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Influence of HLA-DRB1 Alleles on Antibody Responses to PfCP-2.9-Immunized and Naturally Infected Individuals

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Abstract
Introduction The *Plasmodium falciparum* chimeric protein, PfCP-2.9, which consists of apical membrane antigen (AMA)-1(III) and merozoite surface protein (MSP)1–19, is a promising asexual-stage malaria vaccine currently being evaluated in clinical trials. This study attempts to investigate the potential association between human leukocyte antigen (HLA)-DRB1 genotype and antibody response against PfCP-2.9 in healthy population and malaria patients.
Materials and methods We investigated the HLA-DRB1 alleles in 40 participants from phase I trial and 86 malaria patients from southern China by polymerase chain reaction with allele sequence-specific primers. The antibody and cellular response against PfCP-2.9 or its components were measured by enzyme-linked immunosorbent assay and T lymphocyte proliferation assay.
Results In clinical subjects, the anti-PfCP-2.9 antibody response was likely suppressed by HLA-DR6 alleles, which was consistent with the T lymphocyte proliferation assay. Nevertheless, HLA-DR7 positively correlated with antibody responses in naturally infected individuals while DR8 correlated with weaker antibody responses for all the three recombinant proteins. Moreover, parasitemia was significantly lower in samples with higher antibody levels against PfCP-2.9 or rMSP1–19, but not for rAMA-1(III).
Conclusion These data suggest that antibody responses against PfCP-2.9, AMA-1(III), or MSP1–19 elicited by vaccine formulation or natural infection are controlled by different HLA-II alleles. Moreover, the antibody response to MSP1–19 contributed more to protection immunity than AMA-1(III).

Keywords *Plasmodium falciparum* · PfCP-2.9 chimeric protein · malaria vaccine · HLA-DRB1 alleles

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Introduction

Plasmodium falciparum malaria remains a major global health threat, causing more than 1,000,000 deaths each year [1]. The blood stage of the *Plasmodium* is the only one associated with clinical disease, and immunity to the blood-stage parasites is a major goal of malaria vaccine development [2]. Many erythrocytic-stage antigens of *P. falciparum* are being evaluated for vaccine development, including apical membrane antigen (AMA-1) and merozoite surface protein 1 (MSP-1), the two primary malaria vaccine candidates expressed on the surface of merozoite [3, 4]. Domain III of the AMA-1 ectodomain (AMA-1 (III)) and the C-terminal region of MSP1 (MSP1–19) have been suggested to be involved in the merozoite invasion process and are both vaccine targets [5, 6]. High levels of MSP-1

antibodies provide protection from clinical malaria [7, 8]. We previously produced a *P. falciparum* chimeric protein (PfCP-2.9), which consists of AMA-1 (III) and MSP1–19, in *Pichia* yeast and showed high yield, immunogenicity, and antibody-mediated inhibition of parasite growth in vitro [9, 10]. A phase I randomized, single-blind, placebo-controlled, dose-escalation study demonstrated its safety, tolerability, and immunogenicity [11].

Human leukocyte antigen (HLA) class II alleles, including HLA-DR, HLA-DQ and HLA-DP, can modulate antibody production [12, 13]. HLA-DR is the most polymorphic and plays a crucial role in the antibody response, with specific HLA-DR alleles influencing the acquisition of antibodies to various malaria antigens [14–17]. In a phase I trial of PfCP-2.9, we found an overall group effect on the antibody response to the vaccine formulation in volunteers, but there were no significant dose–response relationships in four dose groups, implying that HLA-II alleles may influence antibody levels regardless of the antigen dose [11]. Here, we evaluated the influence of HLA-DRB1 alleles on antibody acquisition to PfCP-2.9 and its constituents, rAMA-1(III) and rMSP1–19, using plasma from participants in phase I trial and malaria-infected individuals and the correlation of antibody levels to protection from *P. falciparum* infection in southern China.

Materials and Methods

Subjects

The details of volunteer recruitment for the clinical trial have been previously published [11]. Briefly, 52 healthy malaria-naive adults in Shanghai (China) were enrolled in this clinical trial and were randomly allocated to five groups. Four groups (ten participants each) received 20, 50, 100, or 200 μg of a vaccine formulation, while the fifth (12 participants) received placebo.

