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Original article

Evaluation of the antigenicity of universal epitopes from PvDBPII in individuals exposed to *Plasmodium vivax* malaria

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Abstract

The Duffy-binding protein (PvDBP) mediates invasion of reticulocytes by the malaria parasite *Plasmodium vivax*. PvDBP has been recognized as a good vaccine candidate due to its ability to induce antibody responses capable of inhibiting target cell invasion after natural infections. For the development of subunit-based vaccines, it is important to identify universal epitopes that could be presented by different HLA-DR alleles to induce effective cellular and humoral immune responses. In this study, the antigenicity of universal epitopes from PvDBPII was evaluated by stimulating peripheral blood mononuclear cells (PBMCs) isolated from individuals with different degrees of *P. vivax* malaria exposure and distinct HLA-DR alleles. Peptides 1635 and 1638 induced lymphoproliferation and stimulated the production of IL-6 and IFN-γ. The results suggest that conserved peptides binding with high activity to red blood cells and with known affinity to HLA-DR proteins could be good components for a *P. vivax* vaccine.

Keywords: *Plasmodium vivax*; Duffy-binding protein; Peptide vaccines; Cytokines; Cell proliferation

1. Introduction

Malaria remains a worldwide health problem [1]. Vaccination is considered one of the most promising strategies for controlling this disease [2]. However, obtaining a fully effective vaccine has not been possible due to the great variety of surface proteins being expressed by the parasite at its life-cycle stages and the high degree of polymorphism existing among them. In addition, immunity acquired after a natural infection is inefficient, gradually acquired, and species, stage, strain, and variant specific [3,4]. The immune response is genetically restricted due to the high degree of polymorphism existing in human leukocyte antigens (HLA), i.e., a given epitope may be recognized by an individual but not by another [5,6], hampering vaccine development.

The identification of universal epitopes is a widely used strategy for defining which peptides should be included in a multiantigenic antimalarial vaccine, as it helps avoiding HLA-associated genetic restrictions of the immune response, and ensures that effective cellular and humoral immune responses are induced against malarial pathogens in a large proportion of the population [6–8].

Malaria is one of the most prevalent parasitic diseases in tropical and subtropical countries. About 500 million of new cases are annually reported, and it is estimated that around 1–2 millions of these cases are fatal [9]. *Plasmodium vivax* is the most widespread malaria species affecting mainly Asian, South and Central American countries, and the second leading cause of malaria as it is responsible for 132–391 million infections per year [10,11].

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Plasmodium species have a complex life cycle involving a mosquito vector and a vertebrate host [12]. During invasion of red blood cells (RBCs) by merozoites (blood stage forms), proteins released from the apical organelles (rhoptries, micronemes, and dense granules) are responsible for the parasite's recognition and the RBCs infection [13]. The family of erythrocyte binding antigens (EBAs) is found within these proteins, which includes the Duffy-binding protein (DBP) essential for invasion of RBCs by *P. vivax* and *Plasmodium knowlesi* merozoites [14,15].

Pv/PkDBP is a 140-kDa protein stored and later on secreted from the micronemes to the membrane where it interacts with the Duffy antigen receptor for chemokines (DARC) to mediate an irreversible binding (formation of the tight junction) between RBCs and merozoites. Up to date, this protein has been the only characterized pathway of merozoite invasion into human RBCs in these two species [14–16]. The functional domain of the binding receptor (called region II or DBPII) is located in the cysteine-rich N-terminal region and consists of approximately 330 amino acids (amino acids 198–522), 12 cysteine residues, highly conserved aromatic amino acids, and highly polymorphic clusters against which the host's immune responses are directed [17–19].

In vitro binding assays have shown that anti-PvDBP antibodies produced during natural *P. vivax* infections or by immunization with recombinant DBP, are capable of blocking the specific interaction between DARC and PvDBPII, and such blockage prevents invasion of human RBCs by *P. vivax* merozoites [20–22]. Studies in different human populations have described that recurrent exposures to *P. vivax* infection increase the levels of naturally-acquired PvDBPII-binding inhibitory antibodies [23,24].

Two PvDBPII universal epitopes (denoted as 1635 and 1638) were reported in previous studies for binding to a wide range of HLA-DR alleles, laying in opposed localizations with respect to polymorphic clusters of PvDBPII, and binding with high capacity to reticulocytes [25,26]. In this study, the antigenicity of these peptides was evaluated by performing in vitro lymphocyte proliferation assays and measuring cytokine levels produced by PBMCs isolated from people with various HLA-DR types and different degrees of exposure to *P. vivax* malaria.

2. Materials and methods

2.1. Population study

Peripheral blood samples were obtained from 35 adults (age range: 17–78 years) who had a previous history of malaria caused by *P. vivax*. These exposed individuals were divided into 5 groups according to the number of *P. vivax* malaria episodes (1, 2, 3, 4 to 5, and more than 5 episodes). Samples were collected from people inhabiting the Municipality of Tierralta, a city of ~63,000 inhabitants located at 51 m above sea level in the Colombian department of Córdoba, a region of high risk for *P. vivax* infection with 26,867 cases being reported in 2007. All individuals signed an informed consent form after being explained about the objective of the study. None of these individuals had a clinical episode of malaria at the time of blood sampling, or had parasites in their blood (as assessed by thick blood smear). Seven individuals with no previous history of exposure to malaria were used as controls. All procedures were evaluated and approved by FIDIC's ethics committee.

