



OPEN Characterization of T and B cell epitopes in PvCyRPA by studying the naturally acquired immune response in Brazilian Amazon communities

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Plasmodium vivax, a challenging species to eliminate, causes millions of malaria cases globally annually. Developing an effective vaccine is crucial in the fight against *vivax* malaria, but considering the limited number of studies focusing on the identification and development of *P. vivax*-specific vaccine candidates, exploring new antigens is an urgent need. The merozoite protein CyRPA is essential for *P. falciparum* growth and erythrocyte invasion and corresponds to a promising candidate antigen. In *P. vivax*, a single study with multiple vaccine candidates indicates PvCyRPA with strong association with protection, outperforming classic malaria vaccine candidates. However, little is known about the specific naturally acquired response in the Americas, as well as the antigen epitope mapping. For this reason, we aimed to investigate the cellular and humoral immune response elicited against PvCyRPA in Brazilian endemic areas to identify the existence of immunodominant regions and the potential of this protein as a single or even a multi-stage specific malaria vaccine candidate for *P. vivax*. The results demonstrated that PvCyRPA is naturally immunogenic in Brazilian Amazon individuals previously exposed to malaria, which presented anti-PvCyRPA cytophilic antibodies. Moreover, our data show that the protein also possesses important immunogenic regions with an overlap of B and T cell epitopes. These data reinforce the possibility of including PvCyRPA in vaccine formulations for *P. vivax*.

Malaria caused by the parasite *Plasmodium vivax* is responsible for around 5 million cases annually with huge burden infections around the globe, especially in Southeast Asia and South America^{1,2}. In Brazil, this species accounted for more than 84% of the 131,224 malaria cases registered in 2022³. Considering this scenario, the development of efficient and low-cost tools is essential to contribute to *P. vivax* malaria elimination, especially because this species possesses important transmission skills, such as the development of early gametocytes in the cycle even before the appearance of symptoms in the human host and its ability to persist as dormant stage parasites for years causing relapses. Together, these characteristics favor *P. vivax* transmission in comparison to other *Plasmodium* species, even the deadly *P. falciparum*. Thus, a vaccine could be a key tool in *P. vivax* malaria control^{1,4,5}.

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Due to the difficulty and urgent need to control malaria's impact on global health, investigators have been widely exploring *Plasmodium* antigens to compose a vaccine, however, the majority of these studies focus on *P. falciparum*, known as the parasite responsible for the majority of world malaria deaths. This strengthens the need of identifying new proteins able to integrate an effective vaccine against *P. vivax* malaria⁶. In this scenario, the Cysteine-rich protective antigen, known as CyRPA has emerged as a promising blood-stage vaccine candidate. This protein was previously described in *P. falciparum* and is localized in the micronemes of merozoites, where it forms a complex with other two proteins: the Reticulocyte binding-like homologous protein 5 (RH5), a protein already known to be a malaria leading vaccine candidate and the RH5-interacting protein (Ripr)⁷. Together, these proteins represent an essential complex for erythrocyte invasion in *P. falciparum*, once their interaction with the host cell receptor basigin triggers Ca^{2+} release and establishes a tight junction that allows cell invasion. It has been already demonstrated that merozoites deficient in PfCyRPA still can bind red blood cells, however, they do not attach irreversibly, making invasion impossible for the parasite⁸. Besides, different studies also demonstrated that specific antibodies elicited against PfCyRPA were capable of inhibiting parasite growth by blockage of erythrocyte invasion both in vitro and in vivo^{7,9,10}.

The protein RH5 is only found in species closely related to *P. falciparum* in *Laverania* subgenus, thus, in *P. vivax*, this tertiary complex does not exist and basigin is not required as a host cell receptor. For *P. knowlesi*, a species closely related to *P. vivax*, it was also demonstrated that both Ripr and CyRPA have independent functions but are still essential for parasite survival in the asexual life cycle¹¹. Regarding the immune role of CyRPA in *P. vivax* infection, there is only one published study, conducted in a cohort of Papua New Guinean children, which tested their seroreactivity against a library of 38 *P. vivax* antigens, including PvCyRPA, and found that its presence in different antigen combinations was strongly associated with protection requiring very low antibody levels, outperforming classical malaria vaccine candidates such as circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1), merozoite surface protein 1 (MSP-1), and reticulocyte-binding protein (RBP)^{12–15}. However, the immune response against PvCyRPA remains largely unexplored within the Americas. Moreover, comprehensive investigations about the T and B cell linear epitope mapping of PvCyRPA have yet to be conducted. The absence of such studies underscores the necessity for examination of the immune dynamics associated with PvCyRPA in the Brazilian Amazon, particularly in elucidating the specificities of T and B cell responses at the epitopic level.

In this context, based on all of the promising characteristics of CyRPA protein, our study aimed to investigate the naturally acquired cellular and humoral immune response against PvCyRPA in Brazilian endemic areas, investigating the profile of antibodies against recombinant PvCyRPA and the existence of immunodominant regions containing either B or T cell epitopes to reinforce its potential as a single vaccine candidate or even a possible candidate in a multi-target vaccine composition.

Results

Clinical and epidemiological profile of the population naturally exposed to malaria

The studied population consisted of 90 individuals that were divided into three study groups: 30 individuals infected with *P. vivax*, 30 cohabitants that have reported previous malaria episodes and live in the same house/region of infected individuals and 30 exposed individuals that live in malaria endemic area, who have not reported malaria in the last 10 years. There were 40 men (44.4%) and 50 women (55.6%), with medians of 27 years of age (interquartile range – IQ 19–38) and of 27,5 years living in endemic areas (IQ 19–38). The population has a median of 3 previous episodes of malaria (IQ 0–9) and from the 60 individuals that have reported previous history of the disease, 51 (85.0%) were infected by *P. vivax*, as shown in Table 1. All the collected epidemiological data was submitted to Kolmogorov-Smirnov test, once the result of the test indicated that the Gaussian distribution was not normal, only nonparametric statistical tests were performed with these data.

Reactivity of IgM and IgG antibodies of individuals naturally exposed to malaria against recombinant PvCyRPA

Of the 90 individual plasma samples tested for recombinant PvCyRPA, observed frequencies were: 20 individuals responding for IgM antibodies (22.2%) and 51 individuals responding for IgG antibodies (56,7%) ($p < 0.0001$). Of these individuals, 60 presented IgM and/or IgG (66,7%) ($p < 0.0001$ considering the frequency of IgM responders) as demonstrated in Fig. 1a. A higher frequency of IgG responders and higher RI's were observed in comparison to IgM responders ($p < 0.0001$) (IgM – RI with a median of 0.591, ranging from 0.249 to 4.660; IgG – RI with a median of 1.159, ranging from 0.247 to 5.490) (Fig. 1b). Epidemiological data provided by study participants was compared between responders and non-responders to IgM antibodies against recombinant PvCyRPA. The time elapsed since the last malaria episode was shorter in responders (median 0, IQ 0–0) than in the group of non-responders (median 9, IQ 0–24.5) ($p = 0.0298$). No statistical differences were observed in other epidemiological parameters between these groups or between IgG responders and non-responders to PvCyRPA.

The frequency and reactivity of IgM and IgG antibodies were also accessed comparing individuals by group (*P. vivax*; cohabitants, and exposed). A higher frequency of responders to IgM antibodies was identified in the *P. vivax* group, where 15 individuals (50.0%) responded to PvCyRPA compared to only two (6.67%) in the cohabitants group ($p = 0.0004$) and three (10%) in the exposed group ($p = 0.0015$) (Fig. 1c). For IgG anti-PvCyRPA response, frequencies remained similar, with 19 responders (63.3%) in *P. vivax*, 17 (56.7%) in cohabitants, and 15 (50.0%) in the exposed group (Fig. 1e). The reactivity index (RI's) of IgM antibodies was compared among groups and it was found that IgM RI's in the *P. vivax* group were higher than those found in both other groups ($p = 0.0001$ for cohabitants and $p = 0.0010$ for exposed) (Fig. 1d). No differences were observed for IgG antibodies RI's (Fig. 1f).

Epidemiological data	(N = 90)
Gender	N (%)
Male	40 (44.4%)
Female	50 (55.6%)
Exposure factors	Median (IR)
A.PI	61.4
Age (years)	27 (19–38)
Time of residence in endemic area (years)	27.5 (19–38)
Time of residence in the present address (years)	19 (12–31)
Months since the last malaria episode	0 (0–18.75)
Number of malaria episodes in the last year	0 (0–1)
Number of previous malaria episodes	3 (0–9)
Previously infecting species	N (%)
<i>P. vivax</i>	14 (15.6%)
<i>P. falciparum</i>	4 (4.4%)
<i>P. vivax</i> and <i>P. falciparum</i>	37 (41.1%)
No infections in the last 10 years	30 (33.3%)
Not reported	5 (5.6%)
Diagnosis (Thick smear and PCR)	N (%)
<i>P. vivax</i>	30 (33.3%)
<i>P. falciparum</i>	0 (0%)
Mixed	0 (0%)
Negative	60 (66.7%)

Table 1. Epidemiological data of malaria-exposed population.

