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Immunogenetic markers associated with a naturally acquired humoral immune response against an N-terminal antigen of *Plasmodium vivax* merozoite surface protein 1 (PvMSP-1)

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Abstract

Background: Humoral immune responses against proteins of asexual blood-stage malaria parasites have been associated with clinical immunity. However, variations in the antibody-driven responses may be associated with a genetic component of the human host. The objective of the present study was to evaluate the influence of co-stimulatory molecule gene polymorphisms of the immune system on the magnitude of the humoral immune response against a *Plasmodium vivax* vaccine candidate antigen.

Methods: Polymorphisms in the *CD28*, *CTLA4*, *ICOS*, *CD40*, *CD86* and *BLYS* genes of 178 subjects infected with *P. vivax* in an endemic area of the Brazilian Amazon were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The levels of IgM, total IgG and IgG subclasses specific for ICB2-5, i.e., the N-terminal portion of *P. vivax* merozoite surface protein 1 (PvMSP-1), were determined by enzyme-linked immuno assay. The associations between the polymorphisms and the antibody response were assessed by means of logistic regression models.

Results: After correcting for multiple testing, the IgG1 levels were significantly higher in individuals recessive for the single nucleotide polymorphism rs3116496 in *CD28* ($p = 0.00004$). Furthermore, the interaction between *CD28* rs35593994 and *BLYS* rs9514828 had an influence on the IgM levels ($p = 0.0009$).

Conclusions: The results of the present study support the hypothesis that polymorphisms in the genes of co-stimulatory components of the immune system can contribute to a natural antibody-driven response against *P. vivax* antigens.

Keywords: *Plasmodium vivax*, MSP-1, ICB2-5, Immunogenetics, Antibodies

Background

According to the World Health Organization, there were an estimated 200 million cases of malaria in 2014 and an

estimated 584,000 resulting deaths worldwide [1]. Among the five species that can cause malaria in humans, *Plasmodium vivax* is the most widely distributed, accounting for most of the cases of malaria in South and Southeast Asia, Latin America and Oceania; there are an estimated 2.5 billion people living in areas at risk of transmission of the disease [1, 2]. Furthermore, although vivax malaria

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has not been considered life-threatening for a long time, reports of severe cases associated with *P. vivax* have been increasingly numerous [3].

The blood stage of the *Plasmodium* lifecycle is responsible for the pathology associated with malaria. In this stage, merozoites released from schizont-infected erythrocytes invade non-infected erythrocytes, resulting in their destruction and the release of more merozoites into the bloodstream. During this brief extracellular period, these free merozoites are exposed to the host immune system, and proteins that are critical for parasite invasion are, therefore, important targets for the development of malaria vaccines. Merozoite surface proteins (MSPs) are among the most studied, especially MSP-1, which is necessary for merozoite attachment to erythrocytes [4] and normal parasite development [5].

The most widely accepted structure of the *P. vivax* merozoite surface protein 1 (PvMSP-1) gene indicates that it consists of six polymorphic blocks (blocks 2, 4, 6, 8, 10 and 12) flanked by seven conserved blocks (blocks 1, 3, 5, 7, 9, 11 and 13) [6]. By analysing the primary structure of PvMSP-1, several seroepidemiological studies have been performed to evaluate its immunogenic potential [7–11]. Although the C-terminal portion of the protein (PvMSP-1₁₉) is the most immunogenic [7, 9, 12], a number of studies showed high prevalence of IgG against N-terminal PvMSP-1 in individuals exposed to *P. vivax* [9, 10, 13, 14]. Furthermore, specific IgG3 antibodies against the N-terminal portion of PvMSP-1 have been associated with clinical protection in two riverine communities of the Brazilian Amazon [9, 11], similar to that observed in *P. falciparum* infection, where persistence of antibodies IgG3 against N-terminal of MSP-1 was related with prolongation time without malaria [15]. In fact, antibodies specific for the asexual blood stage are thought to be involved in clinical protection against malaria vivax. Longitudinal cohort studies have shown correlations between magnitude of antibody responses to *P. vivax* merozoite antigens and protection from malaria [11, 16–18]. Due to the inability in maintaining *P. vivax* in continuous in vitro culture, it is difficult to define the role of antibodies to this species, but few evidences support that it may be related to inhibition of merozoite invasion [19, 20]. Furthermore, complement and FcR mediated mechanisms seem to be important in antibody-mediated protection [21].

