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Induction of Multifunctional Broadly Reactive T Cell Responses by a *Plasmodium vivax* Circumsporozoite Protein Recombinant Chimera

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***Plasmodium vivax* is the most widespread species of *Plasmodium*, causing up to 50% of the malaria cases occurring outside sub-Saharan Africa. An effective vaccine is essential for successful control and potential eradication. A well-characterized vaccine candidate is the circumsporozoite protein (CSP). Preclinical and clinical trials have shown that both antibodies and cellular immune responses have been correlated with protection induced by immunization with CSP. On the basis of our reported approach of developing chimeric *Plasmodium yoelii* proteins to enhance protective efficacy, we designed PvRMC-CSP, a recombinant chimeric protein based on the *P. vivax* CSP (PvCSP). In this engineered protein, regions of the PvCSP predicted to contain human T cell epitopes were genetically fused to an immunodominant B cell epitope derived from the N-terminal region I and to repeat sequences representing the two types of PvCSP repeats. The chimeric protein was expressed in soluble form with high yield. As the immune response to PvCSP has been reported to be genetically restricted in the murine model, we tested the immunogenicity of PvRMC-CSP in groups of six inbred strains of mice. PvRMC-CSP was able to induce robust antibody responses in all the mouse strains tested. Synthetic peptides representing the allelic forms of the *P. vivax* CSP were also recognized to a similar extent regardless of the mouse strain. Furthermore, the immunization regimen induced high frequencies of multifunctional $CD4^+$ and $CD8^+$ PvRMC-CSP-specific T cells. The depth and breadth of the immune responses elicited suggest that immunization with PvRMC-CSP can circumvent the genetic restriction of the immune response to *P. vivax* CSP. Interestingly, PvRMC-CSP was also recognized by naturally acquired antibodies from individuals living in areas where malaria is endemic. These features make PvRMC-CSP a promising vaccine candidate for further development.**

Plasmodium vivax is the most widespread species of *Plasmodium*, causing up to 50% of the malaria cases occurring outside sub-Saharan Africa, with an estimated 2.48 billion people living in areas with risk of malaria transmission (1–4). Unlike *Plasmodium falciparum*, *P. vivax* is able to persist in a latent stage called hypnozoite within infected parenchymal liver cells. Activation of hypnozoites weeks or months after the primary infection leads to new blood stage infections, causing relapses and opportunities for further transmission (5).

A vaccine targeting the *P. vivax* preerythrocytic stages preventing the entry of sporozoites into hepatocytes or inhibiting the liver stage development could block the production of hypnozoites. The most-characterized antigen and one of the few vaccine candidates for *P. vivax* tested in clinical trials is the circumsporozoite protein (CSP). CSP is an attractive target, since anti-CSP antibodies derived from naturally infected patients or from volunteers exposed to irradiated sporozoites have the ability to inhibit the infection of hepatic cells by sporozoites *in vitro* (6). Unlike *P. falciparum*, this protein is expressed in the course of the *P. vivax* exoerythrocytic stage development (7) and is also expressed by hypnozoites (8). *P. vivax* CSP (PvCSP) is characterized by a highly immunogenic central repetitive domain composed by 19 short blocks of nine tandem amino acid repeats. These repeat sequences exhibit three different variants (VK210, VK247, and *P. vivax*-like) that show a universal distribution. The central domain is flanked by two nonrepetitive N- and C-terminal regions containing small stretches of highly conserved sequences that contain T and B cell epitopes (9–11).

Four phase I clinical trials have been reported using *P. vivax* CSP-based vaccines. The first two trials were conducted in the early 1990s using recombinant proteins expressed in *Escherichia coli* or *Saccharomyces cerevisiae* (12, 13). These proteins were poorly immunogenic and unable to elicit inhibitory antibody responses (12, 13). The next two clinical trials used long synthetic peptides representing the immunogenic regions present in CSP (14, 15). The immune responses in both trials were predominantly toward the N-terminal peptide, and a peptide combination achieved a seroconversion rate of 73%, with low antibody titers against the native protein (15).

The poor immunogenicity of PvCSP vaccine formulations and the differences in its recognition could be explained by genetic restriction. In fact, preclinical trials have shown that the immune

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response to *P. vivax* CSP in mice is genetically restricted (16). In humans, seroepidemiological studies have shown that antibody responses against the PvCSP repeats are modulated by HLA class II molecules (17).

The HLA modulation of the antibody response provides evidence of the vital role of the cellular response against malaria. In mouse models, protection against sporozoite challenge seems to be initiated by $CD8^+$ T cells following immunization with irradiated sporozoites and by $CD4^+$ T cells following immunization with a peptide (18). The protective role of $CD8^+$ T cells in humans has also been observed following vaccination with transgenic viral vectors expressing *P. falciparum* multiepitope string fused to thrombospondin-related anonymous protein (PfME-TRAP) (19). High frequencies of $CD4^+$ T cells and gamma interferon (IFN-γ)-secreting T cells have also been correlated with protection in humans after immunization with the *P. falciparum* CSP-based RTS,S (20). Nevertheless, IFN-γ alone is not enough to predict a protective response, as the production of interleukin 2 (IL-2), tumor necrosis factor alpha (TNF-α) (21), and multifunctional T cells also seems to be correlated with protection and memory responses (22).

