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ANTIBODY RESPONSE TO *PLASMODIUM VIVAX* ANTIGENS IN FY-NEGATIVE INDIVIDUALS FROM THE COLOMBIAN PACIFIC COAST

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Abstract. The Duffy antigen (Fy) is necessary for *Plasmodium vivax* invasion of human erythrocytes. Some populations have a highly prevalent Fy-negative phenotype; such persons are naturally protected from *P. vivax* blood infection but are expected to completely support the *P. vivax* pre-erythrocytic cycle, representing a valuable model for studying the immune response during these parasitic stages. We typed 214 individuals, mostly Afro-Colombians, from a *P. vivax*-endemic area for Fy expression and determined the antibody response to *P. vivax* pre-erythrocytic (sporozoites and CS) and blood-stage antigens (blood forms, *P. vivax* merozoite surface protein 1, and *P. vivax* Duffy binding protein [PvDBP]). Antibody titers to *P. vivax* circumsporozoite protein, P11, and N-terminal peptides and the number of responders were similar in Fy-negative and Fy-positive individuals. The number of responders to sporozoites, blood forms, and PvDBP were different between these groups. Thus, Fy-negative individuals from malaria-endemic areas can be used to study the immune response to the *P. vivax* liver phase without interference of the erythrocytic cycle.

INTRODUCTION

Malaria is a mosquito-transmitted parasitic disease widely distributed in tropical and subtropical areas of the world. Its global burden is estimated to be more than 500 million clinical cases every year, produced mainly by *Plasmodium falciparum* and *P. vivax*.[1,2] Both innate factors and acquired immune responses protect humans from the clinical manifestations induced by multiplication of malaria parasites in the blood.[3,4] Of the multiple innate factors that protect individuals from malaria, hemoglobin abnormalities and lack of expression or modifications of erythrocyte receptors for parasite invasion are the most important. One of the red blood cell groups, the Duffy (Fy) antigen, recently renamed the Duffy antigen receptor for chemokines (DARC) because of its role in chemokine regulation,[5] is an indispensable receptor for *P. vivax* invasion of the erythrocyte. Thus, individuals lacking DARC (i.e., Fy-negative individuals) are naturally protected from *P. vivax*–induced clinical malaria.[6]

The expression of Fy antigen (DARC) on red blood cells determines the susceptibility of individuals to infection by *P. vivax* asexual blood forms. This blood group was first detected in a hemophilic patient in 1950 by Cutbush and Mollison,[7] and three different phenotypes have been defined in Caucasians using antibodies to Fy antigen.[8] Miller and others[9] and Gelpi and King[10] later showed that the Fy antigen was necessary for invasion of red blood cells by *P. vivax* and *P. knowlesi* and that most west Africans do not express DARC on their erythrocytes. More recently, the lack of this receptor in African populations is believed to be due to selective pressure induced by *P. vivax* in those populations in earlier times.[11] Because erythrocytes from Fy-negative individuals cannot be invaded by merozoites, such individuals do not have the clinical manifestations of malaria associated with development of the *Plasmodium* asexual erythrocytic cycle. They also do not contribute to the transmission of the parasite by mosquitoes because they do not develop gametocytes.

Until now, there has been little interest in assessing the immune response induced in Fy-negative individuals who have been frequently inoculated with *P. vivax* sporozoites and little is known about the characteristics of their immune responses. To understand the extent of the anti-*P. vivax* humoral immune responses in Fy-negative individuals, we studied individuals in several villages in the Colombian Pacific coastal region. We hypothesized that because the population is mainly composed of Afro-Colombian individuals, prevalence of individuals expressing the Fy-negative phenotype might vary. We typed the study populations for Fy and assessed the presence of antibodies to defined *P. vivax* antigens from the pre-erythrocytic and asexual blood forms. Understanding the immune response of Fy-negative individuals is an important window into the mechanisms of immune responses induced naturally to different phases of the *P. vivax* life cycle.

MATERIALS AND METHODS

Study area. The Colombian Pacific coast is an extensive region characterized by a dense tropical rain forest and is one of the places worldwide with the greatest amount of rainfall (up to 1,200 mm per year). This region is inhabited mainly by people of African origin and has an unstable malaria transmission pattern with minimal seasonal variation. The region accounts for approximately 4% of the total Colombian population but reports between 20% and 30% of the total malaria cases yearly,[12] with variable cumulative incidences of *P. falciparum* and *P. vivax* across the region (Table 1). We studied communities located in Quibdó (Chocó State) and La Delfina, Punta Soldado, and Zacarías (Valle State). These four rural communities focus on fishing, agriculture, logging, and mining activities.