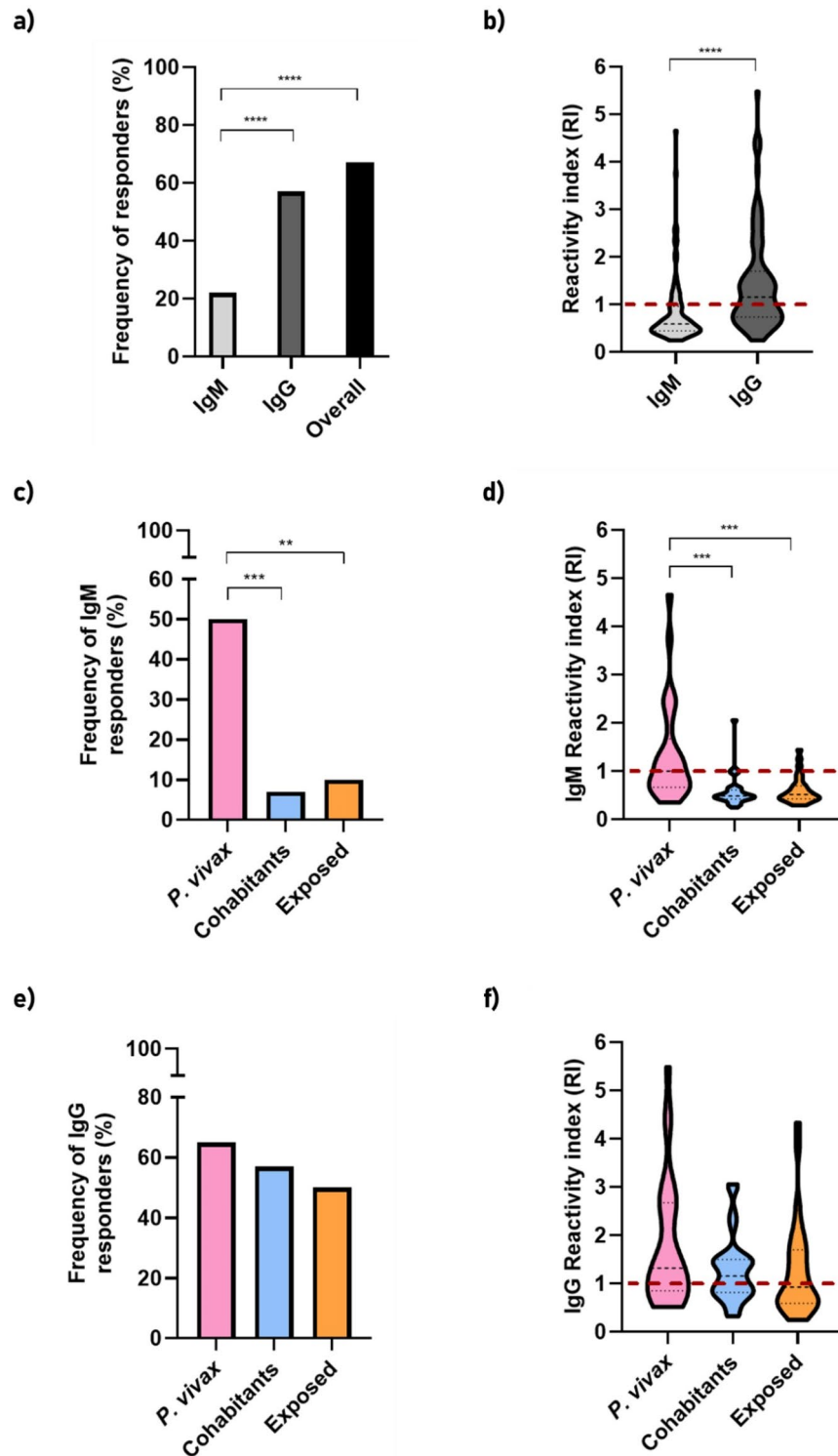
Frequency and magnitude of IgG subclasses (IgG1, IgG2, IgG3 and IgG4) specific for PvCyRPA

Among individuals that presented IgG antibodies anti-PvCyRPA (56.7%), the profile of IgG subclasses induced was investigated. The frequency of IgG1, IgG2, IgG3 and IgG4 responders were 70.6% ($n=36$) for IgG1, 56.9% ($n=29$) for IgG2, 15.7% ($n=8$) for IgG3, and 1.96% ($N=1$) for IgG4, respectively. There were no statistical differences between the frequencies of IgG1 and IgG2, however, both of them were higher than those of IgG3 ($p < 0.0001$) and IgG4 ($p < 0.0001$). The frequency of IgG3 responders was also higher than that of IgG4 responders ($p = 0.031$). Individuals presenting IgG1 antibodies had a median of RI of 1.418 (ranging from 0.512 to 13.157), while those with IgG2 antibodies had a median RI of 1.104 (ranging from 0.367 to 18.542), those who with IgG3 antibodies a median of 0.473 (ranging from 0.265 to 10.430) and IgG4 a median of 0.447 (ranging from 0.276 to 7.418). The IgG1 and IgG2 reactivity was superior to that observed with IgG3 ($p < 0.0001$) and IgG4 antibodies ($p < 0.0001$) (Fig. 2a). Of the 51 IgG responders, 4 individuals did not present any of the investigated subclasses. Considering RI's of IgG subclasses against PvCyRPA in each specific group, similar profiles were observed, with a general predominance of IgG1 and IgG2 RI's in comparison to the other 2 subclasses (Fig. 2b–d).

Evaluation of predicted B cell linear epitopes immunogenicity

Here, we evaluated the recognition of seven B-cell linear epitopes previously predicted in the PvCyRPA full-length¹⁶ (Table 2). The predicted epitopes were composed of 9 to 19 amino acids, which were predicted by at least three different algorithms, and named according to the position of the first and the last amino acid on the sequence as B-PvCyRPA_(81–91), B-PvCyRPA_(119–129), B-PvCyRPA_(134–151), B-PvCyRPA_(181–192), B-PvCyRPA_(241–257), B-PvCyRPA_(257–272) and B-PvCyRPA_(312–330). Among these predicted epitopes, PvCyRPA_(134–151) was the unique sequence predicted by all five algorithms. As shown in Fig. 3, all predicted epitopes were exposed in the PvCyRPA 3D structure.

Varying frequencies of responders to synthetic biotinylated peptides were observed among the 51 individuals with IgG antibodies against recombinant PvCyRPA. ELISA assays indicated that the most frequently recognized epitope was B-PvCyRPA_(312–330), detected in 20 individuals (39.2%), followed by B-PvCyRPA_(81–91) in 15 individuals (29.4%) and B-PvCyRPA_(181–192) in 13 individuals (25.5%). Lower frequencies were observed for B-PvCyRPA_(119–129) and B-PvCyRPA_(257–272), each recognized by 9 individuals (17.6%), B-PvCyRPA_(241–249) by 8 individuals (15.7%) and B-PvCyRPA_(134–151) by 5 individuals (9.8%). The epitope B-PvCyRPA_(81–91) presented a higher frequency of responders when compared to epitope B-PvCyRPA_(134–151) ($p = 0.0232$). Additionally, epitope B-PvCyRPA_(312–330) had a higher frequency of responders in comparison to all epitopes except B-PvCyRPA_(81–91) and B-PvCyRPA_(181–192) with p -values of 0.027 (B-PvCyRPA(119–129)), 0.001 (B-PvCyRPA(134–151)), 0.014 (B-PvCyRPA(241–249)) and 0.027 (B-PvCyRPA(257–272)), as demonstrated in Fig. 4a. Regarding the RI's against all the epitopes, B-PvCyRPA_(81–91) and B-PvCyRPA_(312–330) (frequencies of 29.4% and 39.2% respectively) presented higher RI's in comparison to epitopes B-PvCyRPA_(119–129) ($p = 0.0084$ and $p = 0.0248$), B-PvCyRPA_(134–151) ($p < 0.0001$ and $p = 0.0001$) and B-PvCyRPA_(257–272) ($p = 0.0004$ and $p = 0.0015$). Besides, epitope B-PvCyRPA_(181–192) presented higher RI's when compared to epitope B3 ($p = 0.0115$) as demonstrated in Fig. 4b.



From all responders against the recombinant full-length PvCyrpa, 25 were not classified as responders for any of the 7 tested B cell epitopes. Within 26 responders to at least one of the tested peptides, 11 individuals (42.3%) were capable of recognizing at least 4 of the 7 tested peptides as demonstrated in the heat map (Fig. 5). Interestingly, all these 11 individuals presented IgG antibodies against epitopes B1 and B7, sequences with the best performance when it comes to higher frequencies and higher RIs. Of these 11 individuals, only 2 were capable of recognizing all of the investigated epitopes. 15 individuals (57.7%) were capable of recognizing at least 1 epitope but less than 4 epitopes. Among them, nine individuals presented IgG antibodies against epitope B7. Epidemiological parameters were evaluated among these 2 groups, however, no statistical differences were observed.

