Plasmodium vivax spleen-dependent genes encode antigens associated with cytoadhesion and clinical protection

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Edited by Louis H. Miller, National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD, and approved April 16, 2020 (received for review November 23, 2019)

Plasmodium vivax, the most widely distributed human malaria parasite, causes severe clinical syndromes despite low peripheral blood parasitemia. This conundrum is further complicated as cytoadherence in the microvasculature is still a matter of investigation. Previous reports in Plasmodium knowlesi, another parasite species shown to infect humans, demonstrated that variant genes involved in cytoadherence were dependent on the spleen for their expression. Hence, using a global transcriptional analysis of parasites obtained from spleen-intact and splenectomized monkeys, we identified 67 P. vivax genes whose expression was spleen dependent. To determine their role in cytoadherence, two Plasmodium falciparum transgenic lines expressing two variant proteins pertaining to VIR and Pv-FAM-D multigene families were used. Cytoadherence assays demonstrated specific binding to human spleen but not lung fibroblasts of the transgenic line expressing the VIR14 protein. To gain more insights, we expressed five P. vivax spleen-dependent genes as recombinant proteins, including members of three different multigene families (VIR, Pv-FAM-A, Pv-FAM-D), one membrane transporter (SECY), and one hypothetical protein (HYP1), and determined their immunogenicity and association with clinical protection in a prospective study of 383 children in Papua New Guinea. Results demonstrated that spleen-dependent antigens are immunogenic in natural infections and that antibodies to HYP1 are associated with clinical protection. These results suggest that the spleen plays a major role in expression of parasite proteins involved in cytoadherence and can reveal antigens associated with clinical protection, thus prompting a paradigm shift in P. vivax biology toward deeper studies of the spleen during infections.

Plasmodium vivax | spleen-dependent genes | cytoadherence | global transcription

H uman malaria caused by Plasmodium vivax infection (vivax malaria) is a major global health issue. It is the most geographically widespread form of the disease, accounting for 7.5 million annual clinical cases, the majority of cases in America and Asia, and estimation of over 2.5 billion people living under risk of infection (1). The general perception toward vivax malaria has shifted recently, following a series of reports, from being viewed as a benign infection to the recognition of its potential for more severe manifestations, including fatal cases (2–4). However, the underlying pathogenic mechanisms of vivax malaria remain largely unresolved.

Central to the pathology in Plasmodium falciparum, the most virulent malaria-causing human species, is the phenomenon of cytoadherence to endothelial receptors mediated by variant surface proteins that facilitate sequestration of parasitized red blood cells (RBCs) deep in microvasculature (5). The absence of mature parasites in peripheral blood of patients is unequivocal evidence of cytoadherence and parasite sequestration in this species. In contrast, proteins encoded by spleen-dependent genes associated with clinical protection in vivax malaria causes severe disease. This conundrum finds an explanation from reports suggesting that the spleen is a place for parasite sequestration. We performed a global transcriptional analysis of parasites that grew in the presence or absence of the spleen in a nonhuman primate model. We identified 67 spleen-dependent genes, including multigene variant families, and functionally demonstrated specific adherence to human spleen fibroblasts by a member of such families. Moreover, we further demonstrated that spleen-dependent Plasmodium vivax genes code for immunogenic proteins during natural infections. Our results indicate that this organ plays an important function in P. vivax malaria and call for deeper studies of the role of spleen in P. vivax infections.


The authors declare no competing interest.

This article is a PNAS Direct Submission.

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Data deposition: All data are freely available through the Gene Expression Omnibus database, https://www.ncbi.nlm.nih.gov/geo (accession no. GPL6667).

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1920596117/-/DCSupplemental.

as infected reticulocytes with mature stages of *P. vivax* are detected in peripheral circulation, for a long time it was amply accepted that this human malaria parasite does not sequester in the microvasculature. Against this dogma, in the last decade, different reports have described in vitro cytoadherence of *P. vivax*-infected reticulocytes to human cells and tissue cryosections (6–8). Moreover, infected reticulocytes were able to cytoadhere under static and flow conditions to cells expressing ICAM-1, a well-known *P. falciparum* receptor, and this binding was partly mediated by VIR proteins (6), a superfamily of variant surface proteins likely 

![Diagram showing genes only expressed in Sp+2 and negative in the splenectomized monkeys for any of the two algorithms used.](image)

**Fig. 1.** Identification of *P. vivax* spleen-dependent genes. (A) Scheme of experimental infections and biological samples used for expression analysis. (B) Diagram showing genes only expressed in Sp+2 and negative in the splenectomized monkeys for any of the two algorithms used.
involved in cytoadherence (9, 10). Therefore, even though the exact molecular mechanisms of cytoadherence are not fully elucidated, these observations prompt a paradigm shift in *P. vivax* biology.