2.2. Peptides synthesis

Peptides used in this study belonged to PvDBPII and were previously reported by Saravia et al. as universal epitopes binding to a wide range to HLA-DR molecules [26]. These peptides were denoted as 1635 and 1638, according to our Institute's serial numbering system and had the following amino acid sequences: ^{398}RDYVSELPTEVQKLKEKCDG^{417} and ^{458}ISVKNAEKVQTAGIVTPYDI^{477} (numbers indicate the position within the Sal-1 strain PvDBP sequence, GenBank accession AAZ81525.1). Peptides were synthesized by the *t*-Boc/Bzl solid-phase multiple peptide synthesis technique [27], analyzed by high-performance liquid chromatography (HPLC), and purified by preparative reverse-phase HPLC to be later characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Peptide 1623 ^{158}SNGQPAGTLDNVLEFVTGHE^{177} was used as negative control due to its high binding activity to reticulocytes but low binding capacity to HLA-DR molecules, as reported by Saravia et al. [26].

2.3. Isolation of PBMCs

PBMCs from exposed individuals and controls were isolated from 10 to 20 ml of heparinized peripheral blood using a Ficoll-Hypaque density gradient (lymphoprep, Nycomed Pharma AS). Cells were diluted in 90% fetal bovine serum (FBS)/10% dimethylsulfoxide *v/v* and cryopreserved in liquid nitrogen until use.

2.4. P. vivax *lysate*

Parasites from the VCG-1 (Vivax Colombia Guaviare 1) strain [28] were cultured by successive passes in *Aotus* spp. monkeys kept at FIDIC's primate station in Leticia (Amazonas, Colombia). Maintenance and care of the monkeys complied with the National Institute of Health guidelines for the use of laboratory animals and was supervised by the Colombian Wildlife Corporation (CORPOAMAZONIA), as previously described elsewhere [28]. Briefly, infected RBCs (primarily at the schizont stage) were extracted from 3 to 4 ml blood samples using a discontinuous Percoll gradient (GE Healthcare), as described elsewhere [29]. The total parasite protein was extracted from the pellet by resuspension in lysing solution (5% *w/v* sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 10 mM phenylmethanesulfonylfluoride and 10 mM iodoacetamide) and then exhaustively dialyzed against phosphate buffered saline (PBS).

2.5. HLA-DRβ typing

Genomic DNA was extracted from 300 μl-peripheral blood samples using the UltraClean™ Blood DNA Isolation Kit (MOBIO Laboratories). Low-resolution MHCII-DR typing was performed with a polymerase chain reaction sequence-specific primer (PCR-SSP) typing system using oligonucleotides previously described to amplify HLA-DRβ1*01, DRβ1*03, DRβ1*04, DRβ1*07, DRβ1*11 and DRβ1*13 [30], if the individual was PCR negative when tested using all primer sets, HLA-DRβ1* typing was reported as non determined (ND). The amplification mixture contained 2.5 mM $MgCl_2$, 0.5 μM of forward and reverse primers, 0.25 mM dNTPs, 1× reaction buffer, 0.5 U Taq polymerase (GoTaq®; Promega) and 2 μl of genomic DNA (final volume: 10 μl). PCR assays were performed in a PerkinElmer 9600 thermocycler according to the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at the annealing temperature standardized for each primer, and 30 s at 72 °C; a final extension step was carried out at 72 °C for 5 min. Amplicons were resolved in a 2% agarose gel stained with SYBR® safe (Invitrogen). Genomic DNA previously typed by hybridization of amplified DNA with the DRβ1 kit (INNO-LiPA kits from Innogenetics) was used as positive control [31], and DNAse, RNAse Free Distilled Water (Gibco) was added instead of DNA as negative control.

2.6. Proliferation assays

PBMCs were thawed at 37 °C and washed twice with incomplete RPMI-1640 medium. A total of 2×10^5 PBMCs dissolved in RPMI-1640 medium were seeded in 96-well culture plates (Corning, New York) in triplicate and supplemented with 2 mM L-glutamine (Sigma), 0.1 mM essential amino acids (Gibco), 100 U/ml penicillin (Sigma), 100 mg/ml streptomycin (Sigma), 0.5 μg/ml amphotericin B (Sigma) and 10% inactivated FBS (Gibco), maintaining a final volume of 200 μl. Cells were pulsed with 10 μg/ml of either peptides 1635, 1638 or 1623, and with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma) plus 1.25 mM of ionomycin (Sigma) or 5 μg/ml of *P. vivax* lysate as positive controls, while unstimulated cells were used as a negative control. After 72 h of culturing at 37 °C with 5% CO_2, the supernatants were collected for cytokine analysis and cells were pulsed by adding 1 μCi of [^{3}H]-thymidine per well for 18 h. Cells were harvested onto glass-fiber filters using a PHD Cell Harvester (Cambridge Technology Inc); filters were air-dried prior to the addition of 2.0 ml of Betamax ES Scintillante (ICN Biochemicals Inc). Activity (expressed in counts per minute, cpm) of [^{3}H]-thymidine was determined using a liquid scintillation counter (Beckman Instruments) and the stimulation indexes (SI) were calculated as the ratio between cpm of stimulated and unstimulated cultures. For all individuals an SI $\geq$2.0 was considered as positive.

