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Antígenos envolvidos na invasão dos reticulócitos pelo *Plasmodium vivax*: variabilidade genética do hospedeiro vertebrado e modulação da resposta imune humoral

por

Letícia de Menezes Torres

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"O correr da vida embrulha tudo.
A vida é assim: esquenta e esfria,
aperta e daí afrouxa,
sossega e depois desinquieta.
O que ela quer da gente é ***coragem.****"*

Guimarães Rosa

Dedico este trabalho à minha sobrinha ***Catarina***

que mesmo antes de mostrar seu rostinho

me trouxe entusiasmo pra lutar por um mundo melhor.

E a todas as crianças que de alguma forma sofrem

com as consequências causadas pela malária.

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Resumo

O *Plasmodium vivax* infecta os reticulócitos por meio de uma via de invasão principal que envolve a interação entre a *Duffy binding protein* (região II, DBP_{II}) e seu receptor nos reticulócitos, o antígeno de grupo sanguíneo *Duffy* receptor de quimiocinas (DARC). Contudo, uma via de invasão alternativa pode envolver uma proteína do parasito recém-descrita, denominada EBP2 (Erythrocyte Binding Protein 2). Apesar da exposição natural ao *P. vivax* induza uma resposta de anticorpos capaz de bloquear a interação DBP_{II}-DARC, a maioria dos indivíduos expostos à malária não desenvolvem anticorpos inibitórios (*binding inhibitory antibodies,* BIAbs). Embora estudos prévios demonstraram que polimorfismos na DBP_{II} contribuem para a baixa imunogenicidade da proteína, a influência da variabilidade genética do hospedeiro vertebrado nesta resposta tem sido pouco estudada. Neste contexto, o presente estudo investigou a contribuição dos polimorfismos genéticos do receptor DARC e do sistema HLA classe II na resposta de anticorpos contra a DBP_{II}. Adicionalmente, caracterizamos a nova proteína EBP2 do *P. vivax*, incluindo avaliação das suas propriedades de ligação ao eritrócito/reticulócito e estudos de imunogenicidade. Para isso, um estudo prospectivo, do tipo coorte aberta, foi conduzido com 620 indivíduos naturalmente expostos ao *P. vivax* na região da Amazônia brasileira (comunidade de Rio Pardo/AM). Este estudo incluiu cinco cortes transversais, sendo três conduzidas no primeiro ano de acompanhamento (linha-de-base, 6 e 12 meses) e dois cortes transversais, 6 e 7 anos depois. Nesta comunidade os alelos mais comuns do receptor DARC (*FY*A, FY*B and FY*B^{ES}*) foram genotipados por PCR (reação em cadeia da polimerase) em tempo-real, e as variantes do HLA II (*loci* DRB1, DQB1 and DQA1) genotipadas por PCR-SSO (PCR com oligonucleotídeos de sequência específica pela tecnologia Luminex). As respostas de anticorpos específicos foram avaliadas nos cortes tranversais por meio da sorologia convencional (anticorpos IgG detectados pelo ensaio de ELISA), bem como por ensaios funcionais de inibição (ensaios *in vitro* para detecção de BIAbs). Em conjunto, os resultados permitiram demonstrar que: *(i)* a variabilidade genética do receptor DARC influencia na resposta BIAbs anti-DBP_{II}, sendo esses anticorpos inibitórios mais frequentes em indivíduos heterozigotos carreadores de um alelo chamado "não-funcional" ou silencioso de DARC (*FY*B^{ES}*); a habilidade dos polimorfismos de DARC em influenciar nas propriedades funcionais dos anticorpos pode ser detectada durante todo o período de acompanhamento (neste caso, 12-meses); *(ii)* a variabilidade genética do HLA II influenciou na aquisição e manutenção da resposta de anticorpos anti-DBP_{II}, sendo que diferentes alelos e haplótipos influenciaram tanto na resposta detectada pela sorologia convencional (ELISA) quanto na de anticorpos funcionais (BIAbs). Estudos de modelagem molecular das variantes HLA-DRB1 permitiram identificar diferenças estruturais, particularmente na fenda de ligação entre peptídeo-HLADRB1, que poderiam explicar os diferentes perfis de respondedores identificados. Com relação a EBP2, *(iii)* foi possível demonstrar que essa proteína se liga preferencialmente a reticulócitos imaturos (CD71^{high}) e DARC positivos; (*iv)* na população estudada, anticorpos anti-EBP2 foram mais frequentes que anticorpos anti-DBP_{II}. De importância, 6 e 7 anos após o início do estudo, o perfil de resposta de anticorpos anti-EBP2 se manteve estável, mesmo com a redução dos níveis de transmissão de malária na região; no mesmo período, anticorpos anti-DBP_{II} diminuíram significativamente. Em conclusão, os resultados aqui encontrados fornecem evidências de que a variabilidade genética do hospedeiro vertebrado deverá ser levada em consideração no desenvolvimento de vacinas baseadas na DBP_{II}. Além

disso, reforça os achados iniciais que sugerem que a EBP2 pode ser um candidato promissor para compor uma vacina contra as formas sanguíneas do *P. vivax*; além de ser altamente imunogênica na população estudada, a proteína apresenta características funcionais compatíveis com uma possível função na invasão de reticulócitos jovens (DARC positivos) pelo *P. vivax*.

Plavras chave: 1. Malária Vivax/transmissão 2. *Plasmodium vivax* /parasitologia 3. Imunidade Humoral/genética 4. Sistema do Grupo Sanguíneo *Duffy*

Abstract

The malaria parasite *Plasmodium vivax* infects the red blood cells through a primarily pathway that requires the interaction between the Duffy Binding protein (region II, DBP_{II}) and its cognate receptor, Duffy antigen receptor for chemokines (DARC). An alternative invasion pathway may involve a recent described protein, named EBP2 (Erythrocyte binding protein 2). Although natural exposure to *P. vivax* induces an antibody response able to block DBP_{II}-DARC interaction, a high proportion of individuals fails to develop DBP_{II} binding inhibitory antibodies (BIAbs). While previous studies demonstrated that DBP_{II} polymorphisms contribute to its low immunogenicity, the influence of host genetic variation has been underestimated. Here, we investigated the contribution of genetic polymorphisms in the DARC receptor and in the HLA class II complex on the antibody responses against DBP_{II}. Additionally, we have characterized the new *P. vivax* protein EBP2, including its binding properties and immunogenicity. For that, an open cohort study was carried out among 620 volunteers from a native Amazonian community (Rio Pardo, AM). The study design included five cross-sectional surveys, three carriedout during the first follow-up year (baseline, 6 and 12 months), and two at 6 and 7 years later. Common *DARC* alleles (*FY*A, FY*B and FY*B*^{ES}) were genotyped by real-time PCR (polymerase chain reaction) and HLA class II allelic variants (DRB1, DQB1 and DQA1 loci) were genotyped by PCR-SSO (sequence-specific oligonucleotide primed PCR, with Luminex technology). Antibody responses were evaluated in all cross-sectional surveys by conventional serology (IgG ELISA-detected antibodies) or binding inhibitory assays (BIAbs in vitro assays). Taken together, the results showed that : *(i)* DARC variability influenced on the DBPII BIAbs response, with inhibitory antibodies found more frequent in heterozygous individuals carrying a DARC-silent allele (*FY*B*^{ES}); the ability of DARC polymorphisms to affect functional properties of DBP_{II} antibodies was detected during the follow-up period (12-months); *(ii)* the variability of HLA class II genes influenced in the development and persistence of DBPII immune responses, with different alleles and haplotypes influenced in both, conventional (IgG-ELISA detected) and functional (BIAbs) antibody responses. Modelling the structural effects of the HLA-DRB1 variants showed a number of differences in the peptide-binding groove, which may explain the differences in subject responses. Related to EBP2, *(iii)* we revealed that this protein binds preferentially to early DARC positive reticulocytes (CD71^{high}), and its antigenicity is distinct from DBP_{II}; *(iv)* in the study population, EBP2 ELISA-detected IG antibodies were more frequent that anti-DBPII antibodies. Of interest, six to seven years later, when malaria transmission had declined in the study area, the profile of EBP2 antibody response remained stable while DBP_{II} antibodies dropped significantly. In conclusion, we provided evidences that host variability may be critical on the development of DBP_{II}-based vaccines. In addition, EBP2 seems to be a promise vaccine candidate; besides being highly immunogenic in naturally exposed population, EBP2 may play a role in the invasion of immature Duffy-positive reticulocytes by *P.vivax.*

Key words: 1. Malária Vivax/transmission 2. *Plasmodium vivax* /parasitology 3. Humoral Immunity/genetics 4. *Duffy blood group*

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1 Introdução

A malária é uma doença causada por protozoários do filo Apicomplexa, família Plasmodiidae e gênero *Plasmodium.* Atualmente cinco espécies são responsáveis mais frequentemente pelo parasitismo humano: o *Plasmodium vivax,* o *Plasmodium falciparum,* o *Plasmodium malariae*, *Plasmodium ovale e o Plasmodium knowlesi*, onde este último, que infecta naturalmente primatas não humanos, têm causado infecções graves em humanos, principalmente no sudeste asiático e no continente africano (Cox-Singh e Singh, 2008; White *et al.*, 2008)

No Brasil, as espécies mais prevalentes, em ordem de importância, são o *Plasmodium vivax, P. falciparum e P. malariae*. Além disso, segundo a organização mundial de saúde, em 2015 foram registrados mais de 143.000 casos de malária (Siqueira *et al.*, 2016; Who, 2016), sendo que 99,6% desses casos foram registrados na região da Amazônia legal, composta pelos estados do Acre, Amazonas, Amapá, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins (Saúde, 2015). Embora a malária ainda seja um grave problema de saúde pública no Brasil, na última década, os casos da doença diminuíram cerca de 70% (Saúde, 2015; Siqueira *et al.*, 2016). Isso se deve, provavelmente, à intensificação das ações de controle da doença, tais como diagnóstico precoce e tratamento dos casos (Tauil, 2011).

Do ponto de vista do controle, a infecção pelo *P. falciparum* tem recebido nos últimos anos significante quantidade de investimentos, o que resultou em um tratamento eficiente (Coartem, Novartis) e em uma vacina em estágio avançado de ensaios clínicos (Revisto por Agnandji *et al.*, 2015; Mahmoudi e Keshavarz, 2017). Por outro lado, o *P. vivax*, apesar de ser menos patogênico, continua apresentando grandes desafios para os programas de controle. Certos fatores relacionados à biologia do parasito contribuem para isto, incluindo: i) formas latentes no fígado (hipnozoítos), responsáveis pelas recaídas da doença (Coatney, 1976; Krotoski, 1989); ii) formação precoce de gametócitos, o que favorece a infecção dos vetores (Mckenzie *et al.*, 2002; Collins *et al.*, 2004); iii) invasão apenas de uma subpopulação de eritrócitos, os reticulócitos, que estão presentes na circulação em baixa percentagens resultando em parasitemias subpatentes (Revisto por Cheng *et al.*, 2015). Além disso, *o P. vivax* tem uma distribuição global, estando presente na

maioria dos continentes (Revisto por Olliaro *et al.*, 2016). Em conjunto, esses fatores contribuíram para uma inversão na incidência mundial dos casos de malária, com diminuição de *P. falciparum* e aumento do *P. vivax.* No Brasil, até os anos 90, a prevalência das duas espécies era similar, enquanto o *P. malariae* se mantinha em níveis muito baixos (menos de 1%) (Loiola *et al.*, 2002; Siqueira *et al.*, 2016). Na última década, embora a incidência dos casos de ambas as espécies tenham diminuído, o *P. vivax* tornou-se a espécie predominante, com mais de 80% dos casos registrados no país. Deste modo, no contexto atual de possibilidade de eliminação da malária em algumas regiões do Brasil (Tauil, 2011), o estudo do *P. vivax* passou a ser considerado prioridade no Brasil e no mundo.

1.1 Ciclo biológico dos parasitos da malária humana

Os plasmódios possuem um ciclo heteróxeno, ou seja, necessitam necessariamente de um hospedeiro vertebrado e outro invertebrado. A fase de reprodução sexuada ou esporogônica ocorre no hospedeiro invertebrado, enquanto a fase assexuada ou esquizogônica ocorre no hospedeiro vertebrado (Figura 1).

Os esporozoítos, formas infectantes do parasito, penetram na pele por meio da picada da fêmea do mosquito do gênero *Anopheles* durante o repasto sanguíneo (Amino *et al.*, 2006). Após migrarem no tecido subcutâneo, por períodos variados, podem alcançar o sistema linfático, rota que parece importante para estimular o sistema imune (Amino *et al.*, 2006). Apenas aqueles esporozoítos que escapam do sistema imune vão atingir a circulação sanguínea e migrar para o fígado, dando continuidade ao ciclo de infecção do hospedeiro vertebrado.

Na malária de mamíferos, pelo menos duas vias parecem ser utilizadas pelos esporozoítos para sair dos capilares sinusóides e invadir o parênquima hepático, uma envolve atravessar as células de Kupffer (Mota *et al.*, 2001; Pradel e Frevert, 2001) e outra as células endoteliais dos capilares sinusóides (Tavares *et al.*, 2013). Uma vez no fígado, o parasito tem a capacidade de migrar por meio dos hepatócitos, até por fim se instalar e se desenvolver em uma das célula hepática (Mota *et al.*, 2001). Além disso, no fígado, o *P. vivax* e o *P.ovale* podem se desenvolver em formas dormentes, denominadas hipnozoítos, que são responsáveis pelas recaídas que ocorrem meses ou anos após a infecção inicial (Coatney, 1976; Krotoski, 1985; 1989).

Nos hepatócitos, os esporozoítos se diferenciam e multiplicam assexuadamente dando origem aos merozoítos, que são liberados na corrente sanguínea por meio de vesículas denominadas merossomos, repletos de merozoítos (Sturm *et al.*, 2006). Ao cair na circulação sanguínea, os merozoítos penetram nos eritrócitos, dando início à fase eritrocítica do ciclo, responsável pela sintomatologia clínica da doença. Uma vez nos eritrócitos os merozoítos se diferenciam em trofozoítos imaturos, maduros e por fim esquizontes. Os esquizontes geram merozoítos que, por sua vez, penetram em outros eritrócitos, reiniciando o ciclo eritrocítico.

Após algumas gerações de merozoítos sanguíneos, alguns deles se diferenciam nas formas sexuais do parasito, os macrogametócitos (feminino) e os microgametócitos (masculinos), os quais amadurecem sem sofrer divisão celular. Estas formas sexuais são ingeridas pelo mosquito do gênero *Anopheles* durante o repasto sanguíneo dando início à fase sexuada do ciclo, que ocorre no interior do hospedeiro invertebrado.

No trato digestivo do mosquito, os gametócitos se diferenciam em gametas masculinos e femininos, e dão origem ao zigoto (Vlachou *et al.*, 2006). Quando o zigoto se desloca em movimentos amebóides, ele é denominado oocineto e, por um mecanismo trans-celular, atinge a camada de células epiteliais do intestino médio onde se aloja entre o epitélio e a membrana basal e passa então a ser denominado oocisto (Zieler e Dvorak, 2000; Baton e Ranford-Cartwright, 2004; Vlachou *et al.*, 2004). A maquinaria de replicação de DNA e de síntese de proteínas está ativada no oocisto permitindo a multiplicação assexuada dos parasitos, e a produção milhares de esporozoítos. Os esporozoítos, por sua vez invadem a hemolinfa do inseto e muitos conseguem invadir as glândulas salivares, podendo, num novo repasto sanguíneo, serem inoculados no hospedeiro vertebrado.

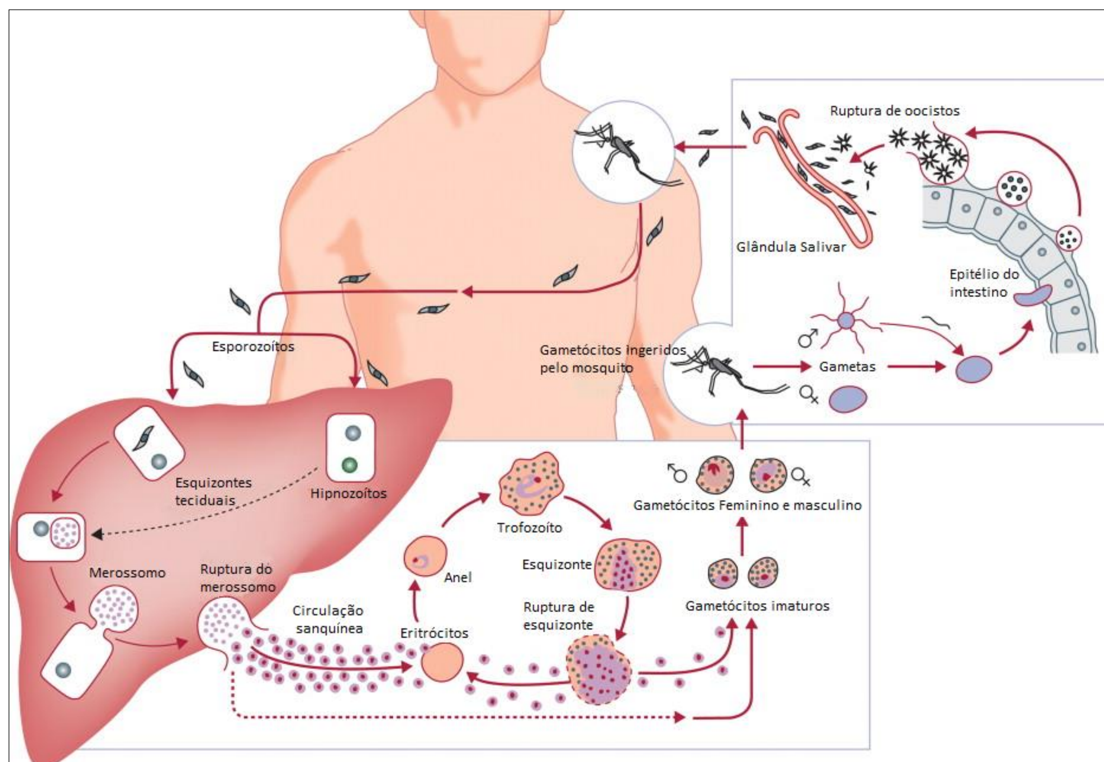

Figura 1 Representação esquemática do ciclo biológico do plasmódio no hospedeiro humano e no vetor Anopheles. Fonte: Modificado de Mueller *et al.*, 2009

1.2 Invasão dos eritrócitos humanos pelo *P. vivax*

A invasão dos eritrócitos pelos parasitos da malária é a parte do ciclo que garante o sucesso da infecção humana e, consequentemente, o desenvolvimento da doença clínica. O processo de invasão é complexo, rápido e envolve várias etapas, como demonstrado na Figura 2.

Em contraste com o *P. falciparum,* que utiliza várias vias de invasão, as quais envolvem vários ligantes do parasito e diferentes receptores no eritrócito (Revisto por Wright e Rayner, 2014; Lelliott *et al.*, 2015), o *P. vivax* utiliza uma via principal de invasão dos reticulócitos (Adams *et al.*, 1992; Lelliott *et al.*, 2015). Sendo assim, o *P. vivax* invade preferencialmente, se não exclusivamente, os reticulócitos (eritrócitos imaturos) e, até o momento, a única via bem caracterizada é mediada pela *Duffy Binding Protein* (DBP), presente nos micronemas do parasito, e seu receptor nos eritrócitos, o antígeno do grupo sanguíneo Duffy/receptor de quimiocinas (DARC) (Miller *et al.*, 1976; Wertheimer e Barnwell, 1989).

Uma vez estabelecida a ligação entre a PvDBP e o DARC o processo de invasão se torna irreversível (Wertheimer e Barnwell, 1989). Dessa forma, indivíduos que não apresentam o receptor na superfície de seus reticulócitos são altamente resistentes à infecção pelo *P. vivax* (Miller *et al.*, 1976). Entretanto, a completa proteção gerada pela ausência do DARC nas células hospedeiras tem sido questionada, uma vez que estudos recentes vêm demonstrando indivíduos DARC negativos parasitados pelo *P. vivax*, o que sugere que este parasito possa utilizar uma via alternativa para invasão dos eritrócitos (Ryan *et al.*, 2006; Cavasini *et al.*, 2007; Menard *et al.*, 2010; Lo *et al.*, 2015).

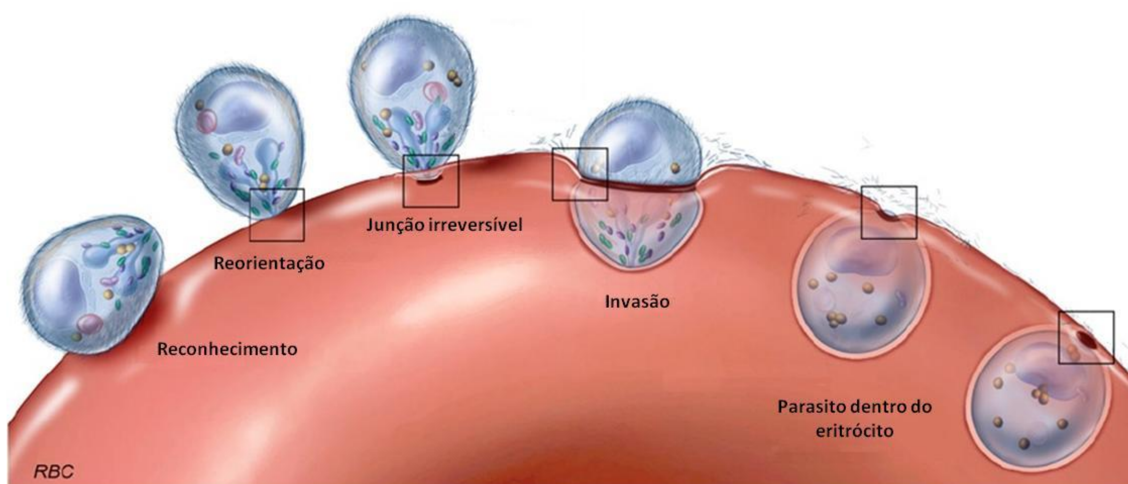

Figura 2 Figura esquemática representando o processo de invasão dos plasmódios. Inicialmente, após encontrar um eritrócito, o merozoíto se adere à superfície do mesmo por meio de ligações de baixa afinidade e reversíveis (reconhecimento); após o reconhecimento, o parasito se reorienta e posiciona sua porção apical perpendicular à superfície do eritrócito e, assim, ocorre uma junção irreversível; após formação da junção, a mesma é deslocada em direção ao pólo posterior do parasito e ao mesmo tempo são liberadas proteínas de adesão situadas em suas organelas apicais (roptrias e micronemas); por fim, o parasito alcança o interior da célula por meio da formação do vacúolo parasitófogo, onde ocorre o desenvolvimento do parasito (Modificado de Srinivasan *et al.*, 2011).

1.3 Proteínas envolvidas no processo de invasão do reticulócito pelo *P. vivax*

Nos últimos anos, vários estudos têm demonstrado várias proteínas envolvidas durante todo o processo de invasão dos reticulócitos, desde a etapa inicial de reconhecimento até o momento final da invasão (Revisto por Beeson *et al.*, 2016). Entretanto, apenas duas proteínas parecem estar diretamente relacionadas à formação da junção irreversível, são elas: a PvDBP (Revisto por Beeson *et al.*, 2016) e a proteína recém descrita EBP2 (Hester *et al.*, 2013).

1.3.1 A Duffy binding protein do Plasmodium vivax (PvDBP)

Como a invasão dos reticulócitos pelo *P. vivax* é altamente dependente da interação entre a DBP e o DARC *(Miller et al., 1976; Adams et al., 1992)*, a ausência desta interação em indivíduos DARC-negativos confere uma elevada proteção contra a infecção (Miller *et al.*, 1976). Assim, esta proteína é a principal candidata a compor uma vacina contra o *P. vivax*. De fato, atualmente, a DBP é o único antígeno de forma sanguínea em fase de triagem clínica (De Cassan *et al.*, 2015; Tham *et al.*, 2016). Em contraste com o *P. falciparum* que atualmente possui vários antígenos em ensaios clínicos (Figura 3) (Revisto por Tham *et al.*, 2016).

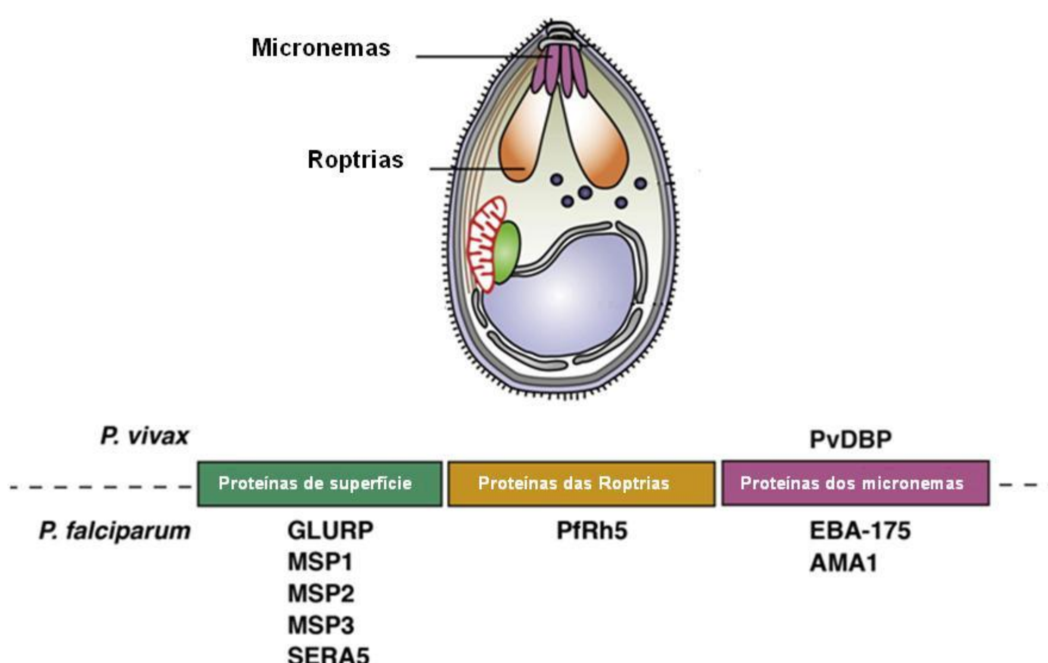

Figura 3 Representação esquemática do merozoíto dos plasmódios ilustrando as principais proteínas de fase sanguínea candidatas a vacina. Os antígenos do *P. vivax* e de P. falciparum que se encontram em ensaios clínicos estão demonstrados de acordo com suas respectivas localizações (Modificado de Tham *et al.*, 2016).

A PvDBP é uma proteína de 140 kDa localizada na porção apical do merozoíto em organelas conhecidas como micronemas (Wertheimer e Barnwell, 1989; Adams *et al.*, 1990; Adams *et al.*, 1992). Esta proteína pertence à família de proteínas homólogas que se ligam aos eritrócitos conhecida como DBL-EBP (*Duffy binding like-erythrocyte binding protein*). Nesta família estão incluídas outras proteínas como, por exemplo, a EBP do *Plasmodium knowlesi* e diferentes proteínas do *P. falciparum,* incluindo a EBA-175 e EBA/BAEBL (Sim *et al.*, 1990; Gilberger *et al.*,

2003; De Sousa *et al.*, 2014). Os membros desta família apresentam similaridades na estrutura do gene e, de acordo com esta homologia, foram definidas seis regiões extracelulares, uma região transmembrana e uma pequena cauda citoplasmática (Figura 4)(Adams *et al.*, 1992; Adams *et al.*, 2001).

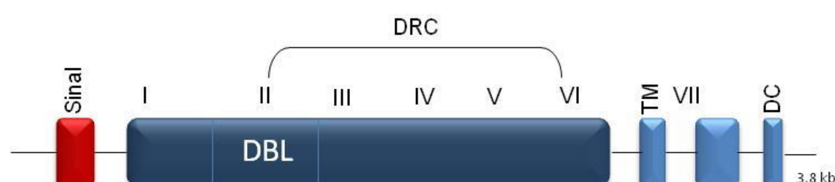

Figura 4 Representação esquemática dos domínios estruturais da proteína *Duffy Binding Protein* do *P. vivax* (DBP). A proteína possui seis regiões de domínios extracelulares (I-VI) e a última região (VII) possui um domínio citoplasmático (DC) e transmembrana (TM). O domínio rico em cisteína (DRC) compreende as regiões II-VI. No domínio II encontra-se a região referente ao ligante (DBL) (Adaptado de De Sousa *et al.*, 2014).

O ligante funcional da PvDBP encontra-se na região II (DBP_{II}), que corresponde ao domínio DBL. Este domínio compreende 330 aminoácidos contendo 12 resíduos de cisteína, onde foi mapeado o sítio de ligação da proteína ao seu receptor nos eritrócitos do hospedeiro. Este domínio está localizado em um segmento de aproximadamente 170 aminoácidos entre as cisteínas 4 e 7, dentro da região II (Adams *et al.*, 1992; Ranjan e Chitnis, 1999; Vanbuskirk, Sevova*, et al.*, 2004; Sampath *et al.*, 2013).

1.3.2 Erythrocyte binding protein 2 do Plasmodium vivax (EBP2)

Embora a maioria das variantes do *P. vivax* utilizem *a* interação PvDBP/ DARC para invadir o reticulócito, estudos recentes tem demonstrando que indivíduos que não expressam o receptor DARC na superfície de suas células eritrocitárias podem se infectar pelo *P. vivax* (Ryan *et al.*, 2006; Cavasini *et al.*, 2007; Menard *et al.*, 2010; Lo *et al.*, 2015). Embora este fato sugira fortemente que este parasito utiliza uma rota alternativa para a invasão dos eritrócitos/reticulócitos, nenhuma outra via de invasão foi caracterizada até o momento (Revisto por Zimmerman *et al.*, 2013).

Por outro lado, um estudo recente publicado por Hester *et al.* (2013) descreveu uma gama de novos genes que não haviam sido descritos por ocasião da

publicação do genoma completo da cepa de referência Sal-1 do *P. vivax* (Carlton *et al.*, 2008). Neste estudo, Hester e colaboradores reanotaram o genoma do *P. vivax,* porém, desta vez utilizando sequências de DNA de parasitos de área endêmica. Entre os vários novos genes descritos está o gene de uma proteína hipotética, a *Erythrocyte Binding Protein 2* (EBP2), que possui características semelhantes às proteínas da família DBL/EBP, que se caracterizam pelo envolvimento no processo de invasão dos merozoítos na célula hospedeira (Hadley, 1986; Adams *et al.*, 1990; Adams *et al.*, 1992). Vale ressaltar que Hester *et al.* (2013) demonstraram que esta proteína é expressa durante o estágio sanguíneo do ciclo do parasito, o que sugere um possível papel desta durante o processo de invasão dos reticulócitos.