Malaria parasites infections induce a dramatic splenic response mostly characterized by variable levels of splenomegaly. This is probably due to the fact that the spleen plays an important dual role in malaria: destruction of infected red blood cells (iRBCs) and expression of parasite antigens, including variant surface proteins involved in pathology (11, 12). Thus, pioneering experiments with *Plasmodium knowlesi* parasites obtained from splenectomized monkeys showed that parasites no longer expressed variant antigens (SICA) on the surface of iRBCs and that immune sera from these animals failed to agglutinate iRBCs with mature stages (13). Upon passage of these parasites into monkeys with intact spleens, however, parasites recovered the expression of SICA antigens, and immune monkey sera showed the agglutinating phenotype. In a more recent study, it has been demonstrated that the spleen plays an important role in controlling the transcriptional and posttranscriptional expression of SICAvar antigens (14). Similar observations on expression of variant proteins were also made in monkey models of *P. falciparum* (15) and *Plasmodium fragile* (16) as well as in a rodent *Plasmodium chabaudi* model (17). In *P. falciparum* splenectomized patients, iRBCs present low expression of surface variant proteins and appearance of mature stages in peripheral blood, likely due to an impairment of parasite tissue sequestration (18, 19). In addition, in immune (19) and nonimmune individuals (20, 21), the absence of the spleen results in increased disease severity.

Altogether, these data support a major role of the spleen in modulating the expression of variant virulent determinants in malaria involved in cytoadherence. We thus hypothesized that *P. vivax* coding genes whose expression is dependent on an intact spleen will allow the identification of antigens involved in spleen cytoadherence and pathogenesis; to test this hypothesis, we used a global transcriptional approach in experimental *P. vivax* infections of spleen-intact and splenectomized *Aotus* monkeys to identify genes whose expression is spleen dependent.

**Results**

**Identification of *P. vivax* Coding Genes with Intact Spleen-Dependent Expression.** To identify *P. vivax* genes whose expression is spleen dependent, a series of experimental infections with the *P. vivax*...
Salvador-1 (Sal-1) strain was performed in splenectomized (Sp−) and spleen-intact (Sp+) Aotus monkeys (Fig. L4). After each infection, Giemsa smears were performed daily to determine the time to patency and peak of parasitemias (SI Appendix, Table S1). At peak parasitemias, mature parasites (multinuclei schizonts) were affinity purified (Fig. L4). Although this purification step drastically reduced the total numbers of iRBCs, it was essential to achieve the highest level of comparability within the dataset. Dual hybridizations comparing the global expression of parasites obtained from the different experimental infections (labeled with Cy5) with a reference pool PvSp-1 obtained from splenectomized monkeys from the Centers for Disease Control and Prevention (CDC) donated by John Barnwell, Malaria Branch, Division of Parasitic Diseases and Malaria, CDC, Atlanta, GA (labeled with Cy3) were performed using an Agilent custom-made array containing 5,038 P. vivax coding genes (one 60-base oligonucleotide at every 2 kb) as annotated (10). Use of this reference pool from the CDC was needed due to the limited amounts obtained from our groups of monkeys Sp−1. Of note, sample Sp−4 was not included in these analyses due to insufficient amount of total RNA obtained. All data are freely available through the Gene Expression Omnibus (GEO) database (GEO accession no. GPL6667).

Two different statistical algorithms were used for expression analysis (SI Appendix, Fig. S1). In the first model, probe intensities were compared with negative controls. Average intensity of each probe was compared with the probability density function of the negative controls. If expression probability of negative controls was less than 0.001 compared with the probe, this probe was classified as "on." If it was greater than 0.05, probe was considered "off." In the second model, a 0.98 quantile of negative controls intensity was used as a cutoff. Probes presenting average intensity at least 10 times greater than this cutoff were considered on, and the ones with average intensity below the quantile were classified as off. For each model, selected genes were those presenting the following expression pattern: off in Sp−1, Sp−2, and Sp−3 and on in Sp+2. Using these criteria, a total of 67 spleen-dependent genes were identified, with close to 50% of them located in subtelomeric regions and around 26% in chromosome 14 (SI Appendix, Table S2). Genes could be grouped into those pertaining to multigene families, such as variant surface proteins (VIR) and Pv-fam genes. In addition, a high number of exported and hypothetical proteins and to a lesser extent, some enzymes and binding proteins were also identified (SI Appendix, Table S2).

