Short communication

Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*

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ABSTRACT

We evaluated the influence of allelic frequency of the human leukocyte antigen (HLA) -DRB1 on the acquisition of antibody response against malaria sporozoite and merozoite peptides in patients with *Plasmodium vivax* malaria acquired in endemic areas of Brazil. IgG antibodies were detected by enzyme-linked immunosorbent assay against four peptides of circumsporozoite protein (CSP) (amino, carboxyl, and VK210 and VK247 repeats) and peptides of merozoite surface protein 1 (MSP-1), apical membrane antigen 1 (AMA-1), and Duffy-binding protein (DBP). We found an association between HLA-DR3 and HLA-DR5 alleles and lack of antibody response to CSP amino terminal, as well as an association between HLA-DR3 and the highest antibody response to MSP1 (Pv200L). In conclusion, we suggest a potential regulatory role of the HLA-DRB1 alleles in the production of antibodies to a conserved region of *P. vivax* CSP and MSP1 in Brazilian population exposed to malaria.

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1. Introduction

Vaccines against malaria are viewed as a potentially cost-effective measure for malaria control and elimination, and, significant effort has been directed toward the identification and characterization of different *Plasmodium* antigens. Antibody response to pre-erythrocytic antigens, such as circumsporozoite protein (CSP); and erythrocytic proteins, such as merozoite surface protein 1 (MSP-1), apical membrane antigen 1 (AMA-1),

and the Duffy-binding protein (DBP); have been systematically evaluated in the literature (Arévalo-Herrera et al., 2010). Various authors have considered the CSP as major target for the development of recombinant vaccines for *Plasmodium vivax* due to the high levels of antibodies elicited against synthetic peptides, which exhibit the same specificity generated in natural infections (Herrera et al., 2005; Rodrigues et al., 2005; Beeson and Crabb, 2007; Penny et al., 2008). Several molecules of asexual blood-stage parasites are being considered as vaccine candidates. Proteins expressed by merozoites play a critical role during the invasion of red blood cells (RBCs) and are responsible for perpetuating the parasite life cycle (Remarque et al., 2008).

Human leukocyte antigen (HLA) class II genes were originally called immune response genes, since their alleles are known to influence antibody production (Germain, 1999). HLA class II

molecules bind antigenic peptides derived from exogenous as well as endogenous peptides, and the HLA class II-peptide complex formed is exposed at the surface of antigen-presenting cells to the T-cell receptor on CD4+ T lymphocytes, and the interaction with the B cell to activate a specific antigen–antibody response. However, since HLA genes are the most polymorphic genetic groups on human population and allelic differences in these molecules can modulate their ability to bind and present antigenic determinants of proteins and, thereby, change the nature of T-cell recognition. Various studies have investigated the influence of HLA alleles on malaria immunology (Banic et al., 2002; Johnson et al., 2004; Oliveira-Ferreira et al., 2004). Some HLA-DR alleles have been associated with a better antibody response to Nt47 (p126 amino-terminal portion) (Banic et al., 2002), AMA-1 (Johnson et al., 2004) of *Plasmodium falciparum*, and VK247 CSP repetition of *P. vivax* (Oliveira-Ferreira et al., 2004). Additionally, previous studies have reported an association between specific HLA alleles and immune response to malaria antigens in human vaccine trials (Nardin et al., 2001; Zhang et al., 2009). Therefore, using plasma samples from Brazilian patients naturally infected with *P. vivax*, we evaluated the influence of the HLA-DRB1 allelic frequency on the antibody response against four different CSP peptides and against three merozoite antigens.

2. Materials and methods

Patients enrolled in this study met the following criteria: presentation to medical care because of clinical symptoms of malaria, age >18 years old, and a positive malaria diagnosis by thick blood film for *P. vivax*. All patients signed a written informed consent. Peripheral blood samples were obtained from individuals from cities or towns in four malaria-endemic states of the Amazon region of Brazil: Macapá, Amapá state; Novo Repartimento, Pará state; Porto Velho, Rondônia state; and Plácido de Castro, Acre state. The malaria epidemiology of these areas has been previously described (Storti-Melo et al., 2011). The samples were frozen, DNA samples were extracted using the Easy-DNA™ extraction kit (Invitrogen, Carlsbad, CA, USA), and a semi-nested polymerase chain reaction (PCR) using specific small-subunit (SSU) rDNA primers (Kimura et al., 1997) was performed to confirm the single *P. vivax* malaria infection. The protocol for this study was reviewed and approved (Process number 235/2006) by the Research Board of the Faculty of Medicine from São José do Rio Preto, São Paulo state, Brazil.

