Fy^a/Fy^b antigen polymorphism in human erythrocyte Duffy antigen affects susceptibility to *Plasmodium vivax* malaria

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Plasmodium vivax **(Pv) is a major cause of human malaria and is increasing in public health importance compared with falciparum malaria. Pv is unique among human malarias in that invasion of erythrocytes is almost solely dependent on the red cell's surface receptor, known as the Duffy blood-group antigen (Fy). Fy is an important minor blood-group antigen that has two immunologically distinct alleles, referred to as Fy^a or Fy^b, resulting from a single-point mutation. This mutation occurs within the binding domain of the parasite's red cell invasion ligand. Whether this polymorphism affects susceptibility to clinical vivax malaria is unknown. Here we show that Fy^a, compared with Fy^b, significantly diminishes binding of Pv Duffy binding protein (PvDBP) at the erythrocyte surface, and is associated with a reduced risk of clinical Pv in humans. Erythrocytes expressing Fy^a had 41–50% lower binding compared with Fy^b cells and showed an increased ability of naturally occurring or artificially induced antibodies to block binding of PvDBP to their surface. Individuals with the Fy^{a+b-} phenotype demonstrated a 30–80% reduced risk of clinical vivax, but not falciparum malaria in a prospective cohort study in the Brazilian Amazon. The Fy^{a+b-} phenotype, predominant in Southeast Asian and many American populations, would confer a selective advantage against vivax malaria. Our results also suggest that efficacy of a PvDBP-based vaccine may differ among populations with different Fy phenotypes.**

Duffy binding protein | resistance

The parasite *Plasmodium vivax* plays a major role in the overall burden of malaria, causing severe morbidity and death (1). At least 80 million individuals worldwide suffer from vivax malaria; indeed, it is the most widely distributed malarial species outside of sub-Saharan Africa (2). Global efforts to eliminate malaria, largely based on reducing transmission, have been considerably less effective with *P. vivax* than with *Plasmodium falciparum* (3, 4), in part because of the former's efficient transmission in diverse ecological settings and its ability to reinitiate blood-stage infection from a dormant liver hypnozoite phase (5). Thus, success at *P. vivax* elimination may depend more on developing vaccines to prevent infection and suppress re-emergent blood-stage parasites.

P. falciparum demonstrates capacity to invade erythrocytes through multiple receptor pathways (6). In contrast, *P. vivax* red cell invasion appears to be primarily dependent on the Duffy antigen (Fy) (7). Although Duffy-independent *P. vivax* infection and disease can occur (8), alternative invasion pathways are not understood. As detailed understanding of host and parasite genetic polymorphisms and immune response inhibition of receptor-ligand interaction is of critical importance for vaccine development, here we have investigated the relevance of the $Fy^a \rightarrow Fy^b$ antigen polymorphism on susceptibility to clinical *P. vivax* malaria.

The gene that encodes the Duffy antigen has two major polymorphisms. A Asp→Gly amino acid substitution (codon 42) in the N-terminal region is associated with the Fy^b and Fy^a blood-group antigens, respectively (Fig. 1*A*). The second polymorphism T→C transition at nucleotide −33 in the Duffy gene promoter ablates Duffy expression on erythrocytes (ES; erythrocyte silent). The blood group and expression phenotypes associated with these polymorphisms have been well characterized; nomenclature and biological properties of Duffy have been summarized previously (8, 9) (Table S1).

Because of the critical role played by the Duffy antigen in *P. vivax* erythrocyte invasion, the corresponding parasite ligand, the Duffy binding protein (PvDBP), which is expressed at the parasite's cellular surface upon invasion, is a major vaccine candidate (10). The binding domain of PvDBP to Fy has been indentified in a 330-aa cysteine-rich region referred to as region II, designated PvDBPII (11, 12). Naturally acquired and artificially induced antibodies to PvDBPII inhibit parasite invasion in vitro (13) and protect against clinical malaria in children (14), supporting PvDBPII as a leading vaccine candidate. The critical residues of Fy, to which PvDBPII binds, map to N-terminal region amino acids 8–42 (Fig. 1*A*) (15, 16). Given studies on non-human primates indicating that Fy^b is the ancestral allele (17, 18), we hypothesized that Fy^a decreased the efficiency of PvDBPII binding, thereby reducing susceptibility to *P. vivax* malaria. Indeed, cross sectional association studies performed in the Brazilian Amazon region suggested that individuals expressing the Fy^b compared with Fy^a antigen may be more susceptible to *P. vivax* infection (19). Additionally, prior studies showed that an orthologous protein expressed by the simian malaria parasite, *Plasmodium knowlesi*, which infects human erythrocytes in a Duffy-dependent manner, preferentially bound Fy^b- compared with Fy^a-expressing erythrocytes, both in vivo and in vitro (20).

