Evaluation of naturally acquired IgG antibodies to a chimeric and non-chimeric recombinant species of Plasmodium vivax reticulocyte binding protein-1: lack of association with HLA-DRB1*/DQB1* in malaria exposed individuals from the Brazilian Amazon

Amanda Ribeiro Ferreira
Oswaldo Cruz Institute

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/oapubs

Part of the Immunity Commons, Immunoprophylaxis and Therapy Commons, Parasitic Diseases Commons, and the Parasitology Commons

Repository Citation
Ferreira AR, Singh B, Cabrera-Mora M, Magri De Souza AC, Queiroz Marques MT, Porto LS, Santos F, Banic DM, Calvo-Calle JM, Oliveira-Ferreira J, Moreno A, Lima-Junior JD. (2014). Evaluation of naturally acquired IgG antibodies to a chimeric and non-chimeric recombinant species of Plasmodium vivax reticulocyte binding protein-1: lack of association with HLA-DRB1*/DQB1* in malaria exposed individuals from the Brazilian Amazon. Open Access Publications by UMMS Authors. https://doi.org/10.1371/journal.pone.0105828. Retrieved from https://escholarship.umassmed.edu/oapubs/2476

Creative Commons License
This work is licensed under a Creative Commons Attribution 4.0 License.
This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in Open Access Publications by UMMS Authors by an authorized administrator of eScholarship@UMassChan. For more information, please contact Lisa.Palmer@umassmed.edu.
Evaluation of Naturally Acquired IgG Antibodies to a Chimeric and Non-Chimeric Recombinant Species of *Plasmodium vivax* Reticulocyte Binding Protein-1: Lack of Association with HLA-DRB1*/DQB1* in Malaria Exposed Individuals from the Brazilian Amazon

Amanda Ribeiro Ferreira¹, Balwan Singh², Monica Cabrera-Mora², Alana Cristina Magri De Souza¹, Maria Teresa Queiroz Marques³, Luis Cristovão Sobrino Porto³, Fatima Santos⁴, Dalma Maria Banic⁵, J. Mauricio Calvo-Calle⁶, Joseli Oliveira-Ferreira¹, Alberto Moreno²,⁷, Josué Da Costa Lima-Junior¹*¹

¹Laboratory of Immunoparasitology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, (FIOCRUZ), Rio de Janeiro, RJ, Brazil, ²Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, United States of America, ³Histocompatibility and Cryopreservation Laboratory, Rio de Janeiro State University, Rio de Janeiro, Brazil, ⁴National Health Foundation, Department of Entomology, Central Laboratory, Porto Velho, RO, Brazil, ⁵Laboratory for Simuliidae and Onchocerciasis, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, (FIOCRUZ), Rio de Janeiro, RJ, Brazil, ⁶Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America, ⁷Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, Georgia, United States of America

Abstract

The development of modular constructs that include antigenic regions targeted by protective immune responses is an attractive approach for subunit vaccine development. However, a main concern of using these vaccine platforms is how to preserve the antigenic identity of conformational B cell epitopes. In the present study we evaluated naturally acquired antibody responses to a chimeric protein engineered to contain a previously defined immunodominant domain of the *Plasmodium vivax* reticulocyte binding protein-1 located between amino acid positions K435-I777. The construct also includes three regions of the cognate protein (F571-D587, I1745-S1786 and L2235-E2263) predicted to contain MHC class II promiscuous T cell epitopes. Plasma samples from 253 naturally exposed individuals were tested against this chimeric protein named PvRMC-RBP1 and a control protein that includes the native sequence PvRBP123-751 in comparative experiments to study the frequency of total IgG and IgG subclass reactivity. HLA-DRB1 and HLA-DQB1 allelic groups were typed by PCR-SSO to evaluate the association between major HLA class II alleles and antibody responses. We found IgG antibodies that recognized the chimeric PvRMC-RBP1 and the PvRBP123-751 in 47.1% and 60% of the studied population, respectively. Moreover, the reactivity index against both proteins was comparable and associated with time of exposure (p<0.0001) and number of previous malaria episodes (p<0.005). IgG subclass profile showed a predominance of cytophilic IgG1 over other subclasses against both proteins tested. Collectively these studies suggest that the chimeric PvRMC-RBP1 protein retained antigenic determinants in the PvRBP123-751 native sequence. Although 52.9% of the population did not present detectable titers of antibodies to PvRMC-RBP1, genetic restriction to this chimeric protein does not seem to occur, since no association was observed between the HLA-DRB1* or HLA-DQB1* alleles and the antibody responses. This experimental evidence strongly suggests that the identity of the conformational B cell epitopes is preserved in the chimeric protein.

Citation: Ferreira AR, Singh B, Cabrera-Mora M, Magri De Souza AC, Queiroz Marques MT, et al. (2014) Evaluation of Naturally Acquired IgG Antibodies to a Chimeric and Non-Chimeric Recombinant Species of *Plasmodium vivax* Reticulocyte Binding Protein-1: Lack of Association with HLA-DRB1*/DQB1* in Malaria Exposed Individuals from the Brazilian Amazon. PLoS ONE 9(8): e105828. doi:10.1371/journal.pone.0105828

Editor: Luzia Helena Carvalho, Centro de Pesquisa Rene Rachou/Fundação Oswaldo Cruz (Fiocruz-Minas), Brazil

Received April 15, 2014; Accepted July 24, 2014; Published August 22, 2014

Copyright: © 2014 Ferreira et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This research was supported by the Brazilian National Research Council - CNPq, Fiocruz, US National Institutes of Health, NIAID grant R01-AI064766, R21AI094402-01 and R21AI095718-01 and the Yerkes National Primate Research Center Base Grant No RR00165 awarded by the National Center for Research Resources of the National Institutes of Health. JMCC’s involvement was supported by NIH-U19-57319. ARF was the recipient of a CNPq Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: alberto.moreno@emory.edu (AM); josue@ioc.fiocruz.br (JCLJ)