HLA-DRB1 alleles genotyping of the 55 DNA samples of the malaria patients was carried out. DNA concentrations were obtained using a spectrophotometer at 260 and 280 nm, and content measured of 100 ng/μL was used for low resolution typing of the HLA-DRB1 by PCR with sequence-specific primers (PCR-SSP) (Micro SSP™ DNA Typing Trays, One Lambda, Inc., United States of America). Following manufacturer's guidelines, 1 μL of sterile water was added to the H1, H4, H7, and H10 wells of each plate. Reactions in these wells served as controls for the assay. Taq polymerase (7.5 U) was added to the DMiX® solution, and 9 μL of this mixture was then added to control wells. We then added 29 μL of DNA (100 μg/mL) to the DMiX® solution, and 10 μL of this final mixture was placed in each reaction well. The reaction plate was placed in a thermal cycler (Applied Byosystems Gene Amp PCR System 9700) with the following settings: an initial phase of 96 °C for 2 min and 63 °C for 1 min; followed by 9 cycles of 96 °C for 10 s and 63 °C for 1 min; followed by 20 cycles of 96 °C for 10 s, 59 °C for 50 s, and 72 °C for 30 s. DNA fragments were visualized on 1.5% agarose gels stained with ethidium bromide.

IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) according to previously published guidelines

(Herrera et al., 2004; Valderrama-Aguirre et al., 2005; Rodrigues et al., 2005; Cerávolo et al., 2005) in the 55 plasma samples. We used four different CSP peptides; amino (N), carboxyl (C), a repetitive region corresponding to the VK210 (R) and a repetitive region corresponding to the VK247 (V) (Herrera et al., 2004); and three merozoite proteins N-terminal fragment of MSP-1 (Pv200L) (Valderrama-Aguirre et al., 2005), recombinant peptide of AMA-1 (Rodrigues et al., 2005), and recombinant peptide of the DBP (Cerávolo et al., 2005). The ELISA IgG cutoff was defined as the mean optical density (OD) plus three standard deviations of the reaction from individuals ($n = 30$) who lived outside the Amazon region and who had never had malaria. The results were expressed as an index of reactivity (IR = OD405 values of tested sample divided by the value of the cutoff). IR values <1.0 were considered negative; IR values IR ≥1.0 were considered positive.

Analyses were performed using R version 2.8.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria, available at http://www.r-project.org). Allele frequencies were calculated using the formula $AF = a/N$, in which a represents the number of positive samples for a specific allele and N represents the total number of alleles in the study population (Garavito, 2003). The heterogeneity of HLA allele frequencies between responder and non-responder groups was evaluated by multiple logistic regression. Differences were considered significant when the p-value was <0.05.

3. Results

The HLA-DRB1 allele frequencies against the four different CSP peptides for responders and non-responders are shown in Table 1. A multivariate regression analysis showed a significant association between non-response to N peptide and the presence of HLA-DR3 ($p = 0.016$) and HLA-DR5 ($p = 0.013$) in individuals infected with *P. vivax*. No significant association, positive or negative, was observed between any HLA-DR molecules and antibody response to C, R, and V peptides.

The HLA-DRB1 frequencies to MSP1, AMA-1, and DBP were similar between responders and non-responders; and no significant association between any HLA-DRB1 allele and antibody response against these proteins was found (Table 2). However, almost 90% of Brazilian malaria patients develop antibodies to the MSP-1 (Pv200L) antigen (Storti-Melo et al., 2011). In our study, only two samples were ELISA negative and, among positive samples we observed high antibody levels with value IR > 10. Thus, we analyzed the influence of HLA-DRB1 alleles on antibody levels to MSP-1, and a significant association between high levels of antibodies to MSP-1 and the presence of HLA-DR3 ($p = 0.042$) was observed by logistic regression analysis. In fact, among individuals with HLA-DR3, the frequency of high responders to MSP-1 (35.7%) was significantly ($p = 0.040$) higher than in patients with other alleles (14.4%). Because the prevalence and levels of anti-MSP-1 antibodies seem related to previous malaria infection (Storti-Melo et al., 2011), we examined the possibility that individuals with HLA-DR3 had been more exposed to malaria, but no difference was observed in the number of previous malaria episodes between these individuals and those without HLA-DR3 ($p > 0.05$).

