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Naturally acquired inhibitory antibodies to *Plasmodium vivax* Duffy binding protein are short-lived and allele-specific following a single malaria infection

I. P. Ceravolo,* B. A. M. Sanchez,* T. N. Sousa,* B. M. Guerra,* I. S. Soares,† E. M. Braga,‡ A. M. McHenry,§ J. H. Adams,§ C. F. A. Brito* and L. H. Carvalho*

*Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, FIOCRUZ, Belo Horizonte, Minas Gerais, †Instituto de Ciências Biológicas, Universidade de São Paulo, São Paulo, SP, ‡Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, and §University of South Florida, Tampa, FL, USA

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Correspondence: L. H. Carvalho, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Malaria Laboratory, Av. Augusto de Lima 1715, 30190-002, Belo Horizonte, MG, Brazil.
E-mail: lhcarvalho@cpqrr.fiocruz.br

Summary

The Duffy binding protein of *Plasmodium vivax* (DBP) is a critical adhesion ligand that participates in merozoite invasion of human Duffy-positive erythrocytes. A small outbreak of *P. vivax* malaria, in a village located in a non-malarious area of Brazil, offered us an opportunity to investigate the DBP immune responses among individuals who had their first and brief exposure to malaria. Thirty-three individuals participated in the five cross-sectional surveys, 15 with confirmed *P. vivax* infection while residing in the outbreak area (cases) and 18 who had not experienced malaria (non-cases). In the present study, we found that only 20% (three of 15) of the individuals who experienced their first *P. vivax* infection developed an antibody response to DBP; a secondary boosting can be achieved with a recurrent *P. vivax* infection. DNA sequences from primary/recurrent *P. vivax* samples identified a single *dbp* allele among the samples from the outbreak area. To investigate inhibitory antibodies to the ligand domain of the DBP (cysteine-rich region II, DBP_{II}), we performed *in vitro* assays with mammalian cells expressing DBP_{II} sequences which were homologous or not to those from the outbreak isolate. In non-immune individuals, the results of a 12-month follow-up period provided evidence that naturally acquired inhibitory antibodies to DBP_{II} are short-lived and biased towards a specific allele.

Keywords: allele-specific, antibody response, duffy binding protein, malaria, *Plasmodium vivax*

Introduction

The Duffy binding protein of *Plasmodium vivax* (DBP) is a critical adhesion ligand that participates in merozoite invasion of human Duffy/Duffy antigen receptor for chemokines (DARC)-positive erythrocytes [1,2]. DBP belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL–EBP) located within the micronemes of *P. vivax* and *P. knowlesi* merozoites [3]. The functional binding domains of DBL–EBP lie in region II, and for *P. vivax* the critical binding residues have been mapped to a central 170-amino acid stretch that includes cysteines 5–8 [4–6]. The gene encoding the *P. vivax* DBP region II (DBP_{II}) is highly polymorphic, and this diversity varies geographically from region to region [7–13]. The pattern of excessive polymorphism is consistent with a high selection pressure on the DBP gene and suggests that allelic variation functions as a mechanism of immune evasion [14,15].

Invasive merozoites are believed to sequester microneme proteins until merozoites contact the target erythrocyte, presumably as a mechanism to reduce exposure of DBP to immune inhibition [16]. Currently, available data on humoral immune responses to DBP in human populations demonstrate that anti-DBP antibodies increase with exposure to *P. vivax* [17–20], and this immune response includes antibody activity that blocks adherence of DBP_{II} to its receptor on erythrocytes [18,21]. The same antibodies that block the DBP_{II}–DARC interaction also inhibit *P. vivax* erythrocyte invasion [22], which is proof-of-concept that anti-PvDBP antibodies can inhibit merozoite invasion. Of importance, children residing in hyperendemic areas for *P. vivax* develop anti-DBP inhibitory antibodies that seem to confer protection against blood-stage infection [23].

As most studies on the DBP antibody response reported to date have been carried out in areas where malaria is highly endemic, there is a scarcity of data on the responses to exposure to a single infection and about the persistence of this antibody response in the absence of reinfection. An outbreak of *P. vivax* malaria, in a village located in a non-malarious area of Brazil, offered us an opportunity to

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investigate the DBP immune response among individuals who had their first and brief exposure to malaria. In the outbreak area, we hypothesized that a first exposure to *P. vivax* malaria induces an anti-DBP antibody response that blocks the interaction between DBP and its receptor on erythrocyte. To analyse this neutralizing antibody response, we used an *in vitro* cytoadherence assay that uses the putative ligand domain of the DBP (region II, DBP_{II}) expressed on the surface of cultivated mammalian cells [18]. To investigate whether neutralizing antibodies recognize DBP_{II} in a strain-specific manner, we analysed polymorphisms within the critical binding motif of *P. vivax* DBP_{II} from the outbreak isolates, and performed inhibition of cytoadherence assays with DBP_{II} sequences which are homologous or not to that from the outbreak area. In this study, carried out with non-immune individuals, we provide evidence that naturally acquired neutralizing antibodies to DBP_{II} can be strain-specific and are relatively short-lived in the absence of reinfection.