We have previously designed and expressed *Plasmodium yoelii* chimeric recombinant proteins that contain cognate predicted human T cell epitopes genetically linked in tandem to a well-characterized B cell epitope as an approach to improve the cellular immunogenicity of vaccine candidates (23, 24). Following this rationale, we report here the design of a chimeric *P. vivax* CSP recombinant protein (PvRMC-CSP). This chimera includes two predicted putative promiscuous T cell epitopes, derived from the C-terminal region of the native CSP protein, arrayed in tandem and genetically fused to an immunodominant B cell epitope derived from the N-terminal region that includes region I (25), followed by amino acid sequences representing the two major variant repeats, VK210 and VK247. To determine whether such a chimeric protein expressed in *Escherichia coli* has the ability to overcome the genetic restriction of the immune response to *P. vivax* CSP reported in mice, we characterized the immunogenicity of PvRMC-CSP in several inbred strains of mice. Our data indicate that this chimeric protein is highly immunogenic irrespective of the major histocompatibility complex (MHC) haplotype eliciting high antibody responses and high frequencies of multifunctional $CD4^+$ and $CD8^+$ T cell responses. To our knowledge, this is the first evidence that a nonvectored vaccine based on CSP can induce robust T cell responses in mice. In addition, we showed that plasma samples derived from individuals naturally exposed to malaria contain high levels of acquired antibodies that recognize the chimeric PvCSP.

MATERIALS AND METHODS

Design and biochemical characterization of the *P. vivax* chimeric CSP. A 636-bp PvRMC-CSP gene was codon optimized and synthesized by Geneart (Regensburg, Germany) (Fig. 1A and B). This synthetic gene encodes a chimeric protein that includes the following: (i) M-A on the N terminus to provide the start signal and decrease degradation in *E. coli* (A) and two additional amino acids (VD) downstream introduced by the cloning strategy (Fig. 1A); (ii) two PvCSP SalI regions predicted to contain promiscuous T cell epitopes (GenBank accession no. AAA29529.1), E_{265}-L_{310} and A_{325}-N_{343}; (iii) the segment containing amino acids G_{37}-G_{62} represents a sequence that includes the conserved region I and is reported to be the target of most of the antibody reactivity elicited by immunization with the complete N-terminal region of the *P. vivax* CSP (25); (iv) amino acid sequences representing different variants of the type 1 repeat sequence (VK210), two copies of the octapeptide GDRAGQPA (GenBank accession no. AAA29535.1) present in North Korean isolates described by Arnot et al. (26) interspaced between two copies of the nonapeptide GD RADGQPA, followed by three copies of the nonapeptide GDRAAGQPA (27); (v) three copies of the nonapeptide ANGAGNQPG representing the type 2 repeat sequence (VK247) (27); (vi) GPGPG spacers, inserted between the described sequences to enhance stability and antigen processing (28). The synthetic gene was digested with NcoI and XhoI and cloned in pET24d(+), which expresses the protein with a C-terminal His_6 tag. The chimeric construct was transformed into *E. coli* BL21(DE3) cells (Novagen, Madison, WI), and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The 213-amino-acid protein, including the His tag added to the protein via the expression vector, was purified with a nickel-nitrilotriacetic acid (Ni-NTA) affinity column according to the manufacturer's instructions (Qiagen, Valencia, CA) and further purified by size exclusion chromatography performed on a fast protein liquid chromatograph (FPLC) (AKTAprime Plus; GE Health Care) instrument using a Sephadex G-75 column.

The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions on 4 to 20% polyacrylamide gels (Lonza, Allendale, NJ). For Western blots, following electrophoresis, the proteins were blotted onto nitrocellulose membranes following standard procedures as described previously (23, 29). The membranes were then incubated with anti-CSP species-specific monoclonal antibodies (2F2 [*P. vivax* allelic form VK210] and 2E10.E9 [*P. vivax* allelic form VK247]) obtained from the Malaria Research and Reference Reagent Resource Center [MR4; ATCC, Manassas, VA]), or anti-His tag antibody at 0.2 μg/ml or polyclonal antibodies derived from rabbits immunized with the chimeric protein (Fig. 1C). The endotoxin levels of the protein were evaluated using the E-Toxate (*Limulus* amebocyte lysate) kit, following the manufacturer's instructions (Sigma, St. Louis, MO).

Synthetic peptides. A library of 49 15-mer synthetic peptides overlapped by 11 residues and spanning the complete PvRMC-CSP chimeric protein sequence was commercially synthesized by the multiple solid-phase technique (Sigma-Aldrich, St. Louis, MO) (Table 1). Peptide pools were used to characterize cellular reactivity: PvRMC-CSP peptide pool A included 13 peptides mainly representing the sequence E_{265}-L_{310}; PvRMC-CSP pool B included 14 peptides representing a region downstream of E_{265}-L_{310} and upstream of the repeats; PvRMC-CSP pool C included 14 peptides that represents the VK210 repeat sequences, and PvRMC-CSP pool D included 8 peptides that represent the VK247 repeat sequences. To test antibody reactivity by an enzyme-linked immunosorbent assay (ELISA), the following linear peptides representing the variant repeats included in the chimeric protein were synthetized by RS Synthesis (Louisville, KY): VK210AD $(GDRADGQPA)_3$, VK210AA $(GDRAAGQPA)_3$, North Korean $(GDRAGQPA)_3$, and VK247 $(ANGAGNQPG)_3$ (Table 1).

