The in Vitro Antigenicity of Plasmodium vivax Rhoptry Neck Protein 2 (PvRON2) B- and T-Epitopes Selected by HLA-DRB1 Binding Profile

Carolina López1,2, Yoelis Yepes-Pérez1,3, Diana Díaz-Arévalo1,4, Manuel E. Patarroyo1,5 and Manuel A. Patarroyo1,6

1 Molecular Biology and Immunology Department, Fundación Instituto de Immunología de Colombia, Bogotá, Colombia, 2 PhD Program in Biomedical and Biological Sciences, Universidad del Rosario, Bogotá, Colombia, 3 MSc Program in Microbiology, Universidad Nacional de Colombia, Bogotá, Colombia, 4 Faculty of Agricultural Sciences, Universidad de CienciasAplicadas y Ambientales, Bogotá, Colombia, 5 School of Medicine, Universidad Nacional de Colombia, Bogotá, Colombia, 6 Basic Sciences Department, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia

Malaria caused by Plasmodium vivax is a neglected disease which is responsible for the highest morbidity in both Americas and Asia. Despite continuous public health efforts to prevent malarial infection, an effective antimalarial vaccine is still urgently needed. P. vivax vaccine development involves analyzing naturally-infected patients’ immune response to the specific proteins involved in red blood cell invasion. The P. vivax rhoptry neck protein 2 (PvRON2) is a highly conserved protein which is expressed in late schizont rhoptries; it interacts directly with AMA-1 and might be involved in moving-junction formation. Bioinformatics approaches were used here to select B- and T-cell epitopes. Eleven high-affinity binding peptides were selected using the NetMHCIIpan-3.0 in silico prediction tool; their in vitro binding to HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101 or HLA-DRB1*1302 was experimentally assessed. Four peptides (39152 (HLA-DRB1*04 and 11), 39047 (HLA-DRB1*07), 39154 (HLADRB1*13) and universal peptide 39153) evoked a naturally-acquired T-cell immune response in P. vivax-exposed individuals from two endemic areas in Colombia. All four peptides had an SI greater than 2 in proliferation assays; however, only peptides 39154 and 39153 had significant differences compared to the control group. Peptide 39047 was able to significantly stimulate TNF and IL-10 production while 39154 stimulated TNF production. Allele-specific peptides (but not the universal one) were able to stimulate IL-6 production; however, none induced IFN-γ production. The Bepipred 1.0 tool was used for selecting four B-cell epitopes in silico regarding humoral response. Peptide 39041 was the only one recognized by P. vivax-exposed individuals’ sera and had significant differences concerning IgG subclasses; an IgG2 > IgG4 profile was observed for this peptide, agreeing with a protection-inducing role against P. falciparum and P. vivax as previously described for antigens such as RESA
More than 50 proteins have been described to date as being involved in malarial parasite's red blood cell (RBC) invasion; most have been identified at molecular level and characterized immunologically in *P. falciparum* (Rozdech et al., 2003; Cowman and Crabb, 2006). Conversely, studying *P. vivax* proteins involved in host invasion has been difficult, mainly due to technical restrictions such as the lack of a continuous *in vitro* parasite culture, leading to inadequate study of parasite biology (Udomsangpetch et al., 2008; Mueller et al., 2009).

Parasites from the phylum *Apicomplexa* have specialized organelles such as rhoptries which contain a large amount of proteins involved in host cell invasion (Counihan et al., 2013). Six *P. vivax* rhoptry neck proteins have been identified to date: *Pv* 34, *PvRON1*, *PvRON2*, *PvRON4*, *PvRALP* and *PvRON5* (Mongui et al., 2009; Arévalo-Pinzón et al., 2011, 2013, 2015; Moreno-Perez et al., 2011; Cheng et al., 2015). They have been described as possible targets for blocking *P. vivax* invasion of RBC (Mongui et al., 2009).

*P. vivax* rhoptry neck protein 2 (*PvRON2*) is 2,204 amino acids (aa) long and is expressed in late schizont rhoptries (Arévalo-Pinzón et al., 2011). It is a highly conserved protein which is secreted by specialized organelles and forms part of the complex of proteins called RONs. This protein, like its orthologs in *T. gondii* (*TgRON2*) and *P. falciparum* (*PfRON2*) interacts directly with the AMA-1 protein. The RON complex is involved in forming the moving junction (MJ) (electron dense ring-shaped structure) which allows parasite entry to a host cell (Aiakawa et al., 1978; Lamarque et al., 2011). RON2’s crucial role during merozoite (Mrz) invasion of erythrocytes, moving junction (MJ) formation and subsequent parasitophorous vacuole (PV) formation (Cao et al., 2009; Collins et al., 2009; Srinivasan et al., 2011) makes it a good vaccine candidate. Moreover, Srinivasan et al. have shown that vaccination with the *PfAMA1-RON2L* complex induce protection in *Aotus* monkeys, mediated by high neutralizing response.

**Keywords: Plasmodium vivax, PvRON2, HLA-DRB1 typing, antigenicity, synthetic peptide, epitope, cellular and humoral response**
antibody titers that prevent the invasion of RBC (Srinivasan et al., 2017).

The development of bioinformatics tools during the last few decades has enabled predicting vaccine candidates based on peptide binding affinity for major histocompatibility complex (MHC) class I or class II (Sturniolo et al., 1999; Nielsen and Lund, 2009; Wang et al., 2010; Zhang et al., 2012; Andreatta et al., 2015). The immune system's function is to recognize and differentiate between self and non-self-antigens so as to trigger cellular and/or humoral immune responses. MHC class II proteins (HLA-II in humans) are expressed on antigen presenting cells’ (APC) surface (i.e., macrophages, dendritic cells and B-lymphocytes). These recognize extracellular antigens and can bind 13- to 18-aa-long peptides. One of the main difficulties in designing a vaccine is the high HLA polymorphism, especially from HLA-DRB1, this being the most polymorphic locus. Antigen binding capability varies from one allele to another, increasing or reducing affinity and driving immune responses. Selecting peptides as good vaccine candidates relies on their ability to be recognized by HLA-DRB1 alleles to ensure a protection-inducing immune response (Stern and Calvo-Calle, 2009). T-cells can trigger stronger immune responses after their APC recognition, depending on the peptides bound to MHC receptors (Blum et al., 2013).

Antigen-antibody interaction plays an essential role in humoral immune responses against pathogens. Bioinformatics tools are extremely useful for identifying antigenic determinants or B-cell epitopes when designing vaccines (Bergmann-Leitner et al., 2013; Panda and Mahapatra, 2017). Predicting linear B-cell epitopes (contiguous aa in a protein sequence) is based on several methods for determining aa physicochemical properties, such as solvent accessibility, hydrophilicity and flexibility (El-Manzalawy et al., 2017; Solihah et al., 2017).