2.7. Cytokines

The levels of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ cytokines were determined using a Cytometric Bead Array (CBA) kit (BD Biosciences). Briefly, 50 μl of capture beads were mixed with 50 μl of human Th1/Th2 PE Detection Reagent II, and 50 μl of supernatants from PBMC cultures stimulated with PvDBP peptides or *P. vivax* lysate. Simultaneously, a 10-point calibration curve was obtained using cytokine standards and beads for each cytokine. The samples were incubated for 3 h at room temperature and kept away from light. They were then washed and centrifuged at 200 g for 5 min and analyzed in a flow cytometer (FACScan, BD).

2.8. Statistical analysis

Nonparametric Friedman's test and Dunn's multiple comparison tests were applied to lymphoproliferation data of the different groups, expressed in terms of SI, using a significance level of 0.05% (two-tailed). The nonparametric Mann–Whitney test was applied to analyze the levels of cytokines, setting the significance level at 0.05% (two-tailed). All analyses and graphs were performed using GraphPad Prism 5.0 and Stata.

3. Results

3.1. Patient population

The exposed individuals were divided into 5 groups according to the number of previous *P. vivax* malaria episodes (1, 2, 3, 4 to 5, and more than 5 episodes). Due to the difficulty of making a follow-up observational study, the number of infections was self-reported by each patient; all patients are adults and were negative for *P. vivax* by thick smear at the time the sample was taken (Supplementary material Table S1). *P. vivax* exposed individuals were assembled in two groups (1–3 and more than 3 episodes) for testing whether there is a relation between age and a greater number of episodes; a Mann–Whitney test was then performed showing no significant difference between the median age of the groups (1–3 episodes: 38 years; >3 episodes: 44 years, *p*-value 0.43).

3.2. Lymphoproliferation assays

The antigenicity of the universal epitopes 1635 and 1638 was evaluated using PBMCs isolated from 35 individuals who lived in a *P. vivax*-endemic region and had different degrees of exposure. The results were compared to those obtained from stimulating PBMCs from 7 individuals with no history of previous exposure to malaria.

For this study, a SI $\geq$2.0 was considered positive. According to this criterion, the universal epitope 1635 induced PBMC proliferation in a high percentage of individuals, being particularly high in individuals who had had more than 5 *P. vivax* episodes, and being low in individuals who had had from 1 to 3 *P. vivax* episodes (Table 1). Stimulation with

Table 1
Lymphoproliferative response induced by *P. vivax* lysate, peptides 1635 and 1638 (universal epitopes of DPB) and peptide 1623 (a DBP peptide with high binding activity to reticulocytes but low affinity to HLA-DRB alleles) in individuals who had been infected with *P. vivax*.

Episodes	1635			1638			1623			*P. vivax*		
	R	SI	SD SI	R	SI	SD SI	R	SI	SD SI	R	SI	SD SI
0	0	0.7	0.1	0	0.7	0.1	0	0.7	0.0	0	0.7	0.3
1	2/7	1.9	0.3	1/7	1.2	0.2	1/7	1.3	0.1	1/7	1.5	0.4
2	0	1.0	0.2	2/7	1.3	0.2	1/7	1.0	0.1	5/7	3.4	0.8
3	1/7	1.3	0.2	0	1.1	0.2	0	1.1	0.2	7/7	3.7	0.7
4–5	5/7	1.8	0.2	3/7	1.5	0.1	0	0.9	0.1	7/7	5.0	0.8
> 5	7/7	3.1	0.2	4/7	2.0	0.2	1/7	1.4	0.1	4/7	4.9	1.0

R: Number of individuals with a Stimulation Index (SI) above the positive threshold (SI >2.0).
SI: Mean of the stimulation index for each exposure group.
SD SI: Standard deviation of the SI for each exposure group.

P. vivax lysate induced strong lymphocyte proliferation in all groups and such proliferation increased with the number of episodes.

Similarly, when comparing the stimulation indexes of exposed and unexposed individuals in response to peptide 1623 (control peptide), there were no statistically significant differences. Although stimulation with either peptide 1635 or peptide 1638 induced PBMC proliferation in both exposed and unexposed individuals, SI values with peptide 1635 were significantly higher in individuals who had had 4–5 and more than 5 episodes, while significantly higher proliferations were only observed in the group which had more than 5 episodes for peptide 1638 (Kruskal–Wallis test $p \leq 0.05$) (Table 1).