Study design. To determine the humoral immune responses to *P. vivax* in both Fy-positive and Fy-negative individuals, we selected 214 men and women 9–92 years of age from *P. vivax* malaria–endemic villages in each of four communities. We invited all individuals participating in a local health campaign to be part of this study; only those who had been residing for more than one year in their village were finally selected. All donors gave informed consent following protocols reviewed

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TABLE 1
Cumulative incidence (%) of malaria cases by village

	Quibdó	Punta Soldado	La Delfina	Zacarías
Plasmodium species	Ranges of annual cases per 100 inhabitants			
P. vivax	0.5–2	1–4	20–57	20–45
P. falciparum	1–5	60–70	1–2	23–45

and approved by the Institutional Review Board of the Universidad del Valle. Whole blood was obtained from individuals by venipuncture of the arm using Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) containing EDTA anticoagulant. Five milliliters of blood were drawn and immediately fractionated. One blood fraction was used to type the individual for the Duffy antigen. The second fraction was centrifuged at 2,000 rpm for 10 minutes to obtain plasma. Plasma aliquots were stored frozen at −70°C and were further tested by an immunofluorescent antibody test (IFAT) and an enzyme-linked immunosorbent assay (ELISA) to determine titers against *P. vivax* whole parasite forms and specific antigens.

Antigens. Three *P. vivax* antigens were available to assess the antibody response: circumsporozoite protein (CS), merozoite surface protein 1 (MSP-1), and Duffy binding protein (DBP). These are among the most widely studied *P. vivax* antigens from the pre-erythrocytic and erythrocytic stages. Two long synthetic peptides corresponding to different regions of the PvCS were used to assess the response to the pre-erythrocytic phase. Peptide N, which encompasses the non-repeat amino flank of PvCS (amino acids 22–125), and a 48-mer peptide composed of tandem repeats of the p11 peptide (amino acids 96–104) were chemically synthesized as described and used as antigens for the ELISA.[13] Recombinant proteins containing region II of the PvDBP, (rPvRII) and a fragment of the amino region of the PvMSP-1 (200L fragment), both expressed on the *P. vivax* asexual blood forms, were used to assess the response to the erythrocytic phase.

The rPvRII fragment was produced at the International Center for Genetic Engineering and Biotechnology (New Delhi, India) as previously described.[14] The 200L fragment corresponding to the N-terminal fragment of PvMSP-1 was produced at the Malaria Vaccine and Drug Development Center (Cali, Colombia) as a chimeric recombinant protein fused with a six-histidine tag (6-his) in *Escherichia coli* and was purified to < 1,000 endotoxin units (EU)/50 μg of protein.

Duffy antigen typing. Duffy antigen expression was typed by a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique using genomic DNA obtained from the blood cell fraction of the patients. The DNA was extracted by a salting-out technique, and 1 μL was used for a nested PCR as previously described.[15]

Antibody response to *P. vivax* antigens. To determine whether Fy-negative individuals responded to specific blood antigens and whether immune antibody response to pre-erythrocytic forms of the parasite differed between Fy-positive and Fy-negative individuals from *P. vivax*–endemic areas, we conducted an IFAT using *P. vivax* sporozoites and blood forms (schizonts), fixed-parasite preparations, and an ELISA for specific antigens.

Immunofluorescent antibody test. Sporozoites and parasite blood forms were collected and used as antigen sources. Glass slides were previously coated with *P. vivax* sporozoites or parasite blood forms. Sporozoites were produced by infection of laboratory-reared *Anopheles albimanus* mosquitoes that fed on *P. vivax*–infected blood. Two weeks after feeding, sporozoites were collected by dissection of the mosquito salivary gland and deposited in a multiwell IFAT slide at a concentration of 2,000 sporozoites/well. Slides were kept frozen at −70°C until use as described elsewhere.[16]

Plasmodium vivax blood forms were obtained from patients with parasitemia levels > 0.5%. These were cultured for eight hours to improve their maturation and to allow schizonts to develop. A pool of sera from healthy volunteers without a history of malaria exposure was used as negative controls. Sera from semi-immune volunteers from malaria-endemic areas were used as positive controls. Fluorescence was read using an epifluorescence microscope (Laborlux 2; Leitz GmbH, Wetzlar, Germany). Antibody titers < 1:80 were considered negative.