Introduction

Malaria is the most relevant parasitic disease and a leading cause of mortality in developing countries. The World Health Organization estimates that malaria was responsible for 207 million clinical cases and 627,000 deaths in 2012 [1]. The enormous progress in the implementation of malaria control measures accounts for a 45% reduction in mortality rates in the past 12 years due to their impact on *Plasmodium falciparum* malaria. These measures include long-lasting insecticidal nets
the amino terminal sequence PvRBP1435-777 within this fragment poorly immunogenic functional domains of are poorly immunogenic or may elicit antibody responses that are transmission or differences in host population genetics. This can be vivax recombinant proteins suggesting differences in malaria reported by Tran et al. was lower than that observed with other Intriguingly, the prevalence of IgG antibodies against PvRBP1 contains the most polymorphic region of the protein suggesting resistance, high prevalence of severe malaria and mortality, the old concept that P. vivax infections are clinically “benign” is not currently accepted. It is therefore imperative to develop novel strategies for malaria control including vaccines. The unique biological features of P. vivax particularly the production of hypnozoites and invasion of reticulocytes have delayed the development of experimental systems to understand parasite-host interactions. Progress toward the development of an effective vaccine has been therefore mainly focused on characterization of proteins orthologous to P. falciparum. Using this approach, vaccine candidates that include the pre-erythrocytic P. vivax circumsporozoite protein (CSP), and the sexual stage 25 kDa protein (PvX25) have been unsuccessfully tested in clinical trials [2–7]. Based on the evidence that P. vivax in contrast to P. falciparum uses the Duffy binding protein (DBP) as a critical invasion ligand, this protein has been broadly studied as a vaccine candidate. DBP is localized within the merozoite’s apical microneme organelles, is a member of the DBP-like erythrocyte binding protein (DBP-EBP) family and is a target of neutralizing antibodies involved in the inhibition of erythrocyte invasion [8]. Data derived from studies in endemic areas of malaria have shown that in natural conditions exposed individuals can develop broadly reactive antibodies that increase with age [9]. However, recent evidence indicates that P. vivax can infect Duffy group-negative individuals [10]. The merozoite proteins PvMSP-3 [11–14] and PvMSP-9 [13,15,16] and the apical pole protein Reticulocyte Binding Protein-1 (PvRBP1) have been also considered as potential vaccine candidates against P. vivax malaria. A less characterized vaccine candidate is the P. vivax Reticulocyte Binding Protein-1 (PvRBP1) that forms a complex with PvRBP2 at the apical pole of the merozoite [17,18]. It has been proposed that PvRBP1s participate in a cascade of events involved in invasion by specific interaction with reticulocytes and subsequent release of the DBP essential for the junction formation step required for merozoite entry into the target host cell [19]. PvRBP1 is a relatively large Type I integral membrane protein that spans over 2,800 amino acids. The amino terminal region of the protein contains a cluster of polymorphic residues suggesting immune selection pressure [20]. The specificity of naturally acquired antibodies reactive against PvRBP1 has been reported in three different malaria exposed populations from the Brazilian Amazon [21]. High prevalence of naturally acquired antibodies against a region that spans 976 amino acids (PvRBP1[131-1407]) was reported in this study [21]. Interestingly, the fragment representing the amino terminal sequence PvRBP1[135-777] within this fragment contains the most polymorphic region of the protein suggesting that it could be the target of functional antibodies [21]. Intriguingly, the prevalence of IgG antibodies against PvRBP1 reported by Tran et al. was lower than that observed with other P. vivax recombinant proteins suggesting differences in malaria transmission or differences in host population genetics. This can be consistent with the finding that Plasmodium polymorphic proteins are poorly immunogenic or may elicit antibody responses that are short-lived in the absence of frequent natural boosting [22]. We have reported that the genetic linkage of cognate T cell epitopes to poorly immunogenic functional domains of Plasmodium proteins can significantly improve their immunogenicity [23,24]. Here we report the design of a chimeric PvRBP1 synthetic gene, codon optimized for expression in E. coli, that encodes a protein that includes three predicted putative promiscuous T cell epitopes, derived from different regions of the native PvRBP1 protein, arrayed in tandem and genetically fused to the immunodominant PvRBP1[135-777] region. Since direct comparisons of the natural antibody response to these antigens provide valuable insight into how such a vaccine might work, we aimed to evaluate if the chimeric protein named PvRMC-RBP1 maintains the structural features that identified the native PvRBP1[135-777] region as a target of naturally acquired antibodies. Comparative seroepidemiological studies were done using PvRMC-RBP1, a non-chimeric control protein PvRBP1[135-777] and a panel of plasma samples from naturally exposed individuals with diverse HLA alleles.

Material and Methods

Study area and volunteers

The study involved 253 different individuals from communities in the malaria endemic region of Rondonia, a state in the western Amazon region of Brazil, where in the last five years P. vivax malaria accounted for more than 70% of all malaria cases. Samples and survey data were collected during the dry months of June-August of 2004 (n = 202) and 2007 (n = 51), coinciding with the period of increased malaria transmission in this region. The majority of the studied population consists of rain forest natives who have resided in the malaria-endemic region for over 25 years or transmigrants from several non-endemic areas of Brazil that have lived in Rondonia for 10 years or more. In addition, we have included as a control 30 individuals living in non-endemic regions of Brazil (Rio de Janeiro) with no history of malaria and who never resided in endemic areas. The study was reviewed and approved by the Oswaldo Cruz Foundation Ethical Committee and the National Ethical Committee of Brazil.

Epidemiological survey

During the active case detection, subjects were informed about the forms of malaria transmission, preventive measures and the research project. Individuals who agreed to participate in our study signed an informed consent document formalizing their participation as volunteers. All volunteers were interviewed to gather personal and epidemiological data with questions related to demographics, time of residence in the endemic area, personal and family histories of malaria, use of malaria prophylaxis, presence of malaria symptoms, and personal knowledge of malaria. Survey data was entered into a database created with Epi Info 2002 (Centers for Disease Control and Prevention, Atlanta, GA).

Human blood samples and malaria diagnosis

Blood samples (10 ml) were collected in heparinized tubes to obtain plasma used in the study. Plasma from all blood samples was separated, stored at –20°C and shipped on dry ice to the Immunoparasitology Laboratory, IOC, Fiocruz. Malaria diagnosis was made on thick and thin blood smears stained with Giemsa (Sigma Chemical Co., St. Louis, USA). Parasitemia for smear positive donors was determined by counting the number of parasites (all species and stages present) per 200 leukocytes in the thick smear. All smear-positive donors were subsequently treated for P. vivax or P. falciparum per the regimen recommended by the Brazilian Ministry of Health.

Recombinant proteins

A 1437 bp synthetic chimeric RMC-Pvrbp1 gene was commercially produced by Geneart (Thermo Fisher, Regensburg, Germany) using proprietary technology platforms. The codon
Figure 1. Schematic representation (A) of the recombinant proteins reported here. The control protein PvRBP123-751 was expressed as an amino terminal thioredoxin (TXR)/6-histidine double fusion protein. PvRMC-RBP1 recombinant protein includes the promiscuous T cell epitopes FYLMQIRKINTETKID, IFIKLKLKEYDMTGDLKNYGVKMNEIHGEFTKSYNLKSECONDARY E tag for protein purification. PvRMC-RBP1 also includes a C-terminal His tag that was added to the protein via the expression vector. (B) Sequence of the PvRMC-RBP1 naturally acquired IgG antibodies against chimeric PvRBP1. Six copies of a P. falciparum sequence, derived from the repeat region of the circumsporozoite protein (NANP), were included at the C-terminus for biochemical characterization and to provide a secondary tag for protein purification.
usage was adapted to the codon bias of *E. coli* genes using a proprietary algorithm (GeneOptimizer). The procedure was optimized to avoid AT-rich or GC-rich sequence stretches, internal TATA boxes and repeat sequences and RNA secondary structures. The optimized gene resulted in a high Codon Adaptation Index (CAI) value of 0.95 that resulted in high and stable expression rates in *E. coli* [25]. The synthetic gene encodes a chimeric protein based on the *P. vivax* RBP1 (GenBank: AAS85750.1), with the topology: MA-F 571-D587-GPGPG-I1745-S1786-GPGPG-L2235-E2263 (Figure 1) designated as a PvRMC-RBP1. The protein topology is similar to that reported by us for the development of a chimeric vaccine construct based on the *P. yoelii* merozoite surface protein-1 [23]. PvRMC-RBP1 includes three putative promiscuous T cell epitopes FYLMQIRKINTEKTKID (F571-D587); IFIKLKLKEYDMTGDLKNYYGVKMNEGFYKSYNLIETHLS (I1745-S1786) and LYLFHQNSDISIVEGGVQNMLAYDKLINE (L2235-E2263) arrayed in tandem at the amino terminus. These regions were predicted to contain promiscuous binding peptide sequences that can bind to several HLA class II alleles as described below. Synthetic peptides were used to validate predicted epitopes as target of T cell recognition (manuscript in preparation). Validated T cell epitopes were then genetically linked to the *P. vivax* RBP1 region K435-I777 Belem strain that overlaps a previously described immune-dominant fragment [21]. GPGPG spacers were inserted between the individual promiscuous T cell epitopes and between L2235-E2263 and the native sequence K435-I777. Six copies of a *P. falciparum* sequence, derived from the repeat region of the circumsporozoite protein (NANP)6, were included at the carboxyl terminal end for biochemical characterization of antigenic integrity and to provide an optional affinity purification tag. The chimeric *Pvrmp1* was excised with NcoI and SacI restriction enzymes and ligated into a linearized C-terminal His tag expression vector (pET24d(+), Novagen).