Higher SI values were found in individuals who had had 4–5, and more than 5 episodes when PBMCs were stimulated with either peptide 1635 or 1638, compared to SI values of PBMCs that were stimulated with peptide 1623 (Fig. 1). SI values of PBMCs stimulated with PMA-ionomycin were significantly higher than those observed for PBMCs stimulated with each of the three DBP peptides in all exposure groups (data not shown).

3.3. Differences in cytokine levels according to the degree of exposure

Polarization responses were evaluated by measuring the levels of three Th1 cytokines (IL-2, IFN-γ, and TNF-α) and three Th2 cytokines (IL-4, IL-6, and IL-10) produced upon stimulation with *P. vivax* lysate and with peptides 1635, 1638 and 1623. Values considered as positive were those higher than the mean plus 3 SD of the control group (unexposed individuals).

In general, cytokine production was observed in individuals with a history of *P. vivax* malaria that were stimulated with parasite lysate or DBP universal epitopes (1635 and 1638), but such response was not detected when PBMCs were stimulated with the control peptide 1623. Significant differences in IFN-γ, IL-2 and IL-6 levels from exposed individuals compared to those from the unexposed ones were found (Table 2), while no statistically significant differences for TNF-α, IL-4 or IL-10 levels were observed (Supplementary material Table S2).

All individuals belonging to exposed groups responded by producing similar amounts of IFN-γ when stimulated with *P. vivax* lysate. On the contrary, IL-6 levels did not show a clear tendency, the response was in general good with 6 out of the 7 individuals within each exposed group responding to the stimulus, but no significant differences among them were found. Finally, IL-2 levels were higher only in individuals who had had 1 vivax episode, but such increase was not statistically significant and this was the only group that showed a high response (6/7 individuals responded to the stimulus) (Table 2, Fig. 2).

Stimulation with peptide 1635 resulted in a very similar IFN-γ response compared to that observed when stimulating PBMCs with *P. vivax* lysate, displaying similar levels among all exposed groups with all individuals inside each group producing IFN-γ levels higher than the cut-off value. A similar behavior was observed for IL-2 production, where 6–7 individuals within each group surpassed the cut-off value, but no statistically significant differences between exposed groups were found. Although IL-6 levels tended to decrease with the number of vivax episodes, no significant differences between exposed groups were found, except for the group of individuals with 4–5 episodes of *P. vivax* malaria; despite this result, it is worth mentioning that only 1 out of the 7 individuals belonging to this group responded to stimulation with peptide 1635 (Table 2, Fig. 2).

Although stimulation with peptide 1638 induced similar IFN-γ levels in all exposed groups, no significant differences between them were found and, in general, individuals showed a poor response, except in the group that had more than 5 episodes where 6 out of the 7 individuals responded to the stimulus. For IL-6, levels varied between exposed groups but without statistically significant differences among them and with 6–7 out of the 7 individuals in each group responding to the stimulus. IL-2 levels remained constant in all groups but were low and only showed significant differences for the groups of individuals with 1, 2 and 3 episodes of *P. vivax* malaria (Table 2).

3.4. Differences in cytokine production according to stimulation with peptides 1635, 1638 and 1623

The levels of IFN-γ, IL-6 and IL-2 cytokines induced by *P. vivax* lysate and peptide 1635 in all groups of exposed

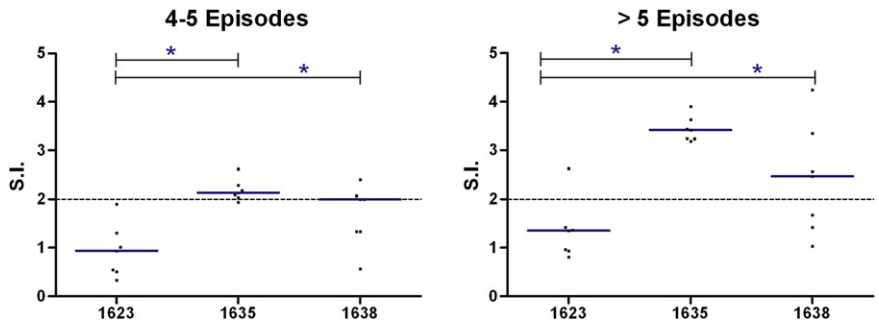

Fig. 1. Stimulation indexes (SI) obtained by pulsing PBMCs from exposed individuals to *P. vivax* malaria (4–5 and more than 5 episodes) with peptides 1623, 1635 or 1638. Statistically significant differences (P-value<0.05) between the groups are indicated by an asterisk *. Each dot indicates one patient in the group. The blue line indicates the median value for each group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

individuals were significantly higher compared to unstimulated samples. Levels of IFN-γ produced in response to stimulation with peptide 1638 were significantly higher than the ones induced by stimulation with PBS in the groups of individuals who had had 4 to 5 and more than 5 episodes, while levels of IL-2 were significantly higher in individuals who had had 1 episode, and levels of IL-6 were significantly higher in all groups of exposed individuals. No significant differences in any of the exposed groups were observed for IFN-γ, IL-6 or IL-2 levels when stimulation with peptide 1623 was compared to stimulation with PBS (Supplementary material Table S3).

