Evaluación de la respuesta inmune frente a los potenciales candidatos a vacuna contra *Plasmodium vivax* PvRON2, Pv12, PvDBP y PvMSP1

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acDC	Ensayo de cocultivo acelerado con DC
ADCI	Inhibición mediada por células dependiente de anticuerpos
ADNg	Ácido desoxirribonucleico genómico
ADPh	Fagocitosis dependiente de anticuerpos
AMA	Antígeno de Membrana Apical
BSA	Albumina sérica bovina
CD	Clúster de diferenciación
CF	Citometría de flujo
CFSE	Carboxyfluorescein diacetate N-succinimidyl ester
CMSP	Células mononucleares de sangre periférica
CPD	Citrato fosfato dextrosa
CSP	Proteína del circumsporozoito
DAPI	4 ',6-diamidino-2-fenilindol
DBP	Proteína de unión a Duffy
DC	Células dendríticas
DCm	Células dendríticas mieloides
DCp	Células dendríticas plasmocitoides
DMSO	Dimetil sulfóxido
EDTA	Ácido etilendiaminotetraacético
ELISA	Ensayo por inmunoabsorción ligado a enzimas
FITC	Isotiocianato de fluoresceína
GAMA	Antígeno de micronemas anclado a GPI
GM-CSF	Factor estimulante de colonia granulocito macrófago
GPI	Anclaje glicofosfatidilinositol
GRp	Glóbulos rojos parasitados
HA	Hemaglutinina
HLA	Antígeno leucocitario humano
IC50	Concentración media inhibitoria
IE	Índice de estimulación
IFA	Inmunofluorescencia indirecta
IFN	Interferón
Ig	Inmunoglobulina
IL	Interleuquina
LB	Linfocitos B
LT	Linfocitos T
MHC	Complejo mayor de histocompatibilidad
mL	Mililitros
Mrz	Merozoítos
MSP	Proteína de superficie del merozoito

NGS	Next Generation Sequencing
NK	Células natural-killer
ON	Óxido nítrico
PAMP	Patrones moleculares asociados a patógenos
PBS-T	Buffer fosfato salino-Tween
pNPP	Substrato líquido fosfatasa alcalina amarillo
Poly I:C	Ácido polinosínico:policitidílico
PRR	Receptores de reconocimiento de patrones
Pv12	Proteína de superficie 12
RBP	Proteína de unión a reticulocitos
RON	Proteína del cuello de las roptrias
RPMI	Roswell Park Memorial Institute
SBN	Sobrenadante de cultivo
SDS-PGE	Electroforesis en gel de poliacrilamida con dodecilsulfato sódico
SP	Sangre periférica
TCM	Linfocito T de memoria central
TCR	Receptor de linfocitos T
TEF	Linfocito T efector
TEM	Linfocito T de memoria efectora
TEMRA	Linfocito T de memoria efectora CD45RA+
Th	Linfocito T ayudador
TN	Linfocito T virgen
TNF	Factor de necrosis tumoral
Treg	Linfocitos T reguladores
TT	Toxoide tetánico

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RESUMEN

La malaria es una enfermedad infecciosa con altos índices de mortalidad alrededor del mundo. Ésta es causada por parásitos del género *Plasmodium*, siendo *Plasmodium vivax* la especie prevalente en el continente americano. En Colombia, para el año 2019 se reportaron 80.415 casos de malaria, de los cuales el 48.7% fueron causados por *P. vivax*, siendo los departamentos de Chocó, Antioquia, Nariño y Córdoba los de mayor incidencia.

El control de esta enfermedad no se ha logrado, debido a la resistencia del parásito a los medicamentos empleados, a la resistencia a los insecticidas adquirida por el vector y a las políticas de salud deficientes. Ante esta situación, la vacunación es considerada como la mejor alternativa de prevención y con mayor potencial para reducir la morbilidad y mortalidad en las poblaciones afectadas.

Dada la dificultad para realizar cultivos continuos *in vitro* de *P. vivax*, la mayor parte de la investigación en malaria se ha enfocado en *P. falciparum*. A pesar de esto, en los últimos años, la Fundación Instituto de Inmunología de Colombia (FIDIC) ha logrado caracterizar varias proteínas de *P. vivax* como posibles candidatos a vacuna; sin embargo, aún se desconoce la capacidad de la mayoría de estas proteínas, y de los epítopes derivados de ellas, para ser reconocidos por componentes de la respuesta inmune humoral y celular.

Con el fin de profundizar en la caracterización de proteínas y/o péptidos como potenciales candidatos, la primera fase del estudio planteó la realización de ensayos de antigenicidad *in vitro* con muestras de individuos expuestos a la infección natural en áreas endémicas de Colombia. Para esto, los epítopes B y T fueron seleccionados *in silico* y la elección de epítopes T fue definida con ensayos de unión *in vitro* a moléculas HLA-DRβ, obtenidas a partir de líneas celulares linfoblastoides B humanas. Los individuos fueron elegidos de acuerdo con la expresión de alelos HLA-DRβ1* de frecuencia intermedia en las poblaciones blanco para una vacuna contra la malaria (HLA-DRβ1*04, *07, *11 y *13).

Durante la segunda fase de este estudio, se evaluó la inmunogenicidad *in vitro* de péptidos nativos y modificados derivados de las proteínas. Para esto, se emplearon linfocitos T CD4+ vírgenes de individuos sanos con restricción a los alelos HLA-DRβ1* antes mencionados, estimulados con células dendríticas como células presentadoras de antígeno profesionales.

Finalmente, se estableció que existe una correlación entre la capacidad de unión a moléculas HLA-DR y la inmunogenicidad de los péptidos modificados derivados de proteínas de *P. vivax*. Los resultados de este estudio generaron datos relevantes para futuros estudios de inmunogenicidad y protección en modelos experimentales, encaminados a la comprensión de los mecanismos de la respuesta inmune frente a *P. vivax* y a la generación de una vacuna efectiva contra la malaria.

INTRODUCCIÓN GENERAL

La malaria es una de las enfermedades infecciosas de mayor prevalencia alrededor del mundo, siendo *P. falciparum* y *P. vivax* los principales agentes causantes. Condiciones medio ambientales de humedad y calor en países tropicales, favorecen el desarrollo del mosquito transmisor; esto, sumado a las condiciones precarias de la población de bajos recursos, han convertido a esta enfermedad en un problema de salud pública global (1). Entidades como la Organización Mundial de la Salud, el Banco Mundial, las Naciones Unidas y la Unicef, han concentrado sus esfuerzos en el control de la infección, implementando estrategias para la reducción de la enfermedad (2).

En el contexto de las estrategias implementadas para el control de la enfermedad, la resistencia que presenta el mosquito frente a los insecticidas, la resistencia por parte del parásito a los medicamentos antimaláricos y que, a la fecha, no se cuenta con una vacuna totalmente efectiva que ayude a disminuir la incidencia de la enfermedad, hacen que se dificulte su erradicación (3). Así, se hace necesario realizar estudios que permitan la identificación de regiones dentro de los antígenos parasitarios que puedan generar una respuesta por parte del sistema inmune del hospedero después de una infección natural para ser empleados en el diseño de una vacuna.

La vacunología reversa emplea herramientas computacionales que permiten identificar estas regiones, abordando la variabilidad antigénica y contribuyendo a la comprensión de la interacción entre las respuestas humoral y celular, y de esta manera podría resolver algunos inconvenientes del enfoque tradicional. Aunque este enfoque se ha empleado en otras enfermedades infecciosas, no ha sido ampliamente explorado en malaria como una alternativa para la predicción de epítopes que puedan ser parte de una vacuna completamente efectiva (4-7).

Si bien se han logrado avances con respecto al desarrollo de una vacuna para *P. falciparum*, en *P. vivax* el diseño se encuentra retrasado (8, 9). Actualmente, dos de los candidatos a vacuna más promisorios para *P. vivax* del estadio intra-eritrocítico, DBP y MSP1, han mostrado conferir sólo protección parcial (10). Por lo tanto, epítopes de nuevas moléculas deben ser identificados. Adicionalmente, ya que los epítopes

naturales podrían no ser protectivos (como se ha observado con PvMSP-1 y PvDBP), modificarlos para optimizar la presentación antigénica sería una buena alternativa para mejorar su eficacia protectora (11).

Por lo anterior, en este estudio se propuso realizar una selección tanto *in silico* como *in vitro* de epítopes de las proteínas PvRON2, Pv12, PvDBP y PvMSP-1 que puedan llegar a ser utilizadas durante el diseño de una vacuna contra *P. vivax*. Adicionalmente, epítopes de dos de las principales moléculas involucradas en el proceso de invasión (PvMSP-1 y PvDBP) fueron modificados buscando mejorar su presentación antigénica en el contexto del Complejo Mayor de Histocompatibilidad (MHC, del inglés Mayor Histocompatibility Complex) clase II gen DRβ.

MARCO TEÓRICO

La malaria es una enfermedad infecciosa causada por parásitos del filo Apicomplexa, género *Plasmodium* sp., que es transmitida por la picadura de un mosquito hembra infectado del género *Anopheles*. Se han descrito cinco especies capaces de producir malaria en humanos: *P. falciparum* y *P. vivax* (responsables del mayor número de casos), *P. malariae*, *P. ovale* y *P. knowlesi* (12-14).

Estos parásitos se distribuyen en áreas tropicales y subtropicales alrededor del mundo. *P. falciparum* se encuentra principalmente en África sub-sahariana y es el responsable de las mayores tasas de morbilidad y mortalidad, asociándose con malaria severa y complicaciones durante el embarazo. Por otra parte, *P. vivax* está presente en el sudeste de Asia, Etiopía, Centroamérica y Suramérica y, a pesar de ser considerado como causa de malaria leve, en los últimos años se han registrado casos de malaria grave por esta especie. Por otra parte, *P. malariae* se distribuye alrededor del mundo con prevalencia en el oeste de África, y *P. ovale* en África y Asia (12) (Figura 1).

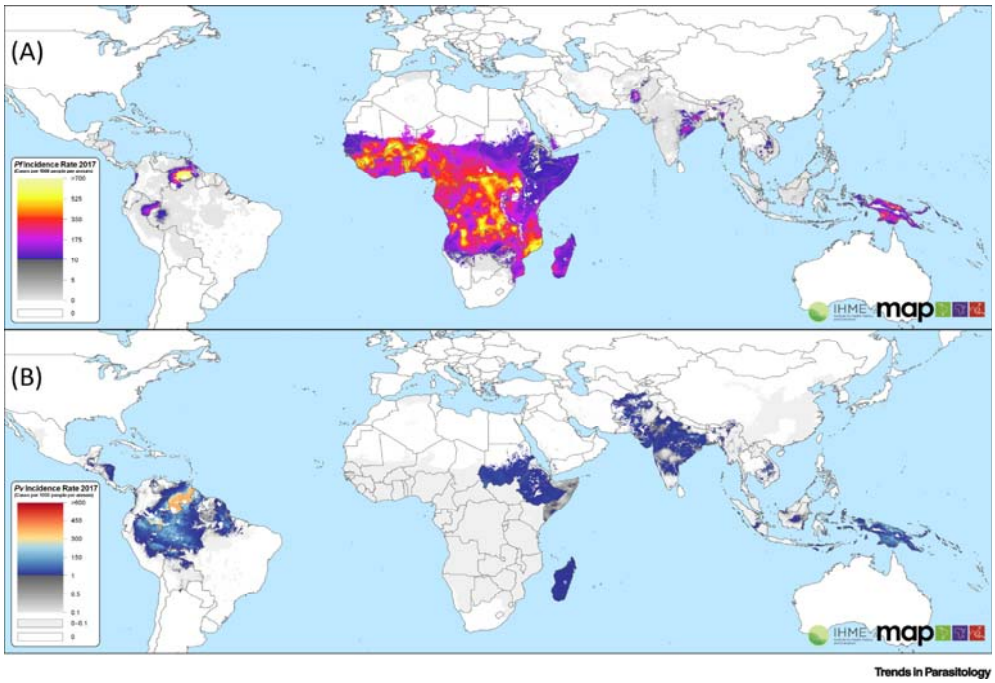


Figura 1. **Tasas de incidencia de malaria por país para el 2017.**
Se muestra la transmisión de malaria causada por (A) *P. falciparum* y (B) *P. vivax*. Tomado de Price R, 2020 (15).

Los síntomas clínicos en el humano son causados por el estadio sanguíneo del ciclo de vida del parásito, y van desde escalofríos, fiebre, dolor de cabeza, malestar general hasta complicaciones generalizadas e incluso la muerte. En el inicio de la infección, la inmunidad innata del huésped es capaz de controlar el aumento exponencial de los parásitos, pero no puede eliminar completamente la infección (16). La infección con *P. falciparum* que no sea tratada a tiempo puede llevar a un síndrome de malaria cerebral, con anemia severa, falla sistémica y acidosis metabólica, síntomas que pueden llevar a la muerte (17). Por otra parte, la infección con *P. vivax* es la causa de mayor morbilidad entre pacientes jóvenes y niños en los continentes asiático y americano.

Epidemiología de la Malaria

Esta enfermedad tiene dos formas de transmisión: la estable, que se caracteriza por ser constante, frecuente y se presenta durante todo el año; y la inestable, que es baja, errática y estacional (14). Para el año 2010, aproximadamente 2570 millones de personas alrededor del mundo estaban en riesgo de adquirir la infección por *P. falciparum*, de éstos, el 56% vivía en áreas de transmisión estable y el 44% en áreas de transmisión inestable (18). Por otro lado, 2480 millones de individuos se encontraban en riesgo de infectarse con *P. vivax*, de los cuáles, el 39% vivía en áreas de transmisión estable y el 61% en áreas de transmisión inestable (19).

Es importante resaltar que las áreas de transmisión estable representan una gran diversidad de niveles endémicos. En cambio, el riesgo es muy bajo en las áreas de transmisión inestable y es poco probable que la incidencia de casos exceda uno por cada 10.000 individuos por año (18, 19). Aunque se venía presentando una reducción de los casos de malaria, para el año 2020 hubo un ligero aumento a nivel mundial, se estimaron alrededor de 241 millones de casos de los cuales 236 millones fueron causados por *P. falciparum* y 4,5 millones por *P. vivax*, este incremento fue asociado con la interrupción de los servicios de salud durante la pandemia de COVID-19 (20).

Aunque la prevalencia global de *P. vivax* es baja comparada con *P. falciparum*, es mayor en las regiones del sudeste asiático, el Pacifico occidental y en la Américas (21). Dentro de las características propias de la biología de *P. vivax* se sabe que: (i) presenta

bajos niveles de parasitemia dada su preferencia por invadir glóbulos rojos inmaduros (reticulocitos) y por su habilidad de migrar más allá de los senos venosos, (ii) produce formas latentes en el hígado conocidas como hipnozoítos, (iii) muestra una aparición temprana de gametocitos durante el estadio sanguíneo de la infección y (iv) la inmunidad natural se adquiere a una edad más temprana (21-23).

Así mismo, en el ciclo de vida del parásito es importante estudiar el origen de los nuevos picos de parasitemia causados por *P. vivax*. Estos pueden atribuirse a: recaída (causada por hipnozoítos hepáticos), recrudescencia (causada por trofozoítos sub-patentes) o reinfección (causada por una nueva inoculación de esporozoítos por parte de los vectores) (24). La tasa y el tiempo en que una cepa causa nuevos picos de parasitemia, se correlacionan con la variación geográfica (25). Las cepas de áreas subtropicales muestran un largo periodo de incubación y por tanto un retraso en los picos, éstas se encuentran en áreas del Mediterráneo, América Central, China y la península coreana. En cambio, las cepas de áreas tropicales presentan tiempos cortos de incubación con intervalos cortos en los picos de parasitemia, y se encuentran en las Américas, el sudeste de Asia y el Pacifico occidental. El África subsahariana y la India muestran frecuencias de picos de parasitemia y periodicidades variables (21).

Ciclo de vida de *Plasmodium* sp.

El parásito presenta un complejo ciclo de vida y dos modalidades de reproducción, la sexual en el mosquito *Anopheles* y la asexual en el hospedero vertebrado (Figura 2). La reproducción sexual ocurre dentro del intestino del mosquito, dando lugar a la formación de ooquinetes, que maduran a oocistos y a partir de éstos se forman los esporozoítos (26). El ciclo de vida intra-eritrocítico depende de la especie parasitaria que invada; para *P. falciparum*, *P. vivax* y *P. ovale* es de aproximadamente 48 horas, para *P. malariae* es de 72 horas y para *P. knowlesi* es de 24 horas.

En el momento en que el mosquito se alimenta de sangre del hospedero infectado, transmite el parásito. El ciclo inicia con la inoculación de esporozoítos móviles, que viajan a los vasos linfáticos y al hígado donde invaden a los hepatocitos. Allí, el esporozoíto expresa diversas proteínas para la invasión de la célula diana. Dentro

del hepatocito, cada esporozoíto produce entre 10.000 a más de 30.000 merozoítos en un tiempo aproximado de 5 a 8 días (27).

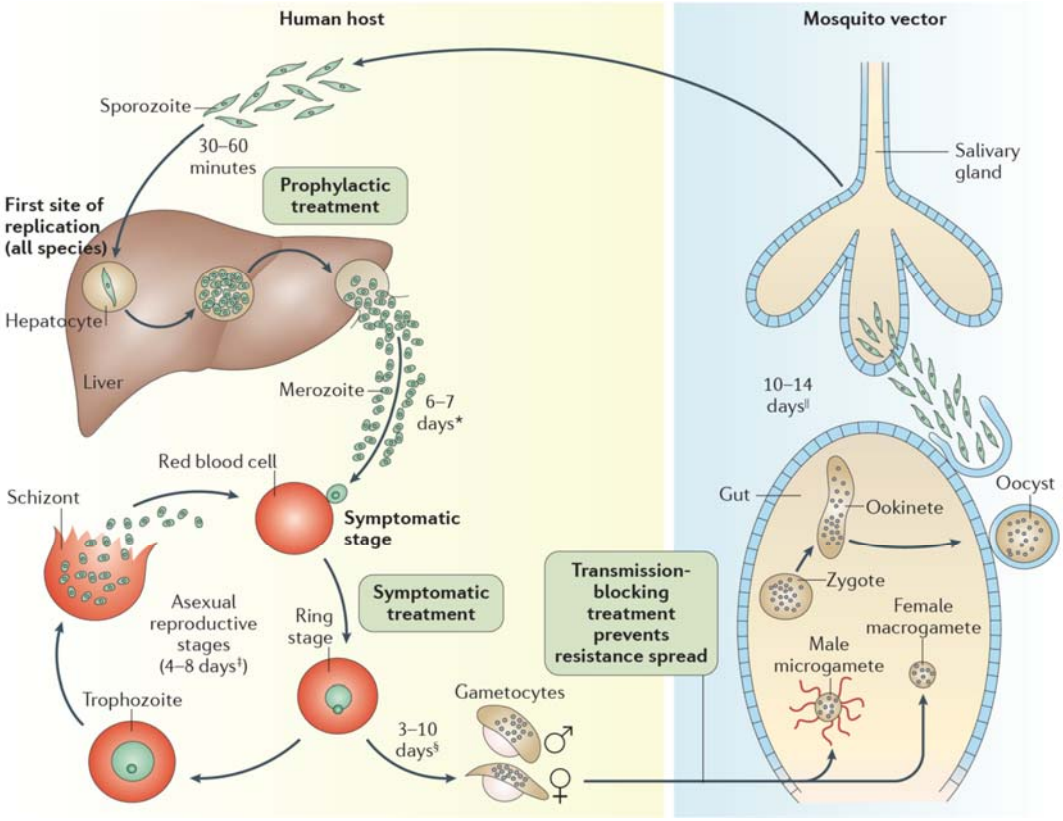


Figura 2. Ciclo de vida de *Plasmodium* sp.
Se muestra el ciclo de vida asexual dentro del hospedero vertebrado con las diferentes formas infectivas: esporozoitos, merozoitos, anillos, trofozoítos, esquizontes y gametocitos; y el ciclo de vida sexual en el hospedero invertebrado con las formas de micro y macrogameto, ooquinete y oocisto de *Plasmodium sp.* Tomado de Phillips MA, 2017 (12).

Posteriormente, el esquizonte hepático explota y libera los merozoítos al torrente sanguíneo para que invadan a los glóbulos rojos. Se han descrito diversas proteínas expresadas por el parásito encargadas de establecer interacciones fuertes con el eritrocito y la posterior formación de la vacuola parasitófora, dentro de la cual el parásito se multiplica (11). Cuando el esquizonte eritrocítico explota, libera de 6 a 30 merozoítos y cada uno puede invadir un glóbulo rojo donde se multiplicará por mitosis. La reproducción asexual del parásito ocurre dentro de los glóbulos rojos, donde el ciclo continua a través de diferentes estadios: anillos, trofozoítos y esquizontes (26). Una pequeña porción de merozoítos se diferencia a formas sexuales llamadas gametocitos (masculinos y femeninos), que son tomados de nuevo por el mosquito para continuar con el ciclo dentro del hospedero invertebrado (14) (Figura 2).

Dos especies, *P. vivax* y *P. ovale*, pueden presentar formas inactivas en el hígado llamadas hipnozoítos, lo que puede causar una recaída de la enfermedad, inclusive años después de la primera infección (14).

Reconocimiento de Péptidos por el Complejo Mayor de Histocompatibilidad (MHC)

Las proteínas del Complejo Mayor de Histocompatibilidad (MHC del inglés, *Major histocompatibility complex*) presentan un alto polimorfismo en los seres humanos; la capacidad de unión al antígeno varía de un alelo a otro, aumentando o disminuyendo su afinidad (28). Una vacuna efectiva debería, en principio, contener múltiples epítopes de unión con alta afinidad a los diferentes alelos frecuentes de MHC de la población diana. Las moléculas del MHC encargadas de la presentación antigénica se dividen en dos grandes grupos: las de Clase I, encargadas de presentar antígenos de origen intracelular y capaces de acoplar péptidos de 8 a 9 aminoácidos de longitud, y las de clase II, que reconocen antígenos de origen extracelular y son capaces de exhibir péptidos de 13 a 18 aminoácidos de longitud (29).

Los genes que codifican las proteínas del MHC clase II en humanos se localizan en el cromosoma 6 y se conocen como el sistema HLA (del inglés, *Human Leukocyte Antigen*). Las proteínas HLA-DP, HLA-DQ y HLA-DR se componen de una subunidad alfa y una beta, un dominio tipo inmunoglobulina y una porción transmembranal corta. La subunidad beta se caracteriza por ser muy polimórfica, mientras la subunidad alfa es monomórfica en humanos (30).

El sitio de unión al péptido está conformado por dos regiones alfa helicales más o menos paralelas, encima de hojas beta. Los péptidos se unen en el surco formado por las hélices y las hojas beta, con sus residuos terminales extendidos (Figura 3). Los péptidos adoptan una conformación poli-prolina tipo II extendida, que expone el esqueleto del péptido a los residuos conservados del MHC con los que establecen puentes de hidrógeno tanto con el esqueleto como con las cadenas laterales. Las cadenas laterales del péptido se unen a los bolsillos del MHC en las posiciones 1, 4, 6 y 9 (a veces 7 también) y aquellas orientadas hacia arriba, hacen contacto con el receptor de los linfocitos T (TCR del inglés, *T Cell Receptor*) (28, 31).

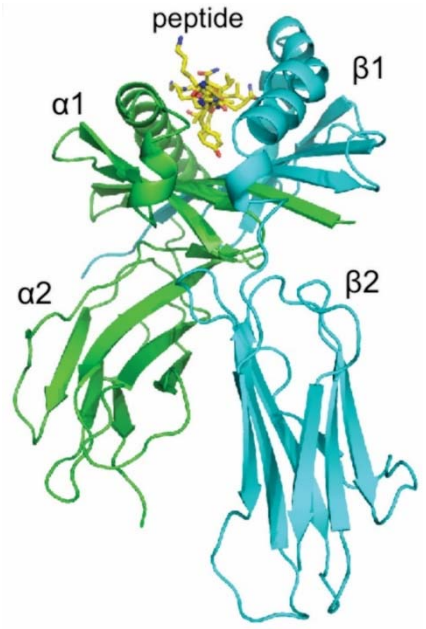


Figura 3. Estructura del complejo péptido-MHC clase II.
Estructura cristalizada del dominio extracelular donde se une el péptido, con las subunidades alfa (verde) y beta (azul); y el dominio tipo inmunoglobulina. Tomado de Stern y Calvo, 2009 (28).

Las moléculas HLA clase II, son expresadas constitutivamente en la superficie de las células presentadoras de antígeno, como las células dendríticas, macrófagos y linfocitos B. Éstas se encargan de reconocer los péptidos de origen extracelular procesados por la vía endosomal/lisosomal, los cuales son editados por la molécula HLA-DM. La presentación antigénica en el contexto del HLA clase II se realiza al TCR de los linfocitos T CD4+ o T ayudadores (28).

Debido al polimorfismo que presentan las moléculas de clase II, es posible generar una respuesta inmune amplia y variada frente a los patógenos. En el caso de la malaria, algunas variantes alélicas se han correlacionado con resistencia o susceptibilidad a la enfermedad. Por ejemplo, la familia alélica HLA-DRβ1*16 se ha asociado con la respuesta de anticuerpos a las regiones repetitivas de CSP (Proteína de Circumsporozoito) variante VK247 en poblaciones expuestas en Brasil, mientras la familia alélica HLA-DRβ1*07 se asocia con la ausencia de anticuerpos a las repeticiones de CSP variante VK210 (32).

Por otro lado, se encontró una asociación entre la familia alélica HLA-DRβ1*03 con altos niveles de anticuerpos contra un fragmento de la proteína MSP-1, mientras que no existe asociación entre el linaje alélico HLA-DRβ1 y la producción de anticuerpos para CSP, AMA-1 y DBP (33). Lima-Junior *y col*., evaluaron la respuesta de anticuerpos IgG a las proteínas MSP-1, MSP-3α y MSP-9 de *P. vivax* y demostraron la relación entre los individuos con la familia HLA-DRβ1*04 y respuesta alta de anticuerpos a $PvMSP3_{CT}$ y $PvMSP3_{NT}$; y de los individuos con la familia HLA-DQβ1*03 con una respuesta frente a $PvMSP3_{CT}$ (34). Para las regiones repetitivas de PvMSP-9 y la región NT, asociaron positivamente la respuesta de IgG con los individuos HLA-DRβ1*04 y DQβ1*03. Esta respuesta de altos niveles de anticuerpos, la asocian con una posible presión selectiva por parte de *P. vivax* en la población amerindia (34).

PREGUNTAS DE INVESTIGACIÓN

¿Los epítopes T de las proteínas PvRON2 y Pv12 de *P. vivax* seleccionados con herramientas bioinformáticas y por ensayos de unión *in vitro* se unen eficientemente a moléculas HLA-DRβ1 y estimulan *in vitro* una respuesta celular de memoria en individuos expuestos a la infección natural?

¿Pueden los epítopes B de las proteínas PvRON2 y Pv12 de *P. vivax* seleccionados por predicción *in silico* ser reconocidos por componentes del sistema inmune humoral de individuos expuestos a la infección natural?

¿Las modificaciones de epítopes seleccionados con herramientas bioinformáticas de las proteínas PvMSP1 y PvDBP de *P. vivax* inducen una expansión y/o activación *in vitro* de linfocitos T CD4+ de individuos no expuestos?

Objetivos

Objetivo General

Evaluar la antigenicidad *in vitro* de péptidos derivados de las proteínas PvRON2 y Pv12, y la inmunogenicidad *in vitro* de péptidos derivados de las proteínas PvDBP y PvMSP1 de *Plasmodium vivax*, para la selección de candidatos a vacuna contra la malaria.

Objetivos Específicos

- Determinar si epítopes T derivados de las proteínas PvRON2 y Pv12 de *P. vivax* seleccionados *in silico* y por su unión *in vitro* a moléculas HLA-DRβ1*, son capaces de generar una respuesta celular *in vitro* por parte de los linfocitos T CD4+ de individuos expuestos a la infección natural.

- Examinar la capacidad de epítopes B de las proteínas PvRON2 y Pv12 de *P. vivax*, de ser reconocidos *in vitro* por componentes de la respuesta inmune humoral de individuos expuestos a la infección natural.

- Comparar la inmunogenicidad *in vitro* entre péptidos nativos y modificados de las proteínas PvDBP y PvMSP1 de *P. vivax* en linfocitos T CD4+ de individuos no expuestos.

Capítulo 1

Antigenicidad *in vitro* de epítopes T de las proteínas PvRON2 y Pv12 de *P. vivax* por su perfil de unión a moléculas HLA-DRβ1*

MARCO TEÓRICO

Inmunidad Adquirida Naturalmente a Malaria

Las moléculas del sistema inmune innato y adaptativo están involucradas, tanto en la patogénesis, como en el control de la enfermedad. La inmunidad clínica a la malaria se puede adquirir durante tres fases: primero, la inmunidad a la enfermedad; segundo, la inmunidad a la infección sintomática; y en tercer lugar, la inmunidad parcial a la parasitemia (35). Durante la infección con *Plasmodium* se desencadena como primera línea de defensa la respuesta inmune innata mediada por células dendríticas (DC), células natural-killer (NK) y macrófagos, junto a la producción de citoquinas, seguida inmediatamente por la respuesta inmune adaptativa, la cual incluye células T, células B y anticuerpos. Durante la picadura, el mosquito inocula esporozoítos en la piel del hospedero, los cuales pueden permanecer en la piel hasta por 6 horas después de la inoculación (36).

Las células dendríticas son las encargadas de la presentación de antígenos a los linfocitos T (LT). Estas células reconocen los antígenos a través de receptores de reconocimiento de patrones (PRR del inglés, *Pattern Recognition Receptor*) los cuáles, reconocen patrones moleculares asociados a patógenos (PAMPs del inglés, *Pathogen-Associated Molecular Patterns*) presentes en el parásito. Este reconocimiento favorece la maduración (incremento de las moléculas de MHC clase II, señales de coestimulación como CD80 y CD86) y migración (incremento en la expresión de CCR7) de las DC hacia los ganglios linfáticos más cercanos para presentar dichos antígenos a los LT (37, 38).

Dentro de las funciones descritas para estas células, se encuentra la activación de linfocitos T y B, la tolerancia inmune, la activación de las células NK y la activación de los macrófagos (39). Las células dendríticas derivadas de la piel (CD103+) tienen un papel importante en la presentación cruzada de antígenos a las células T CD8+; en el caso de malaria, una tercera parte de los esporozoítos son llevados a los nódulos linfáticos regionales, donde son internalizados por las células dendríticas (40).

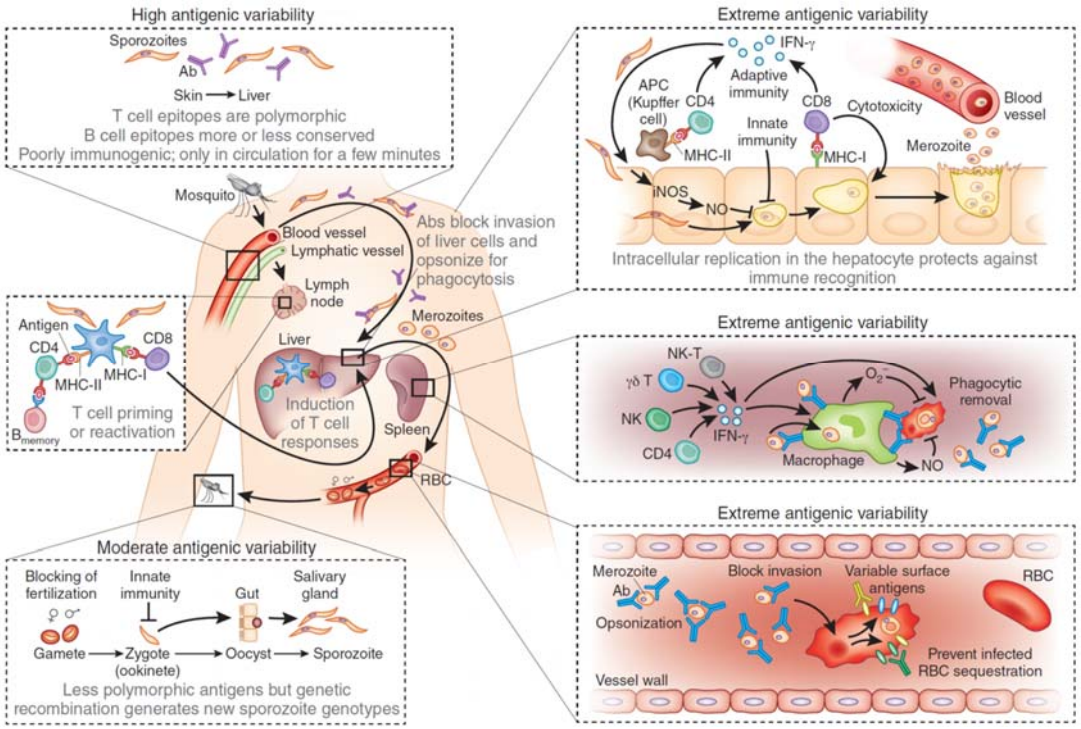


Figura 4. Respuesta inmune para el control de *Plasmodium* sp.
Los esporozoítos son drenados a los ganglios linfáticos o hacia el hígado donde priman a los linfocitos T y B. Tanto los esporozoítos como los merozoítos pueden ser captados por los anticuerpos. Linfocitos T productores de IFN-ɣ pueden activar los macrófagos para que fagociten los GR parasitados. Tomado de Riley & Stewart, 2013 (41).

Durante el estadio eritrocítico, la respuesta inmune generada es protagonizada principalmente por anticuerpos. Mientras que durante el estadio hepático, la respuesta es mediada primordialmente por células (42). Durante el estadio hepático, se da inicio a la presentación de antígenos en la superficie de los hepatocitos, en el contexto de las moléculas del Complejo Mayor de Histocompatibilidad clase I, expresadas en todas las células nucleadas, o por las de clase II presentes en las células presentadoras de antígeno profesionales, los cuales van a ser reconocidos por las células T CD8+ y CD4+, respectivamente (28). Las células T CD4+, las células B y las células NK, tienen un papel importante en la respuesta inmune generada por el parásito durante el estadio eritrocítico, ya que la inmunidad dependerá de la producción y el tiempo de vida de las células B de memoria, después de la infección (Figura 4) (41).

Respuesta celular a malaria

Durante la infección con *P. vivax,* algunos individuos pueden adquirir inmunidad naturalmente. Esta inmunidad está compuesta por la respuesta inmune celular mediada por la producción de citoquinas, de receptores de citoquinas y de enzimas proteolíticas que hacen parte de la respuesta del hospedero frente a la infección, además de anticuerpos tipo IgG. En un área endémica de Colombia pacientes con parasitemias moderadas presentaron niveles altos de IFN-γ y TNF-α, un perfil de citoquinas pro-inflamatorias que se correlaciona con la respuesta encontrada en una región de transmisión inestable; y un balance en la tasa de IL-10/TNF-α, lo cual puede prevenir el aumento de la parasitemia y la patología en el hospedero (43).

En relación con la parasitemia, la respuesta de TNF-α es más fuerte en la infección con *P. vivax* o *P. ovale* que con *P. falciparum*. El incremento de estos factores en la infección con *P. vivax* ayuda a controlar la parasitemia, mientras que en la infección con *P. falciparum* conlleva a complicaciones descritas como la malaria grave mediada por la respuesta del hospedero (44). En otro estudio, cuando se compara la respuesta inmune de pacientes con malaria complicada y no complicada causada por *P. vivax*, la tasa IFN-γ/IL-10 fue más alta en pacientes con enfermedad complicada al igual que los niveles de TNF-α, lo que lleva a concluir que la severidad de la enfermedad por *P. vivax* se correlaciona con la activación de la respuesta inmune proinflamatoria y un desbalance de citoquinas (45).

En la infección con *Plasmodium*, la IL-10 actúa como inmunoregulador mediante el control de los efectos de otras citoquinas producidas por células CD4+ Th1 y CD8+. La sobreproducción de citoquinas como IFN-γ por parte de estas células, ayuda a aumentar la fagocitosis para la eliminación del parásito, pero a la vez produce efectos inmunopatológicos asociados a la enfermedad. Sin embargo, en otro grupo de pacientes con malaria por *P. vivax*, se encontraron niveles altos tanto de IFN-γ como de IL-10 en pacientes con episodios previos. Además, estudios de polimorfismo realizados en el promotor del gen de IL-10 en poblaciones de áreas endémicas, demostraron que los polimorfismos no influyen en la producción de esta citoquina y en su función regulatoria en la respuesta inmune (46, 47).

Para probar la hipótesis de que la infección con *P. vivax* genera una mayor respuesta de citoquinas proinflamatorias que la infección por otras especies de *Plasmodium*, Goncalves y *col*. evaluaron el patrón de citoquinas en infecciones sintomáticas no complicadas por *P. vivax* y *P. falciparum* en un área de baja transmisión de malaria en Brasil. Ellos encontraron una respuesta mayor de citoquinas antinflamatorias pero similar respuesta de citoquinas proinflamatorias que en la infección por *P. falciparum*. La respuesta de citoquinas antinflamatorias como IL-10 y las tasas IL-10/TNF-α, IL-10/IFN-γ e IL-10/IL-6 en malaria clínica por *P. vivax* fue de vida corta, y se correlacionó positivamente con la parasitemia más no con los síntomas, con lo cual, es necesario un balance entre la respuesta de citoquinas inflamatorias y reguladoras (Figura 4) (48).

Proteínas involucradas en el proceso de invasión al glóbulo rojo

A la fecha, se han descrito más de 50 proteínas implicadas en el proceso de invasión de los parásitos de la malaria a los glóbulos rojos; la mayoría se han identificado a nivel molecular y se han caracterizado inmunológicamente en *P. falciparum* (49, 50). Por otra parte, el estudio de las proteínas de *P. vivax* involucradas en los procesos de invasión, se ha visto retrasado debido a la falta de un cultivo continuo *in vitro* del parásito, lo que ha limitado el estudio de la biología del parásito y la identificación de nuevos antígenos junto a su evaluación *in vitro* (51, 52) (Figura 5).

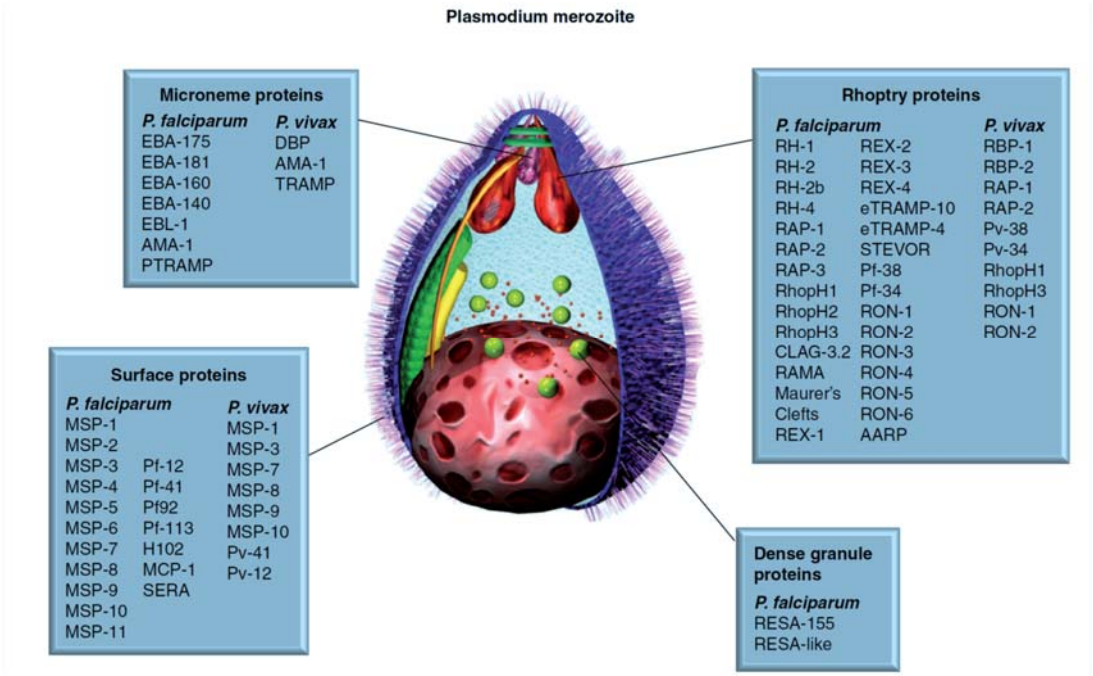


Figura 5. Proteínas descritas para *P. falciparum* y *P. vivax* con su localización.
Proteínas identificadas en merozoíto y su localización en superficie, roptrias, micronemas y gránulos densos. Tomado de Patarroyo MA, 2012 (53).

Proteína del cuello de las roptrias 2 (PvRON2)

La proteína *Pv*RON2 es altamente conservada, secretada por organelos especializados y hace parte del complejo de las proteínas llamadas RONs (del inglés, *Rhoptry Neck*). Esta proteína, como sus ortólogos en *T. gondii* (TgRON2) y *P. falciparum* (PfRON2), interactúa directamente con la proteína AMA-1. El complejo de proteínas RON participa en la formación de la unión estrecha (estructura electrodensa en forma de anillo), que permite la entrada del parásito dentro de la célula diana (54, 55).

La proteína PvRON2 se expresa en las roptrias de esquizontes tardíos y está compuesta por 2.204 aminoácidos (56). PvRON2 es un homólogo de PfRON2; esta última tiene una interacción específica receptor-ligando con la proteína AMA-1, desempeñando un papel crucial durante la invasión de los merozoítos (Mrz) a los eritrocitos, la unión estrecha y posterior formación de la vacuola parasitófora (57-59). La localización de PvRON2 en esquizontes tardíos es similar a la de PfRON2, lo que sugiere que PvRON2 tiene una función homóloga a la de *P. falciparum* (56).

Las proteínas RON son insertadas en la membrana del eritrocito actuando como proteínas de unión a la membrana. En *P. falciparum*, esta unión puede ser bloqueada por anticuerpos anti-AMA-1 y los anticuerpos anti-RON2 pueden bloquear la invasión a los eritrocitos, lo que hace pensar que esta proteína podría también ser uno de los componentes importantes en el diseño de una vacuna frente a *P. vivax* (55, 58, 60-63).

Proteína de superficie 12 (Pv12)

La proteína Pv12 está compuesta de 362 aminoácidos, con una masa molecular de ~41 kDa, posee un péptido señal (PS) en la región N-terminal, un anclaje glicofosfatidilinositol (GPI) en el dominio transmembranal C-terminal y dos dominios de 6 cisteínas (6-Cys) (64, 65). Inicialmente, se caracterizó como una proteína de las roptrias (64), sin embargo, estudios posteriores confirmaron su localización en la superficie de los Mrz al igual que su homóloga en *P. falciparum* (Pf12) (65, 66).

En ensayos con arreglos de proteínas, Pv12 fue reconocida por el 49% de los sueros de individuos con infección activa por *P. vivax*; otro estudio con ensayos de ELISA mostró que el 84,8% de sueros de pacientes con la infección activa tenían respuesta de anticuerpos frente a la proteína (65, 67).

Se ha reportado que varios miembros de la familia de 6-Cys desempeñan un papel como ligando de unión a otras proteínas, lo que sugiere que Pv12 tiene un rol importante en la interacción con otros receptores celulares (65). Estudios genéticos reportan que el gen *Pv*12 es altamente conservado entre las especies de *Plasmodium* y tiene baja diversidad genética entre los aislados, siendo uno de los antígenos más conservados caracterizados hasta fecha. Por lo anterior, Pv12 es un candidato promisorio a vacuna contra este parásito (68, 69).

METODOLOGÍA

El grupo de Biología Molecular-Malaria de la FIDIC ha logrado caracterizar 14 nuevas proteínas de *P. vivax*. Sin embargo, hasta la fecha no se ha realizado un mapeo fino que determine regiones de alta unión a moléculas HLA clase II y la capacidad de estas regiones para ser antigénicas y/o inmunogénicas. En la primera fase de este proyecto se evaluó la antigenicidad *in vitro* de péptidos derivados de las proteínas PvRON2 y Pv12 de *P. vivax*. Primero se seleccionaron epítopes T derivados de estas proteínas, basados en predicciones *in silico* y en ensayos de unión *in vitro* a moléculas HLA-DR, para ser utilizados en estudios de antigenicidad (linfoproliferación) en individuos de dos áreas endémicas en Colombia. De esta manera, se pudo conocer si los péptidos seleccionados por su alta unión a moléculas DR fueron reconocidos por componentes celulares del sistema inmune, de individuos expuestos a la infección natural por *P. vivax*.

Selección de péptidos de las proteínas PvRON2 y Pv12 de *P. vivax*

Predicción *in silico* y síntesis química de epítopes de células T de alta unión a moléculas HLA-DRβ1*

Las secuencias de las proteínas PvRON2 (código de acceso PlasmoDB: PVX_117880) y Pv12 (código de acceso PlasmoDB: PVX_113775), se emplearon para la selección *in silico* de los epítopes T. Para esto, se utilizaron herramientas bioinformáticas para seleccionar los epítopes T con alta afinidad de unión para las familias alélicas HLA-DRβ1*04, *07, *11 y *13 que son las más frecuentes en las poblaciones humanas ubicadas en las áreas endémicas para malaria por *P. vivax* (70). Para esto, se empleó la herramienta NetMHCIIpan-3.1 (71) y se confirmaron con los programas IEDB (72-74) y TEPITOPE (75). Los péptidos seleccionados *in silico* y los péptidos controles biotinilados para los ensayos de unión, fueron sintetizados por el laboratorio 21st Century Biochemicals (Marlboro, MA).

Determinación de perfiles de unión de los péptidos a las moléculas HLA-DRβ1 para la selección de epítopes de células T

Purificación del anticuerpo monoclonal L243 (anti HLA-DR)

El anticuerpo monoclonal anti HLA-DR fue obtenido del sobrenadante (SBN) de cultivo del hibridoma L-243 (ATCC HB-55). Inicialmente, las inmunoglobulinas del SBN se precipitaron por saturación con sulfato de amonio al 45% y el anticuerpo anti-DR fue purificado mediante cromatografía de afinidad con la resina Protein A-Sepharose CL-4B beads (GE Healthcare) en relación 1:2 (v/v) muestra:resina. La pureza de las inmunoglobulinas se determinó mediante electroforesis en gel de poliacrilamida (SDS-PAGE) en condiciones denaturantes y se cuantificaron por el método de ácido bicinconínico con el kit Micro BCA protein assay kit (Thermo Scientific). Finalmente, las fracciones se almacenaron con azida de sodio al 0,02 % (v/v) en alícuotas a -20°C hasta su uso.

Purificación de las moléculas HLA-DRβ1*

Cuatro líneas celulares linfoblastoides B homocigotas fueron suministradas por el International Histocompatibility Working Group (IHWG, Seattle, WA). Cada línea expresa un alelo de la familia HLA-DRβ1* de interés para este estudio: IHW09025 alelo HLA-DRβ1*04:01, IHW09051 alelo HLA-DRβ1*07:01, IHW09043 alelo HLA-DRβ1*11:01 y IHW09055 alelo HLA-DRβ1*13:02. Las células fueron cultivadas hasta obtener $5x10^9$ células para cada línea.

La purificación de las moléculas se realizó por cromatografía de afinidad con la resina Protein A-Sepharose CL-4B (GE Healthcare), la cual tenía acoplado el anticuerpo monoclonal L-243, de acuerdo con lo descrito previamente por Vargas *y col*. (76). La pureza de las moléculas HLA-DRβ1* obtenidas se confirmó por SDS-PAGE nativo (12%) y Western-blot. La concentración fue determinada con el kit Micro BCA protein assay kit (Thermo Scientific) y fueron almacenadas en alícuotas a -80°C hasta su uso.

Ensayos de unión *in vitro* de péptidos sintéticos de las proteínas PvRON2 y Pv12 de *P. vivax* a moléculas HLA-DRβ1*

Para determinar los péptidos de alta afinidad de unión a las moléculas HLA-DRβ1*, se llevaron a cabo ensayos de unión *in vitro* según lo reportado por Vargas *y col*. (76) y Saravia *y col*. (77). Los péptidos seleccionados compiten con péptidos control marcados con biotina reportados como péptidos de alta afinidad de unión a los alelos de interés. Los péptidos empleados como control fueron: Hemaglutinina $HA_{306–318}$ (PKYVKQNTLKLAT) para HLA-DRβ1*04 y HLA-DRβ1*11 (77, 78) y Toxoide Tetánico (TT) (QYIKANSKFIGITE) para HLA-DRβ1*07 y HLA-DRβ1*13 (79).

La molécula HLA-DRβ1* (0,1 µM) se incubó durante 24h con 5 µM de los péptidos biotinados y un exceso de 50 veces el péptido problema (250 µM). Previamente, fueron cubiertos durante 24 horas inmunomódulos Maxisorb NUNC (Thermo Scientific) con el anticuerpo L-243, posteriormente los inmunomódulos fueron lavados y bloqueados. Después, se incubaron con la mezcla anterior durante 2 h, se realizaron los respectivos lavados, se incubaron con Estreptavidina conjugada a fosfatasa alcalina (Vector Labs) y se revelaron con el substrato líquido fosfatasa alcalina amarillo (pNPP) (Sigma-Aldrich). La reacción fue leída a una densidad óptica (DO) de 405 nm con el espectrofotómetro Multiskan GO (Thermo Scientific). El porcentaje de unión del péptido de interés fue calculado con la siguiente fórmula:

$$100 * \left[1 - \left(\frac{\Delta DO\ en\ presencia\ del\ competidor}{\Delta DO\ en\ ausencia\ del\ competidor}\right)\right]$$

Los valores de IC50 fueron calculados sólo para los péptidos capaces de inhibir la unión en más de un 50% del péptido control a un alelo HLA-DRβ1*. El valor de IC50 fue considerado como la concentración del péptido competidor requerida para inhibir el 50% de la unión del péptido control biotinado (77). Para los ensayos de IC50, se evaluaron los péptidos de interés en concentraciones desde 4,2 µM a 250 µM; los valores de IC50 se calcularon como valores relativos con la siguiente fórmula:

$$1 - \left[\frac{(\Delta DO \text{ en presencia del competidor})}{(\Delta DO \text{ en ausencia del competidor})}\right]$$

El valor de IC50 se calculó empleando la fórmula de decaimiento exponencial de dos fases con el software Mathematica v 10.1 (Wolfram Research, Inc., Mathematica, Champaign, IL 2015).

Conformación de los grupos experimentales

Los alelos seleccionados para el estudio cubren aproximadamente el 60% de la población amerindia y el 40% de la población oriental (70), por lo tanto, según los resultados de la tipificación para clase II, se conformaron cuatro grupos experimentales acorde a cada alelo, con máximo 8 individuos para cada uno y el grupo control con 8 individuos pertenecientes a un área no endémica, con los alelos de interés.

Selección de individuos expuestos a la infección natural por *P. vivax*

Se obtuvo sangre periférica (SP) de individuos que vivían en los departamentos de Chocó y Córdoba (áreas endémicas con alta incidencia para malaria por *P.* vivax en Colombia), quienes habían sufrido episodios previos de malaria por *P. vivax*. Para la selección, se tuvo en cuenta los siguientes criterios de inclusión: 1) Mayor de 18 años, 2) Residente de áreas endémicas para *P. vivax*, que hubiera sufrido 1 o más episodios de malaria (el último mínimo hace seis meses) y 3) Que hubiera recibido el tratamiento adecuado para la enfermedad.

Además, se obtuvo muestras de SP de los individuos para conformar el grupo control (provenientes de área no endémica), que cumplieran con los siguientes criterios: 1) Mayor de 18 años, 2) Nunca haber vivido en áreas endémicas para malaria y 3) Nunca haber padecido la enfermedad. Este estudio se llevó a cabo de acuerdo con el marco legal para la investigación en Colombia y la resolución 8430 de 1993 del Ministerio de Salud. Para la toma de muestras de SP, todos los individuos tuvieron el menor riesgo y los datos se manejaron confidencialmente. Las muestras fueron colectadas después que los sujetos firmaran un consentimiento informado.

Toma de muestras

Para la tipificación de los alelos HLA-DRβ1*, se realizó una toma de muestra de 5 mL de SP en un tubo con anticoagulante EDTA (BD Vacutainer Oakville, ON). Para los

ensayos de linfoproliferación, a los individuos que conformaron los grupos experimentales, se les realizó una segunda toma de muestra de SP de 6 mL (en tubos sin anticoagulante con el fin de colectar suero) y de 40 mL (en tubos con citrato fosfato dextrosa (CPD) (BD Vacutainer Oakville, ON) para obtener células mononucleares de sangre periférica (CMSP). Las muestras fueron procesadas en condiciones de esterilidad y en el momento de la toma de muestra se realizó un examen de gota gruesa para confirmar que las muestras fueran negativas para malaria.

Tipificación del locus HLA-DRβ1*

A partir de 300 µL de las muestras de SP, se extrajo el ADN genómico (ADNg) con el kit comercial Wizard Genomic DNA Purification Kit (Promega Corporation), siguiendo las instrucciones del fabricante. El ADNg fue empleado para la tipificación del locus HLA-DRβ1* por alta resolución en Histogenetics (Ossining, NY, USA), a través de la tecnología Next Generation Sequencing (NGS), usando Illumina MiSeq.

Ensayos de antigenicidad con epítopes de células T

Aislamiento de células mononucleares de sangre periférica (CMSP)

Para aislar las CMSP se realizó un gradiente de densidad usando Ficoll-Paque PLUS (GE Healthcare). Brevemente, las muestras se centrifugaron a 2000 r.p.m. durante 5 min., para separar el plasma y el paquete celular. La capa leucocitaria fue diluida con medio RPMI 1640 y se realizó el gradiente de separación conservando una proporción 1:2 ficoll con muestra diluida. Las muestras se centrifugaron a 2000 r.p.m. durante 30 min. a temperatura ambiente. Las células mononucleares fueron recuperadas de la interfase del Ficoll-Paque/medio, y se lavaron dos veces con medio RPMI a 800 x g durante 10 min. La viabilidad celular fue evaluada con el colorante de exclusión azul de tripán y el conteo se realizó en cámara de Neubauer.