Table 1. Predicted population coverage rate for PvRMC-RBP1 as predicted by IEDB.

| Population/Area | Coverage | Average hit | PC90 |
|----------------|----------|-------------|------|
| Amerindian     | 98.45%   | 2.82        | 1.78 |
| Arab           | 95.03%   | 2.21        | 1.23 |
| Asian          | 98.39%   | 2.75        | 1.72 |
| Australian Aborigines | 95.66% | 2.2 | 1.26 |
| Austronesian   | 94.68%   | 2.07        | 1.19 |
| Berber         | 98.71%   | 2.25        | 1.53 |
| Black          | 99.77%   | 3.86        | 2.67 |
| Caucasian      | 99.63%   | 3.13        | 2.16 |
| Hispanic       | 99.80%   | 3.47        | 2.46 |
| Jew            | 97.93%   | 2.42        | 1.49 |
| Kurd           | 97.12%   | 2.28        | 1.38 |
| Melanesian     | 98.99%   | 2.8         | 1.97 |
| Mestizo        | 99.39%   | 3.18        | 2.18 |
| Micronesian    | 97.29%   | 2.43        | 1.45 |
| Mixed          | 99.56%   | 3.22        | 2.23 |
| Mulatto        | 96.16%   | 2.05        | 1.27 |
| Oriental       | 97.17%   | 2.54        | 1.64 |
| Persian        | 98.17%   | 2.56        | 1.6 |
| Polynesian     | 99.33%   | 2.97        | 2.08 |
| Siberian       | 99.01%   | 2.91        | 2.02 |
| Average        | 98.01%   | 2.71        | 1.76 |
| (Standard deviation) | (1.56%) | (0.48) | (0.43) |

*a*Projected population coverage [30].

*b*Average number of epitope hits/HLA combinations recognized by the population.

*c*Minimum number of epitope hits/HLA combinations recognized by 90% of the population.

doi:10.1371/journal.pone.0105828.g001
A control recombinant PvRBP1 protein, with a C-terminal region that overlaps the immunodominant domain E23-V751 was generated by PCR amplification using the *P. vivax* Belem strain as a template [21] and the following primers: forward 5\'GGAGATT-CAGAAATATGACAGAGCAATAGGA3\' and reverse 5\'GGAGATT-CTCTCTCTATATTTAGAAATGTTTGC3\' containing EcoRI restriction sites (underlined). The high fidelity thermostable KOD Thermococcus kodakaraensis KOD1 DNA polymerase system [26] was utilized following the manufacturer’s instructions (Novagen, WI). The insert was confirmed by nucleotide sequencing. The PCR product, kindly provided by Mary Galinski (Emory University), was subcloned into pET32b (Novagen) for expression as amino terminal thioredoxin-6His double fusion protein. Expression of the PvRMC-RBP1 and the non-chimeric PvRBP1 was performed as described [21,23] by re-transforming the positive clones into E. coli BL21 (DE3) cells (Novagen, Madison, WI) with kanamycin selection. Protein expression was induced with 1 mM IPTG for 3 hours, following standard procedures. The recombinant proteins were purified with a Ni-NTA affinity column according to the manufacturer’s instructions (Qiagen, Valencia, CA). Thioredoxin was expressed and purified using similar procedures and used to determine the background antibody reactivity of the fusion protein.

To characterize the PvRMC-RBP1 protein, groups of five female BALB/c mice (8–10 weeks of age; Charles River, Wilmington, MA) were immunized with synthetic peptides representing the T cell epitopes I1745-S1786 and L2235-E2263. The female BALB/c mice (8–10 weeks of age; Charles River, Valencia, CA) were immunized with recombinant thioredoxin-6His protein. Expression of the PvRMC-RBP1 and the non-chimeric PvRBP1 was performed as described [21,23] by re-transforming the positive clones into E. coli BL21 (DE3) cells (Novagen, Madison, WI) with kanamycin selection. Protein expression was induced with 1 mM IPTG for 3 hours, following standard procedures. The recombinant proteins were purified with a Ni-NTA affinity column according to the manufacturer’s instructions (Qiagen, Valencia, CA). Thioredoxin was expressed and purified using similar procedures and used to determine the background reactivity of the fusion protein.

Prediction of T cell epitopes

PvRBP-1 regions containing peptide sequences promiscous for binding to HLA class II molecules were initially predicted by using the ProPred algorithm [28]. The IEDB server (http://www.iedb.org/) was used for subsequent analysis including allele binding score and population coverage [29]. Predictions were performed for: 1) Twenty two DRB1*: 01:01; 01:02; 03:01; 03:02; 04:01; 04:02; 04:03; 04:04; 04:05; 07:01; 08:01; 09:01; 09:02; 11:01; 11:02; 12:01; 13:01; 13:02; 14:01; 15:01; 15:02. 2) Nine DQ/DP: HLA-DQA1*0501/HLA-DQB1*0602; HLA-DQA1*0901/HLA-DQB1*0602; HLA-DQA1*1001/HLA-DQB1*0902; HLA-DQA1*0903/HLA-DQB1*0302; HLA-DQA1*0901/HLA-DQB1*0401; HLA-DQA1*0501/HLA-DQB1*0402; HLA-DQA1*0501/HLA-DQB1*0201. 3) Three DRB3*: HLA-DRB3*0101; HLA-DRB3*0201; HLA-DRB3*0301. 4) One HLA-DRB1*0101 and 5) One HLA-DRB5*0101. Most predictions used the consensus algorithm in which scores equal or lower than 10 are considered to bind to the MHC allele interrogated. The fraction of individuals in human populations responding to PvRMC-RBP1 was calculated by using a computational tool provided by the IEDB server (http://tools.immunepeptide.org/tools/population/). [30]. This server uses MHC allele frequencies in human populations to predict the fraction of individuals that might respond to a given T cell epitope when presented in the context of the MHC allele investigated. Briefly, MHC class II peptide binders were predicted in the PvRMC-RBP1 protein sequence for 22, 10 and 5 major DRB1, DQ and DRB5/65 major MHC alleles, respectively. MHC Alleles predicted to bind sequences in PvRMC-RBP1 were used as “MHC restricted allele” input and “all population groups” presented in ethnicity field for output (Table 1).

Antibody assays

Plasma samples from study participants were screened by ELISA for the presence of naturally acquired antibodies against the recombinant proteins. Briefly, Maxisorp 96-well plates (Nunc, Rochester, NY) were coated with 200 ng of the recombinant protein. After overnight incubation at 4°C, plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with PBS-Tween containing 5% non-fat dry milk (PBS-Tween-M) for 1 hour at 37°C. Individual plasma samples diluted 1:100 in PBS-Tween-M were added in duplicate wells and the plates incubated at 37°C for 1 h. After four washes with PBS-Tween, peroxidase conjugated goat anti-human total IgG (Sigma St. Louis, MO) diluted by 1:1000 was added and plates were incubated and washed as described above. Finally o-phenylene-diamine and hydrogen peroxide were used to reveal bound antibodies. The absorbance was read at 492 nm using a Spectramax 250 ELISA reader (Molecular Devices, Sunnyvale, CA). To determine specific reactivity to the control protein the averaged OD value to thioredoxin alone was subtracted from the averaged OD value to the fusion protein. This procedure was not required for the chimeric PvRMC-RBP1 given that only the His tag was incorporated into C-terminus of the protein. The results for total IgG were expressed as reactivity indexes (RI) that were calculated by dividing the mean optical density of tested samples by the mean optical density plus 3 standard deviations of 5 non-exposed controls tested on each plate. Subjects were scored positive for serum IgG to a particular antigen if the RI was higher than 1. IgG subclasses were determined in individual responders by ELISA as described above where the following peroxidase conjugated monoclonal mouse anti-human antibodies were used: Mouse Anti-Human IgG (hinge)-HRP (clone HP6001, Southern Biotechnology), Mouse Anti-Human IgG2 (Fc)-HRP (clone HP6002, Southern Biotechnology), Mouse Anti-Human IgG3 (hinge)-HRP (clone HP6050, Southern Biotechnology), Mouse Anti-Human IgG4 (Fc)-HRP (clone HP6023, Southern Biotechnology), all diluted by 1:1000. Subclass-specific prevalence for each antigen was determined from OD values using 3 S.D. above the appropriate mean OD of four non-exposed controls as the cutoff for positivity.