Results

Fy^a/Fy^b Polymorphism Affects Binding of PvDBPII to Erythrocytes. To examine whether human erythrocytes expressing Fy^a showed differential binding of recombinant PvDBPII compared with those expressing Fy^b, we screened blood samples from a group of healthy North American volunteers. Using a PCR assay, individual Fy genotypes were established (Table S1). Samples were assayed for degree of recombinant PvDBPII binding to red cells using flow cytometry. We found 40–50% lower binding of

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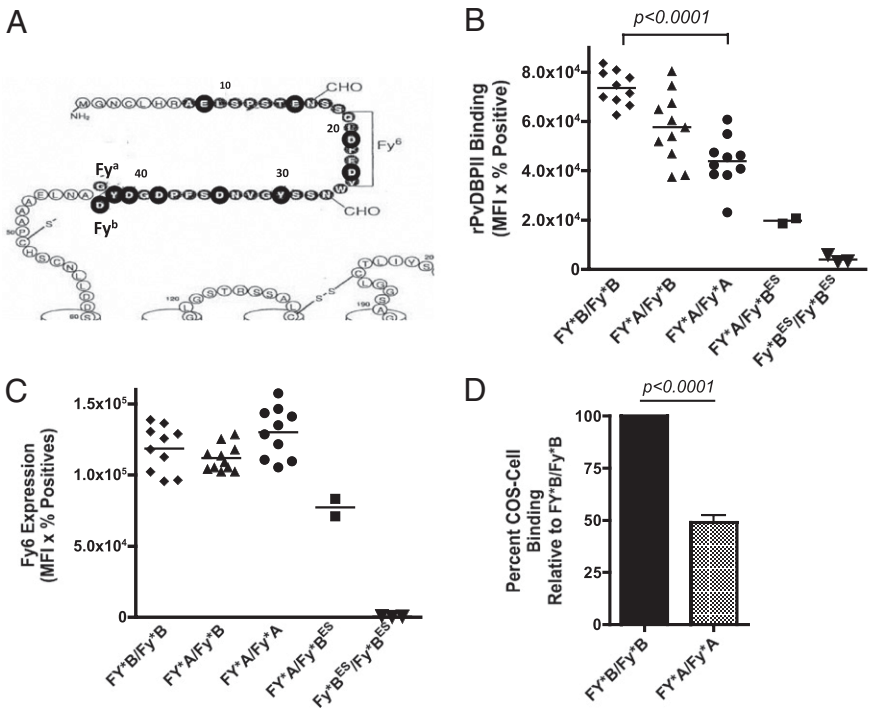

Fig. 1. Relationship of *FY* genotype on binding to PvDBP. (*A*) N-terminal binding domain (black residues) of PvDBP to Fy. Fy6 is a mAb and the corresponding epitope. $Fy^a \rightarrow Fy^b$ is the only polymorphism in the N-terminal region. Red highlights indicate negatively charged amino acids; the overall pI is 3.4 for the N-terminal region. In contrast, the binding domain of PvDBPII [consisting of 330 aa (32)] has a pI of 9.6. (*B*) Flow cytometry assessment of recombinant PvDBPII binding levels to erythrocytes from people differing by Duffy genotype. (*C*) Level of Duffy expression using mAb Fy6. (*D*) Relative binding of PvDBPII expressing COS-cells to erythrocytes of *FY*A/FY*A* ($n = 12$) vs. *FY*B/FY*B* ($n = 12$, $P < 0.0001$) blood donors; combines results of three separate experiments. For example, the mean number of rosettes per 30 high-powered field was 83 ± 11 for *FY*B/FY*B* erythrocytes compared with 46 ± 5 for *FY*A/FY*A* ($P = 0.007$) for one experiment (Fig. S1).