◀ **Fig. 1.** (a) Frequency of IgM and IgG antibodies responders. Bars indicate the percentage of IgM responders (light gray bar), IgG responders (dark gray bar), and both combined (black bar). **** = $p < 0.0001$. (b) Reactivity of IgM and IgG antibodies. RI's of the entire population for both IgM and IgG are demonstrated in this graph. The red dotted line separates responders from non-responders, values of RI's above 1 are considered IgM (light gray) and/or IgG (dark gray) responders. ($p < 0.0001 = ****$). (c) Frequency of IgM responders per group. Bars indicate the percentage of IgM responders in each group: *P. vivax* (pink bar), cohabitants (blue bar) and exposed (orange bar). P values are 0.0015 for ** and 0.0004 for ***. (d) Reactivity of IgM antibodies per group. RI's of each group for IgM antibodies against PvCyRPA is demonstrated in this graph. *P. vivax* group is represented with a pink bottle, the cohabitants group with a blue bottle, and the exposed one with an orange bottle. P values are 0.0010 for *** - *P. vivax* x exposed and 0.0001 for *** - *P. vivax* x cohabitants. (e) Frequency of IgG responders per group. Bars indicate the percentage of IgG responders to in each group: *P. vivax* (pink bar), cohabitants (blue bar) and exposed (orange bar). (f) Reactivity of IgG antibodies per group. RI's of each group for IgG antibodies against PvCyRPA is demonstrated in this graph. *P. vivax* group is represented with a pink bottle, the cohabitants group with a blue bottle, and the exposed one with an orange bottle. The red dotted line separates responders from non-responders, values of RI's above 1 are considered responders. RI's analyzes were done by Kruskal-Wallis test and Dunn's multiple comparison test, while frequencies were compared through Fisher's exact test.

Prediction and testing of potential T cell epitopes present in PvCyRPA

Using the IEDB-MHC-II binding predictions, 10 sequences were identified as potential T-CD4 epitopes in PvCyRPA. Regarding promiscuity, the ability of a peptide to be bound by different HLA alleles, the ten predicted epitopes were evaluated as promiscuous, being recognized by 63–78% of the most usual HLA alleles, presenting percentile rank ranging from 0.29 to 18.77 (Table 3).

To evaluate the immunogenicity of these T-CD4 epitopes, the predicted sequences were synthesized as peptides and combined in 6 different pools containing 4 epitopes each (Pool 1: T-PvCyRPA_(49–63), T-PvCyRPA_(54–68), T-PvCyRPA_(102–116), T-PvCyRPA_(167–181); Pool 2: T-PvCyRPA_(102–116), T-PvCyRPA_(167–181), T-PvCyRPA_(216–230), T-PvCyRPA_(221–235); Pool 3: T-PvCyRPA_(216–230), T-PvCyRPA_(221–235), T-PvCyRPA_(232–246), T-PvCyRPA_(287–301); Pool 4: T-PvCyRPA_(232–246), T-PvCyRPA_(287–301), T-PvCyRPA_(306–320), T-PvCyRPA_(340–354); Pool 5: T-PvCyRPA_(49–63), T-PvCyRPA_(102–116), T-PvCyRPA_(216–230), T-PvCyRPA_(232–246); and Megapool: composed by all the 10 predicted epitopes). These pools were used to perform ELISPOT assays using cells from the 90 studied individuals. Our data showed that the frequencies of individuals who produced IFN- γ against each pool did not present statistical differences, ranging from 21 to 30%, where 19 individuals (21.1%) produced IFN- γ against pool 1, 24 (26.7%) against pool 2, 20 (22.2%) against pool 3, 27 (30.0%) pool 4, 24 (26.7%) against pool 5, and 22 (24.4%) against megapool. Medians of spot forming units (SFU) per 250.000 cells were 13 SFU for pool 1 (IQR 5.0–30.5), 20.75 SFU for pool 2 (IQR 7.0–55.0), 18 SFU for pool 3 (IQR 8.0–43.63), 24.0 SFU for pool 4 (IQR 9.5–50.5), 20.5 SFU for pool 5 (IQR 14.0–44.0) and finally 14.75 SFU for megapool (IQR 6.75–38.0). No statistical differences were observed in frequencies or numbers of SFU among these groups (Fig. 6a).

Next, results of ELISPOT assays were analysed per group (*P. vivax*, cohabitants, and exposed). In the *P. vivax* group medians of IFN- γ SFU were 12.0 for pool 1 (IQR 6.0–22.75), 16.0 for pool 2 (IQR 6.75–39.13), 8.0 for pool 3 (IQR 5.5–18.5), 9.0 for pool 4 (IQR 6.13–29.5), 14.0 for pool 5 (IQR 4.5–22.0) and 14.25 for megapool (IQR 3.63–30.0). In the cohabitants group median of 9.0 for pool 1 (IQR 4.0–28.0), 66.25 for pool 2 (IQR 16.38–91.75), 22.0 for pool 3 (IQR 9.75–48.25), 23.5 for pool 4 (IQR 12.5–41.0), 23.0 for pool 5 (IQR 15.5–53.38) and 14.0 for megapool (IQR 11.0–60.5). In the exposed group medians were 21.75 for pool 1 (IQR 5.5–54.0), 16.25 for pool 2 (IQR 6.38–49.38), 20.5 for pool 3 (IQR 10.5–57.25), 43.0 for pool 4 (IQR 16.0–89.5), 29.75 for pool 5 (IQR 14.38–49.63) and 21.5 for megapool (IQR 8.88–38.38) (Fig. 6 from letter a to letter f). Comparing numbers of IFN- γ SFU among these 3 groups, it was observed that infected individuals presented lower numbers in comparison to exposed individuals for pools 4 and 5 ($p = 0.0072$ and $p = 0.0440$, respectively) as demonstrated in Fig. 6e and f.

Relationship between observed humoral and cellular immune responses and exposure/protection indicatives

Using results obtained from the entire population, the Spearman test was performed to identify correlations between the humoral and cellular immune responses observed for both protein/epitopes and specific epidemiological parameters indicatives of exposure/protection. A correlation between the number of previous malaria episodes was observed with IgG antibodies against epitopes B-PvCyRPA_(81–91), B-PvCyRPA_(241–249), and B-PvCyRPA_(312–330) ($p = 0.036/ r = 0.297$; $p = 0.006/ r = 0.386$; $p = 0.003/ r = 0.417$). No statistical p values were identified comparing all other data. Besides, we have identified several correlations between RI's of IgM, IgG, and its subclasses with different predicted epitopes. First, we have identified that RI's of IgM were correlated with RI's of B cell epitopes B-PvCyRPA_(81–91), B-PvCyRPA_(181–192), B-PvCyRPA_(241–249), B-PvCyRPA_(257–272) and B-PvCyRPA_(312–330) ($p = 0.001/ r = 0.448$; $p = 0.011/ r = 0.355$; $p = 0.013/ r = 0.344$; $p = 0.001/ r = 0.435$; $p = 0.037/ r = 0.293$). A correlation of RI's of IgG1 and IgG3 against recombinant PvCyRPA ($p = 0.002/ r = 0.431$) was also observed. IgG1 RI's presented a correlation with RI's of IgG antibodies against epitope B7 ($p = 0.004/ r = 0.400$) and IgG3 RI's presented a correlation with RI's of IgG antibodies against epitopes B-PvCyRPA_(81–91), B-PvCyRPA_(181–192) and B-PvCyRPA_(241–249) ($p = 0.019/ r = 0.326$; $p = 0.013/ r = 0.344$; $p = 0.019/ r = 0.329$). On the other hand, data of IFN- γ SFU only correlated inversely between megapool and IgG against epitope B-PvCyRPA_(119–129) ($p = 0.034/ r = -0.298$). A correlation matrix was designed to illustrate and compare all these simultaneous associations (Fig. 7).