Absorption treatment ELISA

To ensure that the naturally acquired antibodies detected in ELISA were directed to PvRMC-RBP1 and not to the (NANP)\_n peptide tagged for biochemical characterization, we also performed an IgG absorption ELISA protocol using a synthetic (NANP)\_n peptide. Briefly, flat-bottom plates (NUNC, USA) were coated overnight with 5 μg/mL of the peptide (NANP)\_n. After washing and blocking steps, plasma from 62 randomly selected PvRMC-RBP1 IgG responders were added to the plates at a 1:100 dilution and incubated for two hours. After incubation, plasma samples were transferred to plates coated with PvRMC-RBP1 (200 ng) and the ELISA was performed as previously described.

HLA Genotyping of PBMC

Genomic DNA was isolated from whole blood drawn in EDTA by using a mixture of 5 ml buffer G2 (QIAamp DNA Blood Midi Kit; Qiagen Inc., Chatsworth, CA, USA) and 95 μl proteinase K (20 mg/ml). After incubation at 50°C for 1 h the DNA was ethanol precipitated, collected with a glass stick and transferred into distilled water. DNA concentration and quality was checked with a NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sequence-specific oligonucleotide probes (SSOPs) and Luminex xMAP technology were used to determine the HLA-DRB1 and HLA-DQB1 allelic groups.
of the study populations. Briefly, the system is based on probe arrays bound to color-coded plastic microspheres, and locus-specific biotinylated primers for HLA-DRB1 and HLA-DQB1 loci (LABType, One Lambda Inc, Canoga Park, CA, USA). Biotinylated amplicons were denatured to ssDNA and incubated with DNA complementary probes immobilized on fluorescent coded microspheres (beads) followed by incubation with R-phycocerythrin conjugated to streptavidin. After hybridization, the samples were analyzed using a Luminex Flow Analyzer. The HLA Visual 2.0 software (One Lambda, CA, USA) was used to identify which independent variables (TREA, PMI and TLI) were related to the dependent variable (IgG Reactivity index). Allelic groups were grouped by DR status and data were descriptively summarized using frequencies and percentages for all categorical variables. Overall associations of immunological responses with the alleles from each HLA-DRB1* and HLA-DQB1* loci were evaluated by comparing the allele frequencies between seronegative subjects and seropositive subjects using the Pearson Chi-square test where appropriate. Student’s t test was used to compare the means of normally distributed data or normalized transformations were performed on raw data before testing by one-way ANOVA where appropriate. Differences in the proportions of the frequencies between variables were evaluated by chi-square (χ²) test. Relationships between years of residence in the endemic area and number of past malaria episodes or months since last known malaria episode were assessed with Spearman’s rank correlation. Stepwise multiple linear regressions were also used to identify which independent variables (TREA, PMI and TLI) were related to the dependent variable (IgG Reactivity index). Allelic groups were grouped by DR status and data were descriptively summarized using frequencies and percentages for all categorical variables. Overall associations of immunological responses with the alleles from each HLA-DRB1* and HLA-DQB1* loci were evaluated by comparing the allele frequencies between seronegative subjects and seropositive subjects using standard contingency tables. Each person contributed two observations to the table (one for each allele). Rare alleles, defined as those with less than five occurrences among subjects, were all pooled into a category labeled “other” for analysis. To evaluate global differences in allele distribution, we performed analyses using simulation methods as implemented in the software PASW. This approach randomly generates new cell counts for contingency tables under the null hypothesis of no association, while keeping the margins of the table fixed. We used an approach that compares each allele versus all others combined, resulting in multiple 2×2 tables, and used the maximum Chi-square statistic from this series of tables as a global test statistic (bipartition). All statistical tests were two-sided and HLA analyses were conducted using the PASW software system.

### Results

**PvRBP1 recombinant proteins**

In order to develop a PvRBP1 subunit vaccine candidate, we selected in this large molecular weight protein the region spanning the amino acids 435 to 777. This fragment was selected based on the following considerations: 1) naturally exposed individuals develop antibodies which preferentially recognize this extracellular domain [21]; 2) this fragment includes a cluster of polymorphic residues suggesting that could be the target of protective antibodies and 3) it has been implied to include a functionally relevant binding domain [20]. A caveat to this approach is the loss of regions that elicit T helper responses required for the induction of antibody responses. In fact, the use of a MHC peptide-binding prediction algorithm (ProPred) indicated that although the 435–777 PvRBP1 fragment includes regions recognized by most major MHC class II DR and DQ MHC alleles, this fragment excluded several regions with very high potential for the induction of T helper responses (Table 2). As shown in Table 2, the three selected sequences have mean scores of 0.2 indicating a great potential for binding to a large number of MHC alleles. Peptides with IEDB scores below 10 are considered high binders [29]. Even more important, the predicted alleles cover a large proportion of populations in malaria endemic areas (Table 1).

The synthetic gene encoding the PvRMC-RBP1 was codon optimized for expression in *E. coli*. The gene encoding the PvRBP123-751 control protein was produced by PCR amplification and overlaps the functional domain K435–I777 included in PvRMC-RBP1 (Figure 1). PvRMC-RBP1, PvRBP123-751 and

### Table 2. PvRBP1-435-777 and the three regions selected to design PvRMC-RBP1 are predicted to contain peptide sequences that bind to multiple HLA class II molecules.

| MHC | Alleles evaluated | PvRBP1 region* | Alleles with predicted ≤ 10 scoreb | Geomean score | Min score | Max score |
|-----|------------------|----------------|-----------------------------------|---------------|-----------|-----------|
| DRB1 | 22 | K435-777 | 22 | 4.4 | 0.1 | 10.0 |
|     |     | F_{271-D_{267}} | 19 | 0.2 | 2.4 | 8.8 |
|     |     | l_{1745-S_{1786}} | 17 | 0.2 | 3.1 | 10.0 |
|     |     | l_{2235-F_{2063}} | 16 | 0.2 | 4.8 | 9.9 |
| DQ  | 10 | K435-777 | 9 | 3.4 | 0.1 | 9.9 |
|     |     | F_{271-D_{267}} | 2 | 6.6 | 4.9 | 8.8 |
|     |     | l_{1745-S_{1786}} | 3 | 8.2 | 6.0 | 9.9 |
|     |     | l_{2235-F_{2063}} | 8 | 7.5 | 6.0 | 9.7 |
| DRB1/B5 | 5 | K435-777 | 5 | 2.7 | 0.0 | 9.9 |
|     |     | F_{271-D_{267}} | 4 | 6.1 | 1.6 | 9.4 |
|     |     | l_{1745-S_{1786}} | 1 | 4.2 | 2.2 | 6.6 |
|     |     | l_{2235-F_{2063}} | 4 | 0.7 | 4.8 | 9.1 |

*Score generated by the IEDB server (http://www.iedb.org/ and [29]).

doi:10.1371/journal.pone.0105828.t002
Table 3. Summary of the epidemiological characteristics of studied individuals enrolled in the survey.