Differences between IFN-γ, IL-6 and IL-2 levels produced in response to the different stimuli were also determined. In all groups of exposed individuals, IFN-γ levels were significantly higher when PBMCs were stimulated with either peptide 1635 or 1638, compared to stimulation with peptide 1623, being the levels obtained with peptide 1635 significantly higher compared to those reached with peptide 1638. IL-6 levels obtained upon stimulation with either peptide 1635 or 1638 were only significantly higher in individuals who had had 1 or 2 vivax episodes, compared to levels of IL-6 induced by peptide 1623, and levels achieved with peptide 1635 were significantly higher than those from peptide 1638 in individuals with 1 episode. In addition, exposed individuals from groups with 3, 4–5 and >5 episodes produced significantly higher levels of IL-6 in response to stimulation with peptide 1638, compared to those obtained when stimulation with either peptide 1623 or 1635 was done (Table 2).

Levels of IL-2 were significantly higher in individuals who had had 1 and 2 episodes when PBMCs were stimulated with either peptide 1635 or 1638 when compared to those obtained with peptide 1623, being the levels reached with peptide 1635 significantly higher than those seen with peptide 1638 (Fig. 3).

4. HLA-DRβ1 typing

The individuals enrolled in this study were typed for the dominant alleles in the human population [32]. In general, none of the alleles had a particular prevalence: the HLA-DRβ1*01 allele was most frequently found in the studied population (31%), followed by HLA-DRβ1*03 and HLA-DRβ1*13 (22%), HLA-DRβ1*04 (20%), HLA-DRβ1*07 (14%) and HLA-DRβ1*11 (11%). Since IFN-γ production has been associated with protection against malaria [33–35], the relationship between the expression of a particular HLA-DR allele and cytokine production was analyzed for each individual. Stimulation with peptide 1635 induced high levels of this cytokine despite the HLA-DR allele found or the number of previous *P. vivax* malaria episodes, supporting the notion that this peptide might be presented on a wide range of HLA-DR molecules [26]. With regard to stimulation with peptide 1638, levels of IFN-γ did not change compared to those obtained by stimulation with peptide 1623; however, 4 out of the 7 patients in the group of individuals with more than 5 episodes of *P. vivax* malaria (all having different HLA-DRβ1* alleles) produced higher levels of IFN-γ when being stimulated with peptide 1638, compared to stimulation with peptide 1623 (Fig. 4).

A statistical analysis was performed in order to determine whether there is a different HLA-DR allele frequency between responders and non-responders. A cut-off value of 15 pg/ml of IFN-γ was taken to discriminate responders from non-responders. Patients from different exposure groups were pooled and a Fisher's exact test was then carried out in the STATA software. No statistically significant differences for a given HLA-DR allele were found between responders and non-responders in any of the stimulation treatments (peptides 1635, 1638 or 1623), all of them showing p-values above 0.05 (data not shown). This finding is in agreement with our assumption about the universal character of peptides 1638 and 1635. However, a bigger sample size is required to confirm this result.

5. Discussion

DBP has been widely studied in *P. vivax* since it is the only well characterized mechanism used by these parasites to invade human RBCs [17,36]. The region responsible for the direct interaction between this protein's receptor on the surface of RBCs is known as region II or PvDBPII [37] and numerous studies have demonstrated the key role of anti-

Table 2
Stimulating individuals with different degrees of exposure to *P. vivax* malaria with either *P. vivax* lysate, peptides 1635, 1638 or 1623, induces specific IFN-γ, IL-2 and IL-6 responses.

		P. vivax		1635		1638		1623		PBS	
	Episodes	Median	Response	Median	Response	Median	Response	Median	Response	Median	Response
IFN-γ	0	10		8.8		8.9		10.1		10.3	
	1	43.8*	7/7	38.2*	7/7	15.7*	2/7	13.4	3/7	14.5	0/0
	2	43.0*	7/7	35.6*	7/7	16.12*	4/7	14.6	2/7	15.7	0/0
	3	37.7*	7/7	26.8*	7/7	12.6	1/7	13.7	2/7	11.1	0/0
	4–5	31.2*	7/7	24.4*	6/7	14.7*	2/7	13.0	0/7	11.2	0/0
	> 5	30.3*	7/7	24.6*	7/7	16.7*	6/7	9.8	1/7	12	0/0
	Cut-off	14.6		16.1		16.1		15			
IL-2	0	40.6		24.4		22.6		24.4		22	
	1	119.6*	6/7	84.5*	7/7	67.0*	7/7	43.2*	7/7	43	0/0
	2	54.0*	2/7	48.1*	6/7	36.4*	7/7	25.5	3/7	24.4	0/0
	3	56.4*	3/7	40.6*	7/7	31.2*	5/7	33.7	4/7	29.9	0/0
	4–5	48.2	1/7	43.2*	7/7	31.7	4/7	24.4	3/7	27.2	0/0
	> 5	42.9	0/7	38.2*	6/7	25.4	1/7	25.0	1/7	23.1	0/0
	Cut-off	59.6		29.5		30.4		31.8			
IL-6	0	15.5		16.8		20		18.7		10.5	
	1	424.6*	6/7	482.4*	6/7	322.8*	6/7	60.6	6/7	29.3	0/0
	2	280*	7/7	227.4*	7/7	254.6*	7/7	71.9*	7/7	26.3	0/0
	3	251.1*	7/7	190.6*	7/7	484.4*	7/7	54.2*	5/7	25.6	0/0
	4–5	323*	7/7	52.5*	1/7	172.6*	7/7	41.3	2/7	16.6	0/0
	> 5	490.9*	7/7	70.8*	5/7	416.9*	7/7	62.7	4/7	38.7	0/0
	Cut-off	89.5		63.4		57.6		25.6			