Estimulación *in vitro* de CMSP con epítopes de células T seleccionados

Los ensayos se llevaron a cabo cultivando en placas de 96 pozos fondo redondo (Costar, Corning Incorporated) $2x10^5$ CMSP en 200 µL de medio RPMI-1640

suplementado con glutamina 2 mM, piruvato de sodio 1 mM, $NaHCO_3$ 2 g/L, antibiótico-antimicótico 1X (Gibco) y 10% de plasma autólogo inactivado por calor. Las células se estimularon con los péptidos seleccionados por los ensayos de unión, a una concentración de 10 µg/mL; el mitógeno fitohemaglutinina (PHA) 2% (Sigma) y el lisado de *P. vivax* (5 µg/mL) fueron usados como controles positivos. Las células sin estímulo fueron consideradas como control del ensayo y un péptido de baja afinidad de unión seleccionado en los ensayos de unión, como control negativo. Las células del ensayo se marcaron con CFSE 5µM (carboxyfluorescein diacetate N-succinimidyl ester) para evaluar la actividad linfoproliferativa y se incubaron a 37°C en una atmósfera con 5% de CO_2 durante 5 días, los ensayos se realizaron por duplicado.

Ensayo de linfoproliferación con CFSE

La proliferación celular fue evaluada por citometría de flujo mediante el uso de CFSE (CellTrace CFSE cell proliferation kit, Molecular Probes). El porcentaje de proliferación de las células T CD4+ fue medido por la dilución de CFSE. El índice de estimulación (IE) se obtiene por la pérdida relativa de intensidad de fluorescencia, usando la siguiente fórmula:

$$\frac{\%\text{ de intensidad de fluorescencia de células con CFSE co-cultivadas en presencia del antígeno}}{\%\text{ de intensidad de fluorescencia de células con CFSE co-cultivadas en ausencia del antígeno}}$$

Los datos fueron promediados para cada antígeno en ambos grupos, expuestos y controles. Para evaluar las células marcadas con CFSE, se utilizó el anticuerpo Pacific Blue-labeled mouse anti-human CD4 (RPA-T4 clone), luego las muestran se leyeron en el citómetro de flujo FACS Canto II y los datos se analizaron con el software FlowJo v7.6.5 (Ashland, Oregon, USA).

Determinación de citoquinas en sobrenadante (SBN)

Al finalizar el tiempo de cultivo, se colectaron 100 µL de SBN del cultivo de los linfocitos estimulados y sin estimular. Los sobrenadantes fueron almacenados a -80°C hasta su análisis. Los niveles de citoquinas se determinaron con el kit comercial BD CBA Human Th1/Th2 Cytokine Kit II (San Jose, CA, USA), el cuál cuantifica IL-2, IL-4, IL-6, IL-10, TNF e IFN-ɣ siguiendo las instrucciones del fabricante. El ensayo se leyó en el

citómetro de flujo FACS Canto II y el análisis de los datos se realizó con el software FCAP Array v3.0.1. Los valores obtenidos fueron comparados entre las CMSP no estimuladas y las estimuladas, la producción de las citoquinas se expresó en pg/mL. Como punto de corte para la producción específica de antígeno, se tomó el valor de la media del grupo control más dos desviaciones estándar.

RESULTADOS

Ensayos de antigenicidad de las proteínas PvRON2 y Pv12 de P. *vivax*

Selección de péptidos de las proteínas PvRON2 y Pv12 de *P. vivax*

Se seleccionaron *in silico* once epítopes T para PvRON2 y diez epítopes T para Pv12 de acuerdo con su afinidad de unión predicha a los alelos HLA-DRβ1*04:01, *07:01, *11:01 y *13:02, para un total de 21 epítopes (Tabla 1). Estos epítopes fueron sintetizados y evaluados mediante ensayos de unión *in vitro*, con el fin de conocer su capacidad de unión a los alelos de interés. Se determinaron los porcentajes de unión para los 21 péptidos (Figura 6) y aquellos que presentaban un porcentaje mayor al 50%, fueron elegidos péptidos de alta unión, a los cuáles se les calculó los valores de IC50 (Figura 7).

Tabla 1. Epítopes T seleccionados *in silico* y ensayos de unión *in vitro* para PvRON2.

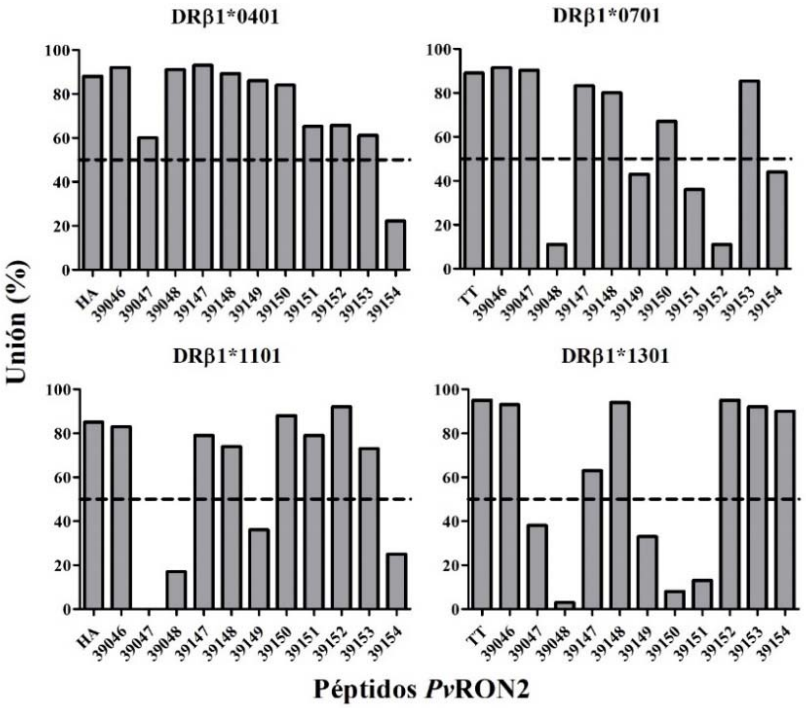
Código del péptido	Secuencia	CoreF	Alelo HLA-DRβ1*	NetMHCII pan 3.1 (%Rank)	Porcentaje de unión*	IC50 µM*	IC50 ratio
39147	LKPFYSLETMLMANS	FYSLETMLM	DRβ1*04:01	0,3	92,6	4,6	0,2
			DRβ1*07:01	2,5	83,2	26,0	1,1
			DRβ1*11:01	10,0	79,3	23,0	4,8
			DRβ1*13:02	34,0	63,0	79,0	10,6
39148	NVRKFFLNDVSSIRH	FFLNDVSSI	DRβ1*04:01	1,0	83,0	11,9	0,6
			DRβ1*07:01	5,0	79,9	39,7	1,7
			DRβ1*11:01	19,0	73,8	83,4	17,5
			DRβ1*13:02	1,4	94,2	7,4	1,0
39149	DKSFISEANSFRNEE	FISEANSFR	DRβ1*04:01	3,5	85,8	26,3	1,4
			DRβ1*07:01	17,0	42,8	ND	ND
			DRβ1*11:01	26,0	36,5	ND	ND
			DRβ1*13:02	24,0	33,5	ND	ND
39150	QTAFRKFFKKIISLG	FFKKIISLG	DRβ1*04:01	17,0	83,6	33,7	1,7
		FRKFFKKII	DRβ1*07:01	6,5	67,2	51,8	2,2
			DRβ1*11:01	1,2	87,6	83,4	17,5
			DRβ1*13:02	48,0	7,7	ND	ND
39151	KLKYIFKRRKTMKKK	FKRRKTMKK	DRβ1*04:01	37,0	65,3	40,0	2,1
			DRβ1*07:01	6,0	36,0	ND	ND
		YIFKRRKTM	DRβ1*11:01	0,1	78,5	51,1	10,7
			DRβ1*13:02	27,0	13,1	ND	ND

39152	LFYVNLFIMSSLSRK	LFIMSSLSR	DRβ1*04:01	3,0	65,8	2,8	0,1
		FIMSSLSRK	DRβ1*07:01	2,0	11,0	ND	ND
			DRβ1*11:01	1,4	91,8	1,9	0,4
			DRβ1*13:02	27,0	94,7	8,0	1,1
39153	MKLLQHIPANLLENI	LLQHIPANLL	DRβ1*04:01	0,5	61,2	57,0	2,9
			DRβ1*07:01	0,1	85,4	7,5	0,3
			DRβ1*11:01	6,5	72,7	52,1	10,9
			DRβ1*13:02	0,1	91,9	10,7	1,4
39154	LKFIVRGNNLKFLNN	IVRGNNLKF	DRβ1*04:01	11,0	22,2	ND	ND
			DRβ1*07:01	4,5	43,7	ND	ND
		FIVRGNNLK	DRβ1*11:01	3,0	24,9	ND	ND
		IVRGNNLKF	DRβ1*13:02	0,2	90,1	6,5	0,9
39046	NYEIYIASSSNIYLM	YIASSSNIY	DRβ1*04:01	0,8	91,8	28,4	1,5
			DRβ1*07:01	0,3	91,5	23,3	1,0
			DRβ1*11:01	13,0	82,9	120,0	25,2
		IYIASSSNI	DRβ1*13:02	0,2	92,8	29,6	4,0
39047	RGPVNYHFSNYMNLD	VNYHFSNYM	DRβ1*04:01	16,0	59,8	54,5	2,8
		YHFSNYMNL	DRβ1*07:01	10,0	90,4	6,0	0,3
			DRβ1*11:01	37,0	0,0	ND	ND
		VNYHFSNYM	DRβ1*1302	13,0	37,8	ND	ND
39048	TPIIVKYDNTHAKNR	IIVKYDNTHA	DRβ1*04:01	12,0	90,7	11,9	0,6
			DRβ1*07:01	41,0	10,5	ND	ND
			DRβ1*11:01	24,0	16,8	ND	ND
			DRβ1*13:02	8,5	3,3	ND	ND

Se muestran el código del péptido y la secuencia; el coreF y el % Rank predicho para los alelos HLA-DRβ1*, de acuerdo con el programa NetMHCIIpan-3.1. Se evalúo el IC50 a los péptidos con un porcentaje de unión ≥50%. IC50 ratio=IC50 del péptido/IC50 péptido control, una razón ≤10 se empleó para determinar péptidos de buena unión. ND: no determinado; significa que un péptido con porcentaje de unión <50% no se le evaluó el IC50. *Datos de este estudio; valores de IC50 fueron calculados para cada péptido control con cada alelo HLA-DRβ1*. Péptidos controles Hemaglutinina (HA), Toxoide tetánico (TT); HA-HLA-DRβ1*04:01 IC50=19,44µM, TT-HLA-DRβ1*07:01 IC50=23,37µM, HA-HLA-DRβ1*11:01 IC50=4,77µM y TT-HLA-DRβ1*13:02 IC50=7,46µM.

Para PvRON2, los resultados de unión *in vitro* mostraron que 10 de los 11 péptidos se unieron al alelo HLA-DRβ1*04:01 (media de unión de 73,45%), 7 de los 11 péptidos al alelo HLA-DRβ1*11:01 (media de unión de 64,47%), 6 de los 11 péptidos al alelo HLA-DRβ1*07:01 (media de unión de 58,33%), 6 de los 11 péptidos al alelo HLA-DRβ1*13:02 (media de unión de 56,55%). Se consideraron como epítopes universales 4 de los 11 péptidos (39046, 39147, 39148 y 39153), dada su unión a los cuatro alelos estudiados. Según estos resultados, la predicción *in silico* y los ensayos de unión experimentales tuvieron una concordancia del 70,45% para los alelos evaluados Figura 6A y Tabla 1).

A) PvRON2



B) Pv12

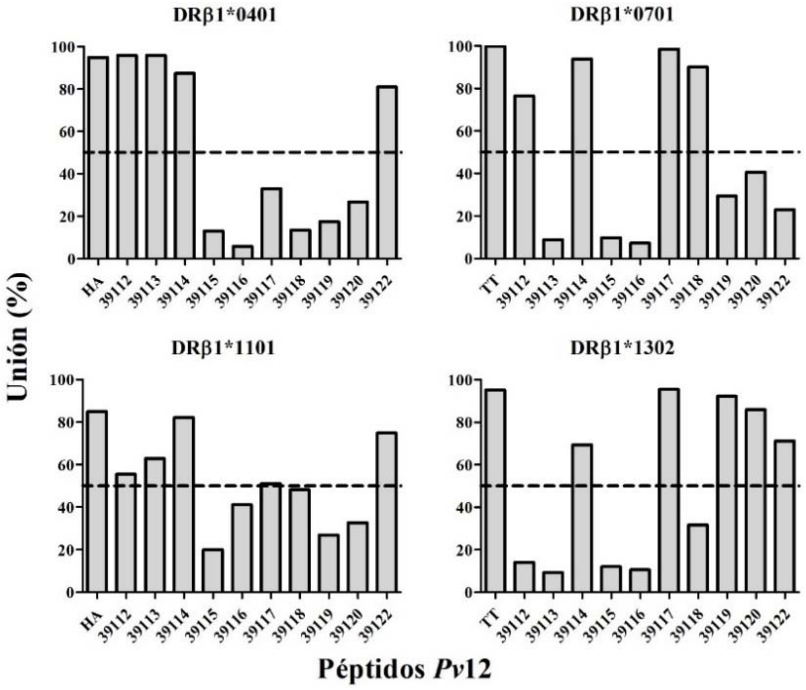


Figura 6. Ensayos de unión *in vitro* de los péptidos de las proteínas PvRON2 y Pv12 a las moléculas HLA-DRβ1*.

Se muestra el punto de corte de unión del 50% para seleccionar los péptidos de alta afinidad de unión, a los cuáles se les determinará el valor de IC50. En cada gráfica, se muestra el porcentaje de unión de cada péptido en estudio y cada péptido control a los alelos HLADRβ1*.

De acuerdo con los ensayos de IC50, el péptido 39152 presentó los mejores valores para las familias HLA-DRβ1*04 (IC50 = 0,15) y HLA-DRβ1*11 (IC50 = 0,4), por lo que fue seleccionado para ambos linajes en los ensayos de linfoproliferación. El péptido 39047 mostró un IC50 de 0,26 para HLA-DRβ1*07 y el péptido 39154 un IC50 de 0,88 para HLA-DRβ1*13, por lo que fueron seleccionados como epítopes alelo-específicos. El péptido 39153 mostró el mejor valor de IC50 para todos los linajes alélicos, por lo que fue seleccionado como epítope universal Figura 7A y Tabla 1).

Para *Pv*12, los resultados de unión *in vitro* mostraron que 4 de los 10 péptidos se unieron al alelo HLA-DRβ1*04:01 (media de unión de 46,94%), 4 de los 10 péptidos al alelo HLA-DRβ1*07:01 (media de unión de 47,81%), 5 de los 10 péptidos al alelo HLA-DRβ1*11:01 (media de unión de 49,54%) y 5 de los 10 péptidos al alelo HLA-DRβ1*13:02 (media de unión de 49,17%). Según estos resultados, la predicción *in silico* y los ensayos de unión experimentales tuvieron una concordancia del 77,5% para los alelos evaluados (Figura 6B y Tabla 2).

Tabla 2. Epítopes T seleccionados *in silico* y ensayos de unión *in vitro* para Pv12.

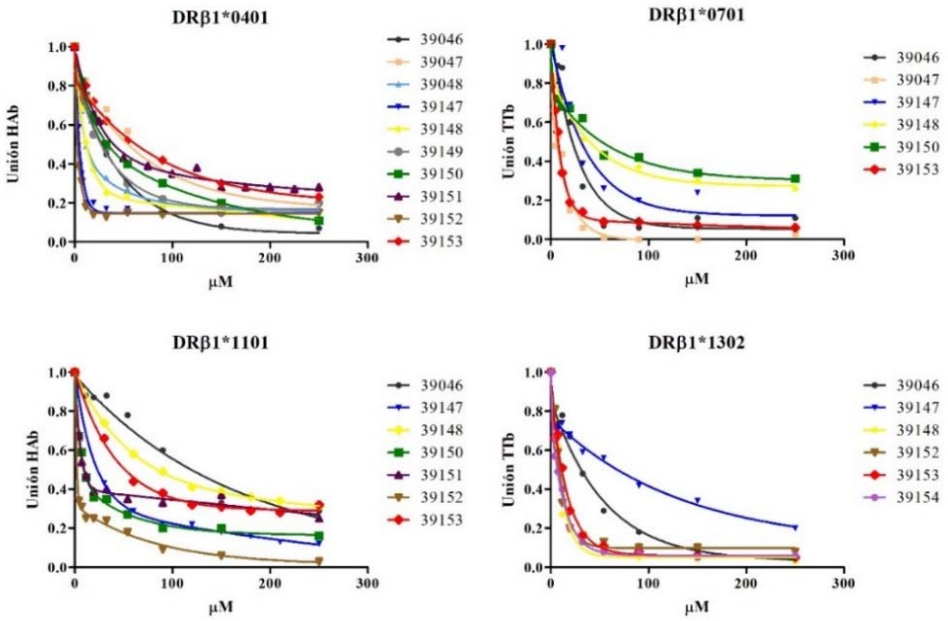
Código del péptido	Secuencia	CoreF	Alelo HLA-DRβ1*	NetMHCII pan 3.1 (%Rank)	Porcentaje de unión*	IC50 µM*	IC50 ratio
39112	RKNYELLPNNCFEQV	YELLPNNCF	DRβ1*04:01	3,5	95,78	7,26	0,37
			DRβ1*07:01	14,0	76,44	29,23	1,25
			DRβ1*11:01	35,0	55,44	250	52,41
			DRβ1*13:02	30,0	14,09	ND	ND
39113	EECFLQGFNLSGKKE	FLQGFNLSG	DRβ1*04:01	3,0	95,81	7,36	0,38
			DRβ1*07:01	24,0	8,89	ND	ND
			DRβ1*11:01	27,0	62,88	38,54	8,08
			DRβ1*13:02	55,0	9,28	ND	ND
39114	YNKIFYARVPQRIYQ	IFYARVPQR	DRβ1*04:01	8,5	87,38	43,18	2,22
		FYARVPQRI	DRβ1*07:01	0,7	93,87	43,09	1,84
		IFYARVPQR	DRβ1*11:01	0,9	82,09	8,28	1,74
		FYARVPQRI	DRβ1*13:02	8,5	69,37	73,30	9,83
39115	KSYDDVSFRVPPNL	SYDDVSFRV	DRβ1*04:01	65,0	12,98	ND	ND
			DRβ1*07:01	65,0	9,86	ND	ND
		DVSFRVPPN	DRβ1*11:01	75,0	20,02	ND	ND

		SYDDVSFRV	DRβ1*13:02	60,0	12,00	ND	ND
39116	NKAKIRVRKRSGEEY	IRVRKRSGE	DRβ1*04:01	95,0	5,76	ND	ND
		KAKIRVRKR	DRβ1*07:01	85,0	7,33	ND	ND
			DRβ1*11:01	13,0	41,12	ND	ND
		IRVRKRSGE	DRβ1*13:02	85,0	10,50	ND	ND
39117	LGIIEVLIPSLPKKI	IEVLIPSLP	DRβ1*04:01	14,0	33,08	ND	ND
		LIPSLPKKI	DRβ1*07:01	8,0	98,56	11,51	0,49
		EVLIPSLPK	DRβ1*11:01	9,5	51,13	133,77	28,04
		LIPSLPKKI	DRβ1*13:02	11,0	95,42	6,65	0,89
39118	LVEYLHGAAAIVKRK	YLHGAAAIV	DRβ1*04:01	3,0	13,48	ND	ND
			DRβ1*07:01	1,2	90,02	15,18	0,65
		LHGAAAIVK	DRβ1*11:01	11,0	48,21	ND	ND
		YLHGAAAIV	DRβ1*13:02	7,0	31,71	ND	ND
39119	GCDFTKNTSPLFTKG	FTKNTSPLF	DRβ1*04:01	6,5	17,45	ND	ND
			DRβ1*07:01	7,5	29,45	ND	ND
			DRβ1*11:01	30,0	26,94	ND	ND
			DRβ1*13:02	4,5	92,18	7,47	1,00
39120	LTDLVMDHYNKIFYA	LVMDHYNKI	DRβ1*04:01	31,0	26,73	ND	ND
			DRβ1*07:01	37,0	40,62	ND	ND
			DRβ1*11:01	36,0	32,68	ND	ND
			DRβ1*13:02	3,5	85,99	38	5,09
39122	KRLVAHFEFATTPDD	FEFATTPDD	DRβ1*04:01	40,0	80,94	58,89	3,03
		LVAHFEFAT	DRβ1*07:01	65,0	23,08	ND	ND
		VAHFEFATT	DRβ1*11:01	65,0	74,87	170	35,64
		LVAHFEFAT	DRβ1*13:02	85,0	71,13	250	33,51

Se muestran el código del péptido y la secuencia; el coreF y el % Rank predicho para los alelos HLA-DRβ1*, de acuerdo con el programa NetMHCIIpan-3.1. Se evalúo el IC50 a los péptidos con un porcentaje de unión ≥50%. IC50 ratio=IC50 del péptido/IC50 péptido control, una razón ≤10 se empleó para determinar péptidos de buena unión. ND: no determinado; significa que un péptido con porcentaje de unión <50% no se le evaluó el IC50. *Datos de este estudio; valores de IC50 fueron calculados para cada péptido control con cada alelo HLA-DRβ1*. Péptidos controles Hemaglutinina (HA), Toxoide tetánico (TT); HA-HLA-DRβ1*0401 IC50=19,44µM, TT-HLA-DRβ1*0701 IC50=23,37µM, HA-HLA-DRβ1*1101 IC50=4,77µM y TT-HLA-DRβ1*1302 IC50=7,46µM.

De acuerdo con los ensayos de IC50, el péptido 39113 presentó el mejor valor para HLA-DRβ1*04 (IC50 = 0,38) y para HLA-DRβ1*11 (IC50 = 8,08), por lo que fue seleccionado como un buen epítope para ambos alelos. El péptido 39117 mostró un IC50 de 0,49 para HLA-DRβ1*07 y de 0,89 para HLA-DRβ1*13, por lo que fue seleccionado como un buen epítope para ambos alelos. El péptido 39114 fue el único epítope que mostro unión mayor al 50% a los alelos evaluados, por lo que fue seleccionado como epítope universal (Figura 7B y Tabla 2).

A) PvRON2



B) Pv12

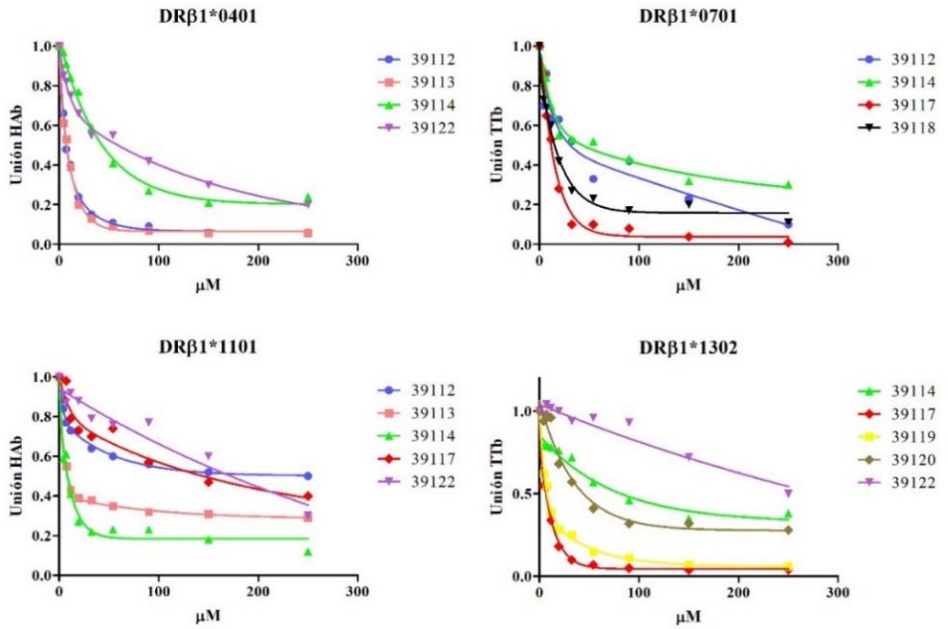


Figura 7. Ensayos *in vitro* para calcular el valor IC50 de los péptidos de alta unión de PvRON2 y Pv12.

Se evaluaron diferentes concentraciones de los péptidos para calcular el valor al cual el péptido control fue desplazado el 50% de la unión (se empleó una función de decaimiento exponencial de segundo orden). Cada punto bajo la curva representa la concentración del péptido evaluado dependiente del péptido control de unión (µM).

Tipificación del HLA-DRβ1* de individuos de áreas no endémicas y endémicas para la infección por *P. vivax* y conformación de los grupos experimentales

Se tipificaron 50 individuos procedentes del departamento de Córdoba, municipio de Tierralta y 29 del departamento de Chocó, municipio de Bahía Solano, expuestos a la infección natural por *P. vivax* (Tabla 3). De igual manera, se tipificaron 50 individuos de un área no endémica en Bogotá. Para conformar los grupos experimentales de los ensayos de antigenicidad, fueron seleccionados 29 individuos expuestos y 8 individuos no expuestos como grupo control (Tabla 4).

Tabla 3. Frecuencia alélica HLA-DRβ1* de los individuos expuestos.

Alelo DRβ1*	n	Frecuencia alélica%
DRβ1*01	14	8,86
DRβ1*03	31	19,62
DRβ1*04	20	12,66
DRβ1*07	19	12,03
DRβ1*08	7	4,43
DRβ1*09	10	6,33
DRβ1*11	13	8,23
DRβ1*12	4	2,53
DRβ1*13	15	9,49
DRβ1*14	3	1,90
DRβ1*15	6	3,80
DRβ1*16	16	10,13
Total	**158**	**100**

Tabla 4. Grupos experimentales ensayos de antigenicidad.

Alelos HLA-DRβ1* Individuos expuestos	Identificación de la Muestra
DRβ1*04	P4BS003
	P4BS004
	P4BS010
	P4BS019
	P4BS025
	P4COR029
	P4COR040
	P4COR047
DRβ1*07	P4BS005
	P4BS013
	P4COR004
	P4COR010
	P4COR015
	P4COR021
	P4COR043
	P4COR046

Alelos HLA-DRβ1* Individuos expuestos	Identificación de la Muestra
DRβ1*11	P4BS008
	P4BS009
	P4BS022
	P4BS026
	P4COR018
	P4COR025
	P4COR033
DRβ1*13	P4BS028
	P4COR011
	P4COR030
	P4COR038
	P4COR039
	P4COR044

Grupo Control	Identificación de la Muestra
	P4BOG003
	P4BOG006
	P4BOG010
	P4BOG016
	P4BOG024
	P4BOG029
	P4BOG043
	P4BOG047

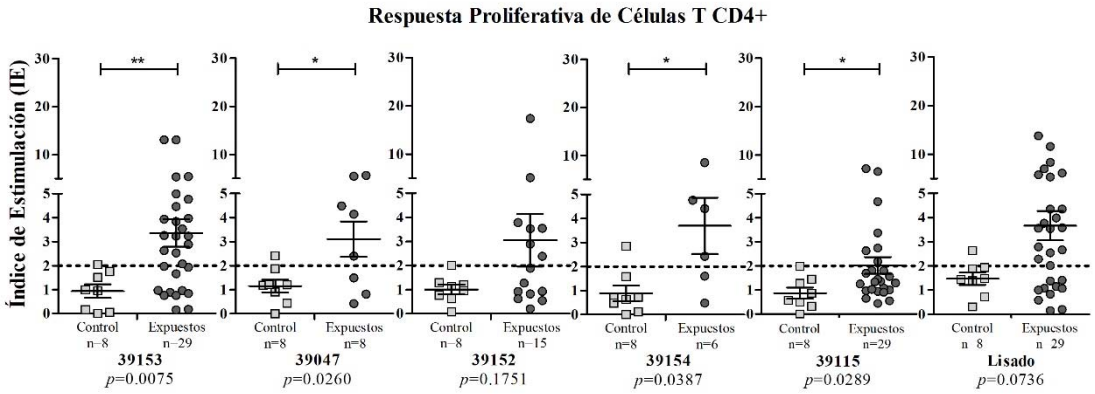
Ensayos de antigenicidad para epítopes de células T

Con la segunda toma de muestra de los individuos expuestos, se realizó la separación de CMSP mediante Ficoll y se estimularon con los péptidos seleccionados según su perfil de unión *in vitro* y el valor de IC50. Para la proteína PvRON2 fueron seleccionados cuatro péptidos: 39152 para HLA-DRβ1*04 y HLA-DRβ1*11; 39047 para HLA-DRβ1*07; 39154 para HLA-DRβ1*13; y el péptido universal 39153. Para la proteína Pv12 fueron seleccionados tres péptidos: 39113 para HLA-DRβ1*04 y HLA-DRβ1*11; 39117 para HLA-DRβ1*07 y HLA-DRβ1*13; y el péptido universal 39114. A las CMSP de los individuos expuestos y del grupo control, se les evaluó la linfoproliferación según el índice de estimulación (IE) y la producción de citoquinas en el sobrenadante (SBN) de cultivo para cada uno de los péptidos de las proteínas estudiadas. El lisado del parásito y un péptido de baja unión de la proteína *Pv*12 (39115) fueron empleados como control positivo y negativo, respectivamente. De acuerdo con el marcaje con CSFE en el día cero

del ensayo, un índice de estimulación superior o igual a dos (IE≥2) fue considerado positivo.

Con todos los péptidos evaluados de la proteína PvRON2 se evidenció linfoproliferación (IE≥2). El péptido 39154 (HLA-DRβ1*07 y *13), el péptido 39047 (HLA-DRβ1*04 y *11) y el péptido universal 39153, presentaron diferencias significativas con respecto al grupo control. (Figura 8A). De la misma manera, todos los péptidos de la proteína Pv12 generaron linfoproliferación (IE≥2). El péptido 39117 (HLA-DRβ1*07 y *13) y el péptido universal 39114, presentaron diferencias significativas comparados con el grupo control. (Figura 8B). El péptido control de βaja unión 39115 presentó el IE más βajo de todos los péptidos evaluados y también presentó diferencias significativas con el grupo control. El lisado de parásito (control positivo) presentó un IE≥2, mostrando una respuesta proliferativa por parte de los linfocitos T CD4+ de los individuos expuestos. Las CMSP del grupo control no presentaron proliferación con ninguno de los péptidos evaluados, ni frente al lisado del parásito.

A) PvRON2



B) Pv12

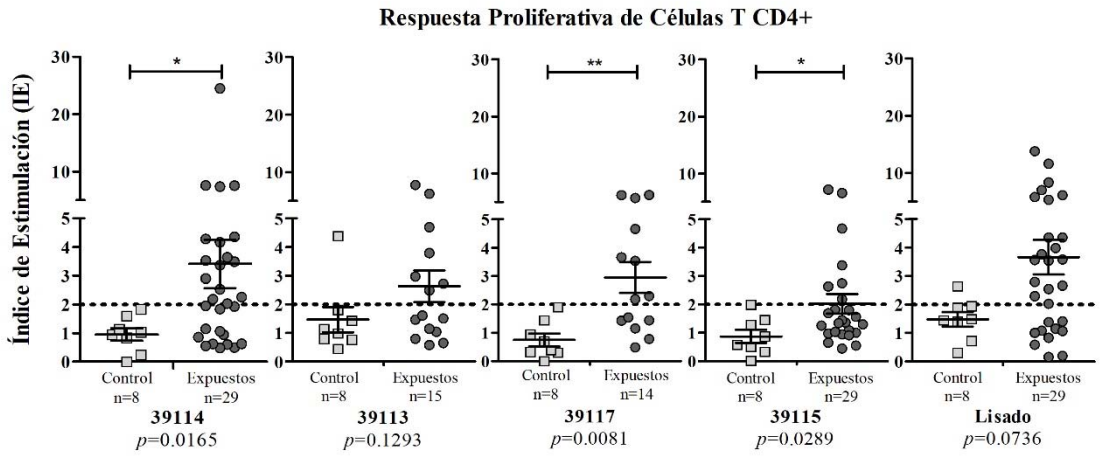


Figura 8. Respuesta proliferativa de linfocitos T CD4+ estimulados con péptidos de las proteínas PvRON2 y Pv12.
(A) Para PvRON2, el péptido universal es 39153 (*n* = 29), el péptido 39152 de alta afinidad de unión para DRβ1*04 y *11 (*n* = 15), el péptido 39047 de alta afinidad de unión para DRβ1*07 (*n* = 8), el péptido 39154 de alta afinidad de unión para DRβ1*13 (*n* = 6), el péptido control de baja afinidad de unión 39115 (*n* = 29) y el lisado de *P. vivax* como control positivo (*n* = 29). (B) Para Pv12, el péptido universal es 39114 (*n* = 29), el péptido 39113 de alta afinidad de unión para DRβ1*04 y *11 (*n*=15), el péptido 39117 de alta afinidad de unión para DRβ1*07 y *13 (*n* = 14), el péptido control de baja afinidad de unión 39115 (*n* = 29) y el lisado de *P. vivax* como control positivo (*n* = 29). Las pruebas estadísticas de Mann-Whitney y Student's t-test fueron empleadas para evaluar las diferencias estadísticamente significativas entre los individuos expuestos y el grupo control ($p \leq 0.05$). Los datos representan la media ± SEM para todos los valores. (*) p<0,05 y (**) p<0,005.

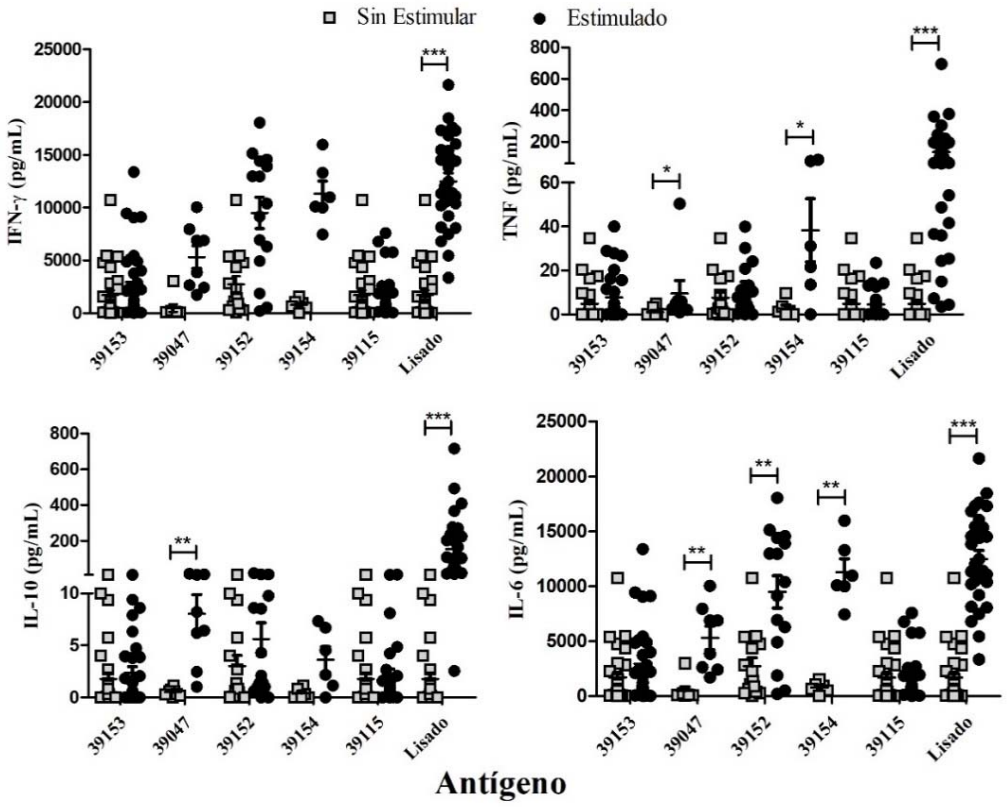
Con el kit CBA Human Th1/Th2 Cytokine Kit II, se evaluaron diferentes citoquinas: IFN-γ, TNF, IL-10 e IL-6. Los niveles de producción de las citoquinas inducidas por los péptidos de las proteínas PvRON2 y Pv12 fueron comparadas entre las CMSP no estimuladas y las estimuladas.

La producción de TNF tuvo diferencias significativas para los péptidos 39154 (HLA-DRβ1*13) y 39047 (HLA-DRβ1*07) de la proteína PvRON2. Y para los péptidos 39113 (HLA-DRβ1*04 y HLA-DRβ1*11) y 39117 (HLA-DRβ1*07 y HLA-DRβ1*13) de la proteína Pv12. La producción de IL-10 fue significativamente mayor para los péptidos 39047 (HLA-DRβ1*07) de PvRON2; 39113 (HLA-DRβ1*04 y HLA-DRβ1*11) y 39117 (HLA-DRβ1*07 y HLA-DRβ1*13) de Pv12. La producción de IL-6 fue mayor para todos los

péptidos específicos y universales, tanto de PvRON2 como de Pv12 con respecto a las CMSP de individuos no expuestos.

Por otra parte, la producción de IFN-γ no presentó diferencias significativas en respuesta al estímulo con ningún péptido, de ninguna de las proteínas. El lisado del parásito mostró una producción significativa de IFN-γ, TNF, IL-10 e IL-6 (Figura 9 A y B).

A) PvRON2



B) Pv12

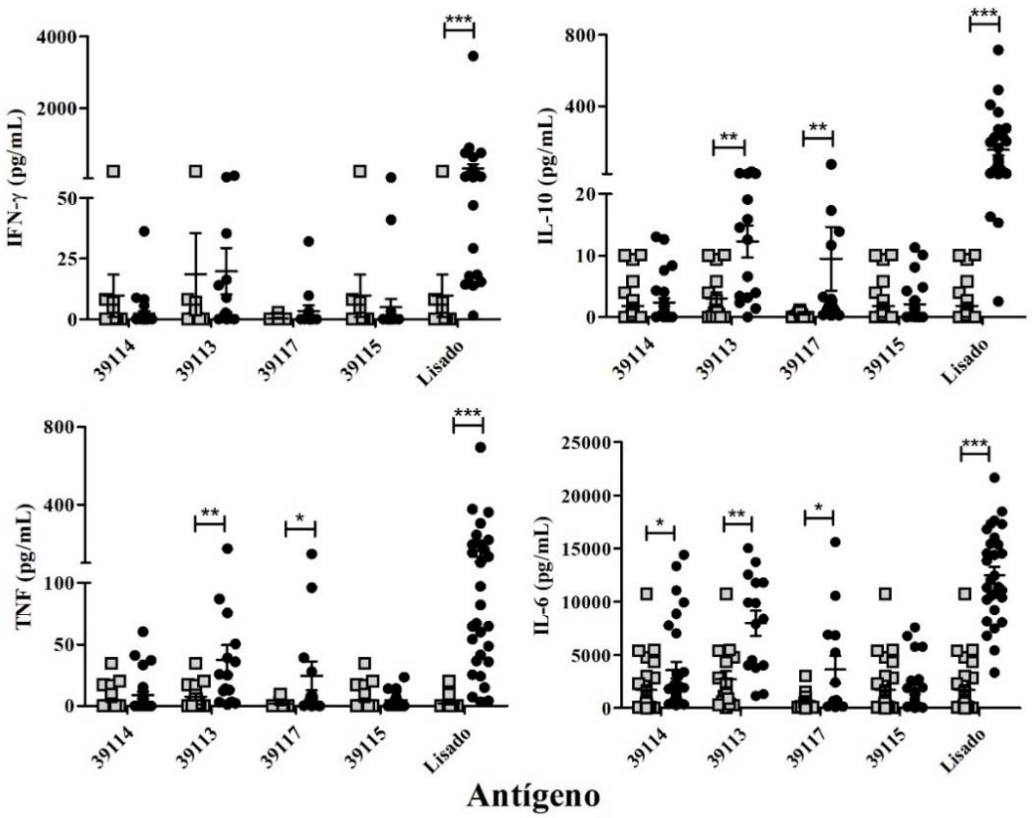


Figura 9. Producción de citoquinas *in vitro* en el sobrenadante de cultivo de CMSP de individuos expuestos para las proteínas PvRON2 y Pv12.
Se muestran el valor medio de los datos individuales de CMSP estimulados y no estimulados con los péptidos de (A) PvRON2: el péptido universal 39153, los péptidos específicos 39047, 39152, 39154; y de (B) Pv12: el péptido universal 39114, los péptidos específicos 39113, 39117. Para ambas proteínas se evaluó lisado de *P. vivax* como control positivo y el péptido de baja unión 39115. Se evaluó la producción de las citoquinas IFN-γ, TNF, IL-10 e IL-6 con el kit CBA y la concentración de las citoquinas se expresó en pg/mL. Los datos representan la media ± SEM para todos los valores y se muestran las diferencias estadísticamente significativas ($p \leq 0,05$). (*) $p<0,05$, (**) $p<0,005$ y (***) $p<0,0005$.

Así mismo, se realizó este análisis con las muestras del grupo control. A diferencia de los individuos expuestos, la producción de IFN-γ fue significativamente mayor con los péptidos específicos 39047 (HLA-DRβ1*07) y 39154 (HLA-DRβ1*13) de la proteína *Pv*RON2 y con los péptidos 39113 (HLA-DRβ1*04 y HLA-DRβ1*11), 39117 (HLA-DRβ1*07 y HLA-DRβ1*13) y el péptido universal 39114 de la proteína Pv12. No se observaron diferencias significativas en la producción de TNF e IL-10, con los péptidos de ambas proteínas. La producción de IL-6 tuvo diferencias significativas para la mayoría de los péptidos de las dos proteínas, excepto para el péptido universal 39153 de la proteína

PvRON2. El lisado del parásito presentó diferencias significativas en la producción de IFN-γ e IL-6.

DISCUSIÓN

Desarrollar una vacuna para *P. vivax* requiere estudios acerca de la respuesta inmune de las proteínas involucradas en la invasión de eritrocitos en pacientes infectados naturalmente. Varios candidatos a vacuna de *P. vivax* han sido caracterizados a la fecha, tales como las Proteínas de Unión a Reticulocitos (RBP) (80), la proteína de unión a Duffy (DBP) (81), el Antígeno de Micronemas anclado a GPI (PvGAMA) (82), algunas proteínas de la familia del antígeno rico en triptófano (PvTRAg) (83), la proteína de superficie de merozoíto 1 (PvMSP1) (84) y el Antígeno de Membrana Apical 1 (AMA1) (85). Adicionalmente, han sido identificados mediadores importantes en la unión al eritrocito, como las proteínas del cuello de las roptrias (RONs), incluyendo PvRON5 (86), PvRON4 (87) y PvRON2 (63). Las proteínas RON han sido asociadas con la formación del "moving junction", ayudando a la entrada de los parásitos al eritrocito; además RON2 es un candidato a vacuna ya que se ha encontrado que anticuerpos anti-AMA1 y anti-RON2 pueden bloquear la invasión al eritrocito (63, 88-91).

En la era post-genómica, las herramientas bioinformáticas y los enfoques de vacunología reversa son muy valiosos. Con esta metodología se pueden identificar los antígenos disponibles de un microorganismo y evaluar sus propiedades; asimismo se puede evitar reacciones cruzadas, seleccionando antígenos que no tengan homología con las proteínas del genoma humano. Además, a un antígeno individual se le puede determinar los epítopes de más alta afinidad que puedan inducir respuestas inmunes protectoras (92).

En este estudio se analizó la secuencia de aminoácidos de las proteínas PvRON2 y Pv12 para determinar *in silico* los epítopes de células T de alta unión a HLA-DRβ1*. Once epítopes de alta afinidad a HLA-DRβ1* fueron predichos para PvRON2 y diez epítopes para Pv12 empleando NetMHCIIpan 3.1 (93), donde por lo menos 1 epítope se unió a una molécula HLA-DRB1*; esto se confirmó por ensayos de unión *in vitro* usando péptidos control biotinilados. Algunos epítopes predichos no se unieron *in vitro* al HLA-DRB1*, esto demuestra que los métodos *in silico* disponibles, tiene diferentes grados de precisión (94), por lo que se hace necesario implementar estudios que correlacionen metodologías de predicción con estudios *in vitro* e *in vivo* (95).

Dentro de los alelos más frecuentes en los grupos amerindios en Colombia, se encuentran el DR4 (*04:03, *04:04, *04:07, *04:11), acompañado por DR2 (DRβ1*16), DR6 (DRβ1*14:02) y DR8 (DRβ1*08:02) (96, 97). Las muestras de sangre de los pacientes usados en este estudio fueron tomadas de Amerindios de Córdoba y Chocó. Estudios previos han sugerido que la sobre dominancia de algunos alelos en una población podría ser debido a la distribución de alelos ventajosos (selección positiva) después de la selección dirigida por el patógeno. Hill y *col*. mostraron en un estudio de alelos del Oeste de África (HLADRβ1*13:02-DQβ1*05:01) que estos no estuvieron presentes en otros grupos raciales y fueron asociados con protección contra anemia severa por malaria, como por ejemplo selección direccional (98).

Varios factores pueden afectar las respuestas inmunes que inducen protección a parásitos de la malaria, y consecuentemente al desarrollo de una vacuna. Dentro de los mecanismos de evasión del parásito se encuentran: la evasión del sistema del complemento, favorecer la infección dependiente de anticuerpos producidos contra proteínas del parásito, el polimorfismo antigénico inmunodominante debido a mutaciones puntuales, inserciones o deleciones en la secuencia codificante, la variación antigénica que es una variación fenotípica que ocurre con el mismo clon genómico del parásito y la diversidad antigénica que se produce cuando los anticuerpos antiparasitarios no inhibidores impiden la acción de los anticuerpos inhibidores (99).

También se ha definido el enmascaramiento de epítope, que es la capacidad de los anticuerpos no específicos del parásito que evitan que los anticuerpos inhibidores específicos del parásito reaccionen con sus epítopes; y la estrategia de cortina de humo para desviar los anticuerpos específicos de un antígeno para que reaccionen contra otro antígeno. Por otra parte, *P. vivax* y *P. ovale* tienen mecanismos adicionales de escape los cuáles son mediados por hipnozoítos de larga duración (100).

En este estudio los individuos expuestos a la infección fueron agrupados de acuerdo con su alelo HLA-DRβ1* con el objetivo de incluir los alelos que ocurren con mayor frecuencia en la población endémica alrededor del mundo, tales como HLA-DRβ1*04, HLA-DRβ1*07, HLA-DRβ1*11 y HLA-DRβ1*13. MHC clase I y II tienen un gran polimorfismo alélico en la región de unión al péptido y la variación en secuencia de

aminoácidos se concentra en esa región, permitiendo que diferentes alelos se unan a diferentes rangos de péptidos (101, 102).

Como se ha descrito en diferentes estudios, la activación de los linfocitos B debe ocurrir de dos maneras, ya sea por el reconocimiento del antígeno por el receptor de las células B o por la interacción con los linfocitos T ayudadores CD4+. Esta última ocurre por la unión del CD40L del linfocito T con el receptor CD40 del linfocito B, por la interacción de ligandos de la familia del receptor de TNF y por la secreción de citoquinas. Los LT ayudadores CD4+ inducen la proliferación de los linfocitos B naïve y dirigen su diferenciación a células plasmáticas productoras de anticuerpos y a linfocitos B de memoria (103-105).

En este estudio, se evaluó la respuesta de los linfocitos T de individuos expuestos a péptidos de alta capacidad de unión de las proteínas PvRON2 y Pv12. Solo los cultivos de CMSP de individuos expuestos mostraron linfoproliferación inducida por los péptidos universales y los péptidos de unión específica, lo cual sugiere que estas proteínas inducen linfocitos T de memoria contra los péptidos de alta afinidad. Cuando se evaluó la producción de citoquinas a los péptidos de la proteína PvRON2, la respuesta de citoquinas de los perfiles Th1 y Th2 fue baja, excepto para IL-6. En otro estudio se ha reportado la baja producción de citoquinas, Silva-Flannery y *col*. encontraron que la inmunización con péptidos monoméricos no generó proliferación de células específicas de antígeno secretoras de IFN-ɣ y no tuvieron una respuesta protectora. También reportaron que los péptidos monoméricos fueron menos tomados por las células presentadoras de antígeno y no pasaban a través del fagolisosoma (106).

Algunas CMSP de los individuos no expuestos tuvieron muy baja secreción de citoquinas, frente al estímulo con los péptidos sintéticos de las proteínas estudiadas. Esto puede deberse a que los macrófagos o las células dendríticas que primaron a los linfocitos T naïve indujeron células T efectoras productoras de citoquinas. La citoquina que se secretó en alta cantidad por parte de los CMSP de los individuos expuestos fue la IL-6; una de las múltiples funciones de esta citoquina es estimular el hibridoma, el crecimiento del plasmocitoma y ayudar en la producción de anticuerpos (107). La IL-6

junto con la IL-12 y el VDR, se han asociado con parasitemia y severidad reducida y la remoción de gametocitos en sangre de individuos expuestos a *P. vivax* (108).

La respuesta de citoquinas inducida por el lisado de *P. vivax* en CMSP de individuos no expuestos, puede explicarse por la producción de citoquinas de parte de las células inmunes innatas, por ejemplo: macrófagos, células dendríticas, células NK, células NKT y células T naïve las cuales se convierten en células efectoras (16). La alta producción de citoquinas en CMSP de individuos sanos comparados con los individuos expuestos a *P. vivax* se puede relacionar con los mecanismos de evasión del parásito por inhibir la respuesta inmune efectiva capaz de eliminar el parásito (99).

La proteína Pv12 hace parte de la familia de proteínas de seis cisteínas (Cys6), se expresa en el estadio sanguíneo y tiene un anclaje a GPI; es conservada entre las especies de *Plasmodium* y los aislados de *P. vivax* (68). Las proteínas de la familia de seis cisteínas se caracterizan porque se expresan durante todos los estadios del ciclo de vida del parásito (merozoitos maduros, gametocitos y estadios pre-eritrocíticos), lo que hace a estas proteínas interesantes candidatos a vacuna (109). Pv12 es homóloga de la proteína Pf12 en *P. falciparum* y ha sido sugerida como posible ligando durante la invasión de los merozoitos a los reticulocitos (110); también ha sido reconocida por el suero de individuos infectados naturalmente (66). Individuos expuestos que viven en áreas endémicas para *P. falciparum* desarrollaron respuesta de anticuerpos a proteínas con anclaje a GPI, y se correlaciona con protección contra los síntomas de la enfermedad (111).

Los ensayos de linfoproliferación con los péptidos de la proteína Pv12 seleccionados por su unión a las moléculas HLA-DRβ fueron capaces de inducir una respuesta linfoproliferativa al ser reconocidos por células T de memoria de los individuos expuestos. Esto se confirmó cuando se comparó con los resultados del grupo control; ellos expresaban los mismos linajes alélicos que el grupo experimental, pero estos no tuvieron una respuesta proliferativa.

Algunos estudios han reportado valores altos de estimulación cuando se evalúa la respuesta proliferativa de proteínas completas o alergenos que han estado en contacto constante con el sistema inmune (112, 113). Los valores de linfoproliferación

con los péptidos de la proteína Pv12 fueron bajos, estos fueron similares a los valores reportados por Martinez *y col.*, donde emplearon péptidos de ~20 a.a. de longitud como epítopes antigénicos (114), esto muestra la importancia de evaluar péptidos (una mínima fracción de la proteína) los cuales fueron capaces de desencadenar una respuesta de células T de memoria.

Los péptidos 39114 y 39117 fueron seleccionados de acuerdo con los ensayos de unión *in vitro*, además de tener ≥ 55 % de respuesta proliferativa (con diferencias significativas con el grupo control) y una media de IE ≥2. Estos resultados indican una respuesta de células T específica para epítopes de Pv12 y comprueba la antigenicidad de estos epítopes cuando son reconocidos por células de memoria del sistema inmune. Además, se correlacionan la proliferación y la producción de citoquinas, como en otros estudios con antígenos de *Plasmodium* (115-117).

Las CMSP estimuladas con los péptidos 39113 y 39117 generaron la producción de IL-6, IL-10 y TNF. La producción de IL6 ha sido asociada con proliferación y diferenciación celular (118), de acuerdo con los resultados donde tres péptidos y el lisado del parásito tuvieron un incremento significativo de esta citoquina. Esta citoquina es considerada multifuncional en la respuesta inmune. La producción de IL-6, IL-10 y TNF ha sido asociada con trombocitopenia en pacientes con malaria por *P. vivax* (119). La producción de IFN-ɣ es asociada con protección en los estadios pre-eritrocíticos de la malaria a través de la producción de óxido nítrico (ON) *in vitro* e *in vivo* después de la infección con esporozoitos de *P. falciparum*, *P. berghei*, *P. yoelii* o *P. chabaudi* (120-122).

Varios estudios han resaltado el papel de las citoquinas proinflamatorias como mediadores de inmunidad protectora contra malaria, por ejemplo TNF junto con IFN-ɣ, actúan sinérgicamente para optimizar la producción de óxido nítrico que es importante para la destrucción del parásito (123). El IFN-ɣ junto con otras citoquinas participa en la inhibición del desarrollo del parásito dentro de los hepatocitos y la activación del macrófago en la fagocitosis del parásito intra-eritrocítico o de merozoitos libres (16).

La IL-10 es otra citoquina con producción diferencial significativa después que las CMSP fueran estimuladas con los péptidos 39113 y 39117. Estudios recientes han mostrado que los linfocitos CD4+ de memoria proliferan durante la segunda infección

de malaria, produciendo una respuesta inmune que controla la carga parasitaria y produce rápidamente IL-10 regulando la inflamación. Esto demuestra la importancia de estos linfocitos en infecciones secundarias donde influencian la naturaleza de la respuesta inmune anamnésica durante la infección secundaria de malaria (124).

Estudios previos han mostrado que CMSP de individuos no expuestos estimulados con glóbulos rojos parasitados con *P. falciparum* (125) o con antígenos de malaria, producen IFN-ɣ (126), principalmente por células inmunes innatas, como las células NK. En otro estudio esas CMSP producen IFN-ɣ e IL-6 después de la estimulación con la proteína CS de *P. falciparum* (127). Por lo anterior, la alta producción de IFN-ɣ e IL-6 de las CMSP del grupo control, sugiere que las células innatas, las células linfoides innatas y las células T naïve reconocieron el lisado del parásito y produjeron citoquinas (128).

