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Promiscuous T-cell epitopes of Plasmodium Merozoite Surface Protein 9 (PvMSP9) induces IFN- γ and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon

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Abstract

Plasmodium vivax merozoite surface protein (PvMSP9) stimulates both cellular and humoral immune responses in individuals who are naturally infected by this parasite species. To identify immunodominant human T-cell epitopes in PvMSP9, we used the MHC class II binding peptide prediction algorithm ProPred. Eleven synthetic peptides representing predicted putative promiscuous T cell epitopes were tested in IFN- γ and IL-4 ELISPOT assays using peripheral blood mononuclear cells (PBMC) derived from 142 individuals from Rondonia State, Brazil who had been naturally exposed to *P. vivax* infections. To determine whether the predicted epitopes are preferentially recognized in the context of multiple alleles, MHC Class II typing of the cohort was also performed. Five synthetic peptides elicited robust cellular responses, and the overall frequencies of IFN- γ and IL-4 responders to at least one of the promiscuous peptides were 62% and 46%, respectively. The frequencies of IFN- γ and IL-4 responders to each peptide were not associated with a particular HLA-DRB1 allelic group since most of the peptides induced a response in individuals of 12 out of 13 studied allelic groups. The prediction of promiscuous epitopes using ProPred led to the identification of immunodominant epitopes recognized by PBMC from a significant proportion of a genetically heterogeneous population exposed to malaria

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infections. The combination of several such T-cell epitopes in a vaccine construct may increase the frequency of responders and the overall efficacy of subunit vaccines in genetically distinct populations.

Keywords

Plasmodium vivax; Merozoite Surface Protein-9; promiscuous T-cell epitopes

1. Introduction

Malaria caused by *Plasmodium vivax* is a major worldwide health problem with an estimated 80 to 300 million cases annually. Although the clinical profile of *P. vivax* malaria is not generally considered severe and a high mortality rate is not common, severe disease and mortality due to *P. vivax* are an increasing concern [1]. Notwithstanding, the substantial epidemiological impact of malaria caused by *P. vivax* can be quantified in terms of its significant economical burden in countries with emerging or developing nations [2–3]. Historically, basic and translational malaria research programs have been broadly focused on *P. falciparum*, and *P. vivax* investigations have received comparatively much less attention and support. In fact, among seventy two malaria vaccine candidates currently in a clinical development pathway only three are based on *P. vivax* antigens [4].

Effective immunity to malaria, whether studying *P. falciparum*, *P. vivax*, or animal model systems, seems to require both humoral and cellular immune responses, although the relative importance of each remains unclear. T helper cells are involved in the regulation of antibody production [5–6] and cytotoxic T lymphocyte (CTL) reactivity [6]. Effector T cells are also needed in the production of IFN- γ , which plays a role in controlling the liver-stage development and parasitemia peaks [7–8]. The identification of human CD4 T-cell epitopes within a protein is important in the context of vaccine design, because optimal T-cell epitopes delivered by vaccination would induce memory cells that could potentially be boosted by natural exposure to the parasite. However, the development of such vaccines is impaired due to the extensive polymorphism in human leukocyte antigens (HLA). The identification of universal T-cell epitopes, with promiscuous profiles of interaction with MHC class II molecules, enhances the possibility of developing subunit vaccines that could elicit immune responses in heterogeneous populations [9]. This will result in efficient response that transcends the barrier imposed by HLA polymorphism [10]. The use of *in silico* tools for mining such peptides circumvents the expensive and laborious experimental screening methods [11]. Because of their variable size, the prediction of peptides binding to HLA class II is more challenging as compared to HLA class I. HLA class II binding peptides are 9–22 amino acids long; with a binding core of 9 amino acids containing the primary anchor residues.

Plasmodium vivax merozoite surface protein-9 (PvMSP9) is a vaccine candidate that is expressed during schizogony and becomes organized on the surface of merozoites in the course of schizont development and segmentation. The *P. vivax*, *P. cynomolgi* and *P. knowlesi* msp-9 gene have typical eukaryotic signal peptides and diverse repeated motifs present immediately upstream of their termination codon. Another feature conserved among these proteins, including the *P. falciparum* MSP9 protein, is the positions of four cysteine residues near the N-terminus, suggesting this conservation maintains structural and perhaps functional characteristics in the MSP-9 family. Rabbit polyclonal antisera raised against recombinantly expressed N-termini of *P. knowlesi* and *P. vivax* MSP-9 cross-react with the counterpart proteins in immunofluorescence and immunoblot assays [12–13].

We have reported that PvMSP9 contains B- and T-cell epitopes recognized by antibodies and T cells from individuals naturally exposed to *P. vivax* in the Brazilian Amazon [14]. Five synthetic peptides derived from the N-terminus of PvMSP9 stimulated T-cells to secrete IFN- γ and IL-4 in from natives from the study population and a migrant population from a malaria free region of Brazil. In the present study we report the identification of peptide sequences containing promiscuous HLA class II epitopes derived from PvMSP9 that are capable of stimulating T cells from donors expressing various HLA genotypes and with confirmed exposure to *P. vivax* infections.