Response: Number of individuals with cytokine levels above the positive threshold.
Median: Median values in pg/ml for each exposure group.
Cut-off: The value of the mean plus 3 SD of the control group (unexposed individuals).
* Statistically significant differences for median levels with regards to the control group (*P*-value <0.05).

PvDBPII antibodies in the natural acquisition of immunity against *P. vivax* malaria [20,22,24,38,39]. This study showed the antigenicity of two universal epitopes from PvDBPII (1635 and 1638), by inducing lymphocyte proliferation and evaluating cytokines produced by PBMCs isolated from individuals living in a *P. vivax*-endemic region who had different HLA-DR alleles and different degrees of exposure to *P. vivax*.

Lymphoproliferation induced by peptides 1635 and 1638 was significantly higher in individuals with more than 5 episodes compared to individuals with no previous exposure to *P. vivax*, and compared to lymphoproliferation induced by peptide 1623 (Fig. 1 and Table 1). However, stimulation of PBMCs with *P. vivax* lysate induced a high rate of proliferation that increased with the number of episodes, which may

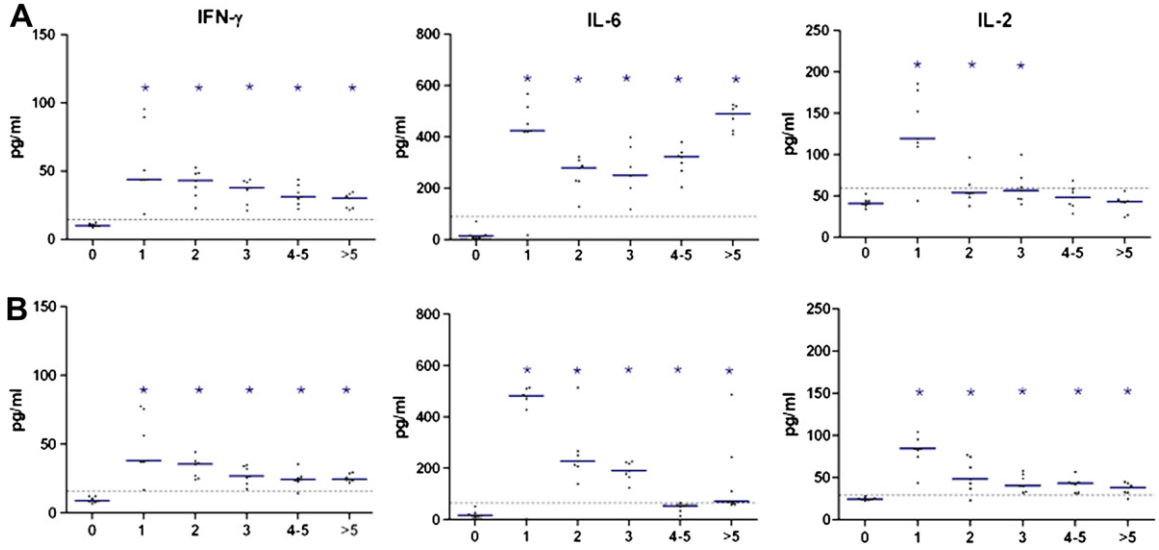

Fig. 2. Comparison of the cytokine levels (in pg/ml) obtained after stimulation with *P. vivax* lysate (A), and peptide 1635 (B). The positive threshold is indicated by the dotted line and represents the mean value for each cytokine in the group of unexposed individuals plus 3 standard deviations. Statistically significant differences between the groups of exposed and unexposed individuals are indicated by an asterisk * (*P*-value<0.05). The blue line indicates the median value for each exposure group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