The 86 malaria patients (mean age 32.6±9.5 years old, four females) participating in this study were residents of Tengchong in Yunnan Province, China. This county is in western Yunnan Province of southern China and is adjacent to Burma. Adult male farmers are at high risk for *P. falciparum* infection as they are more exposed to the mosquito vector, mainly *Anopheles minimus*, because of their occupation. We selected patients that had *P. falciparum* infection with parasitemia between 1,000–30,000 infected red blood cells per microliter of blood, an axillary temperature <40°C, and history of fever. Patients with severe malaria, including cerebral or placental malaria, were excluded from this study. Peripheral bloods were collected with or without anticoagulant for separation of sera and blood clots and were frozen at −20°C until analysis.

HLA Typing

Human genomic DNA was extracted from ethylenediaminetetraacetic acid-peripheral blood using the conventional proteinase K and phenol/chloroform extraction protocol modified by Sambrook et al. [18]. DNA tissue kit (Qiagen) was also used for DNA extraction from blood clots. Class II HLA-DRB1 allele typing was performed using the polymerase chain reaction with the intermediate-resolution sequence-specific primers described by the 11th and 12th International Histocompatibility Workshops [19, 20].

Antibody Measurement

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described [10]. Briefly, microtiter plates (Thermo Lab systems) were coated with PfCP-2.9 at 1.0 μg/ml. Serum samples at various dilutions were added to the plates. Peroxidase-conjugated goat antihuman IgG was then added to the plate at 1:1,000 dilution. Cutoff values were determined as the mean plus three standard deviations for the pre-immune sera.

For the sera from malaria patients, antibody levels against the recombinant proteins, PfCP-2.9, rAMA-1(III), or rMSP1–19 were measured by ELISA for optical density values. The procedure was similar to the ELISA described above, but the sera were all diluted at 1:1,000 in the first antibody reaction.

T Lymphocyte Proliferation

T cell responses to the antigen were measured by a lymphocyte proliferation assay as previously described [10]. Mononuclear cells from peripheral blood were isolated by Ficoll–Hypaque density gradient centrifugation, and the cell suspension with or without the antigen were used to test the incorporation of $[^3H]$TdR. Results are expressed as fold increases in stimulation index (SI), calculated as postvaccination SI divided by pre-immune SI. In this study, SI≥3 were considered as positive reaction.

Statistical Analysis

Statistical analysis was done using SPSS software (Version 10.0). Alleles were grouped by DR status and data were descriptively summarized using frequencies and percentages for all categorical variables. Following the descriptive comparisons, associations were more formally evaluated using logistic regression analyses. Regression variables were created for each allele and were coded as 0, 1, or 2,

according to the number of copies of the allele that a subject carried. Rare alleles, defined as those with fewer than five occurrences among all subjects, were pooled into a category labeled “other”. All global and univariate regression analyses included the design variable as a covariate. Descriptive association between periphery blood parasitemia and antibody levels against various antigens were examined by Pearson correlation analysis.

Results

HLA-DRB1 Alleles and Antibody or Lymphoproliferative Responses to PfCP-2.9 Protein in Clinical Trial Participants

Since there was overall group effect on the antibody response to the PfCP-2.9 vaccine formulation in volunteers [11], the geometric mean of antibody titer values of each immunization group as measured by ELISA may be the best way to come up with a cutoff value. Figure 1a showed that the distribution of ELISA titer values in each immunization group was not all clustered around the cutoff. Therefore, it was proper to adopt the geometric mean as cutoff to classify the “strong” or “weak” antibody responses for each group. Tables 1 and 2 present the results of logistic regression analysis of association between class II HLA-DRB1 alleles and antibody or cellular response to PfCP-2.9 protein, respectively. Global tests revealed no significant association of HLA-DRB1 alleles with PfCP-2.9 specific antibody or lymphoproliferative responses. However, univariate analysis revealed a marginally significant increase in the frequency (16.67%, p=0.063) of the DR6 alleles (DRB1*1301–04 and DRB1*1401–05) among subject with low antibody levels than those with high antibody levels (5.26%; Table 1). Moreover, we found a significant increase in the frequency (18.18%, p=0.041) of the DR6 alleles among subject who demonstrated low SI levels to PfCP-2.9 protein compared to those with significant levels (SI≧3) of PfCP-2.9 specific lymphoproliferative response (2.78%; Table 2).