Enzyme-linked immunosorbent assay. The PvCSP, PvMSP-1 (200L fragment), and PvDBP (rPvRII) antigens were evaluated by an ELISA as previously described[17] with modifications: blocking and diluting buffers contained skim milk (Becton-Dickinson) and bovine serum albumin (BSA) for PvCSP and PvMSP-1, respectively. Absorbance was read at 405 nm in a multichannel spectrophotometer (MRX; Dynex Technologies, Inc., Chantilly, VA). The rPvRII was used at a concentration of 2 μg/mL in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Plates were blocked and incubated with phosphate-buffered saline (PBS), 2.5% BSA for two hours. Serial dilutions were prepared in diluting buffer PBS, 0.1% BSA. Horseradish peroxidase–conjugated

TABLE 2
Baseline sociodemographic characteristics of individuals by Duffy antigen (Fy) status

	Fy-negative n (%)	Fy-positive n (%)
Village		
Delfina	23 (17)	44 (57.1)
PuntaSoldado	15 (11)	8 (10.4)
Zacarías	31 (22.5)	11 (14.3)
Quibdó	68 (49.5)	14 (18.2)
Ethnicity*		
Afro-Colombian	132 (96.4)	27 (35.1)
Other	3 (2.1)	47 (61)
No data	2 (1.5)	3 (3.9)
Village/race		
Delfina/Afro-Colombian	22 (16)	8 (10.4)
Delfina/other	1 (1)	36 (46.7)
Pta Soldado/Afro-Colombian	15 (11)	8 (10.4)
Pta Soldado/other	0 (0)	0 (0)
Zacarías/Afro-Colombian	29 (21)	6 (7.8)
Zacarías/other	0 (0)	3 (3.9)
Quibdó/Afro-Colombian	66 (48)	5 (6.5)
Quibdó/other	2 (1.5)	8 (10.4)
No data	2 (1.5)	3 (3.9)
Sex		
Male	52 (38)	28 (36.4)
Female	81 (59)	46 (59.7)
No data	4 (3)	3 (3.9)
Age group (years)		
<20	21 (15)	10 (13)
20–39	68 (50)	42 (55)
>39	44 (32)	23 (30)
No data	4 (3)	2 (2)

* Ethnicity determined by self definition.

antihuman polyvalent immunoglobulin was used at a dilution of 1:1 × 10^6 in diluting buffer. The plates were incubated for 60 minutes with tetramethylbenzidine/hydrogen peroxide and read at 630 nm. Fifty microliters of stopping solution (2 N H_2SO_4) were added to each well and after 60 minutes they were read again at 450–550 nm.

To define the cut-off value, the antigen was tested using sera from 10 human volunteers with no history of malaria infection. Antibody titers were considered positive when absorbance of the test sera was greater than or equal to the mean absorbance of human control plasma plus 3 standard deviations. ELISA titers < 1:100 were considered negative.

Statistical analysis. To evaluate the association between presence of the Fy (DARC) antigen and elicitation of antibodies against parasite forms or antigens, we calculated prevalence ratios of antibody response for Fy-negative versus Fy-positive individuals using Stata version 8.0 software (Stat Corporation, College Station, TX). Prevalence ratios were adjusted by age as a marker of immune status and village, a proxy for intensity of transmission to control for potential confounders. Sample size was calculated estimating a 50% prevalence of DARC with a variation of 10% and an alpha error = 0.05.

RESULTS

Overall malaria prevalence in the study population. The different villages showed variable cumulative incidence for *P. falciparum* and *P. vivax,* ranging from 0.5 to 45 per 100 inhabitants (Table 1). Individuals from the different villages were tested by IFAT to determine their previous exposure to malaria. A total of 39% of the study volunteers showed responses to *P. falciparum* and 60% to *P. vivax* as determined using as antigen asexual blood parasite preparations from both parasite species. At the clinical interview, 182 (88%) reported previously having malaria. The proportions of individuals with antibodies to blood stages by IFAT were similar (43% and 36%) for *P. falciparum* and (74% and 52%) and *P. vivax* in Fy-positive and Fy-negative individuals, respectively.