Table 1. Demographic, immunological and genetic data of individuals who had been enrolled in the study carried out in the *Plasmodium vivax* malaria outbreak area.

Characteristics	Cases ($n = 15$)	Non-cases ($n = 18$)
Age, years (mean ± s.d.)*	32 ± 13	34 ± 19
Antibody response, *n* (%)†		
MSP1-19	12 (80%)	0 (0%)
$DBP_{II–IV}$	3 (20%)	0 (0%)
DARC functional alleles, *n* (%)‡		
One (*Fy*A* or *Fy*B*)	6 (40%)	6 (33%)
Two (*Fy*A* and/or *Fy*B*)	9 (60%)	12 (67%)
None (Fy^*B^{ES})§	0	0

*Difference not significant ($t = 0·02$, $P > 0·05$). †Number (%) of individuals with a positive antibody response at the time of first cross-sectional survey. ‡The frequencies of individuals bearing the functional alleles *Fy*A* and *Fy*B* (Fy^a and Fy^b antigens on erythrocytes respectively) were similar between cases and non-cases ($P > 0·05$). §Homozygosis for the FY^*B^{ES} (*ES*, erythroid silent) allele abrogates Duffy antigen receptor for chemokines (DARC) antigen expression on the erythrocyte surface, and designates the DARC negative phenotype. DBP, Duffy binding protein; MSP, merozoite surface protein-1; s.d., standard deviation.

Materials and methods

The *P. vivax* malaria outbreak

Between April and May 2003, 25 cases of *P. vivax* malaria were diagnosed for the first time in a small community, Souza, located 70 km from Belo Horizonte, Minas Gerais State, a non-endemic area of Brazil [24,25]. Malaria has never been reported in this area and the Brazilian endemic region, the Amazon area, is 2000 km away. According to the Minas Gerais Department of Health, the source of the infection was a man from the community who had returned from the Amazon, infected by *P. vivax*, in January 2003. The subsequent outbreak in Souza began in April 2003, and entomological surveys incriminated the vector *Anopheles darlingi* as responsible for local malaria transmission [24]. The first human malaria case detected in the outbreak area, named S14, remained at the hospital for about 10 days, until a malaria diagnosis could be established. Because malaria infection had never been reported in the outbreak area previously, the physicians failed to consider malaria on presentation of this patient. After the first case, all patients were treated promptly with chloroquine (1·5 g for 3 days) plus primaquine (30 mg daily for 7 days), and a second round of treatment was given in case of relapses and/or recrudescence (3-day course of chloroquine and 15-day course of primaquine). Control activities also included an active search for acute malaria by thick blood smears and outdoor/indoor spraying of residual insecticide (cypermethrine) [25]. The outbreak was considered of short duration (50–60 days), with the last malaria case diagnosed on 21 May 2003; since then, local/regional Departments of Health have maintained entomological and epidemiological surveillance of the area.

Volunteers and blood collection

Cross-sectional surveys were carried out after discussions with the community about the objectives of the project and its protocols. Individuals who had been infected with *P. vivax* were enrolled in the study if they met the following criteria: (i) informed written consent in accordance with guidelines for human research, as specified by the Brazilian National Council of Health (Resolution 196/96); (ii) residence in the outbreak area; (iii) a minimum age of 15 years; (iv) if women, an indicator of the absence of pregnancy; and (v) a willingness to remain in the outbreak area during the intervening year. As shown in Table 1, a total of 15 individuals met the inclusion criteria (aged 32 ± 13 years). We also included relatives and neighbours who were considered to be exposed to the risk of infection ($n = 18$; 34 ± 19 years). The latter group had had neither symptoms nor blood parasites by direct examination of Giemsa-stained thick smears. Of the 33 volunteers, 32 did not recall previous history of malaria, temporary residence in malaria-endemic areas or travel to the endemic area during their lifetime. A single volunteer with confirmed malaria in the outbreak area (S1) recalled previous malaria infection, temporary residence in the endemic (gold-mining) area, and travelling to the Amazon during the 6 months preceding the outbreak. We collected 5 ml blood samples (ethylenediamine tetraacetic acid) from all subjects. At the time of blood collection, Giemsa-stained thick blood smears were examined for parasites and nested polymerase chain reaction (PCR) assays for malaria diagnosis were conducted later in our laboratory. Blood samples were used to obtain plasma and for DNA preparation. Three, 6, 9 and 12

months after the first survey, four other identical cross-sectional surveys were carried out. The ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human Beings from the Centro de Pesquisas René Rachou/FIOCRUZ (Reports 002/2002 and 07/2006), according to the Resolution of the Brazilian Council on Health-CNS 196/96.