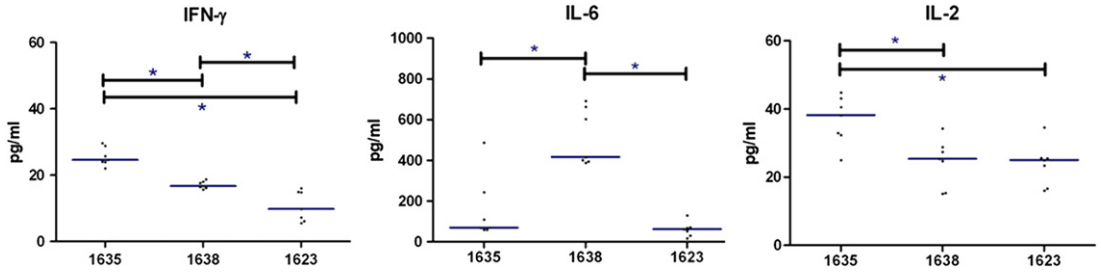

Fig. 3. Comparison between the cytokine levels (in pg/ml) produced by PBMCs from individuals with more than 5 episodes of *P. vivax* malaria in response to stimulation with peptides 1623, 1635 or 1638. Statistically significant differences between cytokine levels induced by each peptide are indicated by an asterisk * (*P*-value<0.05). The blue line indicates the median obtained for each stimulus (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

suggest that a repeated exposure to the parasite is necessary for individuals to acquire a T-cell dependant immune response targeted against conserved universal epitopes of PvDBPII, which could be associated with control of clinical malaria, same has been reported for the antibody response [23,38,40].

The level of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ cytokines was evaluated in order to assess the type of response induced by these peptides. *P. vivax* lysate and universal epitopes induced significantly higher IL-2, IL-6 and IFN-γ levels in individuals previously exposed to *P. vivax* malaria, compared to individuals not exposed. In this study, no recombinant DBP was used due to the technical difficulties involved in obtaining good yields of this purified recombinant protein; instead *P. vivax* lysate was used in order to compare the response obtained by stimulation with universal epitopes of DBP.

The results showed that PBMC stimulation by *P. vivax* lysate induced constant levels of IFN-γ in all groups of exposed individuals, while IL-6 levels, despite being higher than those in the unexposed individuals, did not show a clear trend in exposed groups; on the other hand, IL-2 levels were low in most of the exposed groups except for the group that had 1 vivax episode (Table 2). Because IL-2 is only produced by $CD4^+$ T lymphocytes, the low levels of IL-2 found in this study with respect to those of IFN-γ suggest an imbalance in the Th1 response and the participation of other immune system cell types (Natural Killer cells and $CD8^+$ T lymphocytes) as alternative sources of IFN-γ, like has been reported in other studies in which high IFN-γ and low IL-2 levels have been found in the plasma of patients with severe malaria [41]. The higher levels of IL-6 found here could be associated with increased incidence of febrile episodes, like has been reported for *Plasmodium falciparum* malaria [42]. Because individuals enrolled in this study did not have active *P. vivax* malaria, an association between cytokine levels produced in response to