Prevalence of Fy-negative phenotype. Overall, 74.3% of the 214 individuals we studied were Afro-Colombian, ranging from 64% to 100% of the volunteers in each community. Ninety-six percent of Fy-negative individuals were Afro-Colombian. Among all the individuals studied, only three Fy-negative individuals were from the Mestizo ethnic group. The community with the highest percentage of Afro-Colombians was Quibdó; the majority of Mestizo and indigenous individuals were found in La Delfina. In both groups there were more women than men, and most participants were 20–39 years of age (Table 2).

Responses to pre-erythrocytic antigens. Sera from both Fy-negative and Fy-positive individuals showed antibody responses to sporozoites; however, the percentage of responders was significantly lower in the Fy-negative group compared with the Fy-positive group (adjusted odds ratio = 0.56, 95% confidence interval [95% CI] = 0.40–0.78). The maximum antibody titer was 1:1,280 in both groups. (Figure 1).

We assessed whether this pattern of response was also re-

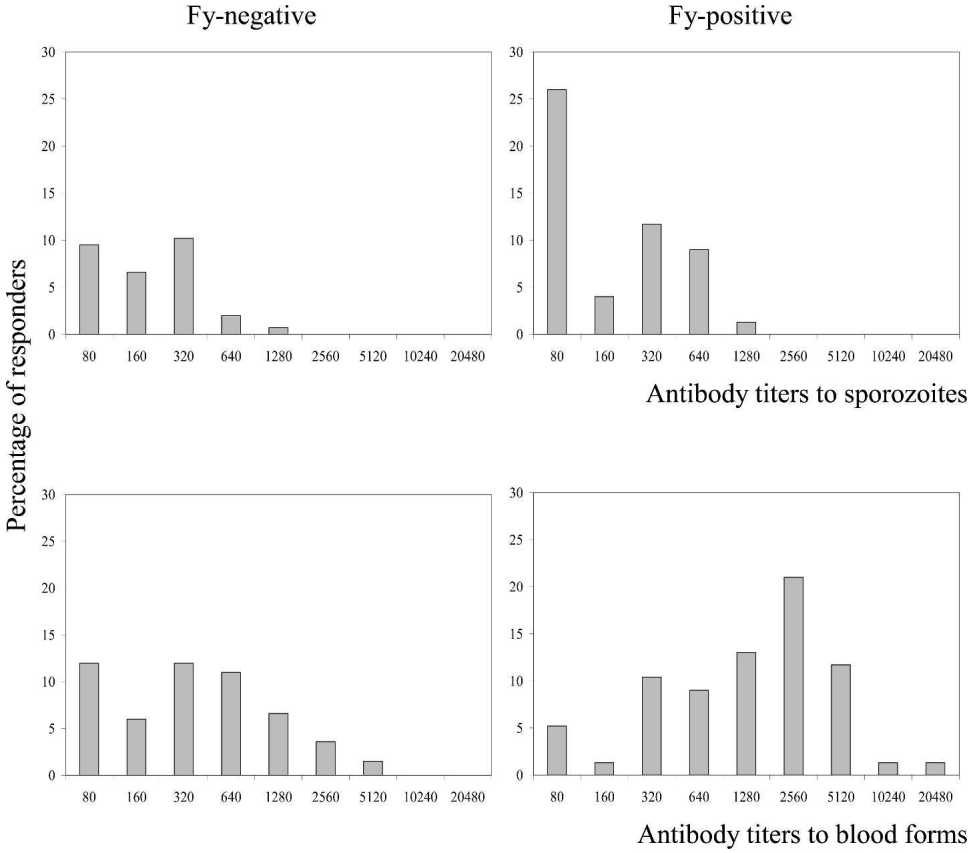

FIGURE 1. Antibody responses against sporozoites and blood forms of *Plasmodium vivax* in 137 Fy-negative individuals and 77 Fy-positive individuals.

flected in the response to specific pre-erythrocytic antigens. For this purpose, we conducted an ELISA with two peptides from PvCS (P11 and N-terminal) (Figure 2). Responses to the N-terminal peptide in Fy-negative and Fy-positive individuals were equal in frequency (adjusted odds ratio = 1.01, 95% CI = 0.75–1.36) and level of antibody titer reached. Also, responses to P11 were not significantly different between the two groups (adjusted odds ratio = 1.18, 95% CI = 0.91–1.55). However, in this group a larger percentage of Fy-negative individuals had each positive antibody titer compared with Fy-positive individuals. Additionally, only one individual in the Fy-negative group reached the highest antibody titer (1:1,600); most of the responders in this group reached titers of 1:200 and 1:400.