This paper describes naturally-acquired T-cell and antibody immune responses to PvRON2 in P. vivax-exposed individuals from two of Colombia’s endemic areas (Córdoba and Chocó), in the search for vaccine candidates. Eleven high-affinity epitopes were selected by NetMHCIIpan-3.0 (Andreatta et al., 2015) in silico prediction and their in vitro binding to at least one of HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101 and HLA-DRB1*1302 was assessed by competition assays. The Bepipred 1.0 tool was used for selecting four B-cell epitopes in silico. A good immune response was observed against two T-cell and one B-cell epitopes; further studies aimed at testing these peptides as components of a subunit vaccine against P. vivax are thus recommended.

**MATERIALS AND METHODS**

**In Silico B-Cell and T-Cell Epitope High Binding Prediction**

The PvRON2 aa sequence (PlasmoDB database code: PVX_117880) was used for predicting T-cell epitopes having high binding affinity for the HLA-DRB1 alleles most frequently occurring in endemic areas worldwide (HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, and HLA-DRB1*1302) (Marsh et al., 1999). NetMHCIIpan-3.0 (Andreatta et al., 2015) was used for predicting these epitopes and confirmed by IEDB (Sturniolo et al., 1999; Nielsen and Lund, 2009; Wang et al., 2010) and TEPITOPE software (Zhang et al., 2012). Three epitopes per HLA-DRB1 were selected for in vitro analysis according to highest predicted binding values (Table 1).

**Bepipred 1.0** (Larsen et al., 2006) and Antheprot 2000 V6.0 (Delage et al., 2001) were used for predicting B-cell epitopes. Four epitopes were chosen as they agreed with average high Parker antigenicity, hydrophilicity and solvent accessibility values obtained with Antheprot software, and the high values obtained with the Bepipred tool (0.35 default threshold and 75% specificity); such peptides were further used for analyzing humoral responses in vitro (Table 2).

**Synthetic Peptides**

Peptides selected in silico were purchased from Twenty First Century Biochemicals Inc. (260 Cedar Hill Street Marlboro, MA 01752 USA) and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The biotinylated peptides used as control for HLA peptide binding in competition assays were synthesized using sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford).

**HLA-DR Molecules Purification**

HLA-DRB1* molecules were purified from human HLA-DRB1*0401 (IHW09025), HLA-DRB1*0701 (IHW09051), HLA-DRB1*1101 (IHW09043) and HLA-DRB1*1302 (IHW09055) homozygous lymphoblastoid B-cell lines (International Histocompatibility Working Group) and cultured in RPMI-1640 (Gibco) with 10% FBS (Gibco), at 37°C in a 5% CO₂ atmosphere. The purification was carried out as previously described by Vargas et al. (2003), briefly 5 × 10⁸ cells were lysed at 1 × 10⁹ cell/mL final density in lysis buffer with 10 µg/mL protease inhibitors [antipain, pepstatin A, soybean trypsin, leupeptin, and chymostatin (SIGMA-ALDRICH)]. The lysate was mixed with Protein A-Sepharose CL-4B beads (GE Healthcare) linked to mAb L243 (ATCC HB-55; anti-DR was confirmed by native SDS-PAGE (12%) and Western-blot; positive aliquots' concentration was determined by the Micro BCA protein assay kit (Thermo Scientific); HLA-DRB1 proteins were stored at −80°C until use.

**In Vitro Peptide-Binding Assays and IC50 Values**

Peptide binding competition assays were performed to test PvRON2 high-affinity binding peptides selected by in silico analysis using NetMHCIIpan 3.0 software. Selected unlabeled peptides competed with biotinylated control peptide in binding to HLA-DRB1*. The biotinylated peptides used were haemagglutinin antigen HA306–318 (PKYVKQNTLKLAT) for HLA-DRB1*04 and HLA-DRB1*11 (Hammer et al., 1994; Saravia et al., 2008) and tetanus toxoid (TT) (QYIKANSKFIGITE) for HLA-DRB1*07 and HLA-DRB1*13 (Doolan et al., 2000).
## TABLE 1 | T-epitopes selected in silico and PvRON2 in vitro binding.