Análises na sequência do gene da EBP2 demonstraram que esta proteína, assim como a PvDBP e todas as outras incluídas na família DBL/EBP, possui: (I) domínio DBL que está relacionado ao processo de invasão eritrocitário; (II) peptídeo sinal; (III) domínio C-terminal rico em cisteínas e; (IV) uma porção transmembrana (Figura 5). Além disso, quando comparada a PvDBP, o gene desta proteína é um pouco menor (2,4 kb versus 3,8 kb), e menos polimórfico, com apenas 11 sítios polimórficos, enquanto a PvDBP possui 32 (Hester *et al.*, 2013).

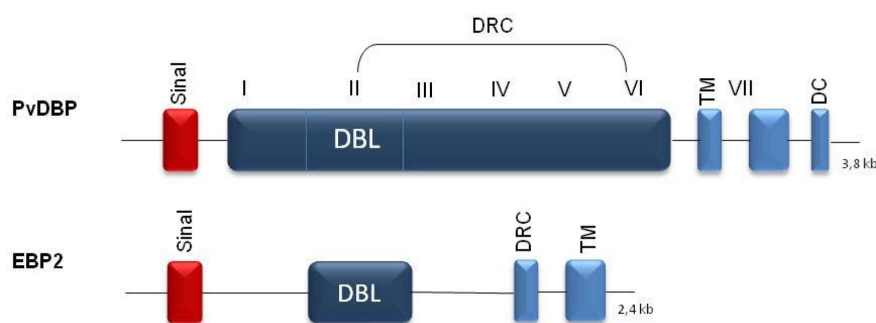

Figura 5 Comparação entre os domínios da proteína predita EBP2 e o gene da proteína PvDBP. Em vermelho está o peptídeo sinal; em azul escuro, o domínio Duffy Binding Like (DBL); em azul claro, o domínio C-terminal rico em cisteína (DCR) e o dominio transmembrana (TM) (Modificado de Hester *et al.*, 2013).

Em análise filogenética, a EBP2 se agrupa apenas com uma EBP de *P. cynomolgi* (Figura 6), sendo, portanto filogeneticamente diferente da DBP do *P. vivax.* Estes dados comprovaram que esta proteína é claramente diferente da maioria das outras DBPs de *Plasmodium* já descritas. Sendo assim, por possuir todas as características chave das proteínas que se ligam ao eritrócito (EBPs) e ser filogeneticamente distinta de DBP do *P. vivax*, tem sido sugerido que a EBP2 possa ter uma função distinta daquela exercida pela PvDBP. Deste modo, a EBP2 constitui

uma forte candidata a participar da invasão dos reticulócitos pelo *P. vivax*, mais especificamente entre aqueles indivíduos DARC-negativos.

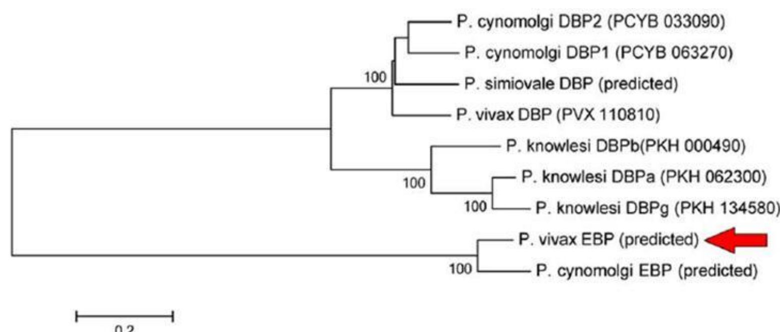

Figura 6 Árvore filogenética demonstrando a relação entre as sequências das proteínas EBPs de *P. vivax*, *P. cynomolgi*, *P. simiovale* e *P. knowlesi*. A posição da EBP2 predita está indicada pela seta vermelha (Modificado de Hester *et al.*, 2013).

1.4 Resposta imune naturalmente adquirida contra a PvDBP, principal antígeno candidato à vacina contra o *P. vivax*

1.4.1 Anticorpos anti-PvDBP detectados pela sorologia convencional

Apesar de a PvDBP ser considerada o principal antígeno candidato a compor uma vacina contra o *P. vivax,* o desenvolvimento desta vacina possui um número significativo de desafios, que incluem: i) o rápido processo de invasão dos reticulócitos pelos merozoítos, em média 30s após o primeiro contato com a célula hospedeira (Gilson e Crabb, 2009); ii) exposição reduzida da PvDBP ao sistema imune, uma vez que a proteína está localizada nos micronemas e somente é exposta no momento da invasão (Adams *et al.*, 1990); iii) alto grau de polimorfismos na região do ligante (DBPII) da proteína, os quais parecem estar relacionados à evasão do parasito ao sistema imune (Vanbuskirk, Cole-Tobian*, et al.*, 2004).

Para o desenvolvimento de uma vacina ou de medidas que visam bloquear a invasão dos eritrócitos, especialmente no caso do *P. vivax*, é necessário entender melhor a resposta imune de indivíduos que são naturalmente expostos ao parasito (Maestre *et al.*, 2010). Atualmente, a maioria dos estudos tem focado em medir

anticorpos contra antígenos recombinantes, já que anticorpos anti-PvDBP parecem estar envolvidos na proteção clínica (Nicolete *et al.*, 2016). Sendo assim, a sorologia convencional (ELISA) tem sido amplamente utilizada a fim de avaliar os níveis de anticorpos IgG em diferente populações. De fato, na região amazônica, alguns trabalhos, incluindo aqueles desenvolvidos pelo nosso grupo de pesquisa em malária, demonstraram que a PvDBP é naturalmente imunogênica, sendo a resposta de anticorpos dependente dos níveis de exposição à malária por *P. vivax* (Ceravolo *et al.*, 2005; Tran *et al.*, 2005; Barbedo *et al.*, 2007; Ceravolo *et al.*, 2008; Souza-Silva *et al.*, 2010; Kano *et al.*, 2012).

Vale destacar que, até o momento, poucos estudos têm avaliado a resposta anti-PvDBP na América Latina e os dados permanecem restritos às áreas endêmicas do Brasil e da Colômbia (Revisto por De Sousa *et al.*, 2014). Nestas áreas, os resultados tem confirmado a baixa imunogenicidade da PvDBP, já que uma frequência relativamente alta de indivíduos com história de longa-exposição ao *P. vivax* (variando de 30 a 70%) não desenvolvem anticorpos anti-PvDBP (Revisto por De Sousa *et al.*, 2014). Porém, em regiões de alta endemicidade como a Papua Nova Guinea (PNG), a resposta de anticorpos anti-PvDBP, como detectada pela sorologia convencional parece ser mais frequente (aproximadamente 70 a 90% de resposta), embora dependente do tempo de exposição à malária (Fraser *et al.*, 1997; Xainli *et al.*, 2003; King *et al.*, 2008; Cole-Tobian *et al.*, 2009).

De relevância, um estudo realizado pelo nosso grupo de pesquisa em malária, em área não endêmica, demonstrou que indivíduos expostos pela primeira vez ao *P. vivax* desenvolvem anticorpos anti-PvDBP que são de curta duração e variante-específicos (Cerávolo *et al.*, 2009). Estes resultados foram posteriormente confirmados por outros autores (Chootong *et al.*, 2012). Esses achados confirmam que o desenvolvimento de uma vacina contra a PvDBP vai depender de desafios, incluindo a baixa imunogenicidade da proteína em áreas como a Amazônia brasileira, onde cada ano de exposição aumenta em apenas 2% a chance de desenvolver anticorpos específicos contra a proteína (Souza-Silva *et al.*, 2010).

Como anticorpos detectados pela sorologia convencional podem não ser bloqueadores da invasão, isto é, impedir a interação entre a PvDBP (região ligante, DBPII) e seu receptor DARC presente na superfície dos eritrócitos (Revisto por De

Sousa *et al.*, 2014), fazem-se necessários estudos para avaliar a funcionalidade dos anticorpos naturalmente adquiridos. Infelizmente, os dados referentes às propriedades funcionais destes anticorpos ainda são limitados (Ceravolo *et al.*, 2008; King *et al.*, 2008; Souza-Silva *et al.*, 2010; Chootong *et al.*, 2012). Esse fato se deve, principalmente, pela complexidade dos ensaios funcionais *in vitro*, já que o cultivo do *P. vivax* ainda apresenta muitos desafios (revisto por Udomsangpetch *et al.*, 2008). Os ensaios atualmente disponíveis utilizam culturas de curta duração do parasito (Grimberg *et al.*, 2007) ou, mais comumente, sistemas indiretos incluindo a expressão do ligante (DBP_{II}) em células de mamíferos transfectáveis (Chitnis e Miller, 1994) ou produção do receptor DARC recombinante em ensaios sorológicos (Shakri *et al.*, 2012).

1.4.2 Anticorpos inibitórios da interação ligante (DBP_{II}) – receptor (DARC)

O objetivo de uma vacina utilizando a DBP_{II} é induzir anticorpos que sejam capazes de impedir a interação entre merozoitos de *P. vivax* e o receptor DARC presente na superfície dos reticulócitos, e, consequentemente, bloquear a invasão (Revisto por Tham *et al.*, 2016). Estudos realizados com indivíduos naturalmente expostos ao *P. vivax*, em diferentes condições de transmissão, tem confirmado ser necessário um longo período de exposição ao parasito para que parte da população possa adquirir anticorpos capazes de bloquear a interação entre DBP_{II}-DARC (Michon *et al.*, 2000; Ceravolo *et al.*, 2008; King *et al.*, 2008; Souza-Silva *et al.*, 2010). De fato, na Amazônia brasileira estes anticorpos inibitórios (BIAbs, binding-inhibitory antibodies) puderam ser detectados em apenas um terço da população com história de longa-exposiçao ao parasito (De Sousa *et al.*, 2014). O mesmo tipo de perfil tem sido observado em áreas hiperendemicas de transmissão, como a Papua Nova Guinea (King *et al.*, 2008). Vale ressaltar que existe apenas moderada associação entre a presença de anticorpos detectados pela sorologia convencional e a resposta de BIAbs (Cerávolo *et al.*, 2008; Souza-Silva *et al.*, 2010).

As razões para a baixa resposta de anticorpos inibitórios contra a DBP_{II} não são conhecidas, entretanto, o fato da proteína se localizar nos micronemas pode contribuir para pouca exposição ao sistema imune. Além disso, a variabilidade genética da PvDBP, principalmente os polimorfismos da região II, parecem

influenciar na resposta de anticorpos inibitórios(Mchenry e Adams, 2006; Sousa *et al.*, 2010). Neste contexto, nosso grupo e outros demonstraram que a resposta imune inibitória contra a DBP_{II} depende da cepa do parasito (Ceravolo *et al.*, 2008; Chootong *et al.*, 2012). Apesar disto, existem evidências de que indivíduos expostos por longos anos ao parasito desenvolvam uma resposta de anticorpos inibitórios que independe da cepa do parasito (King *et al.*, 2008; Souza-Silva *et al.*, 2010). Esses achados demonstram a complexidade da resposta imune anti-DBP_{II}, que envolve tanto anticorpos cepa-específicos quanto anticorpos de ampla reatividade (Cole-Tobian *et al.*, 2009).

Enquanto aspectos relacionados à variabilidade genética da DBP_{II} estão melhor estudados (Nobrega De Sousa *et al.*, 2011), aspectos relacionados ao hospedeiro vertebrado ainda são pouco explorados (Maestre *et al.*, 2010; Storti-Melo *et al.*, 2012). Estes achados fazem com que seja relevante estudar a influência da variabilidade genética do hospedeiro vertebrado na resposta contra a PvDBP, já que a resposta imune contra este antígeno é multifatorial, dependendo tanto de aspectos do parasito quanto do hospedeiro vertebrado.

1.5 Influência de polimorfismos genéticos do hospedeiro vertebrado na resposta imune contra a PvDBP

Embora o receptor DARC seja essencial para o processo de invasão do reticulócitos pelo *P. vivax,* pouco de sabe ainda sobre a influência deste receptor na resposta imune contra a PvDBP (Maestre *et al.*, 2010; King *et al.*, 2011), o que faz com que estudos desta natureza sejam necessários.

Além disso, considerando a variabilidade genética humana, as variações encontradas no antígeno leucocitátio humano (HLA), particularmente no HLA classe II, podem influenciar na resposta imune humoral contra diferentes antígenos sintéticos e/ou recombinantes de *P. vivax* (Banic *et al.*, 2002; Oliveira-Ferreira *et al.*, 2004). Entretanto, até o momento, apenas Storti-Melo *et al.* (2012) avaliaram a influência destes antígenos no desenvolvimento de resposta contra a PvDBP. Assim, estudos para definir a hereditabilidade da resposta imune a DBPII são prioritários.

1.5.1 Antígeno de grupo sanguíneo Duffy/Receptor de Quimiocinas (DARC)

O receptor atípico de quimiocinas 1 (ACKR1, Atypical Chemokine Receptor 1), mais amplamente conhecido como antígeno de grupo sanguíneo DARC é também um receptor para vários membros de quimiocinas das classes CC, CXCs, e foi descrito pela primeira vez por Moore *et al.* (1982). Essa glicoproteína de 40-45 KDa não é restrita a células da linhagem eritrocítica, podendo ser encontrada também em células endoteliais de vênulas pós-capilares de vários tecidos. O gene que codifica o antígeno DARC está localizado no *locus* FY do cromossomo 1 (Figura 7), sendo caracterizado por três alelos principais: *FY*A, FY*B* e *FY*B*^{ES} (*ES*, silenciado na linhagem eritrocítica) (Hadley e Peiper, 1997). Os dois alelos funcionais codominantes, *FY*A e FY*B*, codificam os antígenos Fy^a e Fy^b, respectivamente. Esses alelos se diferem apenas em uma mutação na posição 125 do exon 2 que é responsável pela substituição de uma glicina em Fy^a por um ácido aspártico em Fy^b (Chaudhuri *et al.*, 1995; Iwamoto *et al.*, 1995; Mallinson *et al.*, 1995). Estes antígenos irão definir os fenótipo Fy(a+b-), Fy(a-b+) e Fy(a+b+), sendo que a completa ausência de expressão desses antígenos na superfície dos eritrócitos define o fenótipo DARC negativo Fy(a-b-). Esse último é caracterizado por dois alelos não funcionais *FY*B*^{ES}, cujo silenciamento ocorre devido a uma mutação na região promotora que impede a ligação do fator de transcrição ao promotor GATA1 mutado, não alterando a expressão dessa proteína em outros tecidos (Tournamille *et al.*, 1995). O genótipo *FY*A*^{ES} também pode aparecer caso a mutação no promotor seja no alelo *FY*A,* porém este genótipo é extremamente raro e foi melhor caracterizado na Papua Nova Guiné (Zimmerman *et al.*, 1999).

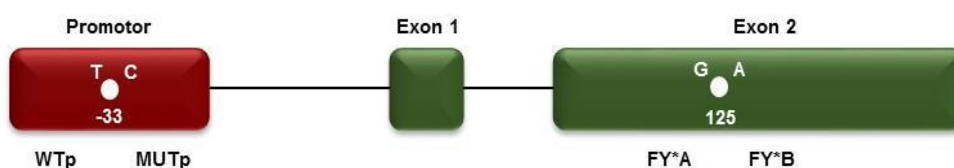

Figura 7 Representação esquemática do gene *FY* que codifica DARC, incluindo a região promotora, dois exons (1 e 2) e o íntron entre os éxons. Na figura: a mutação T-33C no promotor, que diferencia o promotor selvagem (WT) do promotor mutado (MUT); a mutação G125A, no exon 2, que define os alelos FY*A e FY*B, respectivamente (Adaptado de Cavasini *et al.*, 2001).

Com relação à expressão do receptor DARC na superfície dos eritrócitos, têm-se observado que a expressão de DARC varia de acordo com a idade dos eritrócitos, sendo maior nos reticulócitos do que em eritrócitos maduros (Woolley *et al.*, 2000). Além disso, estudos têm demonstrado expressão reduzida desse antígeno nos eritrócitos de indivíduos heterozigotos para a mutação T-33C (alelos FY^*B^{ES} e FY^*A^{ES}). Esses indivíduos expressavam aproximadamente duas vezes menos DARC na superfície dos eritrócitos do que os indivíduos homozigotos para o promotor não mutado (alelos *FY*A, FY*B*) (Zimmerman *et al.*, 1999; Woolley *et al.*, 2000; Yazdanbakhsh *et al.*, 2000). Vale ressaltar que esta diminuição pode estar envolvida na suscetibilidade do indivíduo em se infectar pelo *P. vivax* (Michon *et al.*, 2001; King *et al.*, 2011).

Para elucidar as bases moleculares e o mecanismo de reconhecimento do receptor DARC pela PvDBP, Batchelor *et al.* (2011) desenvolveram um estudo da interação entre a Região II da PvDBP (RII-PvDBP) e a parte N-terminal de DARC. O estudo demonstrou que o sítio de ligação do receptor na RII-PvDBP é formado apenas após a dimerização desta proteína. Os autores demonstraram ainda que esta dimerização é essencial para a posterior dimerização do receptor DARC, sendo o complexo final composto por duas moléculas da PvDBP e duas moléculas do receptor DARC (Figura 8). Deste modo, a PvDBP é liberada das organelas apicais na forma de monômeros sendo os dímeros formados apenas no momento da ligação irreversível ao seu receptor DARC.

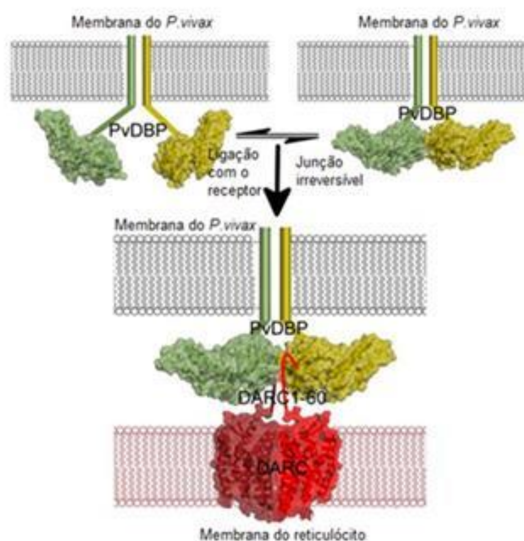

Figura 8 Representação esquemática entre a PvDBP e seu receptor DARC na superfície do eritrócito. Interação entre duas moléculas PvDBP e duas do receptor DARC (modelo predito de dimerização do ligante). Os monômeros da PvDBPII estão em verde e amarelo. O homodímero de DARC é representado pela estrutura em tons de vermelho. A membrana do merozoito do *P. vivax* está representada em preto enquanto a membrana do reticulócito está representada em vermelho (Adaptado de Batchelor *et al.*, 2011).

1.5.2 Antígeno Leucocitário Humano de Classe II (HLA II)

As moléculas de HLA são glicoproteínas de superfície celular heterodiméricas que determinam a natureza humoral ou celular das respostas imunes. As moléculas de HLA exibem antígenos peptídicos em superfícies celulares para ativação de células T e são necessários para o reconhecimento de antígenos pelas células T (Revisto por Afridi *et al.*, 2016).

Em humanos, o sistema HLA ocupa uma região de aproximadamente 4 megabases (Mb) no braço curto do cromossomo 6 (Figura 9). Estes genes são divididos em três regiões genômicas ou classes (I, II e III) (Revisto por Lima-Junior Jda e Pratt-Riccio, 2016). Os complexos gênicos de classe I e II contém os chamados “genes clássicos" sendo responsáveis por respostas imunes específicas. Por outro lado o complexo de classe III incluem “genes não-clássicos” que codificam diferentes produtos incluindo proteínas do soro humano (Revisto por Afridi *et al.*, 2016).

As regiões do HLA diretamente envolvidas na resposta imune tem função distintas, sendo as moléculas HLA de classe I responsáveis pela ativação de células

T CD8+, enquanto as moléculas MHC de classe II ativadoras das células T CD4+. Em condições fisiológicas, as moléculas MHC de classe II são expressas apenas em células apresentadoras de antígenos (células B, macrófagos e células dendríticas), sendo este processo fundamental para o desenvolvimento de uma resposta imune humoral (Revisto por Mcfarland e Beeson, 2002). De importância para o presente trabalho, os genes do complexo de MHC classe II – que codificam as moléculas de HLA classe II envolvidas na resposta humoral – são subdivididos em diferentes *loci*, sendo três considerados clássicos (HLA-DR, HLA-DQ, HLA-DP) e, dois *loci* não clássicos (HLA-DM e HLA-DO) (Figura 8).

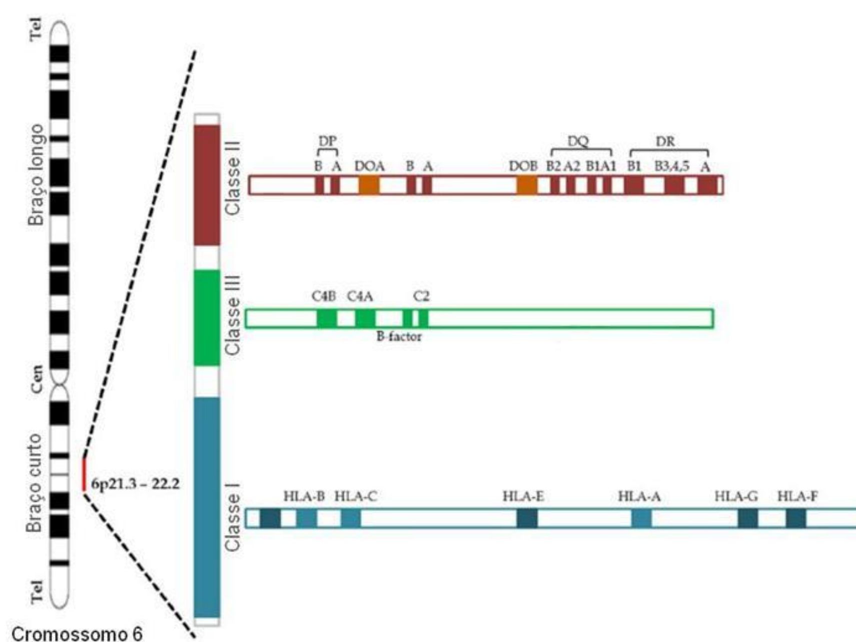

Figura 9 Representação do cromossomo 8 humano com ênfase nos principais genes de HLA. Estão representados os genes do HLA: em vermelho, classe II (DP, DO, DQ e DR); em verde, classe III (C4B, C4A E C2); e em azul, classe I (HLA-B, HLA-C, HLA-E, HLA-A, HLA-G e HLA-F) (Adaptado de Lima-Junior Jda e Pratt-Riccio, 2016).

O papel de moléculas de HLA na susceptibilidade às doenças auto-imunes já está bem estabelecido, incluindo por exemplo, a associação entre DQ2.2 e DQ2.5 e o aumento do risco para doença celíaca (Almeida *et al.*, 2016) e a associação entre HLAB-27 (Classe I) e a predisposição espondilite anquilosante (Brewerton *et al.*, 1973; Mathieu *et al.*, 2008). No caso da malária, vários estudos observaram associações entre alelos de HLA e susceptibilidade e/ou resistência à doença clínica (Revisto por Lima-Junior Jda e Pratt-Riccio, 2016); este é o caso, por exemplo, da associação entre HLAB53 e malária cerebral por *P.falciparum* (Hill *et al.*, 1992). Em

relação aos HLA classe II, inúmeros genes também já foram associados à susceptibilidade e ou proteção contra a malária grave (Hill *et al.*, 1991; May *et al.*, 1999; May *et al.*, 2001). De fato, em diferentes regiões do mundo onde a doença é hiper ou holo-endemica, a malária tem exercido uma enorme pressão seletiva sobre as moléculas de HLA, sendo responsável por modificar o perfil alélico deste complexo em diferentes partes do mundo (Revisto por Garamszegi, 2014).

Na malária, embora as moléculas de HLA tenham sido mais bem estudadas do ponto de vista da doença clínica, particularmente para o *P. falciparum*, espécie mais patogênica, a contribuição deste complexo na resposta imune específica ainda é pouco estudada. Neste contexto, de grande interesse seriam as moléculas de classe II, já que anticorpos têm papel fundamental na resposta imune direcionada às formas sanguíneas do parasito (Langhorne *et al.*, 2008). Neste sentido, estudos com candidatos vacinais demonstram que o HLA de classe II, principalmente HLA-DR, HLA-DQ e HLA-DP, influenciam na produção de anticorpos específicos (Patarroyo *et al.*, 1991; Oliveira-Ferreira *et al.*, 2004) (Patarroyo *et al.*, 1991; Oliveira-Ferreira *et al.*, 2004). Porém, a maioria dos dados relacionados à associação entre HLA classe II e anticorpos na malária foi obtida por meio de estudos transversais, o que pode ter levado a classificação errônea de indivíduos como respondedores (ou não respondedores), já que a resposta de anticorpos pode flutuar ao longo do tempo (Ceravolo *et al.*, 2009; Souza-Silva *et al.*, 2010). Assim, fazem-se necessários estudos longitudinais para que a associação entre HLA classe II e resposta imune possa ser melhor avaliada.

No caso da malária causada pelo *P. vivax*, poucos estudos têm relacionado a influência da variabilidade do HLA II na resposta imune humoral, sobretudo contra a PvDBP (Lima-Junior *et al.*, 2012; Storti-Melo *et al.*, 2012). Vale ressaltar que o único trabalho que avaliou os alelos do HLA II na resposta de anticorpos contra a PvDBP não observou nenhuma associação (Storti-Melo *et al.*, 2012). Entretanto, neste estudo foi incluído um número pequeno de indivíduos, o que inviabiliza conclusões definitivas, já que o HLA classe II é muito polimórfico. Sendo assim, a caracterização dos genótipos de HLA classe II em estudos longitudinais e de base populacional é fundamental para contribuir no entendimento da influência deste fator genético no desenvolvimento de uma resposta contra a PvDBP, principal antígeno candidato a vacina.

2 Justificativa

Nosso grupo de pesquisa em Biologia Molecular e Imunologia da Malária (BMIM/CPqRR) vem desenvolvendo há vários anos estudos que sugerem que a baixa resposta de anticorpos a PvDBP, particularmente a DBP_{II}, pode estar relacionada às características do parasito e do hospedeiro vertebrado (Ceravolo *et al.*, 2009; Kano *et al.*, 2012). Entre as características do parasito incluem-se (i) baixa imunogenicidade e/ou limitada exposição da PvDBP ao sistema imune, uma vez que esta proteína somente é exposta na superfície do parasito no momento exato da invasão; (ii) alto polimorfismo na região do ligante (região II), que resulta em uma imunidade do tipo cepa-específica (Ceravolo *et al.*, 2009). Entretanto, estas características do parasito não explicam o fato de que a maioria dos indivíduos expostos às diferentes variantes do parasito, por um longo período de tempo, não serem capazes de desenvolver anticorpos bloqueadores, isto é, aqueles capazes de inibir a interação do parasito (DBP_{II}) com a sua célula hospedeira (DARC). Com isso, aventa-se a hipótese que fatores genéticos do hospedeiro vertebrado estão contribuindo para esta baixa resposta à PvDBP.

Poucos estudos têm sido conduzidos com o objetivo de verificar a influência de fatores genéticos do hospedeiro vertebrado na resposta imune contra antígenos do *P. vivax* (Maestre *et al.*, 2010; Storti-Melo *et al.*, 2012). Deste modo, embora o receptor DARC seja essencial ao processo de invasão dos eritrócitos pelo merozoíto do *P. vivax*, a influência deste receptor na resposta imune permanece pouco estudada. Até o momento, apenas um estudo realizado na Colômbia avaliou a influência dos genótipos de DARC na aquisição de anticorpos naturalmente adquiridos contra a DBP_{II} (Maestre *et al.*, 2010). Embora o estudo colombiano sugerisse que o perfil de reconhecimento imune à PvDBP na sorologia convencional variou de acordo com o genótipo de DARC, os dados podem não ser conclusivos devido ao pequeno número de indivíduos estudados, e pela ausência de ensaios demonstrando que os anticorpos eram funcionais. Além disso, o estudo colombiano foi realizado em uma região onde a população estudada era pouco susceptível ao *P. vivax,* já que era constituída (na sua maioria) por voluntários afro-descendentes, ou seja, carreadores do fenótipo DARC-negativo que é altamente resistente ao *P. vivax*. Reforçando esta hipótese, estudos do nosso grupo tem demonstrado que a variabilidade genética dos parasitos que circulam na Colômbia é diferente de outras

áreas de transmissão de *P. vivax* do mundo, incluindo a Amazônia brasileira (Sousa *et al.*, 2010). Diante da relevância destes achados, torna-se essencial avaliar a influência do receptor DARC no desenvolvimento de uma resposta imune eficiente, particularmente, no desenvolvimento dos anticorpos bloqueadores da interação entre a DBP_{II} e seu receptor DARC presente na superfície da célula hospedeira.

Como as moléculas de HLA classe II podem influenciar na resposta de anticorpos, particularmente, no caso de vacinas baseadas em subunidades, como aquelas com antígenos recombinantes e/ou sintéticos, torna-se essencial avaliar a associação entre resposta imune contra a PvDBP e HLA classe II. Até o momento, poucos estudos foram realizados nesta direção (Banic *et al.*, 2002; Oliveira-Ferreira *et al.*, 2004; Lima-Junior *et al.*, 2012; Storti-Melo *et al.*, 2012). Diante disso, a genotipagem do HLA classe II em estudos longitudinais de base populacional pode contribuir para o entendimento da influência deste fator genético no desenvolvimento de uma resposta imune eficiente. Estudos nesta natureza podem futuramente auxiliar no aprimoramento de vacinas contra as formas sanguíneas do *P. vivax*.

Por último, embora o complexo DBP_{II}-DARC seja a principal via de invasão dos reticulócitos utilizada pelo *P. vivax*, sabe-se que outra via de invasão parece ser utilizada pelo parasito em indivíduos DARC-negativos *(Ryan et al., 2006; Cavasini et al., 2007; Menard et al., 2010)*. Neste contexto, a busca por outros ligantes do parasito que possam estar envolvidos neste processo parece essencial. Vale ressaltar que, recentemente, foi descrito uma nova proteína de formas sanguíneas do *P. vivax*, denominada aqui *Erythrocyte Binding Protein* 2 (EBP2) (Hester *et al.*, 2013), que possui características estruturais que sugerem um possível papel no processo de invasão celular do *P. vivax*. Assim, fazem-se necessários estudos que possam caracterizar esta proteína do ponto de vista funcional e imunológico.