Mice. Female A/J (H-2^a), DBA/1J (H-2^q), and SJL/J (H-2^s) mice, 6 to 8 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Female BALB/c (H-2^d), C3H (H-2^k), and C57BL/6 (H-2^b) mice, 6 to 8 weeks of age, were purchased from Charles River (Wilmington, MA). The animals were subcutaneously immunized on days 0, 20, and 40 in the base of the tail and in the interscapular area, using 20 μg of the PvRMC-CSP protein emulsified in Montanide ISA 51 (Seppic, Fairfield, NJ). Control groups of mice received phosphate-buffered saline (PBS) alone emulsified in the same adjuvant. All animal protocols were approved by Emory University's Institutional Animal Care and Use Committee and followed accordingly.

ELISAs. (i) Mouse ELISAs. The fine specificity of the antibodies elicited by immunization with PvRMC-CSP in mice was determined by ELISA using Immulon 4HB plates (Thermo Scientific, Waltham, MA) coated with one of the following: 1 μg/ml of PvRMC-CSP or synthetic peptides representing the individual repeat variants or individual overlap-

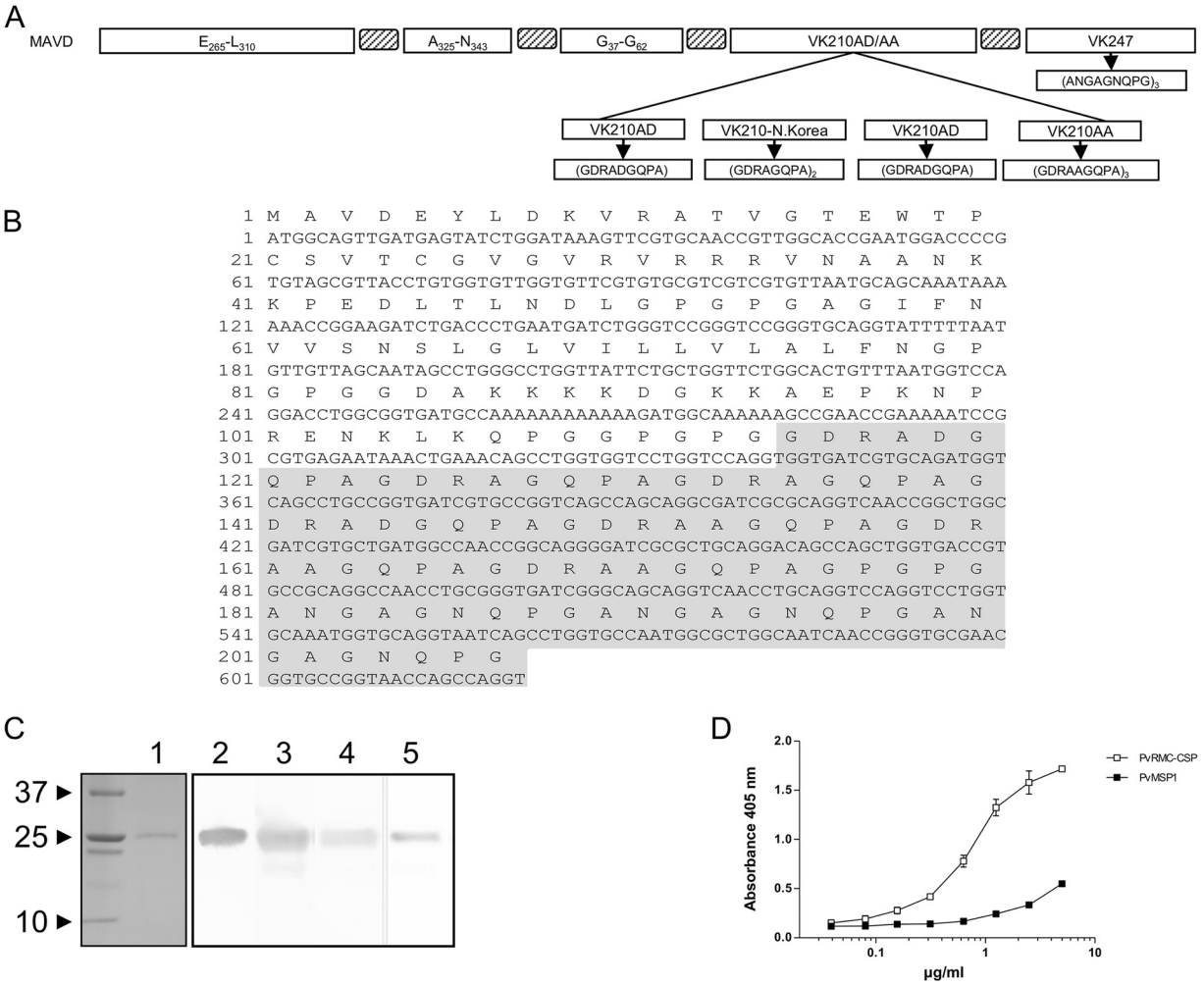

FIG 1 (A) Schematic representation of the recombinant protein reported here. PvRMC-CSP includes the regions of the native CSP that contain predicted promiscuous T cell epitopes E_{265}-L_{310} and A_{325}-N_{343} interspaced with a GPGPG spacer and genetically fused to sequences that are the target of protective antibodies. These sequences include the following: (i) G_{37}-G_{62} from the N-terminal PvCSP including the native region I; (ii) two copies of the octapeptide GDRAGQPA, flanked by one copy of the nonapeptide GDRADGQPA followed by three copies of the GDRAAGQPA sequence (representing the different variants of the VK210 allelic repeat); and (iii) three copies of the nonapeptide ANGAGNQPG (representing the VK247 allelic repeat variant). The two copies of the octapeptide GDRAGQPA were derived from a North Korean isolate (AAA29535.1) (26). GPGPG spacers were also inserted between the three B cell domains (striped boxes). (B) Sequence of the PvRMC-CSP protein. The amino acid sequence is shown in single-letter code. The sequences of the allelic repeat variants are shown on a gray background. (C) Coomassie blue stain after SDS-PAGE separation of the purified protein (lane 1). Western blot analysis of the purified PvRMC-CSP stained with purified IgG antibodies produced in rabbits after immunization with the chimeric protein (lane 2), monoclonal antibody 2F2 that recognizes the VK210 allelic repeat variant (lane 3), monoclonal antibody 2E10 that recognizes the VK247 allelic repeat variant (lane 4), or an anti-6× His tag monoclonal antibody (lane 5). The positions of molecular weight markers (in thousands) (Bio-Rad) are indicated to the left of the gel. (D) Heparan sulfate binding assay. PvRMC-CSP, but not PvMSP1, binds to heparan sulfate in a dose-dependent manner.

ping peptides representing the N-terminal region G_{37}-G_{62}, diluted in PBS as described previously (23). Optical densities (ODs) were determined using a VERSAmax ELISA reader (Molecular Device Corporation, Sunnyvale, CA) with a 405-nm filter. The cutoff was the highest dilution of sera having an OD greater than the mean plus 3 standard deviations obtained using preimmune sera. Results are presented as the reciprocal of the endpoint dilution.