4. Discussion

HLA molecules show huge variability in humans and have an important role in immune response. HLA-DR is the most polymorphic, with specific HLA-DR alleles influencing the acquisition and levels of antibodies to pre-erythrocytic and erythrocytic malaria antigens (Johnson et al., 2004). As previously suggested by Zhang et al. (2009) to improve the efficacy of statistical analysis, we pooled

Table 1
Frequencies of HLA-DRB1 alleles in responders and not responders to four peptides of circumsporozoite protein.

HLA DRB1 alleles	Amino (N)		p-Value	Carboxyl (C)		p-Value	VK210 (R)		p-Value	VK247 (V)		p-Value
	Neg (N = 18)	Pos (N = 92)		Neg (N = 16)	Pos (N = 94)		Neg (N = 52)	Pos (N = 58)		Neg (N = 56)	Pos (N = 54)	
DR1 (DRB1*01)	0.056	0.076	0.467	0.063	0.074	0.868	0.077	0.069	0.295	0.071	0.074	0.468
DR2 (DRB1*15, *16)	0.000	0.098	0.998	0.063	0.085	0.685	0.058	0.103	0.588	0.089	0.074	0.733
DR3 (DRB1*03)	0.222	0.065	0.016ᵧ	0.000	0.106	0.998	0.058	0.121	0.845	0.036	0.148	0.190
DR4 (DRB1*04)	0.278	0.185	0.605	0.250	0.191	0.776	0.212	0.190	0.806	0.196	0.204	0.880
DR5 (DRB1*11, *12)	0.167	0.043	0.013ᵧ	0.000	0.074	0.998	0.038	0.086	0.336	0.036	0.093	0.918
DR6 (DRB1*13, *14)	0.056	0.174	0.211	0.250	0.138	0.095	0.154	0.155	0.520	0.179	0.130	0.462
DR7 (DRB1*07)	0.056	0.174	0.280	0.125	0.160	0.887	0.173	0.138	0.675	0.143	0.167	0.605
DR8 (DRB1*08)	0.111	0.141	0.578	0.188	0.128	0.548	0.173	0.103	0.444	0.196	0.074	0.435
DR10 (DRB1*10)	0.056	0.043	0.753	0.063	0.043	0.787	0.058	0.034	0.853	0.054	0.037	0.814

Neg: non-responders (IR < 1); Pos: responders (IR ≥ 1); N: total number of alleles.

ᵧ $p < 0.05$ by multiple logistic regression.

the alleles identified in our study into a group representing equivalent serological antigens. The N-terminal region of CSP is highly immunogenic and is recognized by T cell clones elicited by vaccination of DR4 (DRB*0401 and *0403) individuals (Parra-López et al., 2006). In our study, HLA-DR3 and HLA-DR5 alleles were associated with failure to produce antibody response against the N-terminal peptide of CSP in naturally infected patients. To our knowledge, no previous associations between HLA-DR3 and malaria antigen response have been reported. In a phase I clinical trial of the Multiple Antigens Peptides (MAP) vaccine, higher anti-sporozoite antibodies titers were restricted to three HLA class II alleles, including the HLA-DR5 (DRB1*1101) plus DRB1*0401 and DQB1*0603, whereas the HLA-DRB1*07 allele failed to elicit high antibodies levels (Nardin et al., 2000). In addition, Oliveira-Ferreira et al. (2004) implicated HLA-DR7 as a poor responder against the VK210 repetitive region of *P. vivax* CSP in Brazilian individuals. In contrast, Zhang et al. (2009) observed an improved antibody response against a chimeric protein (PfCP-2.9) in the presence of HLA-DR7 alleles.