3 Objetivos

3.1 Objetivo Geral

Determinar a influência da variabilidade genética do hospedeiro vertebrado, mais especificamente do receptor DARC e do complexo HLA Classe II, no desenvolvimento da resposta imune humoral contra as formas sanguíneas do *P. vivax*, com ênfase na *Duffy binding Protein* (PvDBP).

3.2 Objetivos específicos

Analisar a influência do polimorfismo genético do receptor eritrocítico DARC na resposta imune convencional e funcional à PvDBP;

Analisar a influência dos polimorfismos do HLA classe II (DQ, DR e DB) na resposta imune convencional e funcional à PvDBP;

Produzir a EBP2 recombinante e avaliar seu potencial imunogênico em modelo animal e em população humana naturalmente exposta ao *P. vivax*;

Testar *in vitro* a capacidade de interação entre a EBP2 e os reticulócitos, bem como com eritrócitos, por meio de ensaios funcionais.

4 Materiais e Métodos

4.1 Área e população de estudo

O estudo foi conduzido no assentamento agrícola de Rio Pardo (1º46’S, 1º54’S, 60º22’O, 60º10’O), localizado no município de Presidente Figueiredo, estado do Amazonas (Kano *et al.*, 2012). A localidade rural de Rio Pardo fica a aproximadamente 160 km de Manaus, com acesso pela rodovia (BR-174) que liga o estado do Amazonas ao estado de Roraima (Figura 10A). O assentamento agrícola foi oficialmente criado em 1996, pelo Instituto Nacional de Colonização e Reforma Agrária (INCRA), como parte dos grandes projetos de colonização da Amazônia focados na agricultura e ocupação humana da região (De Castro *et al.*, 2006). No assentamento de Rio Pardo, a população vive principalmente da agricultura de subsistência e pesca. Grande parte das habitações é considerada inadequada (sem paredes), aumentando assim a exposição ao mosquito vetor. Os serviços de saúde disponíveis são restritos, apenas um posto de diagnóstico de malária, administrado pelo município, fornece o diagnóstico e tratamento gratuito para os moradores da área. Na localidade, a temperatura média anual é de 31ºC, com clima úmido e precipitação média anual de 2.000 mm por ano. Existem duas estações bem definidas: um período chuvoso (Novembro a Maio) e um período de seca (Junho a Outubro). O assentamento é composto por sete áreas denominadas “ramais”: Principal, Samuel, Novo Paraíso, Gusmão, Terra Preta, Taxista e Novo Progresso - que inclui as famílias assentadas de ambos os lados de vias não pavimentadas. Além disto, tem uma população ribeirinha, que vive às margens do Igarapé de Rio Pardo (Figura 9). Nesta área, um recenseamento da população (Outubro a Setembro de 2008) identificou 701 habitantes, onde 360 (51,4%) residiam nas áreas de ramais e 341 (48,6%) no Igarapé.

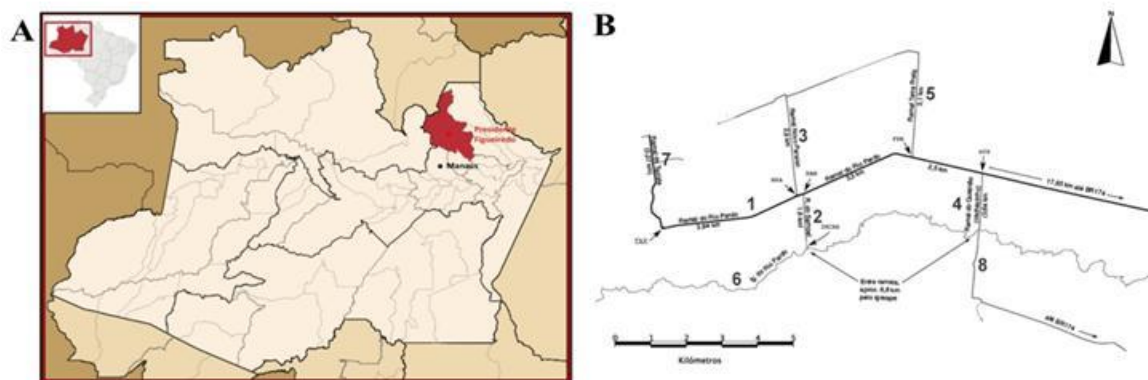

Figura 10 Mapa representando o estado do Amazonas e a localização da área de estudo, o assentamento agrícola de Rio Pardo/AM A) Mapa do Estado do Amazonas, indicando a localização do município de Presidente Figueiredo e a capital do Estado, Manaus. **B)** Plano de assentamento agrícola de Rio Pardo. O assentamento de Rio Pardo é composto por 8 áreas denominadas Ramais: Rio Pardo ou Principal (1), Samuel (2), Novo Paraíso (3), Gusmão (4), Terra Preta (5), Taxista (7), Novo Progresso (8) e a comunidade ribeirinha do Igarapé do Rio Pardo (6) (Adaptado de Kano *et al.*, 2012).

4.2 Desenho experimental do estudo

Os aspectos éticos e metodológicos deste estudo foram aprovados pelo comitê de ética do Centro de Pesquisas René Rachou (Report Nº 007/2006, Nº 07/2009, Nº 12/2010 e Nº26/2013), de acordo com as resoluções do conselho nacional em saúde brasileiro (CNS). Em novembro de 2008, dos 701 residentes do assentamento convidados a participar do estudo, 541 (77,2%) aceitaram por meio de consentimento livre e esclarecido, o qual foi também obtido, em casos de menores, de seus guardiões legais. Além disso, termos de assentimento foram obtidos dos menores, com linguagem apropriada para crianças entre 7-13 anos e adolescentes entre 14-17 anos de idade.

Um estudo de coorte aberta foi iniciado em novembro de 2008 seguindo os seguintes procedimentos para os voluntários da pesquisa: i) entrevista por questionário, para obtenção de dados demográficos, epidemiológicos e clínicos; ii) exame físico, incluindo temperatura corporal e tamanho do baço/fígado; iii) coleta de sangue venoso (indivíduos ≥5 anos; em EDTA, 10mL) ou de sangue em papel de filtro (≤ 5 anos); e IV) diagnóstico de malária por gota espessa. No primeiro corte (Baseline), o DNA genômico foi obtido para estudos moleculares (caracterização das variantes dos parasitos e dos genótipos de DARC do hospedeiro*)* e as amostras de plasmas utilizadas para detectar anticorpos anti- *P. vivax* (n=395).

Seis e 12 meses depois do primeiro corte, dois outros cortes transversais idênticos foram realizados. Além disto, cerca de 6 a 7 anos após o início do estudo,

um subgrupo da população (n=187) pode ser novamente localizado. No corrente trabalho, um total de 620 voluntários participou do estudo, contribuído com amostras de DNA e plasma, alguns deles participaram em mais de um corte transversal; mais especificamente, 395 foram incluídos no *baseline* (Novembro de 2008), 410 no segundo corte (junho de 2009), 407 no terceiro corte (outubro-novembro 2009), 91 no quarto corte (agosto de 2014), e 96 (setembro 2015).

Com relação à subpopulação envolvida no estudo de HLA, um total de 336 indivíduos foi selecionado, sendo estes indivíduos não relacionados. Destes, 222 estavam incluídos no corte I inicial (Baseline), 249 durante o 2º corte (junho 2009) e 239 durante o 3º corte (Outubro-Novembro 2009). Um total de 156 (64%) indivíduos tiveram amostras incluídas nos três cortes transversais (Baseline, 6 e 12 meses depois)

4.3 Genotipagem do antígeno de grupo sanguíneo DARC pela Reação em Cadeia da Polimerase em tempo real

As amostras de DNA extraídas foram processadas em placas óticas de 96 poços (Applied Biosystems, Foster City, CA, USA) para a amplificação dos alelos de DARC. O volume final da reação foi de 20 µL contendo 50-100 ng de DNA genômico, 5 µL SYBR® Green PCR master mix (Biosystems), e 10 pmoles de cada primer (Biosystems). A amplificação e a fluorescência foram detectadas utilizando ABI Prism® 7000 Sequence Detection System (Applied Biosystems). As condições da PCR foram: um ciclo de 95ºC por 10 minutos, seguido de 35 ciclos de 95ºC por 15 segundos e 60ºC por 1 minuto. Após a amplificação, foi realizado um ciclo de dissociação com variação crescente de temperatura (60ºC a 95ºC).

Os iniciadores (Tabela 1) foram desenhados por meio do programa Primer Express® v.2.0 (Applied Biosystems). Dois iniciadores foram modificados da versão descrita por Sousa *et al.* (2007). Foram eles: FGATA e RYA substítuidos por FGATANEW e RYANEW, respectivamente. Os pares de iniciadores FY/RYANEW e FAB/RABGATA foram utilizados na mesma reação, como uma reação de PCR multiplex, bem como os pares FY/RYB e FGATANEW/RABGATA. Os iniciadores RYA e RYB apresentam o polimorfismo G125A na extremidade 3’ e juntamente com o iniciador senso FY amplificam os alelos *FY*A* e *FY*B*, respectivamente. Para detecção da mutação T- 33C na região promotora do gene FY, foram desenhados

os iniciadores FAB e FGATA, que amplificam juntamente com o iniciador RABGATA, o promotor selvagem e mutado, respectivamente. Como os iniciadores FAB/FGATA e RYA/RYB são semelhantes foi introduzida uma alteração no nucleotídeo imediatamente anterior ao nucleotídeo polimórfico, com o objetivo de impedir a amplificação inespecífica dos alelos. A alteração impede o pareamento nucleotídico na extremidade 3' entre o iniciador não-específico e a fita molde de DNA. Uma segunda modificação no iniciador FGATA foi realizada a partir da adição de uma cauda C/G na região 5' do iniciador, com a finalidade de aumentar a diferença de tamanho e temperatura de dissociação dos produtos da PCR amplificados com o par de iniciadores FAB e FGATA.

Tabela 1 Sequência dos iniciadores utilizados para a reação de genotipagem de DARC por PCR em tempo real

Nome dos Iniciadores	Região	Sequência dos iniciadores	Região
FGATANEW	5'	CCC GGG CCC GCC GCC CTCA TTA GTC CTT GGC TCT TGC	3'
FAB	5'	CCC TCA TTA GTC GGC TCT TTT	3'
RABAGATA	5'	A GGG GCA TAG GGA TAA GGG ACT	3'
FY	5'	C TCA AGT CAG CTG GAC TTC GAA GAT	3'
RYANEW	5'	AG CTG CTT CCA GGT TGG CGC	3'
RYB	5'	CTG CTT CCA GGT TGG CGT	3'

(Sousa *et al.*, 2007 com modificações)

4.4 Genotipagem do Antígeno Leucocitário Humano (HLA)

No presente trabalho, a genotipagem dos alelos dos genes do Sistema HLA classe II (DRB1, DQB1 e DQA1) foi realizada pelo método molecular de alta resolução, PCR-SSO (*Polymerase Chain Reaction - Specific Sequence of Oligonucleotides*) com tecnologia Luminex (*One Lambda Inc.,* Canoga Park, CA, USA), conforme recomendações do fabricante. Resumidamente, a amplificação do DNA alvo utilizando iniciadores específicos para os *loci* HLA-DRB1 (alelos 01-16), HLA-DQA1 (alelos 01-06) e HLA-DQB1 (02-06) foi realizada conforme recomendações do fabricante. Posteriormente, a amplificação dos fragmentos de DNA foi verificada em

eletroforese em gel de agarose a 2,5%. A visualização das bandas foi realizada pela coloração com brometo de etídio e exposição à luz ultravioleta. A interpretação dos resultados foi baseada na presença ou ausência de fragmentos específicos de DNA amplificados. Em seguida, os DNAs amplificados foram submetidos à hibridização com microesferas ligadas aos oligonucleotídeos específicos para os alelos de HLA de classe II conjugados à biotina, seguida de incubação com a estreptavidina conjugada com ficoeritrina. A leitura da fluorescência da reação foi realizada em fluorômetro de fluxo utilizando a tecnologia Luminex (One lambda) e as amostras foram analisadas atraves do software HLA FUSION (One lambda Inc., San Diego, CA, USA). Este ensaio foi realizado no Laboratório de Imunogenética da Universidade Estadual de Maringá, em colaboração com a Dra. Ana Maria Sell.

4.5 Proteínas recombinantes

4.5.1 Duffy Binding Protein do *P. vivax*, região II.

As sequências codificantes da região de interesse de cada proteína (PvDBP região II) foram clonadas em um plasmídeo (pET21a+, Novagen) específico para expressão em *Escherischia coli* (*E. coli*). Os plasmídeos resultantes foram usados para transformação em *E. coli* BL21 (DE3) LysE (Invitrogen) e as colônias positivas foram estocadas no glicerol a -80ºC. O protocolo de produção e purificação das proteínas foi realizado conforme estabelecido por (Ntumngia *et al.*, 2012). Resumidamente, uma cultura *overnight* em meio LB (50µg/mL de ampicilina) foi preparada utilizando o estoque de bactérias positivas congeladas. No dia seguinte, a cultura foi diluída 1:10 em meio LB contendo 50µg/mL de ampicilina e mantida a 37ºC sob agitação a 250rpm durante 2 horas. A cultura foi então induzida com 1mM IPTG (isopropyl-β-d-thiogalactopyranoside) até uma densidade ótica a 600nm [OD_{600}] entre 0,8 e 1,0. As células foram centrifugadas e estocadas -80ºC até o momento da purificação.

As proteínas expressas foram purificadas dos corpos de inclusão sob condições desnaturantes. Brevemente, as células foram ressuspendidas em tampão de lise (10mM Tris Buffer [pH 8,0], 1mM EDTA, 3% sucrose, 200 µg/mL de lisozima, 1mM PMSF [phenylmethylsulfonyl fluoride], 0,1M NaCl, 20 µg/mL DNase) à temperatura ambiente. Os corpos de inclusão foram então recuperados por meio de centrifugação e lavados duas vezes com Buffer de lavagem (10mM Tris-HCl [pH

8,0], 3M ureia, 1mM EDTA, 1 mM PMSF), e solubilizados em buffer de solubilização (20mM buffer fosfato [pH 7,8] contendo 8M ureia e 0,5 M NaCl). As proteínas recombinantes obtidas dos corpos de inclusão foram purificadas utilizando cromatografia de afinidade com uma coluna de níquel Sefarose 6 (GE Healthcare). As frações eluídas tiveram sua pureza checada por meio de separação por gel SDS-PAGE e aquelas frações puras foram submetidas ao processo de *refolding* segundo protocolo descrito por Singh *et al.* (2001) singh e colaboradores (2001). Esse processo consiste em uma diluição de 100x em buffer de *refolding* (Buffer fosfato 50mM [pH 7,2], 1mM glutationa reduzida, 0,1 mM glutationa oxidada, 1M uréia e 0,5 M arginina). A proteína permanece no buffer de *refolding* durante 48h a 10ºC sob agitação. Após as 36h, o produto do refolding foi dialisado durante 36h em buffer de diálise (50mM fosfato [pH6,5], 1M ureia) para a remoção da arginina com duas trocas do buffer a cada 12h. O produto dialisado é então concentrado, filtrado em um filtro de 0,2 µm, ajustado a concentração para 1mg/mL utilizando PBS (phosphate-buffered saline) e por fim estocado a -80ºC até a utilização.

Para avaliar a qualidade do *refolding* a proteína recombinante produzida foi submetida ao teste de DTT (dithiothreitol) que consiste em incubar a proteína re-enovelada com o DTT e aplicar em um gel SDS-PAGE. O DTT irá quebrar as ponte de disulfeto formadas durante o processo de re-enovelamento e tornar essa proteína linear. Deste modo ela irá migrar diferentemente da proteína re-enovelada sem o tratamento com DTT.

4.5.2 Duffy Binding Protein do *P. vivax*, região II-IV

O plasmídeo pGEX-2T contendo as sequências codificadoras da região II a IV (aa-177 a 815) da PvDBP foi gentilmente cedido pelo Dr. John Adams (University of South Florida, USF). Este plasmídeo, que gera uma proteína de fusão com a glutationa *S*-transferase de *Schistosoma japonicum* (GST) foi utilizado para transformar bactérias quimicamente competentes, *Escherichia coli* cepa DH5α (Nishimura *et al.*, 1990).

A indução da expressão da proteína recombinante $PvDBP_{II\text{-}IV}$ foi realizada com a adição de 0,1mM de IPTG às culturas bacterianas. Neste caso, a proteína foi expressa na forma solúvel e foi purificada por meio pelo método de cromatografia de afinidade com uma matriz de *glutathione sepharose*® 4B (GS4B)(GST Purification

Modules, Amersham Pharmacia), conforme recomendação do fabricante. Esse processo foi associado à eletroeluição para remoção da proteína contaminante de 70kDa de *E. coli*, provavelmente uma chaperonina, conforme protocolo descrito por (Ceravolo *et al.*, 2005) Posteriormente, as proteínas recombinantes foram quantificadas pelo método de Bradford (Bio-rad) e armazenadas a -20ºC para utilização nos ensaios de ELISA.

4.5.3 Erythrocyte Binding Protein 2 (EBP2) do *P. vivax*, região DBL

A produção e purificação da região DBL da EBP2 seguiu o mesmo protocolo realizado para a $PvDBP_{II}$. Resumidamente, a sequência codificante da região DBL da EBP2 (aminoácidos 159-485) obtida no Genbank (número de acesso KC987954) foi otimizada para expressão em E. coli, comercialmente sintetizada e clonada dentro de um vetor pET21a+ (Novagen) com calda de Histidina para posterior purificação por cromatografia de afinidade. A EBP2 recombinante foi então: I) expressa em bacteria; II) purificada dos corpos de inclusão em meio desnaturante; III) re-enovelada por rápida diluição e; IV) sua conformação foi verificada pelo teste de DTT.

Toda a parte de produção e purificação da proteína recombinante EBP2, bem como ensaios pré-clínicos em animais de laboratório, foi realizada no laboratório do Dr. John Adams, na Universidade do sul da Flórida/EUA, durante o doutorado sanduíche (Tampa, EUA, período agosto de 2014 a abril 2015).

4.6 Imunização dos camundongos com a proteína EBP2

Para a imunização de camundongos da linhagem Balb-c com a proteína EBP2 foram utilizados protocolos bem padronizados pelo grupo do Dr. JH Adams (South Florida University). Antes de iniciar as imunizações foram coletados o soro dos animais (soro pré-imune) para utilização como controle negativo. A partir daí, um grupo de 12 camundongos fêmeas da linhagem Balb-c foram imunizados com 25µg da proteína recombinante emulsificada em 25µL de Titermax Gold (TiterMax), utilizado como adjuvante. Após três semanas os animais foram novamente imunizados com 25µg da proteína recombinante emulsificada em 25µL do adjuvante Titermax. Após 3 semanas, totalizando então 6 semanas, foram coletados o soro dos animais para utilização nos ensaios sorológicos. Como controle da especificidade da proteína, outros grupos de camundondos foram imunizados com a

PvDBP (25 µg) ou com um anticorpo monoclonal (dose) específico para a região II da PvDBP (Ntunmgia *et al.*, 2012).

4.7 Ensaio imunoenzimatico (ELISA) com as proteínas recombinantes

4.7.1 População naturalmente exposta, Rio Pardo/AM

Os ensaios de ELISA foram realizados segundo protocolo já bem estabelecido no nosso laboratório (BMIM/FIOCRUZ), sendo a concentração dos antígenos recombinantes (DBPII e EBP2) e a diluição dos anticorpos primários e secundários determinados previamente por titulação.

Os 96 poços das placas de ELISA (Maxysorp, Nunc, Denmark) foram sensibilizados por 12h a 4ºC com 100µL dos antígenos PvDBP (3 µg/mL) EBP2 (1,5 µg/mL). Posteriormente, as placas foram lavadas com tampão de lavagem [(0,05% tween 20 em PBS, (PBS-T)] e foram adicionados 200µL de tampão de bloqueio [5% de leite em pó (Molico) diluído em PBS-T]. Após 1h de bloqueio a 37ºC, as placas foram lavadas três vezes com PBS-T, e 100µL de soros humanos foram adicionados aos poços, em duplicatas, na diluição de 1:100 (PBS-T adicionado de 1,5% de leite em pó). Após incubação por 1h a 37ºC, as placas foram lavadas com PBS-T por 10 vezes e incubadas novamente, nas mesmas condições, com 100µL/poço do anticorpo anti-IgG conjugado a peroxidase, diluído a 1:1.000 (PBS-T com 1,5% de leite). Após nova lavagem, a reação foi realizada acrescentando-se 100 µL/poço de solução contendo 10 mg de OPD (*o*-phenylenediamine dihydrochloride substrate - Sigma-Aldrich, USA) diluído em 50 mL de tampão citrato de sódio (0,1M pH 5,0) na presença de 40 µL de peróxido de hidrogênio (H202) a 30% (Sigma-Aldrich). A reatividade dos anticorpos foram determinadas pelo valor obtido da absorbância de 492nm (OD492), a partir da leitura das placas em leitor de ELISA (Stat Fax-2.100, Awareness Tecnology, Palm City, FL). O limite de positividade (*cut-off*) entre os resultados positivos e negativos foi estabelecido entre a média observada pelos soros de 30 indivíduos nunca expostos à malária, acrescida de três desvios-padrão. O valor da D.O. (densidade óptica) foram divididos pelo *cut- off* para a obtenção dos valores de IR (Índice de Reatividade). Valores de IR > 1 foram considerados positivos.

4.7.2 Modelo animal

O protocolo de ELISA realizado com soro dos camundongos imunizados seguiu o mesmo protocolo daquele descrito para a população humana (item 4.7.1) com a diferença apenas na diluição dos soros, que no caso dos animais foi realizada uma diluição seriada com início em $1x10^3$ até completa falta de reatividade.

Resumidamente, as placas foram sensibilizadas com as proteínas recombinantes e incubadas por 12h à 4ºC. Após lavagem com PBS-T, as placas foram bloqueadas com tampão de bloqueio por 1h à 37ºC. Após nova lavagem, as placas foram incubadas com soro dos animais nas diluições adequadas e incubadas novamente por 1h a 37ºC. As placas foram lavadas e incubadas com anticorpo anti-IgG (mouse) conjugado com peroxidase, diluído 1:1000. Houve nova incubação por 1h a 37ºC e então as placas foram novamente lavadas. Posteriormente, foram incubadas com solução OPD e a reatividade dos anticorpos foram determinadas pelo valor obtido da absorbância de 492nm (OD492), a partir da leitura das placas em leitor de ELISA.

4.8 Transfecção de células COS-7 e ensaio funcional

Para realização dos ensaios de citoaderência foi utilizada uma linhagem de células de mamíferos permissíveis a transfecção, COS-7, originalmente isolada a partir de células de rim de primata africano, e modificada pelo vírus SV 40 (American Type Culture Collection - ATCC, Manassas, VA). Para realização dos ensaios de citoaderência, células COS-7 foram mantidas em garrafas de cultura de $75cm^2$ (Corning Incorporatioted, EUA) contendo 10mL do meio de cultura Dulbecco's Minimal Eagle Medium (DMEM) (Gibco, Invitrogen Corporation Rockville, MD, EUA). O meio para manutenção das células (meio DMEM completo) continha 5% de soro bovino fetal (SBF) inativado (Invitrogen Life Technologies, Rockville, MD, EUA), 25mM de bicarbonato de sódio, 2mM de L-glutamina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 100UI/mL de penicilina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 100µg/mL de estreptomicina (Gibco, Invitrogen Corporation Rockville, MD, EUA), 25mM de Hepes (Sigma). As culturas foram mantidas em estufa a 37ºC com 5% de CO_2 e 95% de umidade, sendo os repiques realizados a cada dois dias, utilizando-se solução de tripsina e EDTA a 0,25% (Gibco), conforme protocolo padrão (Phelan, 2003).

Para os ensaios de transfecção utilizou-se um plasmídeo construído previamente pelo nosso grupo de pesquisa que representa a variante da DBP_{II} do *P. vivax* mais frequente no Brasil (Souza-Silva *et al.*, 2010). Para os ensaios de transfecção utilizou-se a lipofectamina e reagente *Plus* (Invitrogen Life Technologies, Carlsbad, CA), nas concentrações e protocolos indicados pelo fabricante. Resumidamente, as células COS-7 foram adicionadas às placas de cultura de seis poços (Nunc, Denmark) (1,5 x 10^5 células/poço) e então transfectadas com 0,5µg/poço de DNA plasmidial e complexos de lipossomos (5% de reagente *Plus* e 3% de lipofectamina) em meio de cultura DMEM (Gibco-BRL Life Technologies, Rockville, MD) sem soro bovino fetal (SBF) (meio DMEM incompleto). Este meio incompleto continha 25mM de bicarbonato de sódio, 2mM de L-glutamina (Gibco, Invitrogen Corporation Rockville, MD, EUA) e 25mM de Hepes (Sigma). Após 6h de incubação do complexo lipossoma-DNA (37°C, 5% de CO_2 e 95% de umidade), o meio de transfecção foi substituído por meio DMEM contendo 10% SBF (Gibco), 2mM de L-glutamina (Gibco), 25mM de Hepes (Sigma-Aldrich), 25mM de bicarbonato de sódio (Merck, Darmstadt, Germany), 100UI/mL de penicilina e 100µg/mL de estreptomicina (Gibco), sendo as placas incubadas a 37° C. Após 24 h, o meio de cultura foi novamente substituído por meio DMEM completo, e a eficiência da transfecção verificada por meio da visualização das células em um microscópio de fluorescência. Quarenta e oito horas após a transfecção, as placas foram lavadas com meio DMEM incompleto, e as células incubadas com os soros/plasmas-testes (37ºC, 1h, 5% de CO_2) diluídos em meio DMEM incompleto. A diluição utilizada foi de 1:40, pois em ensaios prévios, essa diluição apresentou a melhor inibição interação ligante-receptor quando diferentes soros foram testados.

Posteriormente, foram adicionados 200µL/poço de uma solução a 10% de eritrócitos humanos O/DARC positivos em meio DMEM completo e as placas incubadas à temperatura ambiente por 2h. Ao final da incubação as placas foram lavadas, três vezes com meio DMEM incompleto, para que os eritrócitos não aderentes fossem retirados. Os resultados foram expressos como porcentagem relativa de inibição (Ceravolo *et al.*, 2008).

4.9 Análises estatísticas

O desenho experimental deste trabalho foi previamente elaborado com a ajuda do médico e epidemiologista Dr. Cor Jesus Fontes (UFMT), uma vez que esta região

já vem sendo estudada pelo nosso grupo há vários anos. Deste modo, um banco de dados foi construído com as informações obtidas dos pacientes (pacote estatístico EpiData 2002).

Levando em consideração a distribuição dos dados amostrais (se seguem ou não uma distribuição normal) testes estatísticos paramétricos ou não paramétricos foram utilizados, respectivamente. Assim, foram utilizados para avaliar a diferença de médias/medianas entre os grupos os testes: *Mann Whitney* (quando comparados apenas dois grupos) e *Krukal-wallis* (comparação de mais de dois grupos), seguido, quando necessário, do teste de *Dunn* como teste *post hoc*. O Qui-quadrado, Z teste ou teste exato de Fisher foi utilizado para comparações de proporções entre os grupos, quando apropriado.

Baseada na resposta imune humoral anti-DBP_{II} nos três cortes transversais, os indivíduos foram categorizados de acordo com sua resposta de longa duração: i) persistente não respondedor (PR), ausência de anticorpos anti-DBP_{II} em todos os três cortes; ii) temporário respondedor (TR), anticorpos detectados em pelo menos 1 dos cortes transversais; iii) persistente respondedor (PR) anticorpos detectados em todos os três cortes transversais.

Com relação às análises de associação entre a resposta imune e os alelos de HLA classe II, foi avaliada a frequência dos alelos entre os indivíduos soropositivos e soronegativos no baseline do estudo utilizando uma tabela de contingência padrão. A associação entre os alelos (ou haplotipos) de HLA e a resposta imune em longo prazo contra a DBP_{II} (PR e PNR) foi avaliada pela tabela de contingência padrão utilizando os testes de qui-quadrado ou exato de Fisher, quando apropriado. Cada indivíduo teve duas observações correspondentes a cada alelo. Aqueles alelos com frequência menor que 0,01 foram excluídos da análise.

Além disso, modelos de regressão logística múltipla foram construídos para descrever associações independentes entre as co-variáveis (idade, gênero, tempo de residência na área, local de residência, episódios prévios de malária e infecção aguda ou recente). Essas análises foram realizadas no software STATA10 (Stata Corporation, College Station, TX).

Em todos os casos, as diferenças foram consideradas estatisticamente significativas com nível de significância de 5% ($p<0,05$).

5 Resultados

A apresentação dos resultados deste trabalho foi dividida em duas partes. A primeira se refere ao estudo da influência da variabilidade do hospedeiro vertebrado na resposta imune contra a DBP_{II}, com ênfase nos polimorfismos do receptor DARC (Artigo 1) e do complexo de HLA classe II (Artigo 2). A segunda parte está relacionada à produção da proteína EBP2 recombinante, bem como avaliação da sua imunogenicidade em camundongos (Artigo 3) e em população naturalmente exposta à transmissão de malária no Brasil (resultados não publicados).

5.1 PARTE I – Influência da variabilidade genética do hospedeiro na resposta imune anti-PvDBP

Artigo 1- “Duffy Antigen Receptor for Chemokine (DARC) Polymorphisms and Its Involvement in Acquisition of Inhibitory Anti-Duffy Binding Protein II (DBPII) Immunity” por Souza-Silva FA*, Letícia de Menezes Torres*, Santos-Alves JR, Tang ML, Sanchez BAM, Sousa TN, Fontes CJF, Nogueira P, Rocha RS, Brito CFA, Adams JH, Kano FS, Carvalho LH. Plos one, 9 (4): e93782, 2014 (Página 50).

Estes autores contribuíram igualmente para o trabalho

Artigo 2- “The Presence, Persistence and Functional Properties of *Plasmodium vivax* Duffy Binding Protein II Antibodies Are Influenced by HLA Class II Allelic Variants” por Kano FS, Souza-Silva FA, Leticia de Menezes Torres, Lima BAS, Sousa TN, Alves JRS, Rocha RS, Fontes CJF, Sanchez BAM, Adams JH, Brito CFA, Pires DEV, Ascher DB, Sell AM, Carvalho LH. PLoS Negl Trop Dis 10(12): e0005177, 2016 (Página 60).

