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Role of *Plasmodium vivax* Duffy-binding protein 1 in invasion of Duffy-null Africans

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The ability of the malaria parasite *Plasmodium vivax* to invade erythrocytes is dependent on the expression of the Duffy blood group antigen on erythrocytes. Consequently, Africans who are null for the Duffy antigen are not susceptible to *P. vivax* infections. Recently, *P. vivax* infections in Duffy-null Africans have been documented, raising the possibility that *P. vivax*, a virulent pathogen in other parts of the world, may expand malarial disease in Africa. *P. vivax* binds the Duffy blood group antigen through its Duffy-binding protein 1 (DBP1). To determine if mutations in DBP1 resulted in the ability of *P. vivax* to bind Duffy-null erythrocytes, we analyzed *P. vivax* parasites obtained from two Duffy-null individuals living in Ethiopia where Duffy-null and -positive Africans live side-by-side. We determined that, although the DBP1s from these parasites contained unique sequences, they failed to bind Duffy-null erythrocytes, indicating that mutations in DBP1 did not account for the ability of *P. vivax* to infect Duffy-null Africans. However, an unusual DNA expansion of DBP1 (three and eight copies) in the two Duffy-null *P. vivax* infections suggests that an expansion of DBP1 may have been selected to allow low-affinity binding to another receptor on Duffy-null erythrocytes. Indeed, we show that Salvador (Sal)1 *P. vivax* infects Squirrel monkeys independently of DBP1 binding to Squirrel monkey erythrocytes. We conclude that *P. vivax* Sal I and perhaps *P. vivax* in Duffy-null patients may have adapted to use new ligand-receptor pairs for invasion.

*Plasmodium vivax* | Duffy blood group antigen | Duffy-binding protein | DNA expansion

In 1975 we identified the failure of *Plasmodium knowlesi* to invade Duffy-null erythrocytes (1). We assumed that the Duffy blood group null phenotype, a common African phenotype, conferred resistance in Africans to *P. vivax*, a Plasmodium closely related to *P. knowlesi*. Subsequent studies demonstrated that African American volunteers who were Duffy-null were resistant to mosquito-transmitted *P. vivax* (2). In addition, African American soldiers in Vietnam who were infected with *P. vivax* were all Duffy-positive (3). Furthermore, African Americans in a village in Honduras who were infected with *P. vivax* were all Duffy-positive whereas those infected with *P. falciparum* were both Duffy-null and -positive (4). We concluded that Duffy null was the basis of resistance to *P. vivax* by Africans.

The molecular basis of Duffy null was a single point mutation in the GATA1-binding sequence in the promoter region 5′ to the Duffy blood group ORF (Fig. 1B) that led to Duffy-blood-group-null erythrocytes (5). Subsequent studies with *P. knowlesi* demonstrated that the invasive merozoites were able to bind and reorient apically with Duffy-null erythrocytes but could not form a junction as occurred in Duffy-positive erythrocytes, indicating that the Duffy blood group was required for *P. vivax* invasion (6). Later, in *P. knowlesi* parasite culture supernatants, the parasite ligand binding to Duffy blood group antigen was identified as the Duffy-binding protein 1 (DBP1) (7). Subsequently, *P. vivax* DBP1 was identified by its ability to bind to Duffy-positive but not to Duffy-null erythrocytes (8). Furthermore the domain within DBP1 that conferred Duffy binding was determined to be the cysteine-rich region 2 of DBP1 (9, 10). Later, Duffy blood group antigen was shown to bind DBP1 through a sulfated tyrosine in its first extracellular domain (Fig. S1) (11).

Recently, Duffy-null individuals were found to be infected with *P. vivax*, both throughout Africa (Kenya, Madagascar, Mauritania, Cameroon, Angola, Equatorial Guinea, Ethiopia, and Sudan) and in South America (12–21), raising the question: How can *P. vivax* invade in the absence of Duffy blood group expression? Is it possible that mutations in the cysteine-rich region 2 of the *P. vivax* DBP1 allows binding to another protein on the surface of Duffy-null erythrocytes, unrelated to the Duffy? Alternatively, the widespread duplication of the gene encoding DBP1 observed in Madagascar (14, 22) and the three and eight copies of DBP1 in the two Duffy-null *P. vivax*-infected patients in Ethiopia (present paper) may allow for low-affinity binding of DBP1 to a new receptor on Duffy-null erythrocytes.