**VIR14 Protein Mediates Specific Adhesion to Human Spleen Fibroblasts.** We have previously hypothesized that variant VIR proteins of *P. vivax* mediate cytoadherence to spleen barrier cells of fibroblastic origin (22). To test this hypothesis, we selected the PVX_108770 gene, encoding for VIR14, as its expression was predicted to be spleen dependent by two different algorithms (SI Appendix, Table S2). In the absence of long-term *P. vivax* in vitro culture, it had been previously used to generate a *P. falciparum* transgenic line (3D7_vir14-human influenza hemagglutinin [HA]) (23). In this transgenic line, VIR14 was localized at the surface of iRBC and showed cytoadhesion to CHO cells expressing the ICAM-1 receptor. To assess the cytoadhesion properties of this VIR protein to spleen fibroblasts, we first tested a commercial spleen fibroblast cell line, Hs 697.Sp (ATCC). This cell line was obtained from a patient who previously had a granulomatous lymph node and Hodgkin's disease without spleen involvement. Compared with the parental strain 3D7, cytoadherence of the transgenic line 3D7_vir14-3HA was significantly higher under flow and static conditions (Fig. 2 A and C). Moreover, adhesion to Hs 697.Sp was five times higher than the previously reported adhesion to CHO-ICAM-1 cells. To avoid confounding due to the pathogenic origin of cell line Hs 697.Sp, we generated a spleen fibroblast cell line (1010T) derived from a transplantation donor with a healthy spleen by culturing homogenized spleen cells for 3 wk. The transgenic strain 3D7_vir14-3HA also presented significantly higher adhesion to 1010T fibroblasts compared with the 3D7 parental strain (Fig. 2 B and C). To further determine the specificity of adhesion to spleen fibroblasts, static adhesion experiments were done using commercial lung fibroblasts (WI-38). No significant adhesion of the transgenic line 3D7_vir14-3HA was found to the WI-38 lung fibroblasts when compared with the 3D7 parental strain (Fig. 2 B and C).

**A Member of the Pv-FAM-D Multigene Family Has No Direct Role in Cytoadherence.** To determine if members of other multigene families whose expression was also dependent on an intact spleen cytoadherence to spleen fibroblasts, we generated a *P. falciparum* transgenic line (3D7_PvFamD-3HA) expressing a member of the Pv-FAM-D multigene family identified by both algorithms (PVX_101580). Moreover, to avoid confounding, this transgenic line was generated using the same expression vector that the one used to generate the 3D7_vir14-3HA transgenic line (Fig. 3A) (23). Expression was validated by RT-PCR (Fig. 3A) and indirect immunofluorescence (Fig. 3 B and C). Colocalization assays with antibodies raised against conserved intracellular acidic terminal segment of PTEMP1 (anti-ATS) revealed that Pv-FAM-D is located at the iRBCs membrane, partially colocalizing with the cytoplasmic domain of PTEMP1 (Fig. 3B). Lack of a more punctuated pattern as seen in 3D7 with anti-ATS antibodies (24) is likely due to a different fixation procedure, which gives images similar to those of 3D7 smears fixed with methanol (25). Surface expression was further validated in live immunofluorescence assays (Fig. 3C).

Cytoadherence assays showed no significant binding of this transgenic line to human endothelial receptors expressed in CHO cells nor to human Hs 697.Sp or 1010T spleen fibroblasts (Fig. 3 D and E). A similar or even lower adhesion than the 3D7 parental strain was found. Additionally, a role in adhesion to lung fibroblasts was excluded as no adhesion of the 3D7_PvFamD-3HA transgenic line was observed to WI-38 lung fibroblasts (Fig. 3E).

