



Plasmodium vivax Cell-Traversal Protein for Ookinetes and Sporozoites: Naturally Acquired Humoral Immune Response and B-Cell Epitope Mapping in Brazilian Amazon Inhabitants

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The cell-traversal protein for ookinetes and sporozoites (CeITOS), a highly conserved antigen involved in sporozoite motility, plays an important role in the traversal of host cells during the preerythrocytic stage of *Plasmodium* species. Recently, it has been considered an alternative target when designing novel antimalarial vaccines against *Plasmodium falciparum*. However, the potential of *Plasmodium vivax* CeITOS as a vaccine target is yet to be explored. This study evaluated the naturally acquired immune response against a recombinant *P. vivax* CeITOS (PvCeITOS) (IgG and IgG subclass) in 528 individuals from Brazilian Amazon, as well as the screening of B-cell epitopes *in silico* and peptide assays to associate the breadth of antibody responses of those individuals with exposition and/or protection correlates. We show that PvCeITOS is naturally immunogenic in Amazon inhabitants with 94 individuals (17.8%) showing specific IgG antibodies against the recombinant protein. Among responders, the IgG reactivity indexes (RIs) presented a direct correlation with the number of previous malaria episodes ($p = 0.003$; $r = 0.315$) and inverse correlation with the time elapsed from the last malaria episode ($p = 0.031$; $r = -0.258$). Interestingly, high responders to PvCeITOS (RI > 2) presented higher number of previous malaria episodes, frequency of recent malaria episodes, and ratio of cytophilic/non-cytophilic antibodies than low responders (RI < 2) and non-responders (RI < 1). Moreover, a high prevalence of the cytophilic antibody IgG1 over all other IgG subclasses ($p < 0.0001$) was observed. B-cell epitope mapping revealed five immunogenic regions in PvCeITOS, but no associations between the specific IgG response to peptides and

exposure/protection parameters were found. However, the epitope (PvCelTOS_{I136-E143}) was validated as a main linear B-cell epitope, as 92% of IgG responders to PvCelTOS were also responders to this peptide sequence. This study describes for the first time the natural immunogenicity of PvCelTOS in Amazon individuals and identifies immunogenic regions in a full-length protein. The IgG magnitude was mainly composed of cytophilic antibodies (IgG1) and associated with recent malaria episodes. The data presented in this paper add further evidence to consider PvCelTOS as a vaccine candidate.

Keywords: PvCelTOS, *P. vivax*, vaccines, epitope mapping, epitope prediction, malaria vaccines, malaria

INTRODUCTION

Malaria remains a major public health problem worldwide. It is caused by protozoan parasites of the genus *Plasmodium*, being responsible for nearly 438,000 deaths and 150–300 million new infections in 2015 (1) and the reason of enormous socioeconomic impact in endemic settings (2). Among the *Plasmodium* species able to infect humans, *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent malaria parasites. *P. falciparum* is extremely prevalent in Africa and is responsible for the majority of cases and deaths worldwide, while *P. vivax* is the most prevalent species outside Africa (3). Despite the reduction in the number of malaria cases and deaths over the past decade (1), the emergence of drug resistance and the significant ongoing burden of morbidity and mortality emphasize the need for an effective malaria vaccine. Unfortunately, potential *P. vivax* vaccine candidates lag far behind those for *P. falciparum* (4). Currently, besides the RTS, S vaccine, there are 30 candidate vaccine formulations in clinical trials against *P. falciparum*, while there is only one against *P. vivax* (5). These data allied to the impact caused by the high *P. vivax* prevalence (2), the severity of the disease (6–11), and the emergence of strains resistant to chloroquine (12–14) and primaquine (15–17), reiterate the importance of identifying and exploring the potential of vaccine candidates against *P. vivax* as an essential step in the development of a safe and affordable vaccine.

Malaria liver-stage vaccines are one of the leading strategies and the only approach that has demonstrated complete, sterile protection in clinical trials. Therefore, vaccines targeting sporozoite and liver-stage parasites, when parasite numbers are low, can lead to the elimination of the parasite before it advances to the symptomatic stage of the disease (18). Corroborating this idea, the sterile protection against *P. falciparum* by immunization with radiation-attenuated sporozoites was demonstrated in several studies (19–21) and the protection lasted for at least 10 months and extended to heterologous strain parasites (22). Based on these findings, sporozoite surface antigens are one of the most promising vaccine targets against malaria, to protect and prevent the symptoms and block its transmission. To date, RTS,S, the subunit vaccine consisting of a portion of *P. falciparum* circumsporozoite protein (CSP), conferred partial protection in Phase III trials and fell short of community-established vaccine efficacy goals (23–26). Conversely, Gruner and collaborators have demonstrated that the sterile protection against sporozoites can be obtained in the absence of specific immune responses to

CSP (27). In addition, a recent study found 77 parasite proteins associated with sterile protection against irradiated sporozoites (28). Collectively, these data reinforce the concept that a multi-valent anti-sporozoite vaccine targeting several surface-exposed antigens would induce a higher protection efficacy.

In this scenario, cell-traversal protein of *Plasmodium* ookinetes and sporozoites, a highly conserved protein among *Plasmodium* species, emerged as a novel target in the development of a vaccine against *Plasmodium* parasites (29). This secretory microneme protein is translocated to the sporozoites and ookinetes surface, being necessary for sporozoites and ookinetes to break through cellular barriers and establish infection in the new host, having a crucial role on cell-transversal ability in both stages (29, 30). The disruption of the genes encoding CelTOS in *Plasmodium berghei* reduces the infectivity in the mosquito host and also the infectivity of the sporozoite in the liver, almost eliminating their ability to cell pass (29). Interestingly, *P. falciparum* CelTOS (PfCelTOS) was naturally recognized by acquired antibodies in exposed populations (31), able to induce cross-reactive immunity against *P. berghei* and inhibit sporozoite motility and invasion of hepatocytes *in vitro* (32). However, the knowledge about *P. vivax* CelTOS (PvCelTOS) has remained limited. Only recently, a study reported PvCelTOS as naturally immunogenic in infected individuals from Western Thailand. Our group, investigating the genetic diversity of genes encoding PvCelTOS in field isolates from five different regions of the Amazon forest, reveals a high-conserved profile. Together, both findings support the potential of PvCelTOS as an interesting target on *P. vivax* sporozoite surface, but further studies are still necessary to consolidate this protein as an alternative in future multitarget vaccines. Therefore, the present study aimed at evaluating the naturally acquired humoral immune response against PvCelTOS in exposed populations from Brazilian Amazon, determining the antibody subclass profile, identifying its B-cell epitopes and verifying the existence of associations between the specific IgG and subclass response against PvCelTOS and epidemiological data that can reflect the exposition and/or protection degree.