We tested the binding of *P. vivax* DBP1 in two Duffy-null individuals living in Ethiopia to Duffy-positive and -null erythrocytes and provide evidence that the former possibility was not the case; these DBP1s did not bind Duffy-null erythrocytes. However, concerning the latter possibility, we also provide evidence for DBP1 and DBP2/Erythrocyte-Binding Protein (EBP) (23) independent infection of Squirrel monkeys by *P. vivax* Salvador (Sal) I.

**Significance**

Duffy-null Africans were thought to be resistant to *Plasmodium vivax* infection. Recently, *P. vivax* infection was observed in Duffy-null Africans. This parasite adaptation is potentially a serious public health problem as the majority of African populations are Duffy-null. This article is aimed at understanding whether mutations or DNA expansion in Duffy-binding protein (DBP) contributes to *P. vivax* Duffy-null infection. Importantly, *P. vivax* infection in Squirrel monkey has an ability to use an invasion pathway that is independent of the DBPs. Thus, *P. vivax* may use a different ligand-receptor pair for its infection in Duffy-null Africans, or some Duffy-negative Africans are not null but express a low level of Duffy blood group antigen.


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Results

**P. vivax DBP1 Polymorphisms in Binding to Duffy-Null Erythrocytes.**

Our study areas in Ethiopia comprise 65% (129 of 200) Duffy-positive and 35% (71 of 200) Duffy-null individuals. The *P. vivax* infections in Duffy-null Ethiopians is less severe than in Duffy-positive Ethiopians (20) as was observed in Madagascar (14). From the Ethiopian sample collection, we identified two Duffy-null individuals infected with *P. vivax*. The blood film from one of the Duffy-null individuals with *P. vivax* shows ring-stage parasites confirming the blood-stage infection in this patient (Fig. 1A). These two Duffy-null individuals had homozygous mutation (C/C) for Duffy-null in the GATA1-binding region upstream of the ORF (Fig. 1B) and had the ribosomal sequence for *P. vivax* and not for *P. falciparum* or *P. ovale* (Fig. 1C and D).

The next set of data was the sequence of the *P. vivax* DBP1 in Duffy-blood-group-null Ethiopians. DBP1s in the two Duffy-null patients were different (Fig. 2A). The two sequences in pRE4 expressed on the COS-7 cell surface did not bind Duffy-null erythrocytes but did bind to Duffy-positive erythrocytes (Fig. 2B and C). Despite a unique sequence for the DBP1 from the Duffy-positive Ethiopian sample, the region expressed in pRE4 bound strongly to Duffy-positive erythrocytes, but did not bind to Duffy-null erythrocytes (Fig. 2B and C).

We tested the *P. vivax* sequence from Madagascar that was found in Duffy-positive and heterozygote Duffy positive/null individuals (Fig. 2A) (22). The DBP1 from Madagascar was duplicated, and both sequences were identical in the DBP1 domain except for a single amino acid difference in the signal sequence. The Madagascar DBP1 region 2 inserted into pRE4 and expressed on COS-7 cells bound Duffy-positive erythrocytes but failed to bind Duffy-null erythrocytes (Fig. 2B and C).

The DBP1 domains were highly mutated in India VII and Brazil I compared with the original *P. vivax* Sal I sequence. In addition to the mutations, the codon for leucine was inserted in each isolate. The blood film from one of the Duffy-null patients was confirmed by PCR (22). A recent publication describes the gene copy number variation of DBP1 in *P. vivax* from Western Thailand (2 and 3 copies), Western Cambodia (2 copies), Papua Indonesia (2 and 3 copies) (24). In our study, to minimize potential issues with primer binding because of different b

**Copy Number Expansion of DBP1 in *P. vivax*-Infected Duffy-Null Patients.** Recently, the whole-genome sequencing of *P. vivax* from a Duffy-positive patient in Madagascar has revealed copy number expansion of Duffy-binding protein 1, and the existence of these two DBP1 copies next to each other was confirmed by PCR (22). A recent publication describes the gene copy number variation of DBP1 in *P. vivax* from Western Thailand (2 and 3 copies), Western Cambodia (2 copies), Papua Indonesia (2 and 3 copies) (24). In our study, to minimize potential issues with primer binding because of different boundaries and heterogeneity of DBP1 duplication lengths, we performed quantitative real-time PCR targeted to DBP1 and found three and eight copies in the two Duffy-null samples from Ethiopia (Fig. 3) compared with the control samples: one copy of PvDBP1 in Sal I (25); one and two copies of PvDBP1 in Cambodia by whole-genome sequencing (24); and one copy of aldolase from Sal I. The sample was negative in the no-DNA control. In addition, the sequences from each DBP1 from the two Duffy-null patients were identical in all of the expanded copies
(sequenced in 32 and 36 DBP1 clones from the two Duffy-null patients).