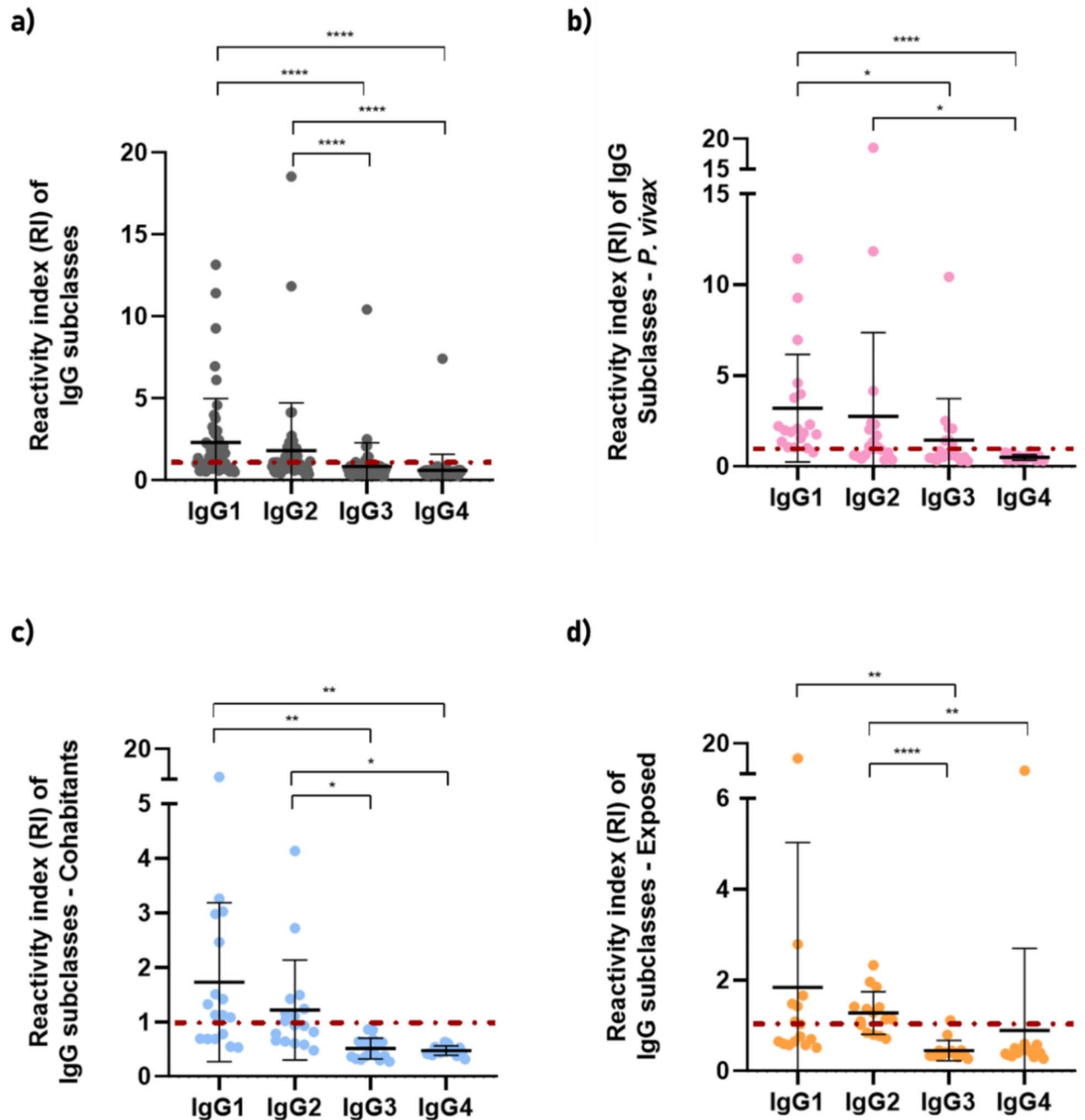


Fig. 2. Reactivity of IgG subclasses. RI's of 90 malaria-exposed individuals for IgG subclasses IgG1, IgG2, IgG3, and IgG4 are shown in this graph. Each dot represents the RI of a patient. The red dotted line separates responders from non-responders, values of RI's above 1 are considered responders to each of the subclasses. $P < 0.0001 = ****$. RI's analyzes were done by Kruskal-Wallis test and Dunn's multiple comparison test.

Discussion

Little is known about the immune response against PvCyRPA. To our knowledge, there is only one paper addressing this topic, so far, in a cohort study of Papua New Guinean children¹². Based on this, the present work represents one of the first reports of immunity against PvCyRPA in the world and the first report in a Brazilian population. As previously described, the studied population is composed of individuals who lived their entire lives in endemic areas and are naturally exposed to *P. vivax* malaria, the predominant *Plasmodium* species in Brazil. Our data demonstrated that PvCyRPA is naturally immunogenic in this population, once several individuals presented both humoral and cellular immune responses to PvCyRPA. IgM antibodies were detected in this population, especially in *P. vivax*-infected individuals, a group that encompasses 75% of IgM

Epitope	Sequence	Length	Ellipro	Bcpred	BepiPred	ABCpred	EMINI
B-PvCyRPA ₍₈₁₋₉₁₎	INSTWETQTTL	11	X	X	X	X	-
B-PvCyRPA ₍₁₁₉₋₁₂₉₎	YKQRSKREGTI	11	X	-	X	X	X
B-PvCyRPA ₍₁₃₄₋₁₅₁₎	NSVTGTIYQKEDVQIDKE	18	X	X	X	X	X
B-PvCyRPA ₍₁₈₁₋₁₉₂₎	SYEYKTANKDNF	12	X	-	X	-	X
B-PvCyRPA ₍₂₄₁₋₂₄₉₎	RISTNNTAR	9	X	X	X	-	-
B-PvCyRPA ₍₂₅₇₋₂₇₂₎	TLDVTNEGKKEYKFKC	16	X	X	-	-	X
B-PvCyRPA ₍₃₁₂₋₃₃₀₎	TEQNAIIVVKPKVQNDDLNG	19	X	X	-	X	-

Table 2. B cell epitopes identified in PvCyRPA.

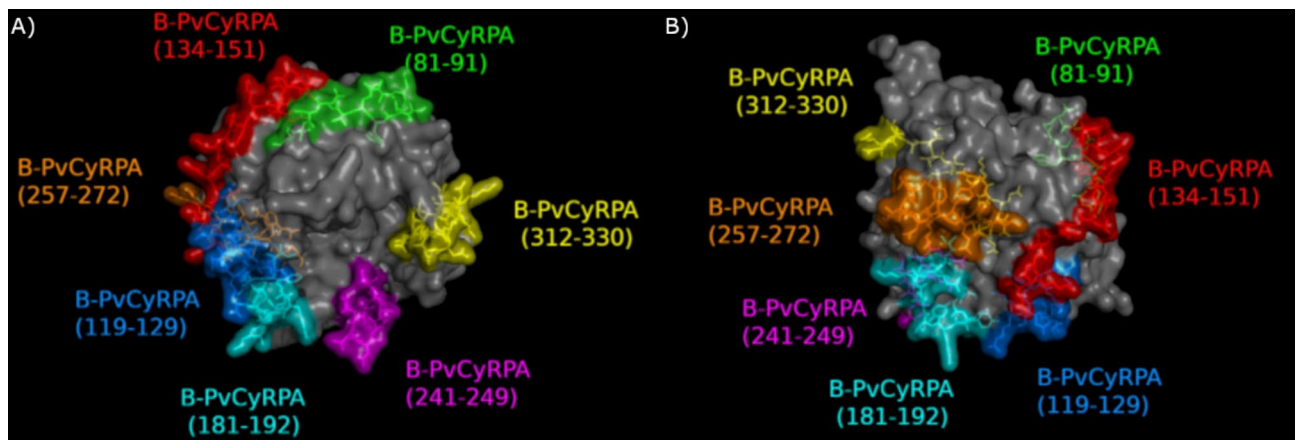


Fig. 3. Location of predicted epitopes in PvCyRPA 3D structure. The protein chain is indicated by a gray and transparent (40%) surface. The locations of epitopes B-PvCyRPA₍₈₁₋₉₁₎, B-PvCyRPA₍₁₁₉₋₁₂₉₎, B-PvCyRPA₍₁₃₄₋₁₅₁₎, B-PvCyRPA₍₁₈₁₋₁₉₂₎, B-PvCyRPA₍₂₄₁₋₂₅₇₎, B-PvCyRPA₍₂₅₇₋₂₇₂₎, and B-PvCyRPA₍₃₁₂₋₃₃₀₎ were indicated by licorice sticks green, blue, red, cyan, magenta, orange, and yellow, respectively. Different rotations of the protein are shown in (A) and (B).

responders. This result may indicate that higher RI's of IgM antibodies against PvCyRPA are possibly indicative of ongoing and/or recent malaria infections, once IgM responders presented an inferior number of months since the last malaria episode compared to IgM non-responders. The observed percentage of IgM responders to PvCyRPA in the *P. vivax*-infected group (50%) is consistent with data obtained from Walker and collaborators using Ghanaian children infected with *P. falciparum* with an observed frequency of 63.2% of IgM responders to PfCyRPA¹⁷. Even though the role of IgM in malaria immune response is less studied than that of IgG antibodies, it is already known that these antibodies are rapidly induced after infection by natural exposure and are long-lived even in the absence of re-infection. Merozoite-specific IgM antibodies also present an important role in the prevention of red blood cell invasion due to a mechanism as it acts as a potent activator of complement in the merozoite surface already observed in patients both adults and children¹⁸.

More than half of the 90 plasma samples obtained from this population presented IgG antibodies against recombinant PvCyRPA ($n=51$). The frequency observed in this paper in *P. vivax*-infected individuals is higher when compared to the frequency observed in Ghanaian children infected with *P. falciparum* where only about 1 in 5 patients presented specific IgG for PfCyRPA (~20%)¹⁹. It is important to highlight that this difference is possibly due to several characteristics, once this comparison is between two different *Plasmodium* species, in different profiles of studied group (children versus adults), living in completely diverse places with different transmission standards. Interestingly, the exposed group presented both a similar frequency of responders and RI's to IgG antibodies in comparison to the other groups (*P. vivax* and cohabitants), in fact, this result was not what was expected, since these individuals have not reported malaria in the last 10 years. However, it is important to highlight that the exposed group have been living in endemic areas for almost their entire lives and could have previous malaria episodes in childhood for example. Previous works have already demonstrated the presence of long-lived antibodies against merozoite antigens such as MSP-1²⁰, MSP-8²¹ and others²². Other possibility is that they had asymptomatic malaria, which is possible since *P. vivax* the predominant species in Brazil, causes submicroscopic infections that are commonly not diagnosed and sometimes not identified as disease because of milder symptoms²³. Next, Looking at the subclass profile of IgG antibodies, we have identified the prevalence of subclasses IgG1 and IgG2. We highlight that IgG1 antibodies are cytophilic antibodies that have been largely described in the scientific literature in *P. falciparum* as protective considering malaria immunity. Besides, IgG1 RI's of our patients were directly correlated with IgG3 RI's, another cytophilic antibody described as an important subclass for the development of clinical immunity²⁴⁻²⁷. Together, IgG1 and IgG3 act in a mechanism capable