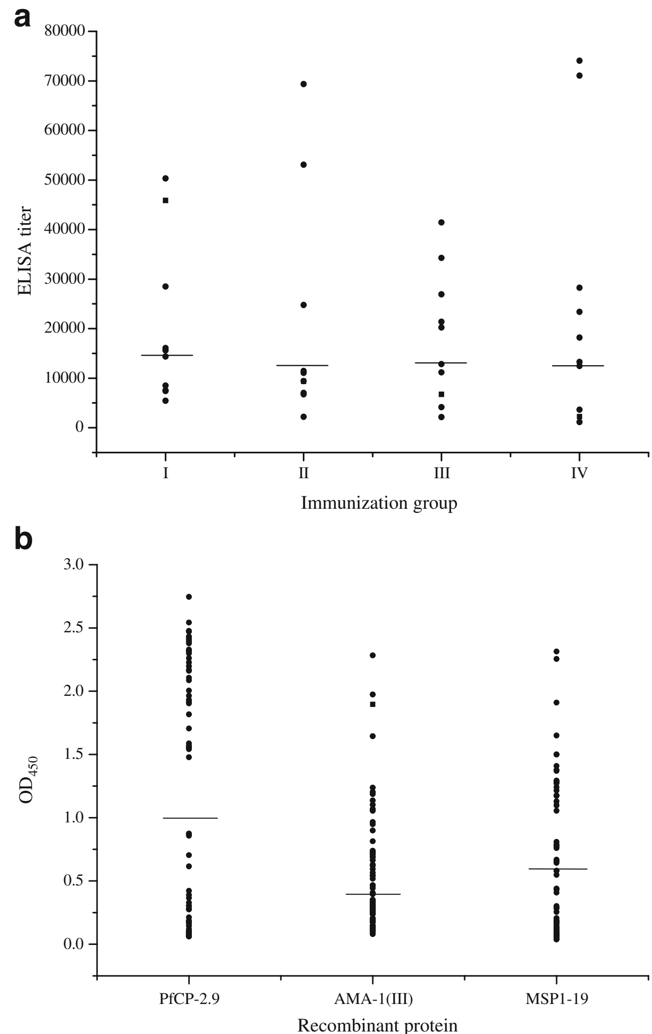

Fig. 1 Scatter plots showing the distribution of the OD_{450} values of ELISA in various groups. **a** Distribution of OD_{450} in four-dose groups of PfCP-2.9 phase I trial. Group I—20 μg; group II—50 μg; group III—100 μg; group IV—200 μg. **b** Distribution of OD450 against PfCP-2.9, rAMA-1(III), and rMSP1–19 in malaria patients from southern China. The geometric mean of ELISA titers and arithmetic mean of OD_{450} for each group was represented by *short horizontal line*

HLA-DRB1 Alleles and Antibody Responses to PfCP-2.9, AMA1-(III), or MSP1–19 in Malaria Patients

We next evaluated the association between HLA-II alleles and antibody responses to PfCP-2.9, AMA-1(III), and MSP1–19 in malaria patients using recombinant proteins expressed in yeast [9]. To assess the “strong” or “weak” antibody responses, the average of optical density (OD) value measured by ELISA were used as cutoff values for classification here. Result showed that the cutoff values of antibody responses against PfCP-2.9, rAMA-1 (III), and rMSP1–19 were 1.0, 0.4, and 0.6 (OD_{450} unit), respectively. Figure 1b showed that the distribution of OD values in each group was not all clustered around the cutoff. Global tests revealed significant associations of HLA-DRB1 alleles with PfCP-2.9- (p=0.029) or rAMA-1(III) (p=0.027)-specific antibody responses and marginally significant association for rMSP1–19 (p=0.075)-specific antibody response. Moreover, univariate analysis showed a significant (p=0.048 for PfCP-2.9, p=0.046 for rAMA-1(III), and p=0.043 for rMSP1–19) increase in the frequency of DR8 (DRB1*0801–0804) among subjects with low antibody levels (12.75% for PfCP-2.9, 13.21% for rAMA-1(III), and 13.21% for rMSP1–19) compared to those with high antibody levels (7.14% for PfCP-2.9; 6.06% for rAMA-1 (III); 6.06% for rMSP1–19; Tables 3, 4, and 5).