**DBP2 of *P. vivax* Structurally Related to DBP1 Binds Duffy-Null Erythrocytes.** Recently, a new ligand, called EBP/DBP2, was identified in a Cambodian isolate and in other African isolates (23). This protein shares a domain structure similar to DBP1. We expressed the protein in COS-7 cells and found that region II of DBP2 binds both Duffy-positive and Duffy-null erythrocytes at low frequency (Fig. 2B and C). EBP/DBP2 may be a ligand for invasion of Duffy-null erythrocytes.

**Invasion of Squirrel Monkey Erythrocytes Is Independent of DBP1 and DBP2.** It was previously shown that *P. vivax* infects Squirrel monkeys despite the failure of DBP1 to bind Squirrel erythrocytes (8, 26, 27). The Duffy blood group of Squirrel monkeys has the GATA1-binding domain that expresses the Duffy blood group, and previous studies have shown that anti-Fy6, an antibody to a
domain upstream of the Duffy-binding region, binds Squirrel monkey erythrocytes (28) (Fig. 1B and Fig. S1). It is surprising to know that, despite the high similarity between the Duffy blood group antigens from Squirrel monkeys, Aotus monkeys, and humans (Fig. S1), Sal I P. vivax DBP1 does not bind Squirrel monkey erythrocytes (Fig. 4A and B). Furthermore, we tested DBP1 from different P. vivax isolates (India VII and Brazil I) and found that DBP1 from India VII and Brazil I formed rosettes with Squirrel monkey erythrocytes at the same frequency as Aotus erythrocytes (Fig. 4A and B). No duplication of DBP1 occurred in the P. vivax Sal I (Fig. 3) (25). DBP2, found in the subtelomeric region of chromosome 2, was deleted in P. vivax Sal I.

**Discussion**

Despite different mutations in DBP1 in two Duffy-null Africans in Ethiopia and in Duffy-positive P. vivax infection in different parts of the world (Madagascar, India VII, Brazil I, and Sal I), none can bind Duffy-null erythrocytes when the variable sequences are expressed on COS-7 cells, although all bind Duffy-positive cells (Fig. 2). The basis of DBP1 DNA expansion in Ethiopia is unknown. Studies on DNA expansion in Plasmodium spp. identify different mechanisms. The simplest to understand is the type where the duplication allows for mutations in the second copy and as a result develops a different function. The six-cysteine in the P. falciparum family often has duplications, and the duplicates vary in sequence. For example, Pfs 48/45 is involved in bringing male and female gametes together to assist in fertilization, and its duplicate, Pf47, permits the ookinetes to pass through the mosquito midgut epithelial cells without being marked for destruction by nitrosylation (29). Another example is the duplication of CLAG3.1 and 3.2 with the worldwide isolates having the identical seven-amino-acid differences between CLAG 3.1 and 3.2 (30). CLAG 3.1/3.2 have a critical function in Plasmodium spp. in that they form a part of a transporter in the erythrocyte membrane (31). Again there is evidence that they may differ in function from the mutational differences between the copies (32).

The second type of DNA expansion that may be relevant to our finding involves selective pressure such that the gene protects the parasite by increasing its expression by DNA expansion. The DNA expansion can involve multiple genes around the selected gene. One example of this is the introduction of an inhibitor for P. falciparum dihydroorotate dehydrogenase (DHODH) that leads to DNA expansion around the gene and always includes a stretch of A’s (nucleotides) on each end of the expansion (33). Similar gene
duplication also occurred in the _P. falciparum_ Multi Drug Resistance 1 (MDR1) gene under pressure from mefloquine (34, 35). In another human infecting parasite _Plasmodium knowlesi_, duplication of DBPrt, the Duffy blood group binding protein, occurred when the parasite was adapted to grow in human erythrocytes (36).

The data are limited on the phenotypic significance of increased DBP1 gene copy numbers in Ethiopia. However, we speculate that the increase in copy number may lead to increased messenger RNA and protein levels. The DNA expansion may facilitate binding to an alternative erythrocyte receptor that has a lower binding affinity to DBP1. The possibility also exists that some Duffy-null individuals may express a low level of Duffy blood group antigen on their erythrocytes despite the inability of GATA1 to bind to the Duffy blood group locus. Three studies have failed to find Duffy blood group antigen on the surface of Duffy-null erythrocytes (14, 37, 38), although extremely low copy number may have been missed. The possibility of low expression of Duffy in _P. vivax_-infected Duffy-null individuals has not yet been excluded.