PARTICIPANTS AND METHODS

Study Area and Volunteers

A cross-sectional cohort study was conducted involving 528 individuals from Rio Preto da Eva (2°50'50"S/59°56'28"W), located north of the Amazon River and 80 km distant from Manaus, the capital of Amazon state. This city has an area of 6,000 km²

and a population of about 22,000 people, who live in rural areas inside the forests. Transmission of malaria in the Amazon occurs throughout the whole year, with seasonal fluctuations with maximum transmission occurring during the dry season from May to October and prevalence of infections by *P. vivax*, responsible for more than 85% of reported malaria cases.

Samples and survey data were collected from November 2013 to March 2015. In addition, we also included, as control subjects, 10 naive individuals living in Manaus, and with no reported previous malaria episodes. Written informed consent was obtained from all adult donors or from parents of donors in the case of children. The study was reviewed and approved by the Fundação Oswaldo Cruz Ethical Committee and the National Ethical Committee of Brazil.

Epidemiological Survey

In order to evaluate the possible influence of epidemiological factors on humoral immunity against PvCelTOS, all donors were interviewed upon informed consent prior to blood collection. The survey included questions related to personal exposure to malaria, such as years of residence in the endemic area, recorded individual and family previous malaria episodes, use of malaria prophylaxis, presence/absence of symptoms, and personal knowledge of malaria transmission. All epidemiological data were stored in Epi-Info for subsequent analysis (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Malaria Diagnosis and Blood Sampling

Venous peripheral blood was drawn into heparinized tubes and plasma collected after centrifugation ($350 \times g$, 10 min). Plasma samples were stored at -20°C and transported to our laboratory. Thin and thick blood smears of all donors were examined for malaria parasites. 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. 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.

Recombinant PvCelTOS Expression in HEK-293T Cells

As previously described (33), the *P. vivax* sequence for CelTOS (Salvador I; Uniprot accession number Q53UB7) was cloned in the expression vector pHLsec, which is flanked by the chicken β -actin/rabbit β -globin hybrid promoter with a signal secretion sequence and a Lys-His6 tag. The protein was expressed upon transient transfection in HEK-293T cells with endotoxin-free plasmids in roller bottles ($2,125\text{ cm}^2$). The secreted protein was purified from the supernatant by immobilized Ni Sepharose affinity chromatography. The presence of proteins in the elution samples was confirmed using 6xHis epitope tag antibody [horseradish peroxidase (HRP) conjugate] in a Western blot. The sample was concentrated using an Amicon Ultra centrifugal filter system (Life Technologies) until reaching 10 ml of final volume. Contaminant proteins and salts were removed from the concentrate by size exclusion purification (SEC) using Superdex

medium in the column. Protein concentration after recovery was tested using a Bradford protein assay, and purity was assessed by silver staining and by Western blotting.

Antibody Assays

Anti-PvCelTOS specific antibodies were evaluated on plasma samples from 528 exposed individuals from Brazilian Amazon and 10 healthy individuals, who had no reported malaria episodes, using enzyme-linked immunosorbent assay (ELISA), essentially as previously described (33, 34). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) were coated with PBS containing $1.5\text{ }\mu\text{g/ml}$ of recombinant protein. After overnight incubation at 4°C , the plates were washed and blocked for 1 h at 37°C . Individual plasma samples diluted 1:100 in PBS-Tween containing 5% non-fat dry milk (PBS-Tween-M) 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 IgG (Sigma, St. Louis) and followed by 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 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 PvCelTOS if the RI of IgG against the recombinant protein was higher than 1. Additionally, the RIs of IgG subclasses were evaluated on responders individuals by a similar method, using peroxidase-conjugated goat antihuman IgG1, IgG2, IgG3, and IgG4 (Sigma, St. Louis).

B Cell Epitope Prediction on PvCelTOS

The prediction of linear B-cell epitopes was carried out using the program BepiPred (35), which is based on hidden Markov model profiles of known antigens, and also incorporates hydrophilicity and secondary structure prediction. For each input FASTA sequence, the server outputs a prediction score for each amino acid. The recommended cutoff of 0.35 was used to determine potential B-cell linear epitopes, ensuring sensibility of 49% and specificity of 75% to this approach. Linear B-cell epitopes are predicted to be located at the residues with the highest scores. In this study, BepiPred was used to predict B-cell linear epitopes and to evaluate the prediction value of peptides containing short amino acid sequences of PvCelTOS.

The Emini surface accessibility (ESA) was used to evaluate the probability of predicted linear B-cell epitopes to be exposed on the surface of the protein. This approach calculates the surface accessibility of hexapeptides and values greater than 1.0 indicate an increased probability of being found on the surface (36). Sequences with BepiPred score above 0.35 and ESA score above 1.0 were considered potential linear B-cell epitopes in regions that could be accessed by naturally acquired antibodies.

B-Cell Epitope Mapping of PvCelTOS

A peptide library of 32 PvCelTOS synthetic 15-mer peptides overlapping by nine amino acids (GenOne Biotechnologies; purity 95% based on HPLC) was synthesized. To evaluate the specific

response to each peptide, the peptide array was performed using MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) coated with PBS containing 5 µg/ml of each peptide in duplicates. After overnight incubation at 4°C, the plates were washed with PBS and blocked with PBS-Tween containing 5% non-fat dry milk (PBS-Tween-M) for 1 h at 37°C. Individual plasma samples were diluted 1:100 with PBS-Tween-M and added in duplicate wells for each sequence and the plates incubated at room temperature for 1 h. After three washings with PBS-Tween, bound antibodies were detected with peroxidase-conjugated goat antihuman IgG (Sigma, St. Louis) followed by addition of *o*-phenylenediamine and hydrogen peroxide. The absorbance was read at 492 nm using an ELISA reader (Spectramax 250, Molecular Devices, Sunnyvale, CA, USA). The results for total IgG were expressed as RIs, which were calculated by the mean optical density of the tested samples plus 3 standard deviations of pools of control individuals. Subjects were scored as positive if the RI was higher than 1.