Capítulo 2

Antigenicidad *in vitro* de epítopes B de las proteínas PvRON2 y Pv12 de *P. vivax*

MARCO TEÓRICO

Los anticuerpos forman parte integral de la respuesta inmune de un individuo contra un patógeno determinado. La estructura de estas moléculas es bifuncional, ya que presentan dos regiones principales, la Fab (que confiere especificidad para el antígeno) y la Fc (que activa una gran variedad de funciones efectoras), que en conjunto finalmente conducen a la eliminación específica del patógeno blanco. Dichas propiedades hacen de los anticuerpos moléculas críticas para el desarrollo exitoso de la respuesta inmune, así como herramientas útiles en investigación experimental, investigación clínica, diagnóstico y terapia clínica [72].

Ensayos de inmunización pasiva demostraron que los anticuerpos son un componente principal involucrado en la inmunidad natural contra malaria [37, 73] y son el principal componente inmune contra las formas sanguíneas [73-80]. Estudios en ratones han demostrado que la inmunización pasiva con anticuerpos dirigidos contra los antígenos de *P. yoelii*, CS, MSP-1, AMA-1 tienen un efecto protector [77, 81-84]. En humanos, se ha demostrado que anticuerpos IgG provenientes de africanos adultos expuestos a *P. falciparum* inhiben el crecimiento parasitario *in vitro*, en cooperación con monocitos y dicha inhibición se correlaciona con la reducción de la parasitemia *in vivo* [79].

Los mecanismos por los cuales los anticuerpos pueden conferir inmunidad protectora contra la infección por *Plasmodium* aún se encuentran en estudio, sin embargo, se han postulado algunos como la intervención estricta en la invasión del merozoito a los eritrocitos [74, 78], inhibición del desarrollo intraeritrocitario de los parásitos [85], inhibición dependiente de anticuerpos mediada por células (ADCI del inglés, *Antibody Dependent Cell-mediated Inhibition*) [79], fagocitosis dependiente de anticuerpos (ADPh del inglés, *Antibody Dependent Phagocytosis*) [86, 87] y prevención de la maduración apropiada o del procesamiento de proteínas de superficie del merozoito [88, 89].

Una serie de estudios han encontrado correlación entre el isotipo del anticuerpo y la protección alcanzada [77, 90-92]. Inmunizaciones pasivas con IgG2a en ratones infectados con *P.berghei* y *P.yoelii*, mostraron una protección mucho mayor en relación

a la inmunización con el isotipo IgG1 [90, 93]. En humanos, los dos isotipos citofílicos IgG1 e IgG3 han sido asociados con protección, ya que actúan en cooperación con células efectoras como monocitos y macrófagos (ADCI y ADCP); son predominantes en individuos constantemente expuestos a malaria y dichos individuos se caracterizan por tener un bajo riesgo de sufrir la enfermedad o por limitar la parasitemia (inmunidad clínica) [81, 94].

La participación de los receptores Fc presentes en la superficie de las células inmunes en la actividad efectora de los anticuerpos contra la malaria, ha sido objeto de varios estudios [95-97] que sugieren que estos receptores son necesarios para inducir un efecto protector cuando el mecanismo involucrado es la ADCI o la ADCP (del inglés, Antibody-Dependent Cellular Phagocytosis).

Respuesta humoral a malaria

En cuanto a la respuesta humoral, en varios estudios se ha demostrado que los anticuerpos tienen un papel importante en la protección a la malaria. Cohen *y col*., demostraron que la transferencia pasiva de anticuerpos de individuos inmunes a malaria hacia niños con malaria clínica severa, son capaces de reducir la densidad parasitaria y los síntomas clínicos de la enfermedad (129).

Las inmunoglobulinas pueden proteger o detener la progresión de la enfermedad por diferentes vías: anticuerpos neutralizantes anti-esporozoíto que pueden bloquear la invasión a los hepatocitos (130-132); los merozoítos pueden ser opsonizados por anticuerpos que activan células NK, o anticuerpos que pueden bloquear las proteínas de unión al glóbulo rojo.

Estudios en áreas endémicas para malaria por *P. falciparum* han evidenciado que altos títulos de anticuerpos citofílicos IgG3 and IgG1 se han asociado con protección (133). Además, estudios *in vitro* han demostrado que los monocitos aumentan su capacidad de destruir parásitos asincrónicos en cultivo en presencia de anticuerpos, y la actividad de los monocitos mejora en presencia de IgG1 e IgG3 (134). Una respuesta predominante de IgG2 con βaja reactividad de IgG4 se ha asociado con resistencia y

resolución a la infección por *P. falciparum*, por lo tanto, una relación alta IgG2/IgG4 parece conferir un papel protectivo (135).

En estudios con proteínas de *P. vivax* como PvMSP8, donde se registra el predominio de anticuerpos IgG2 no citofílicos, altos títulos de esta subclase se han asociado con la resistencia a la enfermedad (136). Por otra parte, la respuesta de anticuerpos contra las proteínas *Pv*CSP-1 (137), *Pv*MSP-1 (138-141), *Pv*RBP1 (142, 143), *Pv*AMA-1 (144) y *Pv*MSP-3α (34, 145) de *P. vivax*, se caracteriza por el predominio de IgG1 e IgG3, las cuales están asociadas a protección frente a la enfermedad (Figura 10).

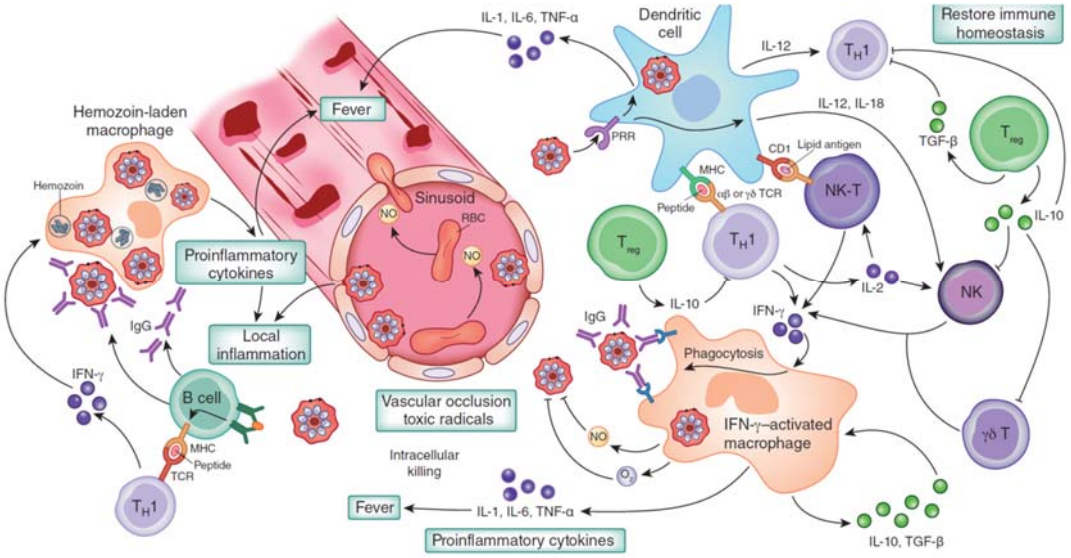


Figura 10. Respuesta inmune humoral y celular generada en malaria.
Por medio de los receptores de reconocimiento de patrones (PRR) se activan las células dendríticas, estas fagocitan los glóbulos rojos parasitados y los presentan a los linfocitos T. Se produce la secreción de citoquinas y se diferencian los LTh1 que promueven la diferenciación de los linfocitos B y la secreción de anticuerpos. Tomado de Riley & Stewart, 2013 (41).

METODOLOGÍA

Para la ejecución de esta fase del proyecto, fue esencial la consecución de muestras de sangre periférica de individuos sanos y de personas con antecedentes de infección por *P. vivax*. Se evaluó la antigenicidad *in vitro* de péptidos derivados de las proteínas PvRON2 y Pv12 de *P. vivax*. Primero se seleccionaron epítopes B derivados de estas proteínas, con base en predicciones *in silico*, y fueron empleados en estudios de antigenicidad en individuos de Córdoba y Chocó en Colombia. De esta manera, se pudo conocer si los péptidos predichos por herramientas bioinformáticas fueron reconocidos por anticuerpos (y su subclase) producidos por individuos expuestos naturalmente a la infección por *P. vivax*.

Ensayos de antigenicidad con epítopes de células B

Selección *in silico* de epítopes B

Epítopes B lineales de las proteínas de interés, fueron seleccionados teniendo en cuenta los siguientes parámetros: promedio de los valores altos de antigenicidad de Parker, hidrofilicidad y accesibilidad al solvente según el programa Antheprot 2000 V 6.0 (146), así como los valores más altos de puntaje según la herramienta web Bepipred 1.0 (147) (umbral 0.35 por defecto y especificidad del 75%).

Reconocimiento de antígenos de *P. vivax* por inmunoblots

Se realizó el protocolo publicado por Rojas-Caraballo *y col*. (148), con algunas modificaciones. Brevemente, se separaron 50 µg de las proteínas recombinantes (*Pv*12 (donada por Moreno-Perez *y col*. (65)) y *Pv*GAMA-CT (Antígeno de micronemas anclado a GPI, donado por Baquero *y col*. (82)) en geles de poliacrilamida al 12% en presencia de SDS, luego fueron transferidas a una membrana de nitrocelulosa. El bloqueo de la membrana se realizó con una solución de PBS-Tween 0.05% y leche descremada al 5%, durante 1 hora en agitación constante a TA. La membrana fue cortada en tiras de 4 mm de ancho y se adicionaron los sueros en dilución 1:50 en solución de bloqueo. Después de 2 horas de incubación en agitación constante a TA, se le adicionó el anticuerpo secundario anti-human IgG (H+L) conjugado con peroxidasa (Vector Labs) en dilución

1:4000 y se reveló con el kit de revelado VECTOR VIP Peroxidase (Vector Labs), según las instrucciones del fabricante.

Detección de anticuerpos específicos de *P. vivax* con ensayos de Inmunofluorescencia Indirecta (IFA)

Se empleó el protocolo publicado por Moreno-Perez *y col*. (65), con algunas modificaciones. Para la obtención de las láminas para IFA, se centrifugaron las muestras de SP de individuos con la infección activa por *P. vivax* a 1.750 x g durante 12 min a TA, se recuperó el plasma y la capa leucocitaria. Los glóbulos rojos se lavaron con solución salina, después se sirvieron en un gradiente de Percoll al 60% y se centrifugaron a 1,750 x g durante 20 min. A partir del gradiente de Percoll, se obtuvieron los glóbulos rojos parasitados (GRp) y fueron diluidos hasta alcanzar una densidad de 5-7 esquizontes por campo y se confirmaron con tinción de naranja de acridina en microscopio óptico. Veinte µL de GRp diluidos fueron servidos en láminas multipozo (Tekdon Incorporated) y se incubaron durante 30 min a TA., el sobrenadante fue removido y las láminas se dejaron secar durante toda la noche.

Al día siguiente, las láminas se bloquearon con solución de bloqueo TBSA (buffer tris salino (TBS) con albumina sérica bovina (BSA) al 1%) durante 30 min a TA y se lavaron tres veces. Luego, se incubaron con los sueros en dilución 1:50 en TBSA durante 1 hora en cámara húmeda. Después, con el anticuerpo secundario anti-human IgG-FITC (Sigma-Aldrich) diluido 1:50 en TBSA durante 45 min en cámara húmeda. El núcleo del parásito fue teñido con 4', 6-diamidino-2-phenylinodole dihydrochloride (DAPI) (0.25 µg/mL) durante 5 min a TA y las láminas se lavaron con TBS-Tween 20 0.05% dos veces y con TBS tres veces, para remover el exceso de reactivo. Las láminas se visualizaron en un microscopio de fluorescencia Olympus BX51, se tomaron las imágenes con el programa DP2-BSW (v2.2 Olympus Corporation) y fueron sobrepuestas con el programa ImageJ 1.51n (National Institutes of Health, USA).

Determinación de anticuerpos para epítopes de células B de las proteínas *Pv*RON2 y *Pv*12 de *P. vivax* con ELISA

Los inmunomódulos Maxisorb NUNC (Thermo Scientific) fueron cubiertos con 1 µg/mL de cada epítope diluido en PBS pH 7.2, y se incubaron toda la noche a 4°C. Al día siguiente, se lavaron con PBS-0.05% Tween 20 (PBST), tres veces y se bloquearon con PBST con leche descremada 2.5% (p/v) durante 1 hora a TA. Se incubaron con el suero en dilución 1:100 (100 µL por pozo) durante 2 horas por duplicado. Se adicionó el anticuerpo secundario anti-human IgG acoplado a peroxidasa (Vector labs) en dilución 1:10.000 y se incubó durante 1 hora a TA. Se adicionaron 100 µL/pozo del revelador TMB 2-Component Microwell Peroxidase Substrate (Sera-Care) para detectar la unión del anticuerpo monoclonal y se detuvo la reacción adicionando 100 µL de ácido fosfórico (H_3PO_4) 1M. La DO se midió a 450 nm en el lector de ELISA Multiskan GO (Thermo Scientific). El punto de corte fue determinado como la media de los controles negativos más dos desviaciones estándar; a las muestras positivas para IgG total se les evaluó las subclases de IgG.

Se realizó el mismo protocolo de ELISA descrito, para evaluar las subclases de IgG con algunas modificaciones: como solución de bloqueo, se empleó PBST con albumina sérica bovina al 3%, se incubaron los sueros en dilución 1:100 durante 2 horas a TA. Se adicionaron los siguientes anticuerpos monoclonales producidos en ratón: anti-human IgG1−biotin (clone 8c/6-39) dilución 1:1.000, anti-human IgG2−biotin (clone HP-6014) dilución 1:15.000, anti-human IgG3−biotin (clone HP-6050) dilución 1:40.000 y anti-human IgG4−biotin (clone HP-6025) dilución 1:60.000 (Sigma Aldrich). Como anticuerpo secundario fue empleado ImmunoPure streptavidin conjugado a peroxidasa (Thermo Scientific), dilución 1:5.000 y como revelador TMB 2-Component Microwell peroxidase, la reacción fue detenida con ácido fosfórico (H_3PO_4) 1M. Finalmente, la DO fue medida a 450 nm en el lector de ELISA Multiskan GO (Thermo Scientific). Los ensayos se realizaron por duplicado.

RESULTADOS

Ensayos de antigenicidad para epítopes de células B

Reactividad a las proteínas recombinantes

Se realizaron ensayos de inmunoblot para el reconocimiento de las proteínas recombinantes Pv12 y PvGAMA, por parte de los sueros de individuos de áreas endémicas y del grupo control. Se realizó la expresión de las proteínas recombinantes Pv12 y PvGAMA a partir de los clonos donados por Moreno *y col*. (65) y Baquero *y col*. (82). La proteína Pv12 fue reconocida por el 33% y PvGAMA por el 70% de los sueros de individuos de área endémica y no fueron reconocidas por los sueros de los individuos del grupo control (Figura 11). Además, se detectaron anticuerpos anti-*P. vivax* adquiridos naturalmente por los individuos de esta misma área, empleando láminas multipozos para IFA cubiertas con glóbulos rojos parasitados (GRp) (Figura 12). En el panel A de la figura 12, se evaluó una muestra de suero de un individuo expuesto de Tierralta que reconoció los GRp; en el panel B, una muestra de suero de un individuo no expuesto (grupo control). Se usó DAPI para teñir los núcleos, anti-IgG humana marcada con FITC para teñir el parásito y las imágenes se sobrelaparon.

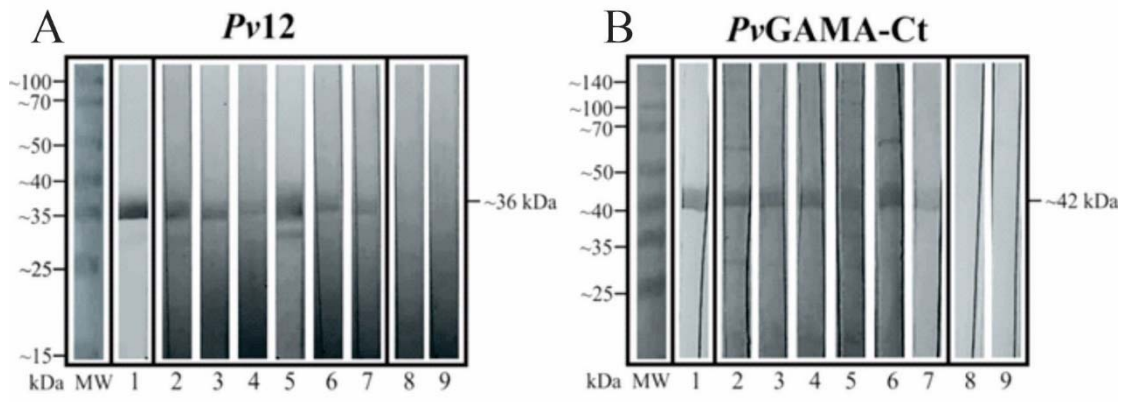


Figura 11. Ensayos de inmunoblot con los sueros de individuos de áreas endémicas en Colombia para *P. vivax* y el grupo control.
(A) Muestras de sueros de individuos expuestos de un área endémica que reconoce la proteína recombinante Pv12, carril 1 a 7; sueros del grupo control que no reconocen la proteína recombinante, carril 7 y 8 (B) Muestras de sueros de individuos expuestos de un área endémica

que reconoce la proteína recombinante control PvGAMA-Ct, carril 1 a 7; sueros del grupo control que no reconocen la proteína recombinante, carril 7 y 8.

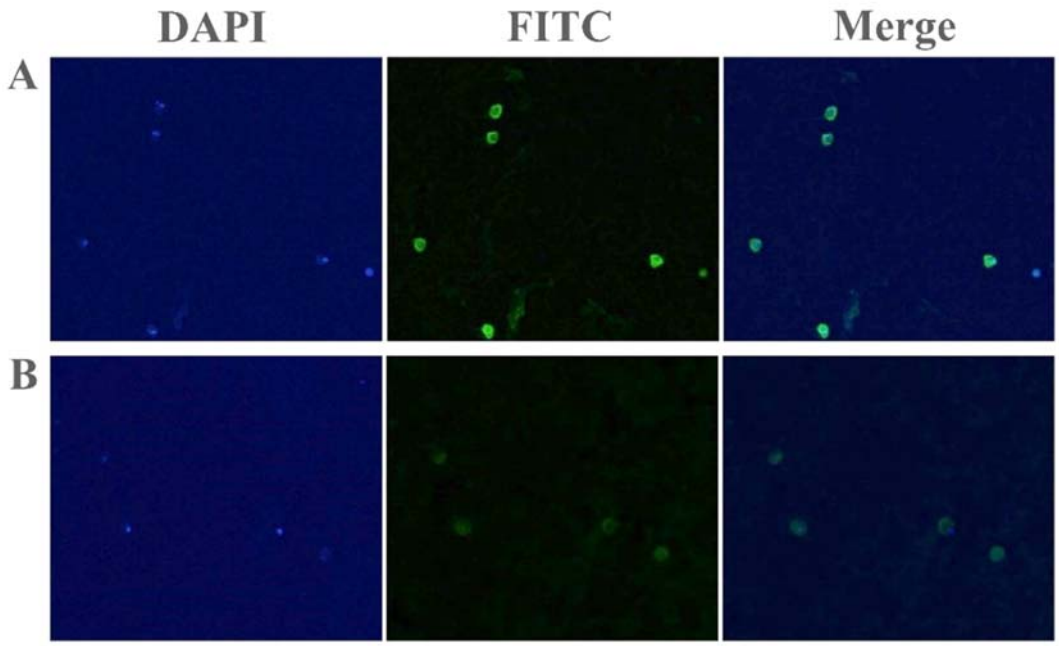


Figura 12. Patrones de Inmunofluorescencia de individuos de áreas endémicas en Colombia para *P. vivax* y el grupo control.
(A) Muestra de suero de un individuo expuesto de un área endémica que reconoce los glóbulos rojos parasitados. (B). Muestra de suero de un individuo no expuesto del grupo control.

Determinación de anticuerpos a epítopes B de las proteínas PvRON2 y Pv12

Con los programas de predicción, se seleccionaron cuatro epítopes B de 20 a.a. de longitud para cada una de las proteínas evaluadas y se realizó la síntesis química de los péptidos correspondientes a cada uno. Se obtuvo muestras de suero de 30 individuos expuestos a la infección natural procedentes de Córdoba y Chocó, y se evaluó la reactividad de los sueros a los epítopes B de las proteínas PvRON2 y Pv12, mediante la técnica de ELISA.

Todos los epítopes B seleccionados *in silico* para las dos proteínas, fueron reconocidos por los anticuerpos IgG de los individuos de áreas endémicas, mientras que el grupo control no los reconoció. Se tomó como punto de corte la media de los

controles negativos, más dos desviaciones estándar. Para ninguno de los epítopes se encontraron diferencias significativas entre las muestras y los controles.

Los resultados para la proteína PvRON2, mostraron que los epítopes 39041 y 39043 presentaron mayor reactividad por parte de los sueros de los individuos de áreas endémicas. Sin embargo, el epítope 39041 presentó la media de reactividad más alta. Tanto el epítope 39041, como el epítope 39043, presentaron diferencias significativas con el epítope 39044, el cual presentó el menor reconocimiento (Figura 13).

Para la proteína Pv12, los resultados mostraron que el epítope 39038 presentó la mayor reactividad por parte de los sueros de los individuos de áreas endémicas. Además, presentó la media de reactividad más alta y diferencias significativas con respecto al epítope 39040 (Figura 13).

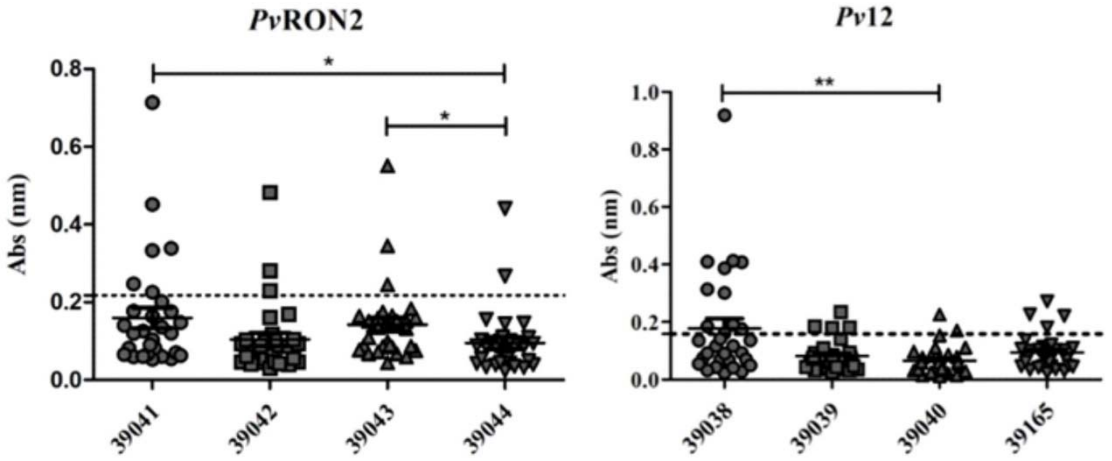
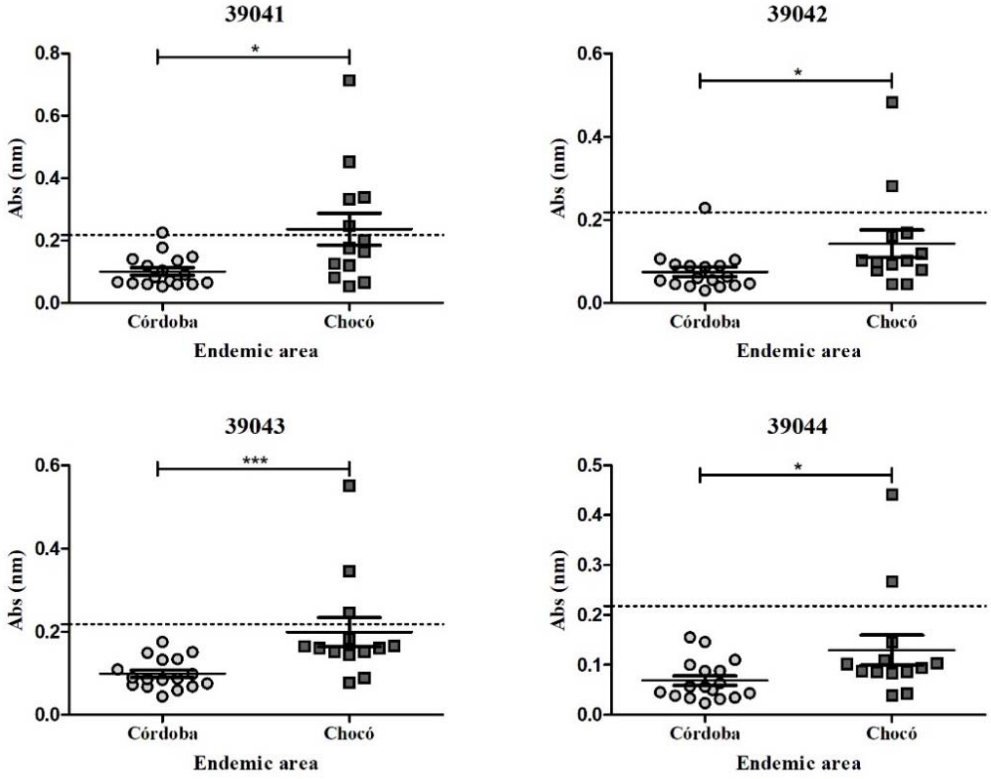


Figura 13. Respuesta de anticuerpos IgG contra los cuatro epítopes de las proteínas PvRON2 y Pv12.
Las muestras seropositivas fueron las que se encontraban por encima del punto de corte (0,218 para *Pv*RON2 y 0,159 para *Pv*12, línea punteada), y se calculó como la media del grupo control más dos desviaciones estándar. Se empleó la prueba de Kruskal-Wallis para analizar las diferencias entre cada respuesta de epítopes B entre las muestras de los individuos expuestos (n = 30, $p \leq 0{,}05$). (*) $p<0{,}05$ y (**) $p<0{,}005$.

Cuando se evaluó la respuesta de anticuerpos por área endémica, se encontró una mayor tendencia de reactividad en las muestras del departamento de Chocó para los cuatro epítopes B de la proteína PvRON2, y para tres epítopes B (39039, 39040 y 39165) de la proteína Pv12 (Figura 14 A y B).

A) PvRON2



B) Pv12

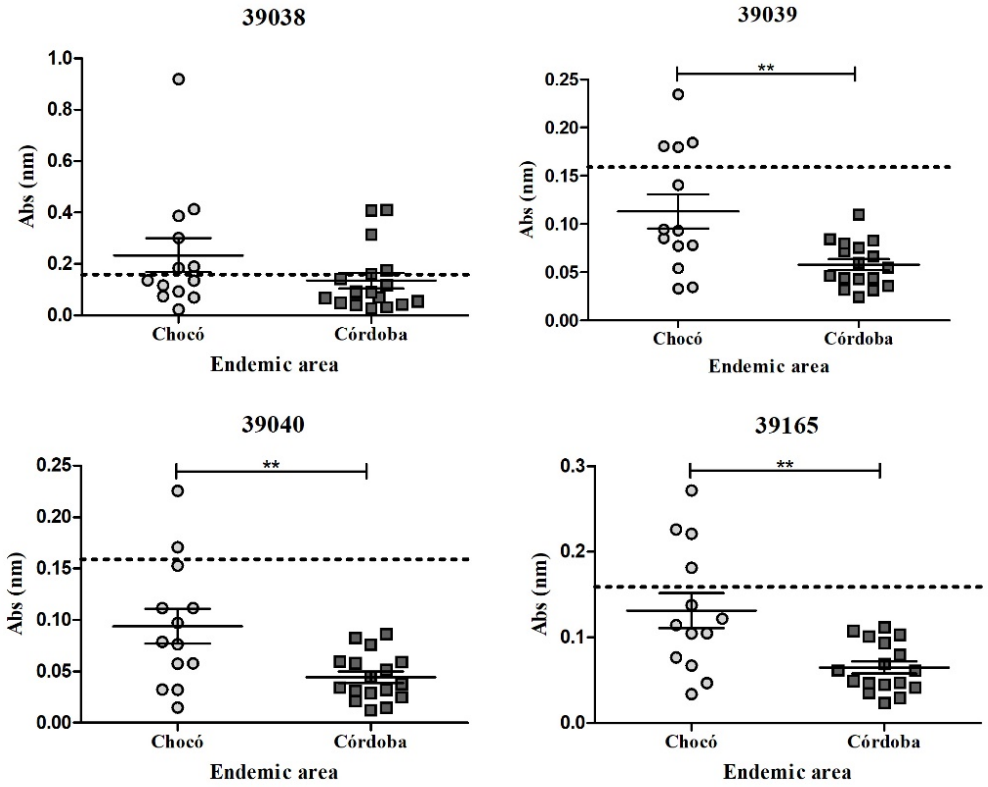


Figura 14. Respuesta de anticuerpos IgG de las proteínas PvRON2 y Pv12 por área endémica. Se empleó la prueba de Mann-Whitney para calcular las diferencias significativas entre las muestras de los departamentos de Chocó (n = 13) y Córdoba (n = 17) ($p \leq 0,05$). (*) $p<0,05$, (**) $p<0,005$ y (***) $p<0,0005$.

Las muestras seropositivas que presentaron un valor superior al punto de corte fueron seleccionadas para evaluar las subclases de IgG. Para PvRON2 el número de muestras evaluadas fueron: para el péptido 39041 n=6, 39042 n=3, 39043 n=3 y 39044 n=2; y para Pv12, el número de muestras evaluadas fueron: para el péptido 39038 n=11, 39039 n=4, 39040 n=2 y 39165 n=4.

Cuando se evaluaron las subclases IgG de los cuatro epítopes de PvRON2 mediante la prueba de Kruskal-Wallis, solamente se observaron diferencias significativas para el epítope 39041, con predominio de IgG2. Los epítopes 39042, 39043 y 39044 de la proteína PvRON2 no presentaron diferencias significativas. Los resultados de absorbancia para las subclases de los epítopes 39042 y 39043 fueron los más bajos (Figura 15A).

Para las subclases IgG de los cuatro epítopes evaluados de Pv12, se encontraron diferencias significativas para el epítope 39038 y el 39040, con predominio de IgG2. Los epítopes 39039 y 39165 de la proteína Pv12 no presentaron diferencias significativas (Figura 15B).

A) PvRON2 B) Pv12

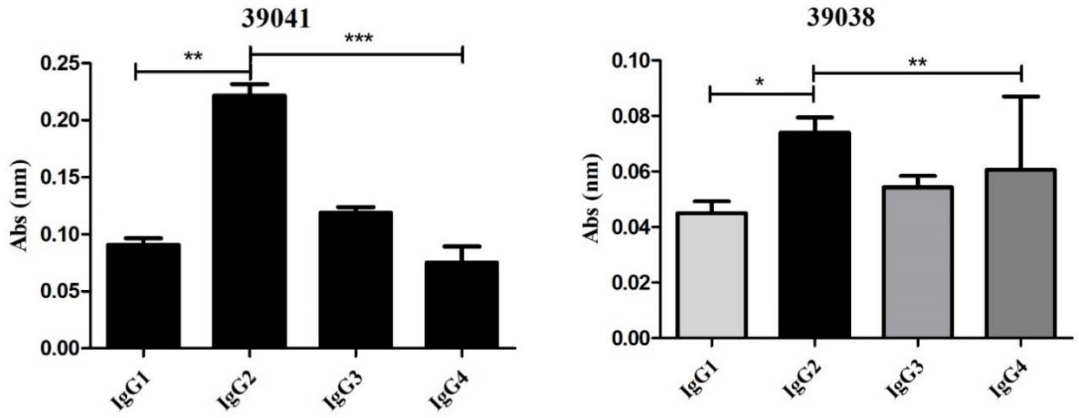


Figura 15. Evaluación de las subclases de IgG a los epítopes de las proteínas PvRON2 y Pv12. Se empleó la prueba de Kruskal-Wallis para analizar las diferencias entre la respuesta de cada subclase de IgG en muestras de individuos expuestos a *P. vivax*, para (A) PvRON2 (n = 6) y para (B) Pv12 (n = 11) $p \leq 0,05$. (*) $p<0,05$, (**) $p<0,005$ y (***) $p<0,0005$.

DISCUSIÓN

Anticuerpos contra proteínas del estadio sanguíneo de *P. vivax* son elementos importantes para bloquear la invasión de los glóbulos rojos (149); estos anticuerpos tienen un papel importante en identificar y validar candidatos a vacuna contra *P. vivax* (33, 150-153). En este estudio se confirmó la presencia de una respuesta humoral adquirida naturalmente contra cuatro epítopes B de PvRON2, los cuales fueron reconocidos por anticuerpos IgG y las subclases. Se observó baja frecuencia de respondedores a epítopes B de PvRON2: 20% al 39041, 10% al 39042, 6,6% al 39043 y al 39044; las bajas respuestas también han sido reportadas para otros estadios sanguíneos tales como PvMSP8. Se ha observado una pérdida de la respuesta media a una proteína blanco a medida que ha transcurrido el tiempo, cuando la respuesta promedio ha sido unas diez veces mayor a la proteína recombinante que a epítopes lineales en pacientes con infección aguda (136). Esto ha sido asociado con anticuerpos de vida corta, debido a respuestas de memoria de corta duración o alteraciones de las células B inducida por el parásito (100) y variaciones genéticas del parásito o en las poblaciones expuestas.

Diferencias en la respuesta de anticuerpos contra epítopes B de la proteína PvRON2 entre individuos expuestos de Chocó (83%) y Córdoba (17%), podrían ser atribuidos a varios factores, incluyendo el nivel de parasitemia y el número de episodios (154). Los últimos 3 reportes de SIVIGILA (2015-2017), mostraron una alta incidencia de la infección de *P. vivax* en Chocó a diferencia de Córdoba (Instituto Nacional de Salud, 2017). Otros factores intrínsecos de los respondedores tales como su HLA, sexo, edad, estrés fisiológico, nutrición y otras enfermedades infecciosas podrían estar también involucrados en tales diferencias (155).

Dos de los epítopes B 39042 y 39044, fueron localizados sobre un motivo coil alfa helical de la proteína y otros estudios han mostrado que péptidos seleccionados *in silico* relacionados con estos motivos han sido reconocidos por anticuerpos adquiridos naturalmente y han sido inmunogénicos en ratones (56, 156); sin embargo, los epítopes 39042 (10%) y 39044 (6,6%) tuvieron los más bajos valores de reconocimiento. El epítope 39041 tuvo diferencias significativas en las subclases de IgG; predominó IgG2 mientras que niveles bajos de IgG4 e IgG1 fueron observados. En estudios previos una

respuesta predominante de IgG2 y baja reactividad de IgG4 ha sido asociada con resistencia a la infección y resolución, la relación entre IgG2/IgG4 fue asociada con un papel protector (135). Resultados similares se han encontrado en estudios de PvMSP8 en cuanto a la predominancia del anticuerpo no citofílico IgG2 el cual ha sido asociado con resistencia a malaria por *P. vivax* (136).

Cuando se realizaron ensayos de inmunoblot con la proteína recombinante Pv12 se comprobó el reconocimiento por parte de los sueros de los individuos expuestos naturalmente a la infección por *P. vivax*, lo que indica que es capaz de desencadenar una respuesta inmune humoral después de la infección mediada por memoria. Estos resultados confirmaron el papel antigénico de esta proteína y coincidieron con los estudios realizados por Li *y col.*, y Moreno *y col.* (64, 65).

Mediante el ensayo de ELISA se evaluó el reconocimiento de la proteína Pv12 por parte de los sueros de los pacientes expuestos y se evidencio una positividad del 33%, en otros estudios realizados por Chen y col. y Lin y col., utilizando la técnica de matriz de proteínas, encontraron que el 15% y el 49% de los sueros de expuestos reconocían la proteína (64, 67). En este estudio, aunque la reactividad de los sueros frente a la proteína y a los epítopes fue baja, se observó una mayor reactividad de los sueros expuestos frente al grupo control. Estos datos coincidieron con un estudio de Jangpatarapongsa y col., quienes reportaron niveles de anticuerpos bajos contra P. vivax, pero reactividad específica de los individuos inmunes contra el parásito (157).

La respuesta promedio de IgG del suero de los individuos expuestos a rPv12 fue mayor que la respuesta promedio a los epítopos B evaluados. Se observaron diferencias con respecto a los subtipos de IgG al evaluar la respuesta de las subclases de IgG a la proteína recombinante y a los epítopos B. Se observó una clara tendencia con respecto a la respuesta de IgG3 a la proteína total, mientras que hubo una alta respuesta de IgG2 a los epítopos B con respecto a la baja respuesta a IgG4.

El epítope 39038 tuvo el mejor reconocimiento de anticuerpos adquiridos naturalmente de los cuatro epítopos B evaluados en este estudio; estudios previos han

informado que es inmunogénico en conejos (65). Este péptido puede considerarse un buen inductor de la respuesta inmune humoral y se propone como candidato a vacuna debido a su papel potencialmente protector.

Capítulo 3

Evaluación de la inmunogenicidad de péptidos de las proteínas PvDBP y PvMSP1 de *P. vivax*

MARCO TEÓRICO

Presentación de antígenos en Malaria

En los humanos las células dendríticas se dividen en dos grupos principales: las mieloides y las plasmocitoides. Estas se caracterizan por tener diferentes funciones, por su ubicación y por la expresión de diferentes Receptores de Reconocimiento de Patrones (PRR del inglés, *Pattern Recognition Receptors*). Las células dendríticas mieloides (DCm), tienen marcadores de linaje negativos, son HLA-DR positivos, expresan CD11c y se dividen en $CD1c^{+}$ (BDCA1) y $CD141^{+}$. Se encuentran en casi todos los tejidos y migran a través de la linfa al ganglio linfático, o por la sangre al bazo; producen IL-12 la cual es importante para la diferenciación de las células Th1 (158, 159) (Figura 16).

El otro grupo son las células dendríticas plasmocitoides (DCp), también tienen marcadores de linaje negativo, son HLA DR positivo y expresan CD123, CD303 (BDCA2) y CD304. Residen principalmente en los ganglios linfáticos y producen diferentes citoquinas, cuando maduran IFN-α, y cuando se activan IL-12. La interacción entre el sistema inmune innato y el adaptativo depende de las células dendríticas; estas células se encargan de activar, diferenciar y expandir las células T naïve en células Th1 o Th2, y de esa forma regulan el desarrollo de la respuesta inmune adaptativa (158, 159).

Uno de los puntos importantes en la homeostasis inmune es la interacción entre las células dendríticas y los linfocitos T reguladores (Treg). Durante una infección de malaria aguda, las DCs se encargan de fagocitar los glóbulos rojos infectados, esto lleva a su maduración y activación. En los casos de malaria no complicada por *P. vivax* o *P. falciparum*, se producen cambios en los números absolutos y proporciones de las poblaciones de células Treg y DCs; en la infección por *P. vivax* se produce una alteración en la maduración de las CDs (160, 161).

En un estudio transversal y longitudinal, se evaluaron los subconjuntos de las DCs en pacientes con malaria aguda sin complicaciones, causada por *P. vivax* y *P. falciparum* y se compararon con adultos expuestos no infectados y asintomáticos. Se concluyó que

la pérdida de DCs funcionales en sangre periférica se asocia con apoptosis espontánea significativa debido principalmente a los altos niveles de IL-10 en plasma (162).

En otro estudio transversal en Papua (Indonesia), se evaluó el número y la activación de las DCs en niños y adultos afebriles infectados con *P. vivax* o *P. falciparum*. Los resultados mostraron que las DCp se expandieron en la infección asintomática por *P. vivax*, sugiriendo que estas células pueden ayudar en el control de la parasitemia. En cambio, en estos pacientes se mantuvieron estables las DCm (163).

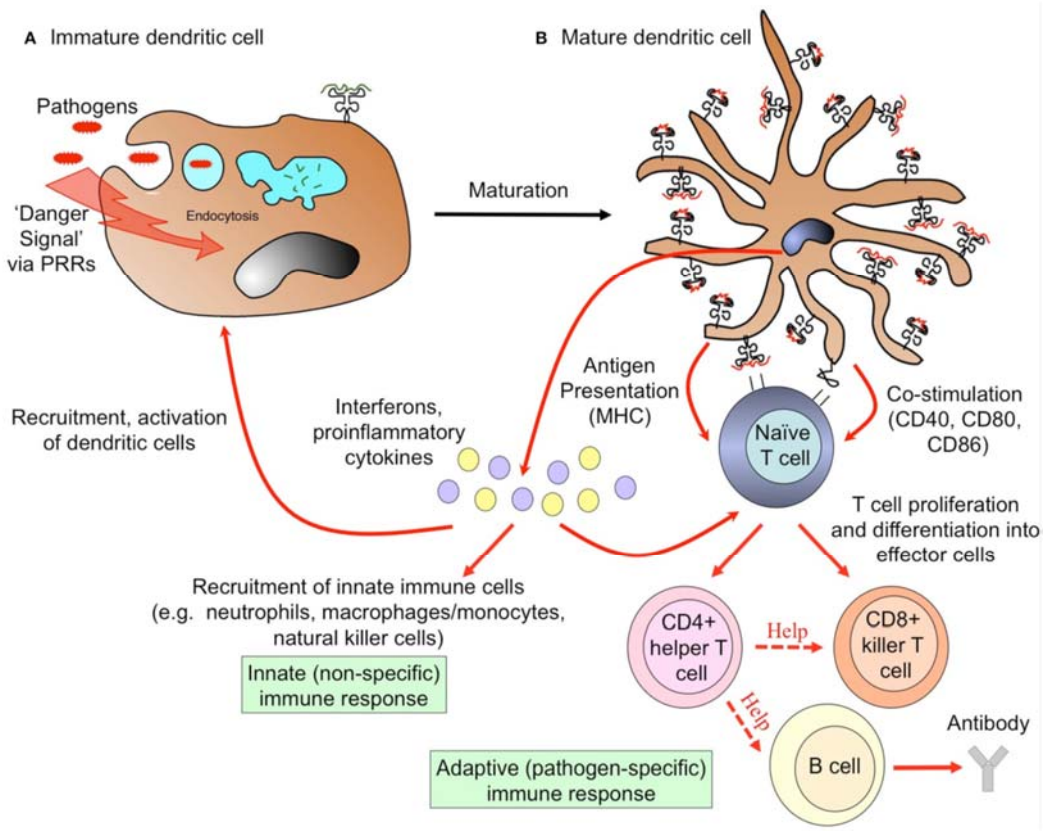


Figura 16. Función de una célula dendrítica como puente entre la inmunidad innata y adaptativa.
La maduración de las DCs se evidencia por cambios morfológicos y funcionales gracias a la captura de patógenos, se genera la formación de dendritas, disminuye su capacidad fagocítica y la redistribución de moléculas del MHC. Las DCs maduras migran a los ganglios linfáticos para presentar los antígenos a los linfocitos T y B, además secretan interferones y citoquinas que inician la cascada de la respuesta inmune adaptativa. Tomado de Yap XZ., 2019 (164).

Células dendríticas aceleradas

Diversos estudios han mostrado que las DC tienen como funciones principales la captura y procesamiento del antígeno, así como la inmunoestimulación de los linfocitos T. Esto depende de la etapa de maduración en la que se encuentren, como células inmaduras captan y procesan el antígeno; y cuando maduran se convierten en inmunoestimuladoras de las células T naïve (TN). A partir de los monocitos de sangre periférica se puede diferenciar *in vitro* a DCs inmaduras, con la adición de IL-4 y GM-CSF (165). Convencionalmente el proceso de maduración de DCs dura siete días, lo cual se obtiene adicionando un coctel de citoquinas proinflamatorias que contiene IL-1β, IL-6, TNF-α y PGE-2 (coctel estándar). Dauer *y col.*, desarrollaron por primera vez DCs derivadas de monocitos maduras en un periodo de 48 horas. Estas células presentaron características fenotípicas y funcionales similares a las CD derivadas en 7 días y al igual fueron capaces de presentar e inducir una respuesta inmune de linfocitos T naïve de la misma forma que lo hacían las DCs de 7 días (166).

Otros estudios han descrito una combinación de citoquinas para la polarización funcional de las DCs denominadas DCs polarizadas tipo 1 o DCs α polarizadas tipo 1 (αDC1), capaces de inducir una fuerte respuesta de células T CD4+ de tipo Th1 principalmente por su alta capacidad de producir IL-12p70. Este coctel incluye entre otras citoquinas IFN-α y ácido polinosínico:policitidílico (poly I:C) (167).

Evaluación de la respuesta inmune *in vitro*

Con el fin de evaluar la inmunogenicidad de las vacunas, es necesario identificar y caracterizar los linfocitos T específicos de antígeno dentro de un repertorio naïve. Se han desarrollado tetrámeros para identificar repertorios de clase I o multímeros para caracterizar repertorios de clase II, pero estos pueden evaluar un solo epítope a la vez e identificar células que se unen pero que no necesariamente reconocen el antígeno procesado. Un método alterno es emplear DCs pulsadas con antígenos para que lo procesen y presenten al repertorio de linfocitos T naïve (168).

Moser *y col.*, publicaron un estudio en el que estandarizaron un co-cultivo de DCs derivadas con citoquinas más linfocitos T CD4+ autólogos, como resultado obtuvieron

que estas DCs fueron capaces de inducir una respuesta primaria de células T ayudadoras contra una variedad de antígenos protéicos. Estos cultivos produjeron una respuesta efectora y poblaciones de células T de memoria, que podrían mantenerse hasta por 6 semanas *in vitro* (169).

La falta de ensayos de rutina para evaluar células T es debido a la baja cantidad en sangre de linfocitos T específicos a un antígeno (0.1% - 0.001%) reportado por Mallone *y col.* (170). En un estudio publicado en 2011 por Martinuzzi *y col.*, estandarizaron un ensayo en donde obtenían DCs que procesaban y presentaban antígenos a linfocitos T *in situ*, empleando CMSP o sangre total. Fueron llamados ensayos de DCs de co-cultivo acelerado (acDC del inglés, *accelerated cocultured DC assays*). Esta estrategia permite amplificar las respuestas de linfocitos T específicos de antígeno *in situ* (171).

Poblaciones de Linfocitos T

Las células T naïve están compuestas por una población homogénea que no ha tenido contacto con antígenos, tiene una vida media de 6 a 10 años y expresan en su superficie CCR7, CD62L y CD45RA, y no expresan CD45RO. CCR7, CD62L están encargados de localizar los linfocitos T en órganos linfoides secundarios; mientras que CD45RA y RO se encargan de la transducción de señales del TCR (172). Las células T naïve se dividen y se transforman en células de memoria y posteriormente en células efectoras (TEF), gracias a las señales del TCR y de moléculas co-estimulatorias como el CD28. Estas se pueden diferenciar a células Th1 productoras de IFN-ɣ, en presencia de IL-2 y generar la expresión de T-bet, o a células Th2 en presencia de IL-4 con expresión de GATA-3 (173).

Al cabo de dos semanas, la mayor parte de las células efectoras mueren y queda una población de células de larga vida, llamadas células de memoria. Esta población es heterogénea e inactiva, pero inclusive en ausencia de estímulo pueden auto renovarse y sobrevivir a largo plazo. La formación de la población de células T CD4+ de memoria, inicia cuando se produce la sinapsis inmunológica. Esto ocurre en los órganos secundarios linfoides, cuando el TCR de los linfocitos T naïve se une al complejo péptido-

Complejo Mayor de Histocompatibilidad clase II (pMHCII) de las células presentadoras de antígeno.

Las células de memoria efectora (TEM) producen IFN-ɣ, IL-4 e IL-5 horas después de la estimulación del TCR, y expresan receptores que permiten la migración a lugares no linfoides. Las células de memoria central (TCM) no producen citoquinas inmediatamente, pero secretan IL-2, proliferan y generan tiempo después citoquinas efectoras. Las TCM expresan CD62L y CCR7, que les permite migrar a los ganglios linfáticos y a los órganos linfoides de mucosas (173).

Una subpoblación de linfocitos T CD4+ son las células T foliculares ayudadoras (del inglés, *T follicular helper cells* TFH), estas proporcionan ayuda a los linfocitos B y generan células B de memoria de alta afinidad. Poseen en superficie el ligando CD40, que se une al CD40 de los linfocitos B que junto a la secreción de IL-21, promueven la diferenciación y el cambio de clase de inmunoglobulina de los linfocitos B. Expresan el receptor de quimioquinas CXCR5 que guían su migración dentro de los folículos, y en alta cantidad el receptor inhibitorio inmune PD-1, el cual regula las TFH en los centros germinales (174).

Las células TFH expresan CXCR3 o CCR6, y se dividen en 3 grupos principales: células TFH1 (CXCR3+ CCR6-), expresan el factor de transcripción T-bet y producen citoquinas de respuesta Th1 como IFN-ɣ. Células TFH2 (CXCR3- CCR6-), expresan el factor de transcripción GATA3 y producen citoquinas Th2 entre las que se encuentran IL-4, IL-5 e IL-13. Por último, células TFH17 (CXCR3- CCR6+) que expresan el factor de transcripción ROR-ɣ y producen citoquinas de respuesta Th17 como la IL-17a y la IL-22 (174).

Proteínas de interés

Proteína de la superficie del merozoíto-1 (PvMSP-1)

La proteína MSP-1 es codificada por el gen *pv200* (175), tiene un peso molecular de aproximadamente 200 kDa. El perfil de procesamiento proteolítico genera 4 fragmentos: 83 kDa, 30 kDa, 38 kDa, y 42 kDa, y a partir de la hidrólisis del fragmento de 42 kDa (región C-terminal), se genera un polipéptido de 33 kDa que se libera al torrente sanguíneo, mientras el fragmento de 19 kDa restante permanece unido a la fase de anillo recién formado después de la invasión al reticulocito (176, 177).

El fragmento de 19 kDa perteneciente a la región C-terminal es uno de los más estudiados de la proteína *Pv*MSP-1 (Pv200). Resultados reportados por Kaslow y Kumar, demostraron la capacidad inmunogénica de la proteína recombinante $Pv200_{19}$ en ratones vacunados (175). Estudios posteriores, se enfocaron en evaluar la respuesta inmune adquirida a esta proteína en personas expuestas a la infección natural, en un área endémica de Brasil. Soares *y col*., mostraron que *Pv*MSP-1 es altamente inmunogénica en humanos y que la región C-terminal contiene dos dominios tipo EGF (del inglés, *Epidermal Growth Factor*) que son inmunogénicos, induciendo tanto respuesta de anticuerpos, como de células T en humanos (178). Fernandez-Becerra *y col*. evaluaron las subclases de IgG en niños, encontrando una respuesta predominante de tipo IgG1 contra la región C-terminal, y porcentajes bajos de IgG3 e IgG4. En adultos, los anticuerpos predominantes fueron de tipo IgG3, observándose una correlación de anticuerpos con la edad (179).

La proteína recombinante completa fue inmunogénica en monos tití (*Callithrix jacchus jacchus*), sin embargo, la respuesta fue variable según el adyuvante utilizado (180). En otro estudio, monos *Aotus* sp. fueron vacunados con dos polipéptidos recombinantes pertenecientes al fragmento de 33 kDa de la región C-terminal de MSP-1. Los *Aotus* sp. produjeron anticuerpos capaces de reconocer la proteína nativa y lograron controlar la parasitemia (181). Un estudio adicional usando las misma mezcla de recombinantes, pero inmunizando con tres dosis en vez de dos, mostró que los niveles de IFN-γ encontrados se relacionaban con el control de la parasitemia y que la

eficacia protectora era superior a la obtenida previamente con dos dosis, siendo cercana al 80% (182).

En infecciones naturales, los anticuerpos anti-*Pv*MSP-1 fueron predominantemente de tipo IgG, los cuales reconocían la región N-terminal, más no la región C-terminal, a pesar de que el reconocimiento frente a esta última incrementa con las exposiciones sucesivas al parásito (178, 183, 184). Estos resultados demostraron la asociación de anticuerpos frente a la región N-terminal de *Pv*MSP-1 tipo IgG3, con la reducción del riesgo de infección y la protección clínica (177).

El fragmento de 42 kDa de la proteína MSP-1 también ha sido estudiado por su potencial como candidato a vacuna. Su inmunogenicidad fue evaluada en ratones, y altos niveles de anticuerpos IgG1, IgG2a e IgG2b fueron observados, mientras la respuesta de tipo IgG3 fue baja. También se encontró una alta respuesta proliferativa a la estimulación con *Pv*MSP-1 p42, detectándose altos niveles de IL-2, IL-10 e IFN-γ en los sobrenadantes de los cultivos. Los resultados mostraron que esta proteína indujo una respuesta celular y humoral en ratones (185). Cuando se evaluó la respuesta inmune humoral naturalmente adquirida contra las proteínas de 19 kDa y 42 kDa de la región C-terminal, se encontró un mayor reconocimiento de la proteína $MSP1_{19}$ que de la $MSP1_{42}$ (186).

Por su demostrada capacidad inmunogénica y protectora en modelos experimentales, además de su antigenicidad e inmunogenicidad en humanos, *Pv*MSP-1 continúa siendo uno de los candidatos más promisorios y estudiados, sin embargo, su elevado polimorfismo, hace que el diseño de vacunas basadas en esta proteína requiera un diseño cuidadoso.

Proteína de Unión a Duffy (PvDBP)

La Proteína de Unión a Duffy (del inglés, *Duffy Binding Protein* (DBP)) de *P. vivax* es una proteína de 140 kDa aproximadamente, secretada por los micronemas de merozoítos de *P. vivax* y *P. knowlesi* (187). DBP hace parte de la superfamilia de proteínas que poseen un dominio denominado DBL (del inglés, *Duffy Binding-like*).

Contiene un dominio extracelular que se divide en 6 regiones, la región 7 contiene el dominio transmembranal y el citoplasmático (188). La región I tiene un gran número de residuos cargados, la región II en el extremo 5' contiene residuos de cisteínas y aminoácidos aromáticos. La región VI también es rica en cisteínas, precede la región transmembranal y comparte las mismas características con la región II (81, 187, 189). Durante la invasión, la proteína DBP está expuesta en la superficie del merozoíto para unirse al receptor del eritrocito; esto la hace visible al sistema inmune, que puede reconocerla y desencadenar una respuesta de anticuerpos capaz de bloquear su interacción con el receptor (190).