The normal growth of Sal I _P. vivax_ in Squirrel monkeys despite the absence of binding of Squirrel monkey erythrocytes to DBP1 (Fig. 4) and the deletion of DBP2 raises questions about the receptor used by the Sal I _P. vivax_ in Squirrel monkey erythrocytes. The same receptor may be used for _P. vivax_ invasion of Duffy-null erythrocytes. In addition to EBPD/BP2 that binds Duffy-null erythrocytes, Reticulocyte Homology (RH) genes that are similar in structure to those described in _P. falciparum_ are found in _P. vivax_ (39, 40). _P. falciparum_ RH genes in the _P. vivax_ RH family were shown to be critical for invasion of erythrocytes (41), and it is possible that one of the _P. vivax_ RH genes may be involved in invasion of Duffy-null erythrocytes. There are many other ligands that may explain the findings, including those yet to be described. _P. vivax_ is not yet adapted to Duffy-null Africans in that it now causes a less severe infection than in Duffy-positive people (14, 20). The concern is that _P. vivax_ may adapt to infection in Duffy-blood-group–null Africans such that it may become a new cause of severe disease (42–44).

**Materials and Methods**

DBP1 and DBP2 (EBP) Constructs in pRE4 Vector. For the expression of DBP1 mutants from different _P. vivax_ strains in COS-7 cells (American Type Culture Collection), the erythrocyte-binding domain (region II) (10) of different DBP1 mutants [Sal I: PVX110810, one _Ethiopia_ Duffy-positive and two _Ethiopia_ Duffy-null patients; India VII: Broad Institute database ID PVyii_046801.0; Brazil I: Broad Institute database ID PVbG_050601.0 and Madagascar (22)] was synthesized and cloned into the pRE4 vector (kind gift from Gary Cohen and Roselyn Eisenberg, School of Dental Medicine, University of Pennsylvania, Philadelphia) in unique Apai and PvuII restriction enzyme sites. The EBPD/BP2 gene sequence (511–1,578 bp) without the signal sequence for the DNA expansion. The sequence was cloned in the pRE4 vector and does not bind human erythrocytes.

**COS-7 Cells–Erythrocyte-Binding Assay.** The assay was performed in an eight-well chambered cover glass (LAB-TEK, Thermo Fisher Scientific). COS-7 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and non-essential amino acids at 37 °C with 5% CO2. The day before transfection, the cells were plated on an eight-well chamber. Later the cells were transfected with 200 ng of DBP1-Rl from Salvador I, Ethiopia (one Duffy-positive and two Duffy-null samples), Madagascar, India VII, Brazil I, and EBPD/BP2 (Cambridge) using Lipofectamine LTX plus reagent (Invitrogen) according to the manufacturer’s protocol. The negative control used in this study is the pRE4 vector. Transfection efficiency using the pRE4 vector, which did not bind to either Duffy-positive or -null erythrocytes. The transfected cells were kept at 37 °C for 48 h for the surface expression of DBP1-Rl, EBPD/BP2, and Rhs in transfected COS-7 cells. After 48 h, the transfected COS-7 cells were incubated with human Duffy-positive and Duffy-null erythrocytes or Squirrel monkey or Aotus monkey erythrocytes (10% hematocrit) for 2 h at 37 °C. After incubation, the cells were washed three times with incomplete RPMI. In total, 20 µL of the DBP1-Rl for the 90 s with a final 5-min extension at 72 °C. PCR products were cloned using pDrive vector (Qiagen). For the two Duffy-negative individuals, 32 and 36 clones were sequenced to determine the sequence for the DNA expansion. Plasmids were sequenced in both forward and reverse directions using the M13 primers (forward: 5′-GGTTAGTACGCAAGCTGTTTGC-3′; reverse: 5′-GGTACCTTGAGCGGGGCTGTTGTGGGGG-3′). The sequence was archived in GenBank (K965309). For negative control, the PRRHS (Plasmid DB ID: PF07_0424100) gene sequence (64–1,578 bp) without the signal sequence was cloned in the pRE4 vector and does not bind human erythrocytes.