PvDBPII (0.2 μg per 10^6 red cells) (Fig. S2) to erythrocytes from *FY*A/FY*A* (i.e., phenotypically Fy^{a+b-}) compared with *FY*B/FY*B* (i.e., phenotypically Fy^{a-b+}) blood donors (Fig. 1*B*) ($P < 0.0001$). Erythrocytes from *FY*A/FY*B* donors displayed intermediate binding (Fy^{a+b+}). Our observed differences in PvDBPII binding could not be attributed to levels of Fy expression, which were similar for *FY*B/FY**B, *FY*A/FY*A*, and *FY*A/FY*B* genotypes (Fig. 1*C*). *FY*A/FY*B*^{ES} cells expressed approximately half the levels of Fy compared with *FY*A/FY*A* cells; as expected, their binding was significantly reduced compared with cells from corresponding *FY*A* homozygotes. Duffy-negative erythrocytes (*FY*B*^{ES}*/FY*B*^{ES}) failed to express Fy and did not bind PvDBPII. We also performed erythrocyte rosetting assays, where PvDBPII was surface-expressed on COS cells. In these studies, cells from *FY*A/FY*A* donors bound COS cells at a 50% lower level compared with erythrocytes from *FY*B/FY*B* donors (Fig. 2*D* and Fig. S2).

To investigate mechanisms responsible for the differential binding, we looked at differences in electrostatic charge, as well as tyrosine (Tyr) sulfation between Fy^a and Fy^b (Fig. 1*A*). The less-efficient parasite binding of Fy^a may be a result of the charge neutrality of Gly42 (replacing Asp42), because the N-terminal region of Fy is negatively charged but PvDBPII is positively charged.

Prior studies have shown that the degree of sulfation of Tyr41 markedly affected binding of PvDBPII to Fy (21). Although Choe et al. observed no substantial difference in sulfation of their Fy^a vs. Fy^b constructs [60 codons Duffy amino terminus joined to human IgG1 Fc domain (20)], no data were provided to compare PvDBPII interaction with their constructs corresponding to Fy^a vs. Fy^b or native Fy^a vs. Fy^b antigens on the red cell surface. To make these comparisons, we treated erythrocytes with the enzyme arylsulfatase, which selectively and partially removes sulfate groups from Tyr (Fig. S2) (22). Interestingly, enzymatic treatment of *FY*A/FY*A* erythrocytes reduced PvDBPII binding by 42% (Fig. 2*A*) ($P = 0.0004$), but had no effect on PvDBPII binding to *FY*B/FY*B* erythrocytes (Fig. 2*C*). Enzymatic treatment did not affect quantitative expression of Fy on erythrocytes (Fig. 2 *C* and *D*, and Fig. S2*B*). Arylsulfatase concentrations that would result in complete removal of sulfate groups could not be used because it caused erythrocyte lysis. These results suggest that Fy^a may be more susceptible to loss of sulfate groups from tyrosines compared with Fy^b.

Binding Inhibitory Antibodies Show Greater Blocking of PvDBPII to Fy^a- Compared with Fy^b-Expressing Erythrocytes. Previous studies have shown that PvDBPII-specific antibodies inhibit *P. vivax* erythrocyte invasion in vitro (13) and correlate with protection against blood-stage infection in vivo (14). To determine whether antibodies that inhibit *P. vivax* invasion would bind differentially to Fy^a- vs. Fy^b-expressing cells, a binding-competition assay was performed using antibodies directed against PvDBPII. We found that similar concentrations of either naturally acquired (Fig. 3 *A* and *B*) or artificially induced inhibitory antibodies (Fig. 3 *C* and *D*) effected 200–300% greater inhibition of PvDBPII binding to erythrocytes from *FY*A/FY*A* compared with *FY*B/FY*B* donors. For these experiments, PvDBPII-specific antibodies were affinity-purified from human and rabbit sera. Therefore, the concentration of these PvDBPII antibodies would be higher than in circulating blood. Of note, antibody preparations were affinity-purified to enrich for antibodies directed to PvDBPII, and thus antibody concentrations used are unlikely to represent circulating antibody levels in individuals. Overall, these results suggest that binding inhibitory antibodies directed against PvDBPII may