| Peptide code | Sequence            | CoreF | HLA-DRB1* allele | NetMHCIIpan 3.0 (%Rank) | Binding percentage* | IC50 µM* | IC50 ratio |
|--------------|---------------------|-------|-----------------|-------------------------|---------------------|---------|----------|
| 39147        | LKFPYSELTLMANS      | FYSLETLM | DRB1*0401       | 0.3                     | 92.6                | 4.6     | 0.2      |
|              |                     |       |                 |                         | DRB1*0701           | 2.5     | 83.2     | 1.1      |
|              |                     |       |                 |                         | DRB1*1101           | 10.0    | 79.3     | 4.8      |
|              |                     |       |                 |                         | DRB1*11302          | 34.0    | 63.0     | 10.6     |
| 39148        | NVRKFLNDVSSIRH      | FFLNDVSSI | DRB1*0401       | 1.0                     | 83.0                | 11.9    | 0.6      |
|              |                     |       |                 |                         | DRB1*0701           | 5.0     | 79.9     | 39.7     | 1.7      |
|              |                     |       |                 |                         | DRB1*11101          | 19.0    | 73.8     | 83.4     | 17.5     |
|              |                     |       |                 |                         | DRB1*111302         | 1.4     | 94.2     | 7.4      | 1.0      |
| 39149        | DKSRSEANSFRNNEE     | FISEANSFR | DRB1*0401       | 3.5                     | 85.8                | 26.3    | 1.4      |
|              |                     |       |                 |                         | DRB1*0701           | 17.0    | 42.8     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*11101          | 26.0    | 36.5     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*111302         | 24.0    | 33.5     | ND       | ND       |
| 39150        | QTAFFKFKKIIISLG     | FFFKIIISLG | DRB1*0401       | 17.0                    | 83.6                | 33.7    | 1.7      |
|              |                     |       |                 |                         | DRB1*0701           | 6.5     | 67.2     | 51.8     | 2.2      |
|              |                     |       |                 |                         | DRB1*11101          | 1.2     | 87.6     | 83.4     | 17.5     |
|              |                     |       |                 |                         | DRB1*111302         | 48.0    | 7.7      | ND       | ND       |
| 39151        | KLKYIFKRRTKMKK      | FKRRTKMKK | DRB1*0401       | 37.0                    | 65.3                | 40.0    | 2.1      |
|              |                     |       |                 |                         | DRB1*0701           | 6.0     | 36.0     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*11101          | 0.1     | 78.5     | 51.1     | 10.7     |
|              |                     |       |                 |                         | DRB1*111302         | 27.0    | 13.1     | ND       | ND       |
| 39152        | LFYVNLIMSSLRKP      | LFIMSSLR | DRB1*0401       | 3.0                     | 65.8                | 2.8     | 0.1      |
|              |                     |       |                 |                         | DRB1*0701           | 2.0     | 11.0     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*11101          | 1.4     | 91.8     | 1.9      | 0.4      |
|              |                     |       |                 |                         | DRB1*111302         | 27.0    | 94.7     | 8.0      | 1.1      |
| 39153        | MKLLHQIPANLENI      | LLQHIPANL | DRB1*0401       | 0.5                     | 61.2                | 57.0    | 2.9      |
|              |                     |       |                 |                         | DRB1*0701           | 0.1     | 85.4     | 7.5      | 0.3      |
|              |                     |       |                 |                         | DRB1*11101          | 6.5     | 72.7     | 52.1     | 10.9     |
|              |                     |       |                 |                         | DRB1*111302         | 0.1     | 91.9     | 10.7     | 1.4      |
| 39154        | LKFKVRGNLKNFLNNN    | IVRGNLNF | DRB1*0401       | 11.0                    | 22.2                | ND      | ND       |
|              |                     |       |                 |                         | DRB1*0701           | 4.5     | 43.7     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*11101          | 3.0     | 24.9     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*111302         | 0.2     | 90.1     | 6.5      | 0.9      |
| 39046        | NYEYMASSSSNLYM      | YASSSNIY | DRB1*0401       | 0.8                     | 91.8                | 28.4    | 1.5      |
|              |                     |       |                 |                         | DRB1*0701           | 0.3     | 91.5     | 23.3     | 1.0      |
|              |                     |       |                 |                         | DRB1*11101          | 13.0    | 82.9     | 120.0    | 25.2     |
|              |                     |       |                 |                         | DRB1*111302         | 0.2     | 92.8     | 29.6     | 4.0      |
| 39047        | RGPVNYHFSYMNLD      | VNYHFSYNL | DRB1*0401       | 16.0                    | 59.8                | 54.5    | 2.8      |
|              |                     |       |                 |                         | DRB1*0701           | 10.0    | 90.4     | 6.0      | 0.3      |
|              |                     |       |                 |                         | DRB1*11101          | 37.0    | 0.0      | ND       | ND       |
|              |                     |       |                 |                         | DRB1*111302         | 13.0    | 37.8     | ND       | ND       |
| 39048        | TPIWYDKIYDTHAKNR    | IIVKYDNTHA | DRB1*0401       | 12.0                    | 90.7                | 11.9    | 0.6      |
|              |                     |       |                 |                         | DRB1*0701           | 41.0    | 10.5     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*11101          | 24.0    | 16.8     | ND       | ND       |
|              |                     |       |                 |                         | DRB1*111302         | 8.5     | 3.3      | ND       | ND       |

Peptide code and sequence are shown; core and %rank according to NetMHCIIpan 3.0 predicted HLA-DRB alleles and values. Binding assays and IC50 values were obtained from the methodology for all peptides. IC50 was assessed for peptides having ≥50% binding. IC50 values were expressed as ratios (IC50 peptide/IC50 control peptide), and good binders were considered when their ratio was ≤10. Specific peptides were selected as those having the lowest ratio value for each allele and a universal peptide had to have the lowest mean ratio value.

*Data from this study; ND means that a peptide had less than 50% binding so that its IC50 value was not evaluated. %Rank values were considered as follows: weak binders rank ≤10 and strong binders ≤2. IC50 values were calculated for each control peptide with each DRB1* allele, the controls HA-DRB1*0401 IC50 = 19.44 µM; TT-DRB1*0701 IC50 = 23.37 µM; HA-DRB1*1101 IC50 = 4.77 µM; TT-DRB1*1302 IC50 = 7.46 µM. Peptides having a IC50 ratio ≤10 were considered good binders.
HLA-DR molecules (0.1 µM) were incubated for 24 h with 5 µM biotinylated HA or TT peptides and a 50-fold excess of unlabeled peptide (250 µM). The mix was incubated for 2 h in Maxisorb NUNC-immune modules (Thermo Scientific) covered with anti-DR. The complex was incubated with alkaline phosphatase streptavidin (Vector Labs) and as substrate alkaline phosphatase yellow (pNPP) liquid substrate (Sigma-Aldrich). Optical density (OD) was determined at 405 nm using a Multiskan GO (Thermo Scientific, Waltham, Massachusetts, USA) ELISA reader. Inhibition was calculated as a percentage, by using the following formula:

\[
100 \times \left(1 - \frac{\text{OD in the presence of competitor}}{\text{OD in the absence of competitor}}\right)
\]

IC50 values (50% concentration inhibition) were determined for peptides able to inhibit high-affinity control peptide binding to a particular HLA-DR by more than 50% (Saravia et al., 2008). The peptides and control peptides were tested in 5–250 µM serial dilutions for the competition assays; Mathematica (version 10.1) software (Wolfram Research, Inc., Mathematica, Champaign, IL, 2015) was used for calculating IC50 values, using two-phase exponential decay. IC50 values were calculated as a relative value using the following formula:

\[
1 - \left[\frac{\text{OD in the presence of competitor}}{\text{OD in the absence of competitor}}\right]
\]

**TABLE 2 | Humoral response to PVRON2 B-cell epitopes.**

| B epitope code | Amino acid sequence | Average response |
|----------------|----------------------|------------------|
| 39041          | YGRTRINKRYMHPFEGKYYKG | 0.159 (SE = 0.026) |
| 39042          | KLOQEGNELKEERQGQEN   | 0.104 (SE = 0.016) |
| 39043          | GEQEEEDDDPFGNSKNGK   | 0.142 (SE = 0.018) |
| 39044          | EKIRKGEHEEERTINQGRA  | 0.094 (SE = 0.015) |
| PVRON2-CT      | 434–749 aa            | 0.475 (SE = 0.071) |

(HG absorbance readings for individuals exposed to natural P. vivax infection from endemic areas of Colombia. B-cell epitope code, as sequences, control protein and average response (OD) are shown. SE, standard error.