**PCR and Cloning of DBP1 to Identify the Similarities/Differences Between Group Antigen Sequencing.** Clinical samples from Jimma, Ethiopia, were collected to determine _P. vivax_ infection and Duffy blood type. Scientific and ethical clearance was obtained from the institutional scientific and ethical review boards of Jimma University, Ethiopia, and the University of California, Irvine. Written informed consent/assent for study participation was obtained from all consenting heads of households, from parent/guardians (for minors under the age of 18), and from adults for each individual who was willing to participate in the study. For each malaria symptomatic or febrile patient, three to four blood samples, equivalent to ~50 µL each, were blotted on Whatman 3MM filter paper (Sigma). Parasite DNA was extracted from dried blood spots by the Saponin (Fluka)/Chelex (Bio-Rad) method (46), and genomic DNA was eluted in a total volume of 200 µL TE buffer.

Diagnosis of _P. vivax_ and Duffy Blood Group Antigen Sequencing. Clinical samples from Jimma, Ethiopia, were collected to determine _P. vivax_ infection and Duffy blood type. Scientific and ethical clearance was obtained from the institutional scientific and ethical review boards of Jimma University, Ethiopia, and the University of California, Irvine. Written informed consent/assent for study participation was obtained from all consenting heads of households, from parent/guardians (for minors under the age of 18), and from adults for each individual who was willing to participate in the study. For each malaria symptomatic or febrile patient, three to four blood samples, equivalent to ~50 µL each, were blotted on Whatman 3MM filter paper (Sigma). Parasite DNA was extracted from dried blood spots by the Saponin (Fluka)/Chelex (Bio-Rad) method (46), and genomic DNA was eluted in a total volume of 200 µL TE buffer. Slides were examined under microscopes using a 100× objective. All slides were stained in duplicate by two independent microscopists at the same sample collection. A nested amplification of the 185 RNA gene of _Plasmodium_ ( _P. falciparum_, _P. vivax_, _P. malariae_, and _P. ovale_) was performed to identify positive infection and parasite species using published protocols (47, 48). Genomic DNA of each sample was amplified in duplicate for verification. In addition, parasite DNA content was estimated using the SYBR Green qPCR detection method using sppecific primers to target the DBP1 and DBP2 (EBP) genes (20, 49). All PCR assays included positive controls of both _P. falciparum_ 7G8 (MR4-MRA-926) and HB3 (MR4-MRA-MRA-155) isolates as well as _P. vivax_ Pakchong (MR4-MRA-342G) and Nicaragua (MR4-MRA-340G) isolates (MR4, https://www.beiresources.org/About/MR4.aspx), in addition to negative controls, including uninfected samples and water.

For all _P. vivax_-positive samples, an ~500-bp fragment of the human Duffy blood group antigen that encompasses the 33rd nucleotide position in the GATA1 transcription factor-binding site of the gene promoter was amplified and sequenced following published protocols (5, 50). The point mutation T-33C leads to failure of Duffy antigen expression on the surface of erythrocytes, and individuals with homozygous (C/C) are Duffy-null (5).
followed by 45 cycles at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min with a final 95 °C for 10 s. This was then followed by a melting curve step of temperature ranging from 65 °C to 95 °C with a 0.5 °C increment to determine the melting temperature of each amplified product. Each assay included an internal reference gene, the P. vivax aldolase, which is known to be a single copy gene in P. vivax, as well as negative controls (uninfected samples and water). The \( pDBP1 \) copy number \( (n) \) in each sample was quantified based on the threshold cycle (CT) using the following equation: \( n = 2^{\Delta \Delta CT} \) where \( \Delta CT = (CT_{\text{sample}} - CT_{\text{ref}}) - (CT_{\text{positive control}} - CT_{\text{ref}}) \). \( C_{\text{aldolase}} \) and \( C_{\text{bupsect}1} \) are threshold cycle values for the aldolase gene and the \( pDBP1 \) gene, respectively, whereas \( C_{\text{ref}} \) is an average difference between \( C_{\text{aldolase}} \) and \( C_{\text{bupsect}1} \) obtained for the positive control Salvador T that contains a single copy of \( pDBP1 \) and aldolase gene fragments. SD is SD calculated as follows: \( SD = \sqrt{(S_{\text{aldolase}}^2 + S_{\text{bupsect}1}^2 + S_{\text{ref}}^2)} \), where \( S_{\text{aldolase}} \) and \( S_{\text{bupsect}1} \) are the SDs from the average CT calculated for these replicates in the \( pDBP1 \) and \( paldolase \) amplifications, and \( S_{\text{ref}} \) is an average SD of the \( \Delta CT \) values for the calibrator.

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