Root Mean Square Fluctuation (RMSF) and Electrostatic Potential Surface Calculation

Molecular dynamics simulations were carried out using GROMACS 5.1.2 (37) software package. Gromos53a6 (38) force field was used. Simple point charge water model (39) was used to solvate the system. Charges were neutralized using Na⁺ and Cl⁻ ions. Steepest descent method was used for energy minimization. Further, 100 ps temperature equilibration was carried out at a temperature of 300 K in the presence of position restraints of 1,000 KJ/mol and the pressure coupling of 1,000 ps at 1 bar of atmospheric pressure. After equilibration, the simulation of 200,000 ps (200 ns) without position restraints was carried out. All simulations were run three times, and consistent results were recorded. RMSF was analyzed from simulation trajectory using GROMACS utilities. The Electrostatic potential surface for the PvCelTOS was calculated using APBS (40) and visualized in PyMOL (Pymol LLC) and the electrostatic potential surfaces for the contours from $-3kT/e$ (red) to $+3kT/e$ (blue) were visualized. The figures were rendered using PyMol.

Statistical Analysis

All statistical analyzes were carried out using Prism 5.0 for Windows (GraphPad Software, Inc.). The one-sample Kolmogorov–Smirnov test was used to determine whether a variable was normally distributed. The Mann–Whitney test was used to compare RIs of IgG against recombinant PvCelTOS between studied groups. Differences in proportions of the RI of IgG subclasses and epidemiological parameters were evaluated by Fisher's exact test and associations between antibody responses and epidemiological data were determined by Spearman rank test. A two-sided *p* value <0.05 was considered significant.

RESULTS

Epidemiological Profile of Studied Individuals

Most studied individuals were adults and naturally exposed to malaria infection throughout the years (Table 1). Age ranged

TABLE 1 | Summary of the epidemiological data of the studied population.

	Overall	PvCelTOS IgG responders	PvCelTOS IgG non-responders (NRs)
Gender—N (%)			
Male	284 (53.8%)	55 (58.5%)	229 (52.8%)
Female	244 (46.2%)	39 (41.5%)	205 (47.2%)
Total	528	94	434
Malaria exposure—median (IR)			
Age (years)	36 (25–50)	38 (21–55.5)	36 (21–50)
Time of residence in endemic area (years)	33 (19–49)	35 (21–55)	33 (19–48)
Number of previous malaria episodes (<i>n</i>)	4 (2–10)	4.5 (2–10)	4 (2–10)
Time since the last malaria episode (months)	51 (24–91)	60 (13.7–89.2)	51 (24–90.5)
Frequency of recent malaria episodes (%)	12.7%	16.0%	13.1%
Previous malaria species contracted—N (%)			
Never infected	7 (1.3%)	0 (0%)	7 (1.6%)
<i>Plasmodium falciparum</i>	32 (6.1%)	5 (5.3%)	27 (6.2%)
<i>Plasmodium vivax</i>	125 (23.7%)	25 (26.6%)	100 (23%)
Both species	158 (29.9%)	31 (33%)	127 (29.3)
Not reported/remember	206 (39%)	33 (35.1%)	173 (39.9%)

Values of age, time of residence in endemic areas, number of previous malaria episodes, and time elapsed from the last malaria episode represent the median (interquartile range), while the parameter “frequency of recent malaria episodes” represents the percentage of individuals who reported malaria episode in the last year. The frequency of individuals who present recent malaria episodes was compared by Fisher's test, and other epidemiological parameters were compared by Mann–Whitney test. No statistical difference was observed between epidemiological parameters of responders and NR individuals.

from 10 to 89 years with an average of 36.9. The proportion of men was significantly higher (53.8%) than for women (46.2%; $\chi^2 = 5.761$, $p < 0.0164$). Regarding the previous personal history of malaria, only seven individuals reported no malaria episode (1.3%). Among those who remembered the *Plasmodium* species, the majority (29.9%) reported infections by *P. falciparum* and *P. vivax*. The number of past malaria episodes also varied greatly among donors, ranging from 0 to 50 (mean = 7.74 ± 16.5). Finally, the time elapsed since the last malaria episode ranged from 0 to 480 months (mean = 71.7 ± 77.9). Interestingly, a correlation trend was observed between the time of residence in the endemic area and the number of previous malaria infections ($p = 0.0003$; $r = 0.153$). Collectively, the epidemiological inquiry indicated that the studied population had different degrees of exposure and/or immunity.

PvCelTOS Is Naturally Immunogenic with the Prevalence of Cytophilic Antibodies in Brazilian Amazon Individuals

To test if the PvCelTOS is a target for naturally acquired antibodies in Amazon individuals, we first assessed the IgG reactivity profile against the recombinant protein. The plasma samples collected from the 528 individuals living in the endemic area reveal a low frequency of responders to PvCelTOS, since only 17.8% of the studied population (94 individuals) presented