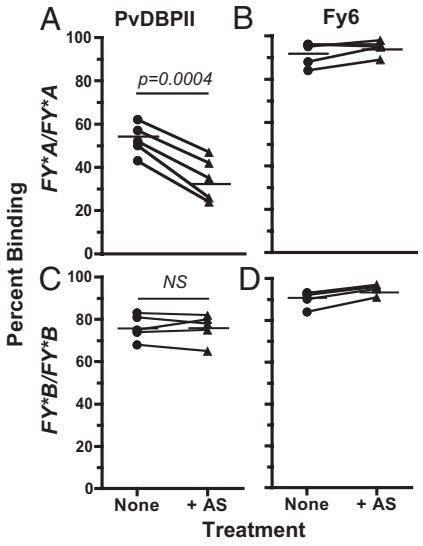

Fig. 2. Effect of arysulfatase treatment on binding of PvDBPII to erythrocytes from blood donors with different *FY* genotypes. (*A*) Treatment of erythrocytes from *FY*A/FY*A* donors selectively and partially removes sulfonate groups from tyrosine residues (Fig. S3) and reduced binding by PvDBPII. (*C*) Identical treatment of erythrocytes from *FY*B/FY*B* donors did not reduce PvDBPII binding. (*B* and *D*) Enzyme treatment of erythrocytes does not affect Fy expression on erythrocytes. Symbols (circle to triangle) paired by lines compare red blood cells from each individual before and after treatment with 500 million units of arylsulfatase.

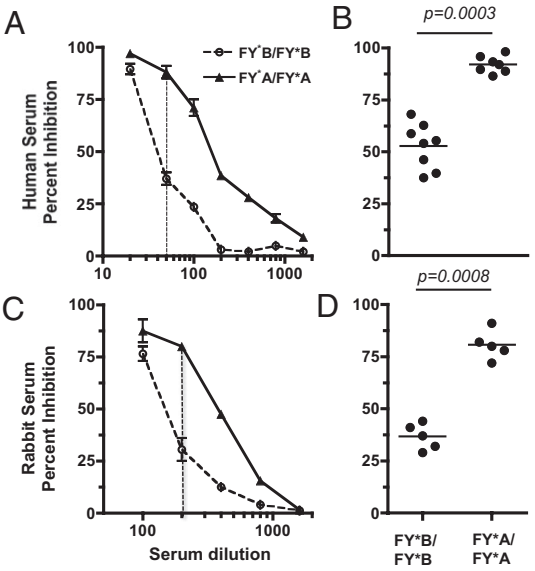

Fig. 3. Fy^a/Fy^b polymorphism affects binding inhibitory antibody blocking activity of recombinant PvDBPII to erythrocytes. (*A*) Inhibition of PvDBPII by different serum dilutions of pooled affinity-purified human binding inhibitory Abs of PvDBPII binding to erythrocytes from an *FY*A/FY*A* compared with an *FY*B/FY*B* blood donor. (*B*) Binding inhibitory Abs from human serum (1:50 dilution) show consistently greater blocking of PvDBPII binding to erythrocytes from *FY*A/FY*A* (n = 7) compared with *FY*B/FY*B* (n = 8) blood donors. (*C* and *D*) Affinity-purified rabbit serum generated against PvDBPII also shows a greater capacity to inhibit binding of PvDBPII to erythrocytes from *FY*A/FY*A* (n = 5) compared with the *FY*B/FY*B* (n = 5) individuals at a serum dilution of 1:200. Each datapoint (*A* and *C*) represents mean (± SD) percent inhibition for individuals with Fy^a vs. Fy^b. Each dot (*B* and *D*) represents means of duplicate or triplicate binding assays for one donor. Statistical comparison uses a Student t test.

inhibit more efficiently when *P. vivax* is accessing the red blood cell through contact with Fy^a compared with Fy^b.