La región II de *Pv*DBP es la encargada de la adhesión y el reconocimiento del receptor del antígeno del grupo sanguíneo Duffy (DARC, del inglés *Duffy Antigen Receptor for Chemokines*), contiene 12 residuos de cisteína, cuyas posiciones son conservadas entre las dos especies (*P. vivax* y *P. knowlesi*), además tiene aminoácidos aromáticos altamente conservados. Se localiza hacia la región N terminal de la proteína y es un dominio característico de las proteínas de la familia EBA, conformado por aproximadamente 330 aminoácidos. Es responsable de la unión irreversible entre el eritrocito y el merozoíto, siendo la ruta predominante de invasión en *P. vivax* (27, 191).

A la región II se le ha llamado el dominio funcional de unión y, a pesar de presentar baja inmunogenicidad durante una infección natural, se ha reportado que exposiciones sucesivas a la proteína son necesarias para que el sistema inmune dirija una respuesta de anticuerpos capaz de bloquear la interacción receptor-ligando, por lo cual esta región es potencial candidato para el desarrollo de una vacuna (190, 192). En estudios de inmunización, *Pv*DBPII es capaz de producir una alta concentración de anticuerpos que inhiben la invasión, al impedir su interacción con el receptor de los eritrocitos en ensayos de unión *in vitro* (193).

En infecciones naturales, se ha observado que los anticuerpos generados frente al dominio de unión a DARC (DBPII) reconocen epítopes altamente polimórficos, lo que confiere una inmunidad cepa-específica (194). Por lo tanto, la eficacia de DBPII como candidato a vacuna dependerá, no solo de la inducción de anticuerpos, sino también de optimizar el reconocimiento de epítopes conservados (195).

Identificación, caracterización y regresión de clústeres: CITRUS

El estudio del comportamiento de los subconjuntos celulares sirve como un marcador de estatus o de resolución clínica de una enfermedad. El método empleado para identificar esas subpoblaciones en una muestra es la identificación manual, pero este análisis es laborioso y dependiente del analizador, lo cual produce sesgos cuando se examina un conjunto de datos. Bruggner *y col.* desarrollaron un método automatizado para identificar los subconjuntos de células estratificadas (CITRUS), el cual identifica grupos de células con características fenotípicas similares, determina el comportamiento de esos grupos con métricas biológicamente interpretables y emplea algoritmos para identificar los subconjuntos con comportamiento predictivo de punto final (196).

Son varios los resultados obtenidos por CITRUS, primero genera un registro de las propiedades de la aglomeración celular que están asociadas con el punto final del ensayo; además crea unas gráficas que muestra la calidad de predicción del modelo, el fenotipo de los grupos celulares identifica los grupos que diferencian las muestras analizadas, el fenotipo de esos grupos en histogramas y la abundancia de esos grupos entre las muestras contrastadas. Los resultados se interpretan en tres pasos: 1) evaluación estadística de las asociaciones detectadas entre las propiedades del grupo y los puntos finales del ensayo; 2) relación entre las propiedades de grupo estadísticamente significativas y el punto final del ensayo; y 3) establece el fenotipo de las aglomeraciones celulares de las cuáles se calcularon las características significativas de aglomeración.

METODOLOGÍA

En la tercera fase del proyecto, se seleccionaron HABPs de las proteínas PvMSP1 y PvDBP mediante análisis evolutivos se determinaron las regiones conservadas entre las diferentes cepas de *P. vivax* y sus residuos críticos fueron modificados para mejorar su estabilidad en el MHC clase II. Posteriormente, se caracterizaron por citometría de flujo las diferentes subpoblaciones (subpoblaciones de memoria, TFH, quiescentes) y el nivel de activación (CD69) de LT CD4+ en respuesta a la estimulación *in vitro* con CD pulsadas con los péptidos tanto nativos como modificados.

Análisis evolutivo de las proteínas PvMSP1 y PvDBP de *P. vivax*

Con el propósito de determinar regiones conservadas con potencial para el diseño de candidatos a vacunas, se seleccionaron secuencias de los genes *pvmsp1* y *pvdbp* no idénticas que tuvieran al menos un 90% de cobertura de secuencia. El alineamiento de las secuencias se realizó con el algoritmo MUSCLE (197) y se corrigió manualmente. Se ejecutaron los análisis con MEGA X v10.1.7 (198) y se construyeron los árboles usando del método de Maximun Likelihood.

Dado que la conservación en sí misma no es indicador suficiente de restricción funcional, adicionalmente se determinó si las regiones conservadas estaban también sometidas a una presión selectiva negativa. Así, se identificaron codones específicos bajo selección natural entre especies utilizando enfoques de Wu & Li (Ka/Ks) (199), bayesianos y de máxima verosimilitud basados en codones (SLAC, FEL, REL (200), MEME (201) y FUBAR (202)), después de la recombinación por el método GARD (203). Los métodos basados en codones estiman la tasa de evolución (ω) en cada codón utilizando una prueba estadística para determinar si ω es significativamente diferente de 1 (evolución neutra). El algoritmo REL de Branch-site (204) se utilizó para identificar linajes bajo selección positiva episódica (la selección tiene ocasionalmente períodos transitorios de evolución adaptativa enmascarados por selección negativa o evolución neutral). Los análisis se efectuaron usando solo las secuencias de las cepas representativas previamente descritas de *P. vivax*. Adicionalmente, con el fin de evaluar la constancia de tal restricción funcional con respecto a especies relacionadas del mismo

linaje de *P. vivax*, se realizó un análisis incluyendo secuencias de referencia para ambos genes de *P. cynomolgi*, *P. knowlesi* y *P. inui*. El servidor web Datamonkey se utilizó para realizar estos análisis (205).

Selección de péptidos de las proteínas PvMSP1 y PvDBP de *P. vivax*

En la selección de los sectores candidatos para la determinación de péptidos candidatos se incluyó como criterio la capacidad de unión de las secuencias propuestas con eritrocitos, determinándose su posible calidad de HABPs (del inglés, *High affinity binding peptide*) según lo reportado por Rodríguez *y col*. (206) y Ocampo *y col*. (81) También se consideró reportes previos sobre la presencia de epítopes T y B.

Teniendo en cuenta las secuencias reportadas en PlasmoDB para PvDBP código de acceso PVX_110810 y PvMSP1 código de acceso PVX_099980, se determinó la presencia de potenciales epítopes B usando los métodos AAP (207), BCPRED (208), FBCPRED (209), ABCPRED (210) , IEDB B-cell epitope tools (211) , BEPIPRED 1.0 (212) y BEPIPRED 2.0 (213). Las zonas con potenciales epítopes B fueron asignadas usando una estrategia de consenso entre los métodos usados.

La estructura de las proteínas analizadas fue obtenida de la base de datos Protein Data Bank, con los códigos 2NPR para PvMSP1 (214) y 3RRC, 4NUU para PvDBP (215, 216), sometidas a un proceso de mejoramiento. A partir de estas estructuras se determinó el grado de exposición al solvente de las regiones de interés, como criterio adicional para evaluar la capacidad de interacción molecular de las mismas. Se usaron los software GETAREA (217) y POLYVIEW (218) para este fin.

A partir de los resultados se seleccionaron las regiones en donde convergían las condiciones de ser de baja variabilidad, presión selectiva negativa, condición de ser HABPs y alta exposición al solvente. Las secuencias propuestas serian posteriormente modificadas para generar epítopes T de alta afinidad a los alelos de HLA-DR objeto de estudio, preservando las zonas de potenciales epítopes B sin modificación.

A partir de las regiones seleccionadas según los criterios anteriormente descritos, se diseñaron péptidos de 20 a.a. preservando la zona de interés como

HAPB/Epítope B, considerando adicionalmente una secuencia contigua para modificarse, empleando el menor número de sustituciones posibles, con el fin de obtener un epítope T viable. Definiendo un marco de unión putativo de 9 a.a. para el MHC-DR (28, 219) y se realizaron sustituciones en cada posición (-1 a 10) por todos los aminoácidos de ocurrencia natural, excepto cisteína (la síntesis de péptidos con este aminoácido produce resultados indeseados, dada la formación de puentes disulfuro). Se evaluaron en una primera ronda de cálculo 1151569 de péptidos únicos por cada región seleccionada, correspondientes a la combinatoria para 1, 2 y 3 sustituciones.

Se realizó un tamizaje *in silico* de los epítopes T con alta afinidad de unión para las familias alélicas HLA-DRβ1*04, *07, *11 y *13 que son las más frecuentes en las poblaciones humanas ubicadas en las áreas endémicas para malaria por *P. vivax* (70), con el programa NetMHCIIpan4.0 (220), esta plataforma predice la unión de péptidos utilizando redes neuronales artificiales entrenadas con un conjunto de datos de mediciones de afinidad de unión (BA) y espectrometría de masas de ligando eluido (EL). El resultado del modelo es una puntuación de predicción de la probabilidad de que un péptido sea presentado de forma natural por el receptor MHC II elegido. El resultado provee un rango de puntuación, que normaliza la puntuación de predicción comparándola con la predicción de un conjunto de péptidos aleatorios. Se definieron a los péptidos como de unión fuerte si mostraban un rank ≤ 1 y de unión débil a los que muestran un rank ≤ 5. Los péptidos seleccionados deberían ser de unión fuerte tanto para EL como para BA; adicionalmente, se debía preservar el marco de unión putativo seleccionado. En caso de que alguno de los sectores no se detectaran péptidos suficientes con estas características, una cuarta sustitución fue efectuada sobre aquellos péptidos con mejores puntajes a MHC clase II.

Los péptidos obtenidos fueron analizados en la herramienta de Proteotypic Peptide Analyzing Tool[1] para verificar la facilidad de síntesis de éstos y otras características fisicoquímicas relevantes. Se verificó la estructura secundaria de los

[1] *Peptide analyzing tool: Thermo fisher scientific - US*. Peptide Analyzing Tool | Thermo Fisher Scientific - US. (n.d.). Retrieved November 10, 2021, from https://www.thermofisher.com/co/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html.

péptidos para evitar aquellos con tendencias helicoidales (para garantizar su ajuste estructural a la molécula del HLA-DR) (221) con el programa PEP2D (222) y se realizó un modelamiento 3D *ab initio* usando la plataforma PEP-FOLD (223). Finalmente, se verificó que los péptidos candidatos no presentaran alta homología con el proteoma humano, para lo que se realizó un pBlast contra la base UniprotKB/swissprot. Los péptidos que por consenso llenaran los requisitos señalados fueron seleccionados.

Selección de individuos de áreas no endémicas para la infección por *P. vivax*

Se colectaron muestras de SP de 50 individuos que vivían en áreas no endémicas para malaria por *P. vivax*, que cumplían con los siguientes criterios de inclusión: 1) Mayor de 18 años, 2) Que no haya vivido en áreas endémicas para malaria, 3) Que no haya padecido la enfermedad. Este estudio se llevó a cabo de acuerdo con el marco legal para la investigación en Colombia y la resolución 8430 de 1993 del Ministerio de Salud. Para la toma de muestras de SP, todos los individuos tuvieron el menor riesgo y los datos se manejaron confidencialmente.

Las muestras se colectaron después que los sujetos firmaban un consentimiento informado. Se realizó la tipificación de los alelos HLA-DRβ1 de interés, según el protocolo descrito, para conformar cuatro grupos experimentales (uno por cada alelo), compuestos por dos individuos cada uno.

Toma de Muestras

Para los ensayos de estimulación *in vitro* de linfocitos T, a los individuos que conformaron los grupos experimentales, se les realizó una segunda toma de muestra de SP de 450 mL para la obtención de CMSP y derivación de células dendríticas. Las muestras fueron colectadas por personal del Banco Distrital de Sangre del Instituto Distrital de Ciencia, Biotecnología e innovación en Salud-IDCBIS. Se colectaron en bolsas cuádruples de donación con anticoagulante CPD y los Buffy coat se procesaron en condiciones de esterilidad.

Ensayos de Inmunogenicidad con Péptidos Nativos y Modificados

Aislamiento de Células Mononucleares de Sangre Periférica (CMSP)

A partir de la segunda toma de muestra, se aislaron las CMSP con el gradiente de densidad Ficoll-Paque PLUS (GE Healthcare), según el protocolo descrito previamente. Se evaluó la viabilidad celular con el colorante de exclusión azul de tripán y se realizó el conteo en cámara de Neubauer. Las CMSP se criopreservaron en medio de congelación que contenía 50% de RPMI-1640, 40% de suero fetal bovino (SFB) y 10% de dimetil sulfóxido (DMSO) a una concentración de 10^7 células/mL y fueron almacenadas en tanque de nitrógeno líquido (-170°C) hasta su uso.

Diferenciación de CMSP a células dendríticas inmaduras (CDi)

Se cultivaron 2 x 10^6 CMSP por pozo en 500 µL de medio AIM V (Gibco), el medio contenía L-glutamina, sulfato de estreptomicina (50 µg/ml) y sulfato de gentamicina (10 µg/ml) y fue suplementado con SFB al 1%. El proceso de diferenciación y maduración se realizó según lo publicado por Martinuzzi y cols y Chamucero y cols (171, 224). Las placas se incubaron a 37°C en una atmósfera con 5% de CO_2 durante 24 horas (Figura 17).

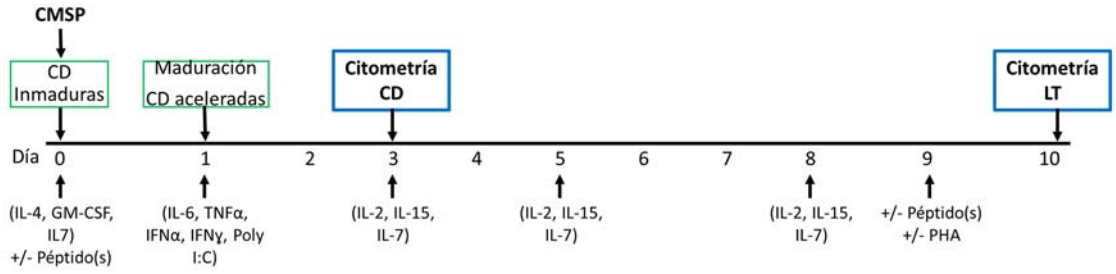


Figura 17. Esquema de cocultivos acelerados para la obtención de CD y estimulación de LT. Diagrama de flujo donde se emplearon células mononucleares de sangre periférica (CMSP) para la obtención de CDs aceleradas y la estimulación con los péptidos para la evaluación de las poblaciones de LT de memoria.

Caracterización fenotípica de las CD por citometría de flujo

El fenotipo de las CD inmaduras y maduras se evaluó con el uso de marcadores de superficie. Se determinó el grado de expresión de las moléculas asociadas a su

maduración: CD80, CCR7, CD83 sobre expresadas y CD209 (DC-SIGN, BD) expresada a la baja, en el citómetro de flujo FACS Canto II y los datos se analizaron con el software FlowJo X v10.0.7r2 (Ashland, Oregon, USA).

Estimulación *in vitro* de LT CD4 con epítopes nativos y modificados

A las CMSP se le adicionaron 10 µg/mL de cada péptido de interés el día 0. A los 3, 5 y 8 días se remplazó la mitad del medio AIM V suplementado con SFB al 1% y se adicionó 25 ng/mL de IL-15, 100 UI de IL-2 y 0.5 ng/mL de IL-7. A los 9 días se adicionó 10 µg/mL de cada péptido de interés y 10 µg/mL de PHA como control positivo. Finalmente, a los 10 días se recolectaron los SBN de cultivo y se almacenaron a -80°C para posteriormente realizar la cuantificación de las citoquinas Th1/Th2 con el kit de CBA (BD). Las CMSP estimuladas con PHA y con un péptido de TT fueron empleadas como controles positivos y las CMSP sin estímulo como control negativo. Al término del periodo de incubación, se recolectaron las células y se marcaron con los anticuerpos monoclonales para determinar poblaciones de linfocitos T CD4, luego las muestras se leyeron en el citómetro de flujo FACS Canto II.

Caracterización fenotípica de las subpoblaciones de LT CD4+ por citometría de flujo

La pureza de las células se comprobó con el uso de marcadores de superficie por citometría de flujo. Se evaluaron los marcadores de las subpoblaciones de linfocitos CD4+ con los anticuerpos monoclonales: anti-CD4 Pacific blue, anti-CCR7 Alexa fluor 488, anti-CD45RA PeCy 5.5, anti-CD69 PeCy7, anti-PD1 APC, anti-CXCR5 PE, anti-CXCR3 APCCy7. Las muestras se incubaron a 4°C durante 20 min, fueron adquiridos 150.000 eventos por muestra en el citómetro de flujo FACS Canto II (BD Biosciences) y el análisis manual de los resultados fue realizado con el software FlowJo X v10.0.7r2 (Ashland, Oregon, USA). Los resultados fueron normalizados con respecto al valor del control negativo, se emplearon pruebas no paramétricas para determinar diferencias estadísticas entre las CMSP estimuladas con los péptidos nativos y las estimuladas con los péptidos modificados, esto se realizó con el software GraphPad Prism v7 (San Diego, CA, USA). Además se hizo un análisis automatizado con el software CITRUS v0.8 (del

inglés, *cluster identification, characterization, and regression*) (196) en la plataforma R (225) con el fin de identificar grupos de células estratificadas que se expresaran diferencialmente para cada condición experimental.

Determinación de citoquinas en sobrenadante

Al finalizar el tiempo de cultivo (10 días), se colectaron 100 µL de SBN del cultivo de los linfocitos estimulados y sin estimular. Los niveles de citoquinas se determinaron con el kit comercial BD CBA Human Th1/Th2 Cytokine Kit II (San Jose, CA, USA), siguiendo las instrucciones del fabricante. El ensayo se leyó en el citómetro de flujo FACS Canto II y el análisis de los datos se realizó con el software FCAP Array v3.0.1. Los valores obtenidos fueron comparados entre las CMSP no estimuladas y las estimuladas, la producción de las citoquinas se expresó en pg/mL.

RESULTADOS

Ensayos de inmunogenicidad de las proteínas PvMSP1 y PvDBP de *P. vivax*

Análisis evolutivo de las proteínas

Fueron seleccionadas 59 secuencias del gen *pvmsp1* no idénticas con el 90% de cobertura de secuencia. Se realizó el alineamiento de las secuencias con el algoritmo MUSCLE (197) y se corrigió manualmente. Se ejecutaron los análisis con MEGA X v10.1.7 (198) y se construyó un árbol usando el método de Maximun Likelihood con el modelo de sustitución JTT+G+I y un BIC 5254.1 Se realizó el mismo procedimiento para el gen *pvdbp*, seleccionándose 137 secuencias en total. Se ejecutaron los análisis con MEGA, el análisis se realizó con el mismo método y el modelo JTT+G con un BIC 8488.1. Resultado de este análisis, se observó que usando las secuencias de seis cepas representativas se puede evaluar la diversidad para ambos genes en *P. vivax*: VCG-I, Sal-I, Brasil-I, India-VII, Mauritania-I y Corea del Norte (226).

Selección *in silico* de péptidos nativos y modificados para los alelos HLA-DRβ1* de interés

Se sintetizaron los epítopes T nativos y modificados, según el protocolo descrito para emplearlos en los ensayos de inmunogenicidad. Para cada una de las proteínas PvMSP1 y PvDBP, se sintetizaron dos péptidos nativos y un epítope T modificado para cada alelo (HLA-DRβ1*04:01, *07:01, *11:01 y *13:02) de acuerdo con los criterios mencionados. Se obtuvo un total de 16 epítopes para las dos proteínas (Tabla 5,Tabla 6).

Tabla 5. Péptidos nativos y modificados seleccionados *in silico* para PvMSP1.

Código del péptido	Secuencia	Core F	Alelo HLA-DRβ1*	Proteína PvMSP1	Rank-BA	Binder-EL
43466	NEVKSSGLL**EKLMKSKLI**KE	EKLMKSKLI		Nativo 1		
43467	NEVKSSGLL**Y****KLMKSNLD**KE	YKLMKSNLD	DRβ1*04:07	Modificados	0,05	<=SB
43468	NEVKSSGL**KY****KLT****KSKLI**KE	YKLTKSKLI	DRβ1*07:01		0	<=SB
43469	NEVKSSGLL**Y****KLMK****K****KLG**KE	YKLMKKKLG	DRβ1*11:01		0	<=SB
43470	NEVKSSGLL**I****KLN****KSKLV**KE	IKLNKSKLV	DRβ1*13:02		0	<=SB
43471	NPFKYSSSGE**YIIKDPYKL**L	YIIKDPYKL		Nativo 2		
43472	NPFKYSSSGE**YII****I****DT****A****KD**L	YIIIDTAKD	DRβ1*04:07	Modificados	0,28	<=SB
43473	NPFKYSSSGE**YII****TK****PYKL**P	YIITKPYKL	DRβ1*07:01		0,05	<=SB
43474	NPFKYSSSGE**YII****I****D****K****YK****G**L	YIIIDKYKG	DRβ1*11:01		0,43	<=SB
43475	NPFKYSSSGE**YII****NK****PYKL**R	YIINKPYKL	DRβ1*13:02		0,21	<=SB

Tabla 6. Péptidos nativos y modificados seleccionados *in silico* para PvDBP.

Código del péptido	Secuencia	Core F	Alelo HLA-DRβ1*	Proteína PvDBP	Rank-Ba	Binder-El
43476	PDRR**YQLSMKELT**NLVNNTD	YQLSMKELT		Nativo 1		
43477	PDRR**YQLS****KT****EL****D**NLVNNTD	YQLSKTELD	DRβ1*04:07	Modificados	0,46	<=SB
43478	PDRR**YQLS****HS****ELV**NLVNNTD	YQLSHSELV	DRβ1*07:01		0	<=SB
43479	PDRR**YQL****IK****KEL****G**NLVNNTD	YQLIKKELG	DRβ1*11:01		0,04	<=SB
43480	PDRR**YQL****NK****K****N****LV**NLVNNTD	YQLNKKNLV	DRβ1*13:02		0,27	<=SB
43481	DRLKQE**LDEFNEVAF**ENEIN	LDEFNEVAF		Nativo 2		
43482	DRLKQE**FI****EFN****TVA****D**ENEIN	FIEFNTVAD	DRβ1*04:07	Modificados	1,3	<=SB
43483	DRLKQE**LDYT****NA****VAF**ENEIN	LDYTNAVAF	DRβ1*07:01		3,22	<=SB
43484	DRLKQE**FR****EFN****RVA****G**ENEIN	FREFNRVAG	DRβ1*11:01		1,66	<=SB
43485	DRLKQE**L****RIN****NEVAF**ENEIN	LRINNEVAF	DRβ1*13:02		0,84	<=SB

Tipificación de individuos de áreas no endémicas para la infección por *P. vivax* y conformación de los grupos experimentales

De los 56 individuos tipificados en Bogotá (área no endémica) (Tabla 7), se seleccionaron 8 individuos no expuestos (dos por cada alelo de interés - resaltados) para conformar los grupos experimentales para los ensayos de inmunogenicidad (Tabla 8).

Tabla 7. Frecuencia alélica HLA-DRβ1 de los individuos no expuestos.

Alelo DRβ1*	n	Frecuencia alélica%
DRβ1*01	11	9,82
DRβ1*03	4	3,57
DRβ1*04	34	30,36
DRβ1*07	11	9,82
DRβ1*08	9	8,04
DRβ1*10	2	1,79
DRβ1*11	7	6,25
DRβ1*12	1	0,89
DRβ1*13	15	13,39
DRβ1*14	5	4,46
DRβ1*15	10	8,93
DRβ1*16	3	2,68
Total	**112**	**100**

Tabla 8. Grupos experimentales ensayos de inmunogenicidad.

Alelo HLA-DRβ1* Individuos no expuestos	Identificación de la muestra	Tipificación HLA-DRβ1*		Serotipo HLA-DR	
DRβ1*04:07	BOGINM011	04:07:01G	14:02:01G	DR53	DR52
	BOGINM010	04:07:01G	15:01:01G	DR53	DR1
DRβ1*07:01	BOGINM002	07:01:01G	08:04:01	DR53	DR8
	BOGINM003	13:01:01G	07:01:01G	DR52	DR53
DRβ1*11:01	BOGINM007	11:01:01G	07:01:01G	DR52	DR53
	BOGINM001	11:01:01G	12:01:01G	DR52	
DRβ1*13:02	BOGINM006	13:02:01	08:02:01	DR52	DR8
	BOGINM012	13:02:01	04:07:01G	DR53	DR52

Ensayos de inmunogenicidad con péptidos nativos y modificados

Obtención y estimulación de células dendríticas aceleradas (aCDs)

Inicialmente se purificaron monocitos de sangre periférica por el método de selección negativa usando el kit Pan Monocyte Isolation Kit (Miltenyi Biotec), según las indicaciones del fabricante. Cuando se evaluó el rendimiento de la purificación por citometría de flujo, fue de menos del 30%. Razón por la cual se decidió realizar la inducción y maduración de las CDs a partir de las CMSP para la presentación antigénica y activación de linfocitos T *in situ*, al mismo tiempo se pulsaron con los péptidos seleccionados *in silico*, de acuerdo con la metodología empleada por Martinuzzi *y col.* con algunas modificaciones (171).

Las CMSP estimuladas únicamente con IL-4 y GM-CSF (células dendríticas inmaduras, CDi) y las CMSP estimuladas con las mismas citoquinas de diferenciación y el coctel de citoquinas proinflamatorias (células dendríticas maduras, CDm), fueron empleadas como controles de diferenciación y maduración de células dendríticas, respectivamente. Se les realizó un seguimiento por microscopía óptica y al cabo de tres días se evaluaron por citometría de flujo, tanto los parámetros de complejidad y tamaño (SSC-A vs FSC-A, respectivamente) como la expresión de los marcadores de superficie CD80, CCR7, CD83 sobre expresadas y CD209 (DC-SIGN) expresada a la baja en CD maduras.

En las células dendríticas maduras se observaron cambios en la morfología celular, pasaron de ser células redondas a alargadas y el número de dendritas aumentó, posterior al cóctel de maduración (Figura 18A). Además, por citometría de flujo se observó un incremento en el patrón de expresión de CD80, CCR7 y CD83 y la disminución de CD209, fenotipo acorde con células dendríticas maduras aceleradas (Figura 18B).

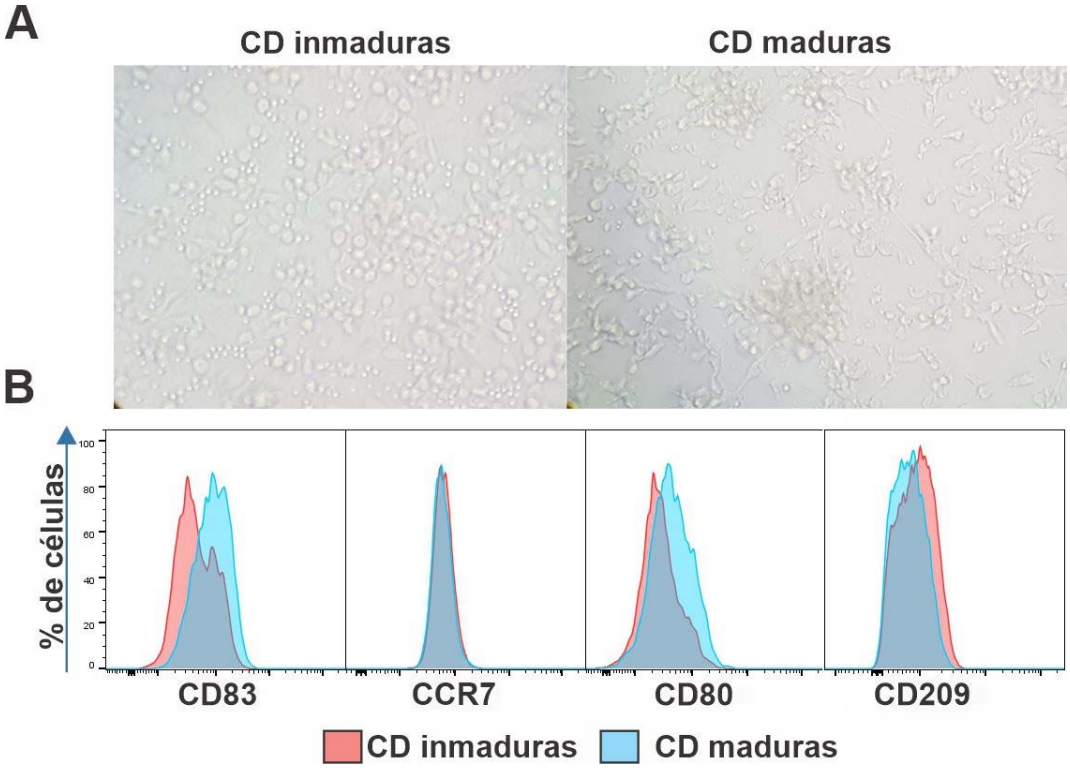


Figura 18. **Generación de células dendríticas aceleradas.**
(A) Fotografías por microscopía óptica de CD inmaduras (panel izquierdo) y maduras (panel derecho) de dos días (40X), obtenidas *in situ* a partir de CMSP de donantes sanos. (B) Fenotipo de maduración de CD de cocultivos acelerados evaluados por citometría de flujo. Histogramas con el patrón de expresión de los marcadores: CD83, CCR7, CD80 y CD209 en CD inmaduras (histogramas rosados); CD maduras de dos días (histogramas azules).

Estimulación *in vitro* de LT CD4 con epítopes nativos y modificados

Con la segunda toma de muestra de los individuos no expuestos, se realizó la separación de CMSP mediante Ficoll y se indujo la diferenciación de los monocitos para obtener las CD aceleradas *in situ*, al mismo tiempo se estimularon con los péptidos seleccionados *in silico*. Para la proteína PvMSP1 fueron seleccionados dos péptidos nativos. El primer péptido nativo denominado 43466 con cuatro péptidos modificados, 43467 para HLA-DRβ1*04:07, 43468 para HLA-DRβ1*07:01; 43469 para HLA-DRβ1*11:01 y 43470 para HLA-DRβ1*13:02. El segundo péptido nativo de la proteína PvMSP1 denominado 43472 con cuatro péptidos modificados, 43472 para HLA-DRβ1*04:07, 43473 para HLA-DRβ1*07:01; 43474 para HLA-DRβ1*11:01 y 43475 para HLA-DRβ1*13:02.

Para la proteína PvDBP fueron seleccionados dos péptidos nativos, el primer nativo 43476 con cuatro modificados, 43477 para HLA-DRβ1*04:07, 43478 para HLA-DRβ1*07:01; 43479 para HLA-DRβ1*11:01 y 43480 para HLA-DRβ1*13:02. El segundo péptido nativo de la proteína PvDBP 43481 con cuatro péptidos modificados, 43482 para HLA-DRβ1*04:07, 43483 para HLA-DRβ1*07:01; 43484 para HLA-DRβ1*11:01 y 43485 para HLA-DRβ1*13:02.

Al cabo de 10 días de cultivo, se analizaron por citometría de flujo las subpoblaciones de LT CD4+ de memoria central (TCM), naïve (TN), de memoria efectora (TEM) y efectoras terminales (TEF) y se evaluó la expansión de las células mediante análisis manual con FlowJo y por análisis automatizados con CITRUS. Además, se evaluó la producción de citoquinas en el sobrenadante (SBN) de cultivo para cada uno de los péptidos de las proteínas estudiadas.

La estrategia empleada para el análisis manual fue la siguiente: primero se utilizaron los parámetros FSC-A vs FSC-H y SSC-H vs SSC-W para seleccionar las células individuales y descartar las dupletas. A partir de ellas, con los parámetros SSC-A y FSC-A para seleccionar complejidad y tamaño, se delimitó la población de linfocitos. A partir de la población de linfocitos, se definió los Linfocitos T CD4+ (LT CD4+), de estos se evalúo la población de Linfocitos T foliculares helper (TFH) con los marcadores PD1 y CXCR5, y la activación o quiescencia de estas células con PD1 y CCR7 (Figura 19A). Por otro lado, a partir de los LT-CD4+ se definieron las subpoblaciones de memoria: naïve (TN), de memoria central (TCM), de memoria efectora (TEM) y efectoras finales (TEF) con los marcadores CD45RA y CCR7 (Figura 19B). A todas las poblaciones se les evaluó el nivel de activación mediante la expresión del marcador CD69 (Figura 19C).

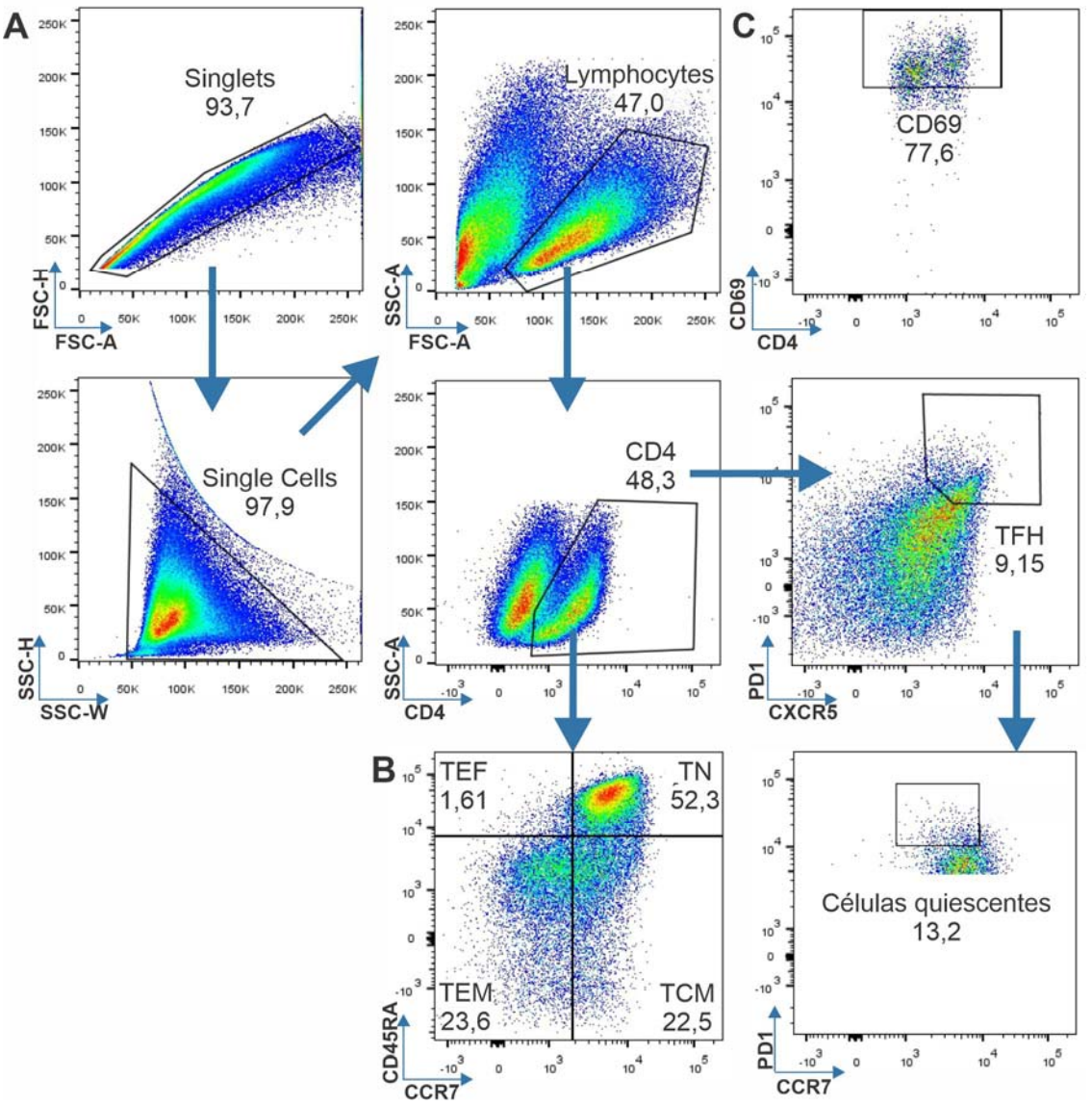


Figura 19. Estrategia para el análisis manual de las subpoblaciones de LT CD4 por citometría de flujo.
(A) Selección de células individuales (FSC-A vs FSC-H y SSC-H vs SSC-W), definición de la población de linfocitos (FSC-A y SSC-A), definición de linfocitos T CD4+ (CD4+) a partir de éstos, linfocitos T foliculares helper (TFH CXCR5+ PD1+); población TFH en quiescencia (PD1+ CCR7-). (B) Definición de subpoblaciones de memoria: T naive (TN), T de memoria central (TCM), T de memoria efectora (TEM) y T efectoras (TEF) mediante la expresión de CD45RA y CCR7; y (C) Activación de cada una de las poblaciones tanto de memoria como totales, mediante el marcador CD69.

A continuación, se describen los resultados más sobresalientes obtenidos mediante el análisis manual por FlowJo en cada una de las muestras procesadas, comparando los porcentajes de las poblaciones descritas del cultivo estimulado con el péptido nativo vs el modificado.

Para el alelo DRβ1*04:07 se analizaron las muestras de dos donantes. Al primer donante BOGINM011 se le evaluó su respuesta contra la proteína MSP1, se observó un aumento en la frecuencia de las poblaciones de las TFH, las TN, las TN activadas (péptidos 43467 y 43472) y las TEF para el péptido 43472. Por otro lado, para la proteína DBP se observó un aumento en la frecuencia de las poblaciones de las TFH, y las TN activadas (péptidos 43477 y 43482) las células quiescentes activadas para el péptido 43482 (Tabla 9).

Cuando se evaluó la respuesta contra las proteínas MSP1 y DBP con la muestra del otro donante (BOGINM010), se observó un aumento en la frecuencia de la población de las células quiescentes activadas con los péptidos 43472 y 43477 respectivamente. Finalmente, se observó un aumento en la frecuencia de la población de las TEF con los péptidos 43477 y 43482 de la proteína DBP, y un incremento de la frecuencia de células con fenotipo TEM estimulados con el péptido 43482 de la proteína DBP (Tabla 9).

Tabla 9. Frecuencia de poblaciones para las muestras del alelo DRβ1*04:07

Alelo DRβ1*04:07

				Frecuencias de Poblaciones					
Donante	Proteína	Péptidos	Código	TFH		TN	TN CD69		TEF
BOGINM011	MSP1	Nativo	43466	4,67		22	41,3		
		Modificado	43467	7,04		24,4	46,1		
		Nativo	43471	3,22		19,1	30,4		1,44
		Modificado	43472	9,22		23,3	48,2		1,93
				TFH	Cel quies CD69		TN CD69		
	DBP	Nativo	43476	4,41			40,6		
		Modificado	43477	9,03			49,4		
		Nativo	43481	8,51	73,3		47,7		
		Modificado	43482	9,37	85,4		51,6		

				Frecuencias de Poblaciones				
Donante	Proteína	Péptidos	Código		Cel quies CD69			
BOGINM010	MSP1	Nativo	43471		68,4			
		Modificado	43472		78,3			
					Cel quies CD69		TEM	TEF
	DBP	Nativo	43476		21,2			2,9
		Modificado	43477		23,1			4,28
		Nativo	43481				13,7	2,95
		Modificado	43482				15	3,55

Para el alelo DRβ1*07:01 se analizaron muestras de dos donantes, la muestra BOGINM002 con la proteína DBP, donde se observó un aumento en la frecuencia de las células CD4+ TEF con perfil de activación para el péptido 43478. Para la muestra BOGINM003 cuando se evaluó la respuesta para la proteína MSP1 y la proteína DBP, se observó un aumento en la frecuencia de las TFH con perfil de activación y las células quiescentes (péptidos 43473, 43478 y 43483). Para la proteína MSP1, se vio un aumento en la frecuencia de las TN y de las TEF con un perfil de activación para el péptido 43473 (Tabla 10).

Tabla 10. Frecuencia de poblaciones para las muestras del alelo DRβ1*07:01

Alelo DRβ1*07:01

				Frecuencias de Poblaciones	
Donante	Proteína	Péptidos	Código		TEF CD69
BOGINM002	DBP	Nativo	43476		26,5
		Modificado	43478		29,2

				Frecuencias de Poblaciones			
Donante	Proteína	Péptidos	Código	TFH CD69	Cel quies CD69	TN CD69	TEF CD69
BOGINM003	MSP1	Nativo	43471	14,4	13,7	3,88	0,83
		Modificado	43473	17,2	17,7	4,88	1,06
				TFH CD69	Cel quies CD69		
	DBP	Nativo	43476	9,68	10,6		
		Modificado	43478	15,1	16,5		
		Nativo	43481	12,3	10,5		
		Modificado	43483	14,9	15,2		

Para el alelo DRβ1*11:01 se analizaron dos muestras, la BOGINM007 para los péptidos de la proteína MSP1, se observó un aumento en la frecuencia del perfil de activación de los LT CD4+ (péptido 43474). Tanto para los péptidos modificados de MSP1 como de DBP, aumentó la población de las TFH y el perfil de activación de las TN (péptidos 43474 y 43479 respectivamente). Finalmente, con la estimulación in vitro con el péptido modificado de la proteína DBP (péptido 43479) aumentó la población de las células quiescentes. Por otro lado, para la muestra BOGINM001 con un péptido de la proteína MSP1, aumentó el perfil de activación de las células quiescentes y de las TN (péptido 43469 y 43474 respectivamente); con el péptido 43469 de la proteína MSP1 aumentó la frecuencia de las células TN. Finalmente, en este donante con la estimulación con el péptido de DBP (péptido 43479), se observó un ligero incremento

en la frecuencia de células con perfil TFH y con el péptido 43484 un incremento en la frecuencia de células con perfil TN(Tabla 11).

Tabla 11. Frecuencia de poblaciones para las muestras del alelo DRβ1*11:01

Alelo DRβ1*11:01

				Frecuencias de Poblaciones				
Donante	Proteína	Péptidos	Código	CD4 CD69	TFH			TN CD69
BOGINM007	MSP1	Nativo	43471	31,2	3,93			27,3
		Modificado	43474	35,6	6,05			33,8
					TFH	Cel quiesc		TN CD69
	DBP	Nativo	43476		5,62	20,6		32,4
		Modificado	43479		6,36	23,1		36

				Frecuencias de Poblaciones				
Donante	Proteína	Péptidos	Código			Cel quies CD69	TN	TN CD69
BOGINM001	MSP1	Nativo	43466			78,9	38,7	
		Modificado	43469			85,3	44,2	
		Nativo	43471					8,39
		Modificado	43474					9,37
					TFH		TN	
	DBP	Nativo	43476		4,29			
		Modificado	43479		4,63			
		Nativo	43481				36,3	
		Modificado	43484				42,2	

Para el alelo DRβ1*13:02 se analizaron muestras de dos donantes, en los cuales fue posible detectar un aumento en la frecuencia de las TFH, tanto en respuesta a la estimulación con un péptido modificado de la proteína MSP1 (péptido 43475) en ambos donantes (BOGINM006 y BOGINM012). Adicionalmente, se observó una respuesta similar a un péptido modificado de la proteína DBP (43480) en el donante BOGINM012 (Tabla 12).

Tabla 12. Frecuencia de poblaciones para las muestras del alelo DRβ1*13:02

Alelo DRβ1*13:02

				Frecuencias de Poblaciones
Donante	Proteína	Péptidos	Código	TFH
BOGINM006	MSP1	Nativo	43466	
		Modificado	43470	
		Nativo	43471	3,71
		Modificado	43475	4,46

				Frecuencias de Poblaciones
Donante	Proteína	Péptidos	Código	TFH
BOGINM012	MSP1	Nativo	43471	5,51
		Modificado	43475	6,5
				TFH
	DBP	Nativo	43476	3,37
		Modificado	43480	4,63

Para el análisis estadístico de los datos normalizados se encontró diferencias significativas con un aumento entre el grupo de péptidos modificados vs los péptidos nativos en la población de células TN de los donantes BOGINM011 alelo DRβ1*04:07 y BOGINM002 alelo DRβ1*07:01 con un valor de *p*=0.0286. Al igual que en el perfil de activación de las células TEM de los donantes BOGINM010 alelo DRβ1*04:07 y BOGINM012 alelo DRβ1*13:02 con un valor de *p*=0.0286 (Figura 20).

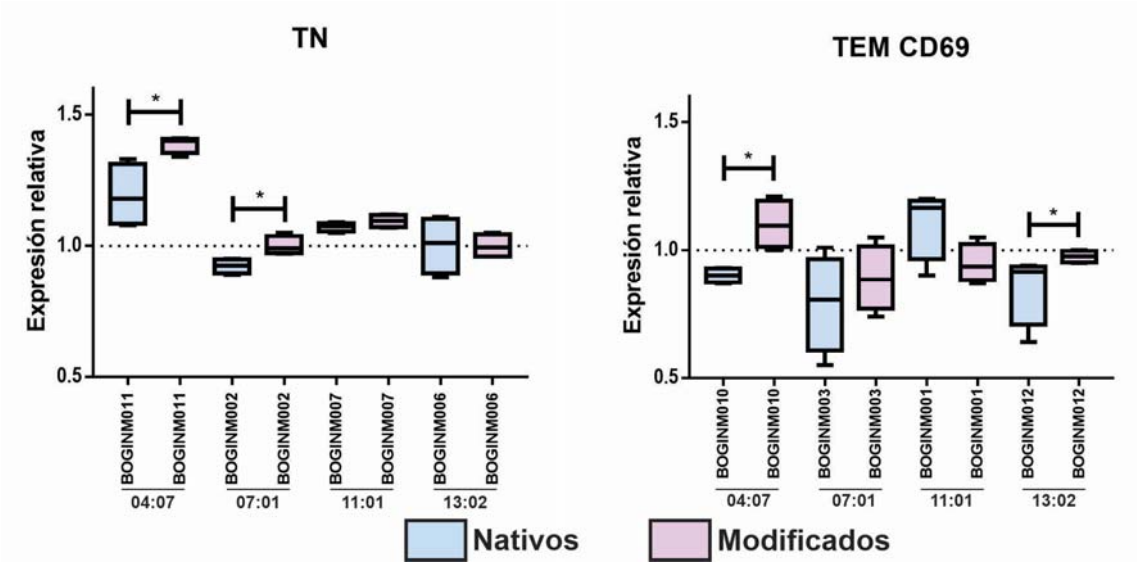


Figura 20. Análisis de las subpoblaciones de LT con aumento entre los péptidos modificados vs los nativos.

Se muestra las subpoblaciones de LT CD4+ que presentaron diferencias significativas cuando se estimularon con los péptidos modificados vs los nativos (cajas rosadas vs azules, respectivamente), la dispersión de los datos se muestra en cajas y bigotes (5-95%). Los datos

fueron normalizados restando el valor del control negativo (sin estimular) a cada una de las muestras. Panel izquierdo población de TN y panel derecho TEM activadas. Se aplicó la prueba no paramétrica de Mann-Whitney, *p<0,05.

También se encontraron diferencias significativas con un aumento entre el grupo de péptidos nativos vs los péptidos modificados del donante BOGINM002 alelo DRβ1*07:01 en el perfil de activación de las células CD4+, en el perfil de activación de las células TFH y en el perfil de activación de las células quiescentes con un valor de p=0.0286. Además, hay diferencias significativas en el donante BOGINM007 alelo DRβ1*11:01 con un aumento entre el grupo de péptidos nativos vs los péptidos modificados en las células TCM con un valor de p=0.0286 (Figura 21).

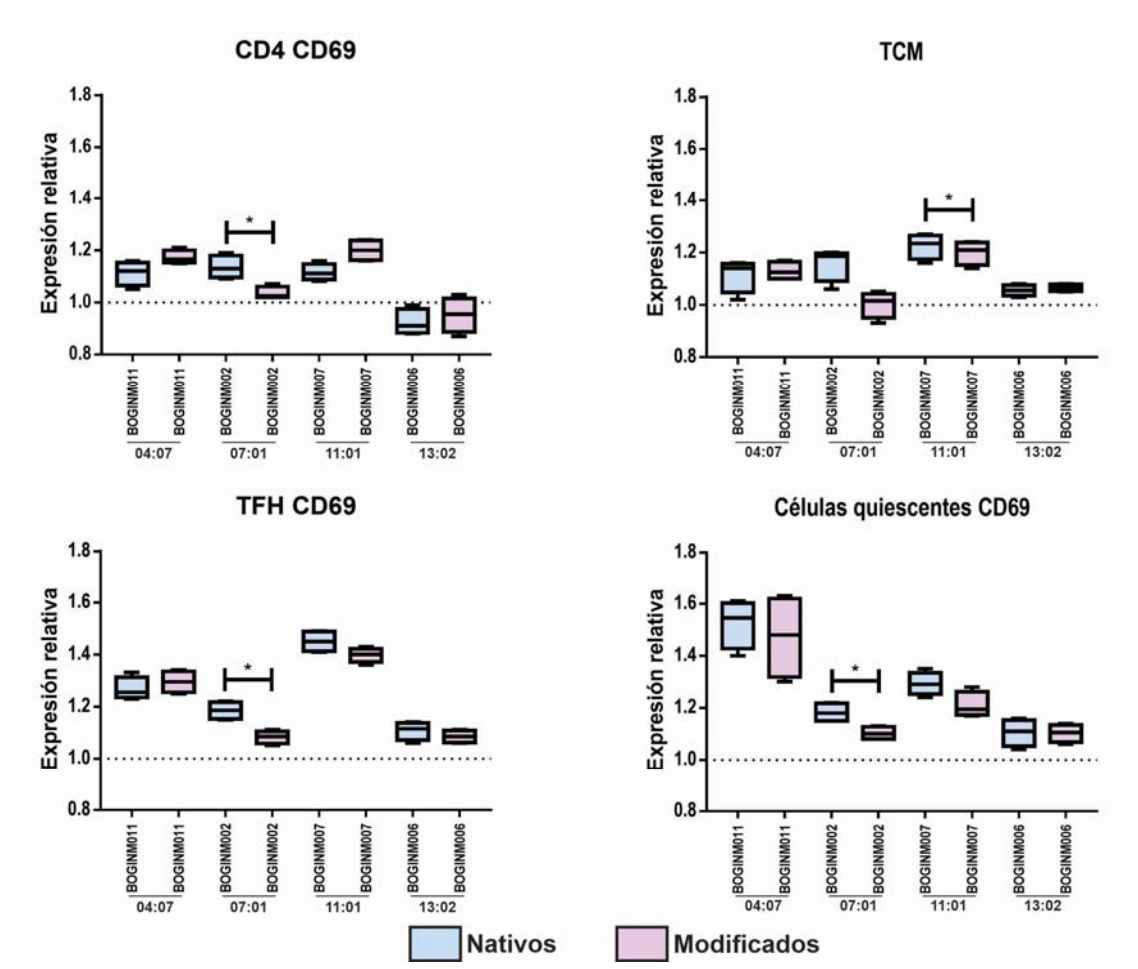


Figura 21. Análisis de las subpoblaciones de LT con aumento entre los péptidos nativos vs los modificados.
Se muestra las subpoblaciones de LT CD4+ que presentaron diferencias significativas cuando se estimularon con los péptidos nativos vs los modificados (cajas azules vs rosadas, respectivamente), la dispersión de los datos se muestra en cajas y bigotes (5-95%). Los datos fueron normalizados restando el valor del control negativo (sin estimular) a cada una de las muestras. Se aplicó la prueba no paramétrica de Mann-Whitney, *p<0,05.

A partir de los SBN de los cultivos se evaluó la secreción de las siguientes citoquinas: IFN-γ, TNF, IL-10, IL-6, IL-4 e IL-2 mediante el kit CBA Human Th1/Th2 Cytokine Kit II (BD). Los niveles de producción de las citoquinas inducidas por los péptidos de las proteínas PvMSP1 y PvDBP fueron normalizados y analizadas con GraphPad. En las CMSP del donante BOGINM001 con el alelo DRβ*11:01 se encontró diferencias significativas en la secreción de IL-2 entre las CMSP estimuladas con los péptidos modificados vs los nativos, con un valor de *p*=0.028 (Figura 22).

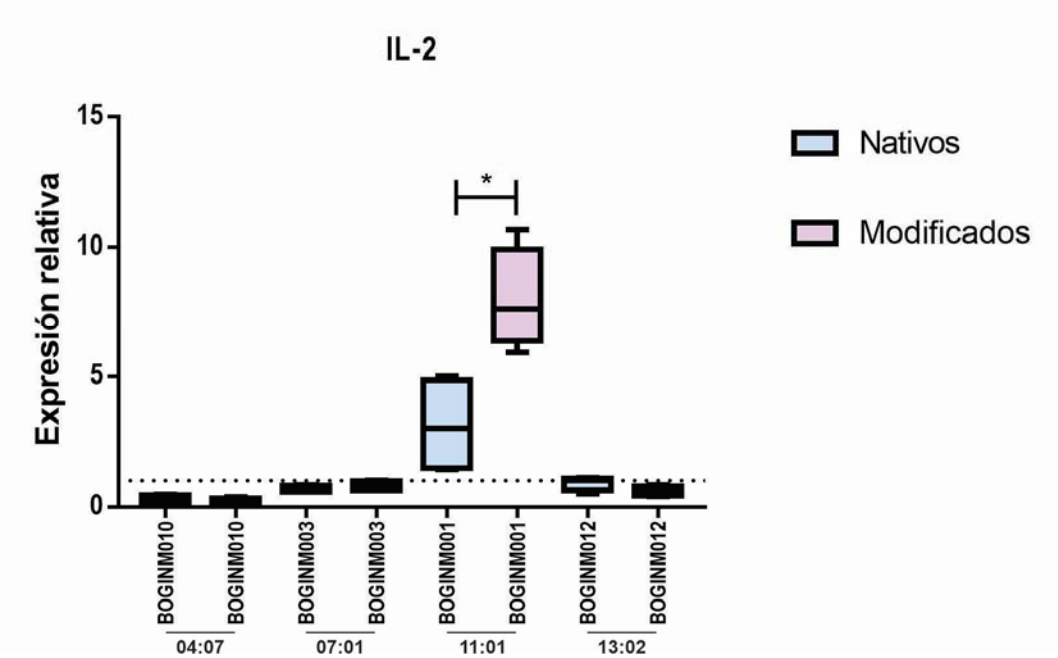


Figura 22. Evaluación de la secreción de las citoquinas de perfil Th1/Th2.
Se muestra la producción de IL-2 que presentó diferencias significativas cuando se estimularon los LT con los péptidos modificados vs los nativos (cajas rosadas vs azules, respectivamente), la dispersión de los datos se muestra en cajas y bigotes (5-95%). Los datos fueron normalizados restando el valor del control negativo (sin estimular) a cada una de las muestras. Se aplicó la prueba no paramétrica de Mann-Whitney, **p*<0,05.