**Naturally Acquired Immune Responses of *P. vivax* Spleen-Dependent Antigens.** To determine if *P. vivax* spleen-dependent antigens are targets of naturally acquired immune responses, a list of genes for complementary DNA (cDNA) amplification and cloning into the pVEXGST1.4d vector was selected for expression as glutathione S-transferase (GST)-tagged proteins using the wheat germ in vitro expression system (SI Appendix, Table S3) (26). Selection criteria were all members pertaining to subtelomeric multigene families (vir and Pv-fam) genes by any of the two algorithms used. In addition, we also selected the rest of the genes predicted by the two algorithms, excepting those annotated as enzymes or RNA binding proteins. Unfortunately, despite several different attempts, most of the genes, including PVX_101580, were either unclonable or expressed as insoluble products. Therefore, only five genes representing three different multigene families VIR14 (PVX_108770), Pv-FAM-A (PVX_112670), and Pv-FAM-D (PVX_112910); one membrane transporter SECY (PVX_000960); and one hypothetical protein HYP1 (PVX_114580) were expressed as soluble products in the wheat germ system (SI Appendix, Fig. S2) (26). We also expressed a GST-tagged MSP1-19 protein as a control of exposure as this is a highly immunogenic protein in natural vivax infections (27). Immune sera from a retrospective study (28), including 383 children from 1.2 to 5.6 y of age from Papua New Guinea (PNG), were used in multiplex assays as described (26). Recurrence of *P. vivax* in this cohort was high with 91.3 and 76.5% of children.
experiencing a PCR- or light microscopy-detectable *P. vivax* by week 6, respectively (28).

At baseline, 26.4% (HYP1) and 75.6% (VIR14) of children had antibodies to the spleen-dependent antigens, whereas 67.1% of children had antibodies to the nonspleen-dependent MSP1-19 antigen (Fig. 4). Antibodies to PvFAM-D2, SECY, and VIR14 were significantly less commonly observed at week 6 than week 0 (*P* < 0.01) but recovered to pretreatment level at week 40 for PvFAM-D2 and Secy. The prevalence of antibodies to MSP1-19, PvFAM-A2, and HYP1 did not change from week 0 to 6 but decreased significantly by week 40 (*P* ≤ 0.03) (Fig. 4). The prevalence of antibodies to VIR14 and HYP1 increased significantly with age (*P* ≤ 0.002), indicating continued natural acquisition of immunity (Fig. 4B), whereas antibodies to PvFAM-D2 and MSP1-19 were significantly more common in children with concurrent *P. vivax* infections (as detected by light microscopy and/or PCR) (Fig. 4C). After adjusting for difference in exposure, children with antibodies against HYP1 (hazard ratio [AHR] = 0.65, confidence interval [CI]95 [0.46, 0.92], *P* = 0.01) showed significant association with protection against clinical
P. vivax episodes during follow-up (SI Appendix, Table S4). Children with antibodies to the spleen-dependent PvFAM-D2, however, tended to be at increased risk of acquiring new P. vivax during follow-up (light microscopy [LM] positive: AHR = 1.23, CI95 [0.99, 1.52], \( P = 0.056 \)), although not reaching statistical significance. No associations with risk of P. vivax infection or disease were observed for antibodies to any of the other antigens.

**Discussion**

Analysis of infections in splenectomized hosts has demonstrated that the spleen plays a major role in modulating expression of malaria variant multigene families involved in cytoadherence. To understand the role of the spleen in the expression of P. vivax genes, we used experimental P. vivax infections in splenectomized and spleen-intact Aotus monkeys and performed global transcriptional analyses of highly pure and synchronous parasite populations. We demonstrated that 67 coding genes largely located at subtelomeric regions are dependent on the spleen for expression and that such antigens are targets of naturally acquired immune responses.