Microscopy and *Plasmodium* diagnosis by nested PCR

Well-trained microscopists examined 200 fields of Giemsa-stained thick blood smears. DNA was extracted from 300 μl of individual whole-blood samples by using a genomic DNA purification kit (Puregene, Gentra Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Parasite species identification was performed by nested PCR amplification of the small subunit ribosomal RNA (18S SSU rRNA) genes, as described previously [26].

The DARC genotyping

Extracted DNA was used to detect the three common alleles at the *FY* locus – *FY*A*, *FY*B*, FY^*B^{ES} (ES, erythroid silent) – using real-time PCR with allele-specific primers, essentially as we have described recently [27].

Recombinant proteins and serological assay

Two recombinant *P. vivax* proteins were used to detect total immunoglobulin G (IgG) antibodies. The recombinant DBP, which includes amino acids 132–771 (regions II–IV, $DBP_{II–IV}$), was expressed as a soluble glutathione S transferase (GST) fusion protein of 140 kDa, as described previously [17,20]. The recombinant protein representing the 19-kDa C-terminal region of the merozoite surface protein-1 of *P. vivax* (MSP1-19), which represents amino acids 1616–1704 of the MSP1 of *P. vivax*, has been described elsewhere [28]. To assess IgG antibodies against $DBP_{II–IV}$ and MSP1-19, an enzyme-linked immunosorbent assay (ELISA) was carried out, as described previously [20], with serum samples at 1 : 100. For the recombinant proteins $DBP_{II–IV}$ (5 μg/ml) and MSP1-19 (1 μg/ml), the final optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST (antigen control). The results were expressed as an index of reactivity ($IR = OD_{492}$ values of test sample divided by the value of the cut-off). Cut-off points were set at three standard deviations above the mean OD_{492} of sera from 30 individuals who had never been exposed to malaria. Values of $IR > 1{\cdot}0$ were considered positive.

The *P. vivax* DBP_{II} amplification and sequencing

Extracted DNA was used as a template in the PCR to amplify the fragment corresponding to nucleotide positions 870–1545 (amino acids 290–515) of the DBP_{II}, as described previously [13]. Platinum high fidelity *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) was used in PCR to reduce possible nucleotide mis-incorporation. Amplicons were purified using the GFX-96 PCR kit (Amersham Biosciences, Little Chalfont, UK) and sequenced directly using DYEnamic™ ET dye terminator kit (Amersham Biosciences) and MegaBace 500 automated DNA sequencer (Amersham Biosciences). The sequences were analysed using Bioedit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify DBP_{II} polymorphisms relative to the SAL-1 sequence [29].

The DBP–pEGFP constructs

Region II of DBP (DBP_{II}) from a *P. vivax* laboratory reference clone (Sal-1) [29] has been subcloned previously into the pEGFP–N1 plasmid (Clontech, Mountain View, CA, USA), with a flanking signal sequence from the herpes simplex virus glycoprotein D1 (HSVgD1) [18]. This targets expression to the surface of the transfected COS cells as a green fluorescent protein (GFP) fusion protein. An additional GFP construct with the DBP_{II} sequence from the outbreak *P. vivax* isolate was made by subcloning a fragment corresponding to aa 198–522 of region II into pEGFP–HSVgD1 plasmid, using primers described previously [30]. Recombinant plasmids were purified by use of an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA, USA).

COS cell transfection and erythrocyte-binding assays

Recombinant plasmids were transfected into COS-7 cells (American Type Culture Collection, Manassas, VA, USA) using lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, COS-7 cells in six-well culture plates ($1{\cdot}5 \times 10^5$ cells/well) were transfected with plasmids (0·5 μg/well)–liposome complexes (5% plus-reagent and 3% lipofectamine) in Dulbecco's modified Eagle medium (DMEM; Sigma, St Louis, MO, USA) without serum. After 6 h of cell exposure to DNA-liposome complexes (37°C, 5% CO_2), transfection medium was replaced by DMEM with 10% of fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA). At 24 h after transfection, the efficiency of transfection was assessed by fluorescence; the recombinant protein expression levels were similar between the Sal-1 and outbreak DBP_{II} variants (data not shown). Forty-eight hours after transfection, the erythrocyte-binding assays were performed as described previously [21]. For this, anti-serum was added at 1 : 20 (1 h at 37°C, 5% CO_2) followed by incubation with 10% of human O^+ erythrocytes suspension (2 h, room temperature). Unbound erythrocytes were removed by washing and binding was quantified by counting rosettes (10–20 fields, 200×). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the cell surface.