**Study Population**

Peripheral blood was obtained from 79 people living in the Colombian departments of Chocó and Córdoba (known P. vivax malaria endemic areas, having the highest case incidence) who had suffered previous episodes of malaria. Inclusion criteria consisted of being over 18 years-old, residing in a P. vivax-endemic area, having had 1 or more episodes of P. vivax malaria (the last one 6 months beforehand) and having received suitable treatment for the disease. Although a stronger immune response would have been expected in acutely-infected P. vivax individuals, the construction of study groups required a prior HLA typing and thus, a second sample had to be taken from individuals matching the alleles of interest to assess antigenicity. Taking this into account, the antigenicity sample was taken from people that had suffered P. vivax malaria at least 6 months earlier. A control group of 50 individuals was selected; this consisted of healthy adults residing in Bogotá, Colombia, who had never lived in malaria-endemic areas and who had never experienced malarial infection. This study was performed according to the legal framework for research in Colombia and Ministry of Health’s Resolution 8430 of 1993. The patients had the least risk, all data were kept confidential and were rigorously protected. The samples were collected after all individuals signed an informed consent form; all procedures were evaluated and approved by FIDIC’s ethics committee.

**HLA-DRB1 Typing**

Genomic DNA (gDNA) from 300 µL peripheral blood samples was extracted using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA), following the manufacturer’s instructions. gDNA was used for high resolution HLA-DRB1 typing by Histogenetics (Ossining, NY, USA) through Next Generation Sequencing (NGS) technology using Illumina MiSeq.

**PBMC Isolation**

Twenty-nine people carrying HLA-DRB1 typing for HLA-DRB1*04, HLA-DRB1*07, HLA-DRB1*11 and HLA-DRB1*13 alleles were selected from P. vivax endemic areas of Colombia’s Córdoba and Chocó departments. Eight people carrying the same HLA-DRB1* alleles from a non-endemic area formed the control group. About 40 mL peripheral blood was collected in citrate phosphate dextrose (CPD) tubes and 6 mL peripheral blood in BD vacutainer serum collection tubes (BD Vacutainer Oakville, ON). Thick blood smears were used for confirming samples negative for malaria. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Paque PLUS (GE Healthcare) gradient centrifugation. Briefly, theuffy coat was resuspended in RPMI 1640 (Gibco) and separated by Ficoll, spinning at 1,000 g for 30 min at room temperature (RT). Mononuclear cells were collected, washed and spun at 800 g for 10 min, twice. Cell viability was evaluated by trypan blue exclusion test and cells were counted in a Neubauer chamber.

**T-Cell Proliferation**

Briefly, 2 × 10^5 PBMC were cultured in 200 µL RPMI-1640 (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, 2 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin (all Gibco) and 10% heat-inactivated autologous plasma in 96-well round-bottomed plates (Costar, Corning Incorporated). Proliferation activity was evaluated by flow cytometry using carboxyfluorescein diacetate N-succinimidyl ester (CFSE, 5 µM) (CellTrace CFSE cell proliferation kit, Molecular Probes, Eugene, Oregon, USA) reduction in replicating cells.

The cells were left without stimulation (unstimulated control) or were stimulated by co-culture with synthetic peptides (10 µg/mL) or 2% mitogen phytohemagglutinin (PHA) (Sigma) or 5 µg/mL P. vivax lysate as positive controls. Pv12 low binding peptide was selected (39115) by binding assay and used as negative control (manuscript in preparation). The 96-well plates
were incubated in 5% CO₂ at 37°C for 5 days; 100 µL culture supernatant was then collected per well and stored at −80°C until analysis for cytokine production. Duplicate assays were carried out.

The CD4-Pacific Blue-stained cell stimulation index was calculated by proliferative cells’ relative percentage loss of carboxyfluorescein succinimidyl ester (CFSE) in the presence of antigen, divided by percentage relative CFSE loss for proliferative cells without antigen (Racanelli et al., 2011). Data was averaged for each antigen and for both exposed individuals and control groups. SI ≥ 2 was taken as antigen-specific positive proliferation. A Pacific Blue-labeled mouse anti-human CD4 (RPA-T4 clone) antibody (BD Biosciences), was used for CFSE-cell cluster measure. The samples were then read on a FACS Canto II flow cytometer; FlowJo software (v7.6.5, Ashland, Oregon, USA) was used for analyzing the results.

Cytokine Secretion
IFN-γ, TNF, IL-10, and IL-6 levels in lymphocyte culture supernatant were determined with a BD CBA Human Th1/Th2 Cytokine Kit II (San Jose, CA, USA), following the manufacturer’s instructions. Supernatants were read on a FACS Canto II flow cytometer; FCAP Array software (v3.0.1) was used for analyzing the results. Results were expressed in pg/mL for each cytokine; data were compared between unstimulated and stimulated PBMC supernatant culture. Two standard deviations higher than that for control group were taken as positive antigen-specific production.

Indirect Immunofluorescence Assays (IFA)
IFA followed that previously described by Moreno-Pérez et al. (2013) with some modifications. Briefly, blood samples from individuals having active P. vivax infection were spun at 1,750 g for 12 min at RT. Both plasma and Buffy coat were recovered and parasite red blood cells (pRBC) were washed with saline solution. pRBC were passed through a 60% Percoll gradient and spun at 1,750 g for 20 min. P. vivax-pRBC were diluted until 5–7 schizonts per field and confirmed by acridine orange. Twenty µL of diluted pRBC were placed into multilist microscope slide wells (Tekdon Incorporated) and incubated for 30 min. The supernatant was then removed, and microscopic slides left overnight (ON) at room temperature (RT) to air dry. The slides were then blocked for 30 min at RT with tris-buffered saline (TBS) 1% bovine serum albumin (TBSA) solution and washed three times. The serum samples from 30 exposed individuals and 8 sera from the control group were diluted in TBSA at 1:50 dilution and incubated for 1 h in a humid chamber. Reactivity was observed by fluorescence microscopy using anti-human IgG-FITC antibody (Sigma-Aldrich) diluted 1:50 in TBSA for 45 min in a humid chamber. The parasite nuclei were stained with 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (0.25 µg/mL) for 5 min at RT and washed twice with 0.05% TBS-Tween 20 and three washes with TBS to remove excess reagent. The slides were visualized on an Olympus BX51 fluorescence microscope, using 100X oil immersion objective; DP2-BSW software (v2.2 Olympus Corporation) was then used to take images and ImageJ 1.51n software (National Institutes of Health, USA) for merging images.