Posteriormente se empleó el algoritmo CITRUS para evaluar la expresión *in vitro* de los marcadores analizados por citometría en todas las muestras que conformaban los grupos experimentales. Con el objetivo de establecer un fenotipo de células que permitiera diferenciar los estímulos en estudio. Primero se validaron los modelos para cada una de las muestras y después se analizaron las aglomeraciones celulares (clústers/modelos) que diferencian los grupos de comparación nativos vs modificados.

Para el alelo DRβ1*04:07 se analizaron dos muestras, la BOGINM011 donde el algoritmo valida estadísticamente (tasa de error baja y de falsos descubrimientos Figura 23A), un incremento en dos clústeres (79969 y 79989) en el grupo de péptidos modificados vs los péptidos nativos (Figura 23B) caracterizados por tener un fenotipo compatible con LT-CD4+ TFH activados. El clúster 79969 se caracteriza por el siguiente perfil CD4+, CD69+, CD45RA+, CXCR5+, CCR7+, CXCR3+ y PD1+ y el clúster 79989 CD4+, CD69+, CD45RAint, CXCR5+, CCR7+, CXCR3+ y PD1+ (Figura 23C).

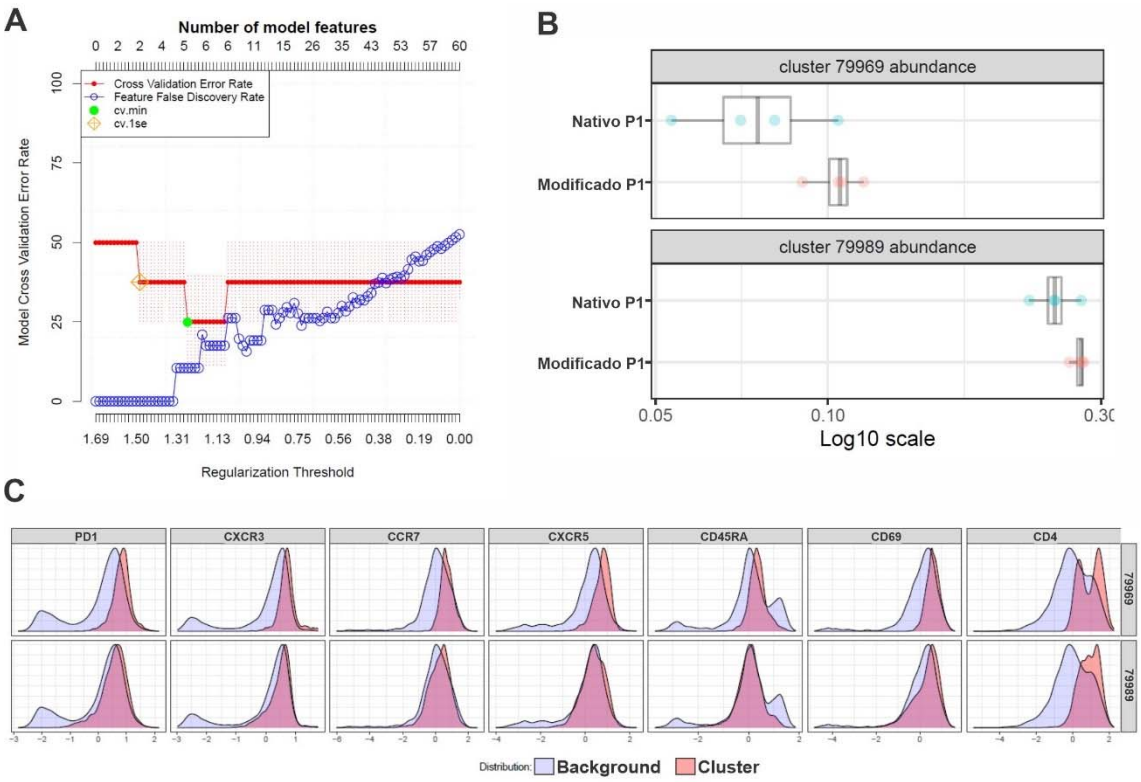


Figura 23. Resultados del análisis de CITRUS para las muestras del alelo DRβ1*04:07.
(A) Curva de modelo predictivo, en rojo se muestra la tasa de error estimada del modelo y en azul la tasa de falsos descubrimientos. Se muestra una validación adecuada (baja tasa de falsos descubrimientos con una baja rata de error de validación). Se muestran los dos modelos predictivos aplicables a los datos obtenidos: cv.min, círculo verde (menor tasa de error) y cv.1se diamante naranja (tasa de error establecida de 1). (B) Abundancia relativa de los clústers 79969 y 79989 en una gráfica de caja y bigotes. (C) Histogramas de expresión de cada marcador, en columnas de izquierda a derecha PD1, CXCR3, CCR7, CXCR5, CD45RA, CD69 y CD4, en los grupos determinados en el panel B, comparando la expresión basal (histograma azul) con la expresión del clúster específico (histograma rojo).

Para la muestra BOGINM010 según el modelo muestra una tasa de error satisfactoria (Figura 24A), el grupo con expresión diferencial significativa es el clúster el 79997 (Figura 24B) con fenotipo compatible de LT CD8+ con el siguiente perfil CD4-, CD69int, CD45RAint, CXCR5int, CCR7int, CXCR3- y PD1- (Figura 24C). La abundancia de esos grupos es diferente, con una mayor expresión en el grupo de péptidos modificados vs los péptidos nativos.

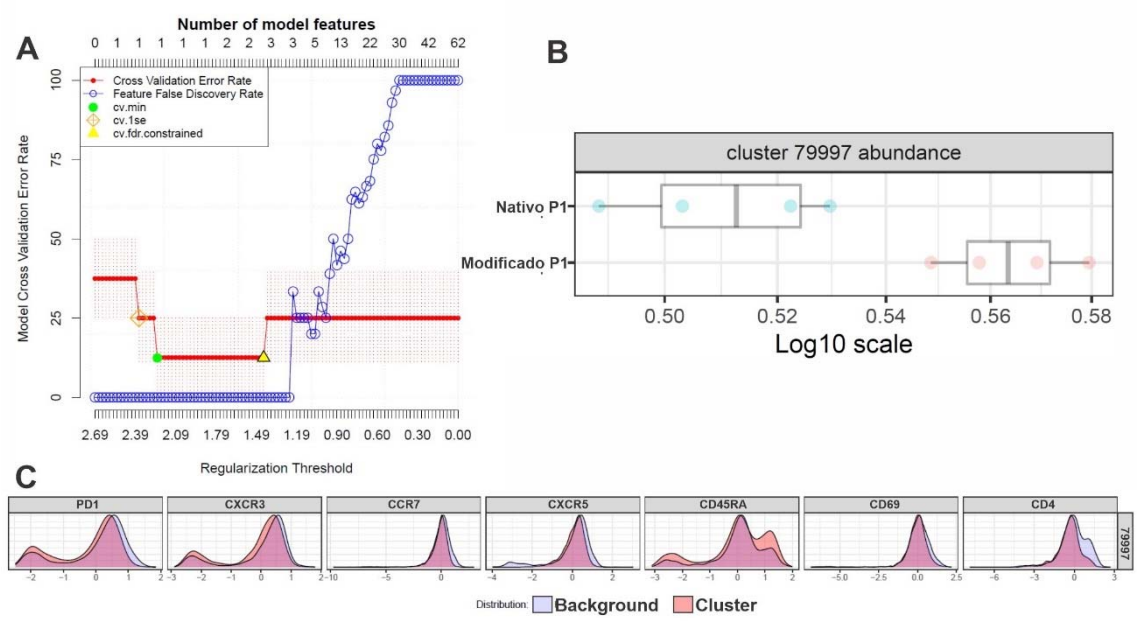


Figura 24. Resultados del análisis de CITRUS para las muestras del alelo DRβ1*04:07.
(A) Curva de modelo predictivo, en rojo se muestra la tasa de error estimada del modelo y en azul la tasa de falsos descubrimientos. Se muestra una validación adecuada (baja tasa de falsos descubrimientos con una baja rata de error de validación). Se muestran los dos modelos predictivos aplicables a los datos obtenidos: cv.min, círculo verde (menor tasa de error) y cv.1se diamante naranja (tasa de error establecida de 1). (B) Abundancia relativa del clúster 79997 en una gráfica de caja y bigotes. (C) Histograma de expresión de cada marcador, en columnas de izquierda a derecha PD1, CXCR3, CCR7, CXCR5, CD45RA, CD69 y CD4, en el grupo determinado en el panel B, comparando la expresión basal (histograma azul) con la expresión del clúster específico (histograma rojo).

Para el alelo DRβ1*07:01 se analizaron dos muestras, la BOGINM002 donde el algoritmo valida estadísticamente la prueba (Figura 25A), un incremento en dos clústeres (79993 y 79996) (Figura 25B) en el grupo de péptidos nativos vs los modificados con un fenotipo compatible de LT-CD4+ TEM activados y de TCM, respectivamente. El clúster 79993 se caracteriza por el siguiente perfil CD4int, CD69int,

CD45RA-, CXCR5-, CCR7-, CXCR3- y PD1- y el clúster 79996 se caracterizan por el siguiente perfil CD4+, CD69-, CD45RA-, CXCR5-, CCR7int, CXCR3int y PD1- (Figura 25C).

Para la muestra BOGINM003 según el modelo muestra una tasa de error no satisfactoria.

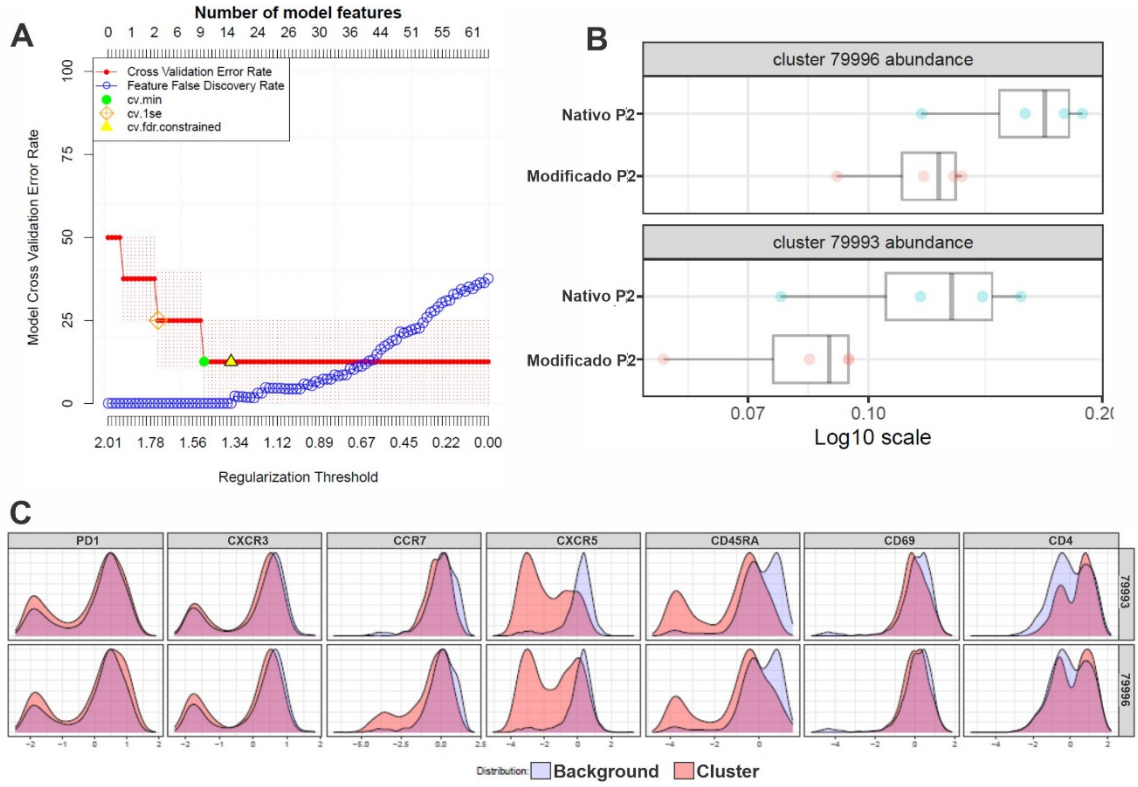


Figura 25. Resultados del análisis de CITRUS para las muestras del alelo DRβ1*07:01.
(A) Curva de modelo predictivo, en rojo se muestra la tasa de error estimada del modelo y en azul la tasa de falsos descubrimientos. Se muestra una validación adecuada (baja tasa de falsos descubrimientos con una baja rata de error de validación). Se muestran los dos modelos predictivos aplicables a los datos obtenidos: cv.min, círculo verde (menor tasa de error) y cv.1se diamante naranja (tasa de error establecida de 1). (B) Abundancia relativa de los clústers 79996 y 79993 en una gráfica de caja y bigotes. (C) Histogramas de expresión de cada marcador, en columnas de izquierda a derecha PD1, CXCR3, CCR7, CXCR5, CD45RA, CD69 y CD4, en los grupos determinados en el panel B, comparando la expresión basal (histograma azul) con la expresión del clúster específico (histograma rojo).

Para el alelo DRβ1*11:01 se analizaron dos muestras, la BOGINM007 según el modelo muestra una tasa de error no satisfactoria. Para la muestra BOGINM001 donde el algoritmo valida estadísticamente (Figura 26A), un incremento en dos clústeres (79893, y 79997) (Figura 26B) en el grupo de péptidos nativos vs los péptidos modificados caracterizados por tener un fenotipo de LT CD4+ TFH y TEF,

respectivamente. El clúster 79893 se caracteriza por el siguiente perfil CD4+, CD69-, CD45RA-, CXCR5int, CCR7+, CXCR3+ y PD1+, y el clúster 79997 con el siguiente perfil CD4int, CD69-, CD45RA-, CXCR5-, CCR7-, CXCR3- y PD1- (Figura 26C). Además, hay un incremento en dos clústeres (79990 y 79986) (Figura 26B) en el grupo de péptidos modificados vs los péptidos nativos caracterizados por tener un fenotipo de LT CD4+ TN. El clúster 79990 con el siguiente perfil CD4int, CD69-, CD45RA+, CXCR5-, CCR7+, CXCR3- y PD1- y el clúster 79986 con el siguiente perfil CD4int, CD69-, CD45RA+, CXCR5-, CCR7int, CXCR3- y PD1- (Figura 26C).

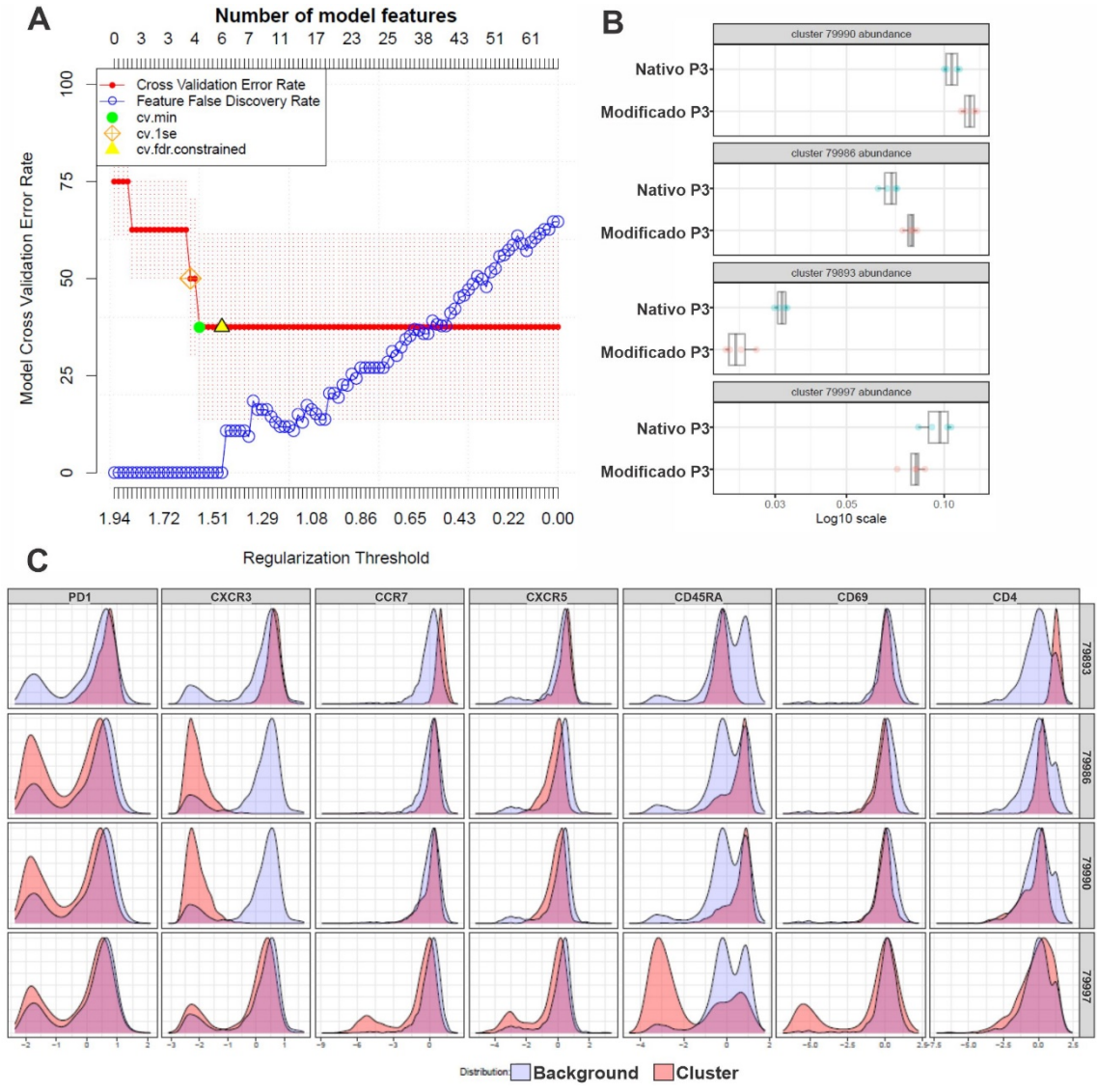


Figura 26. Resultados del análisis de CITRUS para las muestras del alelo DRβ1*11:02.
(A) Curva de modelo predictivo, en rojo se muestra la tasa de error estimada del modelo y en azul la tasa de falsos descubrimientos. Se muestra una validación adecuada (baja tasa de falsos descubrimientos con una baja rata de error de validación). Se muestran los dos modelos predictivos aplicables a los datos obtenidos: cv.min, círculo verde (menor tasa de error) y cv.1se

diamante naranja (tasa de error establecida de 1). (B) Abundancia relativa de los clústers 79990, 79986, 79893 y 79997 en una gráfica de caja y bigotes. (C) Histogramas de expresión de cada marcador, en columnas de izquierda a derecha PD1, CXCR3, CCR7, CXCR5, CD45RA, CD69 y CD4, en los grupos determinados en el panel B, comparando la expresión basal (histograma azul) con la expresión del clúster específico (histograma rojo).

Para el alelo DRβ1*13:02 se analizaron dos muestras, la BOGINM006 donde el algoritmo valida estadísticamente (Figura 27A), un incremento en un clúster (79965) (Figura 27B) en el grupo de péptidos modificados vs los nativos caracterizados por tener un fenotipo de LT-CD4+ TFH activadas. El clúster el 79965 se caracteriza por el siguiente perfil CD4+, CD69+, CD45RA+, CXCR5+, CCR7+, CXCR3+ y PD1+ (Figura 27C). Un incremento en el clúster (79992) (Figura 27B) en el grupo de péptidos nativos vs los péptidos modificados caracterizados por tener un fenotipo de LT-CD4+ TN activadas. El clúster 79992 se caracteriza por el siguiente perfil CD4+, CD69int, CD45RAint, CXCR5-, CCR7+, CXCR3int y PD1- (Figura 27C).

Para la muestra BOGINM012 según el modelo muestra una tasa de error no satisfactoria.

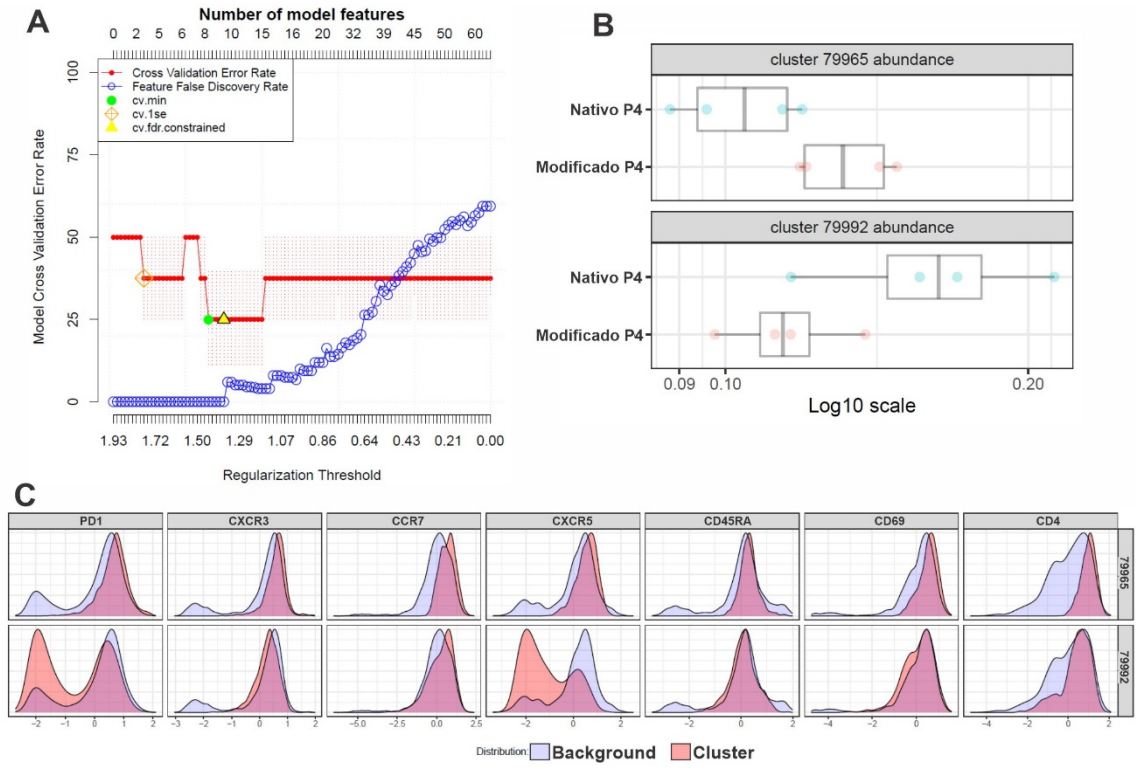


Figura 27. Resultados del análisis de CITRUS para las muestras del alelo DRβ1*13:02.

(A) Curva de modelo predictivo, en rojo se muestra la tasa de error estimada del modelo y en azul la tasa de falsos descubrimientos. Se muestra una validación adecuada (baja tasa de falsos descubrimientos con una baja rata de error de validación). Se muestran los dos modelos predictivos aplicables a los datos obtenidos: cv.min, círculo verde (menor tasa de error) y cv.1se diamante naranja (tasa de error establecida de 1). (B) Abundancia relativa de los clústers 79965 y 79992 en una gráfica de caja y bigotes. (C) Histogramas de expresión de cada marcador, en columnas de izquierda a derecha PD1, CXCR3, CCR7, CXCR5, CD45RA, CD69 y CD4, en los grupos determinados en el panel B, comparando la expresión basal (histograma azul) con la expresión del clúster específico (histograma rojo).

De acuerdo con el análisis de las subpoblaciones de linfocitos T CD4+ realizada mediante el algoritmo CITRUS, se encontró una diferencia estadística en la población de TFH activados cuando las muestras fueron estimuladas con los péptidos modificados comparado con la estimulación con los péptidos nativos, en donantes con dos de los cuatro alelos evaluados. En cambio, en los modificados se encontró una disminución con diferencia estadísticamente significativa en las subpoblaciones de memoria compuesta por TCM, TEM activadas, TN y TEF cuando las muestras fueron estimuladas con los péptidos modificados (Tabla 13).

Tabla 13. Recopilación de los análisis realizados con CITRUS.

Alelo DRβ1	Donante	Clúster	Incremento	PD1	CXCR3	CCR7	CXCR5	CD45RA	CD69	CD4	Fenotipo
04:01	BOGINM011	79969	Modificados	+	+	+	+	+	+	+	TFH activado
		79989	Modificados	+	+	+	+	Int	+	+	TFH activado
	BOGINM010	79997	Modificados	-	-	Int	Int	Int	Int	-	LT CD8+*
07:01	BOGINM002	79996	Nativos	-	Int	Int	-	-	-	+	TCM
		79993	Nativos	-	-	-	-	-	Int	Int	TEM activado
11:02	BOGINM001	79990	Modificados	-	-	+	-	+	-	Int	TN
		79986	Modificados	-	-	Int	-	+	-	Int	TN
		79893	Nativos	+	+	+	Int	-	-	+	TFH
		79997	Nativos	-	-	-	-	-	-	Int	TEF
13:02	BOGINM006	79965	Modificados	+	+	+	+	+	+	+	TFH activado
		79992	Nativos	-	Int	+	-	Int	Int	+	TN activado

* No se tiene la marcación con CD8 y/o CD3.

DISCUSION

Para algunas de las proteínas más estudiadas de *P. vivax* se han identificado y caracterizado los péptidos de alta capacidad de unión a reticulocitos (HABPs) y sus respectivos residuos críticos de unión. En el caso de *P. falciparum* se ha descrito que los HABPs de proteínas involucradas en la invasión son poco inmunogénicos y no inducen respuesta inmune protectora, razón por la cual se han modificado algunos de los residuos críticos en la unión, con el propósito de mejorar su ajuste a moléculas HLA clase II y a su vez su capacidad inmunogénica y protectora (77, 227-229).

Durante el desarrollo de una vacuna es necesario analizar la respuesta inmune generada, esto se lleva a cabo mediante ensayos celulares capaces de medir la especificidad y frecuencia de las subpoblaciones de memoria, además de evaluar la calidad de las respuestas protectoras. Una de las técnicas más empleadas para el monitoreo de la respuesta antígeno específica es el cultivo celular, por medio del cual se puede evaluar la población de los linfocitos T naïve y predecir la antigenicidad a las vacunas. Pero debido a su baja frecuencia y pobre respuesta, se hace necesario emplear técnicas para el procesamiento y presentación de antígenos por CPA profesionales (230).

En este estudio se generaron células dendríticas en cocultivos acelerados inducidas *in situ*, empleando las citoquinas de diferenciación IL-4 y GM-CSF y el coctel de maduración descrito. Los resultados obtenidos mostraron que las CD aceleradas tuvieron las mismas características morfológicas de las CD de siete días derivadas de monocitos, fueron capaces de estimular los linfocitos T naïve y de activar los linfocitos T específicos de antígeno de acuerdo con los resultados obtenidos por Martinuzzi y Dauer y cols. (166, 171). El establecimiento de un cultivo de alta densidad donde se generaron células dendríticas a partir de CMSP, tiene ventajas como la optimización en el tiempo de cultivo y en la purificación de las células, además de mantener el contacto entre las células de tal forma que se estimule la presentación antigénica, la proliferación celular y la diferenciación funcional de los linfocitos T CD4+ (231).

Algo que favorece la respuesta de las células T efectoras es la producción de IL-2, ya que los LT CD4+ que la secretan actúan como reservorios de células de memoria potencialmente efectoras. Dentro de las funciones de esta citoquina se ha descrito que genera la proliferación de linfocitos T CD4 y CD8, programa los LT CD8+ para mejorar la capacidad de memoria y la función efectora, además mejora la actividad de las células Natural Killer para controlar tempranamente la infección (232). En un estudio realizado en individuos naïve inmunizados con la vacuna RTS,S/AS, se encontraron las subpoblaciones TCM y TEM con una frecuencia significativamente mayor en los individuos protegidos vs los no protegidos. Después de estimular *in vitro* las CMSP con péptidos de CSP de *P. falciparum* el porcentaje de células TEM que generaron IL-2 fue mayor que las TCM, además se encontró una asociación entre la producción de esta citoquina y los títulos de anticuerpos específicos de CSP y se correlacionó con protección (233). Nuestros resultados mostraron un aumento significativo en la producción de IL-2 entre las CMSP estimuladas con los péptidos modificados vs los nativos, para el alelo DRB1*11:01. Lo que puede señalar que las modificaciones realizadas a los péptidos favorecen la producción de IL-2 por parte de los LT específicos a estos antígenos, actuando como un factor de crecimiento de los linfocitos TFH y que probablemente favorezca la proliferación y diferenciación de células B en los CG.

En un área endémica de baja exposición en Brasil se evaluó un grupo de pacientes con malaria no complicada por *P. vivax*, en donde encontraron un aumento de células T naive y de células de memoria TCM con una disminución de las TEM, asimismo los LT de memoria produjeron más IL-10 e IFN-ɣ, citoquinas responsables de modular la respuesta inmune durante la infección (234). En una zona holoendémica en Kenia occidental se evalúo la respuesta de CMSP a la proteína recombinante MSP-1_{42}, se observó una mayor frecuencia de células T CD4+ TCM y TEM en adultos residentes mientras que en los niños fue mayor las TN, la proporción de TEF fue similar en ambos grupos; la respuesta de LT CD4+ de memoria no se correlacionó con la producción de anticuerpos a la proteína (235).

Para el control de los parásitos de la malaria son necesarias dos tipos de células, los linfocitos T ayudadores CD4+ (Th1) productores de IFN-ɣ, T-βet+ los cuales estimulan las células fagocíticas para atrapar y eliminar a los parásitos; y los linfocitos T foliculares

ayudadores (TFH) encargadas de promover la diferenciación de los linfocitos B naïve en células plasmáticas de larga vida o en linfocitos B de memoria, la maduración de la afinidad y la proliferación de estos en los centros germinales (CG). Las TFH se caracterizan por la producción de IL-21, esta citoquina ayuda a formar de manera óptima los CG y fomenta de manera autocrina la supervivencia de los TFH (236, 237).

Dentro de las células TFH se encuentra el subgrupo PD-1+, CXCR3− y CXCR5+ circulantes, las cuales tienen mayor similitud tanto en función como en fenotipo con las TFH de CG; este subgrupo de células TFH tiende a polarizarse a las subclases Th2 o Th17 conocidas como mejores ayudadoras de los linfocitos B. Estas proporcionan una ayuda más eficiente a los linfocitos B en el cambio de clase y en la producción de los diferentes isotipos de inmunoglobulinas (238, 239). Un estudio longitudinal realizado en una población de niños de Malí, donde hay transmisión intensa de *P. falciparum* mostró que durante la malaria aguda la respuesta de las TFH se dirige hacia la subpoblación PD-1+, CXCR3+ y CXCR5+ por lo cual se polariza hacia la población Th1 menos funcional y no se correlaciona con la respuesta de anticuerpos (240).

Cuando se evaluó un grupo de individuos infectados con *P. vivax* antes y después del tratamiento, se observó que la infección aguda aumenta la proliferación de las subpoblaciones de TFH Th1 (CXCR3+ CCR6-) y Th17 (CXCR3- CCR6+) y baja la frecuencia de las Th2 (CXCR3- CCR6-); también incrementan la producción de IL-21 importante en la activación de linfocitos B y en la producción de anticuerpos durante la infección. Además, pacientes con más de 5 episodios de malaria tenían un incremento de células TFH (PD-1+ICOS+CXCR5+CD45RO+CD4+CD3+) y correlaciones fuertes que involucran grupos de células plasmáticas, anticuerpos y células TFH. Mostrando que las infecciones múltiples activan las células TFH de circulación, generan linfocitos B de memoria clásica y aumenta la producción de anticuerpos, produciendo una respuesta humoral efectiva (241).

En individuos con infección asintomática por *P. vivax* se encontraron aumentadas las células TFH CXCR3+ en circulación, estas células también se encontraron en pacientes asintomáticos, lo anterior sugiere que una baja carga de parásitos es suficiente para aumentar esta población celular; además en ese estudio identificaron como predictor de riesgo reducido de infección a los linfocitos T CD4+ de memoria (242).

En estudios realizados a personas inmunizadas con una vacuna de influenza estacional, donde evaluaron muestras de tres cohortes de vacunados (adultos y niños) encontraron que los linfocitos T ICOS+ CXCR3+ CXCR5+ CD4+ inducen la proliferación de células B de memoria y las diferencian a células plasmáticas, además se correlacionaron con la respuesta de anticuerpos protectores preexistentes, pero no con la producción de anticuerpos primarios (243).

Los resultados obtenidos en este trabajo resaltan el efecto de la modificación de los aminoácidos que entran en contacto con el HLA (DRB1*04:01 y 13:02) en los epítopes analizados (proteínas MSP1 y DBP) favoreciendo la respuesta de las TFH hacia el subconjunto (poblaciones caracterizadas por CITRUS) PD1+, CXCR3+ CCR7+ CXCR5+ CD45RA+ CD69+ CD4+). Este efecto se correlaciona con la población celular aumentada en individuos infectados naturalmente por *P. vivax* descritas previamente (235). Lo anterior permite sugerir que estos péptidos modificados fueron capaces de generar la expansión y activación de esta población celular (CD69+).

La población identificada por CITRUS se correlacionó con la evaluada en estudios para monitorear la respuesta inmune a la vacunación por influenza (243), la cual se asoció con el aumento de las células B de memoria y la producción de anticuerpos de alta afinidad, por lo cual estos resultados permiten sugerir que péptidos modificados que induzcan una expansión de TFH activadas como la descrita anteriormente, pueden ser buenos candidatos a vacuna contra la malaria.

En conjunto, los resultados obtenidos en este trabajo van dirigidos a aportar información relevante sobre la modificación de los epítopes importantes para el diseño de vacunas basadas en péptidos, las cuales han llamado la atención de los investigadores en los últimos años. Estas vacunas poseen ventajas como: (i) una fácil producción a gran escala, (ii) no requieren de condiciones especiales de almacenamiento al ser liofilizadas, (iii) se pueden personalizar al alelo de HLA requerido, y (iv) se puede dirigir a un tipo de respuesta inmune específica (244). Sin embargo, poseen algunas desventajas: (i) pueden ser muy sensibles a mutaciones de los epítopes de interés, (ii) poseen restricción genética por la especificidad del TCR, y (iii) presentan baja inmunogenicidad (245).

CONCLUSIONES

Dando cumplimiento a los objetivos planteados en este estudio, se puede concluir lo siguiente:

Para el primer capítulo, se correlacionaron diferentes metodologías como fueron las herramientas bioinformáticas, los ensayos de unión y los ensayos de antigenicidad *in vitro*, para la evaluación de la respuesta de la población de linfocitos T CD4+ provenientes de individuos de zonas endémicas. Este enfoque permitió obtener epítopes T de las proteínas PvRON2 y Pv12 de *P. vivax* que generaron una respuesta linfoproliferativa y la producción de citoquinas por parte de los linfocitos T de memoria de estos individuos, los cuáles pueden ser de interés para ser incluidos entre los antígenos candidatos a vacuna para la malaria.

El segundo capítulo, mediante diferentes ensayos de antigenicidad se evaluó la respuesta de anticuerpos adquirida naturalmente contra epítopes B de PvRON2 y Pv12, en individuos expuestos. Con estos resultados se confirmó el rol antigénico de estas proteínas y que estos epítopes pueden ser evaluados como candidatos a vacuna contra esta enfermedad.

En el tercer capítulo la metodología empleada permitió establecer un modelo *in vitro* que demostró que las modificaciones realizadas a los epítopes indujeron una respuesta de la población de células TFH en donantes sanos. Esto sugiere que una estrategia de vacunación empleando epítopes modificados puede dirigir la respuesta hacia una población de células TFH facilitando la generación de una respuesta inmune efectiva de larga duración contra la malaria.

PERSPECTIVAS

Los resultados de este trabajo demostraron la importancia de evaluar tanto epítopes T como epítopes B, frente a la infección natural causada por *P. vivax*. Además, se implementó un ensayo funcional donde se mostró que las modificaciones fueron capaces de inducir una respuesta de una población celular que favorecería una respuesta inmune eficaz y de larga vida frente a una vacuna.

Para lo cual hay varios aspectos por evaluar, por una parte, la producción de IL-21 por las células dendríticas derivadas in situ a partir de monocitos puede indicar la inducción y calidad de la respuesta de la población de TFH.

Con el fin de complementar el ensayo de inmunogenicidad con los péptidos modificados, planteamos la purificación de linfocitos B autólogos para evaluar la generación de células plasmáticas específicas de antígeno. A la vez, evaluar si se generan linfocitos B de memoria y producen anticuerpos específicos para los epítopes modificados.

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The *in Vitro* Antigenicity of *Plasmodium vivax* Rhoptry Neck Protein 2 (*Pv*RON2) B- and T-Epitopes Selected by HLA-DRB1 Binding Profile

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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 (*Pv*RON2) 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

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and MSP2. The bioinformatics results and *in vitro* evaluation reported here highlighted two T-cell epitopes (39047 and 39154) being recognized by memory cells and a B-cell epitope (39041) identified by *P. vivax*-exposed individuals' sera which could be used as potential candidates when designing a subunit-based vaccine.

Keywords: *Plasmodium vivax*, *Pv*RON2, HLA-DRB1 typing, antigenicity, synthetic peptide, epitope, cellular and humoral response

BACKGROUND

Malaria is one of the most important public health problems in tropical and subtropical regions worldwide. Nearly 3.3 billion people globally are at risk of contracting the disease; 214 million new cases appeared and 438,000 deaths occurred in 2015. Malaria is caused by parasites from the phylum Apicomplexa, genus *Plasmodium*. *Plasmodium vivax* is the second most prevalent known species and has the greatest geographical distribution as it can develop in its vector at lower temperatures and survive at higher altitudes. It also has a latent form known as hypnozoite; this remains in the hepatocytes, enabling parasite survival in a host for a long time (Mueller et al., 2009; Guerra et al., 2010; WHO, 2016).

Although malarial cases in Latin America decreased during the last decade, a rise in cases reported from Venezuela and Colombia has been reported in 2015 and 2016 (PAHO/WHO, 2017), Colombia being listed as the fourth regarding incidence during 2015 (i.e., 10% of malarial events) (WHO, 2016). A passive surveillance study of malarial transmission in Colombia between 2011 and 2013 showed that 50.7% of cases were caused by *P. vivax*, 48.9% by *P. falciparum* and 0.4% mixed infection (Arévalo-Herrera et al., 2015). A severe malaria study on Colombia's Pacific coast showed that *P. vivax* induced acute anemia in children and *P. falciparum* patients had high renal and hepatic damage rates (Arévalo-Herrera et al., 2017).

Since *P. vivax* has wide-scale global distribution, some strategies used to combat malaria involve using insecticide-impregnated mosquito nets and drugs such as sulphadoxine-pyrimethamine, artemisinin, and chloroquine (WHO, 2016); despite such efforts, vector insecticide resistance and parasite resistance to anti-malarial drug has increased during recent years (Rieckmann et al., 1989; Fairhurst and Dondorp, 2016). Administering anti-malarial drugs, together with developing an effective antimalarial vaccine, is considered a relevant control strategy for preventing and eradicating malaria (WHO, 2016).

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* (Bozdech 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, *Pv*RON1, *Pv*RON2, *Pv*RON4, *Pv*RALP and *Pv*RON5 (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 (*Pv*RON2) 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* (*Tg*RON2) and *P. falciparum* (*Pf*RON2) interacts directly with the AMA-1 protein. The RON complex is involved in forming the moving junction (MJ) (electro dense ring-shaped structure) which allows parasite entry to a host cell (Aikawa 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 *Pf* AMA1-RON2L complex induce protection in *Aotus* monkeys, mediated by high neutralizing

Abbreviations: aa, amino acids; AMA, apical membrane antigen; APC, antigen presenting cell; BSA, bovine serum albumin; CBA, Cytometric Bead Array; CFSE, carboxyfluorescein diacetate N-succinimidyl ester; CPD, citrate phosphate dextrose; DAPI, 4′, 6-diamidino-2-phenylinodole dihydrochloride; DNA, deoxyribonucleic acid; DBP, Duffy binding protein; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GAMA, GPI-anchored micronemal antigen; gDNA, genomic deoxyribonucleic acid; GPI, glycophosphatidylinositol; HA, hemagglutinin antigen; HABPs, high activity binding peptides; HLA, human leucocyte antigen; HPLC, high-performance liquid chromatography; IEDB, immune epitope database; IFA, indirect immunofluorescence assays; IFN-γ, interferon gamma; IgG, immunoglobulin G; IL, interleukin; MHC, major histocompatibility complex; MJ, moving junction; Mrz, merozoites; MSP, merozoite surface protein; NGS, next generation sequencing; NK, natural killer; NKT, natural killer T-cells; OD, optical density; *P. falciparum*, *Plasmodium falciparum*; *P. vivax*, *Plasmodium vivax*; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PBST, PBS-0.05% Tween 20; pg, picograms; PHA, phytohemagglutinin; pNPP, p-nitrophenylphosphate; pRBC, parasite red blood cells; PV, parasitophorous vacuole; RALP, rhoptry-associated leucine (Leu) zipper-like protein; RBC, red blood cells; RON, rhoptry neck proteins; RPMI, roswell park memorial institute; RT, room temperature; SE, standard error; SEM, standard error of the mean; SI, stimulation index; *T. gondii*, *Toxoplasma gondii*; TBS, tris-buffered saline; TBSA, TBS-1% BSA; TCR, T-lymphocyte receptor; Th1, T helper 1; Th2, T helper 2; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF, tumor necrosis factor; TRAg, tryptophan-rich antigen; TT, tetanus toxoid; VDR, vitamin D3 receptor; μM, micromolar.

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 *Pv*RON2 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 Bepripred 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 *Pv*RON2 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 (Deléage 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% CO_2 atmosphere. The purification was carried out as previously described by Vargas et al. (2003), briefly 5×10^9 cells were lysed at 1×10^8 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 purified by affinity chromatography using Protein A-Sepharose CL-4B beads) overnight, HLA-DRB1* molecules were obtained by affinity chromatography. HLA-DRB1 protein purity 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 *Pv*RON2 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 $HA_{306-318}$ (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 *Pv*RON2 *in vitro* binding.

Peptide code	Sequence	CoreF	HLA-DRB1* allele	NetMHCIIpan 3.0 (%Rank)	Binding percentage*	IC50 μM*	IC50 ratio
39147	LKPFYSLETMLMANS	FYSLETMLM	DRB1*0401	0.3	92.6	4.6	0.2
			DRB1*0701	2.5	83.2	26.0	1.1
			DRB1*1101	10.0	79.3	23.0	4.8
			DRB1*1302	34.0	63.0	79.0	10.6
39148	NVRKFFLNDVSSIRH	FFLNDVSSI	DRB1*0401	1.0	83.0	11.9	0.6
			DRB1*0701	5.0	79.9	39.7	1.7
			DRB1*1101	19.0	73.8	83.4	17.5
			DRB1*1302	1.4	94.2	7.4	1.0
39149	DKSFISEANSFRNEE	FISEANSFR	DRB1*0401	3.5	85.8	26.3	1.4
			DRB1*0701	17.0	42.8	ND	ND
			DRB1*1101	26.0	36.5	ND	ND
			DRB1*1302	24.0	33.5	ND	ND
39150	QTAFRKFFKKIISLG	FFKKIISLG	DRB1*0401	17.0	83.6	33.7	1.7
		FRKFFKKII	DRB1*0701	6.5	67.2	51.8	2.2
			DRB1*1101	1.2	87.6	83.4	17.5
			DRB1*1302	48.0	7.7	ND	ND
39151	KLKYIFKRRKTMKKK	FKRRKTMKK	DRB1*0401	37.0	65.3	40.0	2.1
			DRB1*0701	6.0	36.0	ND	ND
		YIFKRRKTM	DRB1*1101	0.1	78.5	51.1	10.7
			DRB1*1302	27.0	13.1	ND	ND
39152	LFYVNLFIMSSLSRK	LFIMSSLSR	DRB1*0401	3.0	65.8	2.8	0.1
		FIMSSLSRK	DRB1*0701	2.0	11.0	ND	ND
			DRB1*1101	1.4	91.8	1.9	0.4
			DRB1*1302	27.0	94.7	8.0	1.1
39153	MKLLQHIPANLLENI	LLQHIPANLL	DRB1*0401	0.5	61.2	57.0	2.9
			DRB1*0701	0.1	85.4	7.5	0.3
			DRB1*1101	6.5	72.7	52.1	10.9
			DRB1*1302	0.1	91.9	10.7	1.4
39154	LKFIVRGNNLKFLNN	IVRGNNLKF	DRB1*0401	11.0	22.2	ND	ND
			DRB1*0701	4.5	43.7	ND	ND
		FIVRGNNLK	DRB1*1101	3.0	24.9	ND	ND
		IVRGNNLKF	DRB1*1302	0.2	90.1	6.5	0.9
39046	NYEIYIASSSNIYLM	YIASSSNIY	DRB1*0401	0.8	91.8	28.4	1.5
			DRB1*0701	0.3	91.5	23.3	1.0
			DRB1*1101	13.0	82.9	120.0	25.2
		IYIASSSNI	DRB1*1302	0.2	92.8	29.6	4.0
39047	RGPVNYHFSNYMNLD	VNYHFSNYM	DRB1*0401	16.0	59.8	54.5	2.8
		YHFSNYMNL	DRB1*0701	10.0	90.4	6.0	0.3
			DRB1*1101	37.0	0.0	ND	ND
		VNYHFSNYM	DRB1*1302	13.0	37.8	ND	ND
39048	TPIIVKYDNTHAKNR	IIVKYDNTHA	DRB1*0401	12.0	90.7	11.9	0.6
			DRB1*0701	41.0	10.5	ND	ND
			DRB1*1101	24.0	16.8	ND	ND
			DRB1*1302	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.*

TABLE 2 | Humoral response to *Pv*RON2 B-cell epitopes.

B epitope code	Amino acid sequence	Average response
39041	YGRTRNKRYMHRNPGEKYKG	0.159 ($SE = 0.026$)
39042	KLQQEQNELNEEKERQRQEN	0.104 ($SE = 0.016$)
39043	QEQEEEEDDNDPNGSKKNGK	0.142 ($SE = 0.018$)
39044	EKIRKQEEEEEERINNQRRA	0.094 ($SE = 0.015$)
*Pv*GAMA-CT	434–749 aa	0.475 ($SE = 0.071$)

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

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:

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

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:

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

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, the buffy 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, 5μM) (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. *Pv*12 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% CO_2 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 multitest 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-phenylinodole 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 r*Pv*GAMA (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 (H_3PO_4); 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 2 h 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 (H_3PO_4). 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. $p \leq 0.05$ 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 *Pv*RON2 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**.

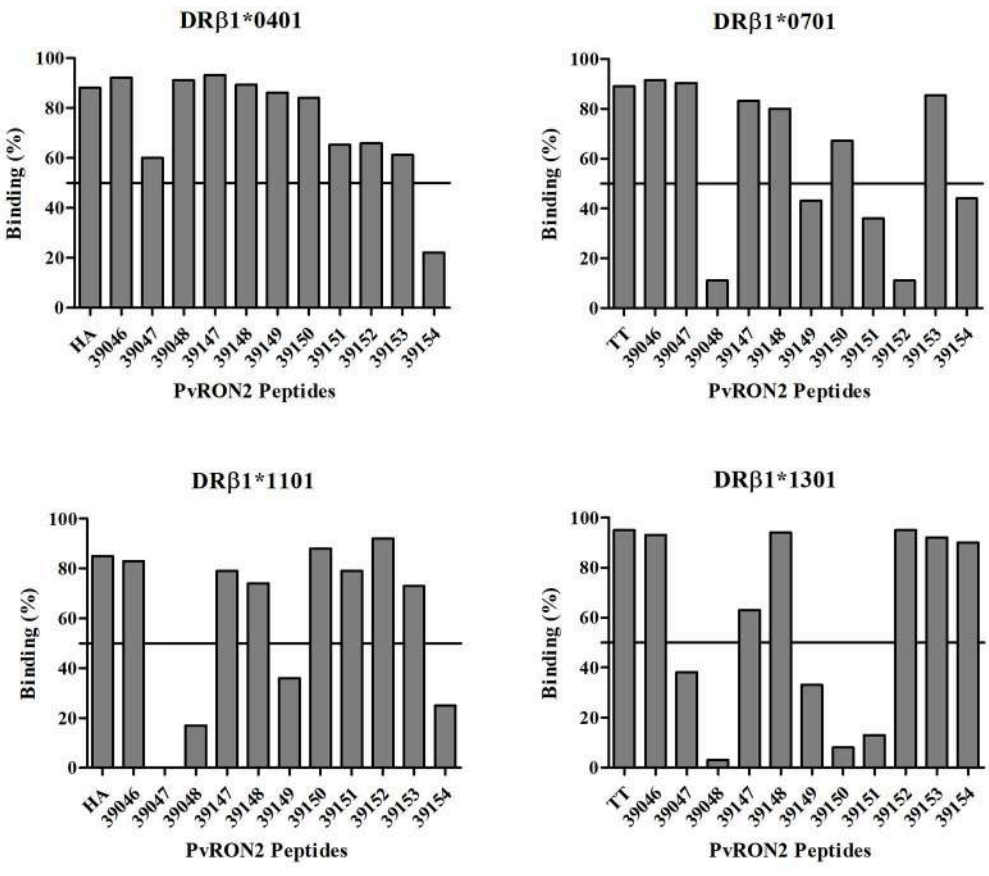

FIGURE 1 | *Pv*RON2 peptides *in vitro* binding to purified HLA-DRB1* molecules. A cut-off line is shown at 50% binding, used for selecting high-binding peptides for further evaluation of IC50 value. Each plot shows percentage epitope binding to HLA-DRB1* in this study and that for their control peptide.

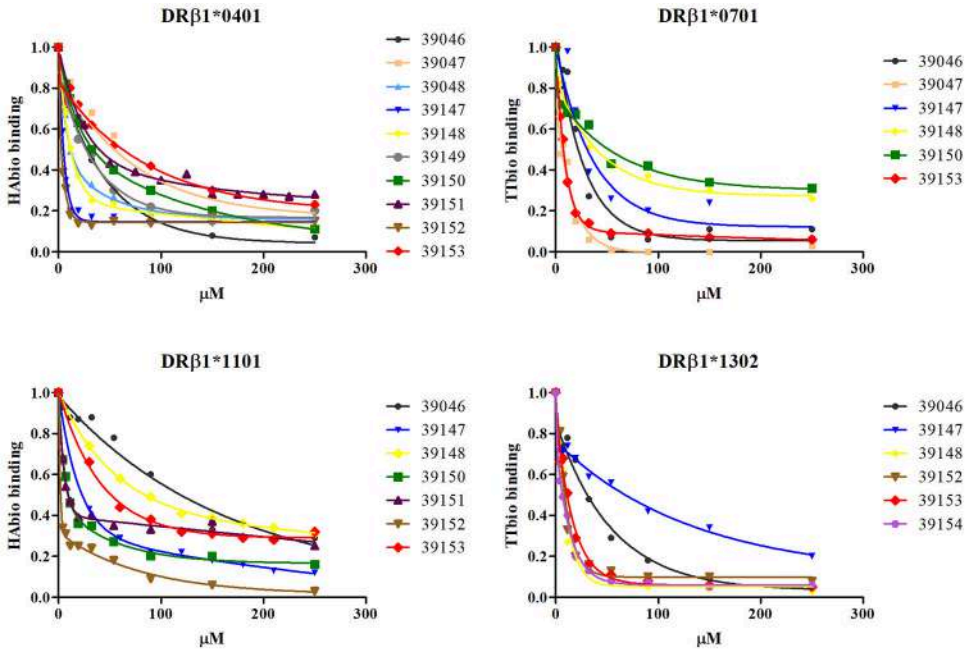

FIGURE 2 | *In vitro* assays for calculating a *Pv*RON2 peptide's IC50 value. Different epitope concentrations were evaluated for calculating the value at which control peptide was displaced by 50% (using a second order exponential decay function). Each point under the curve represents evaluated epitope concentration-dependent control peptide (μM) binding.

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 *Pv*RON2 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).