(ii) Human ELISAs. To evaluate reactivity of naturally acquired antibodies against PvRMC-CSP, plasma samples were selected from 251 individuals from communities in Rondonia, a state in the western Amazon region of Brazil, where malaria is endemic. In the last 5 years, *P. vivax* malaria accounted for more than 70% of all malaria cases in the region as reported (30, 31). The majority of the studied population consists of rainforest natives who have resided in the region where malaria is endemic for more than 25 years or transmigrants from several areas of Brazil where malaria is not endemic who have lived in Rondonia for 10 years or more. The study was reviewed and approved by the Oswaldo Cruz Foundation Ethical Committee institutional review board (IRB) no. 138/01 and 354/06 and the National Ethical Committee of Brazil.

Human plasma samples were tested using Maxisorp 96-well plates (Nunc, Rochester, NY) coated with 200 ng of the recombinant protein. After overnight incubation at 4°C, the plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with PBS-Tween containing 5% nonfat dry milk (PBS-Tween-M) for 1 h at 37°C. Individual plasma samples diluted 1:100 in PBS-Tween-M were added to duplicate wells, and the plates were incubated at 37°C for 1 h. After four washes with PBS-Tween, peroxidase-conjugated goat anti-human total IgG (Sigma-Aldrich) diluted by 1:1,000 was added, and the plates were incubated and washed as described above. Finally, *o*-phenylenediamine and hydrogen peroxide were used to reveal bound antibodies. The absorbance was read

TABLE 1 Peptides used for immunological assays

Assay and peptide used	Sequence
ELISA	
VK210AD	$(GDRADGQPA)_3$
VK210AA	$(GDRAAGQPA)_3$
VK247	$(ANGAGNQPG)_3$
North Korean	$(GDRAGQPA)_3$
Flow cytometry	
Pool A overlapping E_{265}-L_{310}	MAVDEYLDKVRATVG
	EYLDKVRATVGTEWT
	KVRATVGTEWTPCSV
	TVGTEWTPCSVTCGV
	EWTPCSVTCGVGVRV
	CSVTCGVGVRVRRRV
	CGVGVRVRRRVNAAN
	VRVRRRVNAANKKPE
	RRVNAANKKPEDLTL
	AANKKPEDLTLNDLG
	KPEDLTLNDLGPGPG
	LTLNDLGPGPGAGIF
	DLGPGPGAGIFNVVS
Pool B overlapping A_{325}-N_{343} and G_{37}-G_{62}	GPGAGIFNVVSNSLG
	GIFNVVSNSLGLVIL
	VVSNSLGLVILLVLA
	SLGLVILLVLALFNG
	VILLVLALFNGPGPG
	VLALFNGPGPGGDAK
	FNGPGPGGDAKKKKD
	GPGGDAKKKKDGKKA
	DAKKKKDGKKAEPKN
	KKDGKKAEPKNPREN
	KKAEPKNPRENKLKQ
	PKNPRENKLKQPGGP
	RENKLKQPGGPGPGG
	LKQPGGPGPGGDRAD
Pool C VK210 repeats	GGPGPGGDRADGQPA
	PGGDRADGQPAGDRA
	RADGQPAGDRAGQPA
	QPAGDRAGQPAGDRA
	DRAGQPAGDRAGQPA
	QPAGDRAGQPAGDRA
	DRAGQPAGDRADGQP
	QPAGDRADGQPAGDR
	DRADGQPAGDRAAGQ
	GQPAGDRAAGQPAGD
	GDRAAGQPAGDRAAG
	AGQPAGDRAAGQPAG
	AGDRAAGQPAGDRAA
	AAGQPAGDRAAGQPA
Pool D VK247 repeats	PAGDRAAGQPAGPGP
	RAAGQPAGPGPGANG
	QPAGPGPGANGAGNQ
	PGPGANGAGNQPGAN
	ANGAGNQPGANGAGN
	GNQPGANGAGNQPGA
	GANGAGNQPGANGAG
	AGNQPGANGAGNQPG

at 492 nm using a Spectramax 250 ELISA reader (Molecular Devices, Sunnyvale, CA). The results for total IgG were expressed as reactivity indexes (RI) that were calculated by dividing the mean optical density of tested samples by the mean optical density plus 3 standard deviations of 5 nonexposed controls tested on each plate. Subjects were scored positive for serum IgG to a particular antigen if the RI was higher than 1 (30, 31).

