG outlined in the small box shows 100% blocking activity in the nDARCIg assay at a dilution of 1:20, and 55% blocking of rDBPII to mAb 099100 (1:50 dilution). The box and whisker plots (median blocking activity, 25% and 75% interquartile ranges, and 95% confidence intervals) to the right for each country examine the degree to which mAbs 099100 and 087085 compete with serum blocking activity stratified as to whether participants had 80–100% blocking activity versus lower activity of 40–79%. The sample size for the two strata is shown in the scatter plots for each country. Difference in blocking activity was examined using Mann-Whitney U test.



**Figure 12.** MAbs inhibit Pv invasion of reticulocytes. Three experiments were performed in Brazil with 3 different clinical isolates. Four experiments were performed in Cambodia with 4 different clinical isolates. The percent invasion inhibition relative to no added Ab is shown. Median and 95% confidence intervals are shown. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared to 043038 (anti-TTCF) (Mann Whitney U test). Experimental details are provided in Methods.