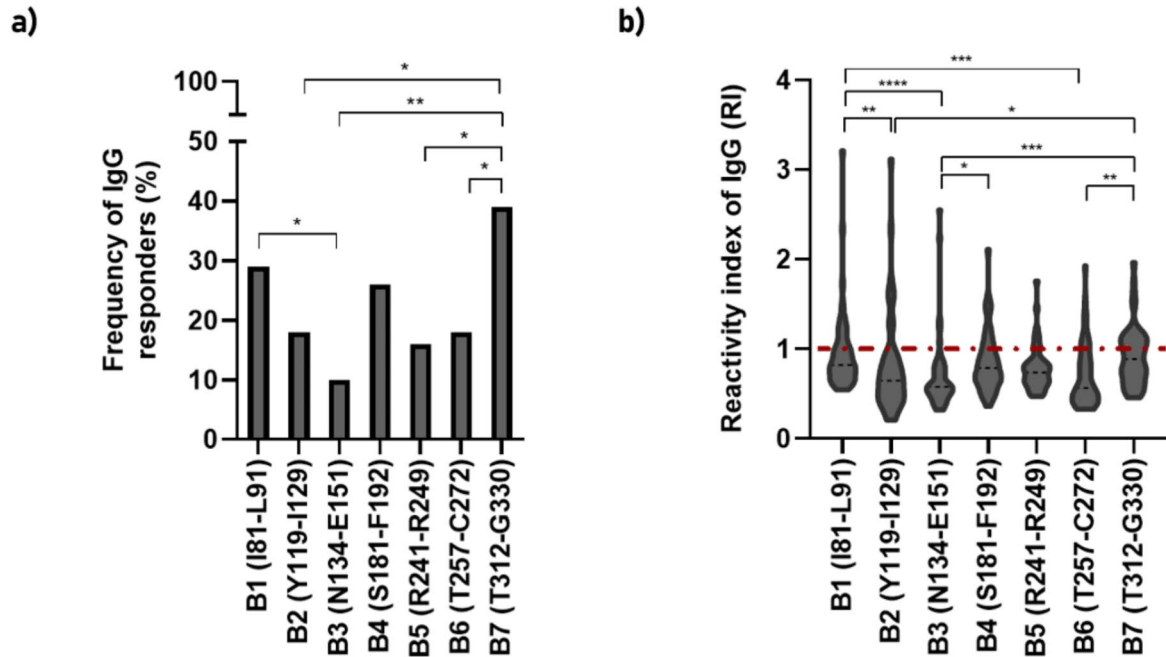


Fig. 4. (a) Frequency of IgG responders to different PvCyRPA B cell epitopes. The percentage of IgG antibody responders to each one of the 7 predicted; (b) Reactivity of IgG antibodies against PvCyRPA B cell epitopes. RI's of 51 individuals that respond with IgG antibodies are demonstrated in this graph for each of the 7 predicted B cell epitopes. The red dotted line separates responders from non-responders, values of RI's above 1 are considered responders to each one of the B cell epitopes. B1 x B2 ($p=0.0084^{**}$), B1 x B3 ($p<0.0001^{****}$), B1 x B6 (0.0004^{***}), B7 x B2 ($p=0.0248^*$), B7 x B3 ($p=0.0001^{***}$), B7 x B6 ($p=0.0115^*$) and B4 x B3 ($p=0.0115^*$). RI's analyzes were done by Kruskal-Wallis test and Dunn's multiple comparison test, while frequencies were compared through Fisher's exact test.

of controlling malaria asexual blood stages through Antibody-Dependent Cell-Mediated Inhibition (ADCI), binding to Fc-gamma receptors on monocytes' surface and triggering the release of cytokines or other soluble mediators that block the division of intraerythrocytic parasites²⁸.

All seven B cell epitopes identified and investigated in this paper were recognized by IgG antibodies present in the plasma of individuals at different levels. In fact, observed frequencies were low, however, we still highlight epitope B-PvCyRPA₍₃₁₂₋₃₃₀₎, predicted with the highest potential comparing prediction scores and identified in this study as the sequence with the highest frequency of responders and median of RI's. A previous paper published by our group investigated the genetic diversity of PvCyRPA in clinical isolates from the Brazilian Amazon and demonstrated a moderate presence of polymorphisms in the same B cell epitopes investigated in this paper, highlighting that they could somehow impact the immune response against the protein. The presence of these different polymorphisms in the predicted B-cell epitopes of PvCyRPA could influence antibody recognition, however, it also suggests that the protein could be under selective pressure by the host's immune system. Finally, it is important to notice that epitope B-PvCyRPA₍₃₁₂₋₃₃₀₎ was one of the regions where polymorphisms weren't identified¹⁶. After all ELISA assays specific to B cell epitopes were performed, it was possible to observe that *P. vivax*-infected individuals presented a higher repertoire of IgG antibodies specific to the studied sequences and that it diminishes when it comes to the cohabitants group, and it's even lower when it comes to exposed group. This observation suggests that, when the *Plasmodium* parasite is present, the immune system is largely stimulated to produce IgG antibodies against several portions of PvCyRPA. Individuals who were not infected by the time of blood collection but were already exposed several times to *Plasmodium* parasites in the last 10 years recognized fewer epitopes than the infected group but still a significant number of sequences in comparison to the exposed group, individuals who are at least 10 years without malaria and consequently without continuous antigen/epitope stimulation by PvCyRPA. Another hypothesis is that maybe the repertoire does not diminish, but the intensity of the observed immune response against them does. For example, França and colleagues demonstrated the ability of PvCyRPA to promote protection with low antibody levels¹², thinking about this reasoning, individuals with more time since the last malaria episode could present low but still protective antibody levels against these sequences that might not be identified in ELISA assays based on the established cut off considering the optical densities of non-endemic patients' plasma samples. It is also important to note that even with a lower repertoire of IgG against B cell epitopes in the exposed group compared to the groups *P. vivax* and cohabitants, IgG antibodies observed in the exposed group were in general directed to epitopes B-PvCyRPA₍₈₁₋₉₁₎ and B-PvCyRPA₍₃₁₂₋₃₃₀₎ and maybe these sequences are capable of eliciting specific memory B

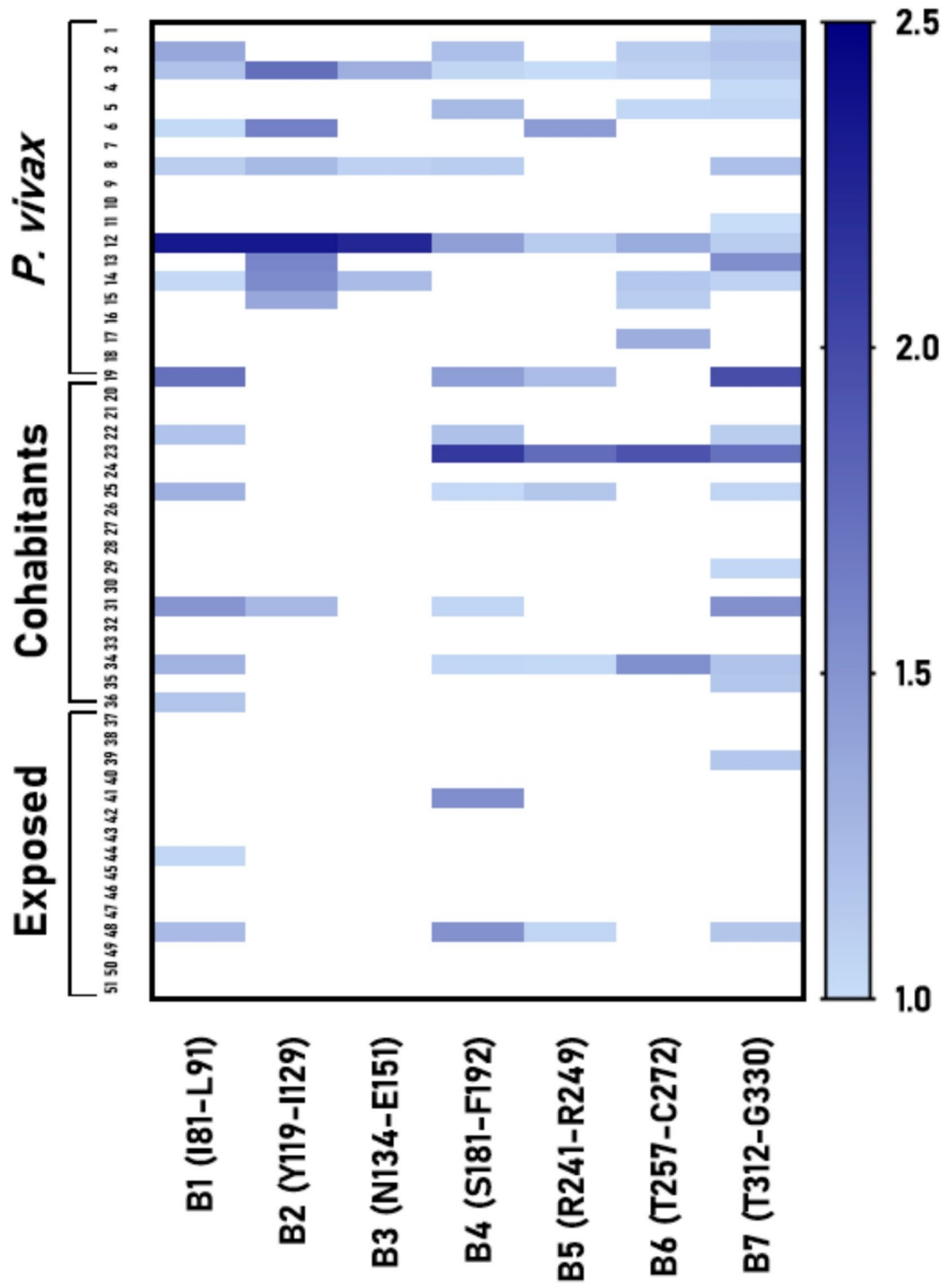


Fig. 5. Heat map with RI's of IgG antibodies against B cell epitopes. RI's of 51 individuals that respond to IgG antibodies are demonstrated in this heat map for each of the 7 predicted B cell epitopes. Each line from 1 to 51 represents one of the tested individuals and different epitopes that were recognized by them. The gradient color bar indicates different values of RI's.