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Duffy Antigen Receptor for Chemokine (DARC) Polymorphisms and Its Involvement in Acquisition of Inhibitory Anti-Duffy Binding Protein II (DBPII) Immunity

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Abstract

The *Plasmodium vivax* Duffy binding protein (PvDBP) and its erythrocytic receptor, the Duffy antigen receptor for chemokines (DARC), are involved in the major *P. vivax* erythrocyte invasion pathway. An open cohort study to analyze *DARC* genotypes and their relationship to PvDBP immune responses was carried out in 620 volunteers in an agricultural settlement of the Brazilian Amazon. Three cross-sectional surveys were conducted at 6-month intervals, comprising 395, 410, and 407 subjects, respectively. The incidence rates of *P. vivax* infection was 2.32 malaria episodes per 100 person-months under survey (95% confidence interval [CI] of 1.92-2.80/100 person-month) and, of *P. falciparum*, 0.04 per 100 person-months (95% CI of 0.007–0.14/100 person-month). The distribution of DARC genotypes was consistent with the heterogeneous ethnic origins of the Amazon population, with a predominance of non-silent *DARC* alleles: *FY*A* > *FY*B*. The 12-month follow-up study demonstrated no association between *DARC* genotypes and total IgG antibodies as measured by ELISA targeting PvDBP (region II, DBPII or regions II–IV, DBPII-IV). The naturally acquired DBPII specific binding inhibitory antibodies (BIAbs) tended to be more frequent in heterozygous individuals carrying a *DARC*-silent allele ($FY{*}B^{ES}$). These results provide evidence that DARC polymorphisms may influence the naturally acquired inhibitory anti-Duffy binding protein II immunity.

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Introduction

Plasmodium vivax is the most widespread *Plasmodium* species and is a potential cause of morbidity and mortality among the 2.48 billion people living at risk of infection [1]. Recent evidence of multidrug-resistant *P. vivax* associated with severe and fatal disease elevates it to one of global health concern [2,3]. *Plasmodium vivax* infects human erythrocytes (RBCs) through a pathway that requires interaction between an apical parasite protein, the Duffy binding protein (PvDBP), and its receptor on reticulocytes, the Duffy antigen receptor for chemokines (DARC) [4–6]. The goal in developing PvDBP as a vaccine against blood stages of *P. vivax* is to elicit an antibody response that inhibits parasite adhesion to DARC-positive human reticulocytes, and thereby prevents merozoite invasion. The importance of the interaction between PvDBP (region II, DBPII) and DARC to *P. vivax* infection has stimulated a significant number of studies of PvDBP antibody responses. Available data demonstrate that naturally occurring antibodies to PvDBP are prevalent in individuals living in *P. vivax* endemic areas [7–9], and these antibodies can block the DBPII/DARC interaction [10–12]. While inhibitory DBPII antibodies confer a degree of protection against blood stage infection [12], these antibodies are biased towards a specific allele [13]. Although anti-PvDBP immune responses have been well characterized, little is known about the association between this immune response and DARC host genotype [14,15].

Although most individuals lacking DARC on their RBCs are naturally resistant to *P. vivax* [4], some infections occur in DARC-negative persons living in vivax malaria endemic areas [16–18]. Beyond being receptors for *P. vivax* and various chemokines [19], DARC proteins have clinical and biological significance and have been reported to be associated with transfusion incompatibility and hemolytic disease of the newborn [20–22]. It is also implicated

in several inflammatory diseases, and cancer, and might play a role in HIV infection and AIDS [23–26]. Recently, a previously unreported function of this receptor has been described in *P. falciparum* infection, in which DARC proteins seem to be essential for platelet-mediated killing of *P. falciparum* parasites [27].

The two common *DARC* alleles in Caucasians, *FY*A* and *FY*B*, differ by a single base substitution (125 G>A) resulting in the replacement at residue 42 in the extracellular domain of a glycine (Fya antigen) for an aspartic acid (Fyb antigen) [28,29]. Another mutation in the DARC gene promoter region abolishes receptor expression on erythroid cells by disruption of a binding site for the GATA1 erythroid transcription factor, resulting in the absence of DARC antigens on RBCs (-33T>C; Fy^{ES}, erythrocyte-silent) [30]. Although most DARC negative individuals carry the GATA mutation in the *FY*B* allele (silent *FY*B* allele), the presence of a *cis*-regulatory mutation within *FY*A* has been described [31]. The overall expression level of erythroid-specific DARC is co-dominant; therefore, DARC-null promoter heterozygosity reduces the DARC expression level by approximately 50 percent [31–33]. Similarly, the susceptibility to *P. vivax* in DARC-positive individuals varies among specific *DARC* genotypes [31,34–36].

In the current study, we present data of the first population-based study of the relationship between DARC genotypes and PvDBP inhibitory antibodies. The methodology included a community-based open cohort study in an agricultural settlement of the Amazon area of Brazil in which 620 individuals were genotyped for DARC, and their PvDBP immune responses were evaluated by conventional serology (recombinant proteins) and binding inhibitory antibodies (BIAb) targeting the DBPII ligand.

Material and Methods

Study area and population

The study was carried-out in the agricultural settlement of Rio Pardo (1°46′S–1°54′S, 60°22′W–60°10′W), Presidente Figueiredo municipality, northeast Amazonas State in the Brazilian Amazon area. Rio Pardo is located approximately 160 km from Manaus, the capital of Amazonas, along the main access to a paved road (BR-174) that connects Amazonas to Roraima State. The settlement was officially created in 1996 by the National Institute of Colonization and Agrarian Reform (INCRA) as part of a large scale colonization project focused on agriculture and wide-ranging human settlement in the Amazon area [37]. The mean annual temperature is 31°C with humid climate and average annual rainfall of the 2,000 mm per year. The rainy season extends from November-May and dry season from June–October. The settlement is composed of areas called 'ramais', which include households on both sides of unpaved roads, and a riverine population called Igarapé. A census in September–October 2008 identified 701 inhabitants, with 360 (51.4%) living in ramais areas and 341 (48.6%) in and around Igarapé. Inhabitants of the area live on subsistence farming and fishing along the small streams of the Rio Pardo River. The study site and malaria transmission patterns have been described in detail elsewhere [38]. Although *P. vivax* and *P. falciparum* are transmitted year round, *P. vivax* is responsible for about 90% of malaria cases [38]. Housing quality is poor, rendering indoor residual spraying ineffective. The availability of curative services is limited, and a government outpost provides free malaria diagnosis and treatment.

Study design and cross-sectional surveys

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 (Report No. 007/2006 and No. 07/2009), according to the Resolution of the Brazilian Council on Health-CNS 196 / 96 after consultation with the community. In November of 2008, of 701 residents of the settlement invited to participate in the study, 541 (77.2%) accepted by giving written informed consent, which was also obtained from the next of kin, caregivers, or guardians on the behalf of participating minors. In addition to the consent form, separate assent forms were obtained from minors, in language appropriate for children ages 7–13 and adolescents from 14 to 17 years of age.

A population-based open cohort study was initiated in November of 2008, with the following procedures [38]: (i) administration of a structured questionnaire to all volunteers to obtain demographical, epidemiological, and clinical data; (ii) physical examination, including body temperature and spleen/liver size, recorded according to standard clinical protocols; (iii) venous blood collection in individuals aged five years or older (EDTA 10 ml), or blood spotted on filter paper (finger-prick) in those aged <5 years; and (iv) search for malaria parasites by light microscopy on Giemsa thick blood smears. Geographical location of each dwelling was recorded using a hand-held 12-channel global positioning system (GPS) (Garmin 12XL, Olathe, KS, USA) with a positional accuracy within 15 m. At initial enrollment, of 541 volunteers, genomic DNA was amplified to *DARC* polymorphisms and plasma samples screened to *P. vivax* antibodies in 395 (73%).

Six and twelve months following the initial survey, identical cross-sectional surveys were carried out. In total, 395 subjects were enrolled at baseline, 410 at the 2nd survey (June 2009), and 407 in the 3rd survey (October-November, 2009). A total of 620 volunteers contributed DNA and plasma samples, some of which participated in more than one cross-sectional survey. One hundred eighty-two (29.4%) and 205 (33%) subjects provided samples in two and three cross-sectional surveys, respectively.

Laboratory diagnosis of malaria

Malaria infection was diagnosed by microscopy of Giemsa-stained thick smears and real-time PCR amplification of a species-specific segment of the multicopy 18S rRNA gene of human malaria parasites. The Giemsa-stained smears were evaluated by experienced microscopists, according to the malaria diagnosis guidelines of the Brazilian Ministry of Health. For real-time PCR, genomic DNA was extracted from either EDTA whole-blood samples (adults and children≥5 years old) or dried blood spots on filter paper (children<5 years) using the Puregene blood core kit B (Qiagen, Minneapolis, MN, USA) or the QIAmp DNA mini kit (Qiagen), respectively, according to manufacturers' instructions. Real-time PCR was performed as previously described [39] using a consensus pair of primers (PL1473F18 [5′- TAACGAACGA-GATCTTAA-3′] and PL1679R18 [5- GTTCCTCTAAGAAGC TTT-3′]).

DARC genotyping

Extracted genomic DNA was used to detect the three common alleles at the *DARC* locus, *FY*A*, *FY*B*, and $FY{*}B^{ES}$ (ES = erythroid silent), using real-time PCR with allele-specific primers, as previously described with minor modification [34]. Essentially, the original FGATA and RYA primers were replaced to FGATANEW (5′ CCC GGG CCC GCC GCC CTCA TTA GTC CTT GGC TCT TGC 3′) and RYANEW (5′ AG CTG CTT CCA GGT TGG CGC 3′), respectively. Each 20 µl reaction mix contained 50–100 ng genomic DNA, 10 µL SYBR Green PCR master mix (Biosystems) and 0.1–1.0 pmoles/µL of each primer (Biosystems). The amplification and fluorescence were detected by ABI Prism 7000 Sequence Detection System (Applied

Biosystems) using a cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, melting curves were observed from the dissociation curve resulting from continuous measurements of fluorescence (F) at 530 nm during which the temperature was gradually increased from 60 to 95°C. Melting peaks of each amplified fragment were visualized by plotting the negative derivative of the fluorescence over temperature versus temperature (–dF/dT° vs. T°). DNA samples from previously well-characterized Brazilian individuals carrying the most common DARC genotypes were included as positive controls. In addition, in c. 10% of samples, a 942-bp fragment of DARC, comprising polymorphic positions −33T>C and 125G>A, was amplified by PCR using the forward primer: 5′-TCAAAACAGGAAGACCCAAG-3′ and reverse: 5′-AGAGG-TCTGAAAAGCATGAA-3′ (Macrogen DNA sequencing services; http://dna.macrogen.com/eng/). No discordant results were obtained.

Recombinant protein and serological assay

The enzyme-linked immunosorbent assay (ELISA) for total IgG antibodies to PvDBP was carried out using two recombinant proteins, covering the region II (DBPII) or regions II–IV (DBPII-IV). The recombinant DBPII, which includes amino acids 243–573 (regions II), was expressed as a 6xHis fusion protein of 39 kDa, as previously described [40]. The recombinant DBPII-IV, amino acids 132–771 (regions II–IV), was expressed as a soluble glutathione S-transferase (GST) fusion protein of 140 kDa, as previously described [7,8]. To assess IgG antibodies to PvDBP, ELISA was carried out as previously described [8] with serum samples at 1:100 and recombinant proteins at a final concentration of 3 μg/mL (DBPII) or 1.25 μg/mL (DBPII-IV). For DBPII-IV protein, the final optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST (antigen control). The results were expressed as reactivity index: RI = OD values of test sample divided by the value of the cut-off. For each recombinant protein, cut-off points were set at three standard deviations above the mean OD of sera from 30 individuals who had never been exposed to malaria. Values of RI>1.0 were considered positive. For statistical purposes, individuals were categorized as high responders (RI>4) or low responders (RI≤4). This criterion was defined based on our previous findings that few individuals develop PvDBP antibody at RI>4 [38].

COS cell transfection and erythrocyte-binding assays

COS7 (green monkey kidney epithelium, ATCC, Manassas, VA) cells were transfected with the plasmid pEGFP-DBPII, which coded for a common DBPII sequence circulating in the Amazon area [38]. This construct allows expression of DBPII as a fusion protein to the N-terminus of EGFP used as a transfection marker as previously described [10]. Transfections were realized with lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocols. Briefly, COS-7 cells in six-well culture plate (1.5×10^5 cells/well) were transfected with plasmid (0.5 μg/well)-liposome complexes (5% Plus-reagent and 3% lipofectamine) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) without serum. After 6 h exposure to DNA-liposome complexes (37°C, 5% CO_2), transfection medium was replaced with DMEM with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD). At 24 h post-transfection, culture medium was again replaced and efficiency of transfection was assessed by fluorescence. Forty-eight hours post-transfection, erythrocyte-binding assays were performed as previously described [11]. Briefly, plasma samples were added at 1:40, and plates were incubated for 1 hr at 37°C in 5% CO_2. Human O^+ $DARC^+$ erythrocytes in a 10% suspension were added to each well (200 μL/well), and plates were incubated for 2 h at room temperature. Since *DARC* genotypes might influence *in vitro* erythrocyte-binding assays [36], as was confirmed here (Figure S1), all *in vitro* binding assays were carried-out with erythrocytes expressing both DARC antigens (Fya and Fyb, *FY*A/FY*B* genotype), which display intermediate binding and predominate in Brazil. After incubation, unbound erythrocytes were removed by washing the wells three times with phosphate buffered saline (PBS). Binding was quantified by counting rosettes observed in 10–20 fields of view (200×). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the COS cell surface. For each assay, pooled plasma samples from Rio Pardo residents characterized as non-responders by ELISA were used as a negative control (100% binding). For this purpose, only plasma that did not inhibit erythrocyte binding, as opposed to samples from unexposed Brazilian donors, was pooled for the negative control (usually, 10 plasma samples/pool). A positive control included a pool of plasma from individuals with long-term exposure to malaria in the Amazon area. The percent inhibition was calculated as 100 × (Rc - Rt)/Rc, where Rc is the average number of rosettes in the control wells and Rt is the average number of rosettes in the test wells.

Statistical analysis

A database was created with Epidata software (http://www.epidata.dk). Linear correlations between two variables were determined by using the Pearson correlation coefficient. Comparisons of two independent proportions were realized by Z-test or chi-square test as appropriate. McNemar's test was used for analysis of correlated proportions. Differences in medians were assessed using Kruskal–Wallis with Dunn's post hoc test to identify differences between groups. The 95% Mid-P exact test was used to estimate incidence ratios (person-time of follow-up) and confidence intervals, considering variables such as duration of malaria-exposure, number of persons exposed, and previous malaria episodes. The level of significance of 5% was adopted. All analyses were performed on Stata software, v12 and OpenEpi (http://www.openepi.com/v37/Menu/OE_Menu.htm).

Results

Malarial infection, enrollment and follow-up, and PvDBP conventional serology

We investigated acute malaria infection in 620 subjects with a median of age 28 years and 1.4:1 male-female (Table 1). Age of subjects basically corresponded to the time of malaria exposure in the Amazon area ($r = 0.91$; $P<0.0001$, Pearson's correlation test). Median time of residency in the Amazon area was 24 years. At the time of first blood collection, 19 (3%) subjects had positive blood-smears, with 17 (90%) of these infections caused by *P. vivax*. Real-time PCR confirmed all microscopically positive samples. In addition, PCR-based protocol identified 17 additional malaria infections, 15 *P. vivax* and two *P. falciparum*. The overall prevalence of malaria was 5.8%, with 32 of the 36 (89%) infections caused by *P. vivax*. No *P. malariae* or mixed *Plasmodium* infections were diagnosed by either microscopy or real-time PCR.

The 620 participants were followed up for an average of 7 months (10 days to 12 months), thus representing 4,646 person-months of follow-up. Based on parasitological-confirmed cases, the incidence rates of *P. vivax* malaria was 2.32 episodes per 100 person-months (95% confidence interval [CI] of 1.92–2.80/100 person-month) and, of *P. falciparum* 0.04 per 100 person-months (95% CI of 0.007–0.14/100 person-month). The temporal

Table 1. Demographic, epidemiological and genetic data of 620 subjects, Rio Pardo Settlement, Amazonas, Brazil.

Characteristic	
Median age, years (IQR)	28 (14–47)
Gender, male:female	1.4:1
Acute malaria infection, n (%)	
Light Microscopy (LM)	19 (3.1)
PCR	17 (2.7)
Total	36 (5.8)
Years of malaria exposure, median (IQR)	24 (13–41)
Previous malaria episodes, median (IQR)	5 (1–11)
***DARC* genotypes, n (%)**	
*FY*A/FY*B*	182^{a} (29.4)
*FY*A/FY*A*	137^{b} (22.1)
$FY{*}A/FY{*}B^{ES}$	125^{b} (20.2)
*FY*B/FY*B*	87^{c} (14.0)
$FY{*}B/FY{*}B^{ES}$	69^{d} (11.1)
$FY{*}B^{ES}/FY{*}B^{ES}$	20^{e} (3.2)
DARC* alleles, n (%)	
*FY*A*	581 (46.8)
*FY*B*	425 (34.3)
$FY{*}B^{ES}$	234 (18.9)

IQR = interquartile range.
LM: 17 P. vivax, two P. falciparum; Real-Time PCR: 15 P. vivax, two P. falciparum.
[a–e]Significant differences (P<0.05, Z-test).
* Proportions differ significantly, $FY{*}A > FY{*}B > FY{*}B^{ES}$, (P < 0.0001, Z-test).
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distribution of vivax malaria episodes according to rainfall and season is illustrated in Figure 1A.

Among the 620 recruited volunteers, 395 were enrolled at the baseline, 410 during the 2nd survey (6 months) and 407 during the 3rd survey (12 months). At the baseline, ELISA IgG antibodies to the main variant of PvDBP circulating in the area (Sal-1) were detected in 32% (region II, DBPII) to 50% (region II–IV, DBPII-IV) of the studied population (Figure 1B). The profile of DBPII antibody response was relatively stable over the course of the cross-sectional studies, and was not associated with the malaria transmission season (Figure 1B). On the other hand, DBPII-IV antibodies fluctuated according to malaria transmission (50% vs. 29% vs. 36% at baseline, 6, and 12 months, respectively; P<0.05 by Z-test). Consecutive serological surveys demonstrated that, while 50% of DBPII responders could be classified as high responders (RI>4), the majority of DBPII-IV responders were low responders (70 to 85%, RI≤4). In this area, presence of PvDBP antibodies seemed not to be influenced by acute malaria infection, as the frequency of responders was similar in infected and non-infected groups (P>0.05; Figure S2). However, the low numbers of acute infections identified in the study preclude conclusions about the association between infection and PvDBP antibody response. Acute infections were not explored in the further analyses.

DARC polymorphisms and generation of *P. vivax* specific antibodies

Three *DARC* genotypes, *FY*A/FY*B*, *FY*A/FY*A* and $FY{*}A/FY{*}B^{ES}$, were frequently observed (Table 1), with heterozygous *FY*A/FY*B* being the most prevalent (182 out of 620, 29.4%). The *DARC* negative genotype ($FY{*}B^{ES}/FY{*}B^{ES}$) was present at low frequency (3.2%, 20 of 620). Accordingly, we found a significant predominance of non-silent *DARC* alleles in the study area, with $FY{*}A > FY{*}B > FY{*}B^{ES}$ ($p<0.0001$ by Z-test). Individuals carrying the different *DARC* genotypes were equally distributed among the demographical and epidemiological variables associated with the risk of malaria infection, such as age, sex, and dwelling location (data not shown).

Anti-PvDBP IgG antibodies by conventional serology

To determine whether DARC polymorphisms influence the PvDBP antibody responses, we initially analyzed PvDBP antibodies as detected by ELISA. DBPII antibodies were analyzed at baseline and after 6 and 12 months, with individuals stratified according to *DARC* alleles, i.e., those carrying two (*FY*A/FY*A*, *FY*A/FY*B*, *FY*B/FY*B*), one ($FY{*}A/FY{*}B^{ES}$, $FY{*}B/FY{*}B^{ES}$), or no ($FY{*}B^{ES}/FY{*}B^{ES}$) *DARC*-positive allele (Figure 2). The frequency of responders among DARC negative individuals ($FY{*}B^{ES}/FY{*}B^{ES}$) was low, and their pattern of response remained the same at all survey times (Figure 2A). In DARC positive individuals, DBPII antibodies were detected in 29–36%, with no difference between individuals carrying one or two *DARC*-positive alleles (Figure 2A). A similar profile of response was detected for DBPII-IV antibodies (data not shown).

Since 205 of 620 individuals provided plasma samples at all three surveys (paired-samples), we analyzed this sub-group for differences in their anti-DBPII serological responses (Figure 2B). In general, the profiles of the DBPII antibody responses were similar between paired and non-paired samples (Figure 2). However, in the paired sub-group, it was possible to detect a slight trend toward a more intense response to DBPII among DARC positives carrying a single positive allele (high responder, RI>4.0) (Figure 2B). However, the difference was significant only at 12 months (Z-test = 3.2; P = 0.0014), with 35% and 47% responders detected among double and single positive carriers, respectively. This tendency was not associated with any particular genotype and was not detected by using another recombinant (DBPII-IV) (data not shown).

DBPII binding inhibitory antibodies (BIAbs)

Further experiments investigated whether DARC polymorphisms altered the functional proprieties of DBPII antibodies. Due to the methodology constraints of performing functional assays, the three cross-sectional measures of DBPII BIAbs responses were performed on a subset of the study population comprising 270 samples corresponding to 90 DARC positive individuals (paired-samples) matched for age, sex, and malaria exposure (16 to 23 volunteers per *DARC*-positive genotype). Plasma samples from DARC negative individuals (n = 21) were used as a negative control, and no DBPII BIAbs could be detected in this group (data not shown). DBPII BIAbs were significantly more frequent in single *DARC*-positive allele carriers than in double positive carriers (Figure 3A). This pattern of acquired immune response was similar in all cross-sectional measures of DBPII BIAbs. Beyond the frequency and levels of inhibitory antibodies, the persistence of response was also higher in the group carrying a single *DARC*-positive allele (Z-test = 3.002; P = 0.0027) (Figure 3B), a result that was not detected by conventional serology (data not shown).

The tendency towards an increased DBPII inhibitory antibody response among single *DARC*-positive allele carriers led us to investigate whether a particular *DARC* genotype or allele could be associated with this response. Persons homozygous for either *FY*A* or *FY*B* alleles were associated with lower DBPII BIAbs; whereas the presence of these alleles in heterozygosis for the *GATA* mutation ($FY{*}A/FY{*}B^{ES}$ or $FY{*}B/FY{*}B^{ES}$) was associated with a

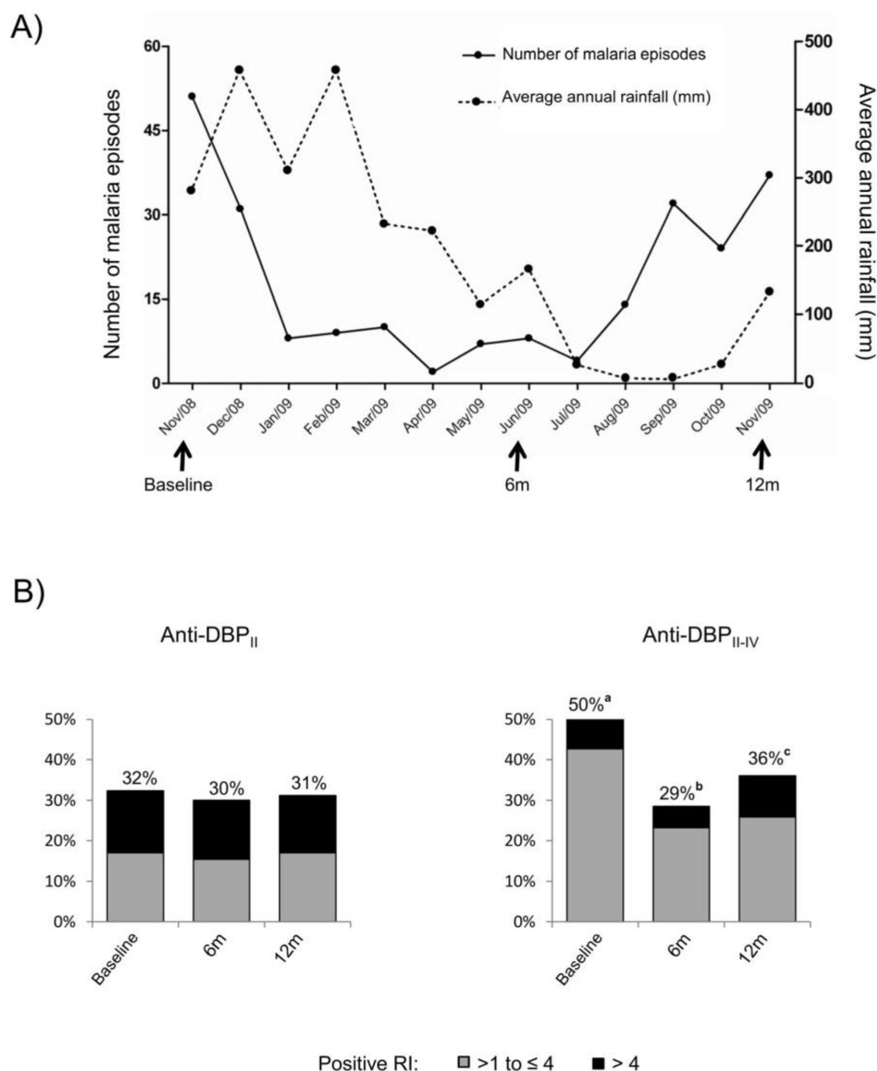

Figure 1. Temporal distribution of *P. vivax* malaria episodes and serological evaluation of ELISA-detected IgG antibodies targeting PvDBP, Amazonas, Brazil (Nov 2008-Nov 2009). (A) Episodes of malaria, as detected by conventional microscopy, varied according to rainfall and season; (B) DBPII-IV antibodies fluctuate with season while anti-DBPII remains stable. ELISA-detected antibody responses were evaluated November 2008, June 2009, and November 2009. Results are expressed as frequency (%) of responders, with Reactivity Index (RI)>1.0 considered positive; different superscripts (a–c) indicate significant differences (P<0.05 by Z-test.)
doi:10.1371/journal.pone.0093782.g001

much higher inhibitory response, and these profiles were consistent in all three surveys (Figure 4). Individuals heterozygous for *DARC*-positive alleles (*FY*A/FY*B*) developed an intermediate level of DBPII BIAbs (c. 38%). These results demonstrated that *DARC* genotype influences the frequency and stability of human DBPII inhibitory antibody response.

Discussion

An important goal of PvDBP vaccine efforts is to inhibit parasite invasion of DARC positive reticulocytes. Since DARC polymorphisms are suspected to affect the ability of PvDBP antibodies to block parasite invasion [36], we carried-out the first follow-up population-based study of the relationship between DARC polymorphisms and DBP antibodies. In the study area, incidence rates for *P. vivax* and *P. falciparum* malaria were 2.32/100 and 0.04/100 person-months, respectively, allowing the classification of the area as hypo- to meso-endemic, consistent with the general profile of malaria transmission in the Amazon region [41]. At enrollment, 32% of the studied individuals showed ELISA-detected IgG antibodies to the ligand region II (DBPII), while a higher seroprevalence (50%) was found by using a recombinant protein covering regions II to IV (DBPII-IV). Although DBPII antibodies were less frequent than DBPII-IV antibodies, the magnitude of the DBPII immune response was significantly higher and included a significant number of high responders (RI>4), possibly because

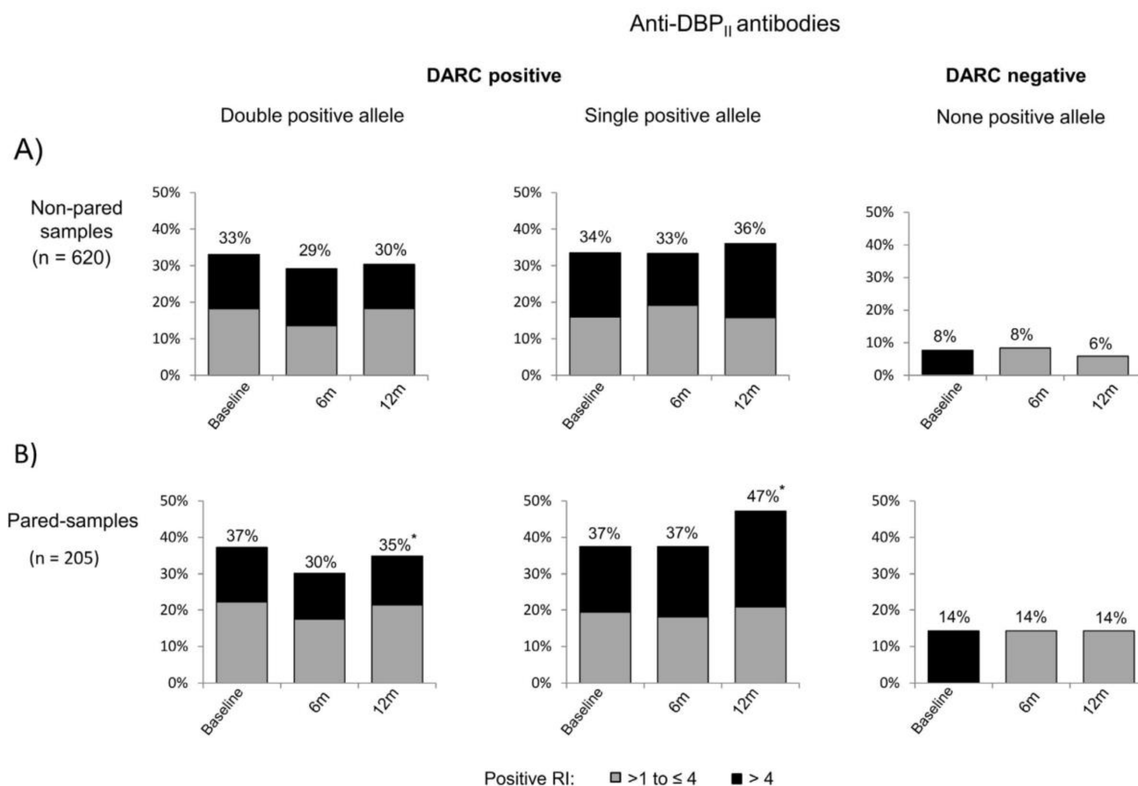

Figure 2. Frequencies of DBPII IgG ELISA-detected antibodies are similar in individuals carrying one or two *DARC* positive alleles. DBPII antibody responses were evaluated in plasma samples from individuals who participated in (A) at least one cross-sectional survey (non-paired samples, n = 620) or (B) in three consecutive surveys (paired-samples, n = 205). DARC positive individuals were categorized as carrying double positive alleles (*FY*A/FY*A, FY*A/FY*B, FY*B/FY*B*) or a single positive allele (*FY*A/FY*B^{ES}, FY*B/FY*B^{ES}*). DARC-negatives do not express DARC antigens on erythrocyte surface (*FY*B^{ES}/FY*B^{ES}*). ELISA results were expressed as frequency of responders (percentages on the top of the figures), with Reactivity Index (RI)>1.0 considered positive. For all frequency comparisons, significant differences were found only between DARC negative and DARC positive groups (P<0.05 by chi-square test).
doi:10.1371/journal.pone.0093782.g002

dominant B-cell epitopes lie on region II [42–44]. Accordingly, the analysis of consecutive serological surveys demonstrates that, while DBPII-IV antibodies fluctuate with season, DBPII antibodies were stable throughout the study period.