Enzyme-Linked Immunosorbent (ELISA) and Subclass IgG Assays
Total IgG antibodies were measured in serum from exposed individuals and control group. Maxisorb NUNC-immune modules (Thermo Scientific) were coated with 1 µg of each epitope and of rPvGAMA (10 µg/mL) (Baquero et al., 2017) in phosphate-buffered saline (PBS), pH 7.2, and incubated ON at 4°C. The immune modules were washed three times with PBS-0.05% Tween 20 solution (PBST) the next day and then blocked with 2.5% (wt/vol) non-fat powdered milk in PBST solution for 1 h at RT. Serum at 1:100 dilution was incubated for 2 h (100 µL per well) in duplicate. Secondary antibody horseradish peroxidase-conjugated goat anti-human IgG (Vector labs) was added at 1:10,000 dilution in blocked solution and incubated for 1 h at RT. TMB 2-Component Microwell Peroxidase Substrate (Sera-Care) was added at 100 µL/well to detect monoclonal antibody binding. The reaction was stopped by adding an equal volume of 1M phosphoric acid (H₃PO₄); OD was measured at 450 nm using a Multiskan GO (Thermo Scientific, Waltham, Massachusetts, USA) ELISA reader. Cut-off value was determined as negative control serum samples’ mean plus two standard deviations; IgG subclasses were determined for positive total IgG serum.

The ELISA protocol described above was followed to evaluate IgG subclasses with minor modifications, as follows: 3% (wt/vol) bovine serum albumin (BSA, Sigma) in PBST was used as blocking agent/solution and serum at 1:100 dilution was incubated for 2h in duplicate. A 1:1,000 dilution of monoclonal anti-human IgG1–biotin antibody produced in mouse (clone 8c/6-39), 1:15,000 monoclonal anti-human IgG2–biotin antibody produced in mouse (clone HP-6014), 1:40,000 monoclonal anti-human IgG3–biotin antibody produced in mouse (clone HP-6050) and 1:60,000 anti-human IgG4–biotin antibody, mouse monoclonal (clone HP-6025) (Sigma Aldrich) were used. ImmunoPure streptavidin, horseradish peroxidase conjugate (Thermo Scientific), at 1:5,000, dilution, was used as secondary antibody and TMB 2-Component Microwell peroxidase as substrate. The reaction was stopped by adding an equal volume of 1M phosphoric acid (H₃PO₄). OD was measured at 450 nm, using a Multiskan GO ELISA reader (Thermo Scientific, Waltham, Massachusetts, USA).

Statistical Analysis
GraphPad Prism software (version 5.0, San Diego, CA, USA) was used for analysis and constructing graphs. A Mann-Whitney test was used for comparing two groups regarding non-parametric data and Kruskal-Wallis test (with Dunn’s multiple comparison post-test) for comparing more than two groups. Student’s t-test was used for comparing two groups of data having a normal distribution. A 95% confidence interval was used. p ≤ 0.05 was considered significant. Significance level has been highlighted on all graphs by asterisks, as follows: *p < 0.05; **p < 0.005, and ***p < 0.0005.
RESULTS

T-Epitope Selection According in Vitro Binding Profile

Table 1 gives PvRON2 antigenic epitope prediction results. Eleven epitopes were selected in silico for HLA-DRB1*04, *07, *11, and *13 alleles (3 epitopes for each allele and 2 epitopes for the *07 allele). These epitopes were evaluated in vitro for their ability to bind all alleles of interest; those having greater than 50% binding were carefully chosen as high-binding peptides (Figure 1).

In vitro results showed that 10/11 (90.9%) peptides bound to the HLA-DRB1*04 allele (73.45% mean binding), being the most promiscuous allele studied; 7/10 (70%) peptides bound to HLA-DRB1*11 (64.47% mean binding); 6/11 (54.5%) of the peptides bound to HLA-DRB1*07 (58.33% mean binding) and HLA-DRB1*13 (56.55% mean binding). Four of the eleven peptides bound to all alleles studied here and were thus considered universal epitopes (39046, 39147, 39148, and 39153). Experimental binding assays and in silico binding predictions agreed in a 70.45% for the alleles studied (Table 1).

The IC50 value was calculated for all high-binding peptides to select the ones displaying higher affinity. Different peptide concentrations (μM) were used and the point at which 50% of the control peptide was displaced was thus calculated. IC50 μM value was calculated using a second order exponential decay function (Figure 2). IC50 assays demonstrated that epitope 39152 had the lowest IC50 ratio for HLA-DRB1*04 (0.15) and HLA-DRB1*11 (0.4), so it was thus selected as a good epitope for both alleles. Epitope 39047 (0.26) was selected as specific epitope for HLA-DRB1*07 and epitope 39154 (0.88) for HLA-DRB1*13. Of the four universal epitopes, peptide 39153 had the lowest IC50 mean value (Table 1, Figure 2). The selected epitopes were screened for antigenicity according to their HLA-DRB1* binding profile in previously typed patients.

Evaluating T-Cell Response Against Selected Epitopes

The people in the study had to have resided in the area for at least the last 5 years (average 29 years) and have had 1 or multiple episodes of P. vivax malaria, the last episodes dated between 2011 and 2015. It is well known that a naturally-acquired response requires a long period of time and multiple exposures to the parasite (Wipasa et al., 2002). HLA-DRB1* allele distribution for the 79 people here typed is shown in the Supplementary Table 1.
Among the alleles of interest, HLA-DRB1*04 had a frequency of 12.66%, HLA-DRB1*07 a frequency of 12.03%, HLA-DRB1*11 a frequency of 8.23% and HLA-DRB1*13 a frequency of 9.49%.

PBMC from individuals exposed to *P. vivax* infection (and control group) were stimulated with 10 µg/mL PvRON2 peptides and incubated for 5 days to evaluate proliferative response. Both universal epitope 39153 and DRB1* allele-specific peptides induced proliferation (≥2 Stimulation Index) in individuals from endemic areas, whereas there was no proliferation regarding specific peptides for each allele or parasite lysate in the control group (Table 3 and Supplementary Table 2). There were statistically significant differences for universal epitope 39153 (p = 0.0075), DRB1*13-specific peptide 39154 (p = 0.0387) and DRB1*07-specific peptide 39047 (p = 0.0260) between the group of exposed individuals and the control group. This response to the different antigens used in the lymphoproliferation assay, was compared with a Kruskal-Wallis test (with a Dunn’s multiple comparison post-test), where no statistically significant differences were found in response to the different peptides.

Low-binding control peptide 39115 showed the lowest proliferative response (SI = 2.018) and was significantly different regarding the control group (p = 0.0289). *P. vivax* lysate also induced a greater proliferative response in exposed individuals, despite no statistically significant differences were observed when compared to the control group (Figure 3). However, peptide 39115 induced T cell proliferation in 7 of the 29 exposed individuals, despite no binding to HLA-DRB1* was either predicted or observed in vitro. Considering that this peptide is a T epitope from the *Pv*12 protein as shown by the lymphoproliferation assays, further analyses to assess whether it is being presented by other class II molecules such as HLA- DP or HLA-DQ are worth carrying out.