Several reports based on global transcriptional analysis using customized microarray platforms (29–31), and more recently, on RNA sequencing (RNA-seq), have been applied to sequence P. vivax isolates (32–34). These expression data analyses have identified stage-specific and differentially expressed genes coding for proteins with functions related to parasitic development, virulence capacity, and/or host–parasite interaction, among others. We customized an Agilent microarray representing all coding genes of the Salvador I reference strain (10) and identified genes whose expression was dependent on the presence of the spleen in experimental P. vivax infections of spleen-intact and splenectomized Aotus monkeys. From the 67 spleen-dependent genes identified, close to 50% were located at subtelomeric regions and pertained to variant multigene families (VIR proteins, Pv-FAM-D proteins, Pv-FAM-A proteins, and Plasmodium exported proteins) (SI Appendix, Table S2). Genes belonging to these multigene families are among the most expressed genes in isolates obtained from different patients, underscoring the importance of variant antigens in natural infections (29–33). Noticeably, gene PVX_108770 (VIR14) pertaining to the vir multigene family and shown here to mediate specific cytoadherence to human spleen fibroblasts was present in the list of 25 vir genes highly expressed in clinical isolates (30). Our previous results showed that P. vivax VIR proteins present different subcellular localizations revealing that they might play different functions. Additionally, we demonstrated that VIR members of subfamilies A and D do not mediate cytoadherence, whereas VIR14, pertaining to subfamily C, does (23). It is therefore tempting to speculate that spleen-dependent VIR proteins play a role in antigenic variation and cytoadherence in its strict sense, whereas the function of nonspleen-dependent VIR proteins

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**Fig. 4.** Prevalence of positive antibody titers according to (A) week of follow-up, (B) age group cut at median, and (C) detectable parasitemia. A positive antibody titer was defined for MFI above mean +2 SD of negative controls. \( P \) values were adjusted for multiple pairwise comparisons using Tukey’s method.
remains to be elucidated. In the absence of other supporting data, this remains to be determined.

To assess if *P. vivax* spleen-dependent proteins are targets of naturally acquired immune responses, we expressed five genes representing three different multigene families, one membrane transporter, and one hypothetical protein as soluble proteins in the wheat germ cell-free system. This system has proved robust and reproducible to express soluble, mostly intact, and correctly folded malarial proteins (35). Immunogenicity was evaluated measuring total immunoglobulin G (IgG) antibody levels of sera from a prospective longitudinal study of children from PNG (28). All proteins were immunogenic, albeit at different levels (Fig. 4) (SI Appendix). PvFAM-A2 and PvFAM-D2 proteins were recognized by a high percentage of sera. These results are in agreement with members of these families being highly expressed in transcriptional analysis of parasite isolates (32). Furthermore, a recent study showed that these two members of PvFAM proteins, when analyzed as recombinant proteins using sera from the Republic of Korea (36), were also immunogenic. The fact that antibodies to PvFAM-D2 and MSP1-19 were significantly more common in children with concurrent *P. vivax* infections clearly indicates that these two proteins could be associated with an active infection. Recent analysis of naturally acquired antibody responses to *P. vivax* MSP1-19 in an area of unstable malaria transmission in Southeast Asia has shown that antibodies to PvMSP1-19 may serve as a serological marker for unstable malaria transmission in that particular study area (37). PvFAM-D2 pertains to a family sharing a conserved N terminus to C terminus protein structure, starting with a signal peptide, followed by Protein Export Elements (PEXEL) motif, antigenic regions containing B cell epitopes, and two transmembrane domains. This topology is only disrupted in two members (PVX_000015 and PVX_118695) where predicted antigenic regions are also observed between the signal peptide and the PEXEL motif (SI Appendix, Fig. S3). Further epidemiological studies should determine the value of the Pv-FAM-D family as markers of exposure. On the other hand, low antibody levels were observed against SECY and HYP1. Noticeably, despite pertaining to the variant vir gene family with more than a thousand genes described to date, PVX_108770 (VIR14) presented the highest positivity of all antigens tested, including the high number of predicted conserved globular domains of unknown function (9, 23, 38). Whether such domains elicit cross-reacting antibodies responsible for this high percentage of positivity remains to be demonstrated.

The number of genetically distinct blood-stage infections acquired over time, also known as the molecular force of blood-stage infection (molFOB), is a direct way to measure individual differences in exposure to *P. vivax* infections (39). This parameter has also been shown to be a major predictor of clinical disease in *P. falciparum* (40) and *vivax* malaria (39). Remarkably, when adjusting for difference in exposure, children with antibodies against HYP1 showed significant association with protection against clinical *P. vivax* episodes during follow-up (SI Appendix, Table S4). HYP1 is 100% conserved among *P. vivax* isolates from Mauritania, North Korea, India, and Brazil and 90% among other species, including *Plasmodium knowlesi*, *Plasmodium cynomolgi*, *Plasmodium coatneyi*, and *Plasmodium knowlesi* (SI Appendix, Fig. S4). These results highlight its value as a target for vaccination against asexual blood stages of *P. vivax* and reinforce the importance of using molFOB in the search for association of clinical protection of vaccine candidates in *P. vivax* in prospective longitudinal studies.