Fy^a/Fy^b Polymorphism Is Associated with Reduced Risk to Clinical *P. vivax*. We then sought to determine if our in vitro findings associated with *FY* genotype correlated with in vivo susceptibility to uncomplicated clinical *P. vivax* malaria. For this study, we analyzed data from 400 individuals (5–74 y of age) living in a *P. vivax*-endemic region of the Brazilian Amazon studied previously (Table S2). All individuals were actively followed for clinical malaria over 14 mo, as determined by blood-smear microscopy and PCR-confirmation during a time when there was a surge in malaria infection (23). Overall, 124 cases of *P. vivax* and 66 cases of *P. falciparum* malaria were diagnosed, with annual incidence rates of 0.31 and 0.17, respectively. Mixed infection was found in 31 cases.

Prior analysis of the complete dataset suggested that duration of residence in the community and distance from the Iquiri River influenced risk for malaria (23). Here we observed a slight reduction in risk for *P. vivax* malaria, with longer duration of residence in the transmission area [risk ratio 0.94, 95% confidence interval (CI) 0.91–0.98, P = 0.01, negative binomial analysis], consistent with development of acquired immunity. Individuals who lived in the high-transmission area close to the river were at increased risk of *P. vivax* malaria (risk ratio 3.24, 95% CI 1.60–6.05, P = 0.001). Similar risk ratios for development of *P. falciparum* malaria were associated with time and location (risk ratio 2.89, 95% CI 1.72–5.67, $P < 0.001$). There was no significant association for *FY* genotype and prevalence of individuals living in high vs. low transmission areas, nor for *FY* genotype and duration of residence (Table S2) (Pearson's χ^2 test, P = 0.12).

Individuals with *FY*A/FY*B*^{ES} and *FY*A/FY*A* genotypes showed the lowest incidence of clinical *P. vivax* (Fig. 4). Negative binominal analysis adjusting for duration of local residence and transmission areas showed respectively, 80% and 29% reduced risk of clinical vivax malaria for individuals with *FY*A/FY*B*^{ES} and *FY*A/FY*A* compared with the *FY*A/FY*B* genotype (Table 1). In contrast, individuals with *FY*B/FY*B*^{ES} and *FY*B/FY*B* genotypes had 220–270% greater risk of vivax malaria compared with the *FY*A/FY*B* genotype (Fig. 3*B*). Interestingly, we observed no significant difference in the percent of subjects with antibody responses against PvDBPII in association with *FY* genotype (see Table 1 for sample sizes, *FY*A/FY*B*^{ES} 16.7%, *FY*A/FY*A* 11.9%, *FY*A/FY*B* 24.4%, *FY*B/FY*B*^{ES} 21.4%, *FY*B/FY*B* 11.3%). Results show a consistent decreased susceptibility

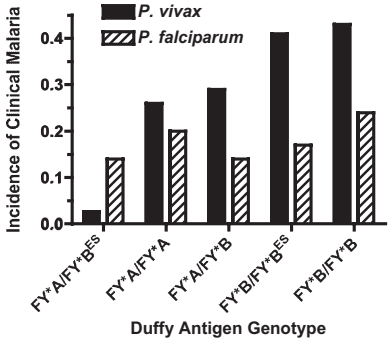

Fig. 4. Effect of *FY* genotype on the unadjusted annual incidence of clinical *P. vivax* (black bars) and *P. falciparum* malaria (striped bars). Incidence is expressed as mean number of clinical episodes per person-years of follow-up. Using a negative binomial analysis, the overall effect was highly significant, $P < 0.001$. The adjusted effect of specific genotype on *P. vivax* risk is shown in Table 1.