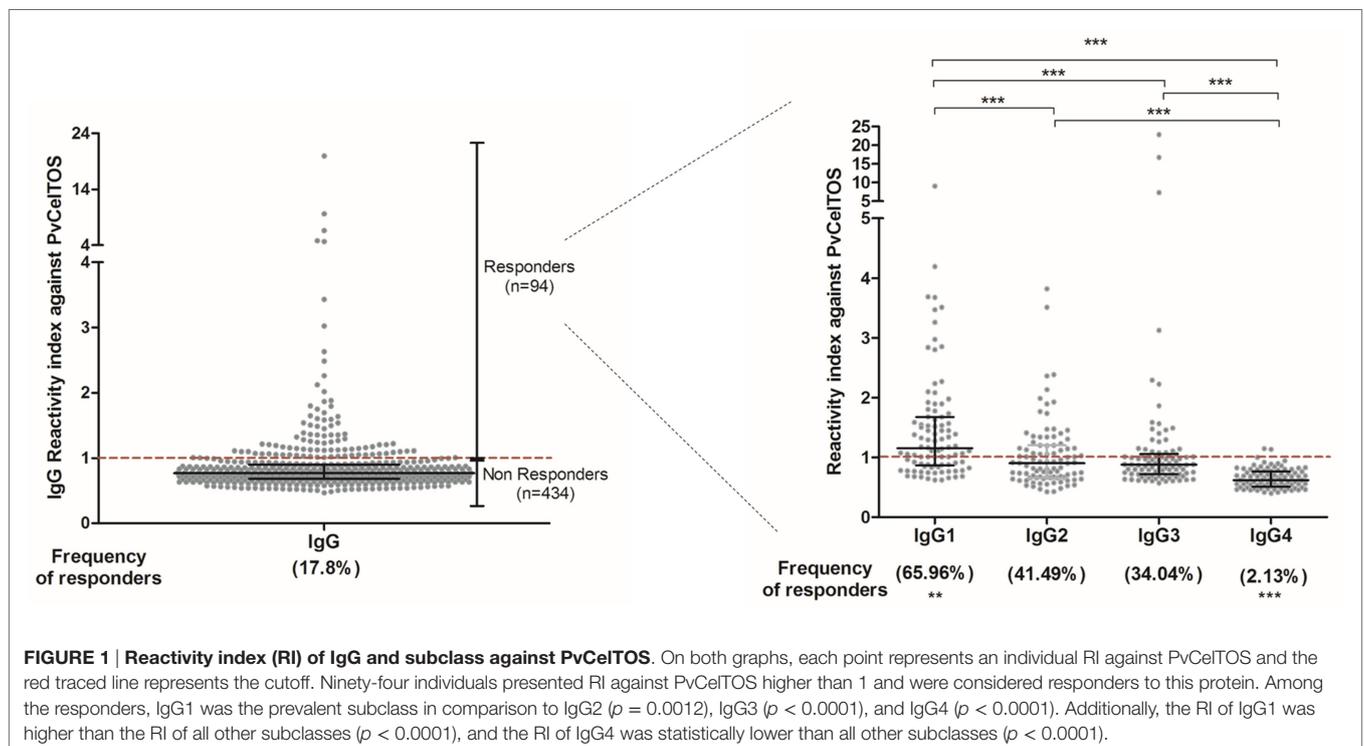
specific IgG antibodies against the protein. Interestingly, the epidemiological data were similar between responders and NRs against this protein (**Table 1**). On both groups, responders and NRs, the age, time of residence in endemic area, the number of previous malaria episodes, the number of recent malaria episodes, the frequency of individuals with recent malaria episodes, and months elapsed from the last malaria episode were similar ($p > 0.05$).

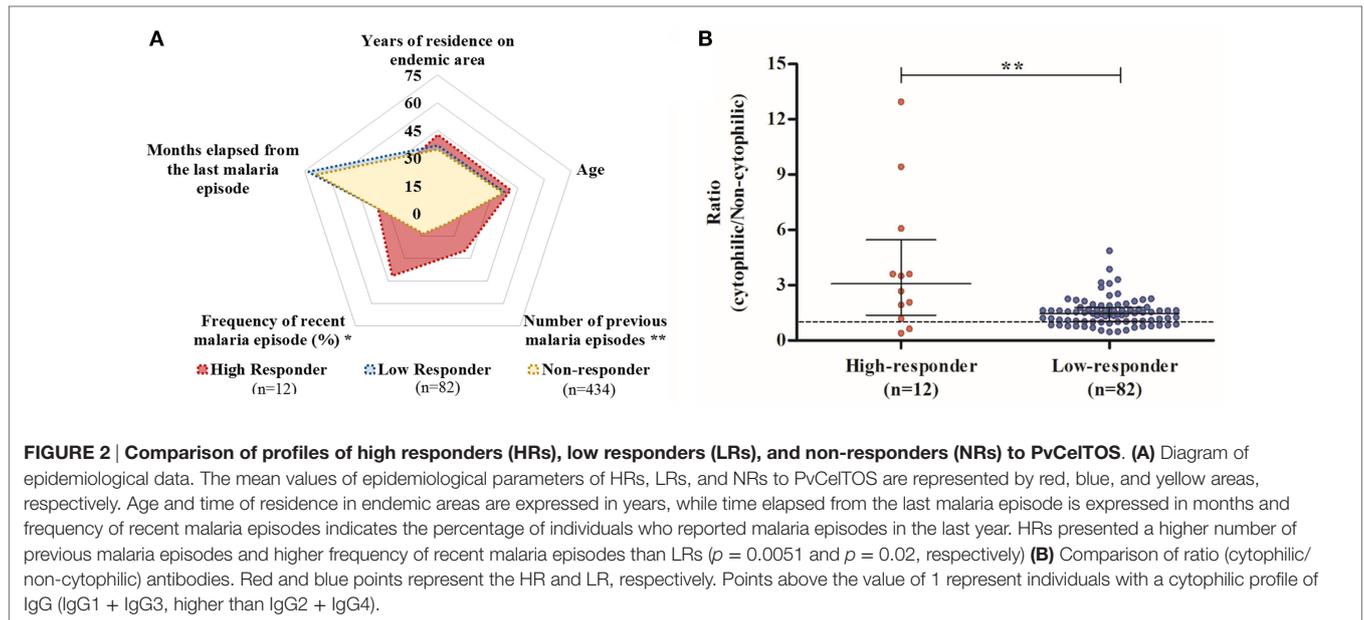
Among the group of responders to PvCelTOS, the RI ranged from 1.01 to 19.93 (median = 1.205; interquartile range = 1.082; 1.552), reflecting a wide spectrum in magnitude of naturally acquired IgG response. The IgG subclass profile was marked by IgG1, the most prevalent subclass, present in 65.96% of responders, and with major RI (median = 1.15; interquartile range = 0.86–1.68) compared to IgG2 (median = 0.9; interquartile range = 0.66–1.2), IgG3 (median = 0.88; interquartile range = 0.72–1.06), and IgG4 (median = 0.62; interquartile range = 0.51–0.76) (**Figure 1**). Moreover, IgG3 RIs were directly correlated to the number of recent malaria episodes ($p = 0.003$; $r = 0.315$; Figure S1A in Supplementary Material) and inversely associated with the time elapsed from the last malaria episode ($p = 0.031$; $r = -0.258$; Figure S1B in Supplementary Material).