*P. vivax* remains the most widely distributed human malaria parasite. Due to specific biological features, including dormancy in the liver and a tropism for reticulocytes, its elimination requires an improved understanding of its biology and pathogenesis (41). One particular open question regards the conundrum of low peripheral blood parasitemia coincident with severe disease. Recent evidence indicates that this seeming discrepancy could be due to the adherent capacity and sequestration of iRBC parasite populations, outside the peripheral blood, and particularly in the spleen (6, 42–46), the bone marrow (47, 48), and with a less degree of certainty, to other organs (3, 6). Our data thus support a model (Fig. 5) in which infected reticulocytes expressing spleen-dependent VIR proteins, represented here by the VIR14 protein, can adhere to the microvasculature of the spleen, in particular to fibrocytic cells expressing ICAM-1. In contrast, as many VIR proteins are not spleen dependent, it is legitimate to speculate that infected reticulocytes expressing such VIR proteins will not cytadhere. Therefore, they will circulate in peripheral blood, eliciting the acquisition of cross-reacting VIR antibodies due to the presence of conserved immunogenic globular domains (10, 23, 38) as well as of antibodies against other variant proteins such as PvFAM-D coexpressed in these infected reticulocytes. Further studies of spleen-dependent *P. vivax* genes could thus help in unveiling mechanistic insights of spleen cytoadherence in *P. vivax* as well as discovering new antigens for vaccination and markers of exposure. Most relevant, together with hypnozoite and other tissue reservoirs such as the bone marrow, these populations may represent an added challenge for malaria elimination, particularly since cytoadherence to the microvasculature of the spleen will represent a privileged niche where parasites can escape (46), thus prompting a paradigm shift in *P. vivax* biology toward deeper studies of the spleen during infections.

**Materials and Methods**

**Ethics Statement.** The serological analyses of plasma samples from cohort participants received ethical clearance by the PNG Institute of Medical Research Institutional Review Board (IRB; IRB 07.20) and the PNG Medical Advisory Committee (07.34). Individual written consent was obtained from the parents or guardians of all children. The consent included a specific approval to investigate their children’s immune responses to different malarial antigens and their association with protection.

The Animal Ethical Committee of Universidad del Valle approved the protocol involving *Aotus lemurinus griseimba* monkeys from the Fundación Centro de Primates in Cali, Colombia. Animals were handled and housed following the National Research Council’s Guide for the Care and Use of Laboratory Animals (50).

**Infections of Aotus Monkeys.** Five groups of *A. lemurinus griseimba* monkeys (*n* = 2 per group), four splenectomized (Sp1–, Sp2–, Sp3–, and Sp4–) and one with spleen (Sp+2), were used. All groups were handled according with animal welfare guidelines and maintained with standard requirements of water and food supplies. Animals were splenectomized by a veterinarian according to standard operational procedures under general anesthesia 4 wk prior to infections.

Spleen-intact (Sp+) and splenectomized (Sp–) animals were infected by intravenous (iv) inoculation of 10⁷ iRBCs (500-µL volume) obtained from a donor monkey previously infected with the *P. vivax* Sal-1 reference strain (10). First recipient group was splenectomized monkeys (Sp–1 group); thereafter, parasites were passaged by iv inoculations through groups Sp2–, Sp3–, Sp4–, and Sp+2. Parasite passages were performed at peak parasitemia (∼3 to 4 wk postinoculation); simultaneously, 3 to 4 mL of peripheral blood was collected via femoral vein in heparinized sterile tubes from each animal, and 1-mL aliquots were processed for RNA extractions.

**Sample Processing for RNA Isolation and Gene Expression Array.** For RNA isolation, blood was first centrifuged at 600 × g for 5 min, and packed RBCs were resuspended at 25% hematocrit in incomplete Roswell Park Memorial Institute medium (RPMI). Mature *P. vivax* parasites were purified using magnetic MACS LS columns (Miltenyi) as described (51) and preserved into 1 mL of TRIzol for later processing. RNA extraction was performed following the manufacturer’s instructions, and 50 ng of each sample was reversely transcribed into cDNA, being subsequently amplified and labeled with Cy5 and Cy3 dye following Agilent’s Two-Color Microarray-Based Gene
Expression Analysis protocol version 6.5 (Agilent Technologies). Spike-in RNA (Agilent Technologies) was used as internal control. Slides were scanned with Agilent's G2565CA Microarray Scanner System. Dye normalized, background subtracted, and log ratios of sample to reference expression were calculated using Agilent’s Feature Extraction Software version 9.5.