2. Material and Methods

2.1 Study area and volunteers

A cross-sectional cohort study was conducted involving 142 individuals from communities in the malaria endemic region of Rondonia state, Brazil, where *P. vivax* malaria accounts for more than 70% of all malaria cases in the last five years (Brazilian Ministry of Health, 2008). The individuals in the study population consist of rain forest natives who have resided in the malaria-endemic region for over 25 years (50%) as well as transmigrants from several non-endemic areas of Brazil who have resided in the region for 10 years or more (38%). Samples and survey data were collected during the dry months of June–August of 2004, coinciding with the period of increased malaria transmission in Rondonia State. Written informed consent was obtained from all adult donors or from parents of donors in the case of minors. The study was reviewed and approved by the Fundação Oswaldo Cruz Ethical Committee and the National Ethical Committee of Brazil.

2.2 Epidemiological survey

To evaluate epidemiological factors that may influence the cellular immune response against PvMSP-9, all donors were interviewed upon informed consent. Questions in the survey 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 recorded and entered into a database created with Epi Info 2002 (Centers for Disease Control and Prevention, Atlanta, GA).

2.3 Collection of human blood samples and malaria diagnosis

Venous peripheral blood was collected into heparinized tubes, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll/Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation and used in the ELISPOT assays within the first 12 hours after collection. Plasma was stored at -20°C and thin and thick blood smears of all donors were examined for malaria parasites. Parasitological evaluation by examination of 200 fields at 1000X magnification under oil-immersion, all slides were examined by a researcher expertise in malaria diagnosis. Donors positive for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated per the chemotherapeutic regimen recommended by the Brazilian Ministry of Health.

2.4 ProPred analysis for PvMSP9 promiscuous T cell epitopes

HLA-DR binding frames along the primary structure of PvMSP9 were detected by ProPred analysis. The amino acid sequence of PvMSP9 was scanned to identify promiscuous MHC binding peptides using virtual matrices designed for 51 HLA-DR alleles [15]. Eleven sequences were identified within the N-terminal region of PvMSP9 which were predicted to bind at least 40% HLA-DR alleles included in the ProPred algorithm at a 3% threshold. Synthetic peptides representing such putative T-cell epitopes were synthesized at the Laboratory of Biochemistry of Proteins and Peptides, Institute Oswaldo Cruz, Fiocruz. The

complete amino acid sequences of five out of 11 synthetic peptides (including 3 overlapping regions) that induced the highest cellular response and the relative amino acid position were: 1) peptide pE (V₁₄₇ - K₁₅₉), VVHKLNNKMKSLK; 2) peptide pH (V₄₃₈- D₄₄₉), VSLMASIDSMID; 3) peptide pJ (K₃₂₅-I₃₃₉), KLKDILLRVLYKTYI; 4) peptide pK (P₄₃₄-I₄₄₈), PAEDVSLMASIDSMI and 5) peptide pL, (A₄₄₃-K₄₅₆), ASIDSMIDEIDFYEK. The following six peptides the frequency of responders was very low (less than 3%) and the relative amino acid position were: 1) peptide pA (M₁-F₁₃), MRVNIIIFSFVVF; peptide pB (V₃₇-C₅₀), VNNYEKLFKVIKC; peptide pC (L₈₆-V₉₉), LMNDLKVVGKNEHV; peptide pD (Y₁₀₀-N₁₁₂), YGPNTNELKKIIH; peptide pF (F₃₄₁-T₃₅₃), LRVLYKTYINYRT; peptide pG (L₃₇₁-R₃₈₃), LELGMKILFNKLR.

2.5 ELISPOT assays

Cells cultures were carried out in duplicate in nitrocellulose 96 well plates (MAHA S4510 - Millipore, Billerica, MA) coated overnight at 4°C with 5 µg/ml capture anti-IFN-γ monoclonal antibodies (MabTech, Stockholm – Clone D1K) or anti-IL-4 (Pharmingen, San Jose, CA - Clone MP4-25D2) in phosphate buffered saline. The plates were blocked with RPMI medium containing 10% fetal calf serum for at least 2-h. 2.5×10^5 cells were added to the ELISPOT plates in the presence of medium alone, 10 µg/ml of each PvMSP9 peptide or 1 µg/ml of phytohemagglutinin. Cells were stimulated for 24 h for IFN-γ or 48 h for IL-4 at 37°C, 5% CO₂ under sterile conditions. After stimulation, plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T) and incubated with either biotin-anti-human IFN-γ Clone 7-B6-1 (MabTech) diluted in PBS or biotin-anti-human IL-4 Clone 12-1 NON0059 (Biosource International, Camarilla, CA) diluted in PBS-T containing 1% fetal bovine serum (PBS-TF) for 3 h at 37°C. The plates were washed four times with PBS-T and incubated with streptavidin-alkaline phosphatase (MabTech) in PBS-TF for 1 h at 37°C. The plates were washed four times with PBS-T before development with 1-step NBT/BCIP (Pierce, Rockford, IL). Development was stopped by the addition of distilled water. IFN-γ and IL-4 secreting cells appeared as blue spots that were counted with an Immunospot reader (Cellular Technology Ltd, Cleveland, OH) using the Immunospot Software Version 3. ELISPOT responses were expressed as spot-forming cells (SFC) per 250,000 PBMCs. PHA (1 µg/ml) was used as a positive control. The assays were subsequently categorized as positive or negative depending on whether the mean number of SFC in the peptide stimulated wells was greater than the mean number plus twice the SD of SFC in the control wells with medium alone from the same donor. Therefore individuals presenting at least 20 for IFN-γ and 10 for IL-4 more SFCs/ 2×10^5 PBMC in the experimental wells than in control were considered responders.