The development of an adequate immune response depends on the fine regulation of lymphocyte activation. For this, in general, lymphocytes require two activation signals. The first signal is antigen-specific, whereas the second signal, called co-stimulation, is generated by the interaction between the surface molecules of T cells and

those of antigen-presenting cells, including B cells. The interaction between CD28 and its ligands, CD80 and CD86, provides the strongest costimulatory signal for T-cell proliferation, whereas CTLA-4 is a negative regulator that plays a key role in T cell homeostasis and in central tolerance [22]. Another member of the CD28 family, Inducible co-stimulator (ICOS), is an important immune regulatory molecule that participates in T-cell activation and T-cell dependent B-cell responses [23, 24]. CD40 is presented on the surface of B-cells and the CD40-CD40L interaction is the major costimulatory signal for B cells to mount a humoral response [25]. B lymphocyte stimulator (BLYS) is produced mainly by innate immune cells and is needed to provide signals for B cell survival and proliferation [26]. Considering the importance of these molecules in development of immune response and because there are currently multiple lines of evidence showing that the genes involved in the immune response can influence antibody production during malaria infection [27–34], the authors hypothesised that polymorphisms in the genes of the co-stimulatory molecules CD28, CTLA-4, ICOS, CD86, CD40 and BLYS are involved in the magnitude of the naturally acquired antibody-driven response against N-terminal PvMSP-1 in individuals infected with *P. vivax* in the Brazilian Amazon.

Methods

Study area and subjects

The present study was conducted in the municipality of Goianésia do Pará, Pará state, Brazil, which is a constituent of the Brazilian Amazon region, where *P. vivax* is responsible for more than 80 % of all malaria cases [35]. Details of the study area have been described elsewhere [36]. Cross-sectional surveys conducted from February 2011 to August 2012 were used to recruit 178 subjects (125 men and 53 women) presenting with classic symptoms of malaria, who sought treatment at the medical service of the municipality; the subjects had an average age of 29.8 years (varying from 14 to 68 years). Exclusion criteria included children under 10 years old, pregnancy, related individuals and anti-malarial treatment within the previous seven days. Diagnosis was performed by microscopy (thick film), and infection with *P. vivax* was subsequently confirmed by nested polymerase chain reaction (PCR) [37]. The geometric mean of parasitaemia was 718.4 parasites/mm³ (95 % CI 487.2–1059.2). All patients with microscopically confirmed infections were given standard treatment of 25 mg/kg of chloroquine in 3 days plus 0.5 mg/kg of primaquine during seven days. All participants were submitted to a questionnaire to obtain epidemiological information. The length of residence in the municipality varied between one month and

37 years (median of 5 years), and 91.9 % of the individuals had previously contracted malaria. Samples from 40 malaria-naive individuals living in a non-endemic area (São José do Rio Preto, Brazil), who had never been to areas of malaria transmission, were used as controls.

Peripheral venous blood (~10 mL) was collected in EDTA tubes, and plasma samples were obtained by centrifugation and stored at -20°C . The protocol of the present study was approved by the Research Ethics Committee of the Medical School of São José do Rio Preto (CEP/FAMERP no. 4599/2011) and by the health authorities of the municipality of Goianésia do Pará. All participants or their legal guardians signed an informed consent form.

Genotyping

DNA was extracted from peripheral blood samples with an Easy-DNA™ extraction kit (Invitrogen, California, USA). All single nucleotide polymorphisms (SNPs) were genotyped by PCR-restriction fragment length polymorphism (RFLP). The SNPs $-372\text{G} > \text{A}$ and $+17\text{T} > \text{C}$ in *CD28* (rs35593994 and rs3116496, respectively), $-318\text{C} > \text{T}$ and $+49\text{A} > \text{G}$ in *CTLA4* (rs5742909 and rs231775, respectively), $+1564\text{T} > \text{C}$ in *ICOS* (rs4404254), $+1057\text{G} > \text{A}$ in *CD86* (rs1129055), $-1\text{C} > \text{T}$ in *CD40* (rs1883832), and $-871\text{C} > \text{T}$ in *BLYS* (rs9514828) were genotyped according to previously described protocols [36, 38, 39]. Primer sequences, restriction enzymes, and the restriction fragments obtained of each SNP are presented in Additional file 1. PCR for the identification of $-1722\text{T} > \text{C}$ and $-1577\text{G} > \text{A}$ in *CTLA4* (rs733618 and rs11571316, respectively) was performed with a final sample volume of 25 μL containing 1X Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.6 pmol of each primer, and 0.5 U Platinum Taq DNA Polymerase (Invitrogen, São Paulo, Brazil). The primers 5' CTTCATGCCGTTTCCAACCTT 3' and 5' CCTTTTCTGACCTGCCTGTT 3' were used for the $-1722\text{T} > \text{C}$ genotyping, whereas 5' CTTCATGCCGTTTCCAACCTT 3' and 5' ATCTCCTCCAGGAA GCCTCTT 3' were used to identify $-1577\text{G} > \text{A}$. Amplifications were performed under the following conditions: first step of 5 min at 94°C , 35 cycles for 30 s at 94°C , 30 s at 52°C and 1 min at 72°C , and a final step for 10 min at 72°C . The PCR products of $-1722\text{T} > \text{C}$ and $-1577\text{G} > \text{A}$ were digested with the enzymes *BbvI* and *MboII* (Fermentas, Vilnius, Lithuania), respectively. Electrophoresis was performed in 2.5 % agarose and stained with Gel-Red™ (Biotium, Hayward, USA) with the exception of the rs5742909 and rs1883832 SNPs, which were performed in 12.5 % polyacrylamide gel after staining with ethidium bromide, and visualized in a UV transilluminator.