High IgG RIs against PvCelTOS Are Driven by Cytophilic Antibodies and Associated with Recent Infections

In order to identify possible factors that could be associated with this large spectrum of reactivity against PvCelTOS in IgG-positive

individuals, we explored epidemiological data among responders. Initially, we observed that the RI against PvCelTOS was directly correlated with the number of previous malaria episodes ($p = 0.047$; $r = 0.227$; Figure S1C in Supplementary Material) and inversely correlated with the time elapsed from the last malaria episode ($p = 0.045$; $r = -0.24$; Figure S1D in Supplementary Material). Based on these findings, responder individuals were divided into two subgroups: high responders (HRs; individuals who had RI of IgG against PvCelTOS higher than 2) and low responders (LRs; individuals who had RI of IgG against PvCelTOS between 1 and 2). **Figure 2A** illustrates the means of epidemiological parameters of HRs, LRs, and NRs to PvCelTOS. Interestingly, while NRs and LRs presented a very similar profile of epidemiological parameters, HRs presented a statistically higher number of previous malaria episodes in comparison to NR and LR ($p = 0.0058$; $p = 0.0051$, respectively). Moreover, despite no statistical differences could be observed on the time elapsed from the last malaria episode ($p = 0.15$ in ANOVA test), the frequency of individuals who reported recent episodes of malaria was higher in HR (41.6%) than LR (12%, $p = 0.02$) and NR (13.1%, $p = 0.016$). Moreover, the proportion of RIs of cytophilic over non-cytophilic antibodies (IgG1 + IgG3/IgG2 + IgG4) presented direct correlation with RI of IgG of responder individuals ($p = 0.0016$; $r = 0.32$), suggesting that higher RI could be associated with a cytophilic profile of humoral response against PvCelTOS. Interestingly, although the proportion of individuals with cytophilic profile was similar in both groups, HR and LR (83% and 78%, respectively), the ratio of (cytophilic/non-cytophilic) antibodies was significantly higher in HR than LR ($p = 0.0076$) (**Figure 2B**).





Five Immunogenic Regions Identified in PvCelTOS and Two Linear B-Cell Epitopes Broadly Recognized by Naturally Acquired IgG Antibodies

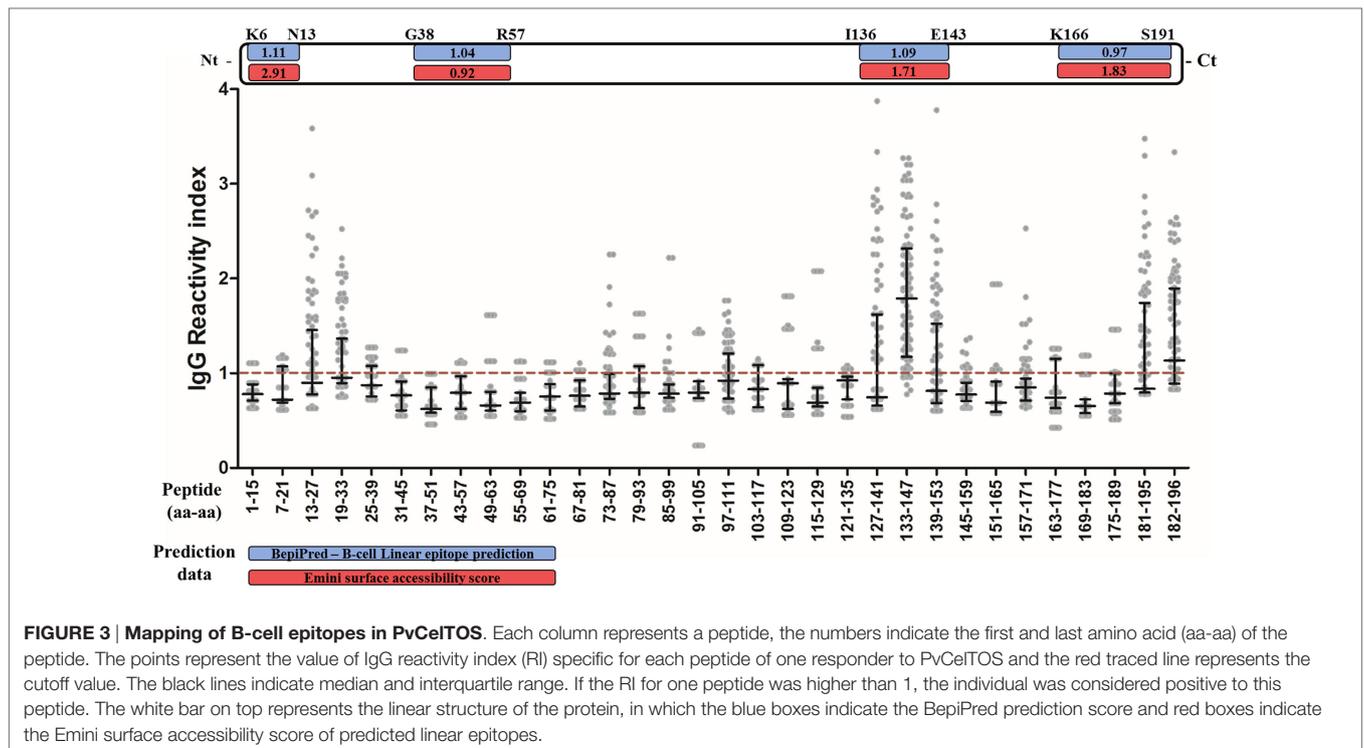
Four B-cell linear epitopes were predicted *in silico* in the entire sequence of PvCelTOS (PvCelTOS_{K6-N13}; PvCelTOS_{G38-R57}; PvCelTOS_{I136-E143}; PvCelTOS_{K166-S191}).

In order to validate the prediction data and identify possible non-predicted immunogenic regions of PvCelTOS, plasma from IgG responders to PvCelTOS was tested against 32 overlapping peptides corresponding to the complete amino acid sequence. First, 10 peptides (N13-L27; S19-V33; E73-I87; L79-K93; S97-A111; P127-V141; I133-G147; P139-V153; L181-L195; E182-D196) were broadly recognized by responders to PvCelTOS (Figure 3). Two of the predicted epitopes (PvCelTOS_{I136-E143} and PvCelTOS_{K166-S191}) were present (partially or entirely) in peptides confirmed as naturally immunogenic. Interestingly, peptides I133-G147 and E182-D196 were recognized by IgG specific antibodies of responders to PvCelTOS in frequencies higher than 50% (92% and 54%, respectively) and presented median of RI higher than 1 (1.79 and 1.14, respectively). In addition, peptides P127-V141, P139-V153, and L181-L195 were located besides the most immunogenic peptides and presented overlapped sequences, which were also recognized by IgG antibodies in moderate frequencies. Peptide I133-G147 (ASTIKPPRVSEDAYF) presented the highest IgG RI ($p < 0.0001$ by ANOVA test) and the highest frequency of recognition (92%) compared to all other peptides. While it contains the entire sequence of predicted epitope PvCelTOS_{I136-E143}, peptides P127-V141 and P139-V153, which contain only the partial sequence of the predicted epitope, presented minor frequencies of recognition (38% and 39%, respectively; $p < 0.0001$ on Fisher's exact test). The peptides L181-L195 and 186-196 were both

partially inserted in the predicted linear epitope PvCelTOS₁₆₆₋₁₉₁ and could be the immune dominant sequence of this longer predicted epitope. These data supported the prediction of linear B-cell epitopes PvCelTOS_{I136-E146} and PvCelTOS_{K166-S191}. Conversely, peptides N13-L27, S19-V33, and S97-A111 also presented frequency of recognition about 40% (38, 40, and 36%, respectively). After the confirmation of five immunogenic regions and two immunodominant epitopes in PvCelTOS, we also compared the RI and frequencies between HR and LR for PvCelTOS. However, no differences were found.