2.6 HLA Typing of PBMC

Genomic DNA was extracted and purified from PBMCs of volunteers using QIAamp blood kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacture recommendation. The amount of DNA obtained was quantified by spectrophotometry. Sequence-specific oligonucleotide probes (SSOPs) were used by Luminex Xmap technology in order to determine the HLA class II allelic groups of studied individuals. 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-Phycoerythrin conjugated to streptavidin. After hybridization, the samples were analyzed with Luminex Flow Analysis equipment. The HLA analysis program deduces the HLA-DRB1 and HLA-DQB1 allelic groups.

2.7 Statistical analysis

Analyses were done using Epi Info 2007 (CDC, Atlanta, GA), Instat or Prism 5 (GraphPad Software, San Diego, CA). Differences in medians for the study population data were tested by non-parametric Mann-Whitney test where appropriate. Student's t test was used to compare means of normally distributed data, and normalized transformations were performed on raw data before testing by one-way analysis of variance where appropriate. Differences in proportions were evaluated by chi-square (χ^2) test. Relationships between years of residence in the endemic area and number of past malaria infections or months since last known malaria episode were assessed with Spearman's rank correlation. Bipartition χ^2 was used to evaluate the relationship between HLA-DRB1 and the frequency of cellular immune response. HLA-DRB1 and -DQB1 alleles were also analyzed for association with the IFN- γ or IL-4 response to PvMSP9 peptides, and when appropriate a relative risk was calculated.

3. Results

3.1 Epidemiological data

The epidemiological and demographic data of the studied population have been described previously [14]. Briefly, the majority of the volunteers are natives of the Amazon forest or residents living in the state of Rondonia for approximately 20 years and transmigrants from non-endemic regions who have lived in malaria endemic regions for at least 10 years. All individuals were exposed to *P. vivax* and *P. falciparum* infections throughout the year. At the time of the blood collection the frequency of malaria infected individuals was very low, 11 individuals were infected with *P. vivax* and 4 with *P. falciparum*. However, the majority of our donors confirmed a prior history of malaria infections.

3.2 IFN- γ and IL-4 responses to PvMSP9 peptides

Five out of the 11 synthetic peptides tested, predicted to be promiscuous, showed that the overall frequencies of IFN- γ and IL-4 responders to at least one of the peptides were 61.2% and 49%, respectively. The frequency of IFN- γ responders was significantly higher than IL-4 for peptides pE ($p=0.0006$), pK ($p=0.0462$) and pL ($p=0.0015$), but no difference was observed for peptides pH and pJ. When the pattern of the responses was examined, significant differences were observed, and the frequencies of positive responses induced by different peptides varied. In evaluating the IFN- γ responses, the peptides pE and pL were more commonly recognized than pH, pJ and pK ($p<0.05$). For IL-4 responses, no differences were observed among the synthetic peptides tested (Figure 1). The mean numbers of adjusted IFN- γ -SFC elicited by all tested peptides (pE= 43 ± 23 ; pH= 39 ± 14 ; pJ= 38 ± 19 ; pK= 41 ± 21 ; pL= 43 ± 21) were significantly higher than IL-4-SFC (pE= 21 ± 8 ; pH= 25 ± 11 ; pJ= 23 ± 8 ; pK= 21 ± 9 ; pL= 22 ± 10). A Venn diagram organizes the relationships among the cellular responses to overlapping peptides pH, pK and pL (Figure 2). Of the 72 responders to pL, 49 individuals also responded to pK and/or pH; of the 55 responders to pK, 43 individuals responded to pH and/or pL; and finally, of the 52 responders to pH, all but 4 individuals responded to the other overlapping peptides.

There were no statistically significant associations between the epidemiological profile of the studied population and either frequency of IFN- γ responders or number of spots. However, the number of IL-4 spots generated after stimulation with all overlapping peptides (pH, pK, pL) were higher in individuals who have lived in malaria endemic areas for more than 20 years when compared with those who have lived in such areas for less than 20 year ($p<0.0129$), and the number of spots generated after pL stimulation was correlated with the time of residence in a malaria endemic area ($r=0.3421$; $p=0.0231$). None of the 30 malaria-naive control samples demonstrated significant IFN- γ or IL-4 cellular responses to the 5

peptides tested. Both the malaria-exposed and malaria-naive groups responded similarly to PHA (577 ± 211 IFN- γ and 198 ± 101 IL-4 SFC).