*Pv*RON2 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 ($p = 0.001$) and *P. vivax*-lysate ($p = 0.0001$). IL-6 responses were significantly greater to peptides 39047 ($p = 0.01$), 39152 ($p = 0.0025$), 39154 ($p = 0.002$) and *P. vivax*-lysate ($p = 0.0001$) (**Figure 4**). Cytokine levels were

TABLE 3 | A summary of PBMC proliferative response to *Pv*RON2 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	3.052 (SE 1.09)	1.004 (SE 0.198)	0.1751
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 *Pv*RON2 B-Epitope Humoral Immune Response in *P. vivax*-Exposed Individuals

Antibody response against four *in silico* selected *Pv*RON2 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). r*Pv*GAMA 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 ($p = 0.0007$) 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 *Pv*RON2 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 *Pv*RON2 B-cell epitopes since there were no significant differences between both areas regarding *Pv*GAMA ($p = 0.4265$), 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 *Pv*RON2 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

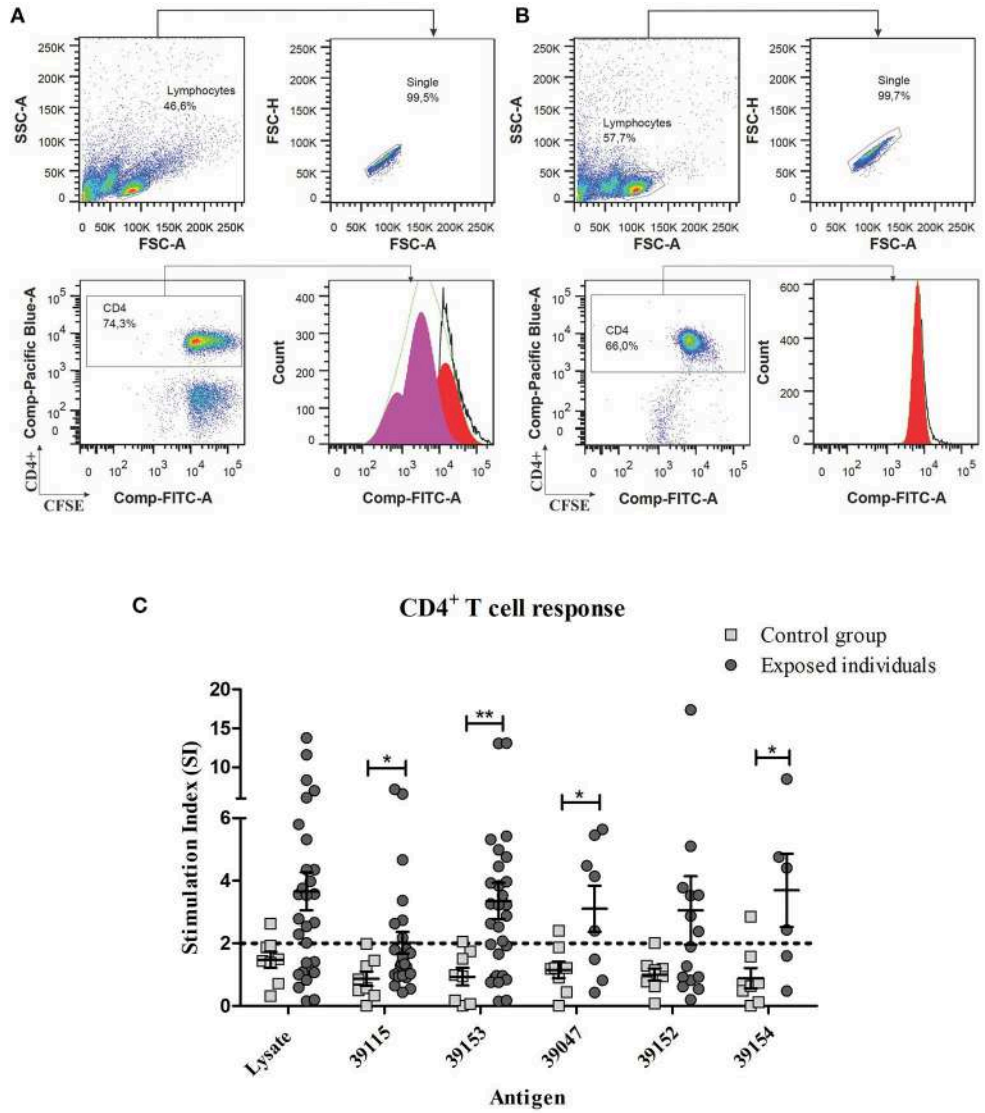

FIGURE 3 | Gating strategy for the proliferation assays and PBMC proliferative response to *Pv*RON2 epitopes from individuals exposed to *P. vivax* infection compared to control group. **(A)** *P. vivax*-exposed individuals' PBMC stimulated with universal peptide (39153). **(B)** Non-stimulated *P. vivax* exposed individuals' PBMCs. **(A,B)** Upper left plot, selected lymphocyte population (SSC-A vs. FSC-A), upper right plot selection of single cells from lymphocyte population (FSC-H vs. FSC-A). The lower left-hand plot shows gated CFSE label lymphocytes (Comp-FITC-A) for analyzing CD4+ T-cells (Comp-Pacific Blue-A). Lower right-hand plot shows CD4+ lymphocyte proliferation analyzed by FlowJo software (v7.6.5, Ashland, Oregon, USA) using 7 peaks or cell generations. **(C)** Mann-Whitney and Student's *t*-tests were used for assessing statistically significant differences between exposed individuals and control group. Universal peptide 39153 ($n = 29$), DRB1*04 and DRB1*11 peptide 39152 ($n = 15$), DRB1*07-specific peptide 39047 ($n = 8$), DRB1*13-specific peptide 39154 ($n = 6$), low-binding control peptide 39115 ($n = 29$) and *P. vivax* lysate ($n = 29$) responses are shown. The CD4+ cells were labeled with Pacific Blue mouse anti-human CD4 (RPA-T4 clone) antibody. Statistically significant differences ($p \leq 0.05$) are shown and data represents the means ± SEM for all values. *$p < 0.05$ and **$p < 0.005$.

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 (*Pv*GAMA) (Baquero et al., 2017), some

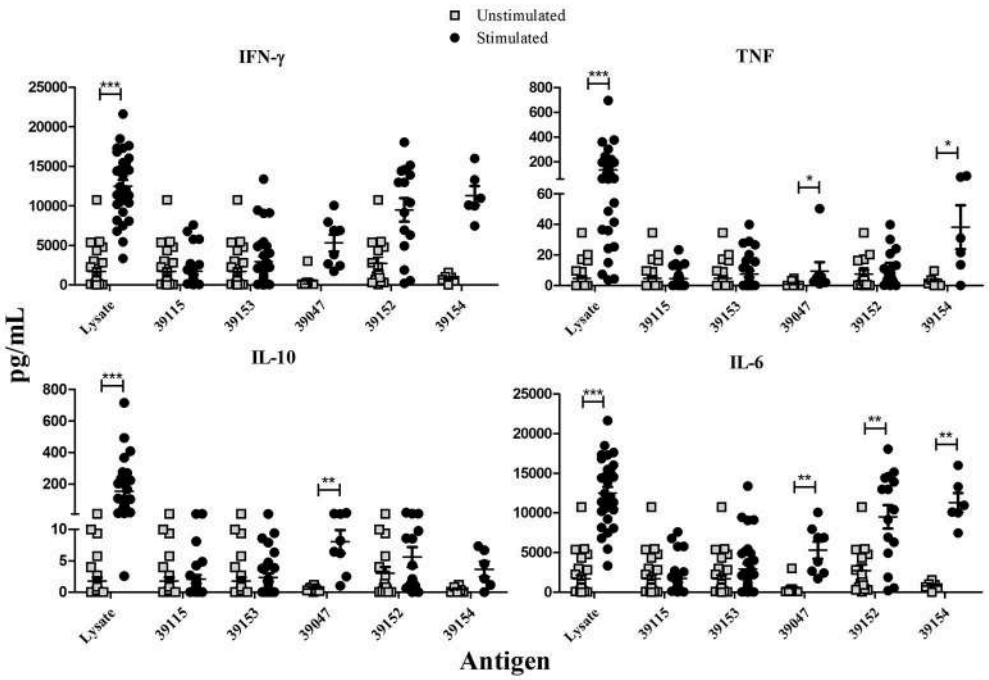

FIGURE 4 | Exposed individuals' supernatant culture *in vitro* cytokine production**.** Individual data shows the mean value of non-stimulated and PBMCs stimulated with universal epitope (39153), specific epitopes 39047, 39152, and 39154, and *P. vivax* lysate. IFN-γ, TNF, IL-10, and IL-6 levels were measured by CBA kit; cytokine concentration is expressed in pg/mL. Statistically significant differences ($p \leq 0.05$) 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 (*Pv*TRAg) family (Zeeshan et al., 2014), merozoite surface protein-1 (*Pv*MSP-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 *Pv*RON5 (Arévalo-Pinzón et al., 2015), *Pv*RON4 (Arévalo-Pinzón et al., 2013) and *Pv*RON2 (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 *Pv*RON2 high-affinity binding-MHC-II DRB1* peptides in *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–pathogen 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 *Pv*RON2 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 (DRBl*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 DRBl 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

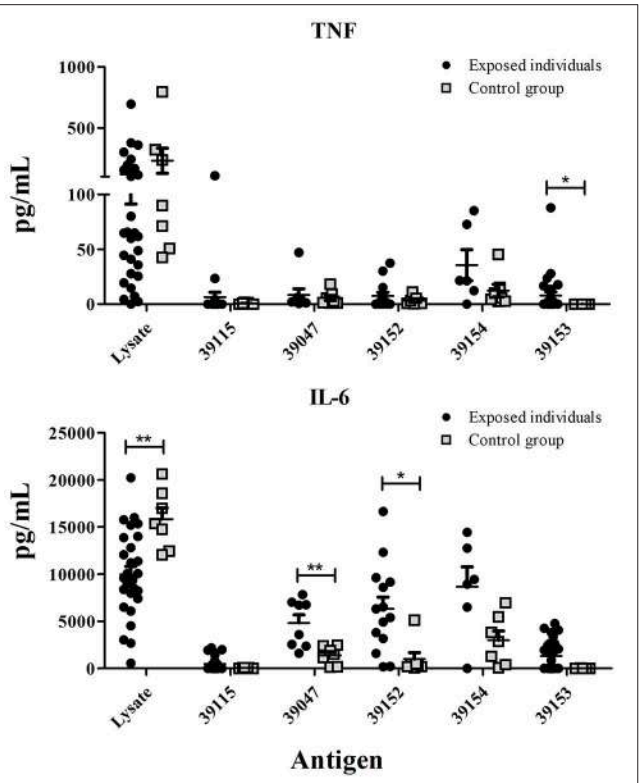

FIGURE 5 | Exposed individuals and control group supernatant culture *in vitro* cytokine production. Individual cytokine values from PBMCs stimulated with universal peptide (39153), specific epitopes 39047, 39152, and 39154, and *P. vivax* lysate. TNF and IL-6 levels were measured by CBA kit and cytokine 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 $^{**}p < 0.005$.

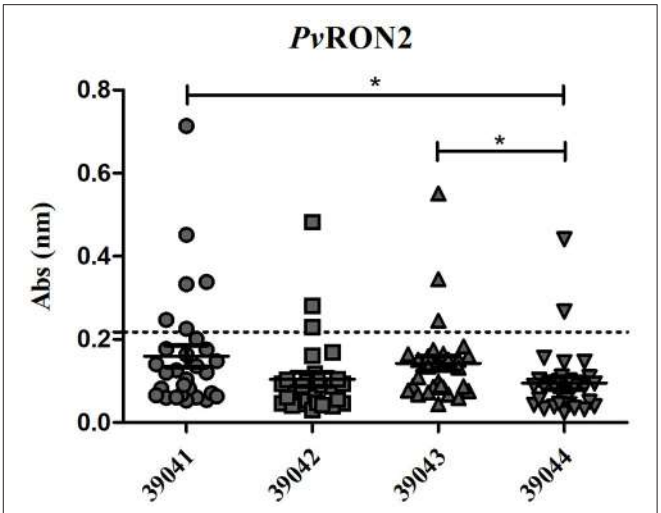

FIGURE 6 | IgG antibody response against four *Pv*RON2 B-cell epitopes ($n = 30$). Seropositive samples were those above the cut-off point (0.218, dotted line), calculated as control group's mean plus two standard deviations. The Kruskal-Wallis test was used for analyzing differences between each B-epitopes response in *P. vivax*-exposed individuals' samples. $^{*}p < 0.05$.

present in other racial groups and were associated with protection against severe malaria, i.e., directional selection (Hill et al., 1991).

Several factors (e.g., parasite evasion mechanisms and immune molecule polymorphism) can affect protection-inducing immune responses to malarial parasites and, consequently, malaria vaccine development. Parasite evasion mechanisms include immunodominant antigen polymorphism, antigenic variation and diversion, epitope masking and the smoke-screen strategy. *P. vivax* and *P. ovale* have additional escape mechanisms which are mediated by long-lasting hypnozoites, as well as using different erythrocyte invasion pathways (Rénia and Goh, 2016). MHC Class I and II have enormous allele polymorphism and aa sequence variation in the peptide binding region, thereby enabling peptides to bind to different alleles (Blum et al., 2013). Such variability hampers a single epitope-based vaccine against *Plasmodium* parasites being developed; however, *in silico* analysis of T- and B-cell epitopes could be useful for identifying vaccine candidates represented by different epitopes which could provide coverage of the whole target population. The exposed patients studied here were grouped according to their HLA-DRB1* to cover the most frequently occurring alleles in the endemic population worldwide, such as HLA-DRB1*04, HLA-DRB1*07, HLA-DRB1*11, and HLA- DRB1*13. Cell-mediated immune 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 *Pv*RON2 B-epitopes which were recognized by IgG antibodies and subclasses. Low individual *Pv*RON2 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 *Pv*MSP8. 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 *Pv*RON2 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).

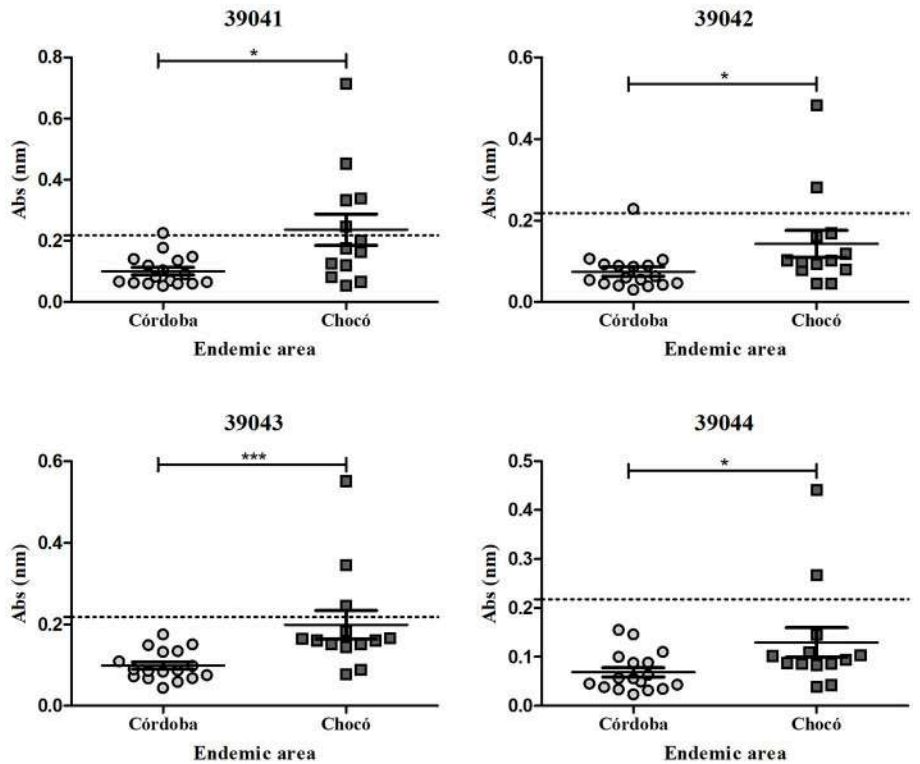

FIGURE 7 | IgG antibody response to *Pv*RON2 B epitopes by endemic area. Significant differences (calculated by Mann-Whitney test) between samples from Colombia's Chocó ($n = 13$) and Córdoba ($n = 17$) departments are shown. The dashed line indicates the cut-off point for seropositive samples. $^{*}p < 0.05$ and $^{***}p < 0.0005$.

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 *Pv*MSP8 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 *Pv*RON2 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 *Pv*RON2 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 *Pv*RON2 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 *Pv*RON2 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

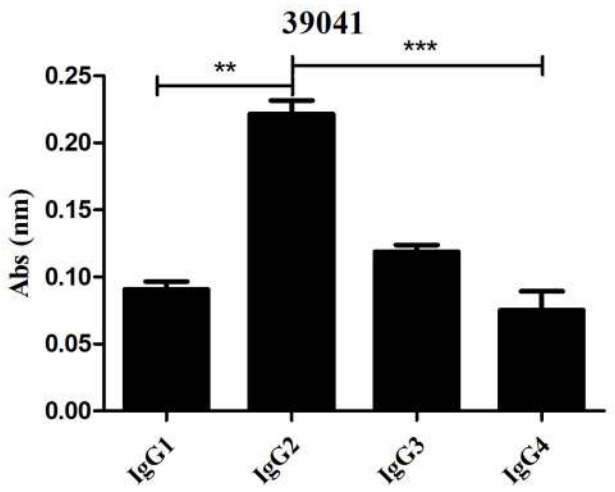

FIGURE 8 | Evaluating IgG subclass response to the 39041 epitope ($n = 6$). The Kruskal-Wallis test was used for analyzing differences between each IgG subclass response in *P. vivax*-exposed individuals' samples. $^{**}p < 0.005$ and $^{***}p < 0.0005$.

gametocytemia clearance in *P. vivax*-exposed individuals (Sortica et al., 2014). *P. vivax* lysate-induced cytokine responses in unexposed individuals' PBMCs could be explained by innate immune cell cytokine production, i.e., macrophages, dendritic cells, NK, NKT and naïve T-cells which become effector cells (Stevenson and Riley, 2004). High cytokine induction in healthy individuals compared to *P. vivax*-exposed individuals might be related to a parasite evasion mechanism for inhibiting effective immune responses able to eliminate the parasite (Rénia and Goh, 2016). 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 1 | Control group supernatant culture *in vitro* cytokine production. Data individual show the mean value of unstimulated and stimulated PBMC (n = 8) with universal epitope (39153), specific epitopes (39047, 39152,

and 39154) and *P. vivax* lysate. IFN-γ, TNF, IL-10, 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 of all values.

Supplementary Figure 2 | Immunofluorescence patterns for exposed individuals from Colombia's *P. vivax*-endemic areas and the control group. The upper panels show one Bahía Solano's exposed individual serum recognition of pRBC. Nuclei were stained with DAPI, the parasite with anti-parasite FITC and then, both were merged. The bottom panels show serum from one non-exposed individual.

Supplementary Figure 3 | IgG antibody response against *Pv*GAMA 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 *Pv*RON2 peptides.

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REVIEW
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What Is Known about the Immune Response Induced by *Plasmodium vivax* Malaria Vaccine Candidates?

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Malaria caused by *Plasmodium vivax* continues being one of the most important infectious diseases around the world; *P. vivax* is the second most prevalent species and has the greatest geographic distribution. Developing an effective antimalarial vaccine is considered a relevant control strategy in the search for means of preventing the disease. Studying parasite-expressed proteins, which are essential in host cell invasion, has led to identifying the regions recognized by individuals who are naturally exposed to infection. Furthermore, immunogenicity studies have revealed that such regions can trigger a robust immune response that can inhibit sporozoite (hepatic stage) or merozoite (erythrocyte stage) invasion of a host cell and induce protection. This review provides a synthesis of the most important studies to date concerning the antigenicity and immunogenicity of both synthetic peptide and recombinant protein candidates for a vaccine against malaria produced by *P. vivax*.

Keywords: malaria, *Plasmodium vivax*, immune response, antigenicity, immunogenicity

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INTRODUCTION

Malaria is one of the most important vector-transmitted diseases, affecting a large part of the world's population. Around 214 million new cases appeared in 2015, and 438,000 people died from the disease. This disease is caused by parasites from the phylum Apicomplexa, genus *Plasmodium*, and

Abbreviations: Spz, sporozoites; RBC, red blood cells; NT, N-terminal; pexel/VTS, *Plasmodium* export element/vacuolar translocation signal; Mrz, merozoites; NFkβ, nuclear factor kappa B; PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; MHC, major histocompatibility complex; NK, natural killer; IFN-γ, interferon gamma; TNF, tumor necrosis factor; LDH, lactate dehydrogenase; TAT, thrombin–antithrombin III; HNE, human neutrophil elastase; IL, interleukin; Th1, T helper 1; Th2, T helper 2; CXCL9, chemokine (C–X–C motif) ligand 9; CCL2, chemokine (C–C motif) ligand 2; CCL5, chemokine (C–C motif) ligand 5; Ig, immunoglobulin; i.v., intravenously; HLA, human leukocyte antigen; CSP, circumsporozoite protein; TRAP, thrombospondin-related adhesive protein; GPI, glycophosphatidylinositol; PNG, Papua New Guinea; LSP, long synthetic peptides; TLR, toll-like receptor; CpG, cytosine and guanine separated by one phosphate; GLA, glucopyranosyl lipid A; TSR, thrombospondin type 1 repeat; MSP, merozoite surface protein; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; HABPs, high activity binding peptides; AMA-1, apical membrane antigen-1; r*Pv*AMA-1, recombinant *Plasmodium vivax* apical membrane antigen-1; FcγR-Fc, Fc-gamma receptors; PP, poly-proline; TCR, T-lymphocyte receptor; ADCI, antibody-dependent cell-mediated inhibition; DBP, Duffy binding protein; DARC, Duffy antigen receptor for chemokines; rDBP, recombinant Duffy binding protein; PBMC, peripheral blood mononuclear cell; RBP, reticulocyte-binding proteins; HARBPs, high affinity reticulocyte-binding peptides.

is transmitted by the bite of a female mosquito from the genus *Anopheles* infected by the parasite (1).

Five species cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malarie*, *P. ovale*, and *P. knowlesi*. Acute febrile disease symptoms appear 10–15 days after the bite of an infected mosquito. Initial symptoms include fever, headache, shivering, and vomiting; if not treated early on, and depending on the species responsible for the disease, severe anemia, metabolic acidosis, or cerebral malaria may be produced, and even lead to death (1).

Studying the proteins involved in *P. vivax* invasion has not been easy, mainly due to technical restrictions such as a lack of continuous culture *in vitro*, meaning that studying the parasite's biology has been limited, as well as the identification of new antigens and their evaluation *in vitro* (2, 3).

Infection by more than one *Plasmodium* species is usually omitted in routine diagnosis by microscopy (4, 5), leading to an overestimation of the amount of cases caused by coinfection in endemic areas and thus to treatment failure (6). Drug resistance since the first report in 1989 (7) has been increasing worldwide throughout Southeast Asia [Indonesia, China, Thailand, Papua New Guinea (PNG)], South America (the Brazilian and Peruvian Amazon region, Colombia), Africa (Madagascar, Ethiopia), Pakistan, and Turkey (8, 9).

Such resistance appears to be related to mutations regarding multidrug resistance 1 (mdr1) gene and variation in the gene's number of copies, presumably due to selective pressure by first-line chloroquine treatment (10, 11).

Even though malaria caused by *P. vivax* has been considered benign (unlike that caused by *P. falciparum*), severe *P. vivax* malaria has emerged during the last few years with some cases leading to death (12–17). In spite of *P. vivax* malaria having a greater global distribution, it is still considered a neglected infection, thereby leading to socioeconomic impact factors being understated in endemic regions, causing more than US$2 billion per year costs worldwide (18). The forgoing means that investment and efforts must be focused on developing a vaccine against *P. vivax* malaria.

Antigenicity studies arise from evaluating the immune response induced in individuals naturally exposed to the infection. On the other hand, immunogenicity assays evaluate *in vitro* or *in vivo* the immune response induced when vaccine candidates are used for immunization (**Figures 1** and **2**).

The present review summarizes classical studies that have been carried out to date concerning the antigenicity and immunogenicity of the most important proteins considered candidates for a vaccine against *P. vivax* malaria. Although the use of a single-stage protein is not enough to provide a successful sterile vaccine, it has represented an important advance in identifying hundreds of malarial antigens that can be combined to develop a multistage, multi-epitope sterile vaccine.

MALARIA: INFECTION BY *P. vivax*

Around 90% of the clinical cases presented are the result of infection by two of the most relevant species: *P. falciparum* or *P. vivax*. *P. vivax* malaria is the second most important around the world and is the most prevalent on the Asian and American continents. Such infection is characterized by relapses several years after the first infection, since a latent form called hypnozoite occurs during hepatic phase. This stage is difficult to diagnose, allowing the parasite to survive in the host for longer (1, 19, 20).

Infection begins with the vector inoculating sporozoites (Spz) into the host's skin; these Spz are motile and travel through the blood stream, later being carried to the liver. Sinnis et al. have named a "skin stage" of infection because they have proposed that this interaction between Spz and cells at the injection site means that Spz may remain in the injection site for 2–3 h, maybe in hair follicles, giving rise to infective merozoites (Mrz) (21, 22). Regarding *P. berghei* expressing GFP (a rodent parasite), it has been observed that Spz have a random gliding-movement. Moreover, Spz glide into the skin, interacting with blood vessel walls. Lymphatic vessels also become invaded to drain lymph nodes near the injection site where some Spz can partially develop into exoerythrocytic stages (23–25).

Sporozoites migrate from the skin to liver cells (these becoming infected first) and then cross/traverse endothelial cells and use cell traversal machinery to pass through the endothelium, thereby beginning the hepatic stage that might go unnoticed clinically (26, 27). Some parasites remain as hypnozoites during this stage, and others go into the blood stream giving rise to the erythrocyte stage where the disease's clinical manifestations are presented.

The severity of the disease during the erythrocyte stage depends on various factors, such as the location of parasitized red blood cells (RBC) in the target organs, the local and systemic action of the parasite's bioactive products, pro-inflammatory cytokine production, as well as innate and adaptive immune system cytokine and chemokine regulators, and the activation, recruiting, and infiltration of inflammatory cells (28).

After invading the hepatocytes, each Spz replicates within the parasitophorous vacuole by a family of parasite proteins having an NT export motif called pexel/VTS (*Plasmodium* export element/vacuolar translocation signal) (29–31). The circumsporozoite protein (CSP) enters the hepatocyte's cytoplasm using the pexel/VTS motif and a nuclear localization signal to go into the nucleus. CSP in the nucleus induces the expression of the host's genes, where the NFkβ transcription factor controls the expression of genes involved in inflammation (32), controlling biological functions such as metabolic transport, the cell cycle, the immune response, and apoptosis, thereby creating a favorable setting for parasite growth (31).

The *Plasmodium* erythrocyte stage begins when an infected hepatocyte ruptures and releases close to 30,000 Mrz into the blood stream, undertaking an initial journey as merosomes to the lungs and then becoming disseminated in the circulation. Each Mrz infects an immature RBC (reticulocyte), which generates 16 new Mrz 48 h later (33).

IMMUNE RESPONSE REGARDING MALARIA

Innate and adaptive immune system molecules are involved in disease pathogenesis and control. Clinical immunity to malaria can be acquired during three phases: immunity to the disease, immunity to symptomatic infection, and partial immunity to

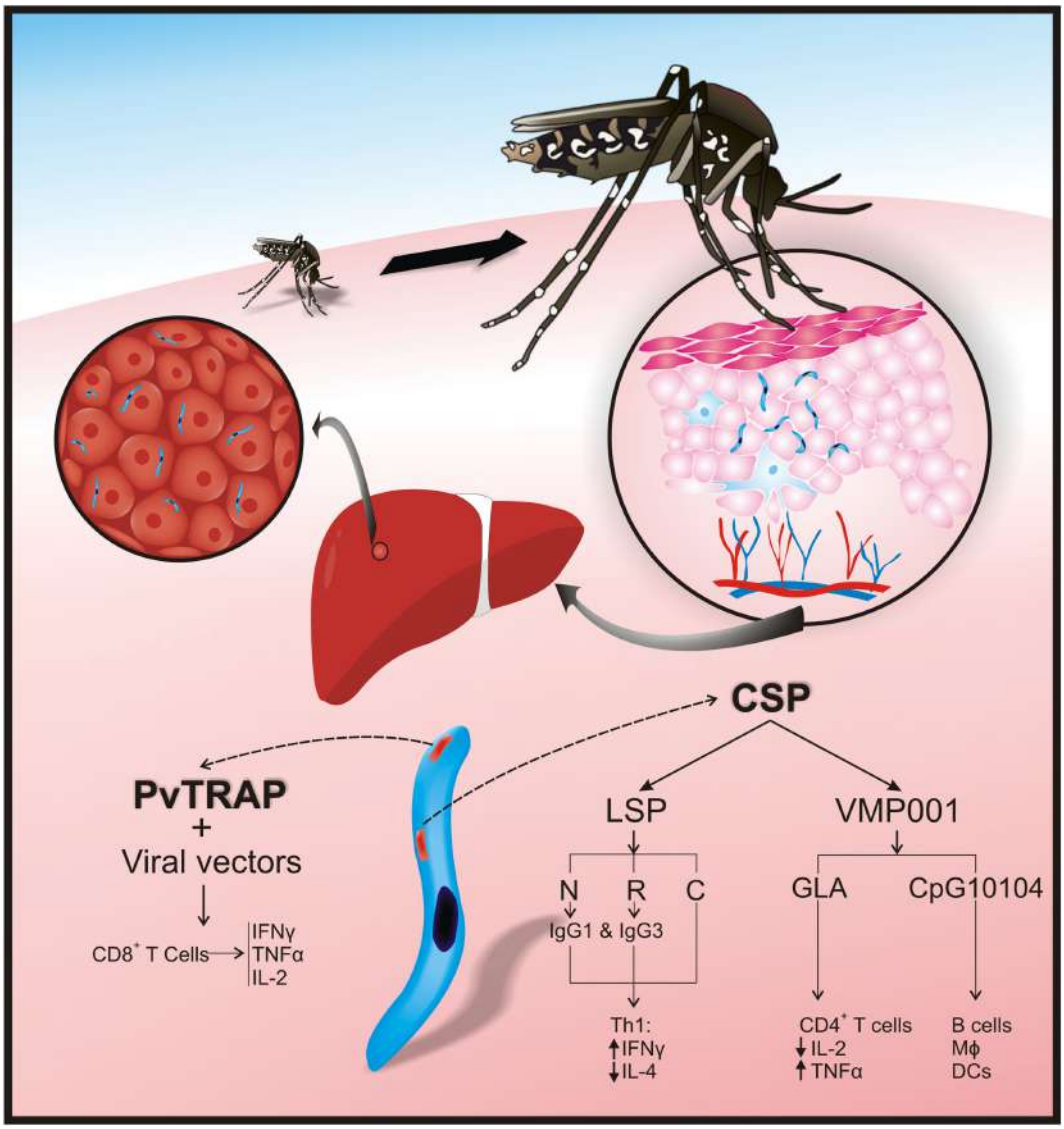

FIGURE 1 | ***Plasmodium vivax* preerythrocyte stage protein immunogenicity**. After sporozoites have been inoculated into the skin by *Anopheles* mosquitoes, they travel to the liver *via* the bloodstream and enter hepatocytes thereby initiating the preerythrocyte stage. *P. vivax* circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (*Pv*TRAP) are involved in hepatocyte recognition and binding in a mammalian host. In CSP, the N-terminal (NT) and repeat region facilitate parasite binding to hepatocytes. Adaptive immune responses against *Pv*CSP and *Pv*TRAP control invasion of hepatocytes by cytokines [CD4+ T-helper 1 (Th1) and CD4+ T-helper 2 (Th2) cells], cytophilic antibodies, and CD8+ T-cells. Interferon gamma (IFN-γ) increases and interleukin (IL)-4 decreases after vaccination with CSP-long synthetic peptides [CSP-LSP-N terminal; CSP-LSP-R (repeat region), and CSP-LSP-N terminal]. Cytophilic antibodies (IgG1 and IgG3) are produced after vaccination with CSP-LSP-N; CSP-LSP-R. Immunization with *Pv*CSP recombinant vaccine (VMP 001) combined with CpG10104 has induced protection and activation of B-cells, macrophages (MΦ), and dendritic cells (DCs). When this recombinant vaccine is formulated with glucopyranosyl lipid A (GLA), there is activation of CD4+ T-cells, production of tumor necrosis factor-alpha (TNF-α), and reduction of IL-2. Immunization with *Pv*TRAP, expressed in viral vectors, induces activation of CD8 T-cells and production of IFN-γ, TNF-α, and IL-2.

parasitemia (28). The premunition (absence of fever with infection and lower densities of parasitemia) is present in places where malaria is endemic and in people that had suffered of several infections through the years (8–15 years), thus acquiring natural immune responses that lower the risk of clinical disease (34). The term was coined early in the 1900s during epidemiological studies with patients from endemic areas that can control the parasitemia and develop a subclinical infection (35). It is characterized by a slow acquisition rate, present just in holo- or hyper-endemic areas, rapidly lost, strain dependent, IgG dependent, and directed toward blood-stage parasites; although the immune response induced is strong, it is not a sterilizing immunity. The protection mechanism has not been completely described, but there is evidence that cytophilic antibodies and memory cells produced after repeated infections with *Plasmodium* variants are responsible for this kind of protection (34, 36–39).

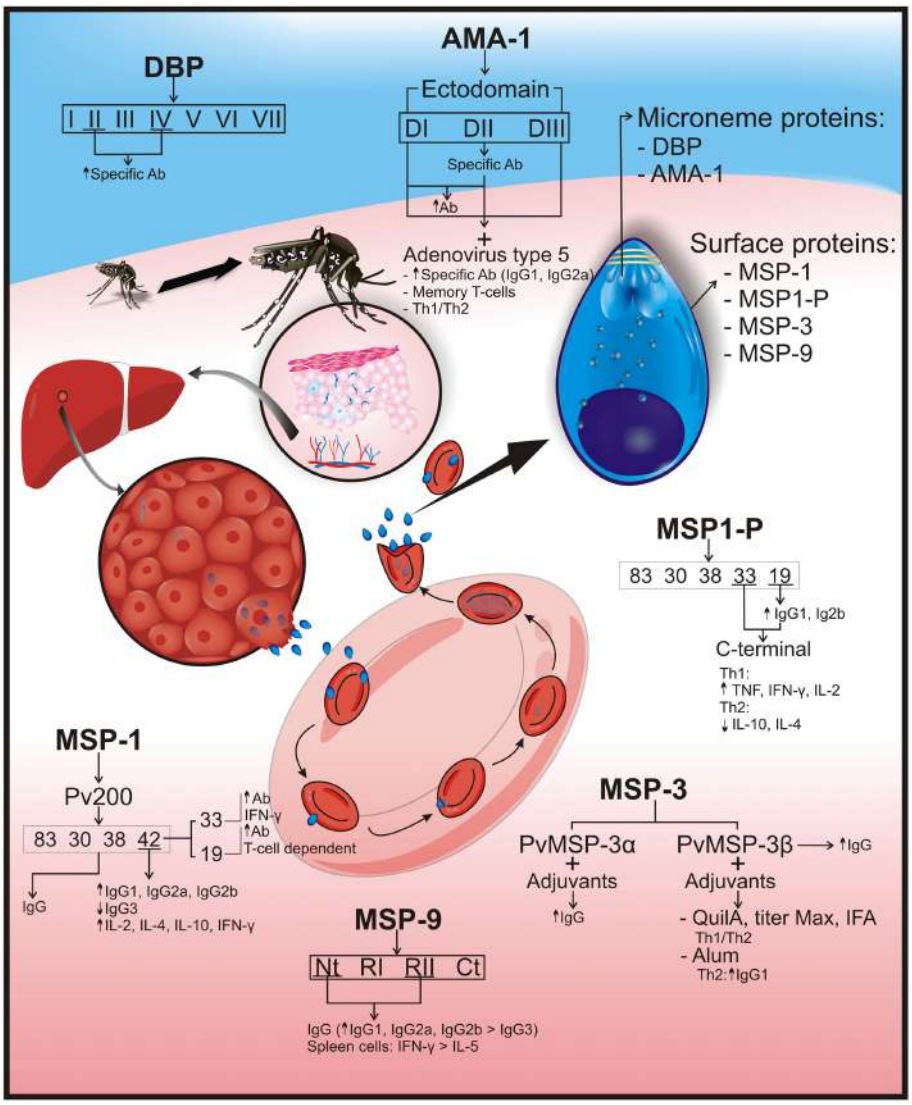

FIGURE 2 | ***Plasmodium vivax* erythrocyte stage protein immunogenicity**. *P. vivax* parasites are differentiated into tissue schizonts in hepatic cells, which, after thousands of replications, are released into the bloodstream as merozoites (Mrz). These Mrz predominantly invade reticulocytes, and their infection cycle is repeated every 48 h. Several surface and microneme merozoite proteins have been identified as vaccine candidates. Surface proteins would include merozoite surface protein-1 (MSP-1/*Pv*200), which is an abundant ligand on merozoite surface and is essential for reticulocyte invasion. MSP-1 was cleaved into 83, 30, 38, 42 (33 and 19) kDa fragments; immunization with the complete protein induced IgG production. MSP-1–42 fragment increased IgG1, IgG2a, and IgG2b production but not that of IgG3, as well as interleukin (IL)-2, IL-4, IL-10, and interferon gamma (IFN-γ) production in vaccinated mice. Immunization with 19-kDa fragments produced high antibody titers that were T-cell dependent. Higher antibody and IFN-γ production was observed after vaccination with the 33-kDa fragment. Another surface protein is merozoite surface protein-1 paralog (MSP1-P), which was also cleaved into 83, 30, 38, 42, 33, and 19 kDa; the last two fragments (C-terminal region) induced a Th1 cytokine response profile, having high tumor necrosis factor (TNF), IFN-γ, and IL-2, but low IL-10 and IL-4 cytokines (Th2 profile). High IgG1 and IgG2b titers were observed in vaccinated animals with 19-kDa fragment. Merozoite surface protein-3 (MSP-3): *Pv*MSP3-α block II is highly immunogenic and induces IgG production. The *Pv*MSP-3β region with Quil A, Titer Max, or IFA adjuvants has produced a balanced Th1/Th2 response and IgG but became directed toward a Th2 response when formulated with Alum. Merozoite surface protein-9 (MSP-9) immune response against NT and repeat region II was mainly IgG, having greater IgG1, IgG2a, and IgG2b titers than IgG3 isotype production. These two regions also induced higher IFN-γ than IL-5 production in spleen cells. The following are microneme proteins: the Duffy binding protein (DBP) is a surface receptor for invading human reticulocytes and is divided into seven regions where regions II (main ligand domain) and IV induce specific antibody production. Apical membrane antigen-1 (AMA-1) is essential during cell host invasion, and its ectodomain defines three subdomains (DI, DII, and DIII). Immunization with AMA-1 induced high IgG antibody titers. Vaccinating mice with human adenovirus type 5 and rAMA-1 have produced long-lived specific antibodies (IgG1 and IgG2a), memory T-cell, and Th1/Th2 balance immune responses. An arrow pointing upwards (↑) indicates an increase in antibody titers or cytokine production; an arrow pointing downwards (↓) shows reduced cytokine or antibody production.

An innate immune response is triggered during *Plasmodium* infection as first line of defense, followed by an adaptive immune response, which includes T-cells, B-cells, and antibodies. A mosquito inoculates Spz into a host's skin when biting; these can remain in the skin for up to 6 h after inoculation (40). Such retention affects the place for antigen presentation and the location and type of response so induced.

Dendritic cells (DCs) present antigens, depending on the anatomic environment and the resulting immune response. These cells, through pattern recognition receptors, recognize the pathogen-associated molecular patterns (PAMPs) exhibited by the parasite. The mechanism of action regarding such recognition triggers intracellular signals enabling DC maturation (41). Three PAMPS have been described in *P. falciparum*: hemozoin, immunostimulatory nucleic acid motifs, and glycosylphosphatidylinositol anchors [glycophosphatidylinositol (GPI) anchors] (42).

The parasite's main source of protein is RBC hemoglobin. Hemoglobin hydrolysis releases lipophilic prosthetic group—heme—which is extremely toxic for the parasite. Heme detoxification is thus necessary and is achieved by converting heme into an insoluble crystalline material called hemozoin (Hz) (43). Regarding *P. falciparum* infection, Hz binds DNA inside host cell phagolysosomes and cytosol, and toll-like receptor (TLR)9 is activated by nucleic acids, NLRP3, AIM2, and other cytosolic sensors (42, 44–46).

In terms of AT content in the genome, *P. falciparum* has the highest AT content (82%) and *P. vivax* the lowest AT content (56%). On the other hand, *in silico* analysis has shown that *P. falciparum* contains ~300 CpG and ~6,000 AT-rich motifs and *P. vivax* ~2,000 CpG and ~5,500 AT-rich motifs. The release of CpG *Plasmodium* DNA into phagolysosomes produces an innate immune response activating TLR9 (42, 44, 47).

Glycophosphatidylinositol anchors connect surface proteins with the protozoan plasma membrane; they are essential toxins for parasite viability (48). They turn on the innate response because they induce cytokine synthesis and are recognized by TLRs such as TLR1–TLR2 or TLR2–TLR6 (depending on GPI anchor activation containing three or two fatty acid chains, respectively) and TLR4 (49–51).

Some functions described for DCs have been T- and B-lymphocyte activation, immune tolerance, natural killer (NK) cell activation, and macrophage activation (52). For example, a third of Spz are drained to regional lymph nodes where they become internalized by skin-derived DCs (CD103+) and presented to CD8+ T-cells (26).

Some studies have shown that infected RBC bind to DCs, inhibit their maturation, and cannot stimulate a T-lymphocyte response in acute *P. falciparum* infection. Other studies have shown that inhibition depends on contacting a larger amount of infected RBC per DC (53, 54).

Antigens begin to be presented on hepatocyte surface during the hepatic stage in context of the major histocompatibility complex (MHC) class I molecules expressed on all nucleated cells to become recognized by CD8+ T-cells (55).

Immune response during the erythrocyte stage is mainly mediated by antibodies while a cellular response predominates during the hepatic stage (56). CD4+ T-, B-, and NK cells also play an important role in the immune response induced by the parasite during the erythrocyte stage since immunity depends on memory B-cell production and lifespan, following infection (57).

During *P. vivax* infection, some individuals can acquire immunity naturally; such immunity consists of a cytokine production-mediated cellular immune response, cytokine receptors, and proteolytic enzymes forming part of the host response to infection, as well as IgG antibodies. Patients having moderate parasitemia in endemic regions of Colombia have high IFN-γ and TNF-α levels, a pro-inflammatory cytokine profile correlated with the response found in an unstable transmission region. The balance in interleukin (IL)-10/TNF-α rate could prevent increased parasitemia and host pathology (58).

A study by Hemmer et al. evaluated the production of lactate dehydrogenase (as hemolysis parameter), TNF-α (which produces a response to parasite products and has antiparasitic activity), thrombin–antithrombin III (pro-coagulant activity parameter), and human neutrophil elastase (its secretion becoming increased by parasite products, having antiparasitic activity) in a population infected by *P. falciparum* and *P. vivax* or *P. ovale* (59).

The parasitemia/response rate of each parameter was greater in patients suffering *P. vivax* or *P. ovale* malaria than *P. falciparum*; regarding parasitemia, TNF-α response was stronger in *P. vivax* or *P. ovale* infection than *P. falciparum*. The increase of these factors in *P. vivax* infection helped to control parasitemia, while they led to complications in *P. falciparum* infection such as host response-mediated severe malaria (59). Studies have shown that *P. falciparum* parasitemia levels decrease when coinfected with either *P. vivax* or other *Plasmodium* species, compared to single infection (60), thereby attributing a possible attenuating role to other *P. falciparum* species (60–62).

Another study that compared the immune response of patients suffering complicated and uncomplicated malaria caused by *P. vivax* reported a higher IFN-γ/IL-10 rate in patients having complicated disease, as well as higher TNF-α level. They concluded that the severity of disease caused by *P. vivax* was correlated with pro-inflammatory immune response activation and cytokine imbalance (63).

Interleukin-10 acts as immunoregulator by controlling the effects of other cytokines produced by CD4+ Th1 and CD8+ T-cells in *Plasmodium* infection. The overproduction of cytokines such as IFN-γ by these cells not only helps to increase phagocytosis for eliminating the parasite but also produces immunopathological effects associated with the disease. However, studies involving another group of patients suffering *P. vivax* malaria found high levels of IFN-γ and IL-10 in patients with previous episodes of malaria. Polymorphism studies regarding the IL-10 gene promoter in populations from endemic regions have shown that polymorphisms neither influence the production of this cytokine nor its regulatory function regarding the immune response (64, 65).

Goncalves et al. evaluated the cytokine pattern in uncomplicated symptomatic *P. vivax* and *P. falciparum* infection in a low malaria transmission region in Brazil to test the hypothesis that *P. vivax* infection causes a greater pro-inflammatory cytokine response than infection by other *Plasmodium* species. They

found a greater anti-inflammatory cytokine response than in *P. falciparum* infection, but similar pro-inflammatory cytokine response. The response of anti-inflammatory cytokines such as IL-10 and IL-10/TNF-α, IL-10/IFN-γ and IL-10/IL-6 ratios in clinical malaria caused by *P. vivax* was short-lived and positively correlated with parasitemia rather than with the symptoms. This means that there must be a balance between inflammatory cytokine and regulator responses (66).

Network analysis was one of the approaches adopted by Mendoca et al. for understanding the interaction between different blood biomarkers for inflammation, tissue damage, and oxidative stress and the immunopathogenesis of malaria. They concluded that when studying uninfected individuals from endemic regions, the network of interactions showed high density between these biomarkers, limiting the symptoms but not the infection. IL-10 and IL-4 have connections with chemokine (C–X–C motif) ligand 9 (CXCL9) in uninfected people and with chemokine (C–C motif) ligand 2 and IFN-γ in people having asymptomatic *P. vivax* infection (67, 68).

Such interactions revealed a protective role for IL-4 and IL-10 cytokines due to their modulating effect on these pro-inflammatory cytokine and chemokine. The network of biomarkers in patients suffering mild malaria consisted of IFN-γ, tumor necrosis factor (TNF), and chemokine (C–C motif) ligand 5, while the interaction occurred between CXCL9 and IL-12 in symptomatic patients. CXCL9 was associated with regulatory cytokines thereby suggesting their role in resistance to infection, as well as being associated at the beginning with symptoms when linked to IL-12. Patients who had a fatal outcome regarding the disease had an interaction between TNF, IFN-γ, and IL-10, the latter modulating the Th1 response, negatively regulating TNF and IFN-γ, due to an IL-12p70 suppression that could lead to death (67, 68).

Cytokine profile variations have been observed in malaria–dengue coinfection. TNF levels have increased in patients with coinfection regarding single infection thereby highlighting the role of IL-6, INF-γ, and IL-7 (68). Regarding the humoral response, antibodies play an import role in protection against malaria, and this has been demonstrated in different studies. Cohen et al. showed that passive transfer of antibodies from malaria-immune individuals to naïve young children suffering severe clinical malaria reduced the parasite density and clinical symptoms related to this disease (69). This experiment was confirmed by further studies where adult patients controlled the clinical symptoms and parasitemia after receiving intravenously injections of sera from people living in malaria-endemic areas (70, 71).

Immunoglobulins can protect or arrest disease progression in different ways; neutralizing anti-Spz antibodies can block Spz from invading hepatocytes (72–74). Mrz can be opsonized in the erythrocyte stage by specific antibodies that activate cell-mediated death or prevent the invasion of RBC and block the proteins responsible for binding to molecules on cell surface.

Studies in malaria-endemic areas have suggested that high IgG3 and IgG1 cytophilic antibody titers are associated with protection (75). *In vitro* studies have shown that monocytes can kill asynchronic malaria parasites in the presence of cytophilic IgG3 and IgG1 antibodies (76). These antibodies facilitate phagocytosis and kill *Plasmodium* parasites since cross-linked FcγR–Fc induce a respiratory burst. Antibody responses against *P. vivax* CSP-1 (77), *Pv*MSP-1 (78–81), *Pv*RBP1 (82, 83), *Pv*AMA-1 (84), and *Pv*MSP-3α (85, 86) have been characterized by IgG1 and IgG3 predominance, which are associated with malaria exposure and malaria protection; **Tables 1** and **2** summaries the results obtained in each study.

MHC Molecules and the Immune Response

Major histocompatibility complex proteins have high polymorphism in human beings; antigen-binding capability varies from one allele to another, increasing or reducing their affinity (55). The forgoing is essential for developing an effective vaccine inducing a protective immune response.

Major histocompatibility complex antigen-presenting molecules are divided into two large groups. Class I present intracellular antigens and can couple eight to nine amino acid-long peptides due to the smaller size of their grooves. Class II recognizes extracellular antigens and can display 13–18 amino acid-long peptides (109).

The genes encoding class II MHC proteins in humans are called human leukocyte antigen (HLA) and are in chromosome 6. They have alpha and beta subunits, an immunoglobulin domain, and a short transmembrane portion. They have genes from three classes of protein: HLA-DP, HLA-DQ, and HLA-DR; the most polymorphic locus is HLA-DR at expense of a highly polymorphic beta subunit, unlike the alpha subunit, which is monomorphic in humans (110).

The peptide's binding site is formed by two almost parallel alpha helix regions above a beta sheet. The peptides are bound in the groove formed by the helices, with their terminal residues extended. The peptides adopt an extended poly-proline type II conformation exposing the peptide backbone to MHC conserved hydrogen-bonding residues covering the groove. Such conformation allows a peptide's side-chains to bind to the groove of the MHC binding site, as well as allowing the side-chains to bind to MHC pockets in positions 1, 4, 6, and 9. The others bind to the T-lymphocyte receptor (TCR)-binding site (55, 111).

Major histocompatibility complex class II molecules, expressed constitutively on antigen-presenting cell surface (DCs, macrophages, and B-lymphocytes) recognize extracellular peptides processed by the endosome/lysosome pathway, which are edited by the HLA-DM molecule. MHC class II presents the antigen to the TCR of CD4+ T-lymphocyte (T-helper) (55).

The CSP is one of the most important proteins described to date in Spz. Previous studies involving individuals residing in *P. vivax* malaria-endemic regions in Brazil have shown low responses for antibodies directed against the repeat region. An association has been reported between the HLA-DR16* allele and antibody response to *P. vivax* VK247 variant CSP repeats, as well as an association between the HLA-DR7* allele and a lack of antibody response to VK210 variant CSP repeats (112).

A study involving an infected population in Brazil evaluated the relationship between HLA-DRB1* alleles and the antibody

TABLE 1 | *Pv*CSP peptide antigenicity.

Antigen	Sequence	Country	*N*	Prevalence of individuals having anti-antigen reactivity (IgG)	Reference
N	SSILLVDLFPTHCGHNVDLSKAINLNGVNFNNVDASSLGAAHVGQSASRGRGLGENPDDEEGDAKKKKDGKKAEPKNPREN KLKQPG	Colombia	80	35%	(87)
R	GDRADGQPA	Colombia	80	61%	(87)
C	YLDKVRATVGTEWTPCSVTCGVGVRVRRRVNAANKKPEDLTLNDLETDVCTMDKCAGIFNVVSNSLGLVILL	Colombia	80	39%	(87)
*Pv*CS-NRC	KAEPKNPREN KLKQPGDRAD	Colombia	42	58%	(88)
	GQPAGDRADGQPA-PEG-KAEPKNPREKLKQPGENGAGDQPGANGAGNQPG-PEG-NNEGANAPNEKSVKEYLDKVRATVGTEWTPC SVTCGVGVRVRRRVNAANKKPEDLTLNDLETDVCTMDKCA	Papua New Guinea (PNG)	32	69%	
*Pv*NR1R2	THCGHNVDLSKAINLNGVNFNNVDASS	Colombia	36	81%	(89)
	LGAAHVGQSASRGRGLGENPDDEEGDAKKKKDGKKAEPKNPRENKLKQPGANGAGNQPGANGAGNQPGANGAGNQPGGDRADGQ PAGDRADGQPAGDRADGQPA	PNG	42	24%	
VMP 001	Recombinant *Pv*CSP with two copies of VK210 repetitive region (GDRA[A/D]GQPA) and one of VK247 (ANGAGNGPG)	Thailand	50	82%	(90)
r*Pv*CSP-c	Recombinant *Pv*CSP with four copies of the VK210 repetitive region (Belem strain) and three of VK247 (PNG strain)	Brazil	40	65%	(91)
		Thailand/Korea	114	73%	(92)

response to CSP, MSP-1, AMA-1, and Duffy binding protein (DBP) peptides. A significant association was found between high MSP-1 antibody levels (especially to the *Pv*200L fragment) and the HLA-DR3* allele; while no association was found between CSP, AMA-1, and DBP antibody production and HLA-DRB1* alleles (113).

During the erythrocyte phase, the merozoite surface protein (MSP) family is the responsible of the interaction between Mrz and reticulocytes. *Pv*MSP-1, *Pv*MSP-3, and *Pv*MSP-9 are potential vaccine candidates since they are exposed to the immune system and are recognized by antibodies from naturally infected individuals. A study by Lima-Junior et al. evaluated IgG antibody response to *P. vivax* MSP-1, MSP-3α, and MSP-9; a relationship between HLA-DRB1*04 individuals and high antibody response to $PvMSP3_{CT}$ and $PvMSP3_{NT}$ and HLA-DQB1*03 individuals, and response to $PvMSP3_{CT}$ was observed (86).

IgG response was positively associated with HLA-DRB1*04 and HLA-DQB1*03 individuals regarding *Pv*MSP-9 repeat regions and the NT region. Such response involving high antibody levels was associated with a possible selective pressure by *P. vivax* in the Amerindian population. Antibody responses for *Pv*MSP-9 were more correlated with the time spent living in a malaria-endemic area and not with a particular HLA-DRB1* allele (86).

Ferreira et al. constructed and expressed a synthetic gene encoding promiscuous T-helper epitopes bound to the $PvRBP1_{435–777}$ sequence. Although it has been observed in clinical assays that candidates for a vaccine against *P. vivax* are poorly immunogenic, they predicted that this chimerical protein (called *Pv*RMC-RBP1) would be recognized by multiple HLA alleles. Epidemiological and serological studies proved the preservation of B-cell conformational epitopes in the chimeric protein. However, no association was found between HLA-DRB1* and HLA-DQB1* alleles and IgG antibody responses to the chimeric or native proteins. This seemed to be because *Pv*RBP1 had multiple promiscuous T-cell epitopes, which did not induce specific genetic restriction (83).

Non-HLA Host Polymorphism

Miller et al. proved (for the first time) the hypothesis that the Duffy negative erythrocytes are resistant to *P. vivax* infection in Africans (114). The Duffy antigen or Duffy antigen receptor for chemokines (DARC) is expressed on RBC surface (115). Its encoding genetic locus having three alleles [*FY*A* (Fy^a) and *FY*B* (Fy^b) with SNP of differences and *FY*O*] has a negative serological phenotype Fy(a−b−) (116, 117). The absence of DARC expression in RBC is due to a point mutation (T46C) in the GATA box of this gene's promoter (118, 119).

Duffy negative patients infected with *P. vivax* have been found during the last few years (56, 120). Mendes et al. reported that Duffy negative individuals from Africa's West Coast were infected with different strains of *P. vivax*, and they concluded that the parasite evolved quickly and used other receptors different to Duffy to invade RBC (121).

The balance between innate and adaptive immune response is important in the development of immunopathology and clinical severity in several infectious diseases. Sohail et al. have investigated polymorphisms in the TNF-α gene promoter region and

TABLE 2 | Erythrocyte phase protein antigenicity.