Main B Cell Epitopes Are Present on PvCelTOS Surface

Peptides that presented overlapped amino acids and were recognized by more than 20% of responders to PvCelTOS (Figure 3) were grouped as immunogenic regions. All peptides inserted in identified immunogenic regions are listed in Table 2 with their respective frequencies of recognition, BepiPred and ESA scores. In this context, we identified five immunogenic regions PvCelTOS_{N13-V33}, PvCelTOS_{E73-K93}, PvCelTOS_{S97-A111}, PvCelTOS_{P127-V153}, and PvCelTOS_{L181-D196}, in which B-cell epitopes could be inserted. Interestingly, the peptides with higher frequency of specific responders (I133-G147, L181-L195, and 182-186) presented a good combination of BepiPred and ESA score. The molecular dynamics and electrostatic potential surface of PvCelTOS indicate regions P127-V153, N13-V33, and L181-D186 as more flexible than E73-K93 and S97-A111 (Figure 4A). Regarding solvent exposure, all immunogenic regions were exposed and accessible in solution. Interestingly, the immunogenic regions L181-D196 and E73-K93 are part of a very negatively charged region, while N13-V33 and P127-V153 are in a mostly neutral-positive region (Figure 4B).



DISCUSSION

Despite significant advances in the understanding of the biology of *Plasmodium* parasites and the immune response elicited by these pathogens, there is not yet a subunit vaccine capable of providing long-lasting protection. The cell-traversal protein for ookinetes and sporozoites (CelTOS) has been considered a potential novel alternative for a vaccine against malaria (29, 32, 41), but the knowledge on *P. vivax* CelTOS potential remains scarce. Unfortunately, many conventional vaccinology strategies applied to *P. falciparum* are especially difficult when dealing with non-cultivable microorganisms such as *P. vivax*. Consequently, seroepidemiological studies have played a significant role in the identification and validation of *P. vivax* vaccine candidates (42–48). Therefore, we confirmed the naturally acquired humoral response against PvCelTOS (IgG and IgG subclass) and identified five B-cell epitopes along the entire PvCelTOS amino acid sequence, which were recognized by IgG antibodies from malaria-exposed populations from Brazilian Amazon.

Plasma samples were collected in three cross-sectional studies with Brazilian Amazon communities between 2013 and 2015. The profile of the studied individuals shows that our population included rainforest region natives and migrants from non-endemic areas of Brazil who had lived in the area for more than 10 years. The majority of individuals reported a prior experience with *P. vivax* and/or *P. falciparum* malaria. Concerning malaria history, the highly variable range of number of previous infections, time of residence in endemic areas, and time since the last infection suggests differences in exposure and immunity, since it is well known that the acquisition of clinical immunity mediated by antibodies depends on continued exposure to the parasite

(49–51). The correlation between time of residence in endemic areas and months since the last infection observed in our study also indicates that this phenomenon could be occurring in low/medium endemic areas like the Brazilian Amazon. Therefore, the selection of these individuals was ideal to detect the presence of antibodies against the new recombinant antigen and distinguish whether the alterations found were related to malaria exposure and/or indicative of protection.

First, we found 94 individuals presenting specific antibodies to PvCelTOS and confirmed the natural immunogenicity of PvCelTOS among exposed individuals from Brazilian Amazon. Recently, Longley and collaborators also reported the first evidence of naturally induced IgG responses to PvCelTOS in human volunteers from Western Thailand (33). Interestingly, the frequency of responders to PvCelTOS observed in our studied population (17.8%) was similar to the frequency observed by Longley on uninfected and clinical malaria individuals (33). Moreover, the low humoral reactivity against PvCelTOS is commonly found in other *Plasmodium* preerythrocytic antigens (48, 52, 53). The short life of specific antibodies, host genetic factors, and/or epidemiological parameters could be possible reasons for the low frequency of responders against PvCelTOS in endemic areas. The short life of specific PvCelTOS humoral response hypothesis does not seem to occur since Longley et al. verified that IgG positivity and magnitude of response were present over the 1-year period in the absence of *P. vivax* infections (33). Our study also describes anti-PvCelTOS antibodies in individuals who reported no malaria in the last 10 years or more. However, in both cases, the contact between human host and sporozoite antigens in transmission areas was not evaluated. In relation to host genetic factors, there is a significant body of

TABLE 2 | Identification of immunogenic regions in PvCelTOS.