Heparan sulfate binding. Heparan sulfate binding assay was performed as previously described by Yadava et al. (32) with some modifications. Briefly, Immulon 4HBX plates (Thermo Scientific, Waltham, MA) were coated with 100 μl/well of a 10-μg/ml solution of heparan sulfate from bovine kidney (Sigma-Aldrich) diluted in water. The plates were incubated overnight at 37°C. Control wells were incubated with 10 μg of bovine serum albumin (BSA)/ml. The plates were washed with a 0.05% Tween 20 (Sigma-Aldrich) in PBS solution and blocked with a 1% solution of BSA in PBS for an hour at room temperature. PvRMC-CSP was diluted in PBS starting at 5 μg/ml, and serial dilutions were plated. The plates were then incubated for 2 h at room temperature. Control wells were incubated with similar dilutions of a recombinant *P. vivax* MSP-1 protein purified using similar protocols. Binding of PvRMC-CSP to heparan sulfate was detected by the addition of mouse antibodies against PvRMC-CSP or antibodies against the recombinant *P. vivax* MSP-1 obtained 20 days after the final immunization for 2 h, followed by anti-mouse IgG labeled with horseradish peroxidase (anti-mouse IgG-HRP) for an hour. The plates were then incubated at room temperature and washed between steps. The reaction was developed by using 2,2′-azinobis(3-ethylbenthiazolinesulfonic acid) (ABTS) (KPL, Gaithersburg, MD), and ODs were read at 405 nm using a VERSAmax ELISA reader (Molecular Device Corporation) after 45 min.

ELISPOT assays. The frequency of PvRMC-CSP-specific T cells was initially determined by IFN-γ- and IL-4-specific enzyme-linked immunosorbent spot (ELISPOT) assays conducted *ex vivo* using spleen cells obtained 20 days after the third immunization. The assays were performed in nitrocellulose microplates (Millipore, Bedford, MA) coated with rat anti-mouse IFN-γ or rat anti-mouse IL-4 (Pharmingen, San Diego, CA) as described previously (23). Freshly isolated spleen cells from six mice were pooled, and triplicate aliquots at 1×10^6 cells/well were plated. T cells were activated by the addition of 10 μg/ml of PvRMC-CSP recombinant protein. Concanavalin A (ConA) was used as a positive control, and splenocytes collected from animals immunized with Montanide ISA 51 and incubated *ex vivo* with PvRMC-CSP were used as a negative control to test specificity. The plates were incubated for 24 h for IFN-γ ELISPOT assays and for 48 h for IL-4 ELISPOT assays. Spot-forming cells (SFC) were calculated after background subtraction of control wells incubated with medium alone.

Flow cytometry assays. Flow cytometry multiparametric analysis of PvRMC-CSP-specific T cells was done using an eight-color panel. The panel was used to simultaneously analyze IL-2, IFN-γ, and TNF-α at the single-cell level in T cells derived from splenocytes obtained 11 days after a single boosting immunization with PvRMC-CSP. A library of 49 synthetic peptides representing the complete amino acid sequence of the chimeric protein was used for *ex vivo* stimulation (Table 1). The cells were stimulated for 6 h with peptide pools or PvRMC-CSP at 2 μg/ml at 37°C in the presence of GolgiPlug (BD Biosciences, San Jose, CA). The cells were then incubated with LIVE/DEAD aqua stain (Life Technologies) followed by surface staining with anti-CD3 (peridinin chlorophyll protein [PerCP]-Cy5.5), anti-CD4 (Alexa Fluor 700), and anti-CD8α (allophycocyanin [APC]-Cy7) for 30 min. The cells were then fixed, permeabilized, and stained with antibodies against IFN-γ (APC), TNF-α (phycoerythrin [PE]), and IL-2 (fluorescein isothiocyanate [FITC]). All the monoclonal antibodies were obtained from BioLegend (San Diego, CA). Flow cytometry analyses were performed using an LSRII flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo (version 9.4.1; Tree Star, Ashland, OR). Analyses of multifunctional T cell responses were done using a Boolean analysis in FlowJo. The lymphocytes were initially gated on the LIVE/DEAD channel, and then $CD3^+$ $CD4^+$ and $CD3^+$

$CD8^+$ antigen-specific cytokine-secreting T cells were identified (see Fig. S2 in the supplemental material). The frequency of antigen-specific cytokine-producing cells was determined by subtracting the percentage of cytokine-producing T cells after incubation with medium alone from the percentage of cytokine-producing T cells after incubation with PvRMC-CSP or the corresponding peptide pools. A threshold for a positive cytokine response was set above the background, and the values for samples that did not meet this requirement were set at zero.

Statistics. Statistical analysis and graphs were made using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). For analysis of the antibody and cellular responses, data were log transformed to conform to the normality and variance requirements of parametric testing and compared using one-way analysis of variance (ANOVA) with *post hoc* Bonferroni's multiple-comparison posttest. To evaluate ELISPOT responses, each group was compared with a control group composed of naive mice from the same strain using Student's *t* test.