Antigen and antibody determination

The glutathione-S-transferase (GST)-tagged recombinant ICB2-5 protein corresponds to the amino acids 170-675 of the N-terminal portion of MSP-1 from the Belém strain of *P. vivax*. The ICB2-5-GST fusion protein was purified on a glutathione-Sepharose 4B column (Amersham Pharmacia), and the protein content was assessed with the Bio-Rad Protein Assay Kit I (Bio-Rad Laboratories, Inc.) [7]. The levels of IgM and IgG subclasses specific for ICB2-5 were measured as described previously with some modifications [7]. The plates were coated with GST-tagged ICB2-5 and GST alone, and all tests were done in duplicate. Briefly, 50 μL of ICB2-5-GST or GST alone (as a control) was coated on ELISA plates at 4 $\mu\text{g}/\text{mL}$ (Costar, Corning Inc., New York, USA) and incubated overnight at 4°C . After washing and blocking the plates with 0.05 % bovine serum albumin-PBS, 50 μL of plasma diluted 1/100 was added to the wells in duplicate and incubated for 1 h at 37°C . Then, 50 μL of peroxidase-conjugated anti-human IgG or IgM antibodies (KPL, Maryland, USA) diluted 1/1000 were added to each well for the detection of total IgG or IgM, respectively. IgG subclasses were detected using mouse monoclonal antibodies specific for each isotype (Sigma, Missouri, USA), diluted according to the tested subclass (1/3000 for IgG1 clone HP6001; and IgG3 clone HP6050, 1/2500 for IgG2 clone HP6014, and 1/5000 for IgG4 clone HP6025). The immobilized monoclonal antibodies were detected with peroxidase-conjugated anti-mouse antibodies for 1 h at 37°C (Sigma, Missouri, USA). Subsequently, the plates were washed and developed in the dark with 50 μL TMB substrate diluted 1/50 with 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.03 % H_2O_2 . The reaction was interrupted after 10 min by adding 50 μL of 2 N H_2SO_4 to each well and read at 450 nm. The determination of positivity for anti ICB2-5 was calculated as described previously, with some modifications [9]. Briefly, firstly the average OD was calculated for each individual, and serum was considered positive if GST-tagged ICB2-5 OD was equal to or greater than cut off for this same protein, measured with sera of individuals who never had a past history of malaria. To confirm the positivity of a serum for anti ICB2-5, the average of GST-tagged ICB2-5 OD was calculated to exclude reactivity against GST. For each serum, the GST-cut off was calculated as average of GST OD plus twice the standard deviations (SD). The positivity for anti ICB2-5 was determined when average of GST-tagged ICB2-5 OD was equal to or greater than its GST-cut off. The results are expressed as the reactivity index (RI), which was calculated by dividing the OD of the sample by the cut-off value. Only the samples that were positive for total IgG (RI > 1) were tested for IgG subclasses.

Statistical analysis

The associations between the SNP genotypes and the antibody response were analysed by means of a logistic regression model, controlling for potential confounders, such as age and gender. Different genetic models (codominant, dominant, recessive and log-additive) were tested with the ‘SNPSassoc’ package [40] implemented for the R statistical software (version 3.1.1). A Bonferroni correction was used to adjust for multiple testing, and the significance was set at $p < 0.005$ (0.05/10). The sample size was calculated using GWApower software [41]. The current sample size ($n = 178$) had 80 % power to detect a variant with about 6 % heritability. Since only 90 samples were assessed for IgG subclasses, the power to detect the same heritability was about 42 %. Genotypic deviations from the Hardy–Weinberg equilibrium (HWE) were evaluated by using the exact test described by Wigginton et al. [42]. Statistically significant differences between the means of continuous variables were assessed with one-way analysis of variance (ANOVA). Correlation coefficients of the antibody levels were estimated with the Pearson correlation. Interactions between SNP pairs were also assessed with the ‘SNPassoc’ package, which determines the effects of interaction by means of log-likelihood ratio tests (LRTs).

Haplotype blocks were determined with Haploview 4.2 using the solid spine of linkage disequilibrium (LD) method [43], and the degree of LD between SNPs was estimated with the D' parameter. The R package ‘haplo.stats’ [44] was used for association tests between haplotypes and antibody levels by means of the ‘haplo.glm’ function, which performs a regression that estimates the effect of each haplotype compared to a reference haplotype by means of a general linear model.