It is well known that DARC-negative individuals are highly resistant to *P. vivax* infection [4] and, in general, present only low levels of antibodies to *P. vivax* blood stages [14]. Therefore, we were particularly interested in investigating the influence of *DARC*-positive alleles on the acquisition of anti-PvDBP immunity. Among *DARC*-positive allele carriers, the frequencies of ELISA-detected IgG antibodies were similar in single (*FY*A/FY*B^{ES}* and *FY*B/FY*B^{ES}*) and double positive (*FY*A/FY*A, FY*B/FY*B* and *FY*A/FY*B*) allele carriers. We observed a weak trend toward increased DBPII IgG antibodies among single positive allele carriers, although this association was not significant in two of the three cross-sectional surveys.

Few studies have investigated the influence of DARC-positive alleles on the presence of conventional PvDBP antibodies acquired post-infection [15,36]. Using the recombinant DBPII expression construct used in the present study, but ELISA assays with plasma samples twice more concentrated, Maestre et al. [15] reported that Colombian subjects with one positive allele were more likely to show DBPII antibodies than were those with two positive alleles. Unfortunately, the low number of ELISA-positive individuals in the Colombian study (DBPII IgG sera, n = 17) precludes solid conclusions with respect to an association between PvDBP antibodies and the *DARC*-positive allele. In accordance with our findings, King et al. [36] reported that *DARC* genotypes were not associated with significant differences in DBPII ELISA-specific antibody responses. In conclusion, we found no clear association between *DARC*-positive alleles and conventional anti-PvDBP immune responses, as detected by ELISA using different recombinant proteins.

In contrast to conventional serology, the present study demonstrated that the frequency and magnitude of DBPII inhibitory antibody responses were significantly lower in adults carrying two *DARC*-positive alleles, especially in individuals homozygous for either the *FY*A* or the *FY*B* allele. Twelve months later, the frequency of persistent DBPII BIAb responders among those bearing double-positive alleles was half that of single-positive carriers. These results suggested that, while individuals with a high level of DARC expression (double positive carriers) exhibited low BIAbs responses, those with a lower level of expression (single positive carriers) exhibited a higher inhibitory response. There is no clear explanation for why individuals carrying a single *DARC*-positive allele exhibit a greater level of

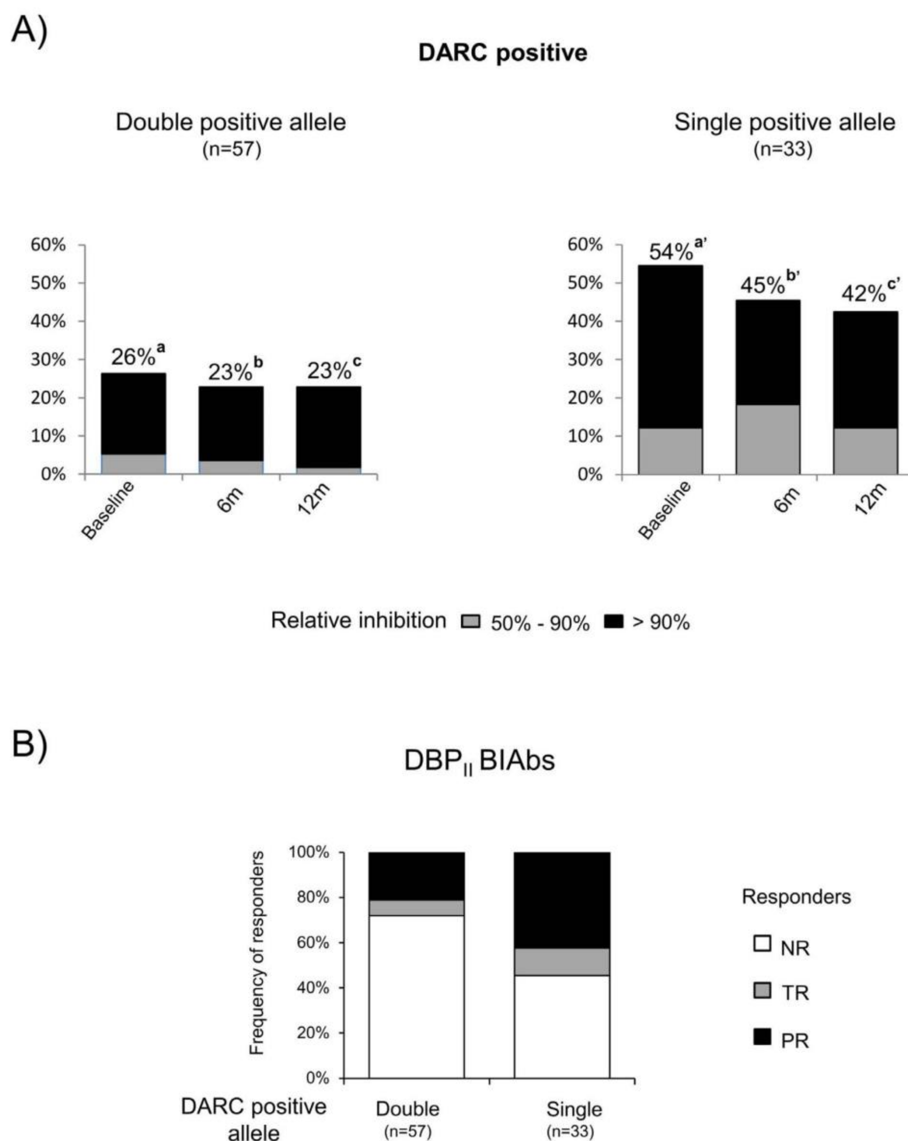

Figure 3. DARC polymorphisms affect the functional properties of DBPII antibodies. DBPII binding inhibitory antibodies (BIAbs) were evaluated in plasma from individuals stratified according to *DARC* alleles, as carrying double positive alleles (*FY*A/FY*A, FY*A/FY*B, FY*B/FY*B*) or a single positive allele (*FY*A/FY*B^ES^, FY*B/FY*B^ES^*). A) Frequencies of BIAbs (top of the columns) at three consecutive surveys showed DBPII BIAbs to be significantly more frequent in single positive allele carriers than in double positive carriers (significant differences between a *vs.* a'; b *vs.* b' and c *vs.* c', as determined by by Z-test, $P<0.05$.); B) After 12-months, the BIAbs response was more persistent in the group carrying a single *DARC*-positive allele (Z-test = 3.002; P = 0.0027). NR = non-responder, no inhibitory antibody response detected at any time-point of the study; TR = temporary responder, inhibitory antibodies in at least one cross-sectional survey; (PR = persistent responder; inhibitory antibodies detected in all surveys. BIAbs were evaluated by COS-7 cyto-adherence assay, with plasma samples at 1:40 and positive results as DBPII –DARC binding inhibition $\geq$ 50%.
doi:10.1371/journal.pone.0093782.g003

inhibitory antibodies that those with two positive alleles, especially as there is no evidence that DARC indirectly down-regulates humoral immune responses against the *P. vivax* blood stage, as has been proposed [15]. Because we and others have shown that low DARC erythrocyte expression reduces the risk of *P. vivax* blood-stage infection [34,35], it can be speculated that limiting access of the *P. vivax* merozoite to the DARC antigen might increase DBPII exposure to the immune system. Considering the quickness of the invasion process [45,46], and that PvDBP appears to be a poor immunogen that remains sequestered in the micronemes until invasion begins [5], it seems to be a feasible mechanism. Despite this, the levels of erythroid-DARC expression per se might not explain our findings, because, while DARC expression has been found to be lower in persons with *FY*B/FY*B* than in *FY*A/FY*A*

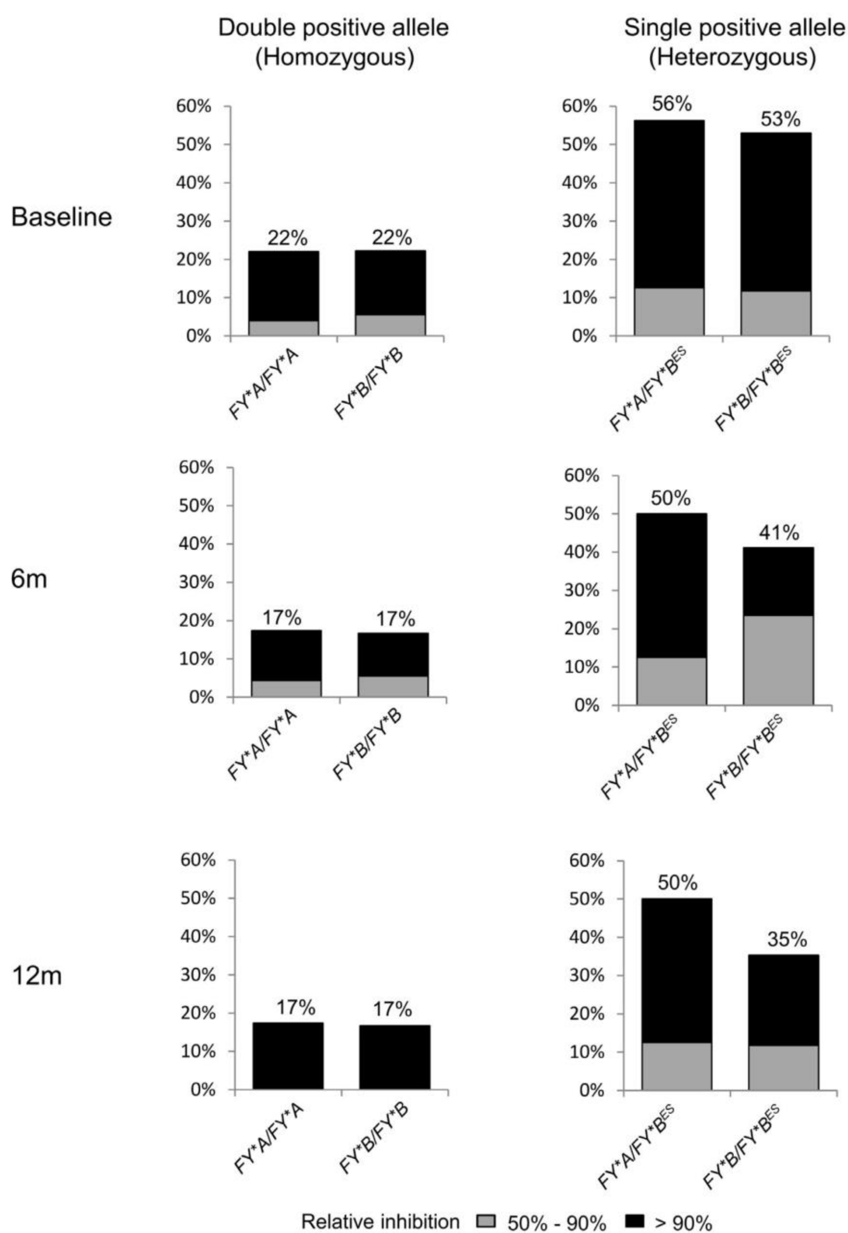

Figure 4. Homozygosis for either the *FY*A* or *FY*B* allele is associated with low frequency of DBPII BIAbs. Plasma samples were grouped according to *DARC* genotype, as described in legend of Figure 3. DBPII BIAbs were evaluated at three consecutive surveys by COS-7 cyto-adherence assay, with plasma at 1:40 and DBPII –DARC binding inhibition ≥ 50% considered positive inhibition.
doi:10.1371/journal.pone.0093782.g004

[47], we found no difference in the inhibitory responses between the *FY*A* and *FY*B* homozygotes. Recent evidence suggests that *FY*A* allelic polymorphism affects the ability of PvDBP antibodies to block parasite invasion [36]. Here, the DBPII/DARC interaction was assessed by using the established COS-7 cyto-adherence assay based upon a multivalent interaction between

DBPII on the surface of COS-7 cells and DARC expressed in RBCs [48]. Because, in the COS-7 assay, antibodies are challenged to inhibit a cell interaction that is likely to be highly multivalent [49], it is possible that small differences in antibody activity might be not detected. Studies to assess the BIAbs through an interaction assay of limited valency, such as the recently described flow cytometry based antibody-inhibition assay in which DBPII expressed on the yeast surface interacts with a dimeric recombinant DARC, would be of interest [49]. Although the inhibitory response in the yeast assay seems to be more amenable to high-throughput analysis, the clinical relevance of an assay with low valency interaction remains to be determined. Although these *in vitro* biological assays have made great contributions to the study of the interaction between DBPII and DARC, it is expected that the successful establishment of a long-term culture of *P. vivax* would lead to insights that could improve our understanding of the role of *DARC* genotypes in invasion and immune response processes. Time-lapse microscopy of live *P. vivax* merozoites grown in the presence of inhibitory anti-sera could shed light on this topic.

Through a 12-month follow-up study in the Amazon area, we demonstrated that DBPII inhibitory antibody responses were approximately 50% lower in *FY*A/FY*A* and *FY*B/FY*B* double-positive individuals when compared with individuals heterozygous for *FY*A or F*B* alleles, suggesting a gene-dosage effect. It is not yet clear if this finding has implications for the acquisition of protection against malaria. Due to the relevance of these findings for vaccine development, it would be pertinent to investigate whether such an association exists in other vivax malaria endemic countries.

Supporting Information

Figure S1 The DBPII binding to erythrocytes is influenced by *DARC* genotype. COS-7 cells, transfected with plasmid expressing the gene encoding DBPII, were incubated with erythrocytes from individuals bearing different *DARC* genotypes. The results were expressed as the median of the number of rosettes (5 to 10 samples per genotype) in erythrocyte-binding assays, as described in material and methods. *Box-plots*: solid line across the box is the median, and the bottom and the top of each box represent the 25th and 75th percentiles, respectively; vertical lines represent the range. DARC-negative erythrocytes were used as a negative control. Significant differences were detected between $FY*B/FY*B$ and $FY*B/FY*B^{ES}$ and $FY*A/FY*B$ and $FY*B/FY*B^{ES}$ ($P<0.01$ by Kruskal–Wallis with Dunn's post hoc test).
(TIF)

Figure S2 The PvDBP antibody response was not shown to be influenced by acute malaria infection. The of DBPII and DBPII-IV ELISA-detected antibodies were evaluated at 0, 6, and 12 months, and individuals were grouped according to the presence (yes) or absence (no) of malaria infection at the time of blood collection. The results are shown as frequency (%) of responders (RI>1.0).
(TIF)

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Author Contributions

Conceived and designed the experiments: LHC FAS LMT FSK JHA. Performed the experiments: FAS LMT JRS MLT TNS BAMS. Analyzed the data: CJFF FSK CFAB. Contributed reagents/materials/analysis tools: PAN CFAB JHA RSR. Wrote the paper: LHC FAS LMT FSK CFAB.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

The Presence, Persistence and Functional Properties of *Plasmodium vivax* Duffy Binding Protein II Antibodies Are Influenced by HLA Class II Allelic Variants

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Abstract

Background

The human malaria parasite *Plasmodium vivax* infects red blood cells through a key pathway that requires interaction between Duffy binding protein II (DBPII) and its receptor on reticulocytes, the Duffy antigen/receptor for chemokines (DARC). A high proportion of *P. vivax*-exposed individuals fail to develop antibodies that inhibit DBPII-DARC interaction, and genetic factors that modulate this humoral immune response are poorly characterized. Here, we investigate if DBPII responsiveness could be HLA class II-linked.

Methodology/Principal Findings

A community-based open cohort study was carried out in an agricultural settlement of the Brazilian Amazon, in which 336 unrelated volunteers were genotyped for HLA class II (*DRB1*, *DQA1* and *DQB1* loci), and their DBPII immune responses were monitored over time (baseline, 6 and 12 months) by conventional serology (DBPII IgG ELISA-detected) and functional assays (inhibition of DBPII–erythrocyte binding). The results demonstrated an increased susceptibility of the *DRB1** 13:01* carriers to develop and sustain an anti-DBPII IgG response, while individuals with the haplotype *DRB1* 14:02-DQA1* 05:03-DQB1* 03:01* were persistent non-responders. HLA class II gene polymorphisms also influenced the functional properties of DBPII antibodies (BIAbs, binding inhibitory antibodies), with three alleles (*DRB1* 07:01*, *DQA1* 02:01* and *DQB1* 02:02*) comprising a single haplotype linked with

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the presence and persistence of the BIAbs response. Modelling the structural effects of the HLA-DRB1 variants revealed a number of differences in the peptide-binding groove, which is likely to lead to altered antigen binding and presentation profiles, and hence may explain the differences in subject responses.

Conclusions/Significance

The current study confirms the heritability of the DBPII antibody response, with genetic variation in HLA class II genes influencing both the development and persistence of IgG antibody responses. Cellular studies to increase knowledge of the binding affinities of DBPII peptides for class II molecules linked with good or poor antibody responses might lead to the development of strategies for controlling the type of helper T cells activated in response to DBPII.

Author Summary

Vaccines are a crucial component of the current efforts to eliminate malaria, and much of the vaccine-related research on *P. vivax* has been focused on the Duffy binding protein II (DBPII), a ligand for human blood stage infection. A high proportion of individuals who are naturally exposed to *P. vivax* fail to develop neutralizing antibodies, but the host genetic factors modulating this immune response are poorly characterized. We investigated whether DBPII responsiveness was dependent on the variability of human leucocyte antigen (HLA) class II cell surface proteins involved in the regulation of immune responses. To obtain a reliable estimate of DBPII antibodies, we carried out a longitudinal study, collecting serum from the same individuals over a period of 12-months. The results confirmed the heritability of the DBPII immune response, with genetic variation in HLA class II genes influencing both the development and persistence of the antibody response. HLA class II genotype also influenced the ability of DBPII antibodies to block the ligand-receptor interaction *in vitro*. Computational approaches identified structural specificity between HLA variants, which we propose as an explanation for differences between a good or poor antibody responder. These results may have implications for vaccine development, and might lead to strategies for controlling the type of immune response activated in response to DBPII.

Introduction

Plasmodium vivax infects human reticulocytes through a major pathway that requires interaction between an apical parasite protein, the Duffy binding protein (DBP), and its cognate receptor on reticulocytes, the Duffy antigen/receptor for chemokines (DARC) [1–3]. Although most individuals lacking DARC on their red blood cells (RBCs) are naturally resistant to *P. vivax* [1], some infections occur in DARC-negative individuals living in vivax malaria endemic areas [4–6, 70]. So far, no alternative ligand facilitating the binding of *P. vivax* to reticulocytes has been identified, which makes the DBP one of the most promising *P. vivax* vaccine targets [8].

The importance of the interaction between DBP (region II, DBPII) and DARC to *P. vivax* infection has stimulated a significant number of studies on DBP antibody responses (reviewed in [8]). The available data demonstrate that naturally occurring antibodies to DBP are

prevalent amongst individuals living in *P. vivax* endemic areas, and that these antibodies can inhibit the DBPII-DARC interaction [7, 9–12]. Even though DBPII-specific binding inhibitory antibodies (DBPII BIAbs) seem to confer a degree of protection against blood stage infection [11], the majority of people naturally exposed to *P. vivax* do not develop a DBPII BIAbs response [8]. In the Amazon Basin, for example, this inhibitory activity was detected in only one third of malaria-exposed subjects [8, 13]. Similarly, less than 10% of children from Papua New Guinea (PNG) with immunity to malaria had acquired high levels of DBPII BIAbs [11]. Given the significant differences in epidemiology and parasite genetics between the Amazon Basin and PNG, the fact that the DBPII BIAbs response is relatively low but also remarkably stable over time is particularly intriguing.

The reasons for the low immunogenicity of DBPII are not clear, but may be linked to a complex immune response driven by genetic diversity in both the parasite and human populations. Several studies have demonstrated the existence of variant specificity in the natural immune response against DBPII, which has been attributed to allelic diversity [12, 14]. On the host side, recent evidence suggests that host genetic polymorphisms might also affect humoral immunity against DBP [15, 16], with DARC polymorphisms thought to affect the ability of DBP antibodies to block parasite invasion [16]. In a previous study, we demonstrated that the naturally acquired BIAbs response tended to be more frequent in heterozygous individuals carrying a DARC-silent allele ($FY^{*}B^{ES}$), which suggested that gene-dosage effect occurred [7]. In this context, we were interested in determining if DBPII non-responsiveness could be associated with variation in the major histocompatibility complex.

While malaria infection represents a key selection pressure for the human leukocyte antigen (HLA), and has left clear evolutionary footprints on the alleles observed in different countries [17], the association between HLA gene expression and responsiveness (or non-responsiveness) to defined malaria antigens has produced contradictory results [18–21]. Beyond the extreme genetic diversity of HLA class II, which hinders interpretation of the role of HLA on antibody responses elicited during malaria, most studies rely on antibody prevalence data collected at a single time-point in cross-sectional analysis of a population. Since malaria transmission is intermittent and seasonal in many endemic areas, it is possible that antibody levels fluctuate over time such that individuals could appear to be non-responders on some occasions and responders on others [20]. In the current study, we present data of the first ongoing population-based study of the relationship between HLA class II genes and DBPII immune response. The methodological approach included a community-based open cohort study in an agricultural settlement of the Brazilian Amazon, in which 336 unrelated volunteers were genotyped for HLA class II (*DRB1*, *DQA1* and *DQB1* loci), and their DBP immune responses were monitored over time by conventional serology (DBPII IgG ELISA-detected) and functional assays (DBPII BIAbs).

Methods

Study area and population

The study was carried out in the agricultural settlement of Rio Pardo (1°46'S—1°54'S, 60° 22'W—60°10'W), in the Presidente Figueiredo municipality, located in the Northeast of Amazonas State in the Brazilian Amazon. The Rio Pardo settlement is located approximately 160 km from Manaus, the capital of Amazonas, along the main access to a paved road (BR-174) that connects Amazonas to Roraima (Fig 1). The settlement was officially created in 1996 by the National Institute of Colonization and Agrarian Reform (INCRA) as part of a large-scale colonization project focused on agriculture and wide-ranging human settlement in the Amazon area [22]. The mean annual temperature is 31°C with a humid climate and an average

Fig 1. Map of the state of Amazonas, Northwestern Brazil, showing the study site. The Rio Pardo settlement is located within the Presidente Figueiredo municipality (grey area in the inset), roughly 160 km from the state capital, Manaus.

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annual rainfall of 2,000 mm per year. The rainy season extends from November to May, and the dry season from June to October. According to a census conducted between September and October 2008, Rio Pardo has 701 inhabitants, most of whom live on subsistence farming and fishing along the tributaries of the Rio Pardo River. The study population was quite stable. Most residents were native to the Amazon region, and their average age of 28 years roughly corresponded to the time of malaria exposure in the Amazon area [7]. In the study area, migration rates were relatively low, as only 28 (8%) of 336 individuals moved out of the village during the follow-up period.

Based on the spleen size of the local children and parasite infection rates, the study area was classified as hypo- to mesoendemic, which is consistent with the general profile of malaria infection for well-established frontier settlements in the Amazon region [23]. The study site and malaria transmission patterns have been described in detail elsewhere [7]. Although *P. vivax* and *P. falciparum* are transmitted year round, *P. vivax* is responsible for about 90% of malaria cases in the region. Similar to other parts of the Brazilian Amazon area [24], a continuous decrease in the number of malaria cases has been reported in the Rio Pardo community; in 2008, the local Annual Parasitological Index (API) was 131 cases per 1000 inhabitants, while in 2009 the API was 54.6 (Health Surveillance Secretariat of the Brazilian Ministry of Health, SVS/MS). In the study area, precarious living conditions, including houses with partial walls and roofs made of tree leaves, increase human-vector contact and reduce indoor residual spraying efficacy [23]. However, while the availability of curative services is limited, a government outpost in the area provides free malaria diagnosis and treatment.

Study design and cross-sectional surveys

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 (Reports No.

007/2006, No. 07/2009, No. 12/2010 and No. 26/2013), according to the Resolution of the Brazilian Council on Health-CNS 466/12. In November of 2008, 541 of the 701 residents of the settlement (77.2%) invited to participate in the study accepted by giving written informed consent, which was also obtained from the next of kin, caregivers, or guardians on the behalf of participating minors.

A population-based open cohort study was initiated in November of 2008, with the following procedures: (i) administration of a structured questionnaire to all volunteers to obtain demographical, epidemiological, and clinical data; (ii) physical examination, including body temperature and spleen/liver size, recorded according to standard clinical protocols; (iii) venous blood collection for individuals aged five years or older (EDTA, 5 mL), or blood spotted on filter paper (finger-prick) for those aged <5 years; and (iv) examination of Giemsa thick blood smears for the presence of malaria parasites via light microscopy. The geographical location of each dwelling was recorded using a hand-held 12-channel global positioning system (GPS) (Garmin 12XL, Olathe, KS, USA) with a positional accuracy of within 15 m. At the time of initial enrollment in the study, 222 out of 541 volunteers had no familial relationships with other volunteers, and were consequently selected for HLA genotype and serological assays.

Six and twelve months after the initial survey, two similar cross-sectional surveys were carried out. In total, 336 unrelated subjects were enrolled in the study, with 222 examined in the baseline cohort, 249 examined during the 2nd survey (June, 2009), and 239 during the 3rd survey (October-November, 2009). A total of 244 (72.6%) subjects had consecutive samples taken, and 156 of these (64%) had samples taken in all cross-sectional surveys (baseline, 6 and 12 month follow-up).

Laboratory diagnosis of malaria

Malaria infections were diagnosed by microscopy of Giemsa-stained thick blood smears, and by Real-Time PCR amplification of a species-specific segment of the multicopy 18SSU rRNA gene of human malaria parasites. The Giemsa-stained smears were evaluated by experienced microscopists, according to the malaria diagnosis guidelines of the Brazilian Ministry of Health. For Real-Time PCR, genomic DNA was extracted from either whole blood samples collected in EDTA, or from dried blood spots on filter paper using the Puregene blood core kit B (Qiagen, Minneapolis, MN, USA) or the QIAmp DNA mini kit (Qiagen), respectively, according to manufacturers' instructions. Real-Time PCR was performed as previously described [25].

HLA genotyping

Molecular amplification of the alleles of *HLA-DRB1*, *HLA-DQB1* and *HLA-DQA1* were performed by the PCR-SSO (polymerase chain reaction, specific sequence of oligonucleotides) technique, with Luminex technology (One Lambda Inc., Canoga Park, CA, USA). Briefly, target DNA was PCR-amplified using group-specific primer sets, after the amplified product was biotinylated, which allowed later detection using R-Phycoerythrin-conjugated Streptavidin (SAPE), and hybridized with microspheres linked to specific conjugated fluorescent probes for each HLA allele group (One Lambda, Canoga Park, CA, USA). The fluorescent intensity varied based on the reaction outcome, with an expected intensity of 1000 or more for positive control probes. Reaction readings were carried out by flow cytometry using Luminex technology (One Lambda). Samples were analyzed through the HLA FUSION software (One Lambda Inc., San Diego, CA, USA).

Recombinant DBPII and ELISA-detected IgG antibodies

A conventional enzyme-linked immunosorbent assay (ELISA) for total IgG antibodies to DBPII was carried out using a recombinant protein that included amino acids 243–573 (region II) of the Sal-1 DBPII variant, which is highly prevalent in the study area [23]; the recombinant protein was expressed as a 39 kDa 6xHis fusion protein, as previously described [26]. ELISA was carried out as previously described [27], with serum samples at 1:100 and DBPII at a final concentration of 3 μg/ml. The results were expressed as reactivity index (RI), calculated by dividing the reading values of the test (OD values) by the cut-off (mean reading for the unexposed group plus 3 SD, n = 30). Values of RI > 1.0 were considered positive.

COS7 cells transfections and DBPII inhibitory binding assays

COS7 cells (green monkey kidney epithelium, ATCC, Manassas, VA) were transfected with the plasmid pEGFP-DBPII, which coded for a common DBPII sequence circulating in the Amazon area [13]. Transfections were performed with lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocols. Forty-eight hours post-transfection, erythrocyte-binding assays were performed as previously described [10]. Briefly, plasma samples were added at 1:40, and plates were incubated for 1 hr at 37°C in 5% CO2. Human O+ DARC+ erythrocytes in a 10% suspension were added to each well (200 μl/well), and plates were incubated for 2 h at room temperature. After incubation, unbound erythrocytes were removed by washing the wells three times with phosphate buffered saline (PBS). Binding was quantified by counting rosettes observed in 10–20 fields of view (x200). Positive rosettes were defined as adherent erythrocytes covering more than 50% of the COS cell surface. For each assay, pooled plasma samples from Rio Pardo residents characterized as non-responders by ELISA were used as a negative control (100% binding). For this purpose, only plasma that did not inhibit erythrocyte binding was pooled for use as the negative control (usually, 10 plasma samples/pool). The positive control included a pool of plasma from individuals with long-term exposure to malaria in the Amazon area. The percent inhibition was calculated as 100 x (Rc—Rt)/Rc, where Rc is the average number of rosettes in the control wells, and Rt is the average number of rosettes in the test wells. Plasma samples with more than 50% of binding inhibition were considered positive.

Prediction of DBPII—HLA class II binding affinity

To predict HLA-DR/-DQ binding affinities (IC50) we used the *P. vivax* DBP sequence (XP_001608387.1) from the NCBI database. Each potential 15-mer sequence frame was scored using the NetMHCIIpan-3.1 server (http://www.cbs.dtu.dk/services/NetMHCIIpan-3.1/), an improved version of the tool that permits a much more accurate binding core identification [28]. Binding affinity was given as the log IC50 value in nanomolar (nM), and the defined thresholds for strong and weak binders were <1.7 nM and <2.7 nM, respectively.

Modelling the structural effects of the HLA class II variants

Homology models of the three DRB1 variants were generated using Modeller and Macro Model (Schrodinger, New York, NY) using an ensemble of previously solved X-ray crystal structures of the HLA II beta chain (PDB IDS: 1IEB Chain D, 3LQZ Chain B and 1SEB Chain B; 76%, 67% and 90% sequence identity respectively). The alpha chain was modelled using an ensemble of available X-ray crystal structures including PDB IDs: 2Q6W and 4AEN (Chain D and A respectively, 100% sequence identity). As previously described [29, 30], the models were then minimized using the MMF94s forcefield in Sybyl-X 2.1.1 (Certara L.P., St Louis, MO),

with the final structure having more than 95% of residues in the allowed region of a Ramachandran plot. The quality of the models was confirmed with Verify3D. The models of the HLA II complex of the alpha and beta chains were built using X-ray crystal structures of the complex (PDB ID: 1IEB, 3LQZ, 1SEB, 2Q6W, 4AEN) to guide protein docking [31]. Two representative DBPII antigenic peptides (H1: FHRDITFRKLYLKRKL; H3: DEKAQQRRKQWW-NESK) were modelled into each HLA II complex variant using the available crystal structures of the HLA II complexes to guide docking. Binding affinities were predicted using CSM-lig [32]. Model structures were examined using Pymol.