| Epidemiological characteristics | N (%) | X²  | P      |
|--------------------------------|-------|-----|--------|
| **Gender**                    |       |     |        |
| Female                        | 103 (40.7%) | 17.46 | p<0.0001 |
| Male                          | 150 (59.3%)  |      |        |
| Total                         | 253 (100%) |      |        |
| **Age (Mean ± SD)**           |       | 35±16.9 |        |
| **Malaria exposure**          |       |     |        |
| Time of residence in malaria endemic area | 30±16.5 | |        |
| Time of residence in Rondonia | 24±15.2 | |        |
| Time of residence in current Address | 8±10 | |        |
| Number of past malaria infections | 6.7±7.58 | |        |
| Past months since the last malaria infection | 156±312 | |        |
| **Previous malaria species contracted** |       |     |        |
| P. falciparum                 | 28 (11.0%) | |        |
| P. vivax                      | 56 (22.1%) | |        |
| Both species                  | 128 (50.5%) | |        |
| Never infected/Not reported   | 41 (16.2%) | |        |
| **Species of the last infection** |       |     |        |
| P. falciparum                 | 67 (26.4%) | |        |
| P. vivax                      | 114 (45.0%) | |        |
| P. falciparum + P. vivax      | 11 (4.3%) | |        |
| Never infected/Not reported   | 61 (24.1%) | |        |
| **Diagnosis**                 |       |     |        |
| P. falciparum                 | 07 (2.7%) | |        |
| P.vivax                       | 18 (7.11%) | |        |
| P.falciparum+P.vivax          | 0 (0%) | |        |
| Not infected                  | 228 (90.1%) | |        |

doi:10.1371/journal.pone.0105828.t003

Figure 2. Naturally acquired IgG antibodies against PvRMC-RBP1 and PvRBP123-751 recombinant proteins. (A) Frequency of IgG responders in the studied population to the recombinant chimeric protein PvRMC-RBP1 and non-chimeric PvRBP123-751. The Chi squared test for proportions analyses was performed to determine statistical differences. The frequency of IgG responders to PvRBP123-751 was significantly higher when compared to PvRMC-RBP1. (B) The median of the IgG reactivity index against both antigens was not significant (Mann Whitney test, p = 0.6833). (C) Reactivity indexes of IgG antibodies against PvRMC-RBP1 and PvRBP123-751 showed significant correlation by nonparametric Spearman test (r = 0.5768; p<0.0001). doi:10.1371/journal.pone.0105828.g002
thioredoxin were purified from *E. coli* lysates by metal chelate chromatography using a Ni-NTA resin. Analyses by SDS-PAGE showed that purified proteins migrated as single bands of the apparent mobility of 55 kDa for PvRMC-RBP1 and 100 kDa for PvRBP123-751 (Figure 1C). Biochemical identity of the recombinant proteins was established by western blot analysis using polyclonal antibodies recognizing the T cell epitopes I1745-S1786 and L2235-E2263 and a His-tag monoclonal antibody (Figure 1G).

**Epidemiological and demographical data**

In this population the majority were adults and all individuals were exposed to malaria infection (Table 3). The age range was 10–85 years with an average of 35 years and the proportion of men was significantly higher (59.3%) than for women (40.7%; \( \chi^2 = 17.46, p < 0.0001 \)). Concerning prior history of malaria infections, 15.9% of all studied individuals did not have or remember previous malaria infections. Among those who reported previous infections, the majority (50.8%) reported previous episodes of *P. falciparum* and *P. vivax* malaria, the two most prevalent species in Brazil [31]. The number of past infections reported by individuals varied greatly among donors, ranging from 0 to 51 (mean = 6.74 ± 7.58). Finally, the fact that the time elapsed since the last infection varied from 0 to 372 months (mean = 156 ± 312) indicated that the studied population have different degrees of exposure and/or immunity.

**Frequency and magnitude of IgG immune response against recombinant antigens**

Assessing the humoral immune response of all 253 studied individuals against PvRMC-RBP1 and the control PvRBP123-751 protein, we observed that both proteins were recognized by naturally acquired antibodies (Figure 2A). Total IgG responses were observed in 47.1% of the population to the chimeric PvRMC-RBP1 and in 60% to the non-chimeric protein PvRBP123-751. There was a significant difference in the response to these two recombinant proteins (\( p = 0.0031 \)). The majority of responders to PvRMC-RBP1 (93.8%) were also responders to PvRBP123-751. Concerning the magnitude of the response against the PvRMC-RBP1 and PvRBP123-751, the reactivity indexes (RI) of IgG antibodies ranged from 0.21 to 7.30 and did not present significant differences in responders (Figure 2B). Moreover, we also observed a significant correlation between the RIs against PvRMC-RBP1 and PvRBP123-751 (Figure 2C). Interestingly, 22 individuals were responders to the PvRMC-RBP1 and were not responders to the non-chimeric PvRBP123-751 (Table 4). Therefore, in order to determine if the (NANP)_6 tag sequence plays a role in the IgG antibody reactivity against PvRMC-RBP1, we performed absorption ELISA experiments. Positive samples from 63 randomly selected individuals were pre-incubated with (NANP)_6 synthetic peptide prior the evaluation of IgG reactivity against PvRMC-RBP1. These experiments showed that, despite that 51% of samples were positive against (NANP)_6 after the absorption step with such synthetic peptide most plasma samples did not differ in their reactivity to PvRMC-RBP1. Consequently these results suggest that the antibodies are specific to PvRBP1 and not to the (NANP)_6 tag (Figure 3).

**IgG subclass profile of responders against PvRMC-RBP1 and PvRBP123-751**

We assessed the overall distribution of the IgG antibody subclass responses to PvRMC-RBP1 and PvRBP123-751 proteins using different comparative analyses. Firstly, we determined subclass-specific prevalence in total IgG positive responders for both antigens (Figure 4A). The results were comparable, IgG1 response was predominant against PvRMC-RBP1 (73.3%) and PvRBP123-751 (86%) when compared respectively to IgG2 (33% and 39%), IgG3 (28% and 36%) and IgG4 (17% and 35%). Secondly, in relation to magnitude of antigen-specific IgG subclasses, we also observed that the RI of IgG1 cytophilic antibodies against PvRMC-RBP1 (1.83) and PvRBP123-751 (1.95) was also significantly higher (\( p < 0.0001 \)) than all other subclasses (Figure 4B). We did not observe differences in the magnitude of the response for IgG subclasses against PvRMC-RBP1 and PvRBP123-751 recombinant proteins.

**Influence of malaria exposure in immune response**

In order to assess whether epidemiological factors influence the naturally acquired immune response against PvRMC-RBP1, different parameters of the population were correlated with the reactivity indexes of total IgG and the IgG subclasses. We first observed a direct correlation between total IgG against PvRMC-RBP1 and age (r = 0.1762, p = 0.003), time of residence in endemic areas (TREA, r = 0.2781, p < 0.0001) and number of previous malaria infections reported also showed a direct correlation with the reactivity indexes against PvRMC-RBP1 (r = 0.1765, P = 0.0049), indicating an additive effect in specific immune response. We also used the time (months) elapsed since the last infection as indicative of protection in order to observe possible evidence of relationship with antibodies against PvRMC-RBP1 or PvRBP123-751. However, we did not observe any significant correlation in the evaluation of total IgG subclasses specific against both antigens. In relation to PvRBP123-751 we also observed a direct correlation between IgG reactivity indexes and exposure factors (age: r = 0.3217, p < 0.0001; TREA: r = 0.2556, p < 0.0001) and number of previous malaria infections (r = 0.2875, p < 0.0001). Lastly, by multiple regression analysis (Table 5) we evaluated the contribution of each independent variable for the IgG magnitude against both antigens. The time of residence in an endemic area had the highest impact on acquired antibody response against the chimeric

---

**Figure 3. Magnitude of IgG immune response against PvRMC-RBP1 before and after (NANP)_6-specific antibody absorption step.** The median of reactivity index of IgG to PvRMC-RBP1 presented no significant difference between before and after absorption (\( p = 0.2525 \)).



---

**Table 4.** Comparison of reactivity indexes of IgG against PvRMC-RBP1 and PvRBP123-751.

| Antigen          | (NANP)_6 Tag Exposed | (NANP)_6 Tag Absorbed |
|------------------|----------------------|-----------------------|
| Reactivity Index | 1.83                 | 1.95                  |

**Table 5.** Multiple regression analysis of the impact of demographic and epidemiological factors on the magnitude of the IgG immune response against PvRMC-RBP1.

| Variable               | Coefficient | p-Value |
|------------------------|-------------|---------|
| Age                    | 0.1762      | 0.003   |
| Time of residence      | 0.2781      | <0.0001 |
| Number of previous malaria infections | 0.1765 | 0.0049 |

---

**Figure 2A** shows that purified proteins migrated as single bands of the apparent mobility of 55 kDa for PvRMC-RBP1 and 100 kDa for PvRBP123-751 (Figure 1C). Biochemical identity of the recombinant proteins was established by western blot analysis using polyclonal antibodies recognizing the T cell epitopes I1745-S1786 and L2235-E2263 and a His-tag monoclonal antibody (Figure 1G).
beta = 0.294; t = 4.545; p = 0.0001) and non-chimeric (beta = 0.251; t = 3.785; p = 0.0001) PvRBP1 proteins, while time since last malaria infection was confirmed as a non-associated variable.