Results

Serological data and single-marker associations

Of the 178 subjects analysed, all infected with *P. vivax*, 90 (50.6 %) exhibited IgG (RI > 1), whereas 53 (29.8 %) exhibited IgM specific for ICB2-5. The antibody levels were higher for IgG1 (mean = 1.08) compared to those of the other subclasses (means of 0.91, 0.79 and 0.79 for IgG2, IgG3 and IgG4, respectively) ($p < 0.001$, ANOVA). The highest correlations between levels of IgG subclasses were found for IgG1 and IgG2 ($r = 0.48$) and IgG1 and IgG3 ($r = 0.43$) (Additional file 2). The levels of ICB2-5-specific antibodies were not influenced by previous exposure to malaria (age, length of residence in the studied area, and previous episodes of malaria) (Additional file 3), except for IgG2, which exhibited higher levels in individuals who reported to have had less than five previous episodes of malaria, compared to those who reported to have had more than five previous episodes [mean

(confidence interval): 0.97 (0.84–1.10) vs. 0.88 (0.76–0.99), $p = 0.01$, ANOVA].

The allelic and genotype frequencies of all the analysed SNPs are listed in Table 1 and Additional file 4. The allele frequencies of these SNPs were previously presented in a larger set of samples (with exception of SNPs rs733618 and rs11571316) [45]. The success rate of SNP genotyping was 100 % for eight of the studied SNPs, whereas rs733618 and rs11571316 were successfully genotyped in 88.2 and 82 % of the samples, respectively, and no deviation from HWE was observed (all p value >0.1). A summary of the statistics for all the evaluated SNPs is listed in Table 1 and Additional file 4. There is not significant association between the polymorphisms and parasitaemia (all p value >0.07, Kruskal–Wallis test, data not show). Three SNPs were significantly associated with the humoral immune response against *P. vivax* ICB2-5. IgM levels were associated with rs35593994 in *CD28*; individuals with the *GG* genotype had lower antibody levels (mean 0.67 vs. 0.88, $p = 0.03$). Based on a recessive model, individuals with a *CC* genotype for rs3116496 in *CD28* exhibited higher IgG1 levels with respect to the other genotypes (mean 3.13 vs. 1.04, $p = 0.00004$). Individuals with a *TC* genotype for rs733618 in *CTLA4* exhibited higher IgG2 levels with respect to homozygous individuals (mean 1.18 vs. 0.87, $p = 0.04$). However, after correction for multiple testing, only association that remained significant was the one with *CD28* (rs3116496).

Linkage disequilibrium analysis and haplotype association

Linkage disequilibrium analysis was performed for all possible pairwise combinations of seven SNPs that were analysed across 255 kb in the 2q33 chromosomal region, which encompasses *CD28*, *CTLA4* and *ICOS*. Two haplotype blocks were defined using the criterion described by Barret et al. [43] (Fig. 1). Three haplotypes of block 1, which contains rs35593994 and rs3116496 in *CD28*, were built, with frequencies varying between 18.5 and 52.2 %, and for block 2, which contains rs733618, rs11571316 and rs5742909 in the promoter region of *CTLA4*, four haplotypes were built, with frequencies varying between 6.3 and 51.8 %. The effects of each haplotype on the antibody levels were estimated and are shown in Table 2. The AT haplotype in block 1 was significantly associated with a 20 % increase in IgM levels ($p = 0.027$) with respect to the reference haplotype (specifically, the most frequent haplotype, i.e., CT). However, no association remained significant after correction for multiple testing.

Interaction analysis

Using the R project package ‘SNPassoc’ [40], the study explored all pairwise SNP–SNP interactions under the codominant model. These interactions are represented

Table 1 Levels of *Plasmodium vivax* ICB2-5-specific IgM, IgG and IgG subclasses with respect to the analysed genotypes from individuals infected with *P. vivax*