RESULTS

Design, expression, and characterization of the chimeric protein PvRMC-CSP. Both antibodies and cellular immune responses against *Plasmodium* CSP have been reported to be involved in protection (33, 34). We have previously reported the use of chimeric recombinant proteins based on *P. yoelii* antigens as an effective delivery system to induce balanced protective immune responses in mice. The chimeric recombinant proteins were designed to optimize the immune response by the genetic linkage of PvCSP regions predicted to contain promiscuous human class II T cell epitopes arrayed in tandem to linear B cell epitopes. Such topology was associated with a significant improvement of the immune response and protective efficacy against *P. yoelii* (23). Although chimeric vaccine constructs based on PvCSP have been reported, our approach is unique in that we aim to develop a universal strategy for the optimal delivery of subunit vaccines. According to this strategy, regions of the protein predicted to bind a large number of MHC class II alleles are fused to protein segments known to contain protective B cell epitopes, eliminating in this manner regions of low immunological value. Using the graphical interphase generated by the ProPred server (35) and *in silico* analysis, we predicted regions with potential binding capacity to reference sets of class I and class II using the Immune Epitope Database and Analysis Resource (IEDB) consensus tool (36–44). The regions contained between amino acids 265 and 310 and amino acids 325 and 343 are predicted to be recognized by multiple MHC class II alleles (see Fig. S1 in the supplemental material). Peptides within PvCSP capable of binding to MHC class I molecules predicted by the IEDB server (http://www.iedb.org/) are present mainly in the signal peptide (amino acids 1 to 19) and in the C-terminal region (amino acids 260 to 340) (Fig. S1). On the basis of this information, we designed the chimeric recombinant protein PvRMC-CSP that comprised the two predicted regions at the C-terminal region of the PvCSP (E_{265}-L_{310} and A_{325}-N_{343}) genetically fused to a previously described B cell epitope that includes region I and sequences that represent the major allelic forms of the repeat region (Fig. 1). The selected sequences are predicted to bind between 5 and 21 human MHC class II alleles in the IEDB reference set that includes 27 alleles.

PvRMC-CSP was purified from *E. coli* lysates by metal chelate chromatography using a Ni-NTA resin. After initial metal chelate purification, the recombinant construct was further purified using analytical gel filtration chromatography on Sephadex G-75; the purified protein migrated as a single peak. SDS-PAGE analyses showed a single band with an apparent mobility of 25 kDa. The biochemical identity of the recombinant protein was established by Western blot analysis using a panel of antibodies recognizing different components of the chimera (Fig. 1C). The monoclonal antibodies 2F2 and 2E10.E9, which recognize the VK210 and VK247 alleles, recognized the chimeric protein and the anti-His tag monoclonal antibody (Fig. 1C). Purified polyclonal antibodies produced in rabbits by immunization with PvRMC-CSP were used as a positive control of reactivity (Fig. 1C), while purified polyclonal antibodies from naive rabbits were used as a negative control of specificity. To assess the presence of bacterial impurities in our construct, endotoxin levels were measured by using *Limulus* amebocyte lysate assay. Endotoxin levels were less than 25 endotoxin units (EU)/mg of protein.

A heparan sulfate binding assay was performed to confirm that the modification on the PvCSP structure in our protein did not affect the functionality of the CSP conserved domains, since the binding of CSP to heparan sulfate proteoglycans on the surfaces of hepatocytes is the main event in the rapid and specific localization of sporozoites to the liver after the infectious anopheline bite (45). Our results indicate that PvRMC-CSP binds to heparan sulfate in a dose-dependent manner (Fig. 1D).

Humoral response in mice immunized with PvRMC-CSP. Murine antibody responses to recombinant PvCSP have been reported to be genetically restricted (16). To test whether the structural changes incorporated in our chimeric protein can act to overcome this limitation, six inbred strains of mice were used to test the immunogenicity of PvRMC-CSP. All serum samples taken before immunization showed no antibodies against PvRMC-CSP (Fig. 2A). After a single immunization, the A/J strain had higher anti-PvRMC-CSP titers than the other mouse strains ranging between 1:81,920 and 1:327,680 and were significantly higher than the titers induced in BALB/c mice (ranging between 1:1,280 and 1:40,960 [$P < 0.01$]) and the SJL mice (ranging between 1:320 and 1:81,920 [$P < 0.01$]). After the second immunization, the antibody titers in the different strains of mice were boosted. The A/J mice continued to produce the highest titer of antibodies with significantly higher IgG titers compared to SJL ($P < 0.01$) and DBA ($P < 0.05$) mice. After the third immunization, all responses were boosted, and the SJL mice produced significantly lower titers than the C57BL/6, DBA, and C3H mice did ($P < 0.05$) (Fig. 2B). However, antibody titers in SJL mice were >49-fold higher than titers obtained after the first immunization and comparable to those recorded for A/J and BALB/c mice.

After confirmation of the broad antibody responses induced by immunization with PvRMC-CSP, the next step was to assess the capacity of the antibodies elicited in mice to recognize the different allelic forms of the *P. vivax* central repeats using synthetic peptides representing the major alleles. All of the mouse strains tested produced antibodies against the variants of the central repeats tested. The antibody titers against VK247 were lower in all the mouse strains tested (Fig. 2C). Nonetheless, this response was higher than 1:320 in all strains tested and reached titers of 1:81,920 in A/J and CH3 mice.