Protein	Protein region	Country	*N*	Prevalence of individuals having anti-antigen reactivity (IgG)	Reference
Merozoite surface protein-1 (MSP-1)	N-terminal (NT) C-terminal	Brazil (Pará)	37	51.4% 64.1%	(78)
MSP-1	C-terminal (*Pv*200$_{18}$—18 kDa fragment)	Republic of Korea (Northern Province of Kyunggi)	421	88.1% (IgG) 94.5% (IgM)	(93)
MSP-1	*Pv*200L	Colombia (Buenaventura)	69	52.2%	(94)
MSP-1	C-terminal (*Pv*200$_{19}$—19 kDa fragment)	Turkey (Province of Sanliurfa)	82	69.5% (IgM) 53.6% (IgG) 7.3% (IgA)	(95)
Merozoite surface protein-1 paralog	C-terminal (*Pv*MSP1P-19) C-terminal (*Pv*MSP1P-33)	Republic of Korea (Province of Gyeonggi Gangwon)	30	73% (IgG3) 43% (IgG1) 57% (IgG1)	(81)
*Pv*MSP3-α	Full length C-terminal NT	Brazil	276	77% 54% 39%	(86)
*Pv*MSP3-α	Full length Repeat block I Repeat block II C-terminal NT	Brazil (Rondonia State)	282	78% 64% 53% 54% 39%	(85)
*Pv*MSP3-α	Repeat block I Repeat block II C-terminal NT	Papua New Guinea (PNG)	264	36% 38% 65% 38%	(96)
*Pv*MSP3-α	FP-1 (aa 359–798)	Brazil (Amazon region)	220	68%	(97)
*Pv*MSP3-β	FP-1 (aa 35–375) FP-2 (aa 385–654) FP-3 (aa 35–654)	Brazil (Amazon region)	220	26% 64.5% 66%	(97)
*Pv*MSP-9	*Pv*MSP9-Nt *Pv*MSP9-RI-RII *Pv*MSP9-Ct	Brazil (Ribeirinha, Colina)	306	74%	(98)
*Pv*MSP-9	*Pv*MSP9-NT V_{147}-K_{159}; V_{438}-D_{449}; K_{325}-I_{339}; P_{434}-I_{448}; A_{443}-K_{456}	Brazil (Rondonia state)	142	61.2% (IFN-γ) 49% (IL-4)	(99)
*Pv*MSP-9	*Pv*MSP9-Nt *Pv*MSP9-RI-RII	PNG	183	45.9% 8.7%	(96)
Duffy binding protein (DBP)	DBPII-IV	PNG (Mandang)	100	60%	(100)
DBP	DBPII-IV	Colombia (Buenaventura)	92	40%	(101)
Apical membrane antigen-1 (AMA-1)	*PV*66/AMA-1	Brazil (North)	221	85% (IgG) 48.5% (IgM)	(102)
AMA-1	DI DII DIII DI–DII DII–DIII Ectodomain	Brazil (Amazonas region)	100	13% 65% 12% 60% 58% 70%	(103)
AMA-1	*Pv*AMA-1	Iran	84	81%	(104)
AMA-1	*Pv*AMA-1	Brazil	1,330	52.5%	(105)
AMA-1	*Pv*AMA-1	Brazil	83	73%	(106)

(*Continued*)

TABLE 2 | **Continued**

Protein	Protein region	Country	*N*	Prevalence of individuals having anti-antigen reactivity (IgG)	Reference
*Pv*RBP1	Full length *Pv*RBP1$_{431–748}$ *Pv*RBP1$_{733–1407}$	Brazil (Rondonia state)	294	66% 41% 47%	(82)
*Pv*RBP1	*Pv*RMC-RBP1 *Pv*RBP1$_{23–751}$	Brazil (Rondonia state)	253	47% 60%	(83)
*Pv*RBP1 *Pv*RBP2	Coiled-coil and C-terminal peptides Repeat sequence peptides NT and repeat sequence peptides Coiled-coil and C-terminal peptides	Republic of Korea	16	68% 62% 68–87% 62–68%	(107)
*Pv*RBP1	*Pv*RBP1a-34 *Pv*RBP1b-32	Republic of Korea	104	34% 39%	(108)

its association with vivax infection in an Indian population. They found that TNF-308A and TNF-1031C were associated with vivax infection (very low frequency) in the study population (122). Other research has shown that IL1B, IL4R, IL12RB1, and TNF genes were associated with susceptibility to *P. vivax* malaria in a population from Brazil's Pará state, reporting −5,839C>T SNP promoter association with *P. vivax* malaria susceptibility (123).

Da Silva et al. have shown an Amazonian population's many host polymorphisms association with susceptibility or resistance to malaria infection. SNPs in the IL-10, CTL4 and TLR4 genes have been significantly associated with lower risk of clinical malaria, while a SNP in the IRF1 gene has displayed an enhanced risk. An intronic SNP in LTA was associated with protection, and one SNP on the TNF promoter was associated with susceptibility to clinical malaria (124).

Another study found no differences regarding IL6-176G>C polymorphism distribution in participants making up the different clinical groups of vivax malaria in a Brazilian population. No association was found between TNF-308G and clinical manifestations of malaria and no haplotype having DDX39B (22 C>G and 348 C>T) and TNF-308G> was identified or polymorphisms increasing the risk of clinical vivax malaria. Moreover, study participants having the genotype combination described here associated with resistance against manifestations of *P. vivax* infection (CG/CC/GG/GG) also had lower levels of pro-inflammatory TNF and IL-6, suggesting that DDX39B confers protection against malaria pathogenesis by reducing inflammatory response (125).

P. vivax PREERYTHROCYTE PHASE PROTEIN ANTIGENICITY AND IMMUNOGENICITY

P. vivax CSP

One of the predominant surface proteins in Spz is the CSP; it is expressed during the preerythrocyte phase and plays a fundamental role during hepatocyte invasion (126). This protein is a candidate for a vaccine against malaria in the preerythrocyte phase since various studies have shown that anti-CSP antibodies block hepatocyte invasion (88, 127, 128).

Circumsporozoite protein in the different *Plasmodium* species has a highly conserved structure; it consists of an NT extreme (N), a species-specific central repeat region (R) located between two conserved regions (region I and region II), and a GPI anchor in the C-terminal extreme (129). The repeat region contains an immunodominant B-cell epitope, which is associated with its immunogenic potential (130).

Furthermore RTS,S/AS01, the most advanced recombinant vaccine to date for preventing malaria caused by *P. falciparum*, is designed from *P. falciparum* circumsporozoite protein (*Pf*CSP) repeat region peptides and C-terminal region T- and B-epitopes, coexpressed with hepatitis B surface antigen. Phase III clinical studies have shown 33–50% efficacy 1-year post-immunization in 5- to 17-month-old infants (89, 92, 131). However, after a 7-year follow-up, the vaccine efficacy declined to 4.4%, with a 16.6% efficacy against all episodes of clinical malaria in the low-exposure cohort and to −2.4% in the high-exposure cohort (132). Furthermore, no vaccine efficacy was observed against severe malaria in children and young infants immunized with RTS,S/AS0 (133). Given that CSP has been widely studied in *P. falciparum*, and among different *Plasmodium* species, this protein is considered as a potential target for designing a vaccine against *P. vivax* (134–137).

Three allele variants of the *P. vivax* circumsporozoite protein (*Pv*CSP) have been described: VK210, VK247, and vivax-like CSP-P, which differ at repeat region sequence level (138, 139). VK210 has greater global distribution, being found in countries like Brazil (140), India (141), Thailand (142), and Peru (143), while VK247 is found in some regions of Colombia and Brazil (144), and vivax-like CSP-P in Brazil (140), Indonesia, Madagascar, and PNG (139).

Antigenicity studies in people exposed to the disease in different endemic regions have found variable prevalence in individuals responding to different *Pv*CSP fragments (87) (**Table 1**). Preclinical studies and phase I clinical assays (**Table 3**) have been carried out regarding *P. vivax* with long synthetic peptides (LSP)

having more than 70 *Pv* amino acids from *Pv*CSP amino terminal (N), carboxyl terminal (C), and repeat (R) regions linked to tetanus toxoid peptide (87). Immunized non-human primates from the genus *Aotus* spp. produced specific antibody response recognizing both LSP and CSP since the first immunization (87). LSP has also induced a Th1-type immune response characterized by increased IFN-γ and reduced IL-4 production in T-lymphocytes stimulated *in vitro* (87, 137).

High IFN-γ production *in vitro* and cytophilic antibodies (IgG1 and IgG3) capable of recognizing fragments from the *Pv*CSP N- and R-regions have been produced by LSP (as in experimental models) in phase I clinical assays. By contrast, the C-terminal region has not been immunogenic in humans (77).

Specific B- and T-cell epitopes must be included to stimulate an immune response thereby enabling recognition by class I and class II MHC molecules. Two modified LSP, *Pv*CS-NRC (137 aa) and *Pv*NR_1R_2 (131 aa) have induced a strong antigen-specific antibody response in immunized mice. They have inhibited Spz invasion of hepatoma cells (HepG2A-16) *in vitro* by 65 and 90% for both *Pv*CS-NRC and *Pv*NR_1R_2, respectively (88, 128). *Pv*CS-NRC has included conserved regions I and II and the repeat region sequence from VK210 and VK247 variants (88, 129). *Pv*NR_1R_2 has been improved by including B-epitopes, T-epitopes, and cytotoxic lymphocytes epitopes (128).

Immunogenic fragments of CSP have been evaluated in a recombinant vaccine (VMP 001) expressed in *Escherichia coli* encoding a *Pv*CSP chimera (149). VMP 001 has triggered a potent immune response in BALB/c mice following a third immunization. The antibodies so produced were capable of agglutinating live Spz, indicating a loss of Spz-infective capability (150). r*Pv*CSP-c, a recombinant protein similar to VMP 001 (91), has shown antibody-specific reactivity against variants VK210 and VK247 (151).

Formulations have been made with adjuvants or TLR agonists to maximize vaccine candidate fragments' immune response. Regarding adjuvants, assays involving BALB/c mice and *Aotus* spp. monkeys have proved that formulation with Freund's, Montanide ISA270, and Montanide ISA51 adjuvants, which have not led to significant differences concerning specific antibody production. However, clinical assays have revealed greater immunogenicity (having greater antibody titers and IFN-γ production) when Montanide ISA 51 adjuvant was used (145). Other adjuvant that has been tested is the inert nanoparticles, which is coated with the *P. berghei* CSP and induced CD8 T cell immunity without pro-inflammatory signals and also induced IFN-γ production levels determined to be required for sterile protection in the *P. berghei* challenge model (152).

*Pv*CSP has been formulated with TLR agonists helping to improve the immune response. *Aotus nancymaae* immunized with VMP 001 plus a TLR9 agonist (CpG 10104) have produced high antibody titers since the first dose, antibodies directed against the C-terminal region, and the VK210 variant repeat region predominating. It was seen that 66.7% of immunized primates became protected following experimental challenge (153), associated with the activation of B-cells, macrophages, and DCs by the CpG 10104 agonist (154).

Other formulations have been evaluated by using new agonists. When using VMP 001 for immunization with the TLR4 agonist (glucopyranosyl lipid A) it was found that this created a CD4+ cell response with high IL-2 production but low TNF levels (90). Fusing a polypeptide covering the *Pv*CSP immunodominant region coformulated with the FliC agonist (*Salmonella typhimurium* flagellin) produced a PAMPs-dependent immune response *via* TLR5 (155).

The results of *Pv*CSP vaccine phase I/II of *Pv*(VMP001/AS01B) have been published recently; it induced an antibody, cell-mediated immune response and delayed the latent period, but it did not induce sterile protection (146). Experience with *P. falciparum* vaccines has demonstrated that single-stage and single-target antigens cannot induce long-lived, sterile protection (133). The next generation *P. vivax* vaccine should include multiple targets, especially those needed for binding to host cells and those blocking transmission.

TABLE 3 | *Plasmodium vivax* vaccine clinical trials.

Stage	Protein	Name	Type	Clinical trial	Reference
Preerythrocytic	*Pv*CSP	CSP-N, -R, -C	LSP	Phase Ib	(77, 145)
	*Pv*CSP	VMP001	Rec	Phase I, IIa	(146)
Transmission blocking	*Pvs*25	Sc*Pvs*25/ ISA51	Rec	Phase I	(147)
	*Pvs*25	*Pvs*25H/ Alhydrogel	Rec	Phase I	(148)
Erythrocytic	*Pv*DBP	ChAd63 *Pv*DBP	Viral vector	Phase Ia	www.clinicaltrials.gov NCT01816113

LSP, long synthetic peptide; Rec, recombinant; CSP, circumsporozoite protein.

Thrombospondin-Related Adhesive Protein (*Pv*TRAP)

The thrombospondin-related adhesive protein (TRAP) has also been evaluated as a potential preerythrocyte phase vaccine candidate. TRAP is a type I transmembrane protein, expressed in the micronemes and translocate to Spz surface during hepatocyte invasion. The ectodomain consists of an A domain, a thrombospondin type 1 repeat (TSR), and a repeat region, which is variable among species. The A and TSR regions are cell adhesion domains that interact with hepatocyte membrane receptors thereby enabling invasion (156–158).

This protein has been studied in *P. berghei* and *P. falciparum* as vaccine candidate; these studies showed a significant reduction in parasites during hepatic phase, mediated by CD8+ cytotoxic T-lymphocytes but involving low antibody production (159, 160) *P. falciparum* (161).

A *P. vivax* study with *Pv*TRAP LSP involved immunizing mice and *Aotus* spp., which were then experimentally challenged. A good antibody response against the peptide was produced in mice, but only 50% recognized Spz. Four immunizations were needed in *Aotus* for obtaining a significant antibody titer, but IFN-γ levels did not increase; four of the six monkeys became protected following experimental challenge (162).

Mice immunized with *Pv*TRAP expressed in viral vectors have induced a better immune response associated with high IFN-γ production and TNF-α by CD8+ T-lymphocytes and the production of high antibody titers specific against *Pv*TRAP. A marked increase in IL-2 production from CD8+ lymphocytes has been seen after inoculating Spz, indicating an active response in the liver (163).

One of the main problems in developing an antimalarial vaccine using *Pv*TRAP has been its high genetic polymorphism observed in different isolates from different regions around the world (164). An approach to preventing these problems would involve studying proteins' conserved regions instead of using immune-dominant antigens, which are highly polymorphic.

P. vivax ERYTHROCYTE PHASE PROTEIN ANTIGENICITY AND IMMUNOGENICITY

Merozoite Surface Protein-1 (MSP-1)

The MSP family has been the most studied candidate from the erythrocyte asexual phase when developing an effective vaccine against malaria. The MSP-1 belongs to this family, being one of the most studied and currently important for both *P. falciparum* and *P. vivax* (165).

The MSP-1 analog in *P. vivax* is encoded by the *Pv*200 gene (166), having a 200-kDa molecular weight (167). The proteolytic processing profile is thought to be similar than for *P. falciparum* MSP-1, leading to 4 fragments: 83, 30, 38, and 42 kDa; further cleavage of the last one (C-terminal region) produces 33 and 19 kDa polypeptides, which are released to the blood stream. A 19-kDa portion remained bound to the recently formed ring phase following reticulocyte invasion (79, 168).

A study in *Pv* exposed individuals found IgG responses to r*Pv*200L (like the *Pf*190L fragment) indicating that the protein has high antigenicity. Sera from immunized animals showed IgG-specific antibodies capable of recognizing this protein *Pv*. The authors highlighted the fact that the observed response had a protective tendency since *Aotus* spp. developed low parasitemia peaks following *P. vivax* challenge (94).

The 19-kDa C-terminal fragment has been one of the most studied from MSP-1 (*Pv*200). Kaslow and Kumar studied $Pv200_{19}$ protein immunogenic capability in mice vaccinated with three doses. An increase in antibodies was observed in sera, which became increased with the second vaccination, this being attributed to a booster for helping epitopes in $Pv200_{19}$. The response was T-cell dependent, suggesting that an immune response to a vaccine based on this protein could be boosted by natural infection (169).

Studies in an endemic area of Brazil by Soares et al. detected IgG antibodies against MSP-1 C-terminal and NT region. Response to the C-terminal region increased according to patients' number of previous episodes of malaria, an increase of up to 80% being observed in patients who had suffered more than four episodes. Moreover, *in vitro* proliferation was observed in 47% of the individuals and IFN-γ production 54% of them. This study suggests that the C-terminal region contains two immunogenic epidermal growth factor (EGF)-like domains which induce T-cell and antibody responses against *P. vivax* during natural infection in humans (78). Later studies with the C-terminal region, specifically $PvMSP\text{-}1_{19}$, have shown that these two EGF-like domains function as a binding portion in *Pv*MSP-1 interaction with erythrocytes (170).

Later studies by Soares et al. found that antibody titers against $PvMSP\text{-}1_{19}$ became rapidly reduced (by up to 13-fold) in infected individuals and those who had received treatment against the disease. Antibody response against the NT region became reduced, even though such reduction was not significant. The decrease of antibodies directed against the C-terminal region could have contributed toward cases of reinfection in high-risk areas (171). Fernandez-Becerra et al. evaluated IgG subclasses in children, finding a predominant IgG1-type response against the C-terminal region and low IgG3 and IgG4 percentages. The predominant antibodies in adults were IgG3, a correlation between antibodies against the C-terminal region and age being observed (172).

High antibody titers against $Pv200_{19}$ have been observed in infected soldiers in the republic of Korea *Pv*, mostly IgG and IgM to a lesser extent; these were maintained for a long period of time (from 4 to 6 months) following recovery from malaria (173). Another study showed that IgG antibody permanence was maintained for more than 5 months, while IgM-type remained negative 2–4 months after the onset of symptoms (93).

The major responses in Turkey (where *P. vivax* is the only *Plasmodium* species present in the area) were IgG, IgM, and IgA to a lesser extent. It is worth highlighting the fact that $PvMSP\text{-}1_{19}$ was highly antigenic in individuals who are naturally exposed to the infection and, since no other Plasmodia are infecting in that area, the response observed cannot be attributable to a crossed reactivity (95).

Rosa et al. characterized the $MSP\text{-}1_{19}$ recombinant protein's antigenic and immunogenic properties together with two T-helper epitopes (the universal pan allelic DR epitope and a new internal MSP-1 epitope from the 33-kDa C-terminal region). It was seen that T-helper epitopes did not modify protein recognition by human IgG. The complete recombinant protein was immunogenic in marmosets (*Callithrix jacchus jacchus*), but only when Freund's adjuvant was used (174).

A study of immune response and protection was conducted in our institute, two groups of *Aotus* spp. (one splenectomized and the other not) were immunized with two recombinant polypeptides ($r PvMSP\text{-}1_{14}$ and $r PvMSP\text{-}1_{20}$) from the MSP-1 33-kDa C-terminal region containing high activity binding peptides (HABPs) to reticulocytes. Most immunized monkeys recognized the $r PvMSP\text{-}1_{14}$, $r PvMSP\text{-}1_{20}$, or the mix of HABPs by enzyme-linked immunosorbent assay (ELISA) and denatured *Pv*MSP-1 42 and 33-kDa fragments by Western blot. Although half the animals immunized with the $r PvMSP\text{-}1_{14}$ and $r PvMSP\text{-}1_{20}$ mixture were protected, some monkeys did not produce antibodies against the vaccine candidate. This suggested that protection was not only mediated by a humoral immune response (175). The next study involved vaccinating *Aotus* spp. monkeys with the two above mentioned recombinant polypeptides but in three doses. *Aotus* spp. produced antibodies capable of recognizing the native protein and managed to control parasitemia in four out of the five immunized

monkeys. Interestingly, some animals produced high IFN-γ levels and controlled parasitemia but displayed low antibody titers; conversely, some other animals were protected having high antibody tires but low IFN-γ levels (176).

Other studies have found that anti-*Pv*MSP-1 antibodies (predominantly IgG), recognizing the NT region, are associated with a reduced risk of infection and clinical protection against the *Pv*MSP-1. Although these antibodies do not recognize the C-terminal region (79), there have been reports that the C-terminal region is immunogenic and capable of naturally stimulating antibody production in new infections (78, 93, 173).

The MSP-1 protein's 42-kDa fragment has also been studied due to its potential as vaccine candidate; its immunogenicity was evaluated in mice. High IgG1, IgG2a, and IgG2b antibody levels were observed while IgG3-type response was low. A high proliferative response also found high IL-2, IL-4, IL-10, and IFN-γ levels being detected in culture supernatants (177). Greater prevalence of recognition of $PvMSP1_{19}$ than $PvMSP1_{42}$ was found when a naturally acquired humoral immune response was reported (178).

An MSP-1 paralog has been identified recently (*Pv*MSP1-P); its immune response was characterized using different protein fragments (83, 30, 38, 42, 33, and 19 kDa). The NT (83 kDa) fragment and two from the C-terminal region (33 and 19 kDa) were recognized by sera from infected patients living in endemic areas (179).

IgG1 and IgG3 (IgG2b in mice) were the predominant responses in patients from endemic regions, as in immunized mice. The C-terminal region induced a predominantly Th1 profile of cytokine response having high TNF, IFN-γ, and IL-2, but low levels of IL-10 and IL-4 cytokines (Th2 profile), showed greater lymphoproliferative response than the MSP1–19 fragment. The correlation between parasitemia and anti-MSP1P antibody level suggest that it does not contribute strongly to inhibiting parasite growth (81).

Due to its colocation with MSP1, it has been thought that it played a similar role in erythrocyte invasion; however, analyzing the sequences has suggested different roles for each protein. Cytoadherence assays demonstrated that MSP1-P could be an essential adhesion molecule regarding *P. vivax* invasion to erythrocytes, is immunogenic in humans, and is a potential vaccine candidate against *P. vivax* (179).

Merozoite Surface Protein-3 (MSP-3)

The *P. vivax* merozoite surface protein-3 (*Pv*MSP3) is a member of the MSP family characterized by having a highly polymorphic alanine-rich central domain (180). It has a relatively conserved N- and C-terminal domain and two central blocks of seven repeats forming tertiary supercoiled helices in their structure (180–182). It is expressed in schizonts and is associated with Mrz surface during the erythrocyte phase (180).

Its homolog in *P. falciparum* has been studied as a vaccine candidate in preclinical (183) and phase I assays, protection was associated with reduced parasitemia by cytophilic antibodies inducing antibody-dependent cell-mediated inhibition of parasite growth mechanism (184). It has been shown to be highly immunogenic in *P. vivax*, having a high prevalence of antibodies directed against *Pv*MSP-3α block II (96).

A correlation has been described between time spent living in an endemic region and the number of previous episodes of malaria, involving an increase in IgG1 and IgG3 anti-*Pv*MSP-3α (85, 86, 96). A naturally acquired response has been found toward 15 antigenic determinants, mainly located in repeat regions (85). Other studies have reported the C-terminal region as being the most antigenic, having a significant increase in IgG in a population from Brazil (97) and PNG (96). However, it has been found the antibodies directed against block II are associated with protection against clinical episodes of *P. vivax* malaria, having greater than 500 parasites/μL parasitemia (96).

Interestingly, no response against *Pv*MSP-3α was produced in immunogenicity studies with C57BL/6 mice, while high antibody titers were produced with PvMSP-3β following the second and third immunization (97). Incorporating adjuvants (Quil A, TiterMax, or IFA) has maximized the response against *Pv*MSP-3α, indicating that another parasite's molecules must act as adjuvant for *Pv*MSP-3α antigenic presentation during natural infection (96, 97). Regarding cellular response, *Pv*MSP-3β with Quil A, Titer Max, or IFA adjuvants have produced a balanced Th1/Th2 response, while the Alum adjuvant directed response toward Th2 with a significant murine IgG1 increase. Alum co-formulated with the TLR9 agonist (CpG ODN 1826) balanced the Th1/Th2 relationship, increasing Th1 response due to pro-inflammatory cytokine production (97).

Merozoite Surface Protein-9 (MSP-9)

The *P. vivax* merozoite surface protein-9 (*Pv*MSP-9) is also a potential vaccine candidate. Some studies have shown that this protein is conserved among *Plasmodium* species infecting humans, rodents, and primates. Furthermore, antibodies produced against *Pv*MSP9 homologs in *P. cynomolgi* and *P. knowlesi* can inhibit Mrz invasion of erythrocytes (180).

The *P. vivax*, *P. knowlesi*, and *P. cynomolgi msp-9* genes encode a hydrophobic signal peptide and repeat motifs upstream of the stop codon and a C-terminal region having two species-specific blocks of repeat amino acids (*Pv*MSP9-RI and *Pv*MSP9-RII). Together with *P. falciparum* has four cysteine residues close to the NT giving the MSP-9 family's structural and functional characteristics. This protein is expressed during schizogony and is organized on Mrz surface during schizont development and segmentation (180, 185).

The cellular and humoral immune response of BALB/c mice immunized with *Pv*MSP9-Nt, *Pv*MSP9-RII recombinants, and the mixture of both recombinants was evaluated for testing *Pv*MSP-9 immunogenicity. Antibody response in mice was determined by ELISA and was mainly IgG; there were greater titers for IgG1, IgG2a, and IgG2b isotypes than IgG3. Regarding cellular response, the amount of spleen cells secreting IFN-γ was higher than those secreting IL-5. Moreover, sera from patients living in an endemic region of Brazil recognized the two recombinant regions, demonstrating that both were immunogenic (186).

A study was carried out on a population, which was naturally exposed to *P. vivax* infection in Brazil, and the immune response against *Pv*MSP9-RIRII and *Pv*MSP-N terminal domains was

evaluated. Of the 306 individuals in this study, 74% had IgG antibodies that recognized at least one of the recombinant proteins, thereby indicating that these proteins are antigenic during natural infection, especially *Pv*MSP9-RIRII. When the IgG subclasses were evaluated, IgG1 was predominant for *Pv*MSP9-RIRII and *Pv*MSP9-N terminus, and IgG2 was prevalent for *Pv*MSP9-RII. Furthermore, five synthetic peptides predicted to bind to HLA-DR alleles were chosen, and the overall cellular response frequency for at least one of the peptides was 58% for IFN-γ and 41% for IL-4. No association was found between IFN-γ production and IgG levels regarding recombinant proteins. Lima-Junior et al. thus concluded that *Pv*MSP9 C-terminal and NT domains are immune response targets for individuals living in *P. vivax* endemic regions. The reactivity index for IgG has been positively correlated with time spent living in an endemic area; conversely, IgG3 reactivity did not predominate regarding response to the recombinant proteins. It has been shown that *Pv*MSP9 NT region peptides induce memory T-cell response where IFN-γ and IL-4 cytokines produced in significant proportion by individuals from endemic regions has indicated the presence of T-cell epitopes (98).

Another study involving volunteers from an endemic region of the Amazon region showed that the response of cells producing IFN-γ was significantly greater than those regarding IL-4. The results obtained for 5 of the 11 peptides selected contained *Pv*MSP9 promiscuous T-cell epitopes. The core sequence (ASIDSMI) shared by three of the peptides was highly immunogenic; another peptide could have had two immunodominant epitopes, one in the overlapping core region and another in the C-terminal region, which produced a cellular response in 23 volunteers (99).

The specific response of IgG to *Pv*MSP9-N terminal has been associated with protection against symptomatic *P. vivax* infection in children aged less than 3 years old in a PNG endemic region. Such antibodies specific for *Pv*MSP9 were prevalent in children suffering frequent infections and have been associated with protection in children who have not had these infections. The authors concluded that two classes of antibodies are produced against the *Pv*MSP9 NT region, one produced by short-lived memory B-cells and the other by long-lived cells (96).

Duffy Binding Protein (DBP)

Plasmodium vivax Mrz requires antigens from the Duffy blood group as surface receptor for invading human reticulocytes (114). *P. vivax* DBP adhesion to its receptor on erythrocytes [Duffy antigen receptor for chemokines (DARC)] is essential for the parasite to continue developing during the asexual phase in human blood (114, 187). *Pv*DBP is a 140-kDa protein, which is located in the micronemes; it has been divided into four important regions: a peptide signal sequence (region I), two cysteine-rich regions separated by a non-homologous hydrophilic region (region II, identified as the erythrocyte-binding domain, and region VI), and transmembrane domain (region VII) (188–191). *Pv*DBP is a main target to use as vaccine candidate since its importance during parasite invasion and its ability to induce antibodies against the parasite's asexual phases (114, 192).

Serological evaluation in a PNG endemic area has shown that a humoral immune response was common and increased with age, suggesting a possible booster effect regarding antibody response in some cases by repeated exposure to the infection (100). A similar pattern has been observed in an endemic region of Colombia where a positive correlation was found between increased antibody response and patients' age. Also, an immunologic boost for DBP was found, even in endemic areas having a low transmission level (101). The forgoing shows that DBP_{II} was naturally antigenic in people residing in endemic regions.

Children having high antibody levels against DBP_{II} has been associated with delayed reinfection time with the same *P. vivax* variant; however, such association was not observed when evaluating MSP1–19 (193). In other studies have been observed that naturally acquired neutralizing antibodies against DBP are short-lived, increasing with acute infection, and are strain specific (194, 195).

Antibodies from plasma from naturally exposed people and from animals immunized with recombinant Duffy binding protein (rDBP) have blocked the specific interaction between the *Pv*DBP ligand domain *in vitro* and its receptor on erythrocyte surface; such inhibitory activity has been correlated with antibody titers (196, 197). The forgoing shows DBP's potential as vaccine candidate due to its essential role as adhesion molecule (196).

The cytokine production of individuals exposed to *P. vivax* was analyzed; IFN-γ, IL-10, and IL-2 induction was observed. The response was seen to depend on individuals' age and the specific DBP_{II} variant, producing partially acquired immunity to *P. vivax* in these populations (198). Similarly, epitopes mapped from the DBP_{II} critical binding region have produced a humoral response, accompanied by increased antibody levels associated with patients' increased age, suggesting recognition through repeated infection. Some individuals recognized $rDBP_{II}$ but not linear epitopes, indicating the presence of conformational epitopes; such cases occur regularly in young people or subjects suffering first acute *P. vivax* infection, suggesting that multiple infections are needed for the recognition of linear epitopes (199).

The DBP binding domain (DBP_{II}) is polymorphic, tending to compromise the efficacy of any vaccine associated with strain-specific immunity (192). Due to the high rate of polymorphism observed in DBP region II, an in-depth investigation was made of the relative importance of conserved and polymorphic residues in this region by directed mutagenesis. The mutations causing the loss of ligand function were mainly produced in discontinuous groups of conserved residues, while almost all mutations in polymorphic residues did not alter RBC binding (200). Such polymorphism has been seen to have a synergic effect on the antigenic nature of DBP (201). Sera from patients reacted to denatured, non-reduced, and native rDBP, indicating immunogenic conserved linear B-epitopes (100). The immune efficacy of a DBP_{II} vaccine depends on inducing antibodies, and this response should be optimized toward conserved epitopes to protect against *P. vivax* (197).

Since DBP region II epitopes have been shown to be immunogenic, studies have established universal epitopes, which can be presented by different HLA-DR alleles inducing an effective cellular and humoral immune response, making them a candidate for a subunit-based vaccine (202). Antigenicity studies using $PvDBP_{II}$ universal epitopes have shown that lymphoproliferation,

IL-6, and IFN-γ production is induced in peripheral blood mononuclear cells (PBMCs) from individuals exposed to infection. Such results have suggested that these epitopes having affinity for HLA-DR molecules can be good components of a vaccine against *P. vivax* (203).

Polymorphisms observed in DARC have also been associated with *P. vivax* infection severity and susceptibility in humans. Individuals with low DARC expression (a single negative allele) have a greater probability of having anti-MSP1 and anti-DBP antibodies than individuals having high DARC expression (double positive alleles). Individuals having high expression of DARC have been found to be associated with greater susceptibility to infection, exhibiting low frequency and magnitude of specific antibody response against *P. vivax* during the blood stage. This could indicate that one of *P. vivax*'s primary mechanisms for evading host immunity works through indirect negative regulation of DARC, influencing the humoral response against erythrocyte invasion and parasite development (204).

Apical Membrane Antigen-1 (AMA-1)

The AMA-1 in *Plasmodium* is a transmembrane protein, which is localized in the micronemes. It seems to be essential during cell host invasion and is present in all *Plasmodium* species (205, 206). Eight disulfide bonds have been identified in the AMA-1 ectodomain of 66 kDa, defining three different subdomains (DI, DII, and DIII) (207). Immune responses induced by AMA-1 from different *Plasmodium* species have shown potent parasite-inhibitory effects both in animals and *in vitro* thus suggesting AMA-1 as a potential vaccine candidate (208).

Plasmodium vivax AMA-1 ectodomain (*PV*66/AMA-1) has been shown to be highly immunogenic in rhesus monkeys, inducing high IgG antibody titers; however, these suffer a rapid decline. A slight reduction in parasitemia has been observed in *P. cynomolgi*-challenged animals previously immunized with *PV*66 (209).

Mice immunized with human adenovirus type 5 and rAMA-1 have produced long-lived specific antibodies (including IgG1 and IgG2a) and memory T-cell proliferative responses. Memory T-cell responses were effector- and central-type, central memory predominating (210). In mice it was observed that response was both Th1 and Th2 following three immunizations and persisted for 1 year following the first immunization. On the other hand, the antibodies produced were capable of recognizing the native protein located on *P. vivax* parasites (211).

When evaluating the immune response against two *Pv*AMA-1 variants (A and B), there were no significant differences regarding the prevalence of IgG response. A marked switching in isotypes, which became increased with age, was also seen. The predominant cytophilic antibodies recognized *Pv*AMA1A (IgG1) and *Pv*AMA1B (IgG1–IgG3). The immune-epidemiological data in this research were similar regarding the two variants, this implied that one of these forms could be used in a universal erythrocyte stage *Pv*AMA-1 antigen-based vaccine (212).

An IgG response was observed in people residing in endemic regions exposed to *P. vivax* in Brazil, IgG1 being the dominant subclass. This antibody response was slightly lower than that observed with $MSP1_{19}$ and increased by 100% in individuals having had more than three episodes. Sequences of *Pv*AMA-1 variable domain from different isolates have been seen to have limited polymorphism in this country (102).

This protein has been seen to be involved in Mrz invasion and contains an extracellular portion containing three different domains (207). When evaluating DI, DII, and DIII, separately or in combination in *P. vivax*-infected individuals, a greater immune response toward proteins containing the domain II was observed. Inhibition assays using the *Pv*AMA-1 ectodomain led to common epitopes being identified within the DI–DII domains, which were recognized by antibodies from people residing in endemic regions. Immunization in mice having the *Pv*AMA-1 ectodomain induced high levels of antibodies, predominantly against DI–II (103).

A linear B-epitope was also identified between amino acids 290–307 (SASDQPTQYEEEMTDYQK) in domain II, this peptide was recognized by sera from individuals naturally infected by *P. vivax* (213).

Recombinant *P. vivax* apical membrane antigen-1 DII was formulated with six adjuvants and was highly immunogenic regardless of the adjuvant used. DII-specific antibodies recognized native AMA-1 protein, demonstrating that it is immunogenic and indicating that this protein region could be evaluated as part of a subunit-based vaccine against malaria caused by *P. vivax* (214).

Reticulocyte-Binding Proteins (RBP)

Reticulocyte-binding proteins include *Pv*RBP1 and *Pv*RBP2 and their variants *Pv*RBP1a and b and *Pv*RBP2a, b, and c, and other family members (215). RBP1 is a homodimer bound by disulfide bonds, binds non-covalently to RBP2, and forms a protein complex (216). They are colocalized in the apical zone in Mrz micronemes and contain a transmembrane domain toward the C-terminal extreme, possessing repeat regions in *Pv*RBP2 and reticulocyte-binding domains (108, 215, 217–219).

It is thought that RBPs could participate in reticulocyte invasion since no infection by *P. vivax* has been observed in mature erythrocytes (220). Their reticulocyte-binding ability has also been reported, but the specific receptors have yet to be identified (219). It has recently been found that only *Pv*RBP2b binds specifically to reticulocytes (221). Due to their participation in infection, they have been studied as erythrocyte phase vaccine candidates, aimed at blocking Mrz invasion of reticulocytes (218).

High affinity reticulocyte-binding peptides (HARBPs) have been identified, 5 in a fragment from the region I of *Pv*RBP1 NT extreme (222) and 24 throughout the whole protein (223). The highly-conserved region III (between amino acids 1,941–2,229) had the greatest amount of HARBPs (223) and, when used as immunogen, it induced high antibody titers in *Aotus nancymaae* monkeys, able to recognize the full *Pv*RBP1 in parasite lysate. T-lymphocytes became activated following the second and third doses, but no protection was obtained after experimental challenge (224). Due to studies involving other *P. falciparum* proteins showing that highly conserved sequences are not immunogenic, in spite of having high binding capability, it has been suggested that changes must be made in some amino acids to increase the immune response and induce protection based on studies of critical binding residues for HARBPs from region III (224).

Antigenicity studies have found a direct relationship between higher anti-*Pv*RBP1 antibody titers and the number of previous episodes, the time spent residing in an endemic region and age (82, 83, 219, 224). The immune response to *Pv*RBP1 has also been associated with greater IgG1 and IgG3 cytophilic antibody presence against fragments from polymorphic regions (82, 83).

Studies regarding different populations where an acquired response to B-epitopes (107) and various fragments from *Pv*RBP1 and *Pv*RBP2 variants (108, 219) have shown high prevalence of IgG antibodies against fragments from repeat region, the supercoiled helix, and *Pv*RBP1 C-terminal region (107) (**Table 2**). Regarding the NT region (including the most polymorphic region of the *Pv*RBP1a and b variants) (108), IgG prevalence was intermediate in a population from Thailand, while no antibody response against *Pv*RBP1b was found in a population from the Republic of Korea (219).

On the other hand, *Pv*RBP2 has been seen to have greater antibody prevalence against NT region, repeat region (107), and *Pv*RBP2c variant fragments (219). *Pv*RBP2c is one of the most polymorphic variants, probably having the greatest global distribution, associated with the prevalence of this variant's recognition (219). *Pv*RBP2b and *Pv*RBP1a have been correlated with lower risk of parasitemia in a cohort study of PNG children (221). Regarding other proteins such as *Pv*DBP, *Pv*RBP antigenicity is much lower (82, 108).

Antigenicity and/or Immunogenicity of Non-Classical Vaccine Candidates

In spite of the technical limitations involved in studying *P. vivax* proteins, other proteins characterized as being promising vaccine candidates have been studied during the last few years. One such is merozoite surface protein-10 (*Pv*MSP10) having NT and C-terminal regions with two EGF-like domains and a GPI anchor (225). Colombian individuals exposed to *P. vivax* infection have shown reactivity to recombinant *Pv*MSP10; r*Pv*MSP10 has also elicited high antibody titers against the protein in immunized *Aotus* monkeys but no protection after challenge (226). Infected Korean individuals had 42% IgG prevalence, IgG1 and IgG3 antibodies predominating. Immunized mice have shown a Th1 response-biased immune response (227).

The *Pv*34 protein was characterized based on homology of the *P. falciparum Pf*34 protein, and antigenicity was evaluated in PBMCs from individuals previously exposed to infection. Stimulation with r*Pv*34 induced proliferation in 71% of individuals and high IL-2, IFN-γ, and IL-4 production (Th1/Th2 profile), such response being attributed to recognition of T- and B-epitopes responsible for a combined immune response (228).

Another protein characterized was *Pv*RON-1, based on its homologous *Pf*ASP protein in *P. falciparum*. This protein's antigenicity was evaluated using sera from people having had previous *P. vivax* infection. The results showed that *Pv*RON-1 was expressed during natural infection and could generate an antibody response in the host (229).

An antigenicity and immunogenicity study of *P. vivax* rhoptry-associated leucine (Leu) zipper-like protein-1 (*Pv*RALP-1) was carried out on patient serum samples and immunized mice. *Pv*RALP-1 was recognized in samples from patient sera, IgG1 and IgG3 being the predominant subclasses, although without significant differences with the other subclasses; Th1/Th2 response was balanced in immunized mice (230).

The CelTOS microneme protein characterized and tested for *P. falciparum,* having 98% homology with *P. vivax,* should be considered for further studies since cross-species protection has been demonstrated in preclinical studies (231). It has been described as vaccine target by blocking transmission infection or preerythrocytic stage due to its cell traversal function in Spz and ookinetes (232). A recent study proved ~20% prevalence of antibodies against *Pv*CelTOS in a Thai population (233). More studies related to immunogenicity, and antigenicity potential are needed and should involve new proteins characterized during the last few years, related to host cell invasion during different life cycle stages, such as rhoptry neck proteins.

TRANSMISSION BLOCKING *P. vivax* VACCINE CANDIDATES

One of the methodologies used for controlling malaria infection has been the search for transmission blocking vaccines, through strategies for preventing ookinete development in the vector (234). The assays conducted for transmission blocking have been focused on two main proteins, *Pv*s25 and *Pv*s28, expressed on gametocyte surface. Anti-sera have recognized *Pv*25 in zygotes and mature ookinetes, and *Pv*28 more in mature ookinetes (235).

Immunogenicity tested with recombinant proteins *Pv*s25 and *Pv*s28 in mice has shown a splenic T-cell proliferative response. Anti-sera from mice immunized with a *Pv*s25–28 chimera had a high antibody titer compared to mice immunized with *Pv*s25 or *Pv*s28 alone. Anti-*Pv*s25–28 and anti-*Pv*s25 had higher transmission blocking than anti-*Pv*s28 (235, 236).

Studies had demonstrated that *P. vivax Sal*I strain recombinant *Pv*s25 and *Pv*s28 had transmission blocking capability, even with natural isolates, thus overcoming genetic polymorphism between isolates (237). Higher transmission blocking has been described as a direct function of antibody titers in sera (238–240).

Different vaccination schemes have been tested, varying adjuvant, dose, expression system (148, 237, 238, 240, 241). Phase I clinical trials determining security and immunogenicity in humans have shown high transmission blocking capability in humans, demonstrating these antigens' potential as vaccine candidates (147, 148).

CONCLUSION

This review has summarized immune responses induced by *P. vivax* vaccine candidates, which are essential in host cell invasion. Classical vaccine development has been focused on immunodominant antigens such as sporozoite and MSPs, which are recognized by sera from partially protected individuals who are naturally exposed to infection. However, surface proteins, for example *Pv*MSP1 and *Pv*CSP, have high allelic polymorphism (164, 242, 243) and are under positive selection by the immune response. After several natural infections, many of these epitopes

have shown an ability to generate a strong immune response in individuals without clinical symptoms, showing an association with IFN-γ effector T-cell activation and generation of cytophilic antibody subclasses. Similar immune responses have been observed in animal models immunized with these polymorphic immunodominant antigens. Nevertheless, the success for this kind of vaccines has been limited, since the cross protectivity obtained for the remaining strains is very low and induces a short-lived immune response (244, 245). Moreover, parasites change their cell targets and molecules during preerythrocytic, erythrocytic, and sexual stages, and single-antigen/single-stage vaccines do not induce sterile protection. It has been observed that *P. falciparum* parasites hide their amino acid conserved domains of the proteins involved in the invasion of host cells, showing immune dominant and polymorphic epitopes to the immune system (246).

Other methodologies are needed to solve these kinds of issue. An alternative is to develop an antimalarial vaccine (246), focused on synthetic peptides designed on conserved regions of Spz and Mrz proteins having high hepatic cell or RBC-binding ability. Although these peptides are not immunogenic, *P. falciparum* and *P. vivax* studies have shown that such peptides can be modified by changing their critical RBC-binding residues for others having similar mass but opposite polarity, making them highly immunogenic and protective (247, 248).

Another problem in the development of an antimalarial vaccine concerns the many haplotypes present in the exposed population. The HABPs can also be modified that fit properly inside the peptide-binding region of MHCII. In studies with *P. falciparum* with a MSP-2 HABP that has been modified to bind to HLA-DRβ1*0403 molecules with high affinity, it was shown that *Aotus* monkeys bearing HLA-DRβ1*0403-like molecules, produced high antibody titers with sterile immunity after challenge with *P. falciparum* FVO. This was a proof-of-concept immune protection-inducing protein structures demonstrating that specifically modified HABPs are able to induce sterile protection against malaria by engaging the proper TCR/pMHCII interactions (249).

Evaluation of conserved epitopes and non-immunodominant antigens important in parasite adhesion and invasion of erythrocytes should be prioritized for multistage, multi-epitope, minimal subunit-based, chemically synthesized antimalarial development, covering a large part of the HLA-DRβ1* population in endemic areas to protect them against malarial parasites.

AUTHOR CONTRIBUTIONS

CL conceived the work and drafted the manuscript; YY-P drafted the manuscript and designed the figures; NH-E and DD-A drafted the manuscript; MP critically revised the manuscript for important intellectual content. All authors have revised the manuscript and given their approval for the version to be submitted.

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RESEARCH ARTICLE

*Plasmodium vivax Pv*12 B-cell epitopes and HLA-DRβ1*-dependent T-cell epitopes *in vitro* antigenicity

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Abstract

Malaria is an infectious disease caused by parasites from the genus *Plasmodium (P. falciparum* and *P. vivax* are responsible for 90% of all clinical cases); it is widely distributed throughout the world's tropical and subtropical regions. The *P. vivax Pv*12 protein is involved in invasion, is expressed on merozoite surface and has been recognised by antibodies from individuals exposed to the disease. In this study, B- and T-cell epitopes from *Pv*12 were predicted and characterised to advance in the design of a peptide-based vaccine against malaria.

For evaluating the humoral response of individuals exposed to natural *P. vivax* infection from two endemic areas in Colombia, BepiPred-1.0 software was used for selecting B-cell epitopes. B-cell epitope 39038 displayed the greatest recognition by naturally-acquired antibodies and induced an IgG2/IgG4 response. NetMHCIIpan-3.1 prediction software was used for selecting peptides having high affinity binding for HLA-DRβ1* allele lineages and this was confirmed by *in-vitro* binding assays. T-epitopes 39113 and 39117 triggered a memory T-cell response (Stimulation Index≥2) and significant cytokine production.

Combining *in-silico*, *in-vitro* and functional assays, two *Pv*12 protein regions (containing peptides 39038, 39040, 39113 and 39117) have thus been characterised as promising vaccine candidates against *P. vivax* malaria.

Introduction

Malaria caused by *Plasmodium vivax* is an important public health problem in many countries worldwide; 13.8 million cases occurred in 2015, causing about 50% of all cases of malaria outside of Africa [1]. According to the PAHO/WHO Epidemiological Alert for malaria, between

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2015 and 2016, an increase in malaria cases caused by *P. vivax* have occurred in the Americas, specially Colombia [2]. Although vector control, antimalarial drug therapy and case management have reduced the amount of events, vaccine development is considered one of the most promising strategies for controlling malaria [1]. Vaccine development strategies include immunisation with irradiated sporozoites (Spz) [3], inducing a protective response with knock-out parasites [4], identifying and expressing proteins which are important in parasite invasion to targeted hosts and searching for immune response-inducing peptides [5].

Given the lack of availability of an *in vitro P. vivax* culture, the development of an effective vaccine has been delayed (compared to progress in the *P. falciparum* field) [6]. However, studying homologous *P. falciparum* proteins expressed during blood-stage has led to potential *P. vivax* vaccine candidates being recognised and characterised [7–12]. Our group has been using a vaccine development strategy based on characterising ~20 amino acid (aa) long conserved peptides involved in target cell invasion and later modifying them to improve their interaction with major histocompatibility complex (MHC) class II molecules, such peptides have been called modified high binding activity peptides (mHABPs). This approach is the basis for developing anti-*P. falciparum* chemically-synthesised vaccines, based on protein subunits from the parasite's development stages (i.e. multi-antigen, multistage) [13].

The use of these peptides as potential vaccine candidates will depend on their ability to act as efficient epitopes that could trigger the cellular immune response mediated by T cells, and/or the humoral response, mediated by B cells. Using algorithms for determining the potential T- and B-cell epitopes helps to save time and resources required for lab experiments of the pathogen's proteins. Such tools have been here used to select *Pv*12-based synthetic peptides as potential vaccines candidates against *P. vivax*.

Previous studies have shown that an immune response against malaria parasites is strongly associated with these peptides' specific binding to MHC DR class II (MHC-DR or HLA-DR (human leukocyte antigen in humans)) [13,14]. This cell surface receptor consists of a protein complex formed by HLA-DRα and HLA-DRβ having high polymorphism in humans, focusing on HLA-DRβ whilst HLA-DRα is virtually monomorphic; for sake of simplicity the complex is referred to as HLA-DRβ hereafter. Each HLA-DR molecule has differences regarding antigen binding affinity, suggesting that peptide-MHC complex formation depends on allele lineage [13,15,16].

Bioinformatics tools have been developed for predicting T-cell epitopes according to their HLA-DR allele binding profile [17–21]. Identifying high-binding HLA-DR peptides (promiscuous or allele-specific) able to trigger an effective immune response forms part of the development of a vaccine covering much of the exposed population's HLA polymorphism [22–24].

Antigen-antibody interaction is the main characteristic of a humoral immune response; however, antibodies only recognise a portion of an antigen (known as antigenic determinant or epitope) [25,26]. Some vaccine development strategies involve using bioinformatics tools for identifying B-cell epitopes in protein vaccine candidates. Mapping linear B-cell epitopes (continuous aa in a protein sequence) through *in silico* methods involves using predictors for analysing a combination of aa physico-chemical properties, such as solvent accessibility, hydrophilicity and flexibility [27,28].

P. vivax proteomic profile studies have led to identifying vaccine candidate proteins involved in parasite invasion [5,9,29]; *Pv*12 is one of such proteins. It consists of 362 aa, having a ~41 kDa molecular mass, an N-terminal signal peptide (SP), a glycosylphosphatidylinositol (GPI) anchor in the C-terminal transmembrane domain (TM) and two 6-Cys domains [30,31]. Although initially characterised as a rhoptry protein [30], later studies confirmed its location on merozoite (Mrz) surface, like *Pf*12 [31,32]. It has been recognised by 49% of sera from individuals with active infection by *P. vivax*'s protein array [30]; another study revealed

84.8% of sera having an antibody response via protein recognition by ELISA (Enzyme-Linked Immunosorbent Assay) [31].

It has been shown that several 6-Cys family members play a role in ligand binding to other proteins, suggesting that *Pv*12 could play an active role in interacting with other cell receptors [31]. Genetic studies have shown that the *Pv*12 gene is highly conserved amongst *Plasmodium* species, having low genetic diversity between isolates and being one of the most conserved antigens encoding genes characterised to date for *P. vivax*. *Pv*12 may thus be a promising candidate for developing a vaccine against this parasite [33].

The present study used bioinformatics tools for selecting *Pv*12 B- and T-cell epitopes and evaluating their *in vitro* antigenicity. The humoral response to *Pv*12 was assessed by ELISA using sera from people living in two *P. vivax* endemic areas in Colombia for identifying IgG (Immunoglobulin G) and subclasses against B-epitopes. T-cell epitopes were evaluated by *in vitro* binding assays to HLA-DRβ1*0401, 1*0701, 1*1101 and 1*1302 molecules; lymphoproliferation and cytokine production assays were then carried out for those peptides displaying high binding affinity, using peripheral blood mononuclear cells (PBMC), according to their HLA-DR profile. Overall, this work has shown that the use of bioinformatics techniques combined with functional experiments yielded fruitful results, offering two regions combining B- and T-cell epitopes from *Pv*12, that can be used as components of a vaccine against *P. vivax*.

Materials and methods

Study area

Peripheral blood (PB) samples were taken from *P. vivax*-exposed individuals living in Colombia's Chocó (Pacific region) and Córdoba (Caribbean region) departments where *P. vivax* malaria is endemic [34]. Colombia has areas having unstable malaria transmission levels, an endemic-epidemic transmission pattern and is mainly rural [35]. The selected *P. vivax*-exposed individuals were over 18 years-old, had had at least one episode of *P. vivax* malaria (the last malarial episode having occurred 6 months previously) and had received treatment. It is known that an *in vitro* cellular response to malarial antigens is suppressed during acute infection; PB samples were taken from exposed individuals [36–38] and thick blood smears were used for confirming that the samples were negative for *P. vivax* infection. This study was carried out in line with Colombian Ministry of Health Resolution 8430/1993 (the legal framework for research in Colombia). The individuals were regarded as being at low risk and all data was kept confidentially and rigorously protected. Written informed consent was signed by all individuals prior to sampling; all procedures were evaluated and approved by FIDIC's ethics committee.

Study population

This study involved typing 79 *P. vivax*-exposed individuals from the aforementioned endemic areas for the HLA-DRβ1 allele; PB samples from 50 individuals living in Bogotá (a non-endemic malaria area) who have never been exposed to malaria were also taken for typing to form the control group. PB samples were taken from *P. vivax*-exposed individuals and the control group and placed in EDTA tubes (BD Vacutainer Oakville, ON, CAN). A Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) was used for extracting genomic DNA (gDNA) from 300 μL whole blood, according to the manufacturer's instructions. The gDNA was verified by 1% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, CA, USA) and sent to Histogenetics (Ossining, NY, USA) for high-resolution HLA sequence-based typing (SBT) of the β1 locus.

Experimental groups

HLA-DRβ1 typing was used for determining experimental and control groups. HLA-DRβ1*04, *07, *11 and *13 allele lineages have been reported as having high frequency in malaria endemic areas worldwide [39]; individuals having at least one allele from the allele lineages were thus selected whilst individuals having two of these lineages at the same time were discarded.

In silico selection and chemical synthesis of *Pv*12 B- and T-cell epitopes

Bioinformatics tools were used for selecting B- and T-cell epitopes from the *Pv*12 aa sequence (PlasmoDB PVX_113775 access number). BepiPred 1.0 [19] and ANTHEPROT 2000 v6.0 [40] were used for selecting B-cell epitopes. The NetMHCIIpan-3.1 tool [41] was used for predicting T-cell epitopes, selecting epitopes according to their HLA-DRβ1*0401, *0701, *1101 and *1302 binding profile. IEDB (Immune Epitope Database) [42–44] and TEPITOPE [45] software was used for confirming T-epitopes prediction.

Selected *in silico* peptides and biotinylated control peptides HA (Haemagglutinin Antigen) and TT (Tetanus Toxoid) were synthesised and purified by 21st Century Biochemicals Inc. (Marlborough, MA, USA).

Immunoblot and immunofluorescence assays (IFA)

PB samples from all endemic area individuals and the control group's antibody response assays were placed in 6 mL BD vacutainer serum collection tubes (BD Vacutainer). *P. vivax* antigens were recognised by separating 50 μg *Pv*12 recombinant protein (r*Pv*12 donated by Moreno-Perez *et al.*) [31] and r*Pv*GAMA (GPI anchored micronemal antigen) control protein (r*Pv*GAMA-CT donated by Baquero *et al.*) [46] on 12% polyacrylamide gel in denaturing conditions; the gel was transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then cut into thin strips and incubated with sera samples diluted 1:50 in blocking buffer. The strips were washed and incubated with a secondary antibody peroxidase anti-human IgG (Vector Labs) at 1:4,000 dilution; a VECTOR VIP peroxidase (HRP) substrate kit (Vector Labs) was used for revealing the assay.The immunofluorescence assays were performed as described by Moreno-Perez *et al.*, [31] with some modifications. Briefly, blood samples from individuals who had active *P. vivax* infection were spun, the buffy coat removed and the plasma recovered.