3.2 HLA genotyping of studied individuals

PBMC of all donors were typed for HLA-DRB1 and HLADQB1 alleles in order to evaluate the promiscuous presentation of PvMSP9 peptides to T cells. The analysis of these 142 donors demonstrates that they represent a heterogeneous group of donors expressing several HLA allelic groups (Figure 3). We found 13 allelic groups in HLA-DRB1* and 5 groups in HLA-DQB1*. There were two predominant HLA allelic groups in our studied population, HLA-DRB1*04 (19% of all HLA-DR genotypes, $\chi^2 = 6.043$; $p < 0.0140$) and HLA-DQB1*03 (47% of all HLA-DQ genotypes, $\chi^2 = 52.450$; $p < 0.0001$). The HLA-DRB1*09 and DQB1*04 presented the lower frequencies with 0.7% and 8.5% respectively.

3.3 Evaluation of PvMSP9 peptides for promiscuous presentation

The stimulation of PBMCs with the five synthetic PvMSP9 peptides induced IFN- γ and IL-4 responses in malaria-exposed individuals with diverse HLA-DR and HLA-DQ backgrounds. Peptides pE, pH, pJ, pK and pL induced IFN- γ and/or IL-4 cellular response in all HLA-DRB1 allelic groups (tables 1 and 2), with the exception of HLA-DRB1*09. However, it is important to note that there was one individual in this group. The frequencies of IFN- γ responders by HLA-DRB1 alleles range from 21.4% (pE in HLA-DRB1*01 individuals; n=28) to 100% (pL in HLA-DRB1*08 individuals; n=10), however the frequency of IFN- γ responders was not associated to a particular HLA-DRB1 allelic group. A similar profile was observed in HLA-DQB1, with a frequency of IL-4 responders ranged from 11.1% (pJ in HLA-DRB1*11 individuals; n=28) to 100% (pH in HLA-DRB1*10; n=2). In evaluation of cellular response by HLA-DQB1, the frequencies of IFN- γ responders ranged from 26.1% (pJ in HLA-DQB1*06; n=46) to 57.1% (pL in HLA-DQB1*02, n=28) and the frequency of IL-4 responders from 18.8% (pJ in HLA-DQB1*05 individuals; n=32) to 41.2% (pH in HLA-DQB1*06 individuals, n=34), but there was no association between the positive or negative individuals and a particular HLA-DQB1 allele. The median number of IFN- γ and/or IL-4 spots generated in responders after stimulation with the five synthetic peptides did not differ significantly among the 13 HLA-DRB1 and the 5 HLA-DQB1 studied alleles.

4. Discussion

Plasmodium vivax merozoite surface protein 9 is a promising vaccine candidate antigen. Previous studies have demonstrated that (i) PvMSP9 is conserved among mice, primate and human *Plasmodium* species [12]; (ii) PvMSP9 recombinant proteins induce high titers of antibodies [13]; (iii) antibodies raised against PvMSP9 are capable of inhibiting merozoite invasion [12]; and (iv) malaria-exposed individuals present high frequency of natural antibody and cellular immune response against different regions of PvMSP9 [14]. Clinical trials based on a few selected malaria antigens have shown limited immunogenicity and a failure to induce long-lasting immunity, possibly due to the lack of effective T-cell epitopes in the constructs used as immunogens [16–17]. Nevertheless, there have been only a few T-cell epitopes reported from malaria antigens [18–24]. A major obstacle for identifying T-cell epitopes is the high level of polymorphism of HLA class II molecules. Thus, one of the most relevant steps for malaria vaccine development is to define T-cell epitopes that can interact promiscuously with a broad range of HLA-DR and/or HLA-DQ molecules.

Here we present the identification of five T cell epitopes in the vaccine candidate PvMSP9 that are capable of stimulating T cells from donors expressing various HLA genotypes and with confirmed exposure to *P. vivax* infections.

Experimental screening methods to evaluate the presence of HLA restriction in immune response to vaccine candidates are expensive and time consuming. Computational prediction methods complement experimental studies, minimize the number of validation experiments, and significantly expedite the epitope mapping process [11]. Such methods have helped identify promiscuous epitopes within *Leishmania* [25], *Mycobacterium tuberculosis* [26] and HIV [27] antigens. Several promiscuous epitopes from pre-erythrocytic [22–23,28], asexual blood-stage [21,24,29], and gametocyte [20] antigens have been predicted and/or experimentally confirmed for *P. falciparum*. In contrast, only limited studies have focused on promiscuous epitopes for *P. vivax* [19,30–32]

In our study, eleven peptides were predicted by the ProPred algorithm to be promiscuous, but only five of them were recognized at high frequency by PBMCs from individuals living in malaria endemic areas. The recall response elicited by at least one of these five peptides was high for both IFN- γ (64.1%) and for IL-4 (50.7%) in comparison with the frequencies observed for other *Plasmodium* antigens such as PvTRAg40 [33], PfTRAP [34], PvDBP [35]. The frequency of T cells reactive to PvMSP9 is comparable to a study by Farouk et al. [36] that measured the cellular response to crude *P. falciparum* antigens by ELISPOT in a Malian population. Evaluating the numbers of IFN- γ and IL-4 SFCs, we observed that IFN- γ had higher numbers of secreting cells than IL-4 for all the synthetic peptides studied. No correlation between IFN- γ response and malaria exposure was observed. However, IL-4 SFC produced upon peptide pL stimulation correlated positively with time of residence in the endemic area and the number of IL-4 spots generated after stimulation with all overlapping peptides (pH, pK, pL) were higher in individuals who have lived in malaria endemic areas for more than 20 years when compared with those who have lived in such areas for less than 20 years. It is possible that variations in exposure may also explain variations in the type of naturally induced TH1 and TH2 immune responses to PvMSP9 [14]. Indeed, data reported by Troye-Blomberg *et al.* [37], showed a strong association between elevated IgG and IgE antibodies to blood-stage antigens with increased numbers of IL-4 secreting cells in individuals less susceptible to malaria infection. Similarly, correlations between the production of IL-4 in response to the *P. falciparum* malaria antigen Pf155RESA and protection against malaria were also reported [38].