Antibody responses to blood-stage antigens. Antibody responses to *P. vivax* blood parasites were present in approximately half of the Fy-negative individuals (Figure 1). However, as expected, responses were less frequent in this group than in Fy-positive individuals (adjusted odds ratio = 0.71, 95% CI = 0.57–0.86). Similarly, Fy-negative individuals had lower titers than Fy-positive individuals.

The Fy-negative individuals also showed antibody responses to blood-stage antigens in concordance with their positive responses to blood parasite forms. For PvMSP-1 (200L fragment), the number of responders (adjusted odds ratio = 0.94, 95% CI = 0.78–1.10) and their maximum antibody titers (1:1,600 for both groups) were not different from those of Fy-positive individuals. However, for PvDBP (rPvRII), Fy-negative individuals responded less frequently than Fy-positive individuals (adjusted odds ratio = 0.61, 95% CI = 0.42–0.91). A lower percentage of responders in the Fy-negative group and one Fy-positive individual

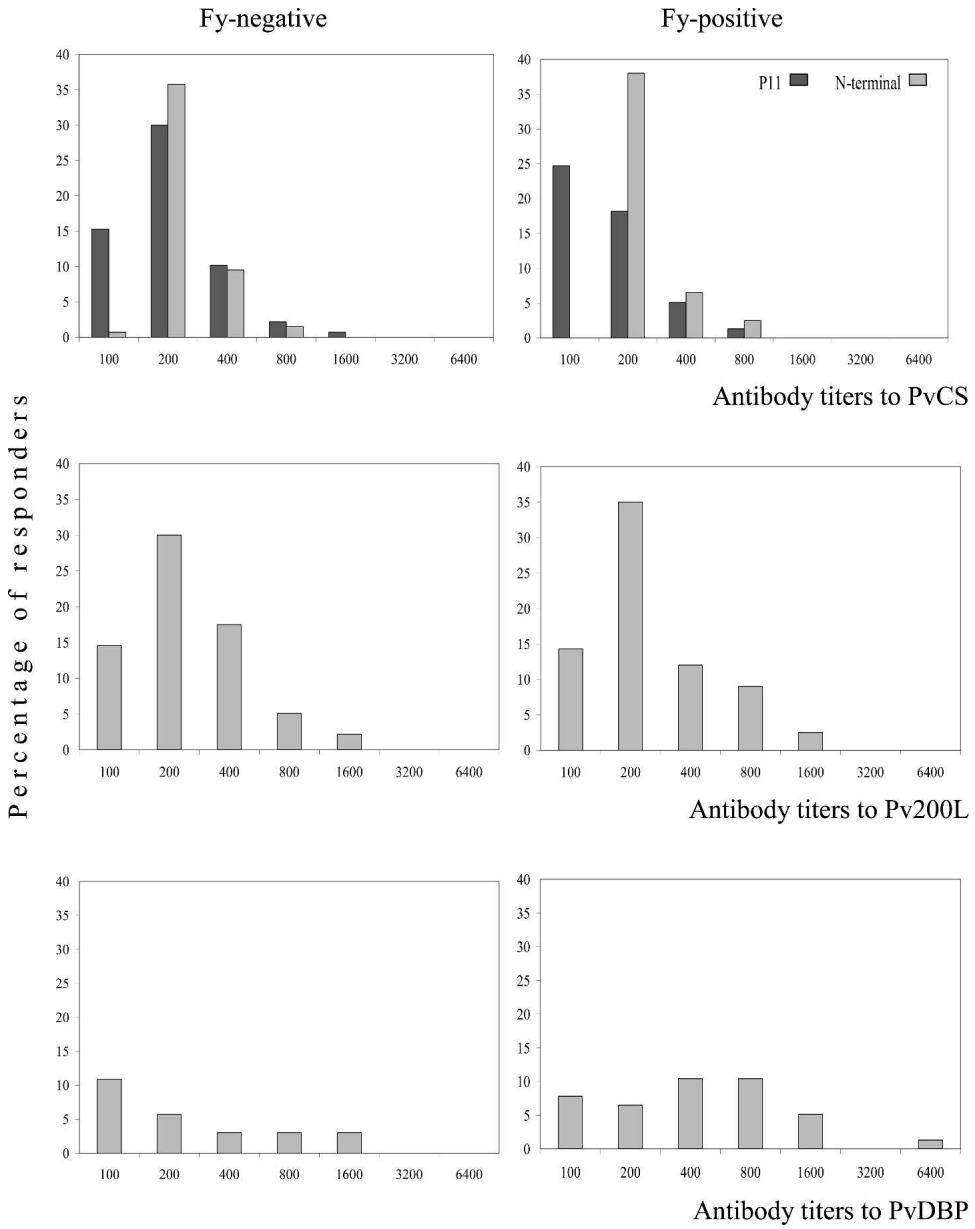

FIGURE 2. Antibody responses against *Plasmodium vivax* circumsporozoite protein (PvCS), Pv200L, and *P. vivax* Duffy binding protein (PvDBP) in 137 Fy-negative individuals and 77 Fy-positive individuals.

had the highest titer (1:6,400) observed in this study (Figure 2).

DISCUSSION

The present study addressed the extent of anti-*P. vivax* humoral immune responses in Fy-negative individuals. These individuals do not contribute to malaria transmission but may represent a valuable model to dissect the immune response to the different parasite forms of the *P. vivax* life cycle. Our study suggests that this assumption might be correct for some *P. vivax* antigens in that both Fy-positive and Fy-negative individuals responded equally in terms of the number of responders and maximum antibody titers to pre-erythrocytic antigens (P11 and N-terminal peptides) and to sporozoites in an IFAT in terms of antibody levels.

Theoretically, Fy-negative individuals are equally exposed to the *P. vivax* pre-erythrocytic cycle as Fy-positive persons in malaria-endemic areas. However, since they do not proceed to the blood phase, FY-negative individuals do not develop malaria symptoms characteristic of this phase of the parasite cycle. They are never exposed to *P. vivax* gametocytes and likely do not have most of the antigens expressed in the asexual blood forms.