P. vivax-exposed individuals and control group serum samples were diluted 1:50 in TBS-BSA and incubated for 1 hour in a humid chamber. Reactivity was observed by fluorescence microscopy using FITC-labelled (Fluorescein isothiocyanate) anti-human IgG (Sigma-Aldrich) at 1:50 dilution in TBS-BSA. Parasite nuclei were stained with 0.25 μg/mL DAPI (4',6-diamidino-2-phenylindole, dilactate) (Life Technologies) and the slides were then washed with 0.05% TBS-T. The slides were visualised on an Olympus BX51 fluorescence microscope (Olympus Corporation); the images were processed using DP2-BSW software (v2.2, Olympus Corporation) and merged using ImageJ 1.51n software (National Institutes of Health, USA).

ELISA assays for B-cell epitopes and recombinant proteins

NUNC immuno-modules (Thermo Scientific) were coated with 1 μg of each epitope and each recombinant protein (r*Pv*12 and r*Pv*GAMA) in PBS pH 7.2 and incubated overnight (ON) at 4˚C. The sera were incubated in 1:100 dilution in blocking solution for 2 hours and peroxidase anti-human IgG (Vector Labs) was used as secondary antibody in 1:10,000 dilution in blocking solution for 1 hour at RT; 100 μL TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate (Sera-Care) was used for revealing the assay. The reaction was stopped by adding an equal volume of 1M phosphoric acid (H_3PO_4) (Merck) and read at 450 nm using a Multiskan GO

(Thermo Scientific, Waltham, MA, USA) ELISA reader. The mean for the control group plus two standard deviations was used as cut-off point.

Positive sera were evaluated for IgG subclasses using 3% BSA in PBS-T as blocking solution, following the ELISA protocol described above. Sera were incubated at 1:100 dilution in PBS-T. The following secondary antibodies diluted in PBS-T were used: anti-human IgG1-biotin (clone 8c/6-39) in 1:1,000 dilution, anti-human IgG2-biotin (clone HP-6014) in 1:15,000 dilution, anti-human IgG3-biotin (clone HP-6050) in 1:40,000 dilution and anti-human IgG4-biotin (clone HP-6025) in 1:60,000 dilution (all from Sigma-Aldrich). Pierce high-sensitivity HRP-conjugated (Horseradish peroxidase) streptavidin (Thermo Scientific) in 1:5,000 dilution in PBS, pH 6.8, and TMB (Sera-Care) were used as substrate. The reaction was stopped and read at 450 nm using an ELISA reader.

Molecular modelling

Pv12 protein (PlasmoDB PVX_113775 access number) 3D structure was inferred using *Pf12* protein structure (PDB: 2YMO) as template, using the I-TASSER server [47]. The most energetically stable model was selected and the error associated with inferred structure was corrected via molecular dynamics simulations to avoid incorrect atomic contacts. NAMD 2.12 [48] was used for molecular dynamics calculations and CHARMM27 force field for molecular dynamics simulations and analysis [49,50] using periodic boundary conditions. Hydrogen atoms were added using Visual Molecular Dynamics (VMD) *psfgen* plugin [51]. The protein was minimised for 1,000 steps, followed by equilibration for 1 ns; the molecular dynamic simulation was run for 20 ns. Molprobity [52] was used for validating the refined model's structural quality. VMD was used to locate the B-epitopes studied here in the resulting *pv12* model. The POLYVIEW protein structure visualisation server [53] was used for calculating relative solvent accessibility and secondary structure annotation.

Purifying anti-HLA-DR monoclonal antibody and HLA-DR complexes

The anti-HLADR monoclonal antibody was obtained from L-243 cell line (ATCC HB-55) culture supernatant [54,55] which was saturated with 45% ammonium sulphate ($(NH_4)_2\ SO_4$) (Merck) for immunoglobulin precipitation and purified by affinity chromatography using Protein A Sepharose.

Four homozygous lymphoblastoid B-cell lines (IHWG) were cultured for each molecule of interest (IHW09025 for HLA-DRβ1*0401, IHW09051 for HLA-DRβ1*0701, IHW09043 for HLA-DRβ1*1101 and IHW09055 for HLA-DRβ1*1302) to obtain HLA-DR complexes. The cells were lysed and molecules purified by affinity chromatography, as described by Vargas *et al.* [56].

Anti-HLADR antibody and HLA-DR purity was verified by 8% polyacrylamide gel and western blot. Amicon Ultra centrifugal filters (Merck) were used for concentrating the positive fractions and quantified using a Micro BCA Protein Assay Kit (Thermo Scientific) which were then stored at -80˚C until use.

In vitro HLA-DRβ1 binding assays and IC50 values

Selected *in silico* peptides' HLA-DRβ1 binding affinity was evaluated by measuring biotinylated control peptide displacement. Biotinylated peptide $HA_{306\text{-}318}$ (PKYVKQNTLKLAT) [23,57] was used as control peptide for assays with HLA-DRβ1*04 and *11 whilst biotinylated peptide TT (QYIKANSKFIGITE) [58] was used for assays with HLA-DRβ1*07 and *13.

The selected peptides' *in vitro* binding capacity was evaluated by competitive ELISA, as described by Vargas *et al.*, [56] and Saravia *et al.* [23]. Briefly, a complex involving 0.1 μM

purified molecules, 5 μM biotinylated control peptide and 250 μM of the peptide to be evaluated (50-fold excess of control peptide) was incubated for 24 hours at RT. The complex was then placed on NUNC-Immuno MicroWell (Thermo Scientific) plates pre-coated with 10 μg/mL anti-HLA-DR, incubated ON at 4˚C and blocked with 3% PBS-BSA for 2 hours at RT. The wells were incubated with alkaline phosphatase streptavidin reagents (Vector Labs) in 1:500 dilution in PBS and subsequently revealed with 200 μL p-NPP (p-nitrophenylphosphate) substrate (Sigma-Aldrich) and read at 405 nm on a MultiSkan GO (Thermo Fisher Scientific, Waltham, MA, USA) ELISA reader. Binding percentages were calculated by using the following formula: 100*[1-(OD in the presence of competitor / OD in the absence of competitor)].

IC50 values were calculated for peptides having more than 50% binding; the aforementioned protocol was followed, varying the concentrations of the peptides to be evaluated by using 4.2 to 250 μM serial dilutions. Binding values were calculated by using the following formula: 1-(OD in the presence of competitor / OD in the absence of competitor). The resulting values were plotted using a second order exponential decay curve using a Mathematica (Wolfram Research, Inc., Mathematica, version 11.0, Champaign, IL (2016)) software formula to calculate the concentration at which the peptide to be evaluated displaced biotinylated control peptide by 50%.

Lymphoproliferation and cytokine quantification assays

P. vivax-exposed individuals and control group PB samples were taken and placed in citrate phosphate dextrose (CPD) solution in Vacutainer tubes (BD Vacutainer); 40 mL blood was used to obtain PBMCs from gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare) to evaluate the proliferative response against *Pv*12 peptides.

Two hundred thousand (2×10^5) PBMCs per well were seeded in 96-well round-bottomed plates (Costar, Corning Incorporated) in RPMI 1640 medium (Gibco) supplemented with inactivated 10% autologous plasma, 2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate and100 U/mL Antibiotic-Antimycotic solution (all Gibco). The PBMCs were labelled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (5μM) (CellTrace CFSE cell proliferation kit, Molecular Probes, Eugene, OR, USA) and stimulated with each peptide at 10 μg/mL final concentration.

Control PBMCs were stimulated with 5 μg/mL parasite lysate and 2% phytohaemagglutinin (PHA) (Sigma) as positive controls. Low binding peptide (LBP) 39115 was used as negative control in experimental binding assays and a well containing PBMCs with just medium (unstimulated) was used as assay control. The cells were cultured at 37˚C in a 5% CO_2 atmosphere for 96 hours; assays were carried out in duplicate. Pacific Blue-labelled mouse anti-human CD4 (RPA-T4 clone) (BD Biosciences) antibody was then used for labelling the cells which were read on a FACS Canto II flow cytometer (BD Biosciences). FlowJo software v7.6.5 (FlowJo, LLC) was used for analysing the data and $CD4^+$ T-lymphocyte proliferation.

Cell proliferation was calculated by relative loss regarding CFSE fluorescence intensity, calculated as the percentage of fluorescence intensity of cells co-cultured with antigen divided by the percentage of co-cultured cells' fluorescence intensity in the absence of antigen [59]. The results were expressed as stimulation index (SI); a $\geq$2 value was considered positive.

CD4+ lymphocyte mean fluorescence intensity (MFI) values were used for additional analysis. Cell proliferation was calculated as the stimulation rate, where the unstimulated CD4+ T-cell population MFI was divided by the stimulated CD4+ T-cell population's MFI. MFI becomes less as the antigen stimulates CD4+ T-lymphocytes, whereby $\geq$1 values indicate cell proliferation [60]. The relative value of proliferation was considered positive, with at least 1% loss in the mean intensity of florescence of the CD4+ population, i.e. $\geq$1.01.

The supernatants were collected and stored at -80˚C. A Human cytometric bead array (CBA) Th1/Th2 Cytokine Kit II (BD Biosciences) was used for measuring cytokines, following the manufacturer's instructions. Samples were read on a FACS Canto II flow cytometry and FCAP Array software v3.0.1 (BD Biosciences) was used for analysing cytokine results (expressed in pg/mL) and sample values were compared to those for wells without stimulation. The cut-off point was calculated as control group mean plus two standard deviations.

Analysing the data

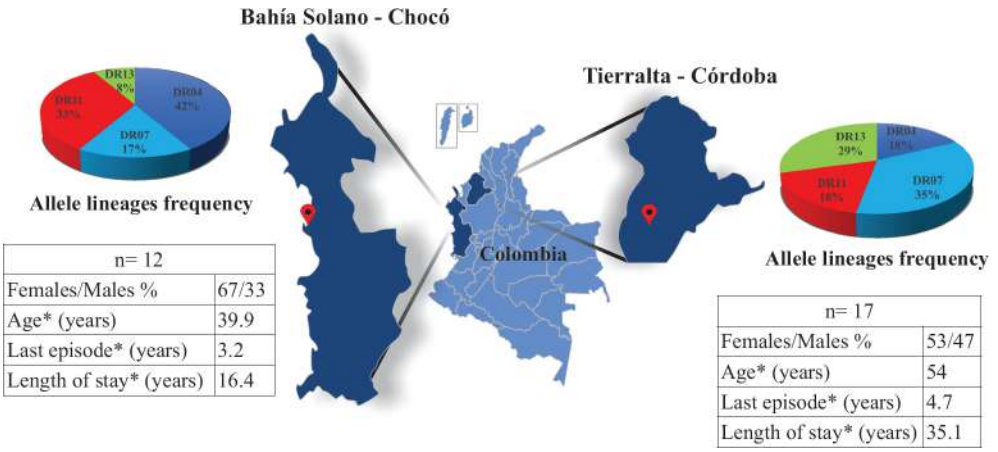
GraphPad Prism v5.01 software (GraphPad Software, Inc.) was used for analysing and plotting the data. The Shapiro-Wilk test was used for tests of normality, the Wilcoxon-Mann-Whitney U-test for comparing two non-parametric variables and Student's t-test for variables having normal distribution. The Kruskal-Wallis test with Dunn's post-test was used for multiple comparisons. Spearman's coefficient (ρ = Rho) was used for calculating correlations between experimental and clinical data. A 95% confidence interval and $p \leq 0.05$ were set as being significant; significance level has been highlighted by asterisks on all graphs, as follows: (*) $p<0.05$, (**) $p<0.005$ and (***) $p<0.0005$.

Results

Study area and study population

Around 70% of malarial cases in Colombia are caused by *P. vivax* [35]; *P. vivax*-exposed individuals living in Bahía Solano (Chocó) and Tierralta (Córdoba) were thus selected (Fig 1). People living in Colombia's Chocó and Córdoba departments have higher *P. vivax* infection incidence; this was reported by the Colombian Public Health Surveillance System (Sistema de Salud Pública–SIVIGILA) in its annual report for 2015 to 2017 [34]. Exposed individuals from these departments were thus selected for this study.

Two samples were needed from the study population for evaluating their immune responses; the first sample was used for typing the HLA-DRβ1* gene in the experimental and control groups. Four experimental groups were clustered according to the selected allele lineages: HLA-DRβ1*04 (n = 8), HLA-DRβ1*07 (n = 8), HLA DRβ1*11 (n = 7) and HLA-DRβ1*13 (n = 6). The second sample was used for determining T- and B-cell antigenicity, analysing the



n= 12	
Females/Males %	67/33
Age* (years)	39.9
Last episode* (years)	3.2
Length of stay* (years)	16.4

n= 17	
Females/Males %	53/47
Age* (years)	54
Last episode* (years)	4.7
Length of stay* (years)	35.1

Fig 1. Study area and study population's demographic parameters. The allele lineage frequency of exposed individuals from endemic areas in Colombia (Chocó and Córdoba departments); n = amount of individuals; *mean value for age, time of last episode and length of stay.

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immune responses of the individuals who had not been infected for at least 6 months to find memory T-cells and long-lived antibodies against conserved peptides.

P. vivax-exposed individuals' antibody reactivity

Western blot was used for evaluating r*Pv*12 and r*Pv*GAMA protein reactivity for determining their antigenicity; r*Pv*12 was recognised by 86% of sera and r*Pv*GAMA (control protein) by 85% of sera from thirty *P. vivax*-exposed individuals (S1A Fig). Control group sera samples were evaluated, no reactivity being observed. *P. vivax*-exposed individuals and control group samples were used for IFA assays. FITC fluorescence was detected in parasitised red blood cells (pRBC) when reacting with the thirty exposed individuals' serum; conversely, no reactivity was observed in the control group's serum samples (S1B Fig).

Antibody response against recombinant proteins and B-cell epitopes

Evaluating the reactivity of natural antibodies from the sera samples taken from the 30 malaria-exposed individuals revealed that 33.3% (10 positive samples) had IgG antibodies against r*Pv*12 (0.2480 mean absorbance); no control group sera recognised r*Pv*12. No significant differences were observed between exposed and control groups (Fig 2A). Regarding IgG subclass, IgG3 significantly predominated over IgG1 and IgG4 antibodies; significant differences were found between IgG2 and IgG4 non-cytophilic antibodies ($p<0.0001$) (Fig 2B). r*Pv*GAMA was used as positive control (0.4757 mean absorbance); 70% (21 samples)

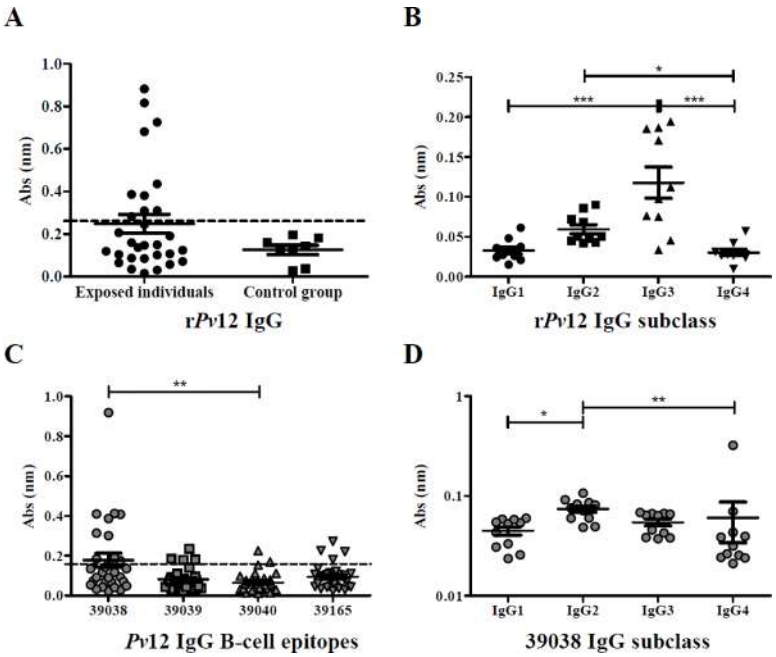

Fig 2. Humoral immune response against *Pv*12. A. IgG antibody response against r*Pv*12. Seropositive samples (n = 30) were those above the cut-off point (0.260, dotted line), calculated as control group's mean plus two standard deviations. **B. Analysing IgG subclass response to r*Pv*12.** The Kruskal-Wallis test was used for analysing differences between each IgG subclass response in *P. vivax*-exposed individuals' samples. **C. IgG antibody response against *Pv*12 B-cell epitopes (n = 30).** Seropositive samples were those above the cut-off point (0.159, dotted line), calculated as control group's mean plus two standard deviations. **D. Evaluating IgG subclass response to the 39038 epitope (n = 11).** The Kruskal-Wallis test was used for analysing differences between each IgG subclass response in *P. vivax*-exposed individuals' samples. (*) $p<0.05$, (**) $p<0.005$ and (***) $p<0.0005$.

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were seropositive and significant differences were observed regarding the control group ($p = 0.0004$) (S2A Fig).

Bioinformatics tools were used for selecting four B-cell epitopes for analysing the humoral immune response. IgG antibody response against selected *Pv*12 B-epitopes was evaluated in sera from individuals exposed to natural *P. vivax* infection. The highest amount of seropositive samples (11 samples / 36.6%) was related to peptide 39038 followed by 39039 and 39165 (4 samples each / 13.3%), and 39040 (2 samples / 6.6%) (Fig 2C).

Peptide 39038 had the highest mean absorbance (0.1780), significant differences being observed regarding 39040 which had the lowest mean absorbance (0.06581) 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 ($p = 0.0027$) (Fig 2C). Although there were no statistically significant differences between exposed individuals and control groups, the exposed individuals had a greater response.

ELISA *Pv*12 peptide results were analysed by endemic area, samples from the Chocó department having greater response compared to those from the Córdoba department. Positive sera reacted to the four peptides, 61.5% of such samples were from the Chocó department. The Mann-Whitney test gave a significantly higher response for peptides 39039 ($p = 0.0028$), 39040 ($p = 0.0045$) and 39165 ($p = 0.0020$) in exposed individuals from the Chocó department compared to exposed individuals from the Córdoba department (S2B Fig).

Seropositive samples were selected for evaluating IgG subclasses; significant differences were only observed for peptide 39038 between IgG subclasses ($p = 0.0012$) whilst other peptides had no significant differences. 39038 had a clear IgG2 predominance regarding other subclasses, having statistically significant differences with IgG1 and IgG4 (Fig 2D).

Using Spearman's test for analysing correlation for non-parametric data revealed tendencies associated with antibody response. One parameter evaluated was time elapsed (in years) since the last episode where decreased antibody response to epitopes 39039 (Rho = -0.40, $p = 0.044$), 39040 (Rho = -0.44, $p = 0.024$) and 39165 (Rho = -0.49, $p = 0.010$) was observed as time elapsed. Reduced response was also observed for *Pv*GAMA protein control (Rho = -0.41, $p = 0.038$).

The other parameter evaluated was exposed people's age, a tendency for decreased antibody response to epitopes 39039 (Rho = -0.44, $p = 0.025$) and 39165 (Rho = -0.44, $p = 0.025$) being observed as age increased.

*Pv*12 T-cell epitope i*n silico* selection

NetMHCIIpan-3.1 was used for selecting ten epitopes from the *Pv*12 aa sequence for HLA-DRβ1*0401, *0701, *1101 and *1302 alleles. These peptides were then evaluated in *in vitro* binding assays, using the same alleles in this study; those having >50% binding were chosen as high-binding peptides (HBP). *In vitro* results showed that 4/10 peptides bound to the HLA-DRβ1*0401 allele (46.94% mean binding), 4/10 peptides bound to HLA-DRβ1*0701 (47.81% mean binding), 5/10 peptides bound to HLA-DRβ1*1101 (49.54% mean binding) and 5/10 peptides bound to HLA-DRβ1*1302 (49.17% mean binding) (Fig 3).

The IC50 ratio values were calculated from the IC50 value (μM) for each peptide (Fig 4) divided by the IC50 value (μM) of its control peptide; ≤10 values were considered good binding peptides (GBP). Experimental assay values agreed 77.5% with the *in silico* prediction for our alleles (Table 1). The peptides having the lowest IC50 values (μM) were selected for the lymphoproliferative assays, peptide 39113 for HLA-DRβ1*04 and *11 allele linages and peptide 39117 for HLA-DRβ1*07 and *13 allele linages. Peptide 39114 was considered a universal peptide (UP) because it was the only one displaying ≥50% binding to the four alleles evaluated here.

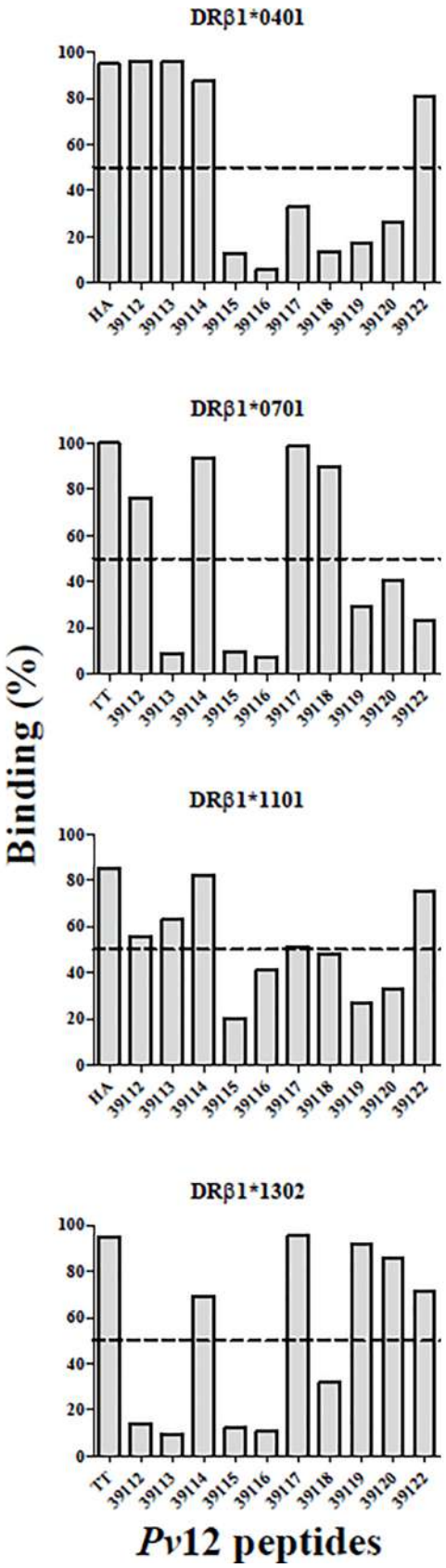
DRβ1*0401
DRβ1*0701
DRβ1*1101
DRβ1*1302
Binding (%)
100
80
60
40
20
0
HA
TT
39112
39113
39114
39115
39116
39117
39118
39119
39120
39122
*Pv*12 peptides

Fig 3. *Pv*12 peptide *in vitro* binding to purified HLA-DRβ1* molecules. A cut-off line is shown at 50% binding for selecting HBP for further evaluation of IC50 value. Each plot shows percentage epitope HLA-DRβ1* binding in this study and that for their control peptides.

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Experimental groups' lymphocyte proliferation

Exposed individuals and control groups' $CD4^+$ T-cell proliferative responses stimulated with UP 39114, GBP 39113 and 39117 were evaluated; LBP 39115 was used as negative control and *P. vivax* lysate and PHA as positive controls.

Exposed individuals having ≥2 stimulation index (SI) were considered responders. Average response to UP 39114 was 3.409 (SE = 0.8458), 2.632 (SE = 0.5563) to GBP 39113, 2.947 (SE = 0.5449) to GBP 39117, 2.018 (SE = 0.3478) to LBP 39115 and 3.664 (SE = 0.6072) to lysate. There was no proliferation in the control group when cells were stimulated with the peptides or *P. vivax* lysate (according to the SI observed) (Fig 5).

Exposed individuals' frequency of response to UP 39114 was 55.2%, 46.7% to GBP 39113, 57.1% to GBP 39117, 28% to LBP 39115 and 65.5% to lysate. Control group response to UP 39114 and GBP 39117 was 0/8, 1/8 to GBP 39113, 0/8 to LBP 39115 and 1/8 to lysate.

There were statistically significantly differences between exposed individuals and control groups regarding UP 39114 ($p = 0.0165$), GBP 39117 ($p = 0.0081$) and LBP 39115 ($p = 0.0289$).

P. vivax lysate induced a greater proliferative response in the exposed individuals (mean SI = 12.47 ± 1.636 SE); however, no statistically significant differences were observed when compared to the control group (mean SI = 5.45 ± 1.138 SE) (Fig 5). All PHA-stimulated PBMCs from exposed individuals and the control group had positive SI, having statistically significant differences ($p = 0.0103$). It was found that 96.6% of the exposed individuals and 100% of the control group responded to PHA (S3 Fig).

Analysing relative proliferation values calculated from MFI data gave similar results, thereby validating data obtained using FlowJo software. There was a maximum 12% variation regarding the amount of exposed responders for lysate, statistical differences being maintained between the exposed and control groups regarding peptides 39114 and 39117. The greatest difference between both analyses was found for LBP 39115 stimulation which gave 50%

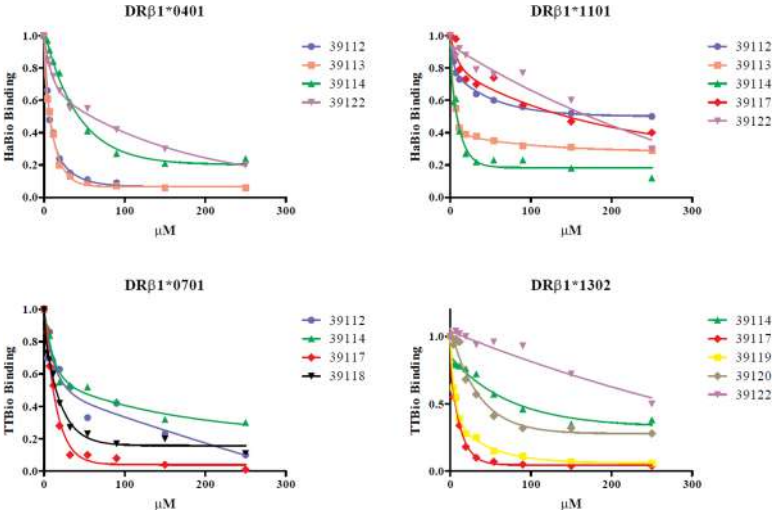

Fig 4. *In vitro* assays for calculating a *Pv*12 peptide's IC50 value. Different epitope concentrations were evaluated for calculating the value at which control peptide was displaced by 50% (using a second order exponential decay function). Each point under the curve represents evaluated epitope concentration-dependent control peptide binding (μM).

https://doi.org/10.1371/journal.pone.0203715.g004

Table 1. T-cell epitopes selected *in silico* and *Pv*12 *in vitro* binding.

Peptide code	Sequence	CoreF	HLA-DRβ1* allele	NetMHCIIpan- 3.1 (%rank)[a]	Binding percentage[b]	IC50 μM[b]	IC50 ratio
39112	RKNYELLPNNCFEQV	YELLPNNCF	DRβ1*0401	3.5	95.78	7.26	0.37
			DRβ1*0701	14.0	76.44	29.23	1.25
			DRβ1*1101	35.0	55.44	250	52.41
			DRβ1*1302	30.0	14.09	ND	ND
39113	EECFLQGFNLSGKKE	FLQGFNLSG	DRβ1*0401	3.0	95.81	7.36	0.38
			DRβ1*0701	24.0	8.89	ND	ND
			DRβ1*1101	27.0	62.88	38.54	8.08
			DRβ1*1302	55.0	9.28	ND	ND
39114	YNKIFYARVPQRIYQ	IFYARVPQR	DRβ1*0401	8.5	87.38	43.18	2.22
		FYARVPQRI	DRβ1*0701	0.7	93.87	43.09	1.84
		IFYARVPQR	DRβ1*1101	0.9	82.09	8.28	1.74
		FYARVPQRI	DRβ1*1302	8.5	69.37	73.30	9.83
39115	KKSYDDVSFRVPPNL	SYDDVSFRV	DRβ1*0401	65.0	12.98	ND	ND
			DRβ1*0701	65.0	9.86	ND	ND
		DVSFRVPPN	DRβ1*1101	75.0	20.02	ND	ND
		SYDDVSFRV	DRβ1*1302	60.0	12.00	ND	ND
39116	NKAKIRVRKRSGEEY	IRVRKRSGE	DRβ1*0401	95.0	5.76	ND	ND
		KAKIRVRKR	DRβ1*0701	85.0	7.33	ND	ND
			DRβ1*1101	13.0	41.12	ND	ND
		IRVRKRSGE	DRβ1*1302	85.0	10.50	ND	ND
39117	LGIIEVLIPSLPKKI	IEVLIPSLP	DRβ1*0401	14.0	33.08	ND	ND
		LIPSLPKKI	DRβ1*0701	8.0	98.56	11.51	0.49
		EVLIPSLPK	DRβ1*1101	9.5	51.13	133.77	28.04
		LIPSLPKKI	DRβ1*1302	11.0	95.42	6.65	0.89
39118	LVEYLHGAAAIVKRK	YLHGAAAIV	DRβ1*0401	3.0	13.48	ND	ND
			DRβ1*0701	1.2	90.02	15.18	0.65
		LHGAAAIVK	DRβ1*1101	11.0	48.21	ND	ND
		YLHGAAAIV	DRβ1*1302	7.0	31.71	ND	ND
39119	GCDFTKNTSPLFTKG	FTKNTSPLF	DRβ1*0401	6.5	17.45	ND	ND
			DRβ1*0701	7.5	29.45	ND	ND
			DRβ1*1101	30.0	26.94	ND	ND
			DRβ1*1302	4.5	92.18	7.47	1.00
39120	LTDLVMDHYNKIFYA	LVMDHYNKI	DRβ1*0401	31.0	26.73	ND	ND
			DRβ1*0701	37.0	40.62	ND	ND
			DRβ1*1101	36.0	32.68	ND	ND
			DRβ1*1302	3.5	85.99	38	5.09
39122	KRLVAHFEFATTPDD	FEFATTPDD	DRβ1*0401	40.0	80.94	58.89	3.03
		LVAHFEFAT	DRβ1*0701	65.0	23.08	ND	ND
		VAHFEFATT	DRβ1*1101	65.0	74.87	170	35.64
		LVAHFEFAT	DRβ1*1302	85.0	71.13	250	33.51

Peptide codes and sequences are shown, as well as NetMHCIIpan-3.1 predicted HLA-DRβ alleles' core and % rank. IC50 was assessed for peptides having ≥50% binding; IC50 values were expressed in μM and peptides were considered GBPs when IC50 ratio was ≤10 (IC50 peptide/IC50 control peptide).

[a]%rank values were considered as follows: LBP≥2 to ≤10 and GBP ≤2. ND (not determined) means that a peptide had less than 50% binding, so that its IC50 value was not evaluated.

[b]Data from this study; IC50 values were calculated for each control peptide with each DRβ1* allele. Control HA-DRβ1*0401 IC50 = 19.44 μM, TT-DRβ1*0701 IC50 = 23.37 μM, HA-DRβ1*1101 IC50 = 4.77 μM, TT-DRβ1*1302 IC50 = 7.46 μM. Peptides having ≤10 IC50 ratio were considered GBP.

https://doi.org/10.1371/journal.pone.0203715.t001

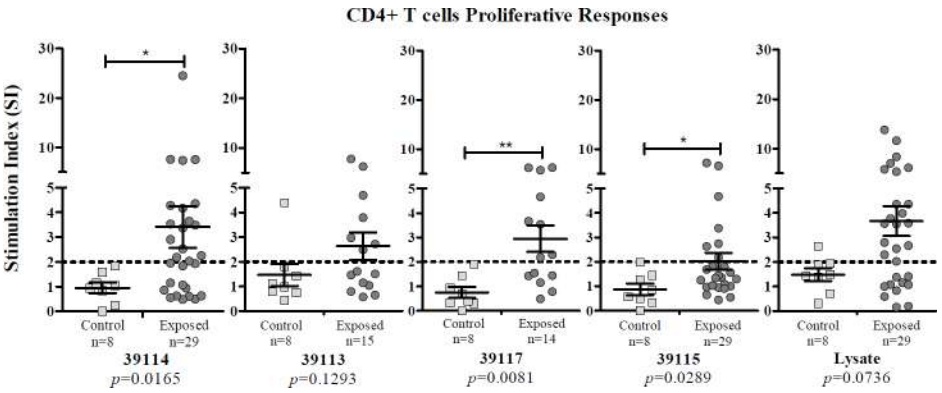

Fig 5. Lymphocyte proliferative response to *Pv*12 epitopes from individuals exposed to *P. vivax* infection compared to control group. UP 39114 (n = 29); DRβ1*04 and DRβ1*11 GBP 39113 (n = 15); DRβ1*07 and DRβ1*13 GBP 39117 (n = 14); LBP / control 39115 (n = 29) and *P. vivax* lysate (n = 29) responses are shown. Mann-Whitney and Student's t-tests were used for assessing statistically significant differences between exposed individuals and control group responses. (*) $p<0.05$ and (**) $p<0.005$.

https://doi.org/10.1371/journal.pone.0203715.g005

responders according to relative proliferation values; however, it had a low mean score/value regarding other antigens and had no statistical differences regarding the control group. No mean relative value for the control group exceeded the cut-off line after stimulation with the different antigens, thereby agreeing with FlowJo software results.

Experimental groups' cytokine profiles

Statistical analysis regarding exposed individuals' unstimulated and stimulated PBMCs showed that IFN-γ (Interferon gamma) production was only significant after stimulation with *P. vivax*-lysate ($p = 0.0001$). TNF (Tumor Necrosis Factor) production was significantly different for GBP 39113 ($p = 0.0048$), GBP 39117 ($p = 0.0183$) and *P. vivax*-lysate ($p = 0.0001$). IL-10 (Interleukin 10) had higher production with GBP 39113 ($p = 0.0054$), GBP 39117 ($p = 0.0012$) and *P. vivax*-lysate ($p = 0.0001$). IL-6 (Interleukin 6) responses were significantly greater to UP 39114 ($p = 0.0108$), GBP 39113 ($p = 0.0025$), GBP 39117 ($p = 0.0366$) and *P. vivax*-lysate ($p = 0.0001$) (Fig 6).

P. vivax-exposed individuals and control groups' cytokine levels were compared, significant differences being found regarding IFN-γ production for UP 39114 ($p = 0.0036$) and *P. vivax* lysate ($p = 0.0246$). Significant differences regarding IL-6 production were found for UP 39114 ($p = 0.0021$), 39113 ($p = 0.0253$), 39117 ($p = 0.0101$) and *P. vivax* lysate ($p = 0.0028$) (Fig 7).

*Pv*12 structure modelling

*Pv*12 structure was modelled using a homology approach using the *Pf*12 structure model as template. Overall the identity between both proteins was 37% with a similarity of 56% (S1 File). The best I-TASSER model was refined using molecular dynamics, and the best B- and T-cell epitopes (39038, 39117 and 39113) were mapped over the 3D model (Fig 8A and 8B). The remaining epitopes were mapped on the primary sequence and secondary structure model (Fig 8C). All B-cell epitopes were found in high solvent accessibility regions of the protein, in contrast, T-cell epitopes were found in low solvent accessibility regions. Two favourable regions were found combining B- and T-cell epitopes, one constituted by the B-cell epitope 39038 and the T-cell epitope 39117 (Inter-domain I, residues 161 to 194, Fig 8) and the other comprises the B-cell epitope 39040 and the T-cell epitope 39113 (6-Cys domain II, residues 250 to 276, Fig 8). In the first region, both the B-cell and the T-cell epitopes were involved in

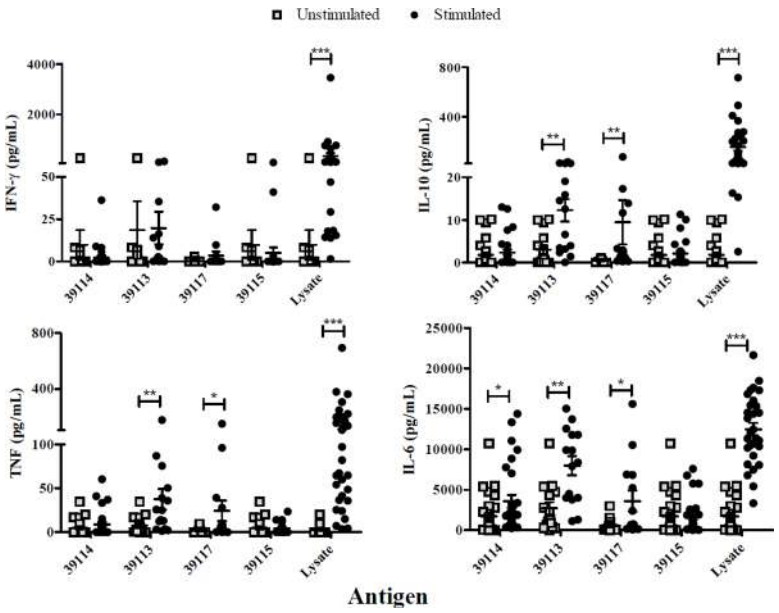

Fig 6. Exposed individuals' supernatant culture *in vitro* cytokine production. Individual data shows the mean value of non-stimulated and PBMCs stimulated with UP 39114, GBP 39113 and 39117 and *P. vivax* lysate. IFN-γ, TNF, IL-10 and IL-6 levels were measured by CBA kit; cytokine concentration is expressed in pg/mL. Statistically significant differences ($p \leq 0.05$) are shown and data represents the mean ± SEM (standard error media) for all values. (*) $p<0.05$, (**) $p<0.005$ and (***) $p<0.0005$.

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good responses, however, in the second region, only the T-cell epitope was suitable, whilst the B-cell epitope showed a low response.

Potential interaction positions between *Pv*12 and *Pv*41 were also mapped (Fig 8C, green triangles) based on the binding regions previously determined between *Pf*12 and *Pf*41 [61,62]. *Pf*41/*Pv*41 were also orthologous proteins (as *Pf*12/*Pv*12), showing an identity of 43% and a similarity of 60% (S1B File). *Pf*12/*Pf*41 interactions involved charged residues, and two were mapped directly in the 39038–39117 region (Asp 174 and Lys 175 into 39038 B-cell epitope) and other two less than 3Å from this region (Gln 48 and Arg 68) (Fig 8). Two other potential interaction positions were mapped into the 39113–39040 region. These could indicate a

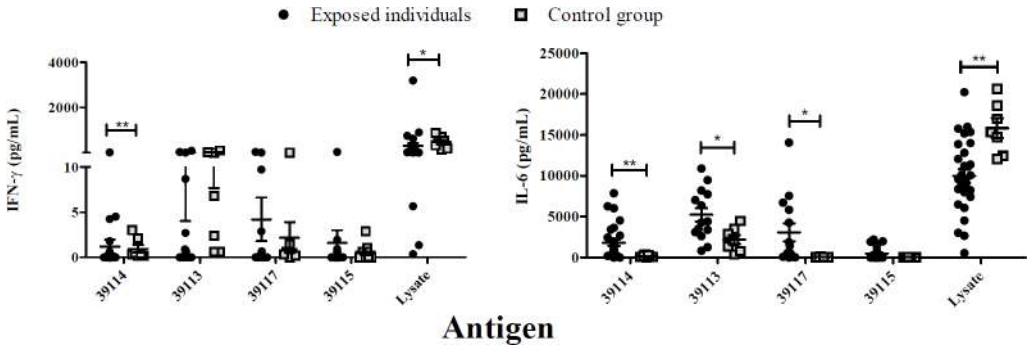

Fig 7. Exposed individuals and control group supernatant culture *in vitro* cytokine production. Individual cytokine values from PBMCs stimulated with UP 39114, GBP 39113 and 39117 and *P. vivax* lysate. IFN-γ and IL-6 levels were measured by CBA kit and cytokine concentration is expressed in pg/mL. Statistically significant differences ($p \leq 0.05$) are shown and data is the mean ± SEM for all values. (*) $p<0.05$ and (**) $p<0.005$.

https://doi.org/10.1371/journal.pone.0203715.g007

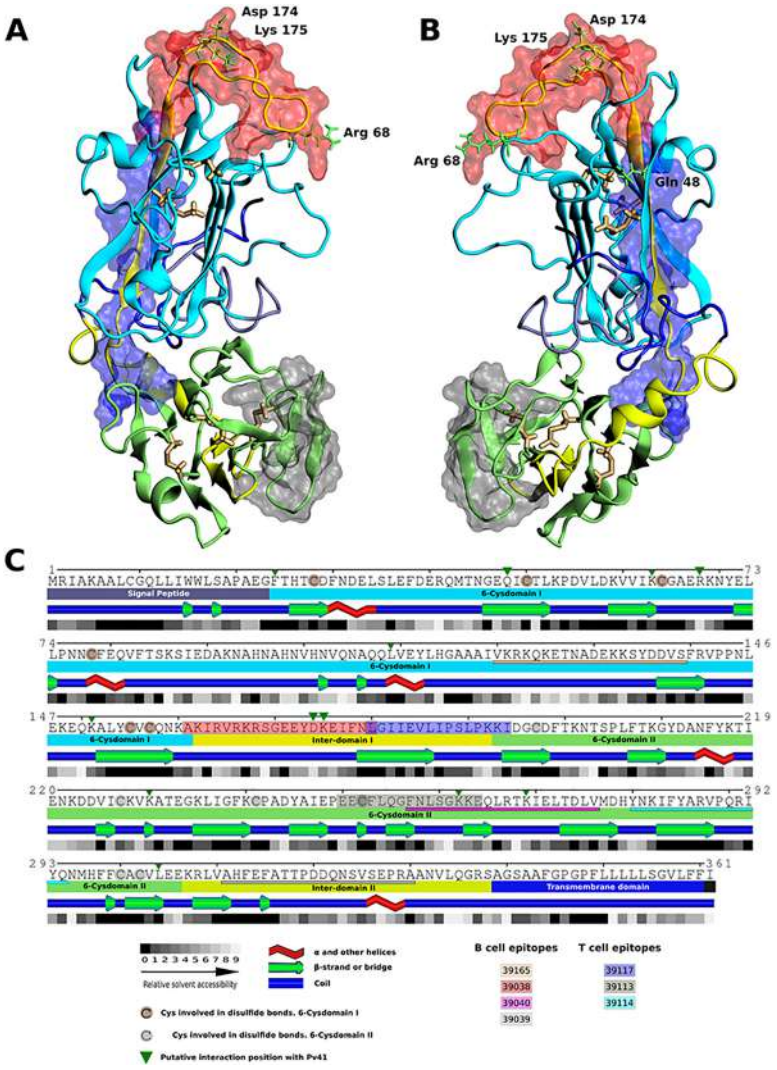

Fig 8. *Pv*12 structure model. A (top view) and B (bottom view). *Pv*12 3D homology model. B-cell epitope 39038 (red) and T-cell epitopes 39117 (blue) and 39113 (grey) are shown as transparent surfaces. Potential interacting positions with *Pv*41 are shown in green and conserved Cys residues in yellow. C. *Pv*12 regions and primary/secondary structure representation, including relative solvent accessibility and potential interacting residues with *Pv*41 (green triangles). Best epitopes are shown (39038- red box, 39117-blue box, 39113-gray box). Other B- and T-cell epitopes are underlined. Conserved cysteine residues are shaded in brown.

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potential blocking activity of antibodies generated against 39038–39117 *Pv*12 region and 39113–39040 region.

Discussion

A particular characteristic of the six-cysteine (Cys6) protein family is that it is expressed during all parasite life-cycle stages (mature Mrz, gametocytes and pre-erythrocyte stages), making such proteins interesting vaccine candidates [63]. The *Pv*12 surface protein is a member of this family, expressed in blood stage and has a GPI anchor; it is one of the most conserved antigens amongst *Plasmodium* species and *P. vivax* isolates [33]. *Pv*12 is homologue of the *Pf*12 protein in *P. falciparum* and (together with other proteins) has been suggested as a possible ligand during Mrz invasion of target cells [64]; it is also recognised by the serum from patients exposed

to natural infection [32]. People from/living in *P. falciparum* malaria-exposed endemic areas develop antibody responses to GPI proteins, correlating with protection against the disease's symptoms, such as fever and anaemia [65].

r*Pv*12 immunoblot assays led to ascertaining *Pv*12 recognition by sera from individuals naturally exposed to *P. vivax* infection, indicating that is capable of triggering a memory-mediated humoral immune response following infection. These results confirmed this protein's antigenic role and agreed with the studies carried out by Li *et al*., and Moreno *et al.* [30,31].

Evaluating ELISA recognition of the whole *Pv*12 in this study showed 33% positivity; likewise, other studies carried out by Chen *et al.* and Li *et al*., involving the protein array technique, found that 15% and 49% of exposed people's sera recognised *Pv*12, respectively [9,30].

Although exposed individuals' sera reactivity to r*Pv*12 and to B-cell epitopes was relatively low, a clear tendency was observed involving greater reactivity by exposed sera, unlike that of the control group. This agreed with a study by Jangpatarapongsa *et al*., who reported low anti-*P. vivax* antibody levels but also highlighted immune individuals having specific reactivity against the parasite [66].

Exposed individuals' sera average IgG response to r*Pv*12 was greater than the mean response to the B-epitopes evaluated here. Differences were observed regarding IgG subtypes when evaluating IgG subclasses' response to the recombinant protein and B-epitopes. A clear tendency regarding IgG3 response to total protein was observed whilst there was high IgG2 response to B-epitopes regarding the low response to IgG4. This type of response (IgG2/IgG4) has been reported previously and has been associated with a protection and resolution role concerning *P. falciparum* infection and protection in that caused by *P. vivax* [67,68].

Epitope 39038 had the best recognition of naturally-acquired antibodies of the four B-epitopes evaluated in this study; previous studies have reported it as being as immunogenic in rabbits [31]. This peptide can be considered a good humoral immune response inducer and is proposed as a vaccine candidate due to its potentially protective role.

Lymphoproliferation assay results showed that the *Pv*12-derived peptides selected for their HLA-DR binding were capable of inducing/triggering a lymphoproliferative response when recognised by memory T-cells. Such response specificity was confirmed when comparing such results to those for the control group; although they had the same allele lineages as the exposed group, they did not have a proliferative response.

Other studies have recorded high stimulation values when evaluating the proliferative response of complete proteins or allergens in constant contact with the immune system [60,69]. Although such values were low in this study, the results were similar to those reported by Martinez *el at*., who used ~20 aa length peptides as antigenic epitopes [22]; this highlights the evaluated peptides' importance which (despite accounting for just a minimal fraction of *Pv*12) were capable of triggering a memory T-cell response.

UP 39114 and GBP 39117 were selected according to *in vitro* binding assays, as well as having a $\geq$55% proliferative response (with significant differences regarding the control group) and a mean SI$\geq$2. These results indicated a specific T-cell response for *Pv*12 epitopes and ascertained these epitopes' antigenicity when recognised by immune system memory cells. They correlated proliferation and cytokine production, as seen in other studies with *Plasmodium* antigens [37,70,71].

The PBMCs stimulated with GBP 39113 and 39117 triggered IL-6, IL-10 and TNF production. IL-6 production has been associated with cell proliferation and differentiation [72], agreeing with our results where peptides (two GBP, the UP and parasite lysate) had a significant increase in this cytokine. This cytokine is considered multifunctional regarding an immune response. IL-6, IL-10 and TNF production has been associated with thrombocytopenia in *P. vivax* malaria patients [73]. IFN-γ production is associated with protection against pre-

erythrocyte malarial stages through both *in vitro* and *in vivo* nitric oxide (NO) production following infection with *P. falciparum*, *P. berghei*, *P. yoelii* or *P. chabaudi* Spz [74–76]. Several studies have highlighted the role of proinflammatory cytokines as mediators of protective immunity against malaria, including TNF which, together with IFN-γ, acts synergistically to optimise NO production which is important for parasite destruction [77]. IFN-γ, together with other pro-inflammatory cytokines, participates in inhibiting parasite development within hepatocytes and macrophage activation in intra-erythrocytic parasite phagocytosis or as free Mrz [78].

IL-10 was another cytokine having significant differential production after being stimulated with GBP 39113 and 39117. Recent studies have shown that memory CD4+ lymphocytes proliferate during secondary malarial infection, producing an immune response which controls parasitic load and rapidly produces IL-10 regulating inflammation. This demonstrates IL-10-producing CD4+ lymphocytes' importance in secondary infections thereby influencing the nature of immune anamnestic responses during secondary malarial infection [79].

Previous studies have shown that PBMCs from naive individuals stimulated with *P. falciparum* parasitised RBC [80] or stimulated with malaria antigens did produce IFN-γ [81], mainly innate cells, such as natural killer cells. PBMCs from non-exposed individuals in another study produced IFN-γ and IL-6 after stimulation with *P. falciparum* CS protein [82]. Accordingly, higher IFN-γ and IL-6 production from control group PBMCs in this study suggested that innate cells, innate lymphoid cells and naive T-cells recognised lysate and produced cytokines [83].

Although stimulation with GBP 39113 had no differences cell proliferation regarding the control group, 46.6% of the exposed individuals' cells proliferated following stimulus and the group's mean remained above the cut-off line. An ideal anti-malarial vaccine candidate would be one which could activate T-cell (Th1 and Th2) response, inducing joint production of cytokines involved in cell proliferation and differentiation of innate and adaptive response, eliminating the parasite and promoting a boosted response by immune system memory cells. We thus propose GBP 39113 (having high binding affinity for HLA-DRβ1*04 and *11 allele lineages) and 39117 (having high binding affinity for HLA-DRβ1*07 and *13 allele lineages) as potential candidates for a vaccine against malaria caused by *P. vivax*.

Bioinformatics tools have proven to be quite useful in the process of vaccine development; predicting B- and T- cell epitopes *in silico* greatly narrows the amount of epitopes to be tested experimentally in *in vitro* antigenicity assays. Humoral and cellular immune responses against predicted B- and T-cell epitopes were here analysed in samples collected from individuals coming from an endemic region for *P. vivax* malaria. A similar approach has been followed in a previous study analysing *Pf*CelTOS protein comparing the *in silico* prediction of B- and T-cell epitopes with the *in vivo* immune response in animal models; such study suggested that optimized algorithms can be useful in vaccine design [26].

A bioinformatics approach combined with functional assays has allowed us to propose two protein regions containing both B- and T-cell epitopes within the *Pv*12 antigen that are able to induce a good immune response. 39038–39117 *Pv*12 region combines the best B- and T-cell epitopes and contains residues that could potentially interact with *Pv*41, standing out as a promising vaccine candidate, since it could trigger antibody/memory responses and block the binding activity to *Pv*41. The 39113–39040 region combines a good T-cell epitope, but a not so good B-cell epitope; however, it also contains potentially interacting residues with *Pv*41, and could be considered as another viable vaccine candidate.

Although the results described in the present study seem promising, caution must be applied considering previous data collected over the years. Several studies have shown that naturally-acquired immunity against *Plasmodium* infection is stage-, strain-, and species-specific.

Studies immunising mice with either the C-terminal region of MSP-1 or AMA1 and then challenging with rodent Plasmodia, have shown only protective efficacy upon challenge with the homologous, but not a heterologous parasite strain [84–86]. A study in *Aotus* using three separate MSP1 fragments formulated in different adjuvants has shown a strain-specific response to challenge [87]. Another study immunising with recombinant AMA-1, assessed the response specificity and showed that AMA-1 was protective when challenging with a homologous *P. falciparum* strain. Nevertheless, AMA-1 was not protective when *Aotus* were challenged with a heterologous strain, suggesting again that the protective response induced by this protein is strain-specific [88]. Our group has invested great efforts in identifying those *P. falciparum* merozoite protein regions that are conserved (non-polymorphic between strains) and also participate in parasite binding to target cells (functionally relevant) by synthesising sequential 20-mer fragments of each, and testing their binding *in vitro*. Peptides displaying high binding have been named HABPs (High Activity Binding Peptides) [89–93]. After finding that conserved HABPs are not immunogenic, nor induce protection in the *Aotus* monkey model, it was decided to modify some amino acids which are critical in target cell binding [94], to allow a better fit into the HLA-DR molecules' peptide-binding region [95,96].

Despite *Pv*12 peptides identified in the present study are antigenic, it is worth highlighting that most of them are conserved and, at least two of them, are functionally relevant (participating in binding to *Pv*41). Based on the observations made in *P. falciparum*, it is likely that modifications to *Pv*12 peptides to further improve their HLA-DR binding could be necessary before carrying out immunogenicity and protective efficacy assays in the *Aotus* monkeys, allowing the proper formation of the MHC-peptide-TCR complex, as has been shown earlier in *P. falciparum* modified HABPs [97].

Supporting information

S1 Fig. Antibody responses. A. Immunoblot assay. r*Pv*12 and r*Pv*GAMA protein recognition by exposed people's sera. Positive control (anti-His) is shown in lane1, lanes 2 to 7 show the sera from 6 individuals living in endemic areas which recognised proteins as expected and lanes 8 and 9 show 2 sera from non-exposed volunteers. **B. Immunofluorescence assay.** a. One Tierralta exposed volunteer's serum sample which recognised pRBC. b. One non-exposed volunteer's (control group) serum sample. DAPI was used for staining nuclei, FITC-labelled anti-human IgG was used for staining the parasite and both were merged.
(PDF)

S2 Fig. IgG antibody response. (A) to r*Pv*GAMA as positive control. Significant differences were observed between exposed individuals (n = 30) and control group (n = 8), (calculated by Mann-Whitney test). The dashed line indicates the cut-off point for seropositive samples. (***) $p<0.0005$. **B. IgG antibody response to *Pv*12 B-epitopes by endemic area.** Significant differences (calculated by Mann-Whitney test) are shown between samples from Colombia's Chocó (n = 13) and Córdoba (n = 17) departments. The dashed line indicates the cut-off point for seropositive samples. (**) $p<0.005$.
(PDF)

S3 Fig. Lymphocyte proliferative response to PHA. PBMCs from exposed and control groups were stimulated with PHA as positive control and Mann-Whitney was used for assessing statistically significant differences between exposed individuals and control group responses (*) $p<0.05$.
(PDF)

S1 File. Interacting residues involved in P12 and P41 binding. The interacting positions, identity and similarity are shown in the sequences **A. *Pf*12 vs *Pv*12.** Primary sequence from *Pv*12, the *Pf*12-PDB (2YMO) crystal structure and *Pf*12 (C6KSX0) reference sequence. **B. *Pf*41 vs *Pv*41.**
(PDF)

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