The frequency and numbers of responders to overlapping peptides shows that the core sequence shared with peptides pH, pK and pL (**ASIDSMI**) is highly immunogenic. However the presence of 23 individuals who present cellular response only to peptide pL suggest that this peptide may have two immunodominant epitopes, one in the overlapping core region and the second one in the carboxy-terminal region that is not shared with pH or pK (DEIDFYEK).

The evaluation of IFN- γ and IL-4 production was used here to measure the recognition and activation of T cells by PvMSP9 putative promiscuous T-cell epitopes. To correlate the cellular response with the prevalence of MHC class II alleles, we determined the HLA antigen distribution among the study population. The observation of 13 allelic groups in the cohort suggests that the study population is heterogeneous, presenting a large variety of allelic groups. It was expected in our study mainly because Brazilian populations have peculiar features of a tri-hybrid populations formed with contribution of Caucasian, African, and native Amerindian origin, in which the phenotypic characteristics of each original population have been highly mixed. However the observation of high frequency of HLA-DR4 and HLA-DQ3 indicates that in this population the Amerindian HLA genotype is conserved [39]. Therefore, previous works already show the association with IgG responders to *Plasmodium* antigens and the HLA-DRB04 in this population [40–41], indeed studies with HLA polymorphism observed in several populations have been attributed to a pathogen induced selection [42–43].

The enrollment of populations with high HLA polymorphism but with a related degree of conservation and association with immune response with *Plasmodium* antigens is ideal to validate the prediction algorithm using PvMSP9. The lack of association with the frequency of IFN- γ and IL-4 cellular responses and the number of ELISpots generated after stimulation with the five PvMSP-9 predicted epitopes supports the recall cellular immune response reported in our previous study [14] and the promiscuous properties of the PvMSP9 derived peptides: pE, pH, pK, pJ and pL.

Several studies have suggested that single-epitope-based vaccines are not potent enough to induce full protection [30,44–45]. However, the identification of immunogenic and promiscuous epitopes within a vaccine candidate antigen is extremely important, since it is possible to formulate a vaccine composed of relevant epitopes from different antigens. Additionally, the combination of multiple B cell and T cell epitopes was shown to increase immunogenicity [46–48]. In conclusion the HLA-DR heterogeneity of the responding subjects and the prediction analysis using the ProPred server strongly suggest that these peptides was presented to T cells promiscuously. Thus the overall results suggest that HLA restriction will not be a problem if these peptides are used in a vaccine candidate.

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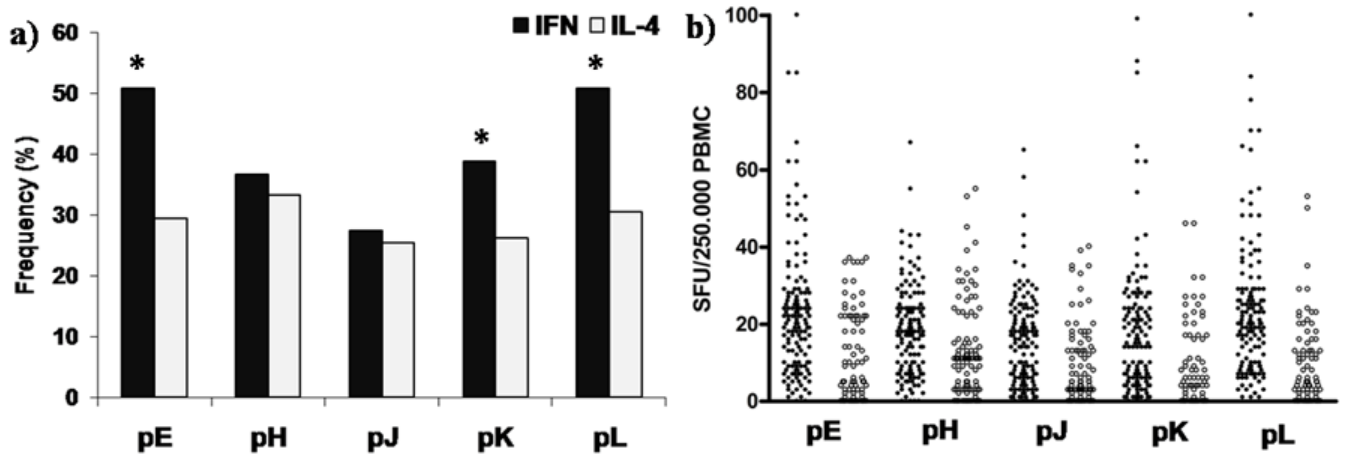


Figure 1.