The structural consequences of each amino acid difference between the DRB1 variants were analyzed to account for all the potential effects of the mutations [33]. The effects of the variations on the stability of DRB1 and the HLA II complex were predicted using DUET [34], an integrated computational approach that optimizes the prediction of two complementary methods (mCSM-Stability and SDM). The effect of the differences on the protein-protein binding affinity between the alpha and beta chains to form the HLA II complex were predicted using mCSM-PPI [35]. The effect of the changes on the binding affinity of the HLA II complex for a model peptide were also analysed using mCSM-PPI, as previously described [36], mCSM-lig [37], and mCSM-AB [38]. These computational approaches represent the wild-type residues structural and chemical environment of a residue as a graph-based signature in order to quantitatively determine the change upon mutation in Gibb's Free Energy of stability or binding.

Statistical analysis

A database was created using Epidata software (http://www.epidata.dk). Linear correlation between two variables was determined by using the Spearman's correlation coefficient. Differences in proportions were evaluated by chi-square (X2) test and, differences in medians were tested using either the Mann-Whitney or Kruskal–Wallis tests, with Dunn's post hoc test, as appropriate. For allelic group comparison, differences in proportion were performed by Z-test or chi-square tests, or Fisher's exact tests, as appropriate. Alleles frequencies for each locus (*DRB1*, *DQA1*, and *DQB1*) were summarized descriptively using frequencies and percentage for immunological categorical variables.

Overall associations with immunological responses and alleles from each locus of HLA class II were evaluated by comparing the allele frequencies between seronegative individuals and seropositive individuals from the baseline study using standard contingency tables. Based on the humoral immune response to DBPII from the three cross-sectional surveys, the long-term immune responses against DBPII were grouped into three categories: (i) *Persistent non-responder* (PNR)—absence of antibodies against DBPII in all three cross-sectional surveys; (ii) *transient responder* (TR)—antibodies detected in at least one cross-sectional survey; (iii) *Persistent responder* (PR)—individuals with detectable DBPII antibodies in all three cross-sectional studies. The association between HLA class II alleles (or haplotypes) and long-term immune response (PR or PNR groups) was analyzed by standard contingency tables (Chi-square and Fisher's exact test, as appropriate), with two observations per subject (one for each allele). Alleles with a frequency of less than 0.01 were not included in the analysis.

Additionally, multiple logistic regression models with stepwise backward deletion were built to describe independent associations between covariates and HLA class II alleles or haplotypes and antibodies to DBPII. Covariates were selected for inclusion in the logistic models if they were associated with the outcome at the 15% level of significance in exploratory unadjusted analysis. Logistic regression models included the following covariates: age, gender, exposure to malaria (time of residence in the endemic area), self-reported malaria episodes,

recent malaria infection and household location within the study area. Multivariate logistic regression was performed using Stata software version 10 (Stata Corporation, College Station, TX). Only variables associated with statistical significance at the 5% level were maintained in the final models. To avoid type II errors due to overly severe correction, statistical adjustment for multiple tests were not used [39, 40]. Type I errors were reduced by using multiple logistic regression models with stepwise backward deletion. Estimated genotype distribution between the observed and expected allelic frequencies was tested using the method described by Guo and Thompson [41] to verify Hardy-Weinberg equilibrium. Because the gametic phase was unknown, maximum-likelihood estimates of haplotype frequencies were obtained from multilocus genotype data and computed using the expectation-maximization (EM) algorithm [42]. Both procedures were performed using Arlequin software version 3.5 (http://cmpg.unibe.ch/software/arlequin35/) [43].

Results

Malaria infection and DBPII-specific antibodies at enrollment

We evaluated DBPII antibody responses in 336 unrelated subjects with a median of age 41 years and a 1.3:1 male-female ratio (Table 1). Age was significantly associated with a subject's time of malaria exposure in the Amazon area (r = 0.82; $p<0.0001$, Spearman's correlation test). At the time of the first blood collection, the overall prevalence of malaria was 5%, with 14 out of the 17 (82%) infections caused by *P. vivax* and 3 (18%) by *P. falciparum*. No *P. malariae* or mixed *Plasmodium* infections were diagnosed by either microscopy or Real-Time PCR. The 336 participants were followed up for an average of 7.5 months (10 days to 12 months), thus representing 2,514 person-months of follow-up. Based on parasitological-confirmed cases, the incidence rates of *P. vivax* malaria were 1.03 episodes per 100 person-months (95% confidence

Table 1. Demographical, epidemiological and immunological data of the study population, 336 unrelated inhabitants of the agricultural settlement of Rio Pardo, Amazonas, Brazil.

Characteristic	
Age, median, years (IQR) [a]	41 (25–51)
Gender ratio, male:female	1.3:1
Years of malaria exposure, median (IQR)[b]	33 (21–48)
Years of residence in Rio Pardo, median (IQR) [c]	7.5 (4–12)
Previous malaria episodes, median (IQR)	5 (1–11)
Acute malaria infection, n (%) [d]	17 (5.0)
P. vivax	14 (4.1)
P. falciparum	3 (0.9)
DBPII antibody response, positive (%) [e]	122 (36)
DBPII binding inhibitory antibodies [n = 164], positive (%)[f]	58 (35.4)

[a] IQR = interquartile range

[b] Time living in the endemic area (Brazilian Amazon)

[c] Time living in the agricultural settlement area

[d] Malaria infection was detected by conventional light microscopy and/or real-time PCR at the time of their first blood-collection (individual baseline); no mixed infections were found in this study

[e] ELISA-detected IgG antibodies targeting DBPII; results are expressed as the number (%) of individuals with a positive antibody response at the time of their first blood-collection

[f] Functional assays with transfected COS cells to detect DBPII binding inhibitory antibodies (BIAbs) were performed on a subpopulation of the study sample

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interval [CI] of 0.69–1.49/100 person-months) and 0.19 per 100 person-months for *P. falciparum* (95% CI of 0.03–0.32/100 person-month).

One hundred and twenty-two (36%) of the individuals enrolled in the study had ELISA-detected IgG antibodies to the main variant of DBPII circulating in the study area (Sal-1) (Table 1). Because not all DBPII IgG antibodies are able to block the interaction between the ligand (DBPII) and its receptor on the RBC surface (DARC), we evaluated the functional properties of the anti-DBPII antibodies. Due to the methodological constraints of performing functional assays, measurement of DBPII binding inhibitory antibodies (BIAbs) was performed on a representative subset of the study population comprising 164 individuals, matched for age, sex, malaria exposure and DARC alleles; 58 (35.4%) of these individuals showed BIAbs response against a predominant DBPII variant circulating in the study area (Table 1).

Evaluation of long-term natural DBPII-specific IgG antibodies

Since the number of malaria cases varied during the course of the study (Fig 2A), we evaluated the long-term antibody response at different levels of malaria transmission. Over three cross-sectional surveys, at 6-month intervals, between 38 to 40% of individuals developed DBPII IgG antibodies, as detected by conventional serology (Fig 2B). Considering the inhibitory antibody response, there was a slight decrease in the frequency of BIAbs at the time of the 3rd cross-sectional survey (34–35% to 25%) (Fig 2C). Finally, the 12-month follow-up study allowed individuals to be classified as persistent non-responders (PNR), transient responders (TR), or persistent responders (PR) for either conventional serology or BIAbs immune response (Fig 2D). For conventional and inhibitory antibody responses, the frequency of acute malaria infections was similar between the PNR, TR or PR groups ($p>0.05$ for all comparisons).

Distribution of HLA class II in the study population and association with ELISA-detected DBPII IgG antibodies

Of the HLA class II loci that were genotyped in the study population, we found 13 *HLA-DRB1*, 6 *HLA-DQA1*, and 5 *HLA-DQB1* allele groups. As expected, *HLA-DRB1* was the most polymorphic locus with 46 alleles identified; there were 21 and 13 *DQB1* and *DQA1* alleles, respectively. For each HLA class II locus, the predominant alleles (frequency ≥ 0.01) were listed in the S1 Fig.

In a preliminary analysis, the effect of HLA class II genes on conventional DBPII antibody response was evaluated at the time of the first blood collection (S1 Table). While three HLA class II alleles (*DRB1*13:01*, *DQA1*01:03*, *DQB1*06:03*) were positively associated with anti-DBPII antibody response, six alleles (*DRB1*10:01*, *DRB1*14:02*, *DQA1*01:01*, *DQA1*05:03*, *DQB1*03:01*, *DQB1*05:01*) were negatively associated. Nevertheless, using multiple logistic regression models, only the *DRB1*13:01* (presence) and *DRB1*14:02* (absence) alleles were significant predictors of anti-DBPII antibodies (Fig 3A).

Since combinations of HLA alleles are inherited together in the genome more often than expected, we further evaluated the association between ELISA-detected DBPII-specific antibodies and HLA class II haplotypes. In total, 126 combinations of specific *DRB1*, *DQA1*, *DQB1* haplotypes were found, and for 27 of them (frequency ≥ 0.01) it was possible to estimate the individual probability of developing DBPII antibodies. Adjusted logistic regression analysis identified a single haplotype associated with poor production of DBPII antibodies, with individuals carrying the haplotype *DRB1*14:02-DQA1*05:03-DQB1*03:01* 5-times less likely to develop a conventional DBPII antibody response (Fig 3A). In addition, for each HLA class II locus analyzed (*DRB1*, *DQA1*, and *DQB1*), the genotype frequencies were confirmed to be in Hardy-Weinberg equilibrium (for the responder vs. non-responder groups).

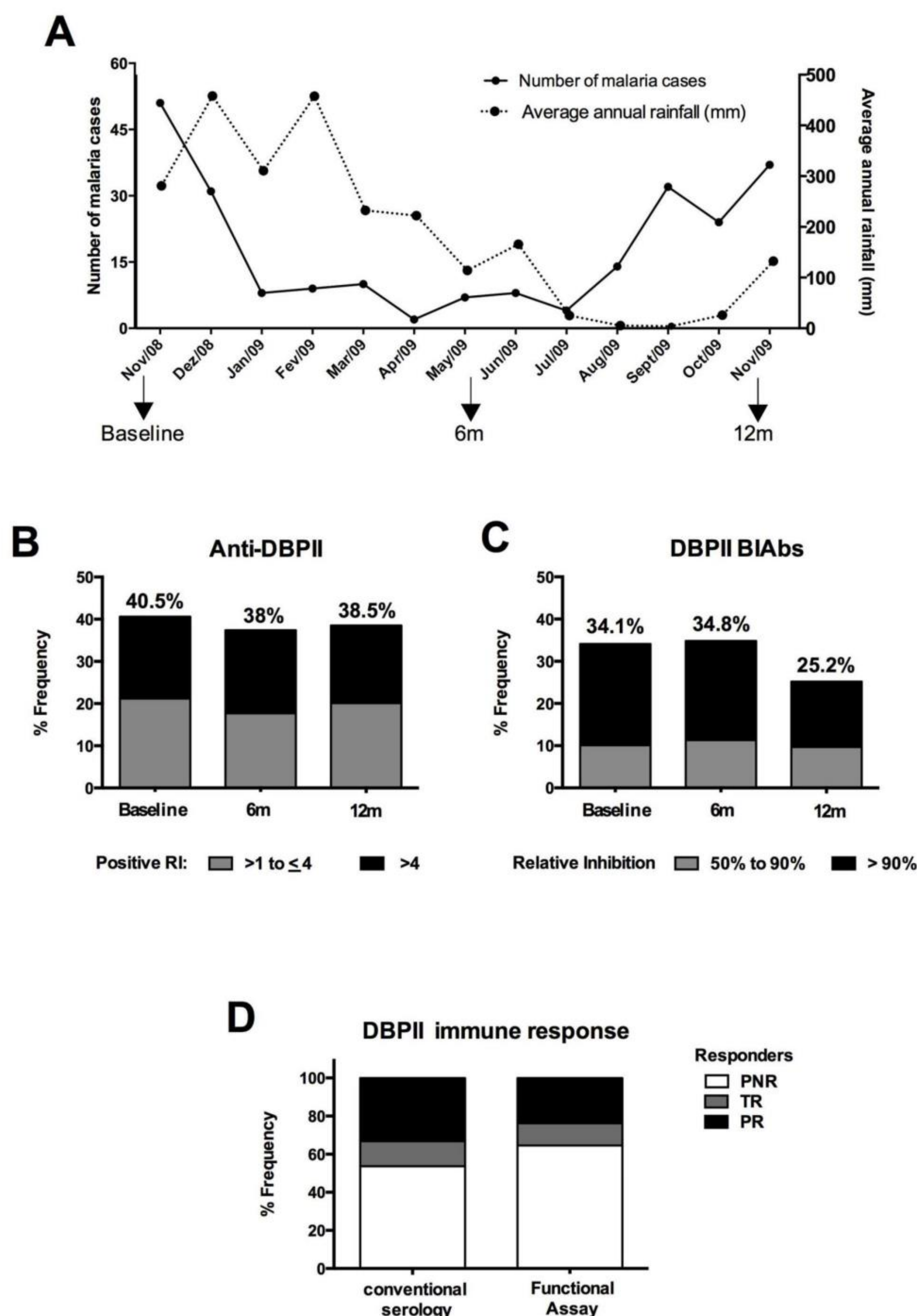

Fig 2. Temporal distribution of *P. vivax* malaria episodes and immune response targeting region II of the Duffy binding protein (DBPII), Rio Pardo Settlement, Amazonas, Brazil (Nov. 2008 –Nov. 2009). **(A)** Monthly malaria notifications in the Rio Pardo settlement (SVS/MS, Surveillance Service database, Brazilian Ministry of Health). Episodes of malaria, as determined by conventional microscopy, varied according to rainfall and season. Arrows indicate the time of the cross-sectional surveys (November, 2008; June 2009; and November, 2009). **(B)**

DBPII IgG ELISA-detected antibody (Anti-DBPII) response remained stable during the follow up period (Baseline, and at 6 or 12 months). Results are expressed as a frequency (%) of responders, and a Reactivity Index (RI) of greater than 1.0 was considered positive. (**C**) DBPII binding inhibitory antibodies (BIAbs) had a slightly decreased frequency at the 12 months follow-up examination. BIAbs were evaluated by COS-7 cytoadherence assay, using plasma samples diluted to 1:40 (n = 164). Positive results were considered as DBPII–DARC binding inhibition of ≥50%. (**D**) Based on conventional (ELISA-detected IgG) and functional serology assays (DBPII BIAbs) at 12 months of follow-up, individuals were classified as *persistent non-responder* (PNR)—no antibody response detected any time-point of the follow-up; *transient responder* (TR)—antibodies detected in at least one cross-sectional survey; or *persistent responder* (PR)–antibodies detected in all cross-sectional surveys.

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Next, we investigated whether the status of persistent responder (PR) or non-responder (PNR) was HLA class II-linked at the time of the 12-month follow-up collection. The frequencies of some HLA class II alleles were significantly different between the PR and PNR groups (S2 Table). An adjusted odds ratio analysis confirmed that two alleles were associated with the status of long-term responder, and a single allele was associated with the absence of an antibody response (Fig 3B). More specifically, while individuals carrying either the *DRB1* 13:01* or *DQA1* 01:03* alleles had an increased probability of a sustained antibody response, the *DQA1* 05:03* allele carriers were associated with the status of persistent non-responders (Fig 3B). It is noteworthy that the *DQA1* 05:03* allele aggregated in a specific haplotype (*DRB1* 14:02-DQA1* 05:03-DQB1* 03:01)* that was primarily associated with the absence of an antibody response (Fig 3A), and this haplotype was in strong linkage disequilibrium (Δ = 1.0; P = 0).

HLA class II variability and DBPII inhibitory immune response

Further experiments investigated whether HLA class II polymorphisms interfered with the functional proprieties of DBPII antibodies. The three cross-sectional measures of DBPII

ELISA-detected DBPII IgG response

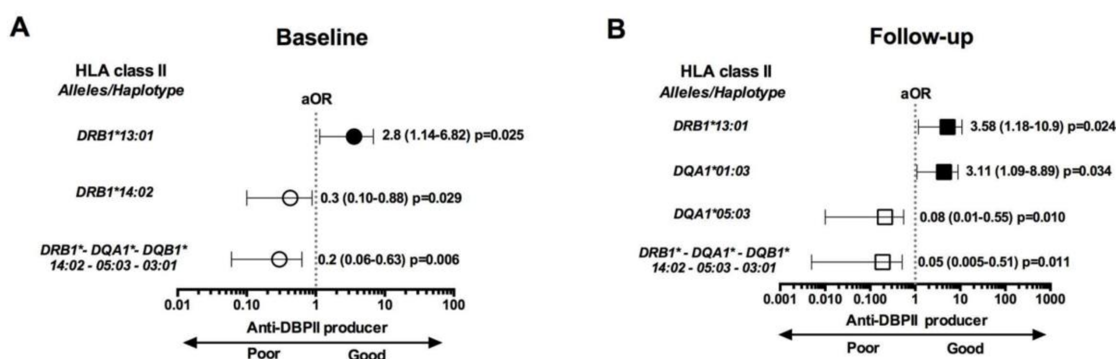

Fig 3. Association between HLA class II alleles (*DRB1*, *DQA1*, and *DQB1* loci) and *Plasmodium vivax* Duffy binding protein (DBPII) antibody responses in individuals naturally exposed to malaria. (**A**) At enrollment (baseline), adjusted Odds Ratio (aOR) analysis for the association of HLA class II (alleles and haplotypes) and the presence (*black circle)* or absence (*white circle*) of DBPII IgG antibody (Ab) response, as detected by ELISA. (**B**) After 12-month follow-up, adjusted Odds Ratio (aOR) analysis for the association of HLA class II (alleles and haplotypes) and the long-term DBPII antibody response; individuals were classified as persistent non-responder (PNR, *white square*) or responder (PR, *black square*), as described in legend of Fig 2. Adjusted ORs analyses were performed using multivariable logistic regression models adjusted for confounding variables (age, sex, previous malaria episodes, and dwelling location). Variables associated with DBPII antibody at 5% level ($p < 0.05$) were maintained in the final models.

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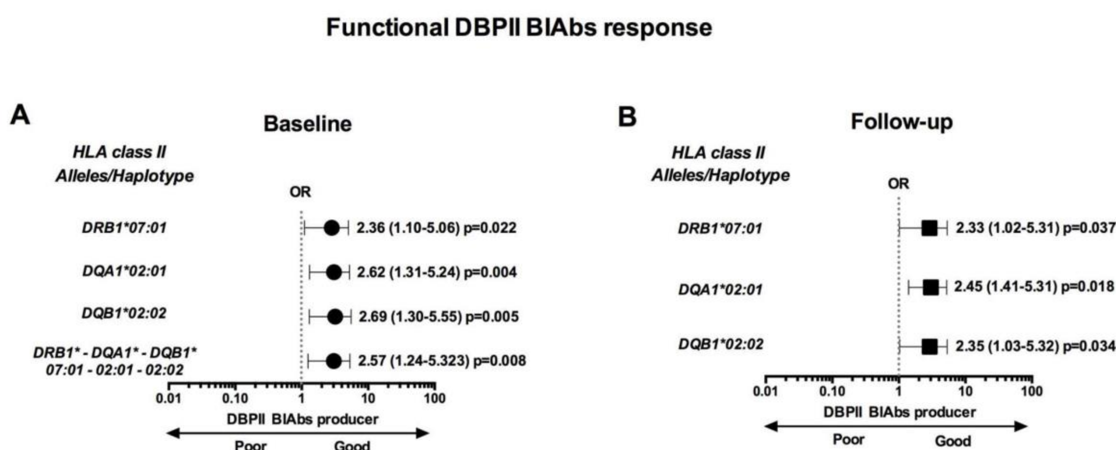

Fig 4. Association between HLA class II alleles (*DRB1*, *DQA1*, and *DQB1* loci) and *Plasmodium vivax* Duffy binding protein (DBPII) binding Inhibitory Antibodies (BIAbs) in individuals naturally exposed to malaria. (**A**) At enrollment (baseline), Odds Ratio (OR) analyses for the association of HLA class II (alleles and haplotypes) and DBPII BIAbs response, as detected by functional assays with DBPII- transfected COS cells; *black circles* represent alleles and haplotypes of HLA class II associated with a positive BIABs response. (**B**) After 12-month follow-up, Odds Ratio analyses for the association of HLA class II (alleles and haplotypes) and long-term DBPII BIAbs response; individuals were classified as persistent non-responder (PNR) or responder (PR, *black square*), as described in legend of Fig 2. Odds Ratio analyses realized using standard contingency tables (p < 0.05; qui-square and Fisher's exact test, as appropriate).

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BIAbs responses were performed on a subset of the study population comprising 164 individuals (Table 1), with responders (n = 58) and non-responders (n = 106) matched for age, sex, and malaria exposure. Three alleles (*DRB1**07:01*, *DQA1**02:01*, and *DQB1**02:02*) were overrepresented in DBPII BIAbs responders (S3 Table), and these same alleles aggregated in a haplotype (Fig 4A), which was in linkage disequilibrium (Δ = 0.90 and 0.94, for responders and non-responders, respectively). Interestingly, the long-term persistence of anti-DBPII responses (determined at the 12 month follow-up analysis) was also associated with those same three HLA class II alleles (Fig 4B). Unfortunately, the small size of the sample precluded use of adjusted odds ratio analyses. Nonetheless, responder and non-responder groups were matched by the confounding variables (age, sex, malaria exposure, and dwelling localization).

In silico binding prediction of DBPII peptides to class II allelic variants

Since persistence and functional properties of DBPII antibodies were influenced by class II allelic variants, we investigated whether differences in the affinity of DBPII peptides for class II molecules might contribute to the observed difference in responses. Based on predicted binding affinity between DBPII peptides and HLA-DR/DQ alleles, we found unexpected differences in affinity in favor of the non-responder allele carriers. Actually, the HLA-DR allele linked to non-responders (*DRB1**14:02)* appeared to have a higher binding affinity for the peptides (low IC50 values) than the HLA–DR alleles that were associated with responders (*DRB1**07:01* and *DRB1**13:01)* (S2 Fig). In spite of that, different HLA-DR binding profiles were found for previously identified DBPII epitopes [44–47]. Of note, the recently described broadly neutralizing DBPII epitopes (2D10/2H2 and 2C6) had low binding affinity for all of the Class II molecules analyzed (S2B Fig). Considering HLA-DQ, the model also showed a higher predicted binding affinity for HLA-DQA1/B1 molecules linked to non-responders.

Structural analysis of HLA-DRB1 variants

Structural analysis of the three HLA-DRB1 variants revealed a number of interesting differences in the peptide-binding groove (S3 Fig), with significant alterations to its electrostatic potential, and reduced affinity for the model peptides (Fig 5A–5C), which we propose as an explanation for the differences in subject responses. The antigenic surface of DBPII has a strong positive charge (S4 Fig), which is suggestive of a binding preference for antibodies targeting positively charged epitopes such as those that will be preferentially bound by the *DRB1*07:01* and *DRB1*13:01* variants. Interestingly, while the *DRB1*14:02* and *DRB1*13:01* variants are the closest in sequence identity, the docked peptides were most similar between the two responder variants (rmsd of the peptides < 3.8 Å), whilst the non-responder did not dock in a similar way (rmsd of the peptides > 8 Å). This supports the suggestion that the non-responder variant leads to reduced antigen presentation.

Overall, while the non-responder-associated variant (*DRB1*14:02*) shares 81.2% and 95.5% sequence identity to *DRB1*07:01* and *DRB1*13:01*, respectively, the sequence identity is lower in the groove region within 5 Å of the presented antigen (*DRB1*07:01*–73.3%; *DRB1*13:01*–89.6%). While the majority of the differences between the variants are located within the peptide-binding domain, this change in the nature of the antigen-binding groove is evident in differences between their isoelectric points, with *DRB1*07:01* and *DRB1*13:01* having slightly acidic pI's (6.5 and 7.0 respectively), and *DRB1*14:02* being basic (7.7). It was also reflected in the energy calculations, with *DRB1*14:02* having an overall Coulomb energy approximately 1.5% lower than either of the responder variants.

One significant difference between the good and poor responder variants was the presence of a glutamine residue at position 99 (247 according to the Protein Data Bank, PDB) in *DRB1*14:02*, compared to aspartic acid in *DRB1*07:01* and *DRB1*13:01*. This residue side-

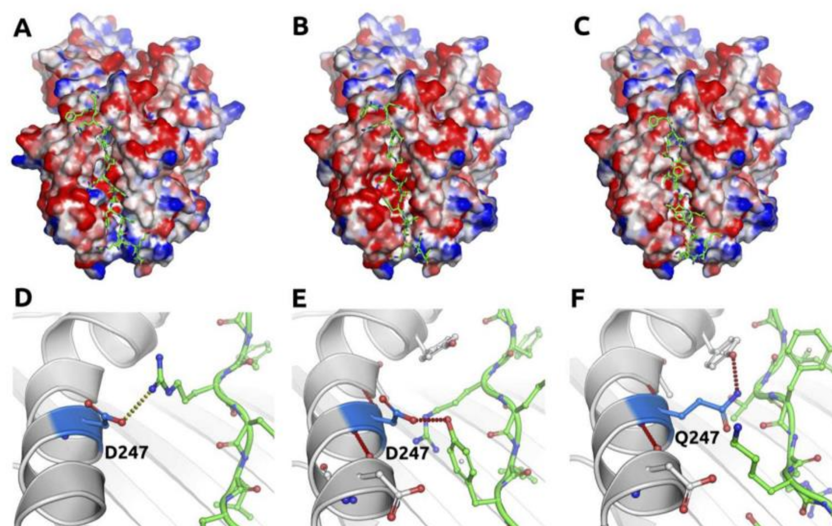

Fig 5. The HLA II complex peptide-binding groove. The figure presents a representation of the HLA II complex electrostatic surface potential, and interactions made by residue 99 of DRB1 for the responder alleles *DRB1* 07:01* (A and D, respectively) and *DRB1* 13:01* (B and E, respectively), and the non-responder allele *DRB1* 14:02* (C and F, respectively). The DBPII H1 peptide is shown inside the binding groove as green sticks. Hydrogen bonds are shown as red dashed lines.

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chain points into the peptide-binding groove and is located approximately 5 Å from the antigen peptide backbone, making a key hydrogen bond (Fig 5D and 5E). Here, loss of the acidic residue in the peptide-binding groove was predicted to reduce the H1 and H3 peptide binding affinity ($\Delta\Delta G < -0.8$ kCal/mol), and is likely to lead to altered antigen binding and presentation profiles, and hence the poor response seen for *DRB1*14:02* variant carriers.

Discussion

The HLA molecules encoded by MHC class II genes are responsible for presenting peptide epitopes to CD4+ T helper cells. Consequently, it is reasonable to postulate that polymorphism in the HLA class II region may account for the variation in DBPII antibody responses. Unfortunately, most antibody prevalence data on malaria have been collected by cross-sectional analysis at a single time point, which might led to misclassification of individual immune responsiveness. Therefore, in this study we conducted a longitudinal study, collecting serum from the same individuals over a period of 12 months, to obtain a reliable estimate of DBPII antibody prevalence. The genetic profile of HLA class II in the study population was similar to other populations of the Brazilian Amazon [48], which are characterized by an interethnic admixture with high proportions of European and Amerindian groups [49]. Accordingly, in the study population 44% had European ancestry, followed by 38% Amerindian, and 18% African ancestry. For other Brazilian regions, the contribution of European ancestry has ranged from 40% in the Northeast to >70% in the Southeast and South [50, 51]. As expected, the Native-American ancestry in the study area (38%) was representative of the Amazonian region, where Amerindian ancestry is much higher that in other Brazilian regions (<10%) [50].

Our current study confirms the heritability of antibody responses to DBPII, with genetic variation in HLA class II molecules influencing both the development and persistence of an individual's anti-DBPII IgG antibody response. Accordingly, multivariate analyses adjusted for potential confounding variables showed effects of alleles linked to the DR and DQ loci on the presence (*DRB1*13:01*) and persistence (*DRB1*13:01* and *DQA1*01:03*) of ELISA-detected DBPII IgG antibodies. On the other hand, two alleles were associated with DBPII-non-responsiveness, *DRB1*14:02* and *DQA1*05:03*, and these comprised a single haplotype (*DRB1*14:02-DQA1*05:03-DQB1*03:01)* that significantly reduced the development of anti-DBPII IgG at any time during the follow-up study (baseline, 6 and 12 months later). Interestingly, the alleles in the aforementioned haplotype were in strong linkage disequilibrium, which demonstrated that these poor-responder alleles are inherited together more often than expected by chance. So far, only a single study has investigated the association between HLA and DBPII antibodies [52]. Although in that case the authors were unable to demonstrate an association between HLA type and ELISA-detected DBP IgG antibodies, and the relatively limited number of responders did not allow any final conclusions about the highly polymorphic HLA class II and DBP antibodies.

Here, the assessment of long-term antibody response was essential to strengthen the conclusion that there was an increased susceptibility of *DRB1*13:01* carriers to develop and sustain their anti-DBPII IgG antibody response. Furthermore, these data confirmed that individuals harboring the haplotype *DRB1*14:02-DQA1*05:03-DQB1*03:01* were persistent non-responders. Due to the overall scarcity of data combining analysis of HLA and immune responses to *P. vivax*, further confirmation of these associations in other malaria endemic areas is needed. Despite the remarkable lack of data on this subject, systematic review and meta-analysis studies have identified a link between the *DRB1*13:01* allele and increased antibody responses to vaccines for other microbial infections, including hepatitis B, influenza virus, serogroup C

meningococcus, and MMR-II (measles and rubella virus) [53, 54]. Additionally, the *DRB1*14* allelic group has been associated with a poor humoral response to HBsAg vaccination [55].

Many field studies examining the immune response to malaria have focused on measuring the concentrations of antibodies to vaccine candidate antigens, while less attention has been paid to complementary approaches defining the functional relevance of these antibodies. By using an *in vitro* assay to quantify inhibition of DBPII–erythrocyte binding [9, 56], we demonstrated that DBPII binding inhibitory antibodies (BIABs) were associated with three alleles (*DRB1*07:01*, *DQA1*02:01* and *DQB1*02:02*), which are in linkage disequilibrium and were found to be part of a single haplotype. Notably, these three alleles were associated with the presence of BIAbs antibodies and were also associated with the persistence of this inhibitory response. Therefore, our observations may explain previous results showing that the majority of people who are naturally exposed to *P. vivax* do not develop antibodies that inhibit the DBPII-DARC interaction, but once they are acquired these BIAbs seem to be stable under continuous exposure to malaria transmission [11, 13].