SNP	Gene	Genotypes	Genotypes (n ^a)	Genotypes (n ^b)	IgG (RI)	IgG1 (RI)	IgG2 (RI)	IgG3 (RI)	IgG4 (RI)	IgM (RI)
rs35593994	CD28	GG/GA/AA	86/80/12	42/39/8	1.02/1.03/1.11	1.11/1.10/0.89	0.92/0.93/0.80	0.76/0.86/0.64	0.77/0.83/0.74	0.66/0.86/1.13 ^c
rs3116496	CD28	TT/TC/CC	118/54/6	60/27/2	1.04/1.02/0.91	1.06/0.98/3.13 ^d	0.91/0.92/0.95	0.82/0.72/0.75	0.82/0.75/0.67	0.83/0.70/0.65
rs733618	CTLA4	TT/TC/CC	135/20/2	72/7/1	1.06/0.89/1.05	1.11/0.97/0.81	0.87/1.18/0.64 ^e	0.80/0.66/0.57	0.80/0.68/0.62	0.82/0.72/1.25
rs11571316	CTLA4	GG/GA/AA	59/75/12	29/39/4	1.04/1.03/0.97	1.17/1.10/0.84	0.96/0.95/0.68	0.74/0.86/0.56	0.73/0.82/0.66	0.81/0.75/0.79
rs5742909	CTLA4	CC/CT/TT	151/27/0	75/14/0	1.15/1.01/-	1.04/1.29/-	0.91/0.93/-	0.80/0.76/-	0.80/0.78/-	0.79/0.78/-
rs231775	CTLA4	AA/AG/GG	75/84/19	40/41/8	1.07/1.00/1.01	0.96/1.22/0.98	0.87/0.98/0.82	0.78/0.82/0.70	0.79/0.79/0.81	0.79/0.75/0.89
rs4675378	ICOS	TT/TC/CC	77/73/27	35/38/16	1.00/1.05/1.09	1.08/1.12/0.99	0.86/0.94/0.96	0.73/0.90/0.67	0.80/0.78/0.80	0.80/0.73/0.85
rs1129055	CD86	GG/GA/AA	110/60/8	53/31/5	1.00/1.08/1.15	1.08/1.09/1.06	0.93/0.90/0.93	0.78/0.82/0.70	0.82/0.76/0.70	0.81/0.73/0.90
rs1883832	CD40	CC/CT/TT	135/37/6	65/20/4	1.01/1.06/1.25	1.11/1.03/0.86	0.92/0.95/0.68	0.81/0.77/0.65	0.81/0.75/0.79	0.80/0.69/1.13
rs9514828	BLYS	CC/CT/TT	103/62/13	48/35/6	0.99/1.07/1.15	0.99/1.23/0.98	0.91/0.94/0.83	0.82/0.69/1.11	0.76/0.81/0.92	0.73/0.83/0.95

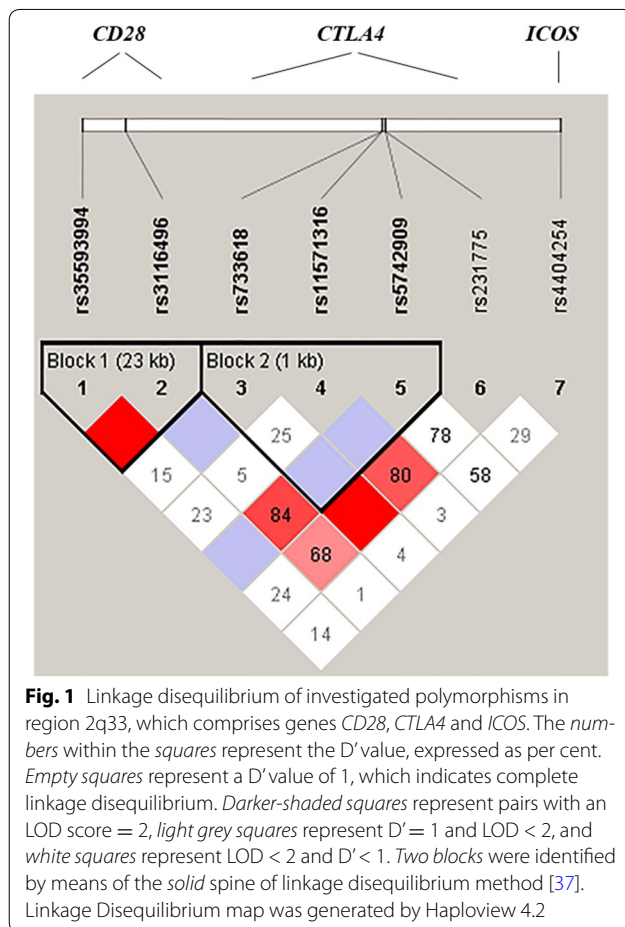
^a Number of individuals evaluated for ICB2-5-specific IgM and total IgG ($n = 178$)

^b Number of individuals evaluated for ICB2-5-specific IgG subclasses ($n = 90$)

^c Significant according to the dominant model ($p = 0.033$)

^d Significant according to the recessive model ($p = 0.00004$)

^e Significant according to the overdominant model ($p = 0.042$)



graphically in Fig. 2, in which the upper part contains the p values for the interaction LRT. Although several significant associations involving rs3116496 in *CD28* and other SNPs (rs1129055, rs5742909 and rs231775) with IgG1 levels were observed, the level of significance was below that found for rs3116496 individually (all p values >0.0001). However, the interaction involving rs35593994 in *CD28* with rs9514828 in *BLYS* was associated with the IgM levels ($p = 0.0009$). A regression analysis was performed to confirm this interaction, and the results of significant associations involving the above two SNPs are listed in Table 3.