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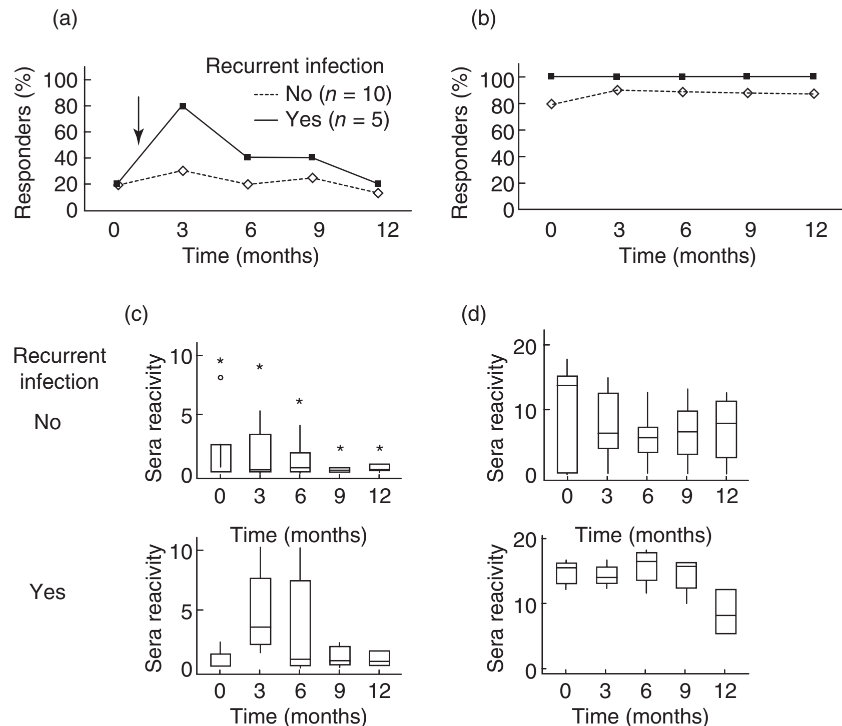

Fig. 1. Antibody responses to Duffy binding protein (DBP_{II-IV}) (a,c) and merozoite surface protein-1 (MSP1-19) (b,d) among individuals who had confirmed *Plasmodium vivax* infection in the outbreak area, and developed ($n = 5$) or not ($n = 10$) recurrent *P. vivax* infections (↓) during the 12-month follow-up period. In (a) and (b), the percentage of responders to DBP_{II-IV} or MSP1-19, respectively, as detected by enzyme-linked immunosorbent assay (ELISA). In (c) and (d), box-plot representations of sera reactivity to DBP_{II-IV} or MSP1-19 respectively; sera reactivity were expressed as index of reactivity (IR) at 492 nm, IR > 1 being considered positive. Box-plots: solid line across the box is the median, and the 25th and 75th percentiles were represented by the bottom and the top of each box respectively; the vertical lines represent the range, with outliers marked by asterisks (S14) or circle (S38).

For each assay, pooled plasma samples from Souza residents, characterized as non-responders by ELISA, were used as a negative control (100% binding). The percentage inhibition was calculated as $100 \times (Rc - Rt)/Rc$, where Rc is the average of the number of rosettes in the control wells and Rt is the average of the number of rosettes in the test wells.

Statistical analysis

Statistical analysis was performed using the Epi-Info 2002 software (CDC, Atlanta, GA, USA) or MiniTab statistical software (Minitab Inc., State College, PA, USA). Differences in means were tested by Student's *t*-test or one-way analysis of variance. Differences in proportions were evaluated by Yates's χ^2 or Fisher's exact tests. *P*-values < 0·05 were considered significant.

Results

Antibody responses to DBP_{II-IV} and MSP1-19 at enrollment

Thirty-three individuals participated in the five cross-sectional surveys, 15 with previously confirmed *P. vivax* infection in the outbreak area (cases) and 18 who had not experienced malaria infection (non-cases). At the first cross-sectional survey, 20% (three of 15) of malaria cases had antibodies to DBP_{II-IV} (Table 1); in contrast, the MSP1-19 was recognized initially by 80% (12 of 15) of these individuals. The remaining 18 individuals (non-case) did not develop a detectable antibody response against either anti-DBP_{II-IV} or anti-MSP1-19. Although 'resistance' to vivax malaria would result from the lack of DARC glycoprotein on red blood cells, none of the individuals studied were homozygous for the allele $FY^{*}B^{ES}$. Therefore, we concluded that absence of DARC on RBCs was not responsible for refractoriness to *P. vivax* infection in this group (Table 1).