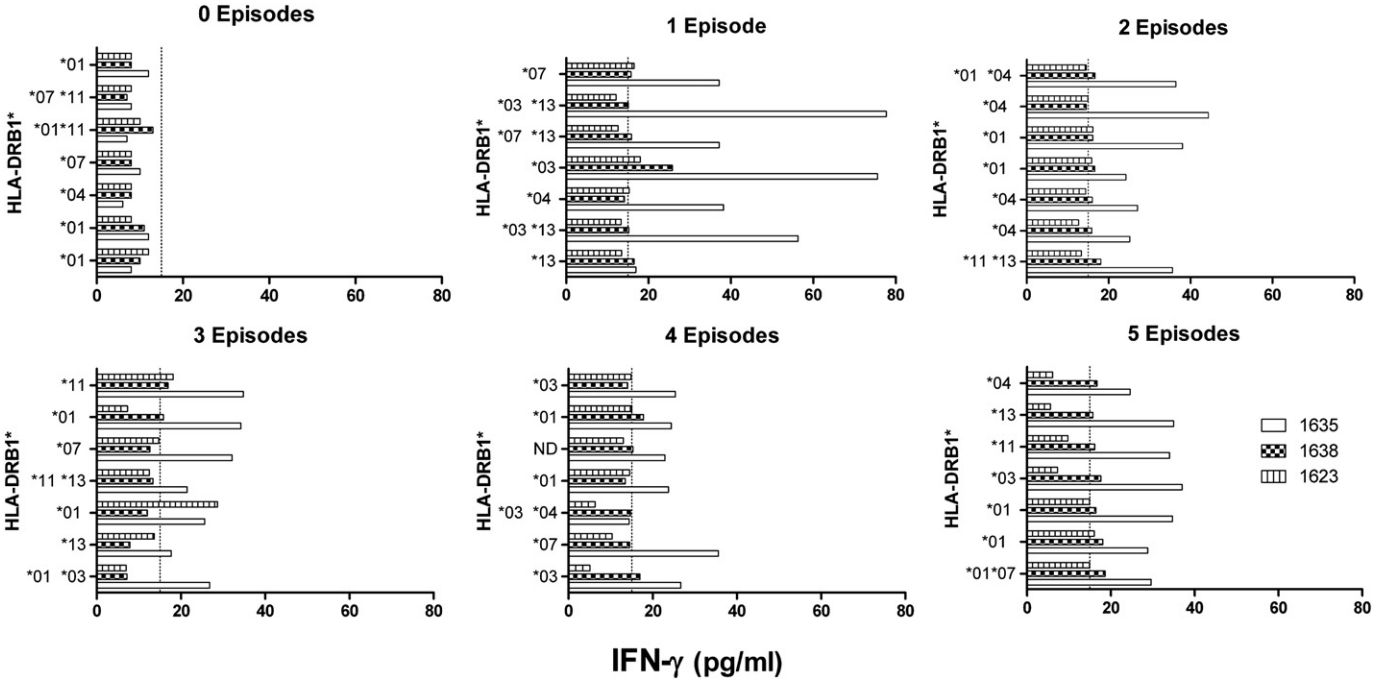

Fig. 4. IFN-γ levels induced by stimulation with peptides 1623, 1635 or 1638, and HLA-DRβ1* typing for each individual. Individuals who were negative for all sets of primers used in the HLA-DRβ1* typing are depicted as not determined (ND). The vertical line shows the cut-off level for IFN-γ in each group (15 pg/ml).

stimulation with *P. vivax* lysate and the degree of exposure to malaria was not found, like has been reported in acute and severe cases of *P. falciparum* malaria [43] and for IFN-γ levels of individuals living in *P. vivax*-endemic areas in response to DBP [43].

Stimulation with peptide 1635 was characterized by inducing a high and constant IFN-γ response in all exposed groups; these levels were significantly higher with respect to stimulation with peptides 1623 and 1638 in the >5 episodes group, while IL-6 levels decreased as the number of episodes increased (Fig. 3 and Table 2). On the other hand, stimulation induced by peptide 1638 produced constant levels of IL-6 with respect to the number of episodes and significantly higher levels compared to stimulation with peptides 1623 and 1635 for the individuals who had had more than 5 episodes, and a poor IFN-γ response with constant low levels in all exposed groups (Fig. 3 and Table 2). Studies in populations living in areas where *P. falciparum* is endemic have found that increased TNF-α and IFN-γ levels respect to IL-6 levels are associated with a reduction in the incidence of clinical episodes caused by *P. falciparum* [42]. The results shown suggest that IFN-γ and IL-6 are important in the immune response of individuals who had been previously exposed to *P. vivax*; however, whether or not this response is associated to protection should be assessed in cohort studies with a higher sample size in order to determine if peptide 1635 could induce a potential protective response.

Few studies have focused on cytokine responses induced by *P. vivax* antigens such as DBP. Individuals from *P. vivax*-endemic regions develop cytokine responses to DBP that slowly increase with age, suggesting that clinical immunity might be directed towards polymorphic regions. However, other authors have reported that the T-cell-mediated immune response against *P. vivax* antigens such as CSP, MSP-1 and AMA-1 does not increase with age, in contrast to what has been observed with the humoral response [44]. Data collected in this study does not show an association between cytokine levels produced in response to stimulation with PvDBPII peptides and the degree of exposure to *P. vivax*. This lack of association might be explained by the low cytokine levels reached, that could be the result of the conserved and universal character of the peptides. Although functionally active protein regions involved in target cell invasion have to remain conserved in order to avoid loss in parasite fitness, these regions have been shown to be often cryptic, poorly antigenic or nonantigenic and nonimmunogenic [45]; this low immunogenicity has been evident for numerous *P. falciparum* proteins [46–48]. The immunodominance of non-functional regions of *P. vivax* can be deduced from a previous study where high titers of naturally-acquired anti-DBP antibodies were found in exposed populations, but a clear correlation between such antibodies and their ability to inhibit DBP binding to RBCs in vitro was not found [39]. This reflects the importance of studying the overall cellular and humoral immune responses induced by the functionally relevant regions of DBP that participate in the invasion process by this parasite species.

The search for epitopes that induce an effective protective response against malaria has been complicated by the genetic restriction mediated by HLA-DR alleles, as has been reported in previous vaccination clinical trials [5]. However, the results of this study indicate that the cytokine response induced by peptides 1635 and 1638 is ubiquitous, as they induced the production of IFN-γ in all exposed groups despite the HLA-DR allele type from each studied individual (Fig. 4). Further support to the idea of testing these peptides as antimalarial vaccine components was established in a previous study in which conserved epitopes of PvDBPII, unlike non-conserved epitopes, were found to induce higher IFN-γ levels [49].

Here we assessed the antigenicity of universal conserved epitopes of PvDBPII that bind with high activity to reticulocytes and highlight them as potential targets to be tested in a future vaccine against *P. vivax* malaria. However, given that *P. vivax* merozoite invasion of *Aotus* spp. RBCs is DBPII-dependent [50], it would be necessary to establish whether these peptides are able to confer protection in this animal model, and determine the immune mechanisms involved in such protection. Despite these promising antigenicity results, caution must be exercised since *P. falciparum* conserved high activity binding peptides to RBCs that also bind to a wide range HLA-DR alleles have been unable to induce high anti-parasite antibody titers, as assessed by indirect immunofluorescence, nor protection in immunized *Aotus* monkeys, requiring of specific amino acid modifications to be rendered into protection-inducers (reviewed in [46–48]).

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Appendix. Supplementary material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.micinf.2010.08.007

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