Discussion

The main strategies used for malaria control are based on prompt diagnosis and treatment and on vector control. However, new resistant parasite strains arise as new drugs are applied, and vector control is also encountering great challenges due to the growing resistance to insecticides, thus justifying research on the development of a vaccine that is effective against malaria. Characterisation of the naturally acquired immune response in different populations is a useful tool for the identification of molecules that can be targeted by anti-malarial vaccines.

The present study was to evaluate the naturally acquired immune response against the N-terminal portion of PvMSP-1. Although the C-terminal portion (MSP-1₁₉) is considered to be the most immunogenic region of the protein [7, 9, 12], there is evidence suggesting that antibodies targeting the N-terminal portion

Table 2 Haplotype frequencies and their association with levels of ICB2-5-specific antibodies

Block	Haplotype	Frequency	IgG		IgG1		IgG2		IgG3		IgG4		IgM	
			Δ%	p	Δ%	p	Δ%	p	Δ%	p	Δ%	p	Δ%	p
Block 1	G-T	0.522	Reference											
	A-T	0.292	2 (-9; 13)	0.72	-3 (-27; 21)	0.80	-3 (-16; 9)	0.61	0 (-15; 14)	0.95	0 (-10; 10)	0.99	20 (2; 38)	0.027
	G-C	0.185	-3 (-16; 9)	0.59	21 (-9; 51)	0.17	0 (-15; 16)	0.96	-8 (-27; 10)	0.38	-7 (-19; 5)	0.26	-6 (-27; 15)	0.58
Block 2	T-G-C	0.518	Reference											
	T-A-C	0.330	-2 (-14; 10)	0.72	-4 (-32; 25)	0.81	-9 (-23; 5)	0.21	2 (-16; 19)	0.85	3 (-9; 16)	0.59	-7 (-28; 13)	0.48
	T-G-T	0.076	12 (-7; 31)	0.22	49 (-23; 121)	0.18	3 (-21; 27)	0.83	-4 (-34; 25)	0.77	-3 (-23; 17)	0.77	-3 (-34; 27)	0.84
	C-G-C	0.063	-17 (-38; 11)	0.27	-10 (-70; 51)	0.75	-14 (-44; 17)	0.38	-14 (-52; 23)	0.45	-11 (-36; 14)	0.38	-23 (-54; 9)	0.16

The effects of each haplotype are relative to the most frequent haplotype, which was used as a reference. Δ% indicates changes relative to the antibody levels compared to reference haplotypes, with 95 % confidence intervals. Block 1 refers to the SNPs rs35593994 and rs3116496, and block 2 refers to rs733618, rs11571316 and rs5742909