Table 1. Effect of Different *FY* genotype on risk of clinical vivax malaria

Genotype	*n*	Risk ratios (95% CI)	*P*
*FY*A/FY*B^{ES}*	35	0.204 (0.09–0.87)	**0.005**
*FY*A/FY*A*	52	0.715 (0.31–1.21)	0.06
*FY*A/FY*B*	140	Comparator	
*FY*B/FY*B^{ES}*	76	2.17 (0.91–4.77)	0.09
*FY*B/FY*B*	87	2.70 (1.36–5.49)	**0.002**

Rate ratios are adjusted for location and duration of residence in endemic area using *FY*A/FY*B* as the comparator groups based on a negative binomial analysis. Boldface indicates statistical significance.

to vivax malaria associated with Fy^{a} and increased susceptibility to vivax malaria with increased expression of Fy^{b}. There was no association between *FY* genotype and risk for *P. falciparum* in the multivariate analysis (overall risk ratio 1.08, 95% CI 0.87–2.38, $P = 0.42$). The analysis was repeated excluding individuals with mixed *P. vivax* and *P. falciparum* infections; *FY*A/FY*B^{ES}* and *FY*A/FY*A* had risk ratios of 0.197 (CI 0.07–0.98), $P = 0.03$ and 0.684 (CI 0.28–1.45), $P = 0.09$ compared with the *FY*A/FY*B* genotype. These results imply that differences in binding of PvDBPII to Fy^{a} compared with Fy^{b} translate to decreased *P. vivax* erythrocyte invasion efficiency, and lower parasitemias in Fy^{a+b-} compared with Fy^{a-b+} individuals. It should be noted that parasitemia levels were not specifically determined in this study.

Geographical Distribution of the Duffy Allelle Frequencies. The global distribution of the three major FY alleles, *FY*A*, *FY*B*, and *FY*B^{ES}*, are shown in Fig. 5. This map was derived from a suite of allele-frequency maps assembled from a database of Duffy blood-typing surveys from 1950 to 2010 (24) (Table S3). The *FY*A* allele appears to be advancing to fixation in many Asian populations. In contrast, *FY*B^{ES}* has followed a different pattern and has achieved fixation in Africa. The *FY*B* allele predominates in European populations. Admixture of *FY*A* and *FY*B* usually occurs in populations from relatively recent European migrations into North Africa and the Americas following the much early migration of populations from Asia.

Discussion

Our study demonstrates that PvDBPII binding is significantly lower to Fy^{a} than the ancestral Fy^{b} antigen (Fig. 1*B*). Concordantly, we found that Fy^{a} was associated with protection, but Fy^{b} was associated with increased infection and disease (Table 1). Although *P. vivax* parasitemia has been shown to correlate with risk of clinical vivax malaria (25), we did not evaluate the relationship of the *FY* genotype with parasitemia, as this feature of infection was not recorded in the Brazilian longitudinal study. Despite our recent findings that *P. vivax* is able to infect human red cells through a Duffy-independent mechanism (8), it is well known that Duffy-negativity (*FY*B^{ES}/FY*B^{ES}*) is responsible for high-level resistance to *P. vivax* blood-stage infection (7), suggesting that this parasite's invasion mechanism is heavily reliant on access to Fy. Given the burden of illness and death associated with vivax malaria (1), and proposals that *P. vivax* originated in Asia following lateral transfers of simian parasites from Old World monkeys (26), the emergence of a major vivax-resistance allele in African populations alone (*FY*B^{ES}*) is puzzling. Therefore, we hypothesize that *FY*A* has also been positively selected to reduce efficiency of *P. vivax* red cell invasion to improve human fitness to *P. vivax* malaria. The observation that *FY*A* has advanced to fixation in many Asian and American populations where vivax malaria is most highly endemic (Fig. 5 and *SI Methods*) supports this conclusion. Although historical record of vivax malaria in Africa is scanty, *FY*B^{ES}* predominates in most African ethnicities and holds vivax malaria at very low prevalence.

Prior studies investigating Fy and other red cell surface proteins as receptors for *P. knowlesi*/*P. vivax* red cell invasion have reported results that both support and vary with our present findings. Although evidence is limited, in vitro infection studies by Miller et al. suggested that *P. knowlesi* displayed lower efficiency in infecting human Fy^{a+b-} compared with Fy^{a-b+} red cells (27). Additional studies did not compare parasite invasion between Fy^{a+b-} and Fy^{a-b+} red cells directly (20, 28, 29) but did