A segment of *P. vivax* CSP N-terminal region that includes region I is also a known target for protective antibodies. Therefore, this segment was included in PvRMC-CSP. To map the epitopes at which the B cell response is directed, we tested several overlapping peptides representing the PvCSP N-terminal amino acids G_{37}-G_{62}. As predicted, most antibodies recognized the conserved region I (KLKQP).

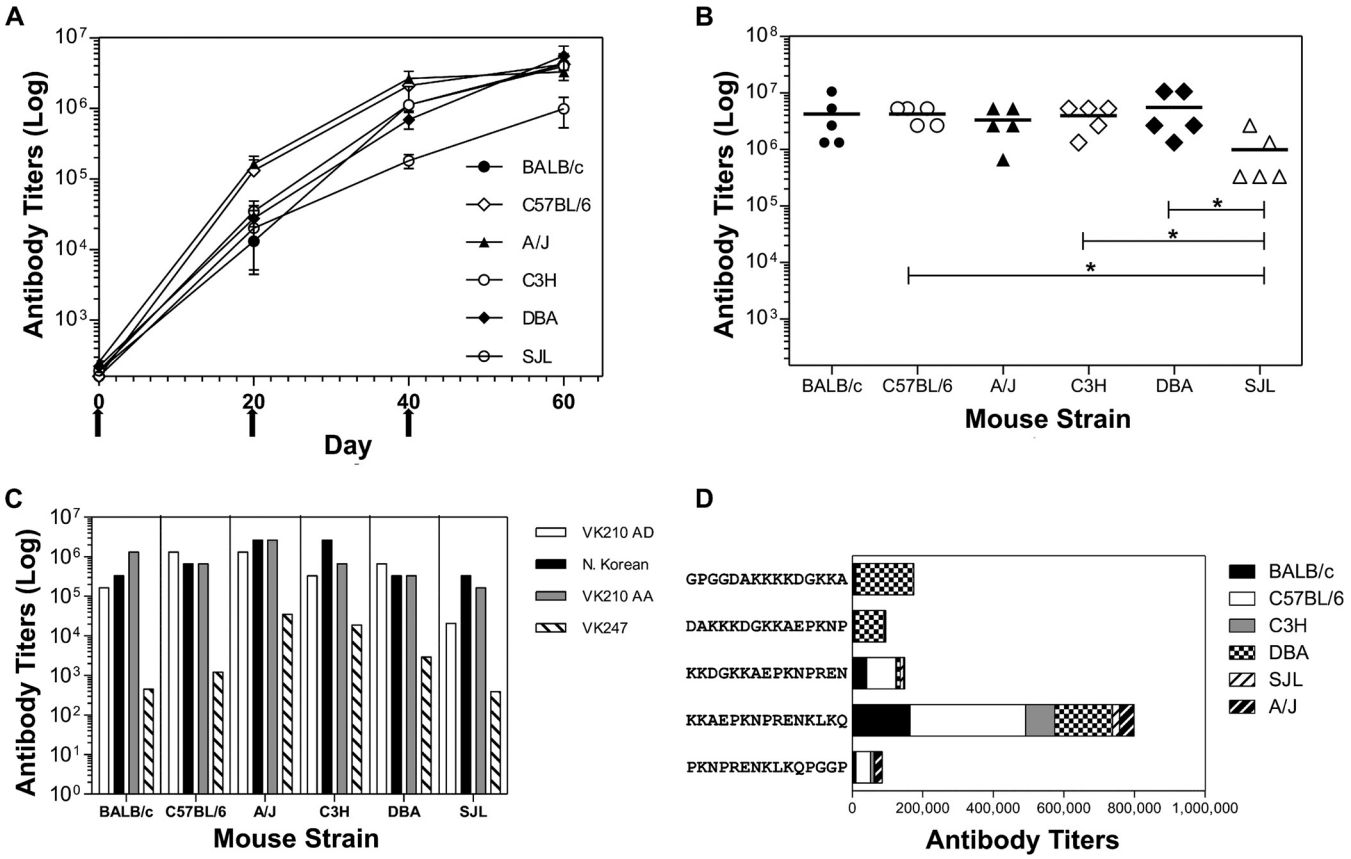

FIG 2 (A) Kinetics of the antibody response against PvRMC-CSP in six different mouse strains with different H-2 alleles determined by ELISAs. Black arrows indicate immunization days. (B) Antibody titers measured 20 days after the final immunization (day 60). Each symbol represents the value for an individual mouse. Each short horizontal line represents the arithmetic mean value for a group. Values that are significantly different ($P < 0.05$) by one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison posttest are indicated by a bar and asterisk below the symbols. (C) Antibody titers to the different alleles of the central repeat domain of the *P. vivax* CSP expressed as arithmetic mean values and determined using pooled sera collected after the third immunization. (D) Mapping of the antibody responses to the *P. vivax* CSP N-terminal sequence that includes region I (G37-G62). The results are presented as stacked columns representing the arithmetic mean titers to individual peptides and determined after the third immunization.

However, the presence of this epitope alone was not enough to induce high antibody titers. Based on the peptide producing the highest antibody response, it seems that a peptide sequence (KKAEPKNPREN) preceding region I is required for efficient recognition (Fig. 2D). It is also important to note that all mouse strains tested were able to recognize the peptide containing region I and the upstream N-terminal sequence with levels ranging between 1:20,480 in SJL mice and 1:327,680 in C57BL/6 mice, showing that the peptide recognition does not depend on the mouse MHC haplotype.