cells since exposed individuals present these antibodies even without recent reports of malaria. Finally, another possibility is that PvCyRPA may be richer in conformational epitopes, once small frequencies of responders were observed for each of the sequences and analysis performed were used exclusively to identify linear B cell epitopes. This hypothesis is also supported by PvCyRPA structure itself, rich in beta strands and random coils

Epitope	Sequence	HLA-alleles binding frequency - N (%)	Percentile rank Median (Min-Max)
T-PvCyRPA _(49–63)	DIYFLFRNELYKTCI	20 (74%)	4.2 (0.69 – 17.78)
T-PvCyRPA _(54–68)	FRNELYKTCIQHVIK	17 (63%)	10.7 (1.41 – 19.51)
T-PvCyRPA _(102–116)	SVFNFIHNDIIVVI	20 (74%)	7.1 (0.53 – 19.3)
T-PvCyRPA _(167–181)	TVKNKKFLLICGILS	18 (67%)	7.5 (0.29 – 17.92)
T-PvCyRPA _(216–230)	LQKGVPIFYLRPIIF	20 (74%)	6.5 (0.69 – 15.71)
T-PvCyRPA _(221–235)	PYFYLRPIIFGDEFG	18 (67%)	8.1 (0.69 – 19.43)
T-PvCyRPA _(232–246)	DEFGFYFYSRISTNN	17 (63%)	4.6 (0.29 – 18.76)
T-PvCyRPA _(287–301)	VTKLNYYITSYVKK	18 (67%)	11.5 (0.94 – 19.34)
T-PvCyRPA _(306–320)	ECYLYYTEQNAIVVK	21 (78%)	7.03 (0.87 – 16.56)
T-PvCyRPA _(340–354)	DESKALFIYSTGYGV	17 (63%)	8.5 (1.8 – 19.59)

Table 3. Predicted T-CD4 epitopes in PvCyRPA.

as it was demonstrated with PSIPRED^{29,30}. It is important to highlight that, the naturally acquired protective immunity mediated by antibodies against *P. vivax* is very challenging to be determined, due to the lack of a well-established continuous in vitro culture. In fieldwork, the association of clinical protection with specific antigens of *P. vivax* has been reported in prospective cohort longitudinal studies, as the first one that described the PvCyRPA potential¹². Unfortunately, the cross-sectional design of our study limited the investigation to retrospective malaria histories, and the best approximation of an individual's protection was the estimated amount of time that had passed since their last malaria episode. However, prospective studies on humoral immune responses against PvCyRPA or biological studies addressing the ability of these antibodies to inhibit merozoite invasion or the development of blood-stage parasites will provide more direct evidence concerning their protective efficacy.

After the investigation of the humoral immune response, cellular immunity was observed using cells of the entire population to perform ELISPOT assays. This type of analysis has been widely used in the study of tropical diseases as it helps to elucidate the role of host-pathogen interaction through the identification of key cytokines³¹. In this scenario, IFN- γ has been largely described in scientific literature as an important piece of malaria immunity. This cytokine is secreted by CD4⁺ Th1 cells and participates in the activation of CD8⁺ T cells, B cells and macrophages, essential components of an effective/protective immune response against *Plasmodium* parasites^{32–34}. Our results demonstrated that all the tested pools containing different combinations of T cell predicted epitopes were capable of inducing IFN- γ production. Higher frequencies of responders were observed for pools 2, 4 and 5 considering all of the studied population. Looking at results per group, pools 4 and 5 presented a similar characteristic, both have higher numbers of SFU in the exposed group in comparison to *P. vivax*-infected group. These pools also share the epitope T-PvCyRPA_(306–320). Looking at both immune responses together, T-PvCyRPA_(306–320) and B-PvCyRPA_(312–330) are localized in the C-terminal region of PvCyRPA and are partially overlapped as they share nine amino acids (TEQNAIVVK). Together, these findings might indicate the C-terminal region of PvCyRPA as the most promising portion of the protein that can induce both humoral and cellular immune response. Considering the potential of PvCyRPA as a vaccine candidate for *P. vivax*, our group has designed and constructed a multistage chimeric protein named PvRMC-1, where PvCyRPA was used in association with the antigens PvCelTOS (cell-traversal protein for ookinetes and sporozoites) and Pvs25 (surface protein). PvRMC-1 was capable to be strongly recognized by naturally acquired antibodies of Brazilian Amazon individuals, with a predominance of cytophilic antibodies IgG1 and IgG3³⁵. Additionally, this chimeric protein was used in different formulations of adjuvants in BALB/c mice immunizations. The observed results suggested that PvRMC-1 is capable to generate both humoral and cellular immune responses in these animals. Besides, generated antibodies recognized not only full-length PvRMC-1 but also linear B-cell epitopes from PvCyRPA, PvCelTOS, and Pvs25 individually. Finally, mice's splenocytes were collected and ELISPOT assays indicated that these cells were activated, producing IFN- γ in response to PvCelTOS and PvCyRPA T-cell epitopes³⁶. All the B-cell and T-cell epitopes of PvCyRPA used to evaluate the immune response against PvRMC-1 were the same synthetic peptides investigated in this work. Together, these findings reinforce PvCyRPA's potential as a vaccine candidate.

In conclusion, this is the first paper demonstrating that PvCyRPA is naturally immunogenic in individuals from the Brazilian Amazon. The humoral and cellular immune responses generated against this protein and its epitopes suggest that PvCyRPA induces cytophilic antibodies such as IgG1 that might have an important role for protection and PvCyRPA's C-terminal region might be a promising region to be explored in new vaccine formulations, due to the presence of B and T cell epitopes, specially epitopes B-PvCyRPA_(312–330) and T-PvCyRPA_(306–320). However, more studies are necessary to understand if antibodies against these epitopes are protective to confirm the full potential of PvCyRPA as a malaria vaccine candidate.

Methods

Study area and volunteers

A cross-sectional cohort study was conducted with 100 volunteer individuals divided into clusters. 90 individuals from the malaria-endemic region of Acre state in Brazil from June to August 2018 were divided into *P. vivax*