Relationship between malaria status and anti-DBP antibodies during a 12-month follow-up period

Although DBP was recognized initially by 20% of individuals who had had a *P. vivax* infection, a secondary boosting could be achieved with a new episode of malaria, making 80% into responders at this time (Fig. 1a). Nevertheless, in those individuals the frequency of responders decreased a few months after the clinical attack. By analysing the levels of anti-DBP antibodies during the 12-month follow-up, we observed a wide range of antibody responses among study participants (Fig. 1c), which made the difference between groups without statistical significance (recurrent *versus* no recurrent infection). Of interest, during the follow-up period, the levels of anti-DBP antibodies were relatively higher in a single individual (Fig. 1c, asterisk in each time-point of the follow-up); this result was not unexpected, because this patient (S14) remained at the hospital for about 10 days until a malaria diagnosis could be established. Despite individual variations, the levels of anti-DBP decreased markedly within the first 6 months of the follow-up.

Table 2. Variant amino acids in Duffy binding protein (DBPII) from the *Plasmodium vivax* outbreak isolates, compared with the *P. vivax* laboratory reference clone Sal-1.

Isolate*	Codon position							
	371	384	385	386	417†	424	437	503
Sal-1, reference clone	K	D	E	K	N	L	W	I
Outbreak								
Primary infection	E	G	K	N	K	I	R	K
Recurrent infection	E	G	K	N	K	I	R	K

*Sal-1 sequence, accession number: M61095; outbreak sequence, accession numbers: EU870443-EU870445. †Grey areas highlight polymorphisms in DBP_{II} that compromise efficiency of rabbit anti-DBP serum to inhibit DBP_{II}–Duffy antigen receptor for chemokines (DARC) interaction [7]. Also, analysis of 122 Brazilian *P. vivax* isolates demonstrates that residues 417 and 424 form part of a cluster surrounding the DARC-binding site (Sousa & Brito, unpublished results).

Altogether, eight of 15 (53%) malaria cases developed anti-DBP antibodies during the follow-up period. The serological response to MSP1-19 was distinctly different (Fig. 1b). Regardless of the occurrence of a relapse and/or recrudescence, the MSP1-19 was a relatively highly immunogenic protein for most individuals who had malaria, with 14 of 15 (93%) positives for anti-MSP1-19 IgG antibodies. However, decreasing levels of antibody reactivity to MSP1-19 was more evident in the group who did not develop a recurrent *P. vivax* infection (Fig. 1d). No one from the uninfected group (non-cases) developed antibodies against either anti-DBP or anti-MSP1-19 (data not shown).

Because the antibody response against DBP decreased few months after the clinical attack, we also investigated antibodies against another *P. vivax* apical antigen and vaccine candidate, the apical membrane antigen-1 (AMA-1) [31]. Our results demonstrated that although the profile of AMA-1 immune response was similar to that obtained with DBP, AMA-1 appears to be more immunogenic, with 53% (eight of 15) of responders at the beginning of the study and with all individuals converting into responders at the time of a new episode of malaria (see *Supporting information*, Fig. S1). However, the frequencies as well as the levels of anti-AMA-1 antibodies were lower in those individuals who did not develop a recurrent *P. vivax* infection (see *Supporting information*, Fig. S1b).

The DBP_{II} polymorphisms and inhibitory activity of naturally acquired anti-DBP antibodies

To characterize the *P. vivax* isolates responsible for the malaria outbreak, we analysed DNA sequences from primary and recurrent infections and identified a single *dbp* allele in the outbreak area (Table 2). This allele differed, at multiple codons, from the *P. vivax* laboratory reference clone Sal-1, including differences in three polymorphic codons (417, 437 and 503) suggested to play a synergistic functional effect on DBP_{II} inhibitory binding [7].