Intriguingly, a significant number of pharmacogenetic studies have identified HLA-*DRB1*07:01* carriers (in less extension, *DQA1*02:01* and *DQB1*02:02)* as being more susceptible to side effects of biological therapy due to the activation of immune response drug-induced [57–59]. Notably, part of the side effect could be explained by the higher production of neutralizing antibodies against drugs (or their metabolites) in the *HLA-DRB1*07:01* and/or *DQA1*02* carriers [59, 60]. Although drug-specific antibodies are undesirable in therapies involving biological proteins, these findings reinforce our results of a much higher frequency and persistence of DBPII neutralizing antibodies in individuals harboring those alleles HLA class II. In future studies, functional analysis of a greater number of individuals might allow for more robust statistical comparisons.

It is noteworthy that the class II alleles associated with DBPII inhibitory activity were not associated with the conventional IgG antibody responses. Likewise, alleles (or haplotypes) associated with ELISA-detected IgG antibodies were not associated with DBPII BIAbs. These results are not completely unexpected because quantitative receptor binding assays distinguish between antibodies that recognize DBPII and those that inhibit binding to DARC receptor. Here, the DBPII/DARC interaction was assessed by using an established cytoadherence assay based upon multivalent interactions between DBPII on the surface of COS-7 cells and DARC expressed in RBCs [56]. As a consequence, we and others have demonstrated a moderate correlation between DBPII BIAbs and ELISA anti-DBPII antibodies (revised in [8]). Overall, our results emphasize the relevance of examining functional aspects of the immune response, particularly in the case of immunogens such as DBPII, in which the goal of vaccination would be to enhance broadly neutralizing antibodies targeting invasion-blocking epitopes.

To gain insights into the difference between good and poor HLA responders, we sought to investigate whether natural HLA-DR/DQ allelic differences could be explained with respect to binding affinity of DBPII epitopes. While predicted DBPII epitopes have a unexpected moderate-to-high affinity for non-responder alleles, the binding affinity of previously described DBPII epitopes [44–47] was much more variable, including low binding affinities of recently described DBPII B-cells epitopes associated with strain-transcending immunity [47]. However, it seems inappropriate to extrapolate our findings to conformational B cell epitopes because the prediction analyses used here were largely determined by the primary amino acid sequence of the peptide-binding core. In this context, the development of tools for reliably predicting B-cell epitopes, particularly for predicting conformational epitopes, remains a major challenge in immunoinformatics [61].

Although the predicting peptide-HLA binding affinity method used here (NetMHCIIpan—www.cbs.dtu.dk/services/NetMHCIIpan-3.1) [62] seems to be a suitable predictive algorithm

for T cell epitopes [28], we performed a further detailed structural in silico analysis of the HLA-DRB1 variants. Significantly, the majority of the differences between HLA-DRB1 variants (good vs. poor responders) were located within the peptide-binding domain, leading to significant changes in the nature of the antigen-binding groove. A striking structural difference between HLA-DRB1 variants was the presence of a glutamine residue at position 99 in the poor responder allele (DRB1*14:02), as compared to aspartic acid in the good responder alleles (DRB1*07:01 and DRB1*13:01). Remarkably, mutation of the corresponding residue has previously been shown to result in loss of the ability of HLA-DP2 to present the metal beryllium to T cells, in genetically susceptible to chronic beryllium- disease [63]. Notably, this single mutation seems to drive helper CD4 T cells in susceptible individuals to secrete Th1-type cytokines, such as gamma-interferon, but not IL-4, leading to beryllium-induced hypersensitivity and chronic beryllium-disease [64, 65]. Consequently, we speculate that the mutation found here in the peptide-binding groove (D247 vs. Q247) is likely to change the outcome of the CD4+T cells immune response. Accordingly, the loss of the acidic residue in the peptide binding groove was predicted to reduce DBPII-specific peptide binding affinity (H1 and H3), which is expected to lead to altered antigen binding and presentation profiles, and hence poor response of carriers of the DRB1*14:02 variant. It strengthens the findings that DRB1*14:02 could be more frequently involved with a poor antibody production [55], while the *DRB1*13*:01 allele produces a much more robust antibody response [53, 54]. Future studies are required to determine differences in the functionalities of DBPII epitopes in the context of different HLA molecules.

Notwithstanding the relevance of our results, the current study has some limitations. As we focused on the highly variable HLA class II genes it may not have been possible to discriminate between causal alleles and variation that is due to the linkage disequilibrium (LD) between alleles. In fact, in most association studies it has been difficult to pinpoint the causal variants within this genetic complex due to strong LD, population heterogeneity, and the high density of immune-related genes [66]. Such studies have proven most successful for diseases with one prominent predisposing genetic factor mapping to either the class I or class II region [67]. In addition, the associations described in the present study are most likely multifactorial, and depend on several additional factors related to the parasite and host environment. Although the structural analysis of DRB1 variants described here suggested that specific alleles might influence anti-DBPII antibody responses, these results indicate a first step towards the understanding of DBPII immune response in the context of different HLA class-II variants. We are confident that future cellular assays can be pursued to confirm and identify mechanisms associated with good and poor antibody responders. Finally, knowledge of the relative binding affinities of DBPII peptides for class II molecules associated with good and poor responses to this major *P. vivax* blood-stage vaccine candidate might lead to strategies for controlling the type of helper T cells activated in response to DBPII.

Supporting Information

S1 Fig. Alleles frequencies of HLA class II (*DRB1*, *DQA1* and *DQB1* loci) of the study population (n = 336), Amazonas state, Brazil. Most frequent alleles of HLA class II (**A**) *DRB1*; (**B**) *DQA1*; and (**C**) *DQB1*. Allele frequencies greater than or equal to 0.01 were included in the figure. For each locus, lowercase letters (a-g) indicate statistically significant differences between allele groups ($p<0.05$, Z-test).
(TIF)

S2 Fig. Binding prediction of *P. vivax* DBP peptides to MHC class II molecules. Binding affinity is depicted for the whole molecule (A) and for the Duffy Binding-Like domain (region

II). (B) Binding affinity is given as the log of the IC50 value (nM) for good (*blue line*) and poor (*black line*) DBPII responders. The predictions for good responders represent the average of IC50 values of the *DRB1*07:01* and *DRB1*13:01* alleles; values for poor responders refer to the prediction for the *DRB1*14:02* allele. The horizontal lines indicate the threshold for defining strong (<1.7) and weak binders (from 1.7 to 2.7) based on predicted affinity. Predicted values of IC50 were specified in a sliding window of 15 amino acids in length. Previously identified epitopes in region II of PvDBP were indicated [44–47]. Boundaries of regions I-VI of the protein were defined as previously described [68]. The three subdomains (SD) in the region are indicated by colored bars: SD1 (green), SD2 (blue) and SD3 (orange) [69].
(TIF)

S3 Fig. Differences in peptide binding groove between HLA class II variants. The *DRB1*14:02* allele is depicted in yellow, *DRB1*07:01* in green (A) and *DRB1*13:01* in blue (B). The peptide is shown as a cartoon in yellow. Residues within 5 Å of peptide for the three variants are shown as sticks. Residues in pink differ between variants.
(TIF)

S4 Fig. DBPII surface electrostatics. This figure presents a representation of the DBPII electrostatic surface potential.
(TIF)

S1 Table. Association between antibody responses against *P. vivax* Duffy binding protein (DBPII) and HLA class II (*DRB1*, *DQA1*, and *DQB1*) alleles of individuals naturally exposed to malaria.
(PDF)

S2 Table. Association between the long-term Duffy binding protein (DBPII) antibody response and HLA class II (*DRB1*, *DQB1* and *DQA1)* alleles of individuals naturally exposed to malaria.
(PDF)

S3 Table. Association between binding inhibitory antibody (BIAbs) response against *P. vivax* Duffy binding HLA class II (*DRB1*, *DQB1* and *DQA1*) exposed to malaria.
(PDF)

S1 File. Data file of HLA class II and antibody immune response.
(XLSX)

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5.2 PARTE II - Avaliação do papel funcional e resposta imune contra a EBP2

Artigo 3 - "A Novel Erythrocyte Binding Protein of *Plasmodium vivax* Suggests an Alternate Invasion Pathway into Duffy-Positive Reticulocytes" por Ntumngia FB, Thomson-Luque R, *Letícia de Menezes Torres*, Gunalan K, Carvalho LH, Adams JH. mBio 7(4):e01261-16 (Página 82).

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OBSERVATION

A Novel Erythrocyte Binding Protein of *Plasmodium vivax* Suggests an Alternate Invasion Pathway into Duffy-Positive Reticulocytes

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ABSTRACT **Erythrocyte invasion by malaria parasites is essential for blood-stage development and an important determinant of host range. In *Plasmodium vivax*, the interaction between the Duffy binding protein (DBP) and its cognate receptor, the Duffy antigen receptor for chemokines (DARC), on human erythrocytes is central to blood-stage infection. Contrary to this established pathway of invasion, there is growing evidence of *P. vivax* infections occurring in Duffy blood group-negative individuals, suggesting that the parasite might have gained an alternative pathway to infect this group of individuals. Supporting this concept, a second distinct erythrocyte binding protein (EBP2), representing a new member of the DBP family, was discovered in *P. vivax* and may be the ligand in an alternate invasion pathway. Our study characterizes this novel ligand and determines its potential role in reticulocyte invasion by *P. vivax* merozoites. EBP2 binds preferentially to young (CD71^{high}) Duffy-positive (Fy$^+$) reticulocytes and has minimal binding capacity for Duffy-negative reticulocytes. Importantly, EBP2 is antigenically distinct from DBP and cannot be functionally inhibited by anti-DBP antibodies. Consequently, our results do not support EBP2 as a ligand for invasion of Duffy-negative blood cells, but instead, EBP2 may represent a novel ligand for an alternate invasion pathway of Duffy-positive reticulocytes.**

IMPORTANCE **For decades, *P. vivax* infections in humans have been defined by a unique requirement for the interaction between the Duffy binding protein ligand of the parasite and the Duffy blood group antigen receptor (DARC). Recent reports of *P. vivax* infections in Duffy-negative individuals challenge this paradigm and suggest an alternate pathway of infection, potentially using the recently discovered EBP2. However, we demonstrate that EBP2 host cell specificity is more restricted than DBP binding and that EBP2 binds preferentially to Duffy-positive, young reticulocytes. This finding indicates that this DBP paralog does mediate a Duffy-independent pathway of infection.**

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Malaria caused by *Plasmodium vivax* is the most predominant form of malaria outside Africa, with over 130 million clinical cases annually (1, 2). Unlike *Plasmodium falciparum* malaria, *P. vivax* blood-stage infection is limited to reticulocytes and individuals who are positive for the Duffy blood group antigen (Fy), also known as the Duffy antigen receptor for chemokines (DARC) (3, 4). Preference for this blood cell type is believed to be mediated by specific ligand-receptor interactions between the parasite merozoites and the host reticulocytes during the invasion process (5, 6). It is believed that the *P. vivax* Duffy binding protein (DBP) on the merozoite interacts with DARC on the reticulocyte surface, precipitating the junction formation step necessary for invasion. Historically, the vital need for the DBP-DARC interaction was evident from the virtual absence of *P. vivax* malaria in populations with a high prevalence of DARC negativity (3, 7). However, recent studies have reported evidence of Duffy (Fy)-independent invasion of human reticulocytes (8, 9). In Madagascar, with a mixture of Duffy-positive (Fy$^+$) and -negative (Fy$^-$) populations of diverse ethnic backgrounds, there was a significant reduction in the prevalence of clinical *P. vivax* malaria in Duffy-negative compared with Duffy-positive individuals (8). Similarly, in the Brazilian Amazon, two cases of clinical *P. vivax* malaria were observed in Duffy-negative samples obtained from Rondonia state (9). From these historically anomalous cases, it is not clear if they represent random isolated infections that have always occurred, if they are new phenomena related to *P. vivax* evolving at this time to use alternate DARC-independent pathways for invasion, or if DBP remains the critical invasion ligand using alternate receptors for invasion.

Recent studies have identified a *P. vivax* DBP homolog erythrocyte binding protein (termed here EBP2) that is the type of novel ligand anticipated in an alternate invasion pathway to DBP (10–12). EBP2 has the key conserved domain features characteristic of the EBP superfamily, including the region II or Duffy binding-like (DBL) ligand domain, considered essential for receptor recognition and merozoite invasion (5). Nonetheless, the *P. vivax* DBP region II (DBPII) has surprisingly stronger similarity to its paralogs in *Plasmodium knowlesi* (≈70%) than to the newly discovered *P. vivax* EBP2 (50%) (see Fig. S1 in the supplemental

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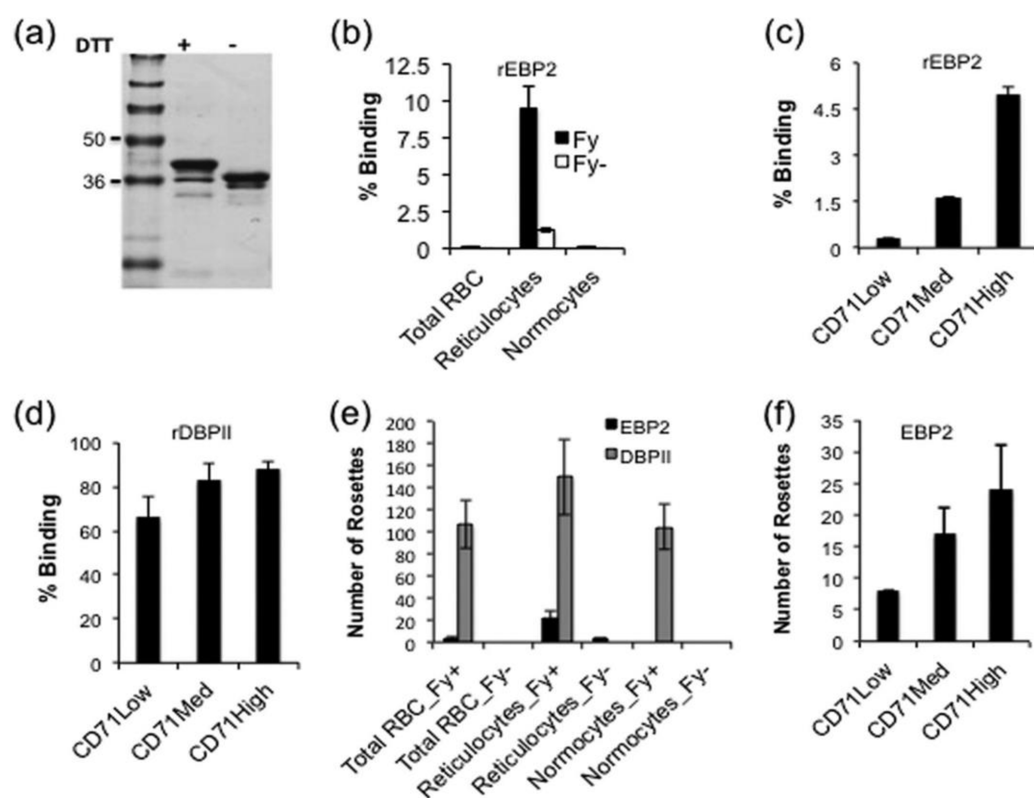

FIG 1 Production and erythrocyte binding properties of recombinant EBP2. (a) Coomassie blue-stained SDS-PAGE gel showing differential mobility of reduced (dithiothreitol-positive [DTT^+]) and refolded (DTT^-) recombinant EBP2. Mobility shift is a simple indicator of native conformation of the refolded antigen. (b to d) Recombinant EBP2 (b and c) and recombinant DBPII (d) binding to different red blood cell types and reticulocyte subpopulations, respectively, by flow cytometry. (e) COS7 cell surface-expressed EBP2 and DBPII binding to different Duffy-positive (Fy^+) and Duffy-negative (Fy^-) erythrocyte types. (f) COS7 cell surface-expressed EBP2 binding to different reticulocyte subpopulations. Bars show mean percentages of red blood cells with bound antigen and mean numbers of rosettes in 30 microscope fields at a magnification of ×20 in the flow cytometry and COS7 assays, respectively. Error bars represent ±standard deviations from two independent experiments. All experiments were performed with blood from at least two different donors. DBPII was used as a control antigen.

material). Therefore, the conserved features of EBP2 suggest that it has a role in invasion while its differences in the key receptor binding domain suggest that it can facilitate an alternate invasion pathway to the DBP ligand. To shed light on this important question of whether EBP2 is a Duffy-negative or DARC-independent ligand, we adapted standard *in vitro* functional assays for DBP to characterize EBP2 receptor specificity. The data presented here provide a better understanding of the biological role of EBP2 in the invasion process and indicate that EBP2 does not explain the observed transmission of *P. vivax* in some Duffy-negative individuals.

Antigen production. The gene sequence coding for the DBL domain of EBP2 (amino acids 159 to 485) (see Fig. S1 in the supplemental material) from GenBank (accession number KC987954) (10) was codon optimized for *Escherichia coli* expression, commercially synthesized, and cloned into an expression vector (pET21a$^+$) with a C-terminal His tag to facilitate purification by affinity chromatography. Recombinant EBP2 was expressed in bacteria, purified from inclusion bodies, and refolded by rapid dilution as previously described (13, 14). The refolded antigen was evaluated for native conformation by separating refolded and denatured antigens by SDS-PAGE. The refolded antigen migrated at the expected size of 37 kDa compared to the denatured antigen at 39 kDa (Fig. 1a). This mobility shift on the gel is a simple indication of the formation of disulfide bonds in the refolded antigen.

EBP2 binds to human reticulocytes. To further analyze EBP2 for functional ligand activity, the refolded recombinant antigen was tested for binding to different erythrocyte types by a highly sensitive flow cytometry binding assay (15). First, reticulocytes were isolated from buffy packs (Interstate Blood Bank, Memphis) by immunomagnetic sorting using CD71 beads (Miltenyi Biotec). Briefly, blood cells (60 to 70 ml) were washed in McCoy's 5A medium (Sigma) by centrifugation at 2,500 × *g* for 5 min. The washed cell pellet was filtered on a NEO1 leukocyte reduction filter (Haemonetics), mixed with an equal volume of cold autoMACS running buffer (Miltenyi Biotec), and incubated with anti-human CD71 microbeads (Miltenyi Biotec) for 30 min at 4°C. Enrichment for CD71-positive cells (reticulocytes) was performed on an autoMACS Pro Separator (Miltenyi Biotec) under positive-selection mode (Posselds), and purity was determined by new methylene blue staining of thin smears. The purity level of the reticulocytes was generally >90%. Subpopulations of the enriched reticulocytes ($CD71^{low}$, $CD71^{med}$, and $CD71^{high}$) were iso-

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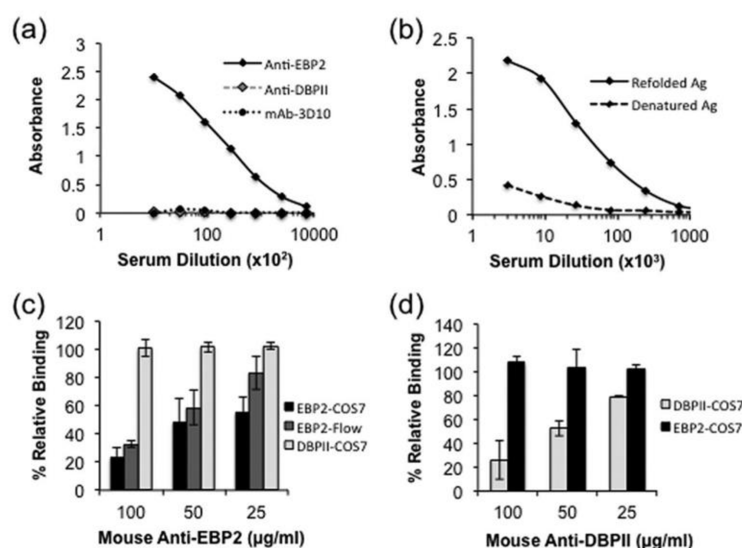

FIG 2 Immunogenicity and functional analysis of anti-EBP2 antibodies. (a and b) Antisera raised in mice (a) and rabbits (b) against recombinant EBP2 were tested by endpoint dilution for reactivity with the homologous antigen (Ag). Antigen preparations were allowed to adsorb onto wells of a microtiter plate and allowed to react with different dilutions of the antisera. Mouse anti-DBPII-Sal1 and mAb-3D10 were used as negative-control antibodies in the ELISA. Each point on the curves represents the mean OD from duplicate wells, while error bars represent ±standard deviations. (c and d) Mouse anti-EBP2 IgG (c) or anti-DBPII IgG (d) was tested against homologous and heterologous antigens in either the COS7 or flow cytometry binding assay for inhibition of EBP2 and DBPII reticulocyte binding. Each bar represents percent binding to reticulocytes in the presence of immune IgG relative to preimmune IgG. Error bars represent ±standard deviations.

lated by staining with CD71-allophycocyanin (APC) (Miltenyi Biotec) and sorted on a BD FACSAria II cell sorter with a 70-μm nozzle (see Fig. S2 in the supplemental material). For the binding assay, 1 μl of total red blood cells, reticulocytes, or reticulocyte-depleted erythrocytes (normocytes) was incubated for 90 min with 5 μg/ml of refolded antigen in 100 μl of phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA) at room temperature (RT) while shaking. Unbound antigen was washed off with PBS-1% BSA, and the cells were incubated with mouse anti-His Alexa Fluor 488-conjugated antibody (Qiagen) in the dark for 1 h at 4°C. After another washing step, the cells were suspended in wash buffer, and erythrocyte binding was quantified by analyzing 100,000 events on a BD Accuri C6 flow cytometer. Recombinant EBP2 bound to $CD71^+$ reticulocytes with a preference for immature ($CD71^{high}$) and Duffy-positive (Fy^+) reticulocytes. Only minimal binding was observed with Fy^- reticulocytes, and no binding was observed with total red blood cells or to normocytes (Fig. 1b and c). Recombinant DBPII showed no significant differences in binding to young or mature reticulocytes (Fig. 1d). The binding specificity was confirmed by transient expression of EBP2 on the surface of COS7 cells as a recombinant transmembrane protein with enhanced green fluorescent protein (EGFP) fused to its cytoplasmic C terminus, as previously described (16, 17). Erythrocytes (reticulocytes or normocytes) were added to each well in a 24-well plate and incubated for 2 h. Nonadherent cells were washed off, and binding was quantified by counting the number of rosettes in 30 microscope fields at a magnification of ×20. The binding pattern was similar to that observed by flow cytometry, with the surface-expressed antigen binding preferentially to immature ($CD71^{high}$) and Duffy-positive (Fy^+) reticulocytes but not to normocytes. Similarly, only minimal binding was observed with Duffy-negative (Fy^-) reticulocytes (Fig. 1e and f). As expected, DBPII, which was used as a control, bound to both Duffy-positive reticulocytes and normocytes (Fig. 1e).

EBP2 is antigenically distinct from DBP and sensitive to antibody inhibition. Antiserum to refolded recombinant EBP2 prepared in mice and rabbits was used to characterize the antigenic properties of EBP2. Antibody titers were determined from immune sera by endpoint titer enzyme-linked immunosorbent assay (ELISA) as the reciprocal of the highest serum dilution giving an optical density (OD) twice that of the preimmune serum. Recombinant EBP2 was highly immunogenic in both animal species, with endpoint titers of 729,000 and 2×10^6 in mice and rabbits, respectively (Fig. 2a and b). There was no reactivity of recombinant EBP2 with serum from DBPII-immunized mice and with a DBPII-specific monoclonal antibody, mAb-3D10 (14) (Fig. 2a). The rabbit polyclonal antibody showed very negligible reactivity with the denatured antigen compared with the refolded antigen, thus confirming the presence of conformational epitopes in the refolded antigen (Fig. 2b). To establish the functional activity of the anti-EBP2 antibodies, their potential to block EBP2-reticulocyte binding was evaluated. COS7 cell surface-expressed EBP2 or refolded recombinant EBP2 in solution was incubated with different concentrations of protein G-purified IgG from mouse serum raised against recombinant EBP2 prior to addition of human Duffy-positive reticulocytes. Binding inhibition was quantified by determining the number of rosettes (COS7 assay) or number of reticulocytes with bound EBP2 (flow cytometry) in the presence of IgG from immune serum relative to IgG from preimmune serum. A concentration-dependent inhibition of EBP2-reticulocyte binding was observed in both assays, but no inhibition of DBPII-reticulocyte binding was observed (Fig. 2c). Similarly, no inhibition of EBP2-reticulocyte binding was observed with mouse anti-DBPII IgG (Fig.

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2d). These data confirm that the character and binding specificity of EBP2 are distinct from those of DBP.

Conclusions. Erythrocyte or reticulocyte invasion by malaria merozoites is central to blood-stage development of all malaria parasites. *Plasmodium vivax* has the distinctive characteristic of invading reticulocytes, with a particular preference for reticulocytes expressing the surface Duffy blood group antigen, and *P. vivax* malaria is rare in populations with high Duffy negativity (3, 4, 7). Not surprisingly, even short-term *ex vivo* culturing of *P. vivax* is difficult when the blood is not Duffy positive and enriched for reticulocytes (18, 19). However, an increasing number of studies in different countries in which malaria is endemic are revealing the common occurrence of *P. vivax* malaria in Duffy-negative individuals, which contradicts the historical paradigm (8, 9). This possibly suggests "discovery" of a preexisting phenomenon previously overlooked or possibly the emergence of an alternative Duffy-independent invasion pathway for *P. vivax* that may lead to an expansion of *P. vivax* malaria into Duffy-negative populations. Whatever the reason for the increasing frequency of these observations, it is a cause for concern.

Recent analysis of *P. vivax* genome from field isolates identified a second putative erythrocyte binding protein, EBP2, with characteristic features similar to those of DBP, which was absent in the initial Sal1 genome sequence (10–12, 20). The primary structure of EBP2 has all the characteristics typical of members of the EBP superfamily (5) but is phylogenetically distant from *P. vivax* DBP (10). Therefore, it is logical to suggest that EBP2 has a functional role distinct from that of DBP in receptor recognition and invasion of reticulocytes. However, a lack of genetic similarity of the genes for EBP2 and DBP suggests that EBP2 is not a recent duplication, its apparent low frequency of single nucleotide polymorphisms (SNPs) indicates that it is unlikely to be under the same level of immune selective pressures as DBP, and lack of conservation of its C-terminal cytoplasmic domain suggests a different internal linkage to the intracellular merozoite invasion motility machinery (10, 11).

In this study, we characterized the functional and antigenic properties of the DBL domain of EBP2 to help determine its potential to be an invasion ligand and mediate a Duffy-independent invasion pathway. Contrary to expectations, the cell binding specificity of EBP2 is more restricted than that of DBP, and accordingly, its functional properties lack those anticipated of a ligand to mediate an alternate, Duffy-independent invasion pathway. Specifically, EBP2 binds exclusively to reticulocytes, in contrast to DBP, which can bind Duffy-positive normocytes as well (Fig. 1). Interestingly, DBP, unlike EBP2, has no preferential binding to the different reticulocyte subpopulations. These combined dual cell binding preferences of EBP2 unexpectedly augment the functional properties of DBP and reticulocyte binding proteins (RBPs), which are the well-characterized ligands of *P. vivax* (21, 22), thereby restricting instead of expanding the *P. vivax* host cell range. Such a binding profile is consistent with the historically defined *P. vivax* invasion phenotype restricted to Duffy-positive reticulocytes, and certainly, the lack of a novel binding preference is in line with the ancient character of the EBP2 gene duplication.

The inability of EBP2 to bind Duffy-positive normocytes indicates that the preference for Duffy-positive reticulocytes is not directly dependent on binding to DARC, as our data also showed minimal binding of EBP2 to Duffy-negative reticulocytes. Similarly, a recent study also showed evidence of EBP2 binding to both Duffy-positive and Duffy-negative erythrocytes, though at low frequency (23). During receptor binding, the DBP ligand domain has been shown to form a homodimer with the N-terminal extracellular domain of DARC (24). The critical functional contact residues of the DBP-DARC interaction have been identified, and especially important is a binding pocket with strong affinity for a sulfotyrosine of the DARC receptor (25–27). Although EBP2 and DBP have highly conserved cysteine residues and many other residues (see Fig. S1 in the supplemental material), only about 15% of the residues that make up the DBP binding site are conserved. A key missing element of EBP2 is the lack of contact residues in one of the regions that make up the sulfotyrosine binding site on DBP (see Fig. S1), which would greatly diminish the affinity of a possible interaction of DARC with EBP2. In addition to the functional differences, DBP and EBP2 are clearly distinct antigenically, as highly inhibitory anti-DBP antibodies did not react with EBP2 and could not inhibit EBP2-reticulocyte binding. Conversely, anti-EBP2 antibodies effectively blocked EBP2-reticulocyte binding (Fig. 2c) but did not affect DBP-DARC interaction. Therefore, it is possible that EBP2 may function as an alternate ligand for *P. vivax* invasion of reticulocytes when the principal ligand, DBP, is blocked by immune antibody.

Our data also show a preference for binding of immature ($CD71^{high}$) reticulocytes by EBP2. This reticulocyte subpopulation is generally restricted to the bone marrow (28) and was recently demonstrated to be the preferred cell type for invasion by *P. vivax* merozoites (29). Interestingly, *P. vivax* bone marrow infections have been reported in patients, even in cases with negative peripheral blood smears (30). Therefore, the observed host cell binding phenotype of EBP2 supports the growing evidence that *P. vivax* merozoite invasion of reticulocytes helps this parasite exploit a niche environment in the bone marrow.

In summary, we have characterized EBP2 as a functionally and antigenically distinct *P. vivax* ligand that binds exclusively to reticulocytes and has a strong preference for Duffy-positive reticulocytes. Based on these data, we propose that EBP2 and DBP are antigenically distinct but functionally redundant ligands that use different receptors while still retaining specificity for invasion of Duffy-positive reticulocytes. If EBP2 mediates an alternate invasion pathway, it seems more likely to be as a secondary pathway when immune inhibition blocks the principal DBP-DARC pathway. It remains to be determined whether the weak interaction of EBP2 with Duffy-negative reticulocytes observed in the *in vitro* functional assays is sufficient to facilitate invasion *in vivo*, although the lack of prevalent SNPs suggests a lack of significant immune exposure. The data presented here are an important step toward a better understanding of the biological role of EBP2 in the invasion process. Further research is necessary to determine the role of EBP2 in binding to Duffy-negative reticulocytes and its potential as a ligand mediating an alternate invasion pathway for *P. vivax* and to identify the receptor for this protein on reticulocytes.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01261-16/-/DCSupplemental.

Figure S1, TIF file, 0.7 MB.

Figure S2, PDF file, 1.9 MB.

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We have no commercial or other association that poses a conflict of interest.