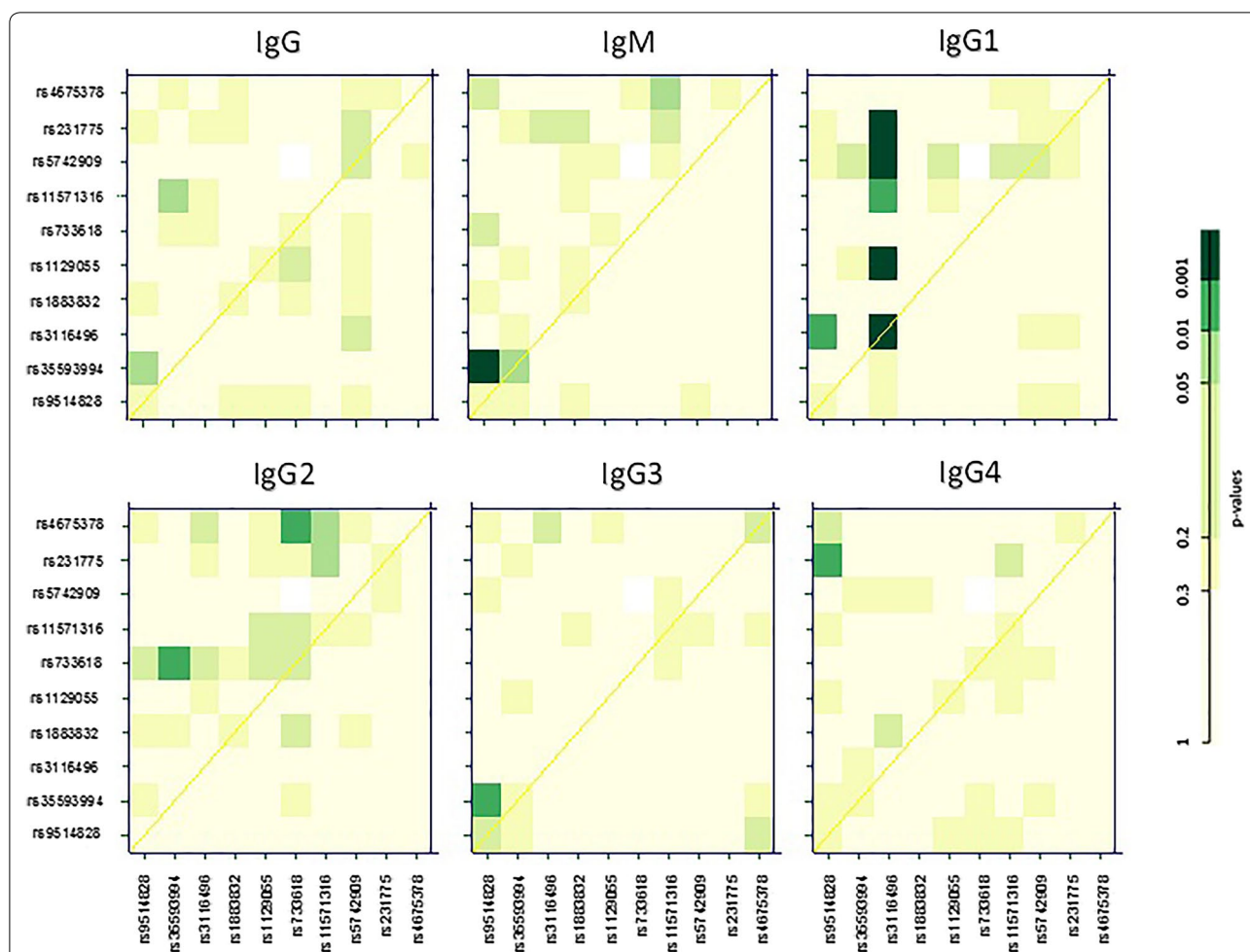


Fig. 2 Interaction between SNPs in the codominant model. The diagonal contains the *p* values of the likelihood ratio test (LRT) for the effect of each SNP individually. The *upper triangle* in the matrix contains the *p* values for the interaction log-LRT. The *lower triangle* contains the *p* values from LRT comparing the two-SNP additive likelihood to the best finding of the single-SNP model [35]

Table 3 Significant interactions between the SNPs rs35593994 and rs9514828 and the levels of IgM specific for ICB2-5

CD28 (rs35593994)	BLYS (rs9514828)	IgM (RI ^a)	Δ ^b	95 % CI	<i>p</i> ^c
GG	CC	0.503	Reference		
GG	CT	1.025	0.523	(0.195–0.850)	0.002
GA	CC	0.916	0.413	(0.126–0.702)	0.005
GA	TT	1.441	0.939	(0.395–1.483)	0.0009

^a Antibody levels are expressed as the mean of the reactivity index (RI)

^b Difference of the RI mean

^c Based on a logistic regression model using individuals exhibiting wild type genotypes (GG and CC) as a reference

of MSP-1 provide clinical protection during infections with both *P. falciparum* [46, 47] and *P. vivax* [9, 11]. The results of the IgG-mediated humoral immune response showed that ICB2-5 was detected by just over half of the studied individuals (50.6 %). This prevalence of responders is similar to that found in individuals infected with *P. vivax* in other places of the Brazilian Amazon [8, 11]. A small percentage (~10 %) of the individuals who reported having had several previous episodes of malaria exhibited high IgM levels, but no IgG was evidenced, suggesting that class switching from IgM to IgG may be impaired. This observation has been reported in previous studies [8, 48], and Soares et al. [8] suggested that this impaired switch from IgM to IgG may be related to deficient CD40/CD40-L interactions. Thus, the authors evaluated

whether co-stimulatory molecule gene polymorphisms are involved in the delayed class switch from IgM to IgG. However, no association was observed.

Regarding IgG subclasses, higher levels of IgG1 were found compared to the other IgG subclasses, which is in contrast to previous studies showing a predominance of IgG3 specific for ICB2-5 [8, 9, 11]. Although there is still no consensus regarding the role of antibody subclasses in clinical protection, it has been suggested that only the cytophilic subclasses, i.e., IgG1 and IgG3, are protective [49, 50]. Two longitudinal studies performed in the Brazilian Amazon, specifically, one in Portu-chuello, near Porto Velho, and the other in a community of Rio Pardo, Amazonas state, have observed that ICB2-5-specific antibodies were associated with clinical protection against malaria caused by *P. vivax* and that IgG3 was detected in all asymptomatic individuals, whereas most symptomatic patients exhibited no IgG3 [9, 11]. If the above results were to be extrapolated to Goianésia do Pará, the site of this study, the fact that a passive collection was performed on patients exhibiting symptoms could explain the low IgG3 levels found. Furthermore, some studies have also shown that IgG2 may be associated with protection. Deloron *et al.* [51] found an association between high IgG2 levels and low risk of acquiring an infection by *P. falciparum*. Although the design of the present study does not allow for the association of the prevalence and levels of clinically protective antibodies, higher IgG2 levels were observed in subjects who reported having had fewer cases of malaria.