**HLA distribution among studied individuals and the associations with IgG response**

We found 13 HLA-DRB1* and 5 HLA-DQB1* allelic groups. There were two predominant HLA allelic groups in our studied population, HLA-DRB1*04 (16% of all HLA-DR genotypes), and HLA-DQB1*03 (40% of all HLA-DQ genotypes). The HLA-DRB1*09, HLA-DRB1*10 and HLA-DRB1*12 were less frequent in HLA-DRB1* and HLA-DQB1*04 was less frequent in HLA-DQB1*. The number of positive individuals for the HLA-DRB1* and HLA-DQB1* alleles and the frequency of each allele are summarized in Table 6.

The association between HLA-DRB1* and HLA-DQB1* alleles and haplotypes and the naturally acquired IgG response to the recombinant proteins was also evaluated. Although 30.2% of the studied individuals did not present detectable antibody titers to PvRMC-RBP1 or PvRBP123-751, genetic restriction to this antigen does not seem to occur, since no association was observed between the HLA-DRB1* or HLA-DQB1* alleles and the frequency of antibody responders to both antigens (Table 6). Moreover, we also evaluated the difference between reactivity index of responders among HLA-DRB1* and HLA-DQB1* allele carriers and no difference was observed.

**Discussion**

A successful malaria vaccine requires the induction of long lasting protective antibodies and robust T cell responses. However, preclinical and clinical trials have shown that a number of vaccine candidates are poorly immunogenic [32–35]. To overcome the poor immunogenicity of subunit vaccines, we have produced chimeric proteins that contain cognate promiscuous T helper epitopes tailored for individual antigens [23,36]. Proof-of-principle studies in mice have shown that such chimeric vaccine constructs induced more robust antibody and T cell responses in comparison to the native protein [23,36]. Relevantly, the magnitude and

![Figure 4. IgG subclass profile in responders against PvRMC-RBP1 and PvRBP123-751 recombinant proteins.](image-url)

**Table 4. Frequency and magnitude of IgG response to PvRMC-RBP1 and/or PvRBP123-751.**

| Frequency | RI (Mean ± SD) |
|-----------|---------------|
| n | % | PvRMC-RBP1 | PvBP123-751 |
| PvRMC-RBP1 (+) and PvRBP123-751 (+) | 98 | 56.0%* | 1.96±1.45 | 2.03±1.97 |
| PvRMC-RBP1 (+) and PvBP123-751 (−) | 22 | 12.6% | 1.36±0.53 | 0.79±0.15 |
| PvRMC-RBP1 (−) and PVP123-751 (+) | 55 | 31.4% | 0.66±0.18 | 1.27±1.03 |
| PvRMC-RBP1 (+) or PVP123-751 (+) | 175 | 100.0% | |

*Frequency of IgG double responders against proteins were significantly higher than PvRMC-RBP1 (X² = 73.25; p = 0.0001) and PvBP123-751 (X² = 21.47; p = 0.0001) single responders.

doi:10.1371/journal.pone.0105828.t004

![Figure 4. IgG subclass profile in responders against PvRMC-RBP1 and PvRBP123-751 recombinant proteins.](image-url)
functionality of the antibody response is significantly improved using this approach [23]. Following this rationale, we predicted promiscuous T helper epitopes in PvRBP1, validated their ability to elicit T cell responses in mice (manuscript in preparation) and generated a synthetic gene encoding such promiscuous T helper epitopes genetically linked to the sequence of the PvRBP1,435-777 domain. Interestingly, sequences of the T cell epitopes included in this chimeric protein (PvRMC-RBP1) were predicted to bind multiple HLA alleles (Table 1). According to these predictions we expect T cell responses in over 95% of the human population (Table 2). Nevertheless, chimeric proteins have the potential of generating neoantigens or masking antigenic domains that are target of protective antibodies. We report here comparative seroepidemiological studies that suggest that the identity of the conformational B cell epitope is preserved in PvRMC-RBP1.

Seroepidemiological studies have played a significant role in the identification of leading vaccine candidates [37–39]. Studies using recombinant proteins representing five different regions of the PvRBP-1 protein have shown that naturally acquired antibodies preferentially recognize the amino-terminal portion of the protein that span 976 amino acids in length [21]. Interestingly, a recombinant protein containing the 517 amino acid-long amino terminal fragment of this region contains 48% of the polymorphic residues reported in the 2,749 amino acid long extracellular domain of the PvRBP-1 protein [21]. This region has also been suggested to include a functionally relevant binding domain [20]. We therefore decided to characterize here the antigenic integrity of the B cells epitopes included in the chimeric PvRMC-RBP1, in comparative experiments with a control recombinant protein that expressed the native PvRBP1,435-777 fragment included in PvRMC-RBP1 by testing naturally acquired antibodies and their association with major HLA-DRB1* and HLA-DQB1* alleles.

Plasma samples were collected in cross-sectional studies with Brazilian Amazon communities in 2004 and 2007. The profile of studied individuals shows that our population included a majority of rainfall region natives and also transmigrants 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. In relation to malaria history the highly variable range of number of previous infections, time of residence in endemic area and time since the last infection suggest 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 [38,40,41]. The correlation between time of residence in endemic areas and months since the last infection observed in our study also indicate that this phenomenon could be occurring in low/medium endemicity areas like the Brazilian Amazon. Therefore, the selection of these individuals was ideal to detect the presence of antibodies against the new recombinant antigen, distinguish whether the alterations found were related to malaria exposure and determine genetic background associated with the HLA allelic groups.

Firstly, we evaluated the naturally acquired humoral immune response mediated by total IgG antibodies against the PvRMC-RBP1 to identify the retention of naturally recognized epitopes previously reported [21]. Our results of humoral immune response mediated by IgG antibodies suggested that PvRMC-RBP1 retains the antigenic identity, being widely recognized by almost half of the studied individuals. Moreover, since P. falciparum infections also occur in this area, we confirmed by absorption assays that there was no significant influence of the P. falciparum (NANP)6 carboxyl terminal tag sequence in the specificity of the antibodies to PvRMC-RBP1. This frequency of responders is comparable to that reported by Tran and colleagues using five PvRBP1 fragments, including a recombinant protein representing the sequence of the fragment PvRBP1,435-777 studied here [21]. However, the frequency of the natural antibody responses remained relatively low when compared to those against other classical P. vivax vaccine candidates, such as MSP-119, MSP-9, MSP-3 and AMA-1, which have frequencies that ranged between 60% and 90% of reactivity against their most immunogenic

Figure 5. Correlation between naturally acquired IgG immune response against PvRMC-RBP1 and exposure to malaria. (A) Spearman’s rank correlation between age and IgG reactivity index against the chimeric recombinant protein in a population naturally exposed to malaria (r = 0.3613; p<0.0001). (B) Spearman’s rank correlation between time of residence in malaria endemic area and IgG reactivity index against the chimeric recombinant protein in a population naturally exposed to malaria (r = 0.2781; p<0.0001).

doi:10.1371/journal.pone.0105828.g005
regions [12,15,42]. On the other hand, the data is still comparable to proteins such as DBP [43] and the N-terminal region of MSP-9 [44].