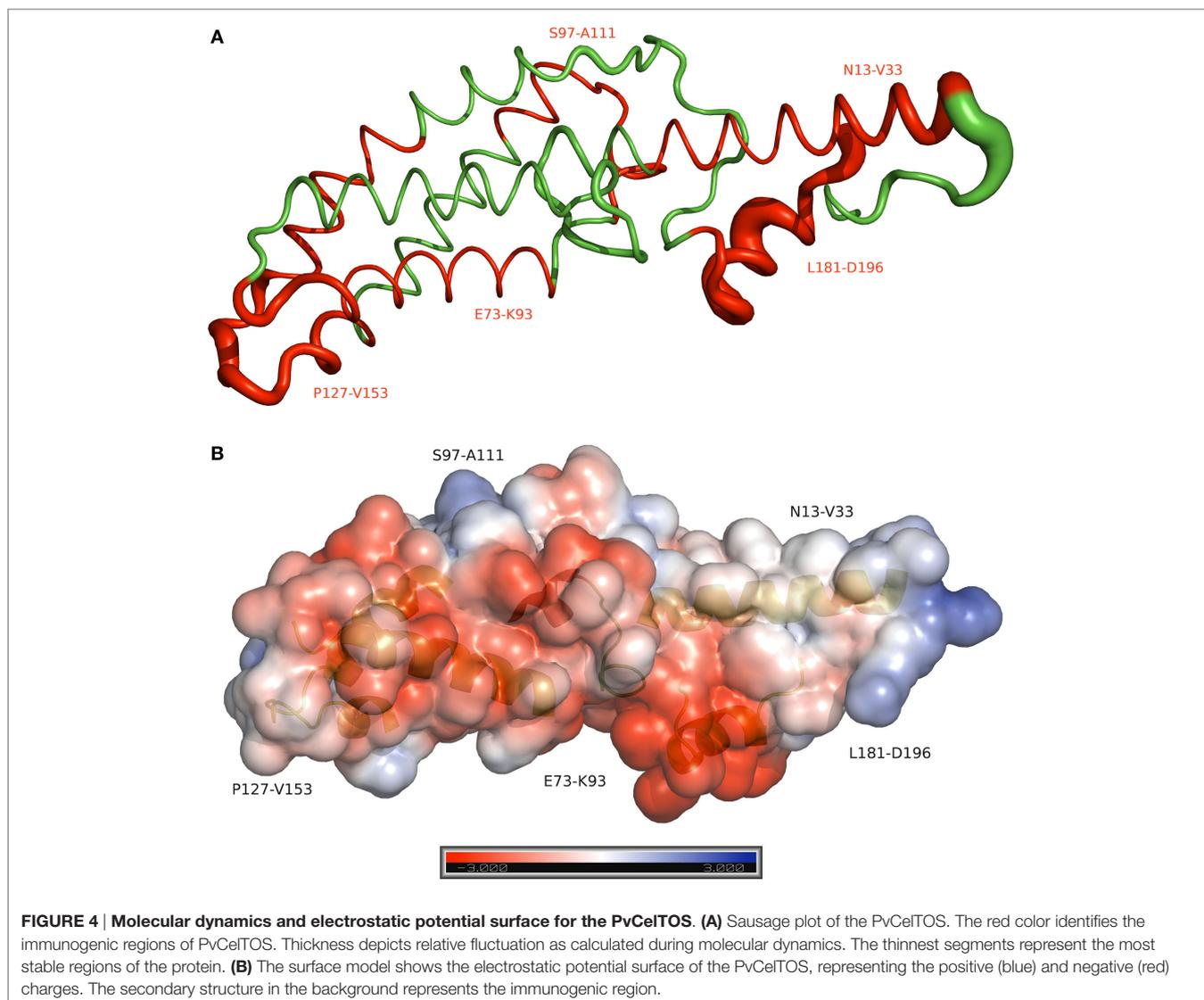
Immunogenic region	Sequence	Reactivity index (RI) Mean (CI 95%)	Responders (%)	Position	Peptide sequence	Specific responders (%)	BepiPred score	ESA score
PvCelTOS _{N13-V33}	NKINRV <u>SIICAF</u> LALFCFVNV	1.17 (1.07–1.27)	45%	13–27	NKINRV <u>SIICAF</u> LAL	38%	-1.52	0.35
PvCelTOS _{E73-K63}	EVIGNELADIANI <u>NEIVSS</u> LQK	0.93 (0.88–0.99)	30%	73–87	<u>SIICAF</u> LALFCFVNV	40%	-2.37	0.07
PvCelTOS _{S97-A111}	SFLOS ^G FDVKTQLKA	0.95 (0.89–1.01)	36%	79–93	LADIANI <u>EVSS</u> LQK	20%	0.14	0.56
PvCelTOS _{P127-V153}	PTEKIVASTIK <u>PRVSEDA</u> YFLLGPW	1.39 (1.27–1.50)	69%	97–111	SFLOS ^G FDVKTQLKA	36%	0.12	0.91
				127–141	PTEKIVASTIK <u>PRV</u>	38%	0.69	0.89
				133–147	IK <u>PRVSEDA</u> YFLLG	92%	0.52	1.14
				139–153	PRVSEDA <u>YFLL</u> GPW	39%	-0.08	0.75
PvCelTOS _{L161-5196}	<u>LEEEEA</u> DEFSDELLD	1.32 (1.21–1.44)	53%	181–195	LEEEEA <u>DEFS</u> DELL	43%	0.95	2.15
				182–196	EEEEAE <u>DEFS</u> DELLD	54%	0.84	2.08

Peptides with overlapping and recognized by more than 20% of responders to PvCelTOS were grouped in immunogenic regions. The RI of an immunogenic region represents the mean of RI of all peptides inserted in that immunogenic region with a confident interval of 95%. The frequency of recognition of immunogenic regions was defined based on the number of individuals with RI to immunogenic region higher than 1. The peptides combined in an immunogenic region were listed with their respective frequencies of recognition, BepiPred score, and Emiri surface accessibility (ESA) score. Overlappedmers were signalized by underlined bold typeface on immunogenic region sequence.

evidences of its influence in malaria outcomes and the capacity to mount a humoral immune response (54–57). To date, associations of HLA class II on humoral immune response to malaria antigens were reported in individuals living in malaria-endemic areas from Brazilian Amazon (58, 59) and in human vaccine trials (60–62). In *P. vivax* preerythrocytic targets, the presence of HLA-DRB1*03 and DR5 was associated with the absence of antibody response to the CSP amino-terminal region (48) and HLA-DRB1*07 was related to the absence of specific antibodies for CSP repeats of VK210 (52). Moreover, Chaves and collaborators reported that PvCelTOS gene sequence is highly conserved among isolates from different Brazilian geographic regions (unpublished data), suggesting a low selective pressure by immune response against PvCelTOS. In our view, the influence of immunogenetic factors in PvCelTOS-specific humoral response are feasible, but more studies are still necessary to confirm this hypothesis.