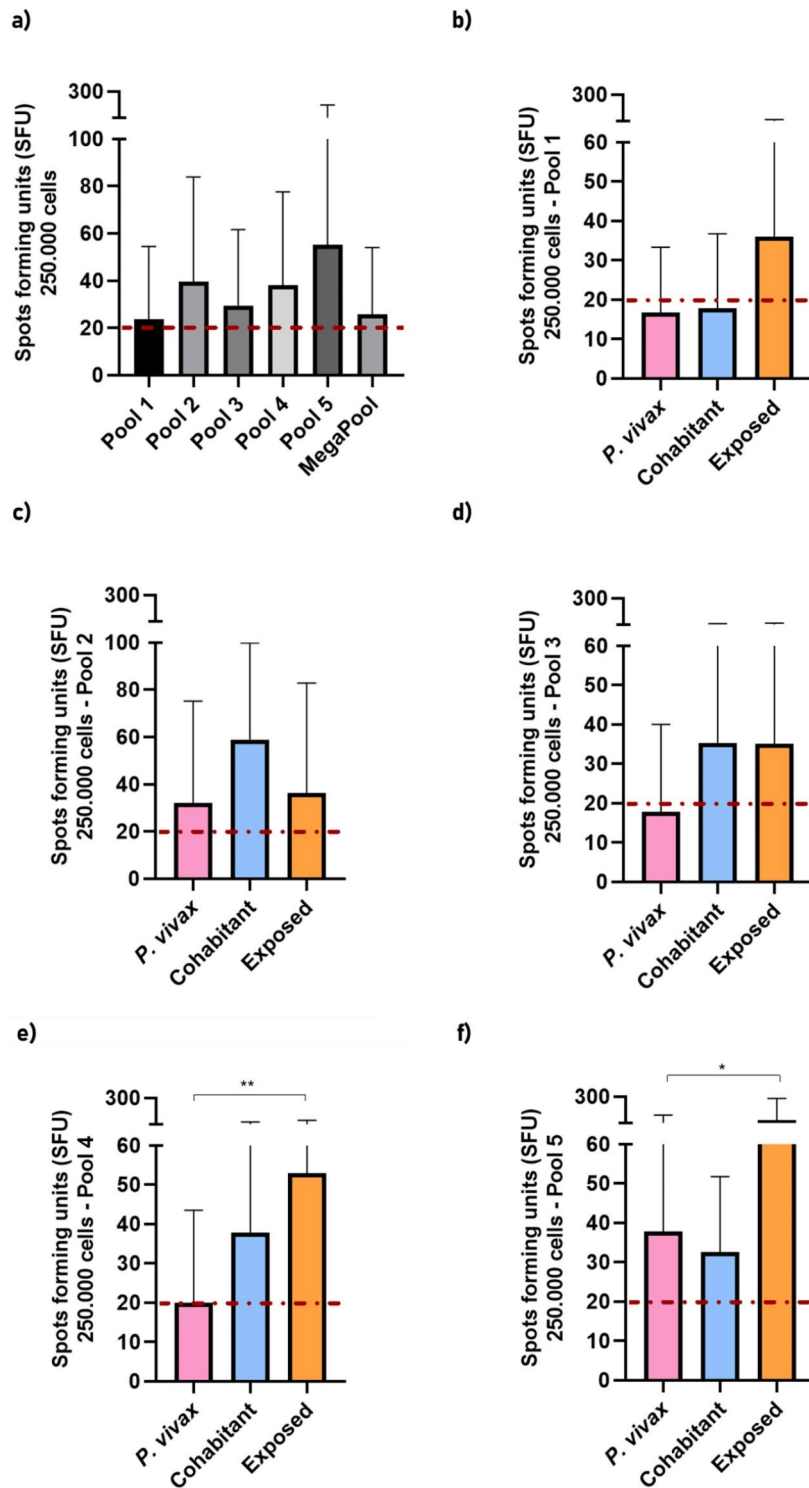


Fig. 6. Numbers of Spots forming units of IFN- γ in the population and per group. The graph demonstrates the numbers of adjusted SFU of IFN- γ for each of the 6 tested pools using the entire population and each studied group. The dotted red line represents the mark of 20 SFU, values above this line indicate individuals capable of producing IFN- γ against the respective pool. The infected group is represented in pink, the exposed with previous malaria infections in blue, and the exposed without previous malaria infections in orange. The red dotted line separates responders from non-responders and represents the mark of adjusted 20 SFU. Analyses using the number of SFU were done by Kruskal-Wallis test and Dunn's multiple comparison test.

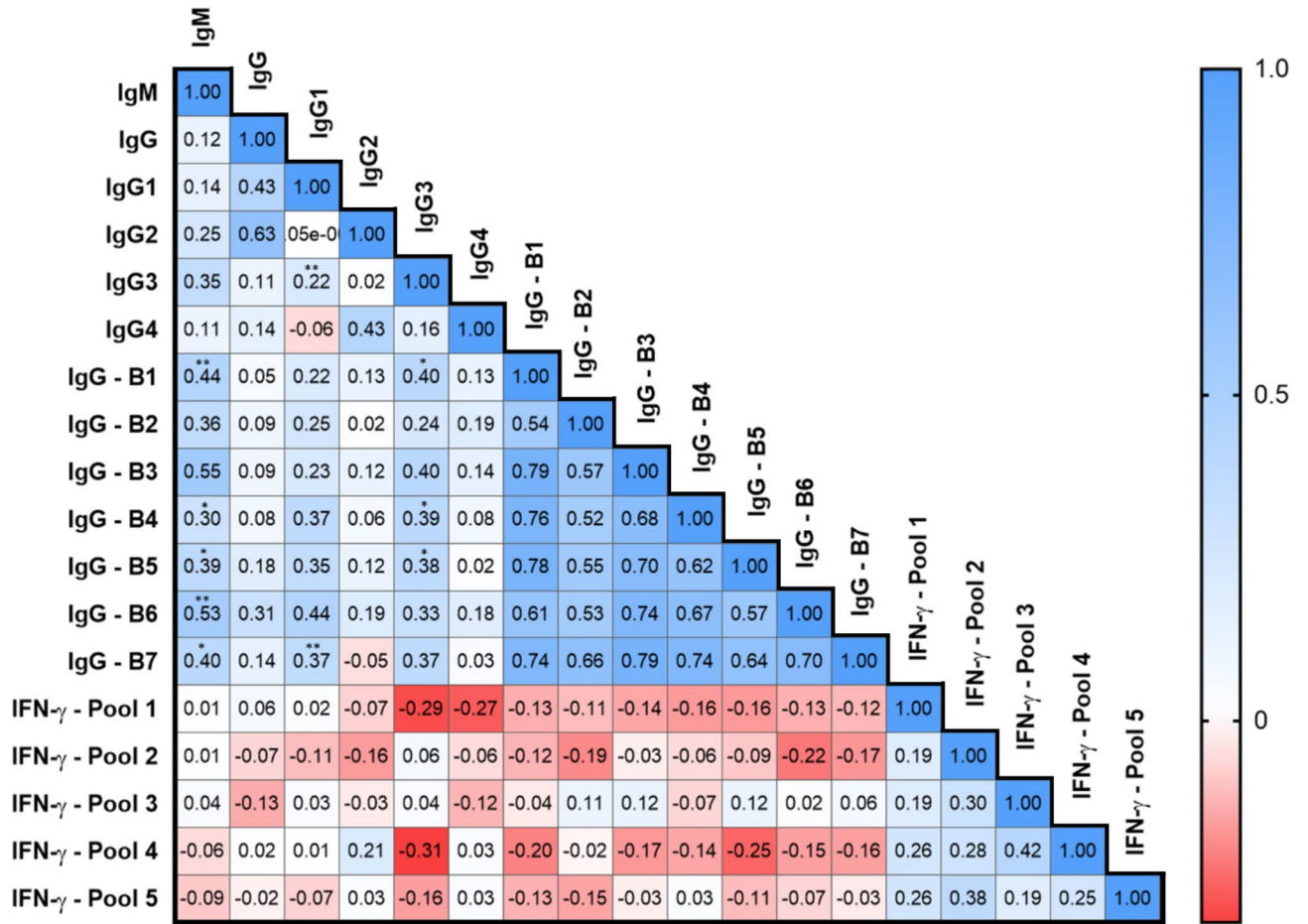


Fig. 7. Correlation matrix of cellular and humoral immune response against PvCyRPA. Results were obtained by Spearman correlation test. Numbers correspond to r value of each correlation. $P \leq 0,05 = *$ and $p \leq 0,01 = **$.

group ($n = 30$; individuals with *P. vivax* diagnosis confirmed by thick blood smears and PCR), the cohabitant group ($n = 30$, composed of individuals not infected by *Plasmodium* spp. at the collection moment, however, that have reported previous malaria episodes and live in the same house/region of infected individuals from the *P. vivax* group) and the exposed group ($n = 30$; composed of individuals that live in malaria endemic area but don't have a previous history of malaria. Lastly, samples and survey data were also collected from 10 individuals living in non-endemic areas of Rio de Janeiro and used as the non-exposed control group to determine cut-offs of experiments. All methods used in the present study were carried out in accordance with the revised Declaration of Helsinki and all the experimental protocols were previously approved by the Fundação Oswaldo Cruz Ethical Committee and the National Ethical Committee of Brazil (CEP-FIOCRUZ CAAE 46084015.1.0000.5248) and written informed consent was obtained from all of the study participants.

Epidemiological survey

To evaluate epidemiological factors influencing humoral and cellular immune responses against PvCyRPA all donors were interviewed. During the survey, participants answered questions related to personal exposure to malaria, for example, years of residence in an endemic area, the number of previous malaria episodes, time since the last malaria episode, presence/absence of symptoms, and use of malaria preventive measures, among others. All epidemiological data collected was stored in Epi-info (Centers for Disease Control and Prevention, Atlanta, GA, USA) for further analysis.

Blood sampling and malaria diagnosis

Venous peripheral blood of participants was drawn into heparinized tubes for peripheral blood mononuclear cells (PBMC) isolation by Ficoll/Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation and stored in liquid nitrogen to perform ELISPOT assays. Plasma was also collected and stored at -20°C and transported to our laboratory for antibody assays. Examination for malaria parasites was performed with thin and thick blood smears of donors. Parasitological evaluations were done by examination of 200 fields at $1,000\times$ magnification under oil immersion and a research expert in malaria diagnosis examined all slides. After the samples were transported to the laboratory, they were submitted to Polymerase chain reaction (PCR) using specific primers for the genus (*Plasmodium* sp.) and species (*P. vivax* and *P. falciparum*) as described in our previous works^{16,35},

in order to heighten the sensitivity of parasite detection. DNA was previously extracted from patients' blood samples by QIAamp DNA blood midi kit (Qiagen, Germantown, MD, USA) as per the manufacturer's instructions. Donors positive for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated using the chemotherapeutic regimen recommended by the Brazilian Ministry of Health. Only samples that were positive for both microscopy and PCR, were included in the *P. vivax* infected group.