Frequency of positive responders (a) and number of spots (b) in ELISpot IFN- γ and IL-4 responses to PvMSP9 synthetic peptides (pE, pH, pJ, pK and pL) in individuals naturally exposed to malaria infections. (*) Significant differences ($p < 0.01$) for the comparison between the frequencies of IFN- γ responders with IL-4 responders, for each peptide calculated using χ^2 test.

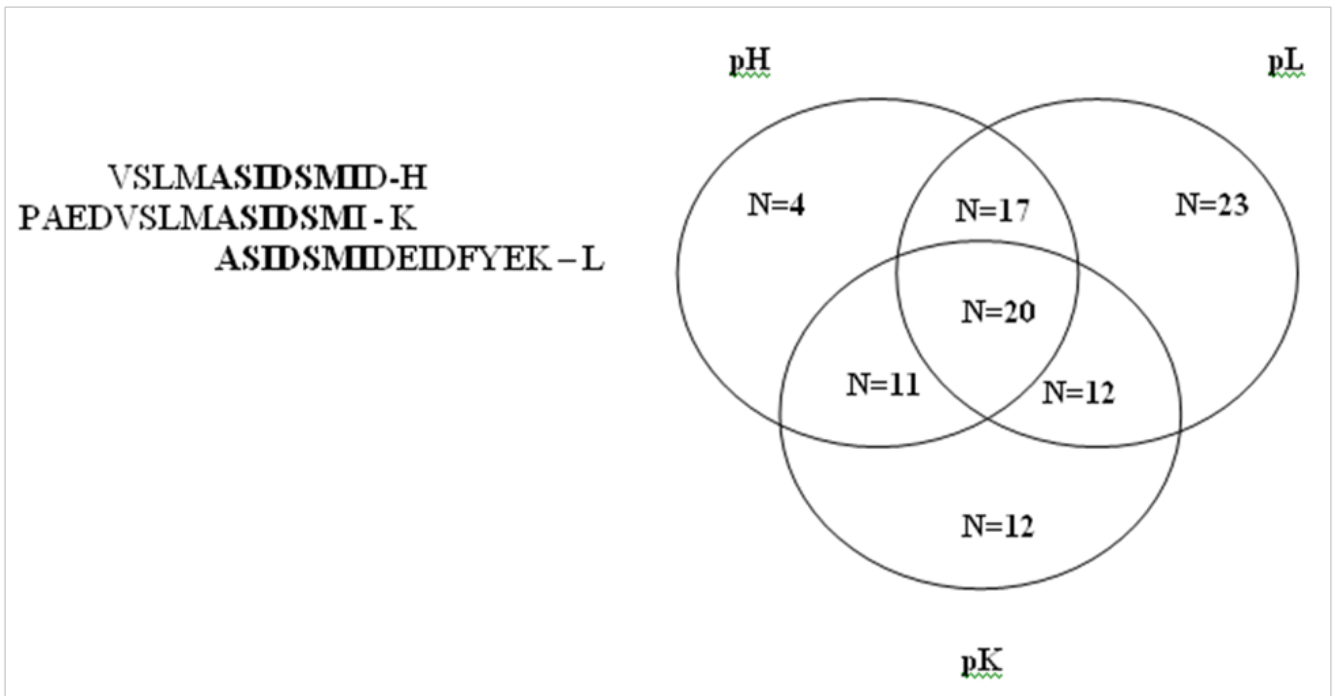


Figure 2.
Venn diagram showing the relationships among the cellular responses to overlapping peptides pH, pK and pL sequences. Intersection points between the responders represent the number of positive individuals for two or three overlapping peptides.