Regarding the influence of epidemiological factors, we initially tried to investigate the associations between exposition to malaria and the frequency of IgG responders to PvCelTOS. Surprisingly, although the association of epidemiological data with specific response against *Plasmodium* antigens was well characterized on several studies (63–65), we observed a similar epidemiological profile between responders and NRs to PvCelTOS. Therefore, we focused on the search of distinct epidemiological and IgG subclass profiles among PvCelTOS responder individuals. The knowledge about the antibody subclass profile is critical to suggest functional antimalarial immunity and to evaluate potential vaccine candidates. Cytophilic antibodies (IgG1 and IgG3) are frequently prevalent on immune serum from high-transmission areas (66–69) and often correlate with protection from disease (70–72). In our study, IgG1 presented higher frequencies of responders and median RI than all other subclasses. Moreover, IgG3 RIs were directly associated with the number of malaria episodes over the last 12 months and inversely correlated with the time elapsed from the last malaria episode, suggesting that recent *P. vivax* infections can raise the levels of anti-PvCelTOS specific IgG3. The sterile protective immunity to malaria was recently associated with a panel of antigens (28), and the relationship of cytophilic antibodies and reduced risk of symptoms are a common finding in high endemic areas (70–74). However, in our study, concerning the higher levels of IgG1 for PvCelTOS and the association of IgG3 levels with recent infections, we cannot confirm or discard its role as part of protective humoral response until more conclusive studies, such as sporozoite inhibition by anti-PvCelTOS specific antibodies, are conducted. In the same way, among responders, IgG RIs were directly correlated with the number of previous malaria episodes and inversely correlated with the time elapsed from the last malaria episodes, suggesting that antibody levels for PvCelTOS could be associated with recent infections.

The influence of epidemiological parameters on immunity to malaria was previously observed in studies from Brazilian Amazon population. Based on previous studies that associated high levels of antibodies with multiple preerythrocytic antigens with reduced risk of clinical malaria in children (75) and decreased risk of infection in adults (68), we also aimed to investigate if



the epidemiological parameters could reveal new findings about the role of exposition on PvCelTOS immunogenicity. Therefore, we subdivided the large spectrum IgG RIs among PvCelTOS responders into HRs (RI > 2) and LR (RI < 2). Although LR and NR to PvCelTOS presented similar exposition factors to malaria, interestingly, HR individuals presented a remarkable higher number of previous malaria episodes, frequency of recent malaria episodes, and a higher ratio of cytophilic/non-cytophilic antibodies than LR. This observation suggested that higher level of exposition to malaria induced a more intense and improved humoral response against PvCelTOS. 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, which presented no significant association with IgG response against PvCelTOS. Prospective studies on humoral immune responses and studies addressing the ability of these antibodies to interfere the motility/

invasion of sporozoites (76, 77) will provide more evidences of the protective role of anti-PvCelTOS antibodies.

Information at the amino acid level about the epitopes of proteins recognized by antibodies is important for their use as biological tools and for understanding general molecular recognition events (78). In this context, epitope prediction programs have been widely used in malaria research (4, 79–81). Nevertheless, the use of chemically prepared arrays of short peptides is a more powerful tool to identify and characterize epitopes recognized by antibodies (46, 82, 83). It is also important to mention that in order to raise antibodies for a peptide, a minimum length of six amino acids is required, and peptides of >10 amino acids are generally required for the induction of antibodies that may bind to the native protein (84). In this context, the synthesis of 15 amino acid peptides, with 9 overlapping, has allowed the identification of PvCelTOS B-cell epitopes encompassed in sequences ranging from 15 to 27 amino acids in length. Therefore, after the confirmation of PvCelTOS as naturally immunogenic in exposed populations, the present

paper describes for the first time the fine B cell epitope mapping of a full-length protein. Initially, 10 peptides were specifically recognized by naturally acquired antibodies from PvCelTOS responders. After a combination of *in silico* approaches and recognition of overlapped peptides, five immunogenic regions were confirmed (PvCelTOS₁₃₋₃₃, PvCelTOS₇₃₋₉₃, PvCelTOS₉₇₋₁₁₁, PvCelTOS₁₂₇₋₁₅₃, and PvCelTOS₁₈₁₋₁₉₆) in different frequencies and RIs. Moreover, the main linear epitope (ASTIKPRVSEDAYF) presented highest IgG RI and frequency compared to all other naturally recognized peptides, suggesting that the majority of naturally acquired antibodies against PvCelTOS are directed to the C-terminal region. Moreover, T cell responses to PvCelTOS may also help to determine the immunodominant repertoire in individuals living in malaria-endemic regions, which could also supply information for the development of a vaccine for PvCelTOS. In humans, PfCelTOS derivate peptides elicited proliferative and IFN- γ responses in *ex vivo* ELISPOT assays using peripheral blood mononuclear cells from naturally exposed individuals living in Ghana (30).

Recently, CelTOS was demonstrated as highly conserved protein across several large groups of apicomplexan parasites including *Plasmodium* spp., *Cytauxzoon*, *Theileria*, and *Babesia* and considered essential to cell infection, traversal, and membrane disruption (85). Despite the genetical differences between PfCelTOS and PvCelTOS, it is important to mention that Bergmann-Leitner and colleagues immunized mice and rabbits with recombinant PfCelTOS and also observed specific antibodies for linear B-cell epitopes at C-terminal (82). These observations suggested that CelTOS could present a similar conformation among species, with similar regions targeted by antibodies. We considered that the exposition of linear epitopes is a critical step to their recognition by circulating antibodies; therefore, the combination of ESA, molecular dynamics, and electrostatic potential surface was used as a complementary approach to predict the exposition of epitope sequences on protein surface. All immunogenic regions identified were exposed and accessible to antibodies. This finding could be important in a future subunit vaccine composition based on these identified regions. However, the potential of these specific antibodies directed main PvCelTOS epitopes in the inhibition of sporozoite motility, invasion, and/or traversal remains to be investigated.

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AUTHOR CONTRIBUTIONS

JL-J did study designing, performed experiments, data analysis, manuscript preparation, and manuscript review. RR-d-S did study designing, performed experiments, data analysis, and manuscript preparation. IS performed experiments. CL-C did recombinant protein expression and manuscript review. JM did molecular dynamics and bioinformatics and manuscript review. DP-d-S performed collection of blood and epidemiological data. AF did fieldwork support. AT performed collection of blood and epidemiological data and diagnosis. FP did fieldwork support. LC performed experiments. LP-R did data analysis and manuscript review. AR-S did recombinant protein expression, data analysis, and manuscript review. DB did study designing, fieldwork support, manuscript review, and data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00077/full#supplementary-material>.

FIGURE S1 | Associations of humoral response and exposition

parameters in responders to PvCelTOS. (A) Spearman correlation between IgG3 reactivity index (RI) and number of recent malaria episodes; (B) Spearman correlation between IgG3 and months elapsed since the last malaria episode; (C) Spearman correlation between anti-PvCelTOS IgG reactivity index and number of previous malaria episodes; and (D) Spearman correlation between anti-PvCelTOS IgG RI and months elapsed since the last malaria episode.

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