Table 1 HLA-DRB1 Allelic Associations with PfCP-2.9-Specific Antibody Response in Phase I Subjects

HLA-DRB1 locus	Allele counts ELISA titer (<cutoff value[b])		Allele counts ELISA titer (≥cutoff value)		Odds of ELISA titer positivity		Locus *p* value	Global *p* value
	N	%	*N*	%	OR	95% CI		
DR2(DRB1*15,*16)	6	14.29	11	28.95	1.761	0.679, 4.566	0.244	0.521
DR4(DRB1*04)	6	14.29	3	7.89	0.466	0.117, 1.855	0.279	
DR5(DRB1*11,*12)	5	11.90	5	13.16	1.164	0.334, 4.052	0.811	
DR6(DRB1*13,*14)	7	16.67	2	5.26	0.199	0.036, 1.091	0.063	
DR7(DRB1*07)	5	11.90	3	7.89	0.587	0.148, 2.325	0.448	
DR8(DRB1*08)	2	4.76	4	10.53	2.192	0.353, 13.612	0.399	
DR9(DRB1*09)	7	16.67	7	18.42	1.616	0.499, 5.229	0.423	
Other[a]	4	9.52	3	7.89	0.759	0.142, 4.075	0.748	

[a] Including DR1(DRB1*01), DR3(DRB1*03), and DR10(DRB1*10)

[b] Geomean value of ELISA titers in each immunization group

We also found a significant (p=0.025) decrease for PfCP-2.9 protein and marginally significant decreases for both rAMA-1(III) (p=0.057) and rMSP1–19 (p=0.052) in the frequency of DR7 (DRB1*0701–0702) among subjects with low antibody levels (0.89% for PfCP-2.9; 1.89% for rAMA-1(III); 1.89% for rMSP1–19) compared to those with high antibody levels (11.43% for PfCP-2.9; 1.89% for rAMA-1(III); 1.89% for rMSP1–19; Tables 3, 4, and 5).

Parasitemia and Antibodies Levels Against PfCP-2.9, AMA-1(III), and MSP1–19 in Malaria Patients

We next investigated the role of HLA class II alleles in susceptibility to *P. falciparum* infection by Pearson correlation analysis of parasitemia and antibody levels against different recombinant antigens in malaria patients from southern China (Fig. 2). Results showed that parasitemia was significantly lower (p=0.026 for PfCP-2.9; p=0.044 for rMSP1–19) in samples with higher antibody levels against PfCP-2.9 or rMSP1–19, but not for rAMA-1(III) (p=0.864).

Discussion

Polymorphism in the HLA-II alleles has the potential to modulate antibody responses with their ability to bind different peptides and thereby change the nature of T cell recognition [12, 21, 22]. For healthy subjects vaccinated with PfCP-2.9, the antibody response against PfCP-2.9 was likely suppressed by HLA-DR6 alleles, which was supported by the T lymphocyte proliferation

Table 2 HLA-DRB1 Allelic Associations with PfCP-2.9-Specific Lymphoproliferative Response in Phase I Subjects

HLA-DRB1 locus	Allele counts lymphoproliferation (SI<3)		Allele counts lymphoproliferation (SI≥3)		Odds of ELISA titer positivity		Locus *p* value	Global *p* value
	N	%	*N*	%	OR	95% CI		
DR2(DRB1*15,*16)	10	22.73	7	19.44	0.807	0.333, 1.958	0.636	0.521
DR4(DRB1*04)	4	9.09	5	13.89	1.107	0.323, 3.801	0.871	
DR5(DRB1*11,*12)	4	9.09	6	16.67	2.048	0.519, 8.086	0.306	
DR6(DRB1*13,*14)	8	18.18	1	2.78	0.102	0.011, 0.910	0.041	
DR7(DRB1*07)	3	6.82	5	13.89	1.442	0.382, 5.438	0.589	
DR8(DRB1*08)	3	6.82	3	8.33	0.903	0.165, 4.939	0.906	
DR9(DRB1*09)	8	18.18	6	16.67	1.16	0.365, 3.684	0.801	
Other[a]	4	9.09	3	8.33	0.878	0.169, 4.553	0.877	

[a] Including DR1(DRB1*01), DR3(DRB1*03), and DR10(DRB1*10)

Table 3 HLA-DRB1 Allelic Associations with PfCP-2.9-Specific Antibody Responses in Naturally Infected Patients

HLA-DRB1 locus	Allele counts ELISA titer (OD<1)		Allele counts ELISA titer (OD≥1)		Odds of ELISA titer positivity		Locus *p* value	Global *p* value
	N	%	*N*	%	OR	95% CI		
DR2(DRB1*15,*16)	17	16.67	8	11.43	0.598	0.299, 1.198	0.147	0.029
DR4(DRB1*04)	7	6.86	5	7.14	0.969	0.351, 2.679	0.952	
DR5(DRB1*11,*12)	20	19.61	11	15.71	0.662	0.350, 1.249	0.203	
DR6(DRB1*13,*14)	26	25.49	14	20.00	0.736	0.440, 1.231	0.243	
DR7(DRB1*07)	1	0.98	8	11.43	13.147	1.378, 125.467	0.025	
DR8(DRB1*08)	13	12.75	5	7.14	0.288	0.084, 0.988	0.048	
DR9(DRB1*09)	9	8.82	4	5.71	0.623	0.228, 1.699	0.355	
Other[a]	9	8.82	15	21.43	1.719	0.812, 3.635	0.157	