Recombinant PvCyRPA expression

The *P. vivax cyrpa* (*pvx_090240*) sequence was synthesized into a pET28 vector and codon optimized for *Escherichia coli* expression by GenScript. The PvCyRPA recombinant protein was expressed with a histidine tag in a *E. coli* STAR BL21(DE3). The protein expression was induced when bacteria cultures reached an absorbance of 0,6, with 0,1 mM isopropil β -d-1-thiogalactopyranoside (IPTG), for 16 h, at 15°C. Recombinant protein was purified through affinity chromatography, on Ni-NTA agarose. Recombinant protein was eluted in a buffer containing 300mM NaCl, 50mM Tris HCl, 500mM imidazole, pH 8,0. Purified protein was checked by SDS-PAGE and western blot with anti-histidin antibody.

PvCyRPA B cell linear epitopes

Recently, our group predicted B cell linear epitopes in PvCyRPA and investigated the antigenic conservation of predicted epitopes among isolates from five different areas of the Brazilian Amazon. Briefly, we explored the protein's data from the Uniprot database (<https://www.uniprot.org>, ID: A5K678) and its respective tridimensional structure modeled by AlphaFold. The prediction of linear B cell epitopes was performed using 5 different prediction algorithms: BCPreds (<http://ailab.cs.iastate.edu/bcpreds/>, accessed on March 8, 2021), BepiPred 1.0 and EMINI Surface Accessibility Prediction from Immune Epitope Database - IEDB (<http://tools.iedb.org/bcell/>, accessed on March 8, 2021), ABCPred (<https://webs.iitd.edu.in/raghava/abcpred/>, accessed on March 8, 2021), and Ellipro (<http://tools.iedb.org/ellipro/>, accessed on March 8, 2021).

BCPreds is a method for predicting linear B cell epitopes using a support machine vector learning method, using as a threshold 75%; BepiPred 1.0, a server based in a combination of Markov model and a propensity scale method, considering scores above 0.35. EMINI Surface Accessibility Prediction calculates the surface accessibility of the linear epitopes and was used with a threshold of 1.0. ABCPred is a server with an artificial network based on the BCIPEP database, that was used with scores higher than 0.84 and Ellipro, that identifies epitopes in a determined sequence using a combination of methods such as Thornton's method, a residue clustering algorithm a modeler program and Jmol viewer, the considered threshold was 0,6.

Antibody assays

Anti-PvCyRPA-specific antibodies were evaluated using enzyme-linked immunosorbent assay (ELISA). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) were coated with PBS containing 1.5 μ g/ml of recombinant protein. After overnight incubation at 4 °C, the plates were washed and blocked with PBS-Tween containing 5% of BSA (PBS-Tween-BSA 5%) for 1 h at 37 °C. Individual plasma samples were diluted 1:100 in PBS-Tween-BSA 2,5% were added in duplicate wells. After 1 h at 37 °C and three washings with PBS-Tween, bound antibodies were detected with peroxidase-conjugated goat antihuman IgM and IgG (Sigma, St. Louis) followed by the addition of *o*-phenylenediamine and hydrogen peroxide. Optical density was identified at 492 nm using a SpectraMax 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The results for total IgM and IgG were expressed as reactivity indexes (RIs), which were calculated by the mean optical density of an individual's tested sample divided by the mean optical density of 10 non-exposed control individuals' samples plus 3 standard deviations. Subjects were scored as responders to PvCyRPA if the RI of IgM and IgG against the recombinant protein was higher than 1. Additionally, the RIs of IgG subclasses were evaluated in the responders' group by a similar method, using peroxidase-conjugated goat antihuman IgG1, IgG2, IgG3, and IgG4 (Sigma, St. Louis). ELISA assays were also performed as previously described, however using 5 μ g/ml of each synthetic biotinylated peptide in PBS to identify levels of IgG antibodies against all of the 7 predicted B cell epitopes, in this experiment, plasma samples were incubated for 2 h at 37 °C.

T cell epitope prediction of PvCyRPA

T cell epitopes present in PvCyRPA were identified using the IEDB analysis resources consensus tool. Considering lengths of 9 mers, predictions scores of the obtained sequences were investigated against 27 of the most frequent HLA alleles circulating worldwide: HLA alleles circulating worldwide: HLA-DRB1*01:01; HLA-DRB1*03:01; HLA-DRB1*04:01; HLA-DRB1*04:05; HLA-DRB1*07:01; HLA-DRB*08:02; HLA-DRB1*09:01; HLA-DRB1*11:01; HLA-DRB*12:01; HLA-DRB1*13:02; HLA-DRB1*15:01; HLA-DRB3*01:01; HLA-DRB3*02:02; HLA-DRB4*01:01; HLA-DRB5*01:01; HLA-DQA1*05:01/DQB1*02:01; HLA-DQA1*05:01/DQB1*03:01; HLA-DQA1*03:01/DQB1*03:02; HLA-DQA1*04:01/DQB1*04:02; HLA-DQA1*01:01/DQB1*05:01; HLA-DQA1*01:02/DQB1*06:02; HLA-DPA1*02:01/DPB1*01:01; HLA-DPA1*01:03/DPB1*02:01; HLA-DPA1*01:03/DPB1*04:01; HLA-DPA1*03:01/DPB*04:02; HLA-DPA1*02:01/DPB1*05:01 and HLA-DPA1*02:01/DPB1*14:01. The T cell epitopes selected presented percentile rank minor than 20 and recognized by at least 14 of used HLA. Ten selected T-CD4 epitopes were synthesized as peptides and organized in 5 different pools containing 4 T-cell epitopes each. The construction of pools followed the natural sequence of the protein with an overlap of the 2 last peptides of the previous pool. An extra pool was also created containing all of the 10 predicted epitopes. Together these pools were used to stimulate T cell lymphocytes in ELISPOT assays.

Peptide synthesis

Seven sequences predicted as antigenic linear B-cell epitopes and ten sequences predicted as promiscuous T-CD4 epitopes were chemically synthesized by the company GenOne Biotechnologies, Brazil. Analytical

chromatography of the peptides demonstrated a purity of approximately > 95%, and mass spectrometry analysis of the peptides indicated their expected mass.

ELISPOT assays

Cell cultures were carried out in duplicate in pre-coated nitrocellulose 96 well plates (MabTech). Plates were blocked with RPMI medium containing 10% fetal calf serum for 30 min and 2.5×10^5 cells were added to the ELISPOT plates in the presence of medium alone to act as a control or with 10 µg/ml of different pool combinations of PvCyRPA peptides. In order to determine IFN-γ secretion, cells were stimulated for 24 h at 37 °C, 5% CO₂ under sterile conditions. After stimulation, plates were washed four times with PBS 1X and incubated with biotin-anti-human IFN-γ Clone 7-B6-1 (MabTech) diluted in PBS 1X containing 0.05% of fetal calf serum for 2 h at 37 °C. The plates were washed four times with PBS 1X and incubated with streptavidin-alkaline phosphatase (MabTech) in PBS 0.05% for 1 h at 37 °C. The plates were washed four times with PBS 1X before development with 1-step NBT/BCIP. Development was stopped by the addition of distilled water. IFN-γ secreting cells appeared as purple spots and were counted with an Immunospot reader (Cellular Technology Ltd, Cleveland, OH) using the Immunospot Software Version 3. ELISPOT, responses were expressed as spot forming units (SFU) per 250,000 PBMCs. Antibody mAbCD3-2 (1 µg/ml) was used as a positive control. The assays were subsequently categorized as positive or negative depending on whether the mean number of SFU in peptide-stimulated wells was greater than the mean number of SFU in control wells with medium alone from the same patient. Individuals presenting at least 20 IFN-γ SFUs/ 2×10^5 PBMC in the experimental wells were considered responders.

Statistical analysis

Statistical analyses were done in GraphPad Prism 8.0 for Windows (GraphPad Software, Inc.). Normality tests were done in all variables using the one-sample Kolmogorov–Smirnov test. The Dunn's test was used to compare RIs of IgG against recombinant PvCyRPA in studied groups (*P. vivax*, cohabitants, and exposed) and RIs of IgG specific to B cell epitopes. Uncorrected Fisher's plus LSD was done to access the differences in proportions of IgG and IgG subclass. Correlations between immune response and epidemiological parameters were evaluated by the Spearman rank test. A two-sided *p*-value < 0.05 was considered significant.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

Study designing: J.C.-L.J. Performed experiments, data analysis and manuscript preparation: I.F.-S. Performed experiments: B.O.-B, A.S.-M, C.M.-R, A.L.-C.A. Field work support: B.O.-B, R. M.-S, H.A.S.-S, E.K.-P.R, J.P.-B, P.R.-T, L.R.-P.R. Recombinant cloning and protein expression: M.A.K.-J and L.-A. Manuscript review and data analysis: R.N.-R.S., K.K.G.-S, C.T.-D.R, P.R.-T, L.R.-P.R. Funding acquisition: J.C.-L.J., P.R.-T, L.R.-P.R. and C.T.-D.R.

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Written consent was obtained for use of plasma and cell samples. Survey data was also collected in accordance to the revised Declaration of Helsinki. Both collection and consent protocols were under approval of Fundação Oswaldo Cruz Ethical Committee and the National Ethical Committee of Brazil (CEP-FIOCRUZ CAAE 46084015.1.0000.5248).

Additional information

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