Fig. 5. Spleen cytoadherence mediated by spleen-dependent VIR proteins. Infections by reticulocyte (Ret)-prone malaria parasites induce spleen remodeling and formation of barrier cells (BCs) of fibroblastic origin where infected reticulocytes (iRets) avoid macrophage (MO) clearance (background scheme) (49). P. vivax iRets expressing spleen-dependent VIR proteins, here represented by VIR14 (PVX_108770), adhere to human spleen fibroblasts expressing ICAM-1 (Right Inset). iRets expressing VIR proteins whose expression is not spleen-dependent will not cytoadhere; thus, reaching peripheral circulation facilitating antigen presentation of conserved immunogenic VIR globular domains (10, 23, 38) as well as other variant antigens such as PvFAM-D (PVX_101580; Left Inset). Partially created by BioRender. RP, red pulp; V, venule.
P. falciparum

of purified plasmid DNA (Qiagen) as previously described (23) using 0.310-kV
both a Neubauer chamber and a Giemsa-stained smear. For adhesion ex-
enriched using a 70% Percoll solution, and parasites were quantified using
stages of cultured
were seeded 5 d before performing the experiment. Mature asexual blood
was run in triplicate. Cells and iRBCs were incubated for 1 h at 37 °C
through the perfusion chamber; then, 1 × 10^7 iRBCs were flowed over for a
total of 30 min, and binding buffer was flowed over for 10 min to remove
the flow rate yielded a wall shear stress of 0.09 Pa, which mimics wall shear stresses in the microvasculature. Static and flow coverslips were
fixated in methanol after the washing process and stained with 10%
Giemsa for 15 min. Adhesions were quantified in an optical light microscope.
Binding experiments were done in triplicate in 3 to 5 independent days.
Statistical analysis was done on GraphPad Prism (version 4), and significance
was determined by an unpaired t test.

P. falciparum Culture, Plasmid Constructs, and Parasite Transfection. P. falciparum parasites were cultured with B+ human erythrocytes (3% hematocrit) in RPMI media (Sigma) supplemented with 10% AB+ human plasma using standard methods (23). Pv-fam-d gene (PXV_101580) was amplified from P. vivax Sal1 genomic DNA (gDNA) using primers F-PvFamD: GGTACCATGAAATAAAACAAAG; R-PvFamD: ttgcatgtctggtcctcttcttttgtt and was cloned in the KpnI-PstI cloning sites of modified transfection vector pARL1a-
HA (23). The plasmid was transformed into E. coli (model KDS120; KD Scientific) to control the flow of the iRBC suspension
was fixed in methanol after the washing process and stained with 10%
Mouse antisera to GST were measured in samples collected at baseline
(rat anti-HA [1:50; Roche] and mouse anti-ATS
cloning, extreme outliers were flagged using Grubb's test recursively (55).

Antibody Measurement by Luminescent Technology. For antibody measurement, 1 μg of each recombinant protein was covalently coated to different Mag-
plex magnetic carboxylated microspheres (Luminescent Corporation) following the manufacturer's instructions. Measurement of total IgG antibodies was
performed by multiplex suspension array using the Luminescent technology as
previously described (54). Briefly, a batch of microspheres, containing 2,000
beads per analyte, was incubated with human plasma samples (1:100 di-
lution) in duplicates and subsequently, with anti-human IgG biotinylated
(Sigma-Aldrich) at 1:4,000 dilution followed by streptavidin-conjugated
R-phycerothrin (R-PE) (1 μg/mL). Beads were acquired on the BioPlex100
system (Bio-Rad), and results are expressed as median fluorescence intensity.
A panel of eight negative controls was included on every plate.
Antibodies to all proteins were measured in a cohort of PNG children aged
1 to 5 years enrolled in a longitudinal cohort study who were randomized to
pretreatment with artesunate (7 d), artesunate (7 d) plus primaquine (14 d),
or no treatment and followed up actively for recurrent Plasmodium infec-
tions and disease for 40 wk. A detailed description of the cohort is given
elsewhere (28). Antibodies were measured in samples collected at baseline
(n = 435), 6 wk after treatment (n = 408), and at the end of follow-up (n = 419). Antibody mean fluorescent intensities (MFIs) were log transformed,
and background values. Tempo of all trends in prevalence of anti-tag
substraction as described (54). Cutoffs for antibody positivity were determined
for each plate separately by calculating mean +2 SD of the negative control values.