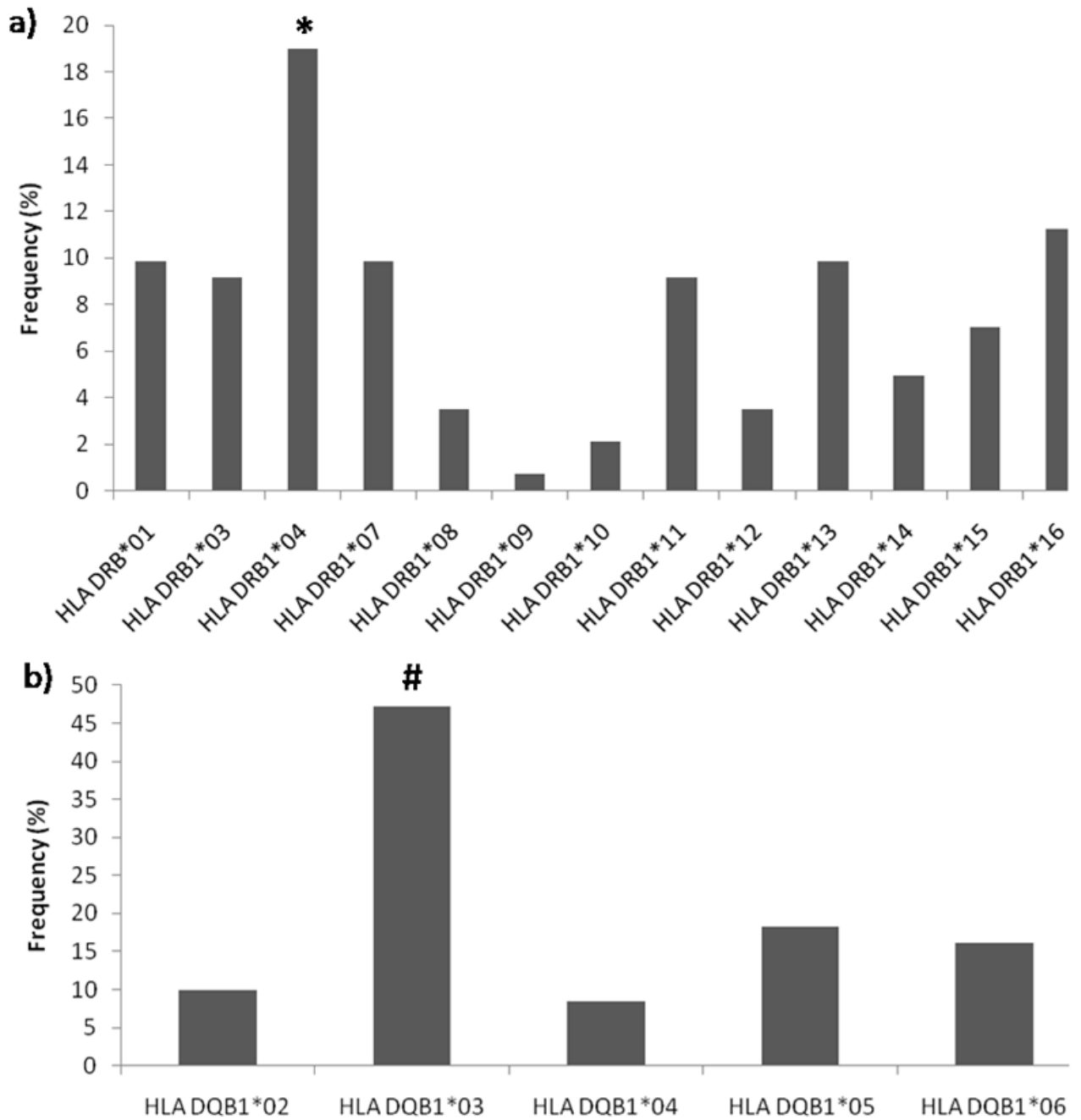


Figure 3.

Frequency of HLA-DRB1* (a) and HLA-DQB1 (b) alleles distribution in the 142 individuals studied. * indicates that the proportion of HLA-DRB1*04 predominate over all others HLA-DRB1* studied alleles ($\chi^2 = 6.043$; $p < 0.0140$) and # indicates that the proportion of DQB1*03 predominate over all other studied DQB1* alleles ($\chi^2 = 52.450$; $p < 0.0001$).

Table 1

Frequency (F) and number (n) of IFN- γ responders to PvMSP9 synthetic peptides by HLA-DRB1* and HLA-DQB1* allelic groups total observations (t). The frequencies of IFN- γ responders to the PvMSP9 synthetic peptides were not associated to a particular HLA-DRB1* or HLA-DQB1* allelic group by bipartition χ^2 test ($p > 0.05$).

| Allelic group | pE | | pH | | pJ | | pK | | pL | |
|---------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a | F (n/t) ^a |
| <i>HLA-DRB1</i> | | | | | | | | | | |
| HLA-DRB1*01 | 57% (16/28) | 36% (10/28) | 21% (6/28) | 50% (14/28) | 43% (12/28) | | | | | |
| HLA-DRB1*03 | 61% (16/26) | 31% (8/26) | 23% (6/26) | 38% (10/26) | 69% (18/26) | | | | | |
| HLA-DRB1*04 ^b | 44% (24/54) | 37% (20/54) | 37% (20/54) | 30% (16/54) | 44% (24/54) | | | | | |
| HLA-DRB1*07 | 57% (16/28) | 43% (12/28) | 29% (8/28) | 50% (14/28) | 71% (20/28) | | | | | |
| HLA-DRB1*08 | 60,0% (6/10) | 40% (4/10) | 40% (4/10) | 80% (8/10) | 100% (10/10) | | | | | |
| HLA-DRB1*11 | 46% (12/26) | 31% (8/26) | 23% (6/26) | 31% (8/26) | 38% (10/26) | | | | | |
| HLA-DRB1*12 | 60% (6/10) | 20% (2/10) | 20% (2/10) | 20% (2/10) | 40% (4/10) | | | | | |
| HLA-DRB1*13 | 43% (12/28) | 50% (14/28) | 29% (8/28) | 36% (10/26) | 50% (n=14) | | | | | |
| HLA-DRB1*14 | 57% (8/14) | 29% (4/14) | 29% (4/14) | 29% (4/14) | 29% (4/14) | | | | | |
| HLA-DRB1*15 | 50% (10/20) | 40% (12/20) | 10% (2/20) | 40% (8/20) | 50% (10/20) | | | | | |
| HLA-DRB1*16 | 44% (14/32) | 37% (12/32) | 31% (10/32) | 37% (12/32) | 44% (14/32) | | | | | |
| <i>Others^d</i> | 50% (4/8) | 25% (2/8) | 25% (2/8) | 50% (4/8) | 50% (4/8) | | | | | |
| <i>HLA-DQB1</i> | | | | | | | | | | |
| HLA-DQB1*02 | 50% (14/28) | 36% (10/28) | 29% (8/28) | 43% (12/28) | 57% (16/28) | | | | | |
| HLA-DQB1*03 ^c | 49% (66/134) | 36% (48/134) | 27% (36/134) | 37% (50/134) | 52% (70/134) | | | | | |
| HLA-DQB1*04 | 58% (14/24) | 42% (10/24) | 33% (8/24) | 50% (12/24) | 50% (12/24) | | | | | |
| HLA-DQB1*05 | 50% (26/52) | 35% (18/52) | 30% (14/52) | 38% (20/52) | 42% (22/52) | | | | | |
| HLA-DQB1*06 | 52% (24/46) | 39% (18/52) | 26% (12/46) | 35% (16/46) | 52% (24/46) | | | | | |