To investigate the specificities of the anti-DBP outbreak plasmas to inhibit the erythrocyte-binding function of the protein, we performed erythrocyte-binding assays using COS-7 cells expressing sequences of DBP_{II} which are identical or not to those of the outbreak isolate. Previously, this *in vitro* assay proved to be a suitable alternative tool for the live-cell invasion inhibition assay [22]. For that, plasma samples of those eight individuals who had developed conventional anti-DBP antibodies, at any time-point of the follow-up period, were tested for inhibition of DBP_{II}–DARC binding (Fig. 2). Three months after the first malaria attack, when the majority of responders were detected, seven of eight individuals had developed inhibitory antibodies against the homologous DBP_{II} sequence, while sera of two (S1 and S31) presented inhibitory activity against the heterologous sequence (Fig. 2a). Despite the occurrence of recurrent infections, most of these individuals lost their anti-DBP inhibitory antibody response within 6 months of follow-up. A single exception was an individual (S1) who had had previous malaria infection during frequent trips to the malaria-endemic area, and who developed inhibitory antibodies against homologous and heterologous DBP_{II} sequences. Beyond the frequency of response, the levels of inhibitory antibodies were also related to the DBP_{II} sequence; the greatest levels were observed with COS cells expressing the homologous DBP_{II} sequence, and no cross-reactivity could be detected at 1 : 40 sera dilution (Fig. 2b).

Discussion

Naturally occurring antibodies to DBP are prevalent in individuals living in areas where vivax malaria is endemic [17,19,20], and these antibodies can block the DBP_{II}–DARC interaction [18,21,23] and inhibit *P. vivax* erythrocyte invasion [22]. In previous studies, carried out in malaria-endemic areas, we and others have found strain-transcendent inhibitory responses to DBP_{II} [21,23]. However, those previous studies could not dismiss the possibility that DBP_{II} cross-variant inhibitory activity reflected only an accumulation of antibodies to strain-specific epitopes. Here, we have examined antibody responses of non-immune individuals after a brief initial malaria infection during a malaria outbreak outside the endemic area. Our study demonstrates that DBP has low immunogenicity

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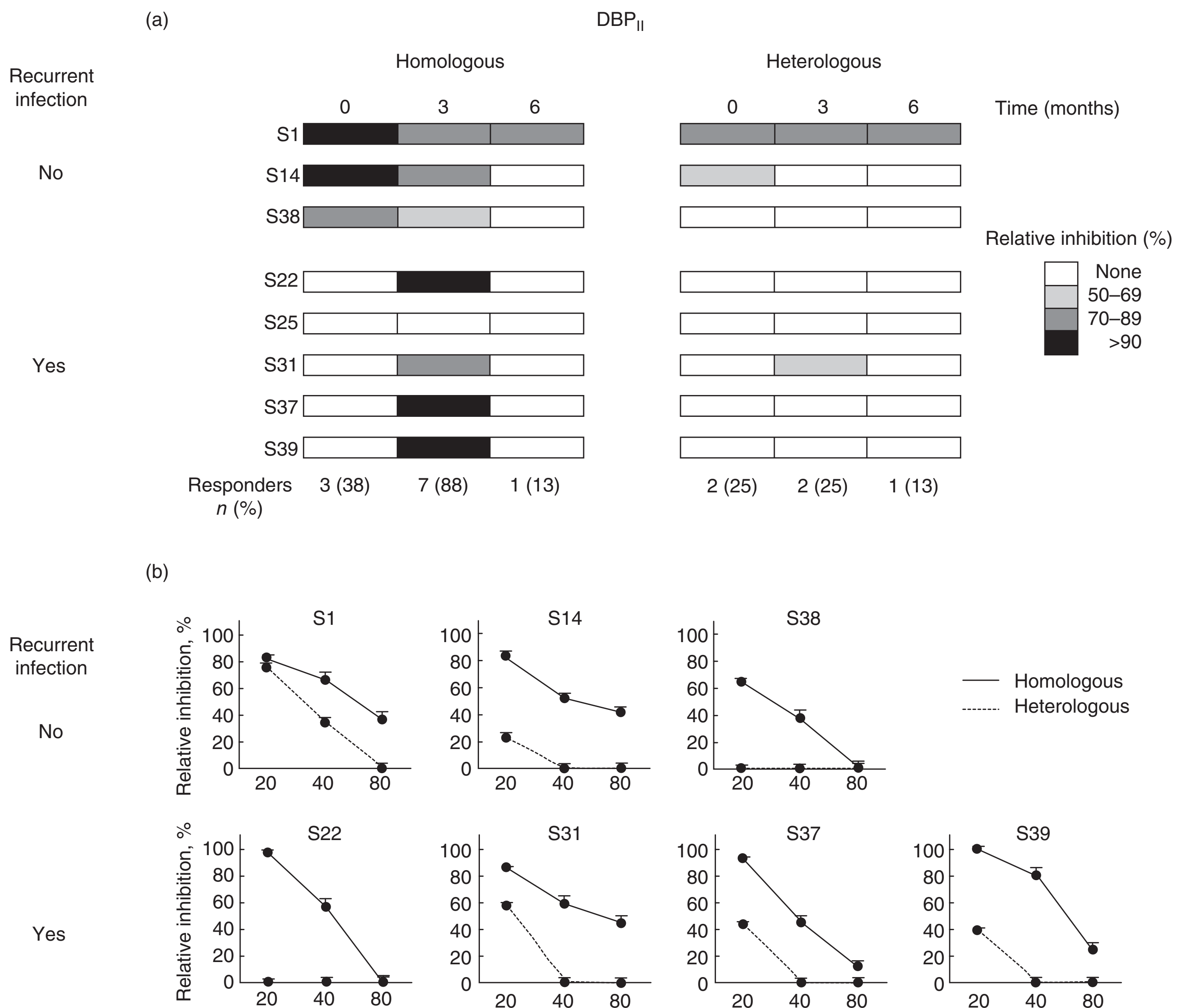