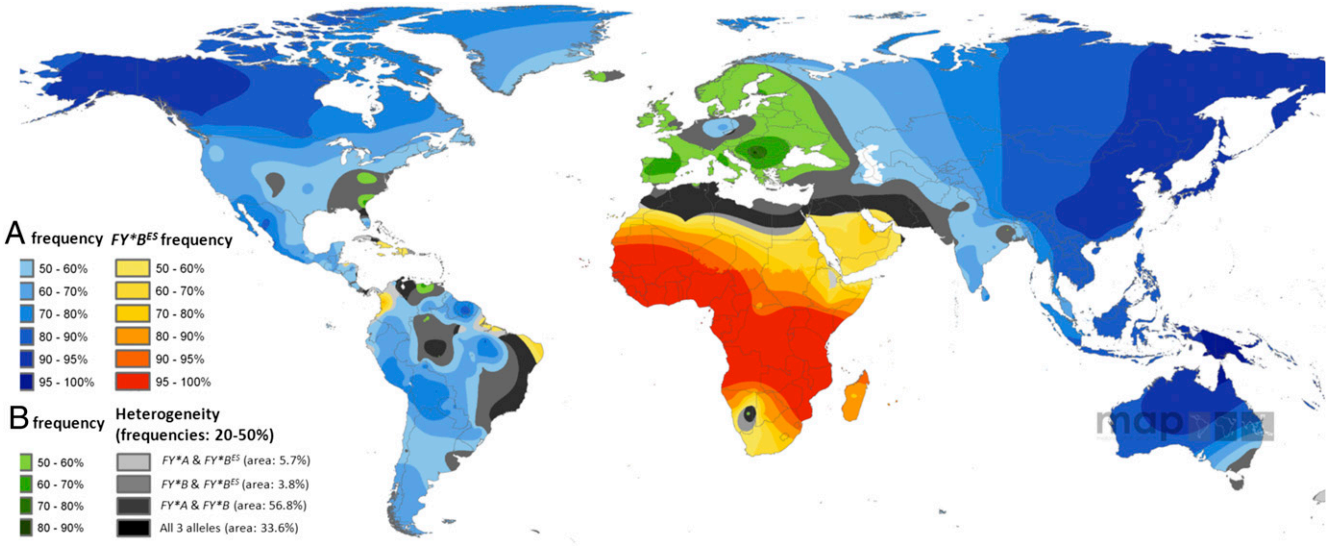

Fig. 5. Global frequencies of the *FY* alleles. Areas predominated by a single allele (frequency ≥ 50%) are represented by a color gradient (blue, *FY*A*; green, *FY*B*; red/yellow, *FY*B^{ES}*). Areas of allelic heterogeneity where no single allele predominates, but two or more alleles each have frequencies ≥ 20%, are shown in gray-scale: palest for heterogeneity between the silent *FY*B^{ES}* allele and either *FY*A* or *FY*B* (when coinherited, these do not generate new phenotypes), and darkest being co-occurrence of all three alleles (and correspondingly the greatest genotypic and phenotypic diversity). Overall percentage surface area of each class is listed in the legend. Refer to *SI Methods* for a methodological summary and further detail about the map surface.

compare binding of parasite proteins to human Fy^{a+b-} and Fy^{a-b+} red cells. Haynes et al. showed that a 135-kDa protein from *P. knowlesi* culture supernatants bound to Fy^{a-b+} erythrocytes much better than to Fy^{a+b-} cells (20). Similar studies found that a 135–140 "native" protein corresponding to the full-length Duffy binding protein from *P. vivax* culture supernatants showed similar binding to Fy^{a+b-} vs. Fy^{a-b+} red cells (30). A subsequent study using the same recombinant PvDBPII construct as the current study and a similar flow cytometry erythrocyte-binding assay showed no significant difference in PvDBPII binding to Fy^{a+b-} vs. Fy^{a-b+} erythrocytes (31). Experiments in this latter study used PvDBPII at concentrations 50-fold higher that those shown here that reveal preferential binding to Fy^{a-b+} vs. Fy^{a+b-} erythrocytes. High-concentration PvDBPII obscure differential binding to Fy^{a-b+} vs. Fy^{a+b-} erythrocytes (Fig. S1). Overall differences and similarities suggest that outcomes of in vitro studies may be sensitive to variation in parasite species/strains and their parasitemias, as well as differences in antigen polymorphism and concentration.