*P. vivax* lysate also induced a greater proliferative response in exposed individuals; however, no statistically significant differences were observed when compared to control group (Figure 3). The mean SI = 12.47 ± 1.636 SE is for exposed individuals and mean SI = 5.45 ± 1.138 SE for the control group. Despite the SI values of PMBCs stimulated with PHA were significantly higher between exposed individuals and control group (p = 0.0103), 96.6% of exposed individuals and the 100% of control group responded to PHA (data not shown).

**PvRON2 Epitope-Dependent Cytokine Secretion**

IFN-γ, TNF (Th1 profile) and IL-10, IL-6 (Th2 profile) production in culture supernatant was quantitatively measured after stimulating PBMCs with peptides selected for HLA-DRB1* by binding assays. *P. vivax*-lysate and PHA were used as positive controls and unstimulated PBMCs as a baseline. Statistical analysis between unstimulated and stimulated PBMCs from exposed individuals showed that IFN-γ was only significant after stimulation with *P. vivax*-lysate (p = 0.0001). TNF production was significantly different for peptides 39047 (p = 0.01), 39154 (p = 0.04) and *P. vivax*-lysate (p = 0.0001). IL-10 had higher production with peptide 39047 (p = 0.0001) and *P. vivax*-lysate (p = 0.0001). IL-6 responses were significantly greater to peptides 39047 (p = 0.01), 39152 (p = 0.0025), 39154 (p = 0.0002) and *P. vivax*-lysate (p = 0.0001) (Figure 4). Cytokine levels were...
TABLE 3 | A summary of PBMC proliferative response to PvRON2 T-cell epitopes.

| Antigen                  | HLA-DRB1*              | Average response (SI) | p-value |
|-------------------------|------------------------|-----------------------|---------|
|                         |                        | Exposed individuals   | Control group |         |
| 39153                   | Universal epitope      | 3.354 (SE 0.578)      | 0.935 (SE 0.282) | 0.0075**|
| 39152                   | DRB1*04 DRB1*11       | 3.062 (SE 1.09)       | 1.004 (SE 0.198) | 0.175   |
| 39047                   | DRB1*07               | 3.108 (SE 0.737)      | 1.156 (SE 0.267) | 0.0260* |
| 39154                   | DRB1*13               | 3.697 (SE 1.17)       | 0.888 (SE 0.327) | 0.0387* |
| 39115                   | Low-binding control peptide | 2.018 (SE 0.347) | 0.87 (SE 0.231) | 0.0289* |
| Parasite lysate         | Positive control       | 3.664 (SE 0.607)      | 1.474 (SE 0.255) | 0.0735  |

Data regarding individuals exposed to P. vivax infection and control group. (SI, stimulation index; SE, standard error). Mann-Whitney’s test and Student’s t-test p-values were determined for exposed individuals compared to control group. *p < 0.05, **p < 0.005.

compared with the Kruskal-Wallis test (with a Dunn’s multiple comparison post-test) between 39115 and all other peptides in exposed individuals, a significantly higher TNF and IL-6 production was observed for peptide 39154 (p < 0.0001).

Control group data was also analyzed; significant differences were found for IFN-γ production after PBMCs had been stimulated with peptides 39047 (p = 0.002), 39154 (p = 0.0006) and P. vivax-lysate (p = 0.0006). IL-6 production was greater after being stimulated with peptides 39152 (p = 0.0006), 39047 (p = 0.002), 39154 (p = 0.01) and P. vivax-lysate (p = 0.0001). This suggests that naïve T-cells and/or other innate cells, such as macrophages, natural killer (NK), natural killer T-cells (NKT) and non-cytotoxic innate lymphoid cells recognized peptides and lysate and produced cytokines (Artis and Spits, 2015) (Supplementary Figure 1).

Cytokine levels were compared between exposed individuals and control group, significant differences being found regarding TNF production for epitope 39153 (p = 0.0127). Significant differences were found for epitope 39047 (p = 0.005) and 39152 epitope (p = 0.010) IL-6 production (Figure 5).

Detecting Antibodies Against P. vivax-Infected RBC by IFA

Naturally-acquired anti-malarial antibodies were detected using Multitest slides (MP Biomedicals) coated with parasitized RBC. The thirty exposed patients’ sera reacted against P. vivax but the control group’s sera did not. Supplementary Figure 2 shows the fluorescence pattern obtained with these sera.

An Analysis of PvRON2 B-Epitope Humoral Immune Response in P. vivax-Exposed Individuals

Antibody response against four in silico selected PvRON2 B-epitopes was evaluated in sera from 30 individuals exposed to natural P. vivax infection (Figure 6, Table 2). The highest number of seropositive samples was for the 39041-epitope (6/30, 20% of samples), followed by 39042 and 39043 (3/30, 10% of samples) and 39044 (2/30, 6.6% of samples). Peptides 39041 and 39043 showed the highest mean response (0.1598 and 0.1422, respectively), and significant differences were observed regarding 39044 which had the lowest mean response (0.0949) out of all four peptides. The Kruskal-Wallis test (with Dunn’s multiple comparison post-test) was used for statistical analysis, there were significant differences regarding epitope response (p = 0.003). Although there were no statistically significant differences between exposed individuals and control group, there was a tendency for a greater response in the first group (data not shown). rPvGAMA was used as positive control, 70% of samples being seropositive (21/30 samples); significant differences with control group were observed (p = 0.0004) (Supplementary Figure 3).

The ELISA results were analyzed by endemic area, showing an evident tendency for a greater response in the samples from the Chocó department compared to the Córdoba department (Figure 7). The Mann-Whitney test gave a significantly higher response for peptides 39041 (p = 0.0135), 39042 (p = 0.0171), 39043 (p = 0.007) and 39044 (p = 0.0492) in P. vivax-exposed individuals. Samples from Colombia’s Chocó (n = 13) and Córdoba (n = 17) departments. From these samples, six reacted positively to at least one PvRON2 B-epitope, where 83% (n = 5) of the samples were from Chocó’s department. Positive sera reacted to the four peptides, 83% were from samples from the Chocó department. Although 39041, 39042, and 39044 peptides had no significant differences between exposed individuals from the Chocó and control group, 39043 had a significantly higher response (p = 0.0186). Differences between endemic areas were only observed in response to PvRON2 B-cell epitopes since there were no significant differences between both areas regarding PvGAMA (p = 0.4265), as it was expected to occur when a whole recombinant protein is used as antigen (several epitopes present within it, could be differentially recognized by exposed individuals, and a similar overall response was thus detected). Seropositive samples were selected for evaluating IgG subclasses.