Fig. 2. Inhibition of Duffy binding protein–Duffy antigen receptor for chemokine (DBP_{II}–DARC) binding by outbreak plasma samples from eight individuals who developed conventional anti-DBP antibodies during the follow-up period. Erythrocyte binding assays were carried out with COS cells expressing the outbreak (homologous) or Sal-1 (heterologous) DBP_{II} sequences, and individual samples were grouped according to the occurrence (S22, S25, S31, S37, S39) or not (S1, S14, S38) of a recurrent *Plasmodium vivax* infection. (a) Results of each subject (at 1 : 20 plasma dilution), at enrollment (time zero), and 3 and 6 months later; values at the bottom of the figure represent the overall frequency of responders for each cross-sectional survey. (b) Titration of the inhibitory antibody responses against homologous or heterologous DBP_{II} sequences, using those seven positive samples from the second cross-sectional survey (3-month follow-up). The *x*-axis represents antibody titers.

and induces a short-term humoral immune response that is lost within 6 months of follow-up. In general, this profile of antibody response is similar to that detected to another apical *P. vivax* vaccine candidate, AMA-1, in which the ELISA positivity rates dropped during the first 9 months of follow-up (*Supporting information*). Nevertheless, during the follow-up period the frequencies of AMA-1 responders were usually higher than those obtained to DBP, especially among those individuals with recurrent *P. vivax* infections. The possibility that AMA-1 is more immunogenic than DBP could be explained by those findings demonstrating that AMA-1 is expressed in both pre-erythrocytic (sporozoite) and erythrocytic (merozoite) stages of malaria parasites [32], while DBP is a merozoite-specific protein.

Of importance, single *P. vivax* exposure appears to induce an anti-DBP inhibitory response that is biased towards a specific DBP allele. The longevity of this DBP inhibitory antibody response is different from that observed among some asymptomatic children residing in a *P. vivax* hyperendemic area [23], in which the anti-DBP inhibitory antibody response was remarkably stable over the 12-month follow-up period. Together, these findings suggest that vaccines based on DBP_{II} should consider short-term antibody responses in non-immune individuals.

The poorer, unstable antibody responses against DBP during the outbreak follow-up period is in contrast to the stronger, stable response to MSP1-19, which is a much more abundant blood-stage molecule than DBP. Regardless of the presence of recurrent *P. vivax* infections, the frequency of responders to MSP1-19 was similar at all five time-points, albeit at a lower magnitude in those without recurrent *P. vivax* infections, as described previously [33]. This longer-term stability of antibodies against *P. vivax* MSP1 has been well documented [34,35], including its persistence for 30 years after malaria exposure [36]. In none of the study individuals did the absence of DARC on erythrocytes play a role in the anti-DBP or anti-MSP1-19 responses.

In the Souza community, where the outbreak occurred, the period of malaria transmission was short (approximately 50 days), being interrupted by treatment of all patients with anti-malarial drugs (chloroquine and primaquine) and the comprehensive spraying of residual insecticide [25]. Considering that the control intervention of the outbreak was so thorough, the origin of the second attack of *P. vivax* in five individuals, about 2 months after the first malaria episode, is unclear. Typically, these infections may have had two origins: (i) a recrudescence originating from asexual blood-stage parasites that survived drug treatment; or (ii) a relapse arising from the dormant liver stages, hypnozoites [37]. The recurrences for the *P. vivax* appear to be more probably relapses, as treatment regimens used in the outbreak area were effective in clearing parasitaemias and there was a long period until the blood-stage infections reappeared. To analyse whether the isolate causing the secondary attack was genetically different from the isolate of initial infections, we compared DNA sequences from primary and recurrent *P. vivax* infections. Molecular analysis demonstrated that a single *dbp* allele was detected in the outbreak area (GenBank Accession numbers: EU870443–EU870445). The *dbp* outbreak allele belongs to allelic family VII, one of the eight DBP_{II} variant families identified in a preliminary analysis of 40 *P. vivax* Brazilian isolates [13].