To assess the ability of the antibodies induced by PvRMC-CSP to recognize the CSP protein in its native form, and given the limited availability of *P. vivax* sporozoites, only pools of sera obtained from C57BL/6 mice (one of the strains that produced high mean antibody titers against the recombinant protein after completion of the immunization regimen) were tested for reactivity against *P. vivax* VK210 sporozoites by immunofluorescence. Serum samples reacted with sporozoites at titers ranging between 1:20,480 and 1:40,960 (data not shown).

Cellular response in mice immunized with PvRMC-CSP. Cellular responses have been shown to play a significant role in protection against a sporozoite challenge. The cellular reactivity to PvRMC-CSP was initially tested *ex vivo* by measuring cytokine production of restimulated splenocytes with the chimeric protein 20 days after the final immunization via an ELISPOT assay. IFN-γ production in all mice tested ranged between 313 and 760 spot-forming cells (SFC)/10^6 splenocytes. The frequency of IFN-γ SFC were significantly higher for all the strains of mice tested compared to cells obtained from the corresponding placebo immunized mice of the same strain which produced between 0 and 72 SFC/10^6 splenocytes (Fig. 3A). These comparative experiments confirmed the specificity of the cellular reactivity was in response to PvRMC-CSP and was not caused by potential traces of residual endotoxin. C3H mice produced significantly higher IFN-γ SFC than the other strains with the exception of SJL (BALB/c mice, $P < 0.01$; C57BL/6 mice, $P < 0.05$; A/J mice, $P < 0.01$; DBA mice, $P < 0.001$). Interestingly, although SJL produced significant lower antibody titers compared to other strains, this strain produced the second highest frequency of SFC after C3H mice ($P < 0.01$).

To further characterize the cellular reactivity induced by immunization with PvRMC-CSP, IL-4-secreting cells were also determined by an ELISPOT assay. The frequencies of IL-4-secreting cells in immunized mice were significantly higher than in the control mice after stimulation with PvRMC-CSP in all strains tested with the exception of C57BL/6 (Fig. 3B). The frequency of IL-4-

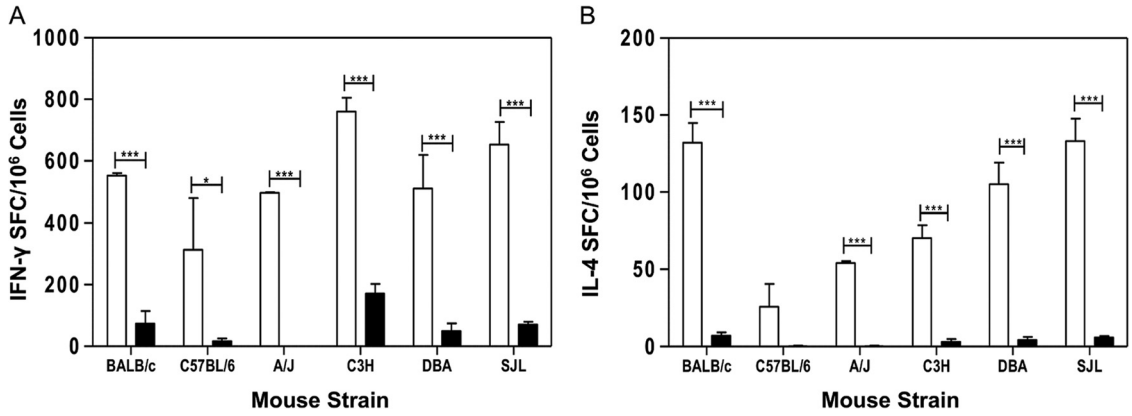

FIG 3 Cells secreting IFN-γ (A) and IL-4 (B) in different mouse strains after immunization with PvRMC-CSP detected by ELISPOT assays conducted *ex vivo* using pools of splenocytes derived from six mice obtained 20 days after the third immunization. Mice immunized with PvRMC-CSP(open bars) and, mice immunized with the adjuvant alone (closed bars) were tested. Results are expressed as the number of spots in the wells stimulated with PvRMC-CSP after the background was subtracted plus standard deviations (SD) using triplicate assays. Values that are significantly different by Student's *t* test are indicated by a bar and asterisks as follows: *, $P < 0.05$; ***, $P < 0.001$.

secreting SFC in PvRMC-CSP-immunized mice ranged between 25 and 133, and the SFC of the placebo-immunized mice ranged between 3 and 7. After *ex vivo* stimulation with PvRMC-CSP, the IFN-γ/IL-4 ratios ranged from 9.1 to 12.1 for C57BL/6, A/J, and C3H mice and from 4.1 to 4.9 for BALB/c, DBA, and SJL mice.

Frequency and functionality of CD4$^+$ T cells after immunization with PvRMC-CSP. Since the ELISPOT assay is unable to differentiate the source of cytokine production or the quality of the cellular immune response (i.e., numbers of multifunctional T cells), flow cytometry and intracellular cytokine staining were performed to further characterize the cellular response induced by PvRMC-CSP (see Fig. S2 in the supplemental material). The frequencies of CD4$^+$ T cells producing IFN-γ, IL-2, or TNF-α after *ex vivo* stimulation with PvRMC-CSP are presented in Fig. 4A. Based on these results, different levels of CD4$^+$ responses dependent on the mouse strain can be inferred. BALB/c mice could be considered high responders, as they produce significantly higher numbers of CD4$^+$ IFN-γ-secreting cells than the other strains do. Accordingly, DBA and C57BL/6 