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5.2.1 Resposta de anticorpos IgG contra a EBP2 em uma população naturalmente exposta ao *P. vivax* (dados não-publicados)

Durante os últimos anos, o nosso grupo de pesquisa em malária (BMIM/CPqRR) vem avaliando aspectos epidemiológicos, imunológicos e genéticos da população amazônica de Rio Pardo. Deste modo, dos 620 indivíduos recrutados inicialmente na comunidade de Rio Pardo (AM), uma subgrupo representativo da população estudada (n=425, 68,5%) foi selecionado para avaliar a presença e persistência de anticorpos IgG naturalmente adquiridos contra a EBP2. Entre os 425 indivíduos selecionados na linha de base, 44 (10%) puderam ser acompanhados por até 7 anos após recrutamento.

Os resultados permitiram demonstrar que, na linha de base, cerca de 50% (213/425) da população apresentou anticorpos IgG anti-EBP2, como detectado pela sorologia convencional (ELISA). Como era esperado, esta resposta de anticorpos aumentou em função de maior exposição ao *P. vivax*, avaliada aqui pelas seguintes variáveis (Figura 10): (i) idade do indivíduo, (ii) tempo de residência na Amazônia (área endêmica)(Figura 10). Nesta área, como a população é nativa da Amazônia, a idade do indivíduo correspondeu ao tempo médio de residência na área endêmica (Correlação de Spearman, r=0,95, p<0,0001). .

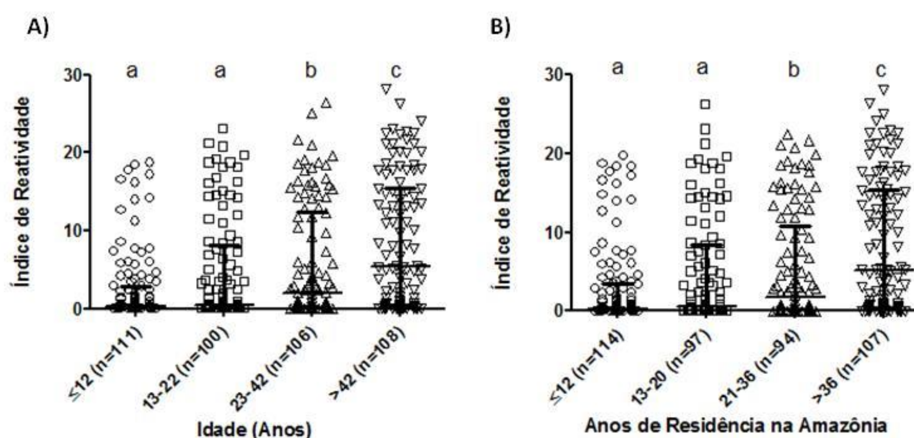

Figura 11 Resposta de anticorpos IgG contra a EBP2 detectados pela sorologia convencional (ELISA) aumentaem função da exposição à malária. Os níveis de exposição à malária foram avaliados em função da (A) idade do indivíduo; (B) tempo de residência na Amazônia brasileira. A resposta sorológica foi avaliada pelo ensaio de Elisa utilizando-se a EBP2 recombinante. Os resultados foram expressos em índice de reatividade (IR), sendo IR >1 considerado positivo. As diferentes letras no topo dos gráficos (a, b e c) indicam as diferenças estatísticas entre as medianas (Krukall wallis test e Dunn test como post hoc). Foi avaliada a correlação entre as variáveis idade e tempo de residência (Correlação de Spearman, r= 0,95, p<0,0001). Foram consideradas estatisticamente significativas as diferenças p<0,05.

O acompanhamento da população estudada, por cerca de sete anos (2008-2015), demonstrou que os níveis de transmissão de malária caíram drasticamente no período (Figura 11A). Assim, buscou-se aqui avaliar a persistência da resposta de anticorpos anti-EBP2, comparando esta resposta com os níveis de anticorpos contra a PvDBP. Os resultados permitiram confirmar a maior imunogenicidade da EBP2 (Fig. 11B) frente à PvDBP (Fig. 11 C). De fato, tanto a frequência de resposta quanto os níveis de anticorpos específicos foram significativamente mais altos contra a EBP2 (média 60%) do que contra a DBP_{II} (média 34%) (*Teste exato de fisher* [p<0,05] *e, Mann Whitney test* [p<0,05]). De relevância, independente dos níveis de transmissão de malária na área estudada, os anticorpos anti-EBP2 foram relativamente estáveis no período. Por outro lado, a frequência de anticorpos anti-DBP_{II}, diminuiu em função da redução da transmissão (Figura 11 C; x^2 de tendência, p=0,04).

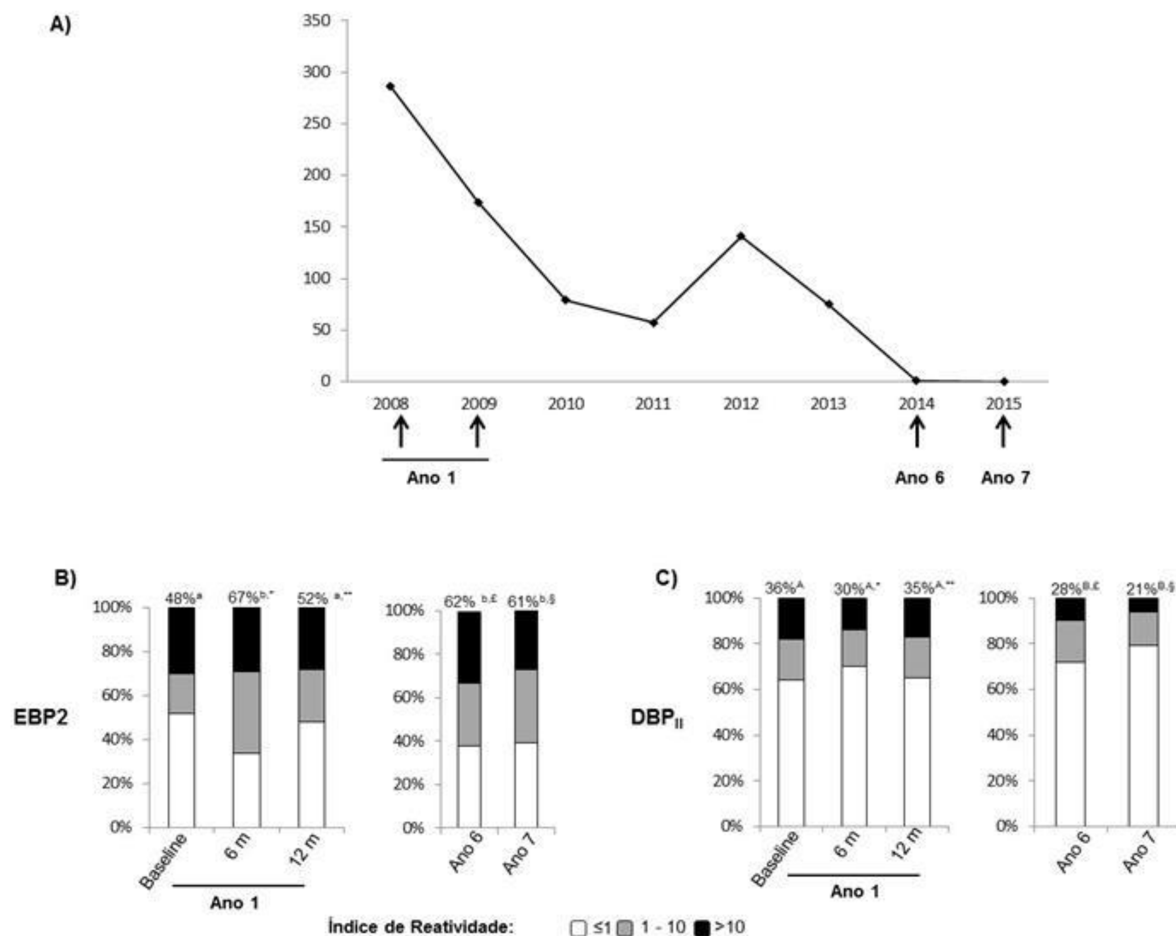

Figura 12 Resposta de anticorpos IgG anti-EBP2 e DBPII em função do número de casos de malária notificados pelo SIVEP (período 2008 a 2015) em Rio Pardo/AM. (A) As setas indicam os meses em que foram realizados os cortes transversais (tempo zero (baseline), 6 meses e 12 meses (Ano 1), 6 e 7 anos depois). A linha preta indica o número de casos de malária notificados pelo SIVEP, em Rio Pardo, durante os anos de acompanhamento. Os gráficos representam a frequência de resposta contra as proteínas (B) EBP2 e, (C) DBPII na população estudada (n=425), ao longo do estudo de coorte. Os resultados estão representados em função da intensidade de resposta, sendo o índice de reatividade (IR) >1 considerado como positivo. Os valores no topo dos gráficos representam a frequência de respondedores (IR>1). Diferenças estatísticas foram obtidas comparando tanto a intensidade de resposta (Mann Whitney test) quanto à frequência de respondedores a cada proteína (letras no topo do gráfico; Teste exato de Fisher). Além disso, os símbolos indicam as diferenças entre a frequência de resposta contra a resposta ant-EBP2 comparada à resposta anti-DBPII (Teste exato de Fisher). Foram considerados estatisticamente significativos os valores de p<0,05.

5.2.2 Influência dos alelos do receptor DARC na resposta imune humoral contra a EBP2

No presente trabalho foi possível demonstrar que a interação *in vitro* entre a EBP2 e os reticulócitos é depende da expressão de antígenos DARC na superfície da célula (Artigo 3, Ntunmgia *et. al.,* 2016). Assim, na próxima etapa fez-se necessário avaliar a influência dos polimorfismos deste receptor na reposta imune contra a EBP2. Para isto, os indivíduos foram divididos em grupos de acordo com o genótipo de DARC, isto é, carreadores de dois alelos funcionais (*FY*A/FY*A, FY*A/FY*B, FY*B/FY*B*), de um alelo funcional (*FY*A/FY*B*^{ES}*, FY*B/FY*B*^{ES}), ou nenhum alelo funcional (FY*B^{ES}/FY*B^{ES}). Os resultados demonstram que a frequência e os níveis de anticorpos IgG anti-EBP2 foram semelhantes entre os indivíduos carreadores de um ou dois alelos funcionais (Figura 12). Embora os níveis de transmissão da doença foram reduzidos drasticamente ao longo do estudo (Figura 11 A), esta resposta de anticorpos se manteve estável por até 7 anos. Como esperado, nos indivíduos DARC-negativos (*FY*B*^{ES}*/FY*B*^{ES}) a resposta anti-EBP2 foi praticamente residual (0-25%). Deste modo, assim como descrito para DBP_{II} (Artigo 1, Sousa-Silva FA, Torres LM *et al.*, 2014), os polimorfismos no genótipo de DARC parecem não influenciar na resposta de anticorpos anti-EBP2 detectados pela sorologia convencional.

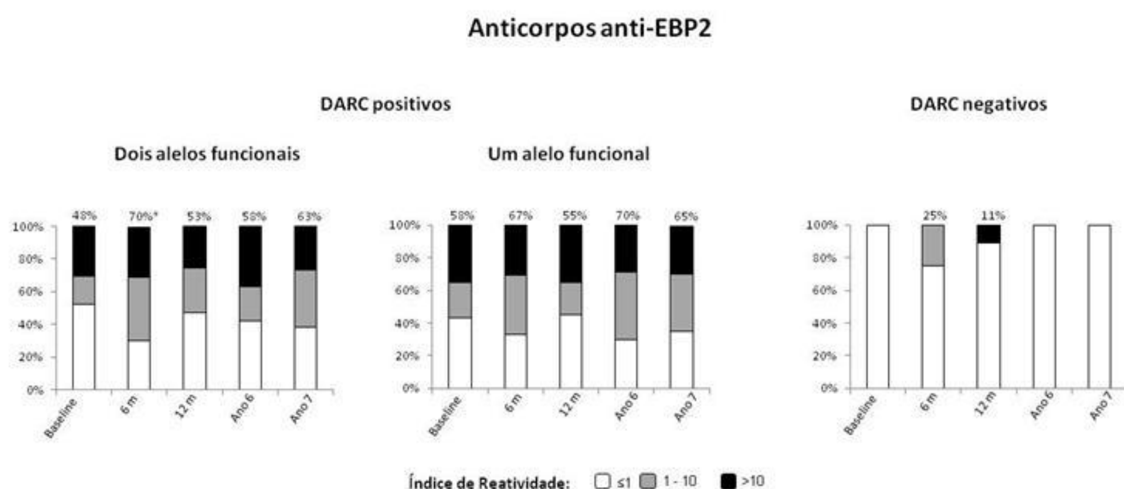

Figura 13 Resposta de anticorpos IgG anti-EBP2 detectados pelo ELISA entre indivíduos carreadores de dois alelos funcionais de DARC (FY*A/FY*A, FY*A/FY*B, FY*B/FY*B), um alelo funcional de DARC (FY*A/FY*BES, FY*B/FY*BES) e nenhum alelo funcional de DARC FY*BES/FY*BES, DARC negativo). Estão representadas as frequências de indivíduos respondedores na população estudada (n=425). O índice de reatividade (IR) > 1,0 foi considerado positivo. Diferenças significativas entre frequências de estão representadas pelo símbolo asterisco (*) (p < 0,05, teste exato de fisher). Os indivíduos DARC negativos foram excluídos das análises uma vez que sua resposta foi muito baixa ou negativa.

6 Considerações finais

6.1 Polimorfismos no hospedeiro vertebrado influenciam na resposta naturalmente adquirida contra a DBP_{II}

Durante os últimos anos, o nosso grupo de pesquisas em malária vem desenvolvendo um estudo pioneiro na Amazônia brasileira, a fim de caracterizar a resposta imune naturalmente adquirida contra proteínas do *P. vivax,* bem como a variabilidade genética dos parasitos circulantes (Revisto em De Sousa *et al.*, 2014). Nestas áreas, foi possível demonstrar que o principal antígeno candidato à vacina contra o *P. vivax,* a PvDBP, é pouco imunogênico e a resposta contra esta proteína é cepa-específica (Ceravolo *et al.*, 2008; Souza-Silva *et al.*, 2010). Vários fatores podem estar relacionados à baixa resposta, incluindo: (i) baixa exposição da PvDBP ao sistema imune, pois esta é uma proteína presente nas organelas apicais do parasito, sendo liberada apenas no momento da invasão (Adams *et al.*, 1990; Adams *et al.*, 1992); (ii) variabilidade genética na região II (ligante) da proteína, já que o polimorsfimo genético pode contribuir para uma resposta variante-específica (Vanbuskirk, Sevova*, et al.*, 2004) e, (iii) fatores relacionados à variabilidade genética do hospedeiro vertebrado (Maestre *et al.*, 2010; King *et al.*, 2011; Storti-Melo *et al.*, 2012). Neste contexto, o presente trabalho teve como objetivo avaliar se a resposta humoral contra a PvDBP (particularmente a região do ligante, DBP_{II}) é influenciada por polimorfismos genéticos do hospedeiro vertebrado; mais especificamente, o gene *DARC*, por se tratar de um receptor utilizado pelo parasito para invadir reticulócitos (Miller *et al.*, 1976; Adams *et al.*, 1992), e genes de HLA classe II, que são altamente polimórficos e fundamentais para o desenvolvimento de uma resposta imune humoral (Afridi *et al.*, 2016). Para isto, foi realizado um estudo longitudinal de base populacional, em uma comunidade rural da Amazônia brasileira, onde indivíduos expostos à malária foram acompanhados por até sete anos.

No geral, os resultados demonstraram que os genótipos de DARC parecem não influenciar na resposta de anticorpos anti-PvDBP, dectados pelo ELISA, uma vez que o perfil de anticorpos foi semelhante nos indivíduos expressando diferentes variantes de DARC. Até o momento, poucos estudos investigaram a relação entre polimorfismos genético de DARC e a aquisição de resposta imune contra a PvDBP, sendo os resultados contraditórios. Em 2010, Maestre e colaboradores demonstraram que na Colômbia indivíduos portadores de um alelo funcional de

DARC (*FY*A* ou *FY*B*) tinham maiores níveis de anticorpos IgG anti-DBP_{II} (ELISA) que aqueles carreadores de dois alelos funcionais. Por outro lado, King *et al.* (2011) não observaram nenhuma associação entre os genótipos de DARC e a aquisição de anticorpos anti-DBP_{II}. No presente trabalho, por meio de estudo longitudinal, foi possível confirmar os achados de King e colaboradores, pois utilizando diferentes proteínas recombinantes demonstramos que anticorpos anti-PvDBP não são influenciados pelos diferentes genótipos de DARC. De fato, a associação encontrada inicialmente nos estudos colombianos (Maestre *et al.*, 2010), pode ter sido em função da metodologia empregada, incluindo baixo número de respondedores (<20 indivíduos) em estudo do tipo transversal. Assim, o corrente estudo reforça a hipótese que polimorfismos de DARC não influenciam na aquisição de anticorpos IgG anti-DBP_{II} detectados pela sorologia convencional.

Em contrapartida, os resultados aqui apresentados demonstraram que anticorpos inibitórios (isto é, aqueles capazes de inibir a interação entre a DBP_{II}/DARC, BIAbs) sofrem influência dos genótipos de DARC. Mais especificamente, os resultados permitiram concluir que nos indivíduos portadores de apenas um alelo funcional de DARC (*FY*A* ou *FY*B*) a frequência e a magnitude dos BIAbs foram significativamente maiores do que no grupo de indivíduos carreadores de dois alelos funcionais. De interesse, este perfil se manteve durante os 12 meses de acompanhamento da população estudada. Embora até o momento não exista uma explicação clara para estes achados, é possível especular que o menor acesso do merozoíto aos eritrócitos dos indivíduos com um alelo funcional possa levar a uma maior exposição da PvDBP ao sistema imune. De fato, o mecanismo de invasão dos eritrócitos pelos parasitos da malária é muito rápido, ocorrendo em um tempo médio de cerca de dois minutos (Dvorak *et al.*, 1975; Gilson e Crabb, 2009). Entretanto, para se investigar esta hipótese seriam necessários experimentos de invasão *in vitro* com reticulócitos expressando diferentes variantes de DARC. Até o momento, estes ensaios têm sido dificultados pela falta de desenvolvimento de uma cultura em longo prazo do *P. vivax*.

Com relação a outros polimorfismos do hospedeiro vertebrado, as variações encontradas no antígeno leucocitátio humano (HLA), incluindo o HLA classe II, estão melhor caracterizadas do ponto de vista de susceptibilidade e/ou proteção contra malária grave (Hill *et al.*, 1991; May *et al.*, 1999; May *et al.*, 2001). Porém pouco se

sabe sobre a influência dos polimorfismo de HLA II na resposta imune humoral contra antígenos candidatos a vacina, sobretudo a PvDBP (Oliveira-Ferreira *et al.*, 2004; Lima-Junior *et al.*, 2012; Storti-Melo *et al.*, 2012). Diante disso, o presente trabalho avaliou ainda influência dos polimorfismos do HLA II na resposta imune humoral de longa duração contra a PvDBP (região do ligante, DBP$_{II}$).

Em conjunto, os resultados aqui apresentados demonstraram que os polimorfismos de HLA II influenciaram tanto na aquisição (DQB1*13:01) quanto na persistência (DQB1*13:01 e DQA1*01:03) de uma resposta de anticorpos contra a DBP$_{II.}$ Por outro lado, alguns alelos foram associados à ausência de resposta contra esse antígeno (DQB1*14:02 e DQA1*05:03) e, além disso, o genótipo DRB1*14:02-DQA1*05:03-DQB1*03:01 foi associado à ausência de resposta anti-DBP$_{II}$ ao longo de todo o estudo (persistente não respondedor). Até o presente momento, apenas um estudo investigou os polimorfismos de HLA e a resposta anti-PvDBP, porém neste estudo nenhuma associação foi encontrada (Storti-Melo *et al.*, 2012). Isso se deve, provavelmente, ao baixo número de respondedores à PvDBP.

Os achados aqui encontrados de associação entre alelos/haplótipos de HLA classe II e produção de anticorpos contra a PvDBP podem ser relevantes para o desenvolvimento de vacinas contra a malária. De fato, muitos dos alelos aqui descritos parecem influenciar na resposta imune protetora contra diferentes vacinas envolvendo vírus ou bactérias. Como exemplo, a relação bem descrita entre o HLA II DRB1*13:01 e produção de anticorpos contra as vacinas de hepatite B e vírus da influenza (Posteraro *et al.*, 2014). Dessa forma, nossos achados reforçam a importância de se identificar indivíduos geneticamente respondedores ou não a PvDBP, pois isto pode permitir aprimorar as vacinas em desenvolvimento.

Os resultados aqui encontrados são relevantes ainda porque foi possível identificar um grupo de alelos, compondo um único haplótipo (DRB1*07:01-DQA1*02:01 -DQB1*02:02) associados à resposta de anticorpos inibitórios da interação ligante-receptor. Esses achados podem explicar o fato da maioria dos indivíduos expostos ao *P. vivax* não desenvolver anticorpos bloqueadores da interação DBP-DARC (Souza-Silva *et al.*, 2010; Chootong *et al.*, 2012). Considerando que uma vacina desenvolvida com a DBP$_{II}$ tenha o objetivo de impedir a invasão por meio do bloqueio da interação entre o parasito e o seu receptor, estudos como o nosso que avaliam o papel funcional da resposta imune humoral

podem ser fundamentais para identificar mecanismos associados ao desenvolvimento de resposta imune protetora.

Uma vez demonstrada a influência do HLA II na persistência e nas propriedades funcionais dos anticorpos anti-DBP_{II}, especulou-se sobre as diferenças estruturais nos alelos de HLA II que poderiam influenciar na afinidade de ligação entre peptídeos e a molécula de HLA. Interessantemente, nossos achados de modelagem molecular demonstraram que, de fato, a maioria das diferenças entre as variantes DRB1 (bons *versus* maus respondedores) estava na fenda de ligação (“groove”) do HLA classe II com os peptídeos específicos, podendo influenciar na apresentação do antígeno. De fato, uma diferença importante entre HLA classe II de bons ou pobre respondedores foi uma troca de aminoácidos – Ácido aspártico para Glutamina -- sendo a perda do resíduo ácido nos pobres respondedores responsável pela menor afinidade dos peptídeos específicos pelo HLA-DR (resultados de predição). Curiosamente, o mesmo tipo de mutação (Glutamina/Ácido aspártico) em indivíduos susceptíveis a doença crônica de berílio faz com que interação entre o HLA-DP2 e as células TCD4+ destes indivíduos desvie a resposta pró-inflamatória (hipersensibilidade) e uma resposta humoral(Dai *et al.*, 2010). Mecanismo semelhante poderia explicar a baixa resposta à PvDBP dos indivíduos aqui descritos como “pobre-respondedores”.

Vale a pena ressaltar que, embora os resultados aqui encontrados entre HLA classe II e resposta imune a PvDBP necessitam ser validados em outras áreas endêmicas, nossos dados abrem perspectivas de novos estudos, principalmente envolvendo resposta celular, que podem ajudar a entender a modulação da resposta de células T no caso, por exemplo, de uma vacina contra o estágio sanguíneo da malária causada pelo *P. vivax.*

6.2 Avaliação do papel funcional da EBP2, bem como da resposta humoral naturalmente adquirida contra esta proteína

Embora o processo de invasão dos eritrócitos pelo *P. vivax* se dá principalmente pela via DBP_{II}/DARC, vários estudos recentes, inclusive no Brasil, tem descrito que indivíduos DARC negativos podem se infectar por essa espécie de *Plasmodium* (Cavasini *et al.*, 2007; Menard *et al.,* 2010; Lo *et al.*, 2015). Apesar da importância destes achados, até o momento, nenhuma outra via de invasão foi descrita para o *P. vivax.* Assim, a segunda parte deste trabalho teve como objetivo produzir e avaliar

um novo antígeno de *P. vivax* com potencial de estar envolvido no processo de invasão dos eritrócitos, a EBP2 (Hester *et al.*, 2013).

Neste contexto, foi avaliado aqui, o possível papel da EBP2 no processo de invasão dos reticulócitos pelo *P. vivax*. De fato, essa proteína foi capaz de interagir com os reticulócitos, porém falhou ao interagir com os eritrócitos maduros. Além disso, diferente do esperado, esta proteína interagiu, principalmente, com células DARC positivas. Contudo, se avaliarmos as similaridades entre os genes da DBP e da EBP2, embora as duas possuam os resíduos de cisteína e vários outros resíduos altamente conservados, apenas 15% da região crítica de ligação entre DBP-DARC é conservada entre as duas. Deste modo, é coerente sugerir que, embora a EBP2 tenha uma predileção por reticulócitos DARC positivos, provavelmente a interação com reticulócitos não seja mediada pelo receptor DARC. Juntos esses achados indicam que, embora a principal via de invasão dos reticulócitos pelo *P. vivax* seja via DBP-DARC, a EBP2 pode participar de uma via alternativa quando a via principal estiver bloqueada, por exemplo, pelo sistema imune.

Interessantemente, foi observado que a EBP2 possui uma predileção por reticulócitos com alta expressão de CD71 (CD71^{high}), correspondente a reticulócitos muito jovens, presentes principalmente na medula óssea. Vale ressaltar que Malleret *et al.* (2015) já haviam demonstrado que o *P. vivax* invade preferencialmente, pelo menos *in vitro*, reticulócitos CD71^{high}. Surpreendentemente, casos de indivíduos com infecção pelo *P. vivax* na medula óssea, mesmo aqueles com gota espessa negativa, já foram descritos (Yoeli, 1948; Imirzalioglu *et al.*, 2006). Esses achados aumentam a evidência de que a EBP2 possa participar da invasão dos reticulócitos na medula óssea.

Com relação ao reconhecimento da EBP2 pelo sistema imune, em modelo animal, esta proteína foi altamente imunogênica, sendo essa resposta antígeno específica. Mais especificamente, soro de animais imunizados com a DBP_{II} , ou mesmo anticorpos monoclonais anti-DBP_{II}, não reagem cruzado com a EBP2. Além disso, os anticorpos contra a EBP2 foram capazes de bloquear a interação EBP-reticulócitos, sugerindo que esta proteína pode induzir uma resposta funcional bloqueadora da interação ligante-receptor. A partir daí, utilizou-se a EBP2 recombinante para avaliar a frequência e a persistência de anticorpos anti-EBP2

naturalmente adquiridos em populações expostas a malária no Brasil (dados não publicados).

Na população de assentamento agrícola da Amazônia (Rio Pardo, AM) foi possível demonstrar que a resposta de anticorpos anti-EBP2 aumenta em função de uma maior exposição à malária, que foi avaliada aqui por diferentes variáveis, tais como, idade, tempo de residência na área endêmica. Além disso, demostrou-se pela primeira vez que essa proteína é altamente imunogênica, já que cerca de 60-70% dos indivíduos naturalmente expostos ao *P. vivax* desenvolveram anticorpos IgG anti-PvEBP2. Estes dados contrastam com aqueles aqui descritos para a DBP_{II}, que foi pouco imunogênica na população de estudo (21-36% de respondedores). De relevância, durante os 7 anos de estudo, pode se demonstrar que a resposta de anticorpos IgG contra a EBP2 é mais estável do que aquela induzida pela PvDBP.

Em conjunto, os resultados aqui apresentados sugerem que a EBP2 não parece explicar a via alternativa de invasão do *P. vivax* em indivíduos DARC negativos. Por outro lado, os resultados descritos até agora sugerem um possível papel na invasão de reticulócitos imaturos, provavelmente, em alguma via envolvendo a medula óssea. Por outro lado, pode-se especular ainda que na presença de elevados níveis de anticorpos anti-PvDBP, o parasito possa invadir os reticulócitos por via secundária, provavelmente, envolvendo a EBP2. Estudos futuros fazem-se necessários para caracterizar estas possíveis vias de invasão do *P. vivax*.

Além disso, uma vez que demonstramos pela primeira vez a imunogenicidade dessa proteína em população naturalmente exposta, mais estudos são necessários para estabelecer se, assim como em modelo animal, parte dos anticorpos anti-EBP2 podem bloquear a interação da ligação entre *P. vivax* e o reticulócito em população naturalmente exposta.

Esses primeiros estudos envolvendo a EBP2 foram particularmente relevantes e têm abrido novas perspectivas de estudos, sobretudo visando (i) estudos in vitro para definir se anticorpos anti-EBP2 são capazes de bloquear a invasão in vitro do *P. vivax*; (ii) estudos imunológicos para identificar mecanismos imunológicos que possam ser responsáveis pelo desenvolvimento de células de memória de longa-duração e, por fim (iii) caracterizar o polimorfismo genético da EBP2 em parasitos circulantes. Neste sentido estudos estão em progressos pelo nosso grupo na Amazônia brasileira.

7 Conclusões

Com este trabalho foi possível concluir que:

1) Polimorfismos genéticos do hospedeiro vertebrado influenciam na resposta imune naturalmente adquirida contra a PvDBP, mais especificamente:

1.1) Anticorpos que bloqueiam a interação DBPII/DARC, mas não aqueles detectados pela sorologia convencional, são influenciados pelos genótipos de DARC, sendo a maior frequência detectada em indivíduos heterozigotos com um alelo DARC silenciado (FY^*B^{ES});

1.2) Poucos indivíduos desenvolvem anticorpos funcionais anti-PvDBP, entretanto, quando estes são adquiridos, os mesmos tendem a se manter estáveis independentemente do genótipo de DARC do indivíduo;

1.3) Alelos e haplótipos de HLA classe II influenciam na aquisição e manutenção de resposta de anticorpos contra a PvDBP, incluindo anticorpos bloqueadores ou não da interação DBP_{II}/DARC;

1.4) Modelos computacionais indicam que diferenças estruturais entre as moléculas de HLA II-DRB1, principalmente nos sítios de ligação com o peptídeo, estão associadas a uma maior (bom-respondedor) ou menor (mau-respondedor) afinidade de ligação do peptídeo ao HLA-DRB1.

2) Com relação a EBP2, experimentos in vitro confirmaram suas propriedades de ligação aos reticulócitos, e estudos sorológicos demonstram elevada imunogenicidade. Mais especificamente,

2.1) A proteína se liga exclusivamente a reticulócitos e, preferencialmente, àqueles com alta expressão de CD71 ($CD71^{high}$) DARC positivos;

2.2) Modelos animais permitiram demonstrar que a EBP2 é altamente imunogênica e induz anticorpos capazes de bloquear a interação da proteína com um receptor ainda desconhecido na superfície de reticulócitos jovens.

2.3.) Na população estudada, indivíduos naturalmente expostos ao *P. vivax* na Amazônia brasileira, a EBP2 foi altamente imunogênica e induziu uma resposta de anticorpos de longa duração, mesmo em condições de baixa transmissão.

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