[a] Including DR1(DRB1*01), DR3(DRB1*03), and DR10(DRB1*10)

Table 4 HLA-DRB1 Allelic Associations with AMA-1(III)-Specific Antibody Responses in Naturally Infected Patients

HLA-DRB1 locus	Allele counts ELISA titer (OD<0.4)		Allele counts ELISA titer (OD≥0.4)		Odds of ELISA titer positivity		Locus *p* value	Global *p* value
	N	%	*N*	%	OR	95% CI		
DR2(DRB1*15,*16)	18	16.98%	7	10.61%	0.521	0.25, 1.084	0.081	0.027
DR4(DRB1*04)	4	3.77%	8	12.12%	2.262	0.704, 7.269	0.171	
DR5(DRB1*11,*12)	17	16.04%	14	21.21%	0.907	0.5, 1.645	0.748	
DR6(DRB1*13,*14)	28	26.42%	12	18.18%	0.642	0.375, 1.097	0.105	
DR7(DRB1*07)	2	1.89%	7	10.61%	5.438	0.954, 31	0.057	
DR8(DRB1*08)	14	13.21%	4	6.06%	0.286	0.083, 0.980	0.046	
DR9(DRB1*09)	9	8.49%	4	6.06%	0.53	0.182, 1.541	0.244	
Other[a]	14	13.21%	10	15.15%	0.938	0.473, 1.859	0.854	

[a] Including DR1(DRB1*01), DR3(DRB1*03), and DR10(DRB1*10)

Table 5 HLA-DRB1 Allelic Associations with MSP1–19-Specific Antibody Responses in Naturally Infected Patients

HLA-DRB1 locus	Allele counts ELISA titer (OD<0.6)		Allele counts ELISA titer (OD≥0.6)		Odds of ELISA titer positivity		Locus *p* value	Global *p* value
	N	%	*N*	%	OR	95% CI		
DR2(DRB1*15,*16)	18	16.98	7	10.61	0.536	0.261, 1.103	0.09	0.075
DR4(DRB1*04)	6	5.66	6	9.09	1.154	0.42, 3.171	0.781	
DR5(DRB1*11,*12)	19	17.92	12	18.18	0.758	0.412, 1.394	0.373	
DR6(DRB1*13,*14)	27	25.47	13	19.70	0.689	0.409, 1.162	0.162	
DR7(DRB1*07)	2	1.89	7	10.61	5.644	0.986, 32.319	0.052	
DR8(DRB1*08)	14	13.21	4	6.06	0.278	0, 080, 0.961	0.043	
DR9(DRB1*09)	7	6.60	6	9.09	0.958	0.381, 2.405	0.927	
Other*	13	12.26	11	16.67	1.06	0.536, 2.098	0.866	

[a] Including DR1(DRB1*01), DR3(DRB1*03), and DR10(DRB1*10)