Statistical Analysis. Clinical malaria was defined as fever (axillary tempera-
ture ≥37.5 °C) or recent history of febrile illness and presence of a con-
comitant Plasmodium sp. parasitemia. Time at risk was counted from the
first day after the last treatment dose was administered and up until
w/low, loss to follow-up, or study completion. Comparison between the time
before and during Plasmodium sp. infection (or clinical episode) and treatment
options were already investigated in a previous study (28) and relied on Cox
proportional hazard regressions using Schoenfeld residuals test to confirm the
proportional hazard assumption. In this study, we also included recoded
antibody titers as explanatory independent covariates in these regressions.

Recovering of Antibody Titers. To account for plate-to-plate variations and to
reflect on antibody level, MFIs were adjusted, platewise, under the as-
sumption that log(Ab_tag) = log(Ab) + log(tag). A linear model was fitted to
each plate and antigen to derive the adjusted MFI value: the minimal tag
value that could be detected was hence considered as background noise for
that experiment.
Adjusted MFI values from negative controls were used to define a standard
reference curve, reflecting the expected values of cross-reactivity that could
be expected in individuals who were never exposed to Plasmodium para-
sites. Assuming log-normal distributions of MFI values in this control pop-
ulation, extreme outliers were flagged using Grubb’s test recursively (55).
Positivity thresholds were defined plate- and antigenwise as 2 SDs above
the mean MFI in negative controls. Patients’ adjusted MFI values were
compared against these thresholds and recoded to binomial outcomes
(positive/negative). As a sensitivity analysis, a more conservative set of
thresholds was computed using 3 SDs. Logistic regression was used to quantify the associations between baseline
positivity and age categories (two groups cut at a median of 3 y old), Plas-
modium sp. parasitemia, and clinical episodes, while adjusting for the
averaged individual molecular force of infection as a proxy for individual
reinfection risk. Time at risk was defined as the period between the first
appearance of Plasmodium parasites and the nadir of antibodies. As a
negative control group, we included individuals with no history of
Plasmodium infection.

ACKNOWLEDGMENTS. We thank John Barnwell (Malaria Branch, Division of
Parasitic Diseases and Malaria, CDC) for the gift of the reference RNA pool
from Plasmodium yoelii obtained from spleenectomized monkeys from the CDC, Artur Scherf
(Biology of Host-Parasite Interactions, Institut Pasteur) for the gift of CHO-
cells expressing different receptors and Marc Nicolau for technical assistance.
We also thank the anonymous reviewers of this manuscript whose criticisms and suggestions significantly improved its content and quality. This work was supported with funding from the Cellex Foundation. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The Barcelona Institute for Global Health (ISGlobal) receives support from the Spanish Ministry of Science, Innovation and Universities through the “Centro de Excelencia Severo Ochoa 2019-2023” Program (CEX2018-000086-S). This research is part of ISGlobal’s Program on the Molecular Mechanisms of Malaria, which is partially supported by the Fundación Ramón Areces. ISGlobal and Germanias Trias i Pujol Research Institute are members of the Centers of Reserca de Catalunya Program, Generalitat de Catalunya.
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Fernandez-Becerra, C; Bernabeu, M; Castellanos, A; Correa, BR; Obadia, T; Ramirez, M; Rui, E; Hentzschel, F; Lopez-Montanes, M; Ayllon-Hermida, A; Martin-Jaular, L; Elizalde-Torrent, A; Siba, P; Vencio, RZ; Arevalo-Herrera, M; Herrera, S; Alonso, PL; Mueller, I; del Portillo, MHA

Title:
Plasmodium vivax spleen-dependent genes encode antigens associated with cytoadhesion and clinical protection

Date:
2020-06-09

Citation:

Persistent Link:
http://hdl.handle.net/11343/244749

File Description:
published version

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