Results from our in vitro studies were consistent with our in vivo observations, suggesting a relationship between Fy^a compared with Fy^b expressed on erythrocytes with the amounts of PvDBPII erythrocyte binding in vitro and further susceptibility to clinical vivax malaria in vivo. Individuals who were Fy^{a+b-} (particularly the $FY*A/FY*B^{ES}$ genotype) had the lowest binding of PvDBPII to their erythrocytes in vitro and the greatest resistance to vivax malaria. Those who were Fy^{a-b+} displayed the highest binding to PvDBPII and the greatest sensitivity to clinical vivax malaria. Interestingly, even though erythrocytes from $FY*B/FY*B^{ES}$ donors express approximately half the amount of Duffy antigen (Table S1) compared with $FY*A/FY*A$ and $FY*A/FY*B$, they were more susceptible to *P. vivax* malaria than the $FY*A/FY*A$ and $FY*A/FY*B$ genotypes (Table 1). The reasons for the relationships between these in vitro and in vivo results are unclear, and may be related to cohort sample size. However, our in vivo findings are consistent with a significant protective effect of the $FY*A$ allele.

The mechanism by which Fy^a-expressing red cells show reduced binding to PvDBPII and reduced susceptibility to vivax malaria needs to be fully elucidated. As the N-terminal region of Fy is negatively charged and PvDBPII is positive, less efficient parasite binding to Fy^a may be because of the electrostatic neutrality of Gly42 (pI = 6) vs. negatively charged Asp42 (pI = 3.1). Additionally, because sulfation of Fy appears to influence PvDBPII binding, observed increased lability of PvDBPII binding to Fy^{a+b-} vs. Fy^{a-b+} following arylsulfatase treatment of donor cells suggests that *P. vivax* red cell invasion efficiency may be susceptible to differences in Fy sulfation.

In conclusion, our observations related to *P. vivax* interaction with Fy^a vs. Fy^b and subsequent development of naturally acquired immunity has important implications for vaccine trials using PvDBPII. In vitro studies demonstrate that both naturally acquired and artificially induced antibodies block erythrocyte binding of recombinant PvDBPII to Fy^a- better than Fy^b-expressing erythrocytes. Although we observed that the FY genotype is not associated with any significant differences in PvDBPII-specific antibody responses, our results suggest that naturally acquired immunity to *P. vivax* infection and disease may be more effective in populations where the $FY*A$ allele predominates. Additionally, our findings indicate that it will be important to test PvDBPII-based vaccine in populations that carry combinations of both $FY*A$ and $FY*B$ alleles (Fig. 5).

Methods

Detailed information is provided in *SI Methods*.

Participants. Erythrocytes for the binding experiments were obtained from malaria uninfected volunteers at Case Western Reserve University. Malaria-exposed subjects were recruited as part of longitudinal cohort study performed in the Brazilian Amazon in 2004–2005, as previously described (23). All work with human samples was performed in accordance with approved Institutional Review Board protocols of the Veterans Affairs Medical Center, Cleveland, OH, University Hospitals, Cleveland, OH, and Ethical Review Board of the Institute of Biomedical Sciences of the University of São Paulo, Brazil.

Binding Experiments. Binding experiments with recombinant Pv Duffy Binding protein and Fy6 mAb that recognizes N-terminal region of Duffy antigen used fresh human erythrocytes from subjects previously genotyped for Duffy and were performed as previously described (13). Binding inhibition levels by anti-PvDBPII were assessed as previously described (13).

Statistical Analysis. A negative binomial regression analysis (SAS version 9.2; SAS Institute) was used because of the overdispersion of the data. Sample size was 400, input risk variables were Duffy genotype, location, and duration of residence in the study area with the primary output being risk of clinical *P. vivax*. There was little interaction between Duffy genotype and location and duration of residence (Table S2).

Mapping Duffy Genotypes. The probability distribution based on a Bayesian model is summarized as a single statistic: in this case, the median value, as this corresponds best to the input dataset, as previously described (24). Median values of the predictions were generated for each allele frequency at a 10 × 10-km resolution on a global grid with GIS software (ArcMap 9.3; ESRI).

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