The Prevalence of IgG Subclass Response Against PvRON2 B-Epitopes in Individuals Exposed to Natural P. vivax Infection

Seropositive samples from exposed individuals recognizing B-epitopes were selected for IgG subclass evaluation. Of the four peptides evaluated, only 39041 had significant differences between IgG subclasses (p = 0.0004) while there were no
significant differences for the other peptides. 39041 had clear IgG2 predominance regarding other subclasses, having statistically significant differences with IgG1 and IgG4 (the latter having the lowest mean response) (Figure 8).

**DISCUSSION**

Developing countries desperately need strategies aimed at preventing malaria (especially that caused by *P. vivax*), such as approaches for developing specific drugs and protective vaccines which are currently unavailable. Although there are *P. vivax* vaccines in phases I and IIa (López et al., 2017), they have not induced sterile protection (Bennett et al., 2016). Developing a *P. vivax* vaccine requires studies analyzing naturally-infected patients’ immune response regarding proteins involved in erythrocyte invasion. Several *P. vivax* vaccine candidates’ binding regions have been characterized to date, such as reticulocyte binding proteins (RBPs) (Urquiza et al., 2002), the Duffy binding protein (DBP) (Ocampo et al., 2002), the *P. vivax* GPI-anchored micronemal antigen (*PvGAMA*) (Baquero et al., 2017), some
FIGURE 4 | Exposed individuals’ supernatant culture in vitro cytokine production. Individual data shows the mean value of non-stimulated and PBMCs stimulated with universal epitope (39153), specific epitopes 39047, 39152, and P. vivax lysate. IFN-γ, TNF, IL-10, and IL-6 levels were measured by CBA kit; cytokine concentration is expressed in pg/mL. Statistically significant differences (p ≤ 0.05) are shown and data represents the means ± SEM for all values. *p < 0.05, **p < 0.005 and ***p < 0.0005.

proteins from the tryptophan-rich antigen (PvTRAg) family (Zeeshan et al., 2014), merozoite surface protein-1 (PvMSP-1) (Rodríguez et al., 2002) and apical membrane antigen-1 (AMA-1) (Arévalo-Pinzón et al., 2017). Important mediators in AMA-1 erythrocyte binding have also been identified, such as rhoptry neck proteins (RONs), including PvRON5 (Arévalo-Pinzón et al., 2015), PvRON4 (Arévalo-Pinzón et al., 2013) and PvRON2 (Arévalo-Pinzón et al., 2011). RONs have been strongly associated with MJ formation, thereby helping parasite entry into erythrocytes; RON2 is a vaccine candidate since anti-AMA1 and anti-RON2 antibodies can block erythrocyte invasion (Arévalo-Pinzón et al., 2011; Lamarque et al., 2011; Srinivasan et al., 2011; Tyler et al., 2011; Vulliez-Le Normand et al., 2017).

The data presented here has described naturally-acquired T-cell immune responses to PvRON2 high-affinity binding-MHC-II DRB1 molecules of P. vivax-exposed individuals from two endemic areas of Colombia. Certain requirements are involved in protection-inducing vaccine design; for example, the proper antigen presentation by MHC-II and MHC-I molecules to T-cell receptors, so as to induce strong immune responses. MHC class II and I molecules are critical in host–parasite interactions as they determine host immune response quality. High-affinity peptide-MHCII-TCR binding time or peptide-MHCII complex amount is critical for mounting protective T-cell responses (Blum et al., 2013; Tubo et al., 2013). Post-genomic era bioinformatics tools and reverse vaccinology approaches have drawn scientists’ attention, since an individual antigen can be screened from one or several microorganisms and the highest affinity epitopes be determined from these might induce protective immune responses (Sette and Rappuoli, 2010).

This study has analyzed the PvRON2 sequence aa to determine high-binding HLA-DRB1* T-cell epitopes in silico. NetMHCIIPan 3.0 (Andreatta et al., 2015) predicted eleven high-affinity HLA-DRB1* epitopes where at least one epitope bound to one HLADRB1* molecule; this was confirmed by in vitro competition assays using biotin-control peptides. However, some predicted epitopes have not bound to HLA-DRB1*, according to other studies (Bergmann-Leitner et al., 2013), while HLA-DRB1*04 bound to 10/11 peptides here. A previous study has shown a strong naturally-acquired humoral response in HLA-DRB1*04 people living in the Brazilian Amazon against 5/9 recombinant P. vivax proteins (Lima-Junior et al., 2012). HLA-DRB1*04 is one of the most frequently occurring alleles in Colombian Amerindian groups, accompanied by DR2 (DRB1*1602), DR6 (DRB1*1402) and DR8 (DRB1*0802). The patients’ blood samples used in this study were mostly taken from Amerindians from Córdoba and Chocó. Tule (5 HLA-DRB1*04 alleles) is the main Amerindian population in Córdoba and the Waunana (4 HLA-DRB1*04 alleles) in the Chocó region, having more diverse DRB1 alleles than other groups (Trachtenberg et al., 1996).

Previous studies had suggested that some alleles’ over dominance in a population could be due to the spread of advantageous alleles (positive selection) after pathogen-driven selection. This has been shown by Hill et al., in a study of West African alleles (HLA-DRB1*1302-DQB1*0501) which were not
FIGURE 5 | Exposed individuals and control group supernatant culture in vitro cytokine production. Individual cytokine values from PBMCs stimulated with universal peptide (39153), specific epitopes 39047, 39152, and 39154, and P. vivax lysate. TNF and IL-6 levels were measured by CBA kit and cytokine concentration is expressed in pg/mL. Statistically significant differences ($p \leq 0.05$) are shown and data is the means ± SEM for all values. $^{*}p < 0.05$ and $^{**}p < 0.005$.

responses were investigated based on MHC class II peptide binding specificity and humoral immune responses by detecting antibody levels against linear B-cell epitopes.

Antibodies against P. vivax blood-stage proteins are important elements for blocking RBC invasion (Wipasa et al., 2002); such antibodies thus play an important role in identifying and validating P. vivax vaccine candidates (Soares et al., 1999; Lima-Junior et al., 2008; Storti-Melo et al., 2012; Changrob et al., 2017; Rodrigues-Da-Silva et al., 2017). We confirmed the presence of naturally-acquired humoral responses against four PvRON2 B-epitopes which were recognized by IgG antibodies and subclasses. Low individual PvRON2 B-epitope responder frequency was observed (20% 39041, 10% 39042, 6.6% 39043 and 39044); such low responses have previously been reported for other blood-stage antigens such as PvMSP8. A loss of mean response to a target protein has been observed as time has elapsed when average response has been about ten times greater to recombinant protein than linear epitopes in acute-infection patients (Cheng et al., 2017). This has been associated with short-lived antibodies, due to short-lasting memory responses or parasite-induced B-cell dysregulation (Rénia and Goh, 2016) and parasite genetic variations or in exposed populations.