In comparative experiments reported here, we observed a 13% higher prevalence of responders against the control protein compared to PvRMC-RBP1. This would indicate that structural changes resulting from the inclusion of the T-cell epitopes modified the antigenic properties of the native protein. However, our results showed that the majority of responders (82%) against PvRMC-RBP1 were also responders against the non-chimeric PvRBP123-751 and the RIs were strongly correlated between both proteins, indicating that Pv-RMC-RBP1 preserves the antigenic domains present in the native protein that are the targets of antibody elicited by natural exposure to \textit{P. vivax} infections. We also corroborated this hypothesis after observing the correlation with the reactivity index against the chimeric antigen with age, the time of exposure to malaria infections and the number of previous malaria infections, reflecting the cumulative effect of the specific immune response to epitopes represented in our chimeric antigen.

Table 5. Multivariate regression analysis of independent variables (PMI, TREA and TLI) associated with the magnitude of IgG immune response against both studied antigens.

|          | Unstandardized Coefficients | Standardized Coefficients |
|----------|----------------------------|---------------------------|
|          | B  | Std. Error | Beta | t     | P values |
| PvRMC-RBP1 | constant | 0.494 | 0.209 | 2.362 | 0.019 |
|          | PMI | 0.033 | 0.012 | 0.177 | 2.788 | 0.006 |
|          | TREA | 0.025 | 0.005 | 0.294 | 4.545 | 0.0001 |
|          | TLI | 0.000 | 0.002 | -0.015 | -2.227 | 0.0821 |
| PvRBP123-751 | constant | 0.841 | 0.262 | 3.213 | 0.002 |
|          | PMI | 0.023 | 0.011 | 0.133 | 2.042 | 0.042 |
|          | TREA | 0.026 | 0.007 | 0.251 | 3.785 | 0.0001 |
|          | TLI | 0.000 | 0.002 | 0.001 | 0.012 | 0.990 |

PMI (Past malaria infections); TREA (Time of residence in endemic area); (Time since the last malaria infection)
doi:10.1371/journal.pone.0105828.t005

Table 6. Frequency (F) and number (n) of IgG responders and non-responders to the PvRMC-RBP1 and Pv-RBP123-751 recombinant proteins tested by HLA-DRB1* and HLA-DQB1* allelic groups from individuals naturally exposed to malaria.

| HLA     | F (n) | PvRMC-RBP1 Responder F | Non Responder F | Pv-RBP123-751 Responder F | Non responder F |
|---------|-------|------------------------|----------------|---------------------------|----------------|
| HLA-DRB1* |       |                        |                |                           |                |
| DRB1*01 | 0.0988(51) | 0.0737 | 0.1213 | 0.0806 | 0.1323 |
| DRB1*03 | 0.0697(36) | 0.0819 | 0.0588 | 0.0709 | 0.0637 |
| DRB1*04 | 0.1627(84) | 0.1885 | 0.1397 | 0.1774 | 0.1323 |
| DRB1*07 | 0.0968(50) | 0.0916 | 0.1029 | 0.0903 | 0.1078 |
| DRB1*08 | 0.0910(47) | 0.1024 | 0.0808 | 0.1032 | 0.0735 |
| DRB1*09 | 0.0155(8)  | 0.0081 | 0.0220 | 0.0225 | 0.0049 |
| DRB1*10 | 0.0116(6)  | 0.0081 | 0.0147 | 0.0096 | 0.0147 |
| DRB1*11 | 0.1046(54) | 0.1024 | 0.1066 | 0.1 | 0.1127 |
| DRB1*12 | 0.0077(4)  | 0.0081 | 0.0033 | 0.0096 | 0.0049 |
| DRB1*13 | 0.1085(56) | 0.1188 | 0.0992 | 0.1064 | 0.1127 |
| DRB1*14 | 0.0775(40) | 0.0819 | 0.0735 | 0.0903 | 0.0588 |
| DRB1*15 | 0.0794(41) | 0.0737 | 0.0845 | 0.0741 | 0.0931 |
| DRB1*16 | 0.0755(39) | 0.0614 | 0.0882 | 0.0645 | 0.0882 |
| HLA-DQB1* |       |                        |                |                           |                |
| DQB1*02 | 0.1375(71) | 0.1311 | 0.1433 | 0.1522 | 0.1421 |
| DQB1*03 | 0.4031(208) | 0.3934 | 0.4117 | 0.4058 | 0.3827 |
| DQB1*04 | 0.1085(56) | 0.1311 | 0.0882 | 0.1225 | 0.0833 |
| DQB1*05 | 0.1705(88) | 0.1598 | 0.1801 | 0.1612 | 0.1711 |
| DQB1*06 | 0.1802(93) | 0.1844 | 0.1764 | 0.1580 | 0.2205 |

doi:10.1371/journal.pone.0105828.t006
The absence of optimized assays to characterize functional antibodies against *P. vivax*, the low frequency of infected individuals and the lack of asymptomatic infections in our population, limited the evaluation of possible associations between anti-PvRMC-RBP1 antibody levels and clinical immunity. Based on the evidence that cytophilic IgG1 and IgG4 antibodies to *P. falciparum* are correlated with protection [45–50], whereas IgG2 and IgG4 even interfere with protective mechanisms, we also evaluated the level of reactivity and profile of IgG subclasses against both proteins. The high prevalence and magnitude of IgG1 against the chimeric and non-chimeric RBP1 recombinant proteins would indicate a protective effect. The lack of differences in frequency profile and magnitude of IgG subclasses between the studied proteins also suggest that the isotype response to the native PvRBP1 is preserved. Moreover, the high frequency of IgG1 responders observed in our work confirms previous findings with PvRBP1 and PvRMC-RBP1, an overlapped fragment of PvRBP1, indicating an IgG1 biased response [21]. In *P. vivax* the association between cytophilic isotypes and protection is not clearly defined. In fact, reports from our group and others already demonstrated a considerable frequency of non-cytophilic antibodies against *P. vivax* MSP-3 [51], MSP-9 [15] and MSP-1 [51] in Brazilian exposed populations.

Since many factors can contribute to the heterogeneity of the immune response to antigens and genetic restriction may influence the generation of protective immune responses to *Plasmodium* target proteins, we also aimed to investigate for the first time the association between HLA-DRB1* and HLA-DQB1* allelic groups and the immunodominant RBP-1 fragment expressed as chimeric and non-chimeric proteins. Moreover we could investigate if the small difference in antibody response against both recombinant proteins was associated with genetic polymorphism of the HLA Class II alleles. Our results demonstrated that the studied population was heterogeneous, presenting 13 HLA-DRB1* and 5 HLA-DQB1* allelic groups. HLA-DRB1*04, HLA-DRB1*11, HLADRB1*13 and HLA-DQB1*03 were the most frequent allelic groups found in the population and the most frequent in native individuals from this Amazon area [52]. Analyses of IgG responders to PvRBP1 and PvRMC-RBP1 showed no association between frequency and specific HLA-DRB1* and HLA-DQB1* allelic group. The lack of associations between HLA allelic groups and *P. vivax* target proteins has also been observed with other surface antigens such as PfAMA-1 and PfDBP [42]. On the other hand, in previous work with individuals from the Southwestern Brazilian Amazon, a high frequency of responders against fragments of PvMSP3 and PfMSP-9 were defined in HLA-DRB1*04 carriers [52] while HLA-DRB1*07 was associated with the absence of antibody responses to VK120 repeats of the CSP [53]. Although computational methods for the definition of T cell epitopes is still far from perfect, these algorithms predicted a relatively large number of promiscuous T cell epitopes in PvRBP1, a finding that agrees with the lack of correlation between antibody responses to this protein and HLA types. In fact, although 40% and 52.9% of the population did not present detectable titers of antibodies to PvRBP1 and PvRMC-RBP1 respectively, we confirmed that genetic restriction to these antigens does not seem to occur, since no association was observed between the HLA-DRB1* and HLA-DQB1* alleles and the antibody response.