P. vivax asexual blood stage antigens have been reported as targets for the production of invasion-inhibitory or growth-inhibitory antibodies. Among these, the MSP-1, the AMA-1, and the DBP have being evaluated for their immune potential (Good et al., 2005). We investigated the possible influence of HLA-DRB1 polymorphisms in antibody production against these antigens but no significant association between HLA-DRB1 allelic frequencies in responders or non-responders was found. In contrast, a higher antibody response against a recombinant AMA-1 of *P. falciparum* has been associated with HLA-DR5 in Cameroon (Johnson et al., 2004). Nevertheless, no previous associations between antibody response to DBP and HLA-DR alleles was reported.

Since most Brazilian patients exposed to malaria produce antibodies to Pv200L, there does not seem to be a limitation in the production of antibodies to this antigen that could be associated with HLA (Storti-Melo et al., 2011). However, a positive association was observed between the highest antibody levels to Pv200L and the presence of HLA-DR3 in this study. This antibody response did not seem related to the number of repeated exposures, since no difference was found between the number of previous malaria episodes and this allele presence. A similar study by Johnson et al. (2004), evaluating the HLA influence in the antibody response to *P. falciparum* asexual-stage antigens, found no association with any HLA-DRB1 or DQB1 alleles for antibody levels to MSP1-190l and to MSA2, but individuals positive for HLA-DR5 (*1201) had the highest antibody levels to PfAMA-1. However, this evaluation was done in Cameroon, an African population, where the genetic composition, the malaria transmission profile, and the *Plasmodium* spp. epidemiology are different from Brazil.

The lack of association observed between HLA-DRB1 alleles and antibody response against some CSP peptides (C, R, and V) and against AMA-1 and DBP in our work may mean either that the HLA-DRB1 does not, in fact, modulate the antibody response against these *P. vivax* antigens, or that, because of the small sample size of our study, we were unable to detect such an association. This finding, therefore, needs to be further investigated. However, the genetic regulation of antigen-specific antibody responses does not seem to be caused only by HLA class II genes. A recent study has shown that genetic factors modulate different antibody isotype and subclass responses to malaria antigens, mainly due to undefined non-HLA-linked genes (Duah et al., 2009). In summary, the association of the HLA-DR3 and DR5 alleles with the absence of antibody response to the N terminal of CSP should be thoroughly investigated before a malaria susceptibility profile can be established. Moreover, our finding that HLA-DR3 caused the highest antibody response to MSP1 needs to be confirmed by analyses using larger sample sizes, given the low frequency of this allele in Brazil.

Table 2
Frequencies of HLA-DRB1 alleles in responders and not responders to merozoite surface protein 1 (MSP-1–Pv200L fragment), apical membrane antigen 1 (AMA-1), and Duffy-binding protein (DBP).

HLA DRB1 alleles	Pv200L		p-Value	AMA-1		p-Value	DBP		p-Value
	Neg (N = 2)	Pos (N = 108)		Neg (N = 12)	Pos (N = 98)		Neg (N = 62)	Pos (N = 48)	
DR1 (DRB1*01)	0	0.074	1.000	0.167	0.061	0.060	0.097	0.042	0.240
DR2 (DRB1*15, *16)	0	0.083	1.000	0.083	0.082	0.795	0.129	0.021	0.087
DR3 (DRB1*03)	0	0.093	1.000	0.167	0.082	0.435	0.081	0.104	0.237
DR4 (DRB1*04)	0	0.204	0.999	0.333	0.184	0.249	0.194	0.208	0.654
DR5 (DRB1*11, *12)	0	0.065	1.000	0	0.071	0.999	0.048	0.083	0.117
DR6 (DRB1*13, *14)	0.5	0.148	0.636	0.083	0.163	0.728	0.113	0.208	0.158
DR7 (DRB1*07)	0	0.157	0.999	0	0.173	0.998	0.145	0.167	0.925
DR8 (DRB1*08)	0.5	0.130	0.176	0.167	0.133	0.547	0.145	0.125	0.520
DR10 (DRB1*10)	0	0.046	1.000	0	0.051	0.999	0.048	0.042	0.740

Neg: non-responders (IR < 1); Pos: responders (IR ≥ 1); N: total number of alleles.

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