The Fy-negative individuals exposed to sporozoite inoculation would proceed to the liver cycle and release merozoites that would be exposed to the immune system. Although the antigens already expressed on the merozoite surface would induce immune responses, the immune system of those individuals will be selectively stimulated by a limited number of *P. vivax* antigens. A model similar to this one has been used by Guerin-Marchand and others to identify *P. falciparum* liver-stage antigens (LSAs).[18] They used sera from individuals exposed for long periods to *P. falciparum*, but who had not entered the blood phase of the cycle because of chemoprophylaxis. Sera form these individuals allowed the selection of multiple LSAs, which are currently being tested as vaccine candidates in clinical trials.

A finding that deserves further analysis is that PvMSP-1 is similarly recognized by Fy-positive and Fy-negative individuals, whereas the PvDBP is mainly recognized by Fy-positive individuals. This may indirectly indicate that MSP-1 is expressed during liver schizogony, as has been shown for *P. falciparum*, and that it might be expressed abundantly enough to stimulate the immune system as much as the repeated merozoite release from the blood cycle does in Fy-positive individuals. Conversely, infection with *Plasmodium* blood parasite forms has been shown to modulate the liver stage immunity to *P. yoelii* and to specific antigens including a fragment of the MSP-1 protein by inducing apoptosis of specific CD4+ T helper cells.[19,20] This modulation might result in lower specific T helper activity to B lymphocytes and consequently lower antibody production in Fy-positive individuals to blood-stage antigens. Additionally, the poor recognition of PvDBP by Fy-negative individuals may be explained by the late expression of this ligand, whose expression appears to be triggered by merozoite binding to the merozoite Fy receptor.

A relationship between expression of DARC and the age of red blood cells has been previously reported[21]; however, previous data about their expression and immune response are not available. In this study, we report a relationship between the expression of DARC and the humoral immune response to different parasite forms of *P. vivax*, as well as to specific antigens in individuals chronically exposed to malaria parasites.

In conclusion, Fy-negative individuals exhibit antibody responses to pre-erythrocytic parasite forms and to a limited number of blood-stage antigens. Selected anti-*P. vivax* responses could be used for continued study of the mechanisms of immunity and for selection of vaccine candidates.

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