Notwithstanding the naturally acquired IgG immune response against chimeric and non-chimeric PvRBP1 and the lack of HLA association with HLA-DRB1*/DQB1* reported here, it remains unknown why only a fraction of the naturally exposed individuals have antibodies against PvRBP1. The lack of natural immune response mediated by IgG in a significant part of studied population could be explained by the presence of polymorphic residues that could be the target of antibodies [20,21]. Moreover, since we observed a correlation with the number of previous infections, and Tran and colleagues reported a low response in recently exposed individuals from a similar area [21], it is also possible that multiple malaria episodes are necessary to induce detectable antibody titers against PvRBP1. Therefore, the presence of multiple promiscuous T cell epitopes in PvRMC-RBP1 in future immunizations could increase the humoral response against *P. vivax* Reticulocyte Binding Protein and overcome the necessity of long time exposure and infections in naturally exposed individuals.

In conclusion, our study provides valuable information concerning the chimeric PvRMC-RBP1. Firstly, the recombinant chimeric construct was broadly recognized by naturally acquired antibodies, which is correlated with time of exposure and number of malaria infections. Moreover, the predominance of the IgG1 cytophilic antibody subclass against the native and the chimeric recombinant protein also indicates a possible role in protective immunity. Lastly, our data suggest, that there was no genetic restriction mediated by HLA-DRB1* and HLA-DQB1* against this immunodominant fragment. Therefore, the confirmation that PvRMC-RBP1 has maintained its functional identity in the context of the immune response will support new studies comparing the immunogenicity in different animal models to test whether the strategy of using cognate promiscuous T cell epitopes to enhance immunogenity can be applied for nonlinear structured domains.

**Acknowledgments**

We are grateful to all individuals who participated in this study, for their cooperation and generous donation of blood, which made this study possible. We thank the Secretary of Health of Rondonia State and the Laboratorio Central – LACEN of Rondonia for supporting fieldwork and Stacey Lapp for critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: JCLJ AM JOF. Performed the experiments: ARF AM JCLJ. Analyzed the data: JCLJ AM JOF LACSP. Contributed reagents/materials/analysis tools: BS DMB JMCC AM. Written the manuscript: JCLJ AM JOF. Fieldwork support: FS.

**References**

1. WHO (2013) World malaria report 2013. In: WHO, editor. Switzerland: World Health Organization.
2. Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, et al. (2005) Phase 1 vaccine trial of Pvs25: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine 23: 3131–3138.
3. Wu Y, Ellis RD, Shaffer D, Foutes E, Malkin EM, et al. (2008) Phase 1 trial of malaria transmission blocking vaccine candidates Pvs25 and Pvs25 formulated with montanide ISA 51. PLoS One 3: e2636.
4. Gordon DM, Cosgriff TM, Schneider I, Wasserman GF, Majarian WR, et al. (1990) Safety and Immunogenicity of a Plasmodium vivax Sporozoite Vaccine. The American Journal of Tropical Medicine and Hygiene 42: 527–531.
5. Harrington DA, Nardin EH, Losowsky G, Bathurst IC, Barr P, et al. (1991) Safety and Immunogenicity of a Recombinant Sporozoite Malaria Vaccine against Plasmodium vivax. Am J Trop Med Hyg 45: 695–701.
6. Arevalo-Herrera M, Soto L, Petaza BL, Cepedes N, Vera O, et al. (2011) Antibody-mediated and cellular immune responses induced in naïve volunteers by vaccination with long synthetic peptides derived from the Plasmodium vivax circumsporozoite protein. Am J Trop Med Hyg 84: 35–42.
25. Sharp PM, Li W-H (1987) The codon adaptation index—a measure of directional synonymous codon usage bias and its potential applications. Nucleic Acids Research 15: 1281–1288.
26. Silva-Flannery LM, Cabrera-Mora M, Jiang JL, Moreno A (2009) Recombinant Plasmodium vivax merozoite surface protein-9 in Northwestern Amazon individuals. Vaccine 27: 6645–6654.
27. Vargas-Serrato E, Barnwell JW, Ingravallo P, Perler FB, Galinski MR (2002) Merozoite surface protein-9 of Plasmodium vivax and related simian malaria parasites is orthogonal to p101/ABRA of P. falciparum. Mol Biochem Parasitol 120: 41–52.
28. Galinski MR, Medina CC, Ingravallo P, Barnwell JW (1992) A reticulocyte-binding protein complex of Plasmodium vivax merozoites. Cell 69: 1213–1226.
29. Rayner JC, Vargas-Serrato E, Huber GS, Galinski MR (2001) A Plasmodium falciparum homologue of Plasmodium vivax reticulocyte binding protein (PVRBP1) defines a trypan-resistant erythrocyte invasion pathway. J Exp Med 194: 1571–1581.
30. Singh BP, Osawa H, Kochen CHM, Puri SK, Thomas AW, et al. (2005) Targeted deletion of Plasmodium knowlesi Duffy binding protein confirms its role in junction formation during invasion. Molecular Microbiology 55: 1925–1934.
31. Rayner JC, Tran TM, Corredor V, Huber CS, Barnwell JW, et al. (2005) Dramatic difference in diversity between Plasmodium falciparum and Plasmodium vivax reticulocyte binding-like genes. The American Journal of Tropical Medicine and Hygiene 72: 666–674.
32. Tran TM, Oliveira-Ferreira J, Moreno A, Santos F, Vaziani SS, et al. (2005) Comparison of IgG reactivities to Plasmodium falciparum merozoite invasion antigen in a Brazilian Amazon population. Am J Trop Med Hyg 75: 244–255.
33. Singh BP, Osawa H, Huber GS, Barnwell JW (2000) Plasmodium falciparum merozoite surface protein-9 naturally acquired antibodies in malaria-exposed individuals from malaria endemic and non-endemic areas. J Infect Dis 208: 479–488.
34. Oliveira-Ferreira J, Pratt-Ricci LR, Arruda M, Santos F, Daniel Ribeiro CT, et al. (2004) HLA class II and antibody responses to circumsporozoite protein 10 of Plasmodium falciparum predict protection from malaria in African children. Infect Immun 73: 2201–2207.
35. Ndungu FM, Bull PC, Ross A, Lowe BS, Kabiru E, et al. (2002) Naturally acquired antibodies in Gabonese newborns. Am J Trop Med Hyg 67: 944–950.
36. Singh B, Cabrera-Mora M, Jiang J, Moreno A (2010) Genetic linkage of autoantibody T cell epitopes in a chimeric recombinant construct improves anti-parasite and anti-disease protective effect of a malaria vaccine candidate. Vaccine 28: 2530–2539.
37. Silva-Flannery LM, Cabrera-Mora M, Jiang JL, Moreno A (2009) Recombinant peptide replicates immunogenicity of synthetic linear peptide chimeras for use as pre-erythrocytic stage malaria vaccine. Microbes and Infection 11: 83–91.
38. Sharp PM, Li W-H (1987) The codon adaptation index—measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Research 15: 1281–1288.
39. Nishida M, Mizuguchi H, Fujisawa S, Komatsu S, Kitahashi M, et al. (2001) Long and accurate PCR with a mixture of KOD DNA polymerase and its exonuclease deficient mutant enzyme. Journal of Biotechnology 88: 119–149.
40. Costa-Casas A, Lai M-C, Shojaee M, Palsson J, Galinski MR, Moreno A (2003) Chimeric epitopes derived by polymeric synthetic linear peptides induce protective immunity to malaria. Microbes and Infection 7: 1324–1337.
41. Singh B, Huang GP (2005) PreProF: prediction of HLA-DR binding sites. Bioinformatics 21: 1265–1267.
42. Wang P, Sidney J, Dow C, Motieh B, Sette A, et al. (2006) A Systematic Assessment of MHC Class II Peptide Binding Predictions and Evaluation of a Method for Predicting Class II HLA-A2 Binding. PLoS Comput Biol 2: e120.
43. Bui H-H, Sidney J, Dinh H, Southwood S, Newman M, et al. (2006) Predicting population coverage of T-cell epitope-based diagnostics and vaccines. BMC Bioinformatics 7: 153.
44. Oliveira-Ferreira J, Lacorda MV, Brasil P, Ladhialu JL, Tanil PL, et al. (2010) Malaria in Brazil: an update. Malar J 9:155.