Antibody response differences against PvRON2 B-cell epitopes between exposed individuals from Chocó (83%) and Córdoba (17%), could be attributed to several factors, including the level of parasitemia and the number of episodes (Druilhe and Pérignon, 1994). The last 3 SIVIGILA reports (2015–2017), showed a higher incidence of P. vivax infection in Chocó regarding Córdoba (Instituto-Nacional-De-Salud, 2017). Other intrinsic factors from the responders such as their HLA, sex, age, psychological stress, nutrition and other infectious diseases could also be involved in such differences (Van Loveren et al., 2001).
Two of the selected B-epitopes (39042 and 39044) were located on an α-helical coiled motif protein and other studies have shown that selected in silico peptides related to these motifs have been recognized by naturally-acquired antibodies and have been immunogenic in mice (Villard et al., 2007; Arévalo-Pinzón et al., 2011); however, 39042 (10%) and 39044 (6.6%) epitopes had the lowest recognition values in our study. 39041 had significant differences in the IgG subclasses analyzed; IgG2 predominated while low IgG4 and IgG1 levels were observed. A predominant IgG2 response and low IgG4 reactivity in previous studies has been associated with P. falciparum infection resistance and clearance, IgG2/IgG4 relationship being associated with a protective role (Aucan et al., 2000). Similar results have been found in PmSP8 studies recording IgG2 non-cytophilic antibody predominance which has been associated with resistance to P. vivax malaria (Cheng et al., 2017). 39041 has been seen to be immunogenic in mice (Arévalo-Pinzón et al., 2011) and its potentially protective role makes this peptide a pivotal PvRON2 epitope for inclusion in a subunit-based vaccine.

It has been thought that antibodies would be enough to protect against malaria and that T-cells do not play an important role during the erythrocyte stage. Advances in immunology-related knowledge have demonstrated that B-cells must be activated by CD4+ T-helper cells to prompt good humoral responses, thereby inducing cytokine, memory cell and antibody production (Batista and Harwood, 2009; Tubo et al., 2013; Yuseff et al., 2013). The role of exposed patients’ T-cell response against PvRON2 high-affinity binding peptides was studied in cytokine proliferation and production assays. Only exposed individuals’ PBMC cultures showed proliferation induced by universal and specific binding peptides, suggesting that PvRON2 induced memory T-cells against high-affinity peptides. However, Th1 and Th2 cytokine responses were low, except for IL-6. Low cytokine responses/production have been observed in other studies; Silva-Flannery et al., found that immunization with monomeric peptide did not result in peptide-specific IFN-γ-secreting cell expansion and was not protective. They also reported that the monomeric peptide was less taken up by antigen-presenting cells and was not going through the phagolysosome (Silva-Flannery et al., 2009).

Cytokine production by unexposed individuals’ PBMCs against PvRON2 synthetic peptides may have been due to dendritic or macrophages cells priming naïve T-cells and inducing effector T-cell cytokine production; nevertheless, secretion was very low for some of them.

Unlike the other cytokines tested here, IL6 was highly secreted by exposed patients’ PBMCs; this was not surprising, since one of IL6’s multi-functions is to stimulate hybridoma and plasmacytoma cell growth and help antibody production (Matsuda et al., 1988). IL6, together with IL12 and VDR, have been associated with reduced parasitemia, its severity and
High cytokine induction in healthy individuals has been demonstrated in studies where conserved high response to a parasite evasion mechanism for inhibiting effective immune responses able to eliminate the parasite (Rénia and Goh, 2004). Nonetheless, the healthy individuals had not been exposed to P. vivax since immunofluorescence assays did not show their antibodies’ reactivity to the parasite.

Taken together, the in silico T-cell and B-cell epitope selection results highlighted two T-cell epitopes (39047 and 39154) and one B-cell epitope (39041) as promising vaccine candidates. Despite the significant differences observed in immune responses evoked in the exposed individuals compared to the control group, overall the responses were relatively low. All selected peptides were conserved among the 11 P. vivax strains which may also explain such low immune responses. This has been demonstrated in P. falciparum studies where conserved high activity binding peptides (HABPs) were poorly antigenic and poorly immunogenic (Patiño et al., 1997; Lougovskoi et al., 1999; Ocampo et al., 2000; Parra et al., 2000; Hensmann et al., 2004). It should be stressed that caution must be taken, since using these 3 promising peptides in a multi-epitope vaccine in their unmodified state would probably mean that they could induce low immunogenicity and not provide long-lasting protection; however, proven approaches have shown that modifying their critical residues should induce a strong and long-lasting protection-inducing immune response (Patarroyo et al., 2010).

HABPs have been seen to be P. falciparum vaccine candidates during the last two decades (Rodriguez et al., 2008); however, they must be modified to make them antigenic and protection-inducing by replacing critical aa with others having the same mass but different polarity (Cifuentes et al., 2008; Patarroyo et al., 2011). Such HABPs can only be used in a tailor-made vaccine targeting a specific HLA-DRB1* endemic population; however, a universal protection-inducing vaccine will require studying other peptides which can bind to other HLA-DRB1* alleles. Future studies should be carried out using modified peptides aimed at assessing immunogenicity and protection-inducing ability in the Aotus experimental model to confirm their suitability as P. vivax vaccine candidates. Likewise, additional peptides should be included to cover all parasite stages, aiming at a 100% protection-inducing, multistage, multi-epitope, minimal subunit-based vaccine.

**AUTHOR CONTRIBUTIONS**

CL: designed and performed the experiments, analyzed the data, drafted the manuscript. YY-P: designed and performed the experiments, analyzed the data, drafted the manuscript, designed the figures. DD-A: drafted the manuscript. MEP: critical suggestions regarding the manuscript. MAP: conceiving the work and drafting all versions of the manuscript. All authors have revised the manuscript and approved the version to be submitted.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00156/full#supplementary-material
Supplementary Figure 3 | IgG antibody response against PvGAMA control recombinant protein. Significant differences (calculated by Mann-Whitney test) are shown between samples from exposed individuals and control. The dashed line indicates the cut-off point for seropositive samples.

Supplementary Table 1 | Exposed-individuals’ HLA-DRB1* allele frequency

Supplementary Table 2 | Non-exposed individuals’ lymphoproliferation assay using PvRON2 peptides.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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