The development of an immune response against *Plasmodium* species is a complex process, and one of the main issues is understanding why individuals differ in their immune responses against the parasite. Thus, the objective of the present study was to investigate the influence of co-stimulatory molecule gene polymorphisms on the production of antibodies specific for an important *P. vivax* vaccine candidate antigen. The most important result was that *CD28* rs3116496 was associated with levels of IgG1 specific for ICB2-5. In addition to the important role of *CD28* in T cell activation, the binding of this receptor to its ligands CD80 and CD86 on the surface of B cells provides bidirectional signals that appear to be important for IgG production by B cells [52]. Thus, *CD28* may be involved in the immune response against malaria. In fact, *CD28* knockout mice infected with *Plasmodium chabaudi* were unable to resolve the infection, maintaining low levels of parasitaemia for weeks after infection [53, 54]. Furthermore, treatment of wild type mice with monoclonal anti-CD86 antibodies abolished IL-4

production and was significantly associated with reduced levels of *P. chabaudi*-specific IgG1 [55].

In the present study, individuals exhibiting the *T* allele for rs3116496 in *CD28* were found to be associated with reduced IgG1 levels. Although the biological functions of this SNP, which is located in the third intron of the gene, are still unknown, it is located near a splice site, at which point mutations can induce abnormal splicing, thus affecting protein expression [56]. The relationship between rs3116496 in *CD28* and susceptibility to several diseases has already been evaluated, and significant associations have been found in type 1 diabetes [57], cervical [58] and breast cancer [59], and rheumatoid arthritis [60]. However, the role of this polymorphism in malaria has not yet been assessed, and even if the presence of the *T* allele, which is associated with lower levels of ICB2-5-specific IgG1, were implicated in higher susceptibility to vivax malaria, further elucidation would still be necessary.

Although it seems likely that immunity against malaria is affected by several genes, the influence of combined polymorphisms is rarely ever investigated. The present study performed analyses of interactions between the SNP pairs and found that *CD28* rs35593994 and *BLYS* rs9514828 together were associated with levels of IgM specific for ICB2-5, especially in individuals with *GA* and *TT* genotypes, respectively. Specifically, they were associated with a ~180 % increase in IgM levels compared to that of wild type genotype individuals. Although the biological mechanisms underlying this interaction are still unknown, *CD28* and *BLYS* may be involved in the production of memory B cells and antibody isotype class switching [61, 62]. Both polymorphisms, i.e., *CD28* rs35593994 and *BLYS* rs9514828, are located in the 5' regulatory region of the gene; thus, differential expression of these genes may change the regulation of the B cells involved in the production of ICB2-5-specific antibodies. Liu *et al.* [63] have shown that despite the generation of memory B cells as a response to vaccination with MSP-1₁₉, the function of these cells was nullified due to a lack of *BLYS* expression in dendritic cells from mice infected with *P. yoelii*.

Although it was observed the influence of these two SNPs in the antibody response to ICB2-5, most of the evaluated polymorphisms did not show any significant differences. In fact, variations in the immune response to malaria can be attributed to several factors, including the environment, previous exposure to malaria and immunogenicity of antigen. Thus, as a complex trait, it is likely that many polymorphisms have small effect in malaria immune response, but large sample size is required to detect it.

Conclusions

In summary, several genes involved in the control of the immune response were investigated, entailing the production of antibodies against a *P. vivax* vaccine candidate antigen. Despite the growing number of studies evaluating human genetic factors that control the immune response to malaria [27–34, 64], it is likely that many genes responsible for the wide inter-individual variation observed in the immune response against malaria remain unknown; studies similar to the present work may help identify subjects who are more prone to respond to a specific vaccine [29]. Although the statistical evidence supporting the described associations is limited by the relatively small sample size and, although it is impossible to tell whether the SNPs studied here are truly causal or are in LD with the causal variants, the results suggest that genetic variations in *CD28* and interactions between polymorphisms in *BLYS* and *CD28* may be involved in the control of the immune response against vivax malaria.

Additional files

Additional file 1. Reaction conditions for the amplification and enzyme digestion of polymorphisms in the studied polymorphisms.

Additional file 2. Correlation between ICB2-5-specific IgG, IgM and IgG subclasses in individuals infected with *Plasmodium vivax* living in an endemic area of the Brazilian Amazon.

Additional file 3. IgM and IgG subclasses levels (RI) for ICB2-5 according to age, length of residence in the studied area and previous episode of malaria.

Additional file 4. Association tests between genetic polymorphisms and *Plasmodium vivax* ICB2-5-specific antibody levels.

Authors' contributions

GCC, RLDM and PAN contributed to the design and conception of the study and wrote the manuscript. AACF, MPC and GCC participated in the molecular analyses. PAN, MEA, DRLB and GCC performed the serum analyses. GCC performed the statistical analyses. LMSM and MMP contributed to data interpretation and reviewed the manuscript. RLDM, GCC and MMP participated in field data collection. All authors read and approved of the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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