^a Each studied individual contributed two HLA alleles observations to this descriptive analysis.

^b The proportion of DRB1*04 predominate over all others DRB1* studied alleles ($\chi^2 = 6.043$; $p < 0.0140$).

^c DQB1*03 predominate over all other studied DQB1* alleles ($\chi^2 = 52.450$; $p < 0.00001$).

^d "Others" includes the following HLA-DRB1* less frequent allelic groups: DRB1*09 (n=2) and DRB1*10 (n=6).

Table 2

Frequency (F) and number (n) of IL-4 responders to PvMSP9 synthetic peptides by HLA-DRB1* and HLA-DQB1* allelic groups total observations (t). The frequencies of IL-4 responders to the PvMSP9 synthetic peptides were not associated to a particular HLA-DRB1* or HLA-DQB1* allelic group by bipartition Chi squared test ($p>0.05$).

| Allelic group | pE | | pH | | pJ | | pK | | pL | |
|---------------------------|-----|--------------------|-----|--------------------|-------|--------------------|-------|--------------------|-----|--------------------|
| | F | (n/t) ^a | F | (n/t) ^a | F | (n/t) ^a | F | (n/t) ^a | F | (n/t) ^a |
| <i>HLA-DRB1</i> | | | | | | | | | | |
| HLA-DRB1*01 | 23% | (6/26) | 31% | (8/26) | 17% | (4/24) | 33.3% | (8/24) | 27% | (6/22) |
| HLA-DRB1*03 | 25% | (6/24) | 25% | (6/24) | 20% | (4/20) | 20% | (4/20) | 30% | (6/20) |
| HLA-DRB1*04 ^b | 21% | (10/48) | 27% | (12/44) | 29% | (12/42) | 29% | (12/42) | 26% | (10/38) |
| HLA-DRB1*07 | 31% | (8/26) | 31% | (8/26) | 23% | (6/26) | 31% | (8/26) | 17% | (4/24) |
| HLA-DRB1*08 | 60% | (6/10) | 60% | (6/10) | 50% | (4/8) | 50% | (4/8) | 50% | (4/8) |
| HLA-DRB1*11 | 18% | (4/22) | 20% | (4/20) | 11% | (2/18) | 11% | (2/18) | 22% | (4/18) |
| HLA-DRB1*12 | 40% | (4/10) | 40% | (4/10) | 40% | (4/10) | 20% | (2/10) | 80% | (8/10) |
| HLA-DRB1*13 | 38% | (10/26) | 43% | (12/28) | 20.0% | (6/30) | 13% | (4/30) | 21% | (6/28) |
| HLA-DRB1*14 | 50% | (6/12) | 33% | (4/12) | 33.3% | (4/12) | 33.3% | (4/12) | 60% | (6/10) |
| HLA-DRB1*15 | 44% | (8/18) | 43% | (6/14) | 43% | (6/14) | 43% | (6/14) | 43% | (6/14) |
| HLA-DRB1*16 | 25% | (8/32) | 29% | (8/28) | 29% | (8/28) | 29% | (8/28) | 17% | (4/24) |
| <i>Others^d</i> | 0% | (0/4) | 50% | (2/4) | 0% | (0/4) | 0% | (0/4) | 50% | (2/4) |
| <i>HLA-DQB1</i> | | | | | | | | | | |
| HLA-DQB1*02 | 38% | (10/26) | 38% | (10/26) | 21% | (6/26) | 38% | (10/26) | 38% | (10/26) |
| HLA-DQB1*03 ^c | 29% | (38/132) | 31% | (38/124) | 28% | (34/120) | 23% | (28/120) | 26% | (28/108) |
| HLA-DQB1*04 | 33% | (8/24) | 38% | (10/24) | 33% | (8/24) | 25% | (6/24) | 33% | (6/24) |
| HLA-DQB1*05 | 24% | (10/42) | 22% | (8/36) | 19% | (6/32) | 31% | (10/32) | 33% | (10/30) |
| HLA-DQB1*06 | 28% | (10/36) | 41% | (14/34) | 19% | (6/32) | 25% | (8/32) | 37% | (12/32) |

^aEach studied individual contributed two HLA alleles observations to this descriptive analysis.

^d“Others” includes the following HLA-DRB1* less frequent allelic groups: HLA-DRB1*09 (n=2) and HLA-DRB1*10 (n=2).