Although the activation of heterologous hypnozoite populations seems to be the most common cause of relapse in patients with vivax malaria [38,39], the presence of a single *dbp* outbreak allele is consistent with either a relapse or a recrudescence. In contrast to previous studies in Asia, the *P. vivax* transmission in the outbreak area originated from a single patient who had had a *P. vivax* relapse after returning from the Amazon area [24,25]. In fact, our results are similar to a previous study of *P. vivax* relapses in Brazil which demonstrated, using the MSP1 molecule as a genetic marker, that parasites from the primary attack were identical to those in relapses [40].

An important finding of our study is the discovery of how parasite genetic diversity relates to naturally acquired neutralizing antibodies against DBP. The results demonstrated that the phylogenetically distant Sal-1 variant was significantly less sensitive to immune inhibition of its DARC binding activity than was the homologous effect against the DBP_{II} allele of the outbreak variant. Significant antibody cross-reactivity was observed in a single individual (S1), a result which was attributed to past cured infections in a gold-mine worker who had a history of previous malaria illnesses in a malaria-endemic area. Although it is not possible at this time to characterize the fine specificity of the inhibitory anti-DBP antibodies, these data demonstrate that variation in few polymorphic residues compromising the inhibitory efficacy of these antibodies. Further work will be necessary to identify the main epitopes recognized by naturally acquired antibodies to DBP in humans.

Altogether, our results indicate that polymorphisms change DBP antigenic character and can compromise immune inhibition, as suggested previously using rabbit immune sera [7]. Of importance, the outbreak and Sal-1 alleles do not share the trio of polymorphic residues (at codons 417, 437 and 503) shown to collectively alter sensitivity to inhibitory antibodies. Overall, these results point towards strain specificity in the natural immune response against DBP. Consistent with this hypothesis, the only individuals in the Amazon area who were observed previously to acquire anti-DBP antibodies that inhibit binding of different DBP_{II} variants to erythrocytes were people who had had long-term exposure [21]. Consequently, it is not surprising that only 9% of asymptomatic children residing in a *P. vivax* hyperendemic area had acquired a significant anti-DBP inhibitory antibody response that transcended strain-specificity [23].

Even though the current data demonstrate that individuals exposed briefly to *P. vivax* developed anti-DBP antibodies which exert a receptor-blocking effect, the magnitude of the inhibitory antibody response was very low compared with that from individuals with long-term exposure to malaria; in the outbreak area, inhibitory activity was achieved with immune sera diluted up to 1 : 80, whereas in the Amazon endemic area inhibitory antibodies could still be detected at a 1 : 1280 sera dilution [21]. It is possible that the low levels of immune response in the outbreak area could be due to the short and brief exposure to parasite blood stages. In fact, in this area, a secondary antibody boost was achieved with a recurrent *P. vivax* infection. Also, our previous data in the Amazon area indicate accumulative exposure to *P. vivax* as the strongest predictor of the presence of anti-DBP antibodies [21]. None the less, it is currently unclear how effective such natural antibody responses may be in preventing disease in this population. A long-term prospective study in a non-immune population is needed to determine the protective nature of the inhibitory anti-DBP_{II} antibodies in terms of anti-disease immunity.

Recently, it has been predicted that the hypervariable region of DBP_{II} is located on sites remote from the DARC binding site, implying that polymorphism cannot alter the capacity of the protein to bind DARC-positive erythrocytes [41,42]. Another line of evidence suggests that those few

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polymorphic residues surrounding the DARC binding domain might elude binding of inhibitory antibody [7,16]. The second model seems to explain why antibodies to DBP can inhibit reticulocyte invasion by *P. vivax* effectively [22]. The results presented here provide strong evidence that the DARC and antibody binding sites have sufficient overlap for antibodies to inhibit binding and provide support for the role of allelic diversity in anti-DBP immune responses.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Antibody responses to the apical membrane antigen-1 (AMA-1) among individuals who had confirmed *Plasmodium vivax* infection in the outbreak area, and developed ($n = 5$) or not ($n = 10$) recurrent *P. vivax* infections (↓) during the 12-month follow-up period. (a) The percentage of responders to AMA-1, as detected by enzyme-linked immunosorbent assay (ELISA); (b) box-plot representations of sera reactivity; sera reactivity were expressed as index of reactivity (IR) at 492 nm, $IR > 1$ being considered positive. Box-plots: solid line across the box is the median, and the 25th and 75th percentiles were represented by the bottom and the top of each box respectively. The recombinant protein AMA-1 was produced as described previously [31].

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