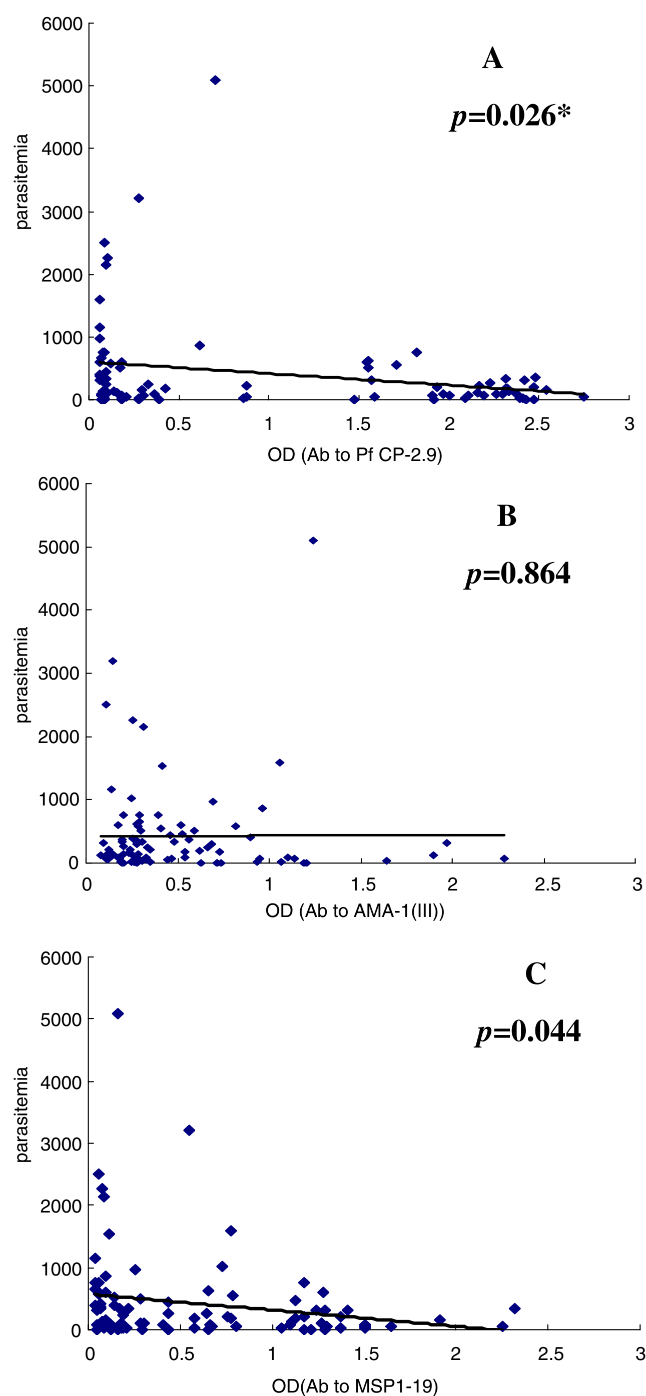

Fig. 2 Association between parasitemia and levels of antibodies against recombinant PfCP-2.9, AMA-1(III), and MSP1–19 in malaria patients. Regression analysis between parasitemia (parasites per 100 leukocytes) and antibody levels to **a** PfCP-2.9, **b** AMA-1(III), and **c** MSP1–19 were shown with regression line, respectively. *Asterisk* The *p* value of Pearson correlation analysis

assay. However, HLA-DR8 alleles were associated with weaker antibody levels in naturally infected malaria patients. This result indicates that PfCP-2.9 may contain additional epitopes besides those derived from AMA-1 (III) and MSP1–19 domains, which could potentially influence the association findings. Another study on epitope identification for PfCP-2.9 protein in our laboratory has found some such additional epitopes (unpublished data).

On the other hand, the differences in antibody response patterns between immunized and naturally infected patients may result from the small sample size in the clinical trial. To improve the efficacy of statistical test, the allele level typing results have been combined into groups that are the equivalent of broad serological antigens from DR1 to DR10. Admittedly, the artificial grouping in the clinical trial with small size may hide some allele-specific correlations. Therefore, these are the preliminary data of association between PfCP-2.9-specific immune responses and host MHC genotype under the limitation of small size in this trial and need to be validated with more samples in the next trial.

In malaria patients, HLA-DR7 alleles correlated with improved antibody responses against both domains, but HLA-DR8 had the contrary effect on AMA-1(III) and MSP1–19, which were also present in the PfCP-2.9 assay. A similar study in Cameroon, Africa, indicated that individuals with HLA-DR5 (DRB1*1201) alleles had higher antibody responses to a variant of recombinant AMA-1 produced in a baculovirus expression system, but there was no association between HLA-II alleles and MSP1–19 antibody levels [23], implying a complex response process that involves more than just HLA-II alleles, e.g., population genomic, age, and multiple infections [8]. Finally, antibody responses to PfCP-2.9 or MSP1–19, but not AMA-1(III), positively associated with protective immunity, implying that the antibody response to MSP1–19 contributes more to protective immunity than AMA-1 (III).

In summary, we have evaluated the associations between HLA-II alleles and antibody responses to PfCP-2.9, AMA-1(III), and MSP1–19 in immunized humans and naturally infected humans. Our findings suggest that HLA-DR7 may participate in an enhanced antibody response, and HLA-DR6 and DR8 perhaps inhibit it. In the future, we will further investigate the association between HLA alleles and antibody responses using more clinical trial samples, which could direct the design of immunization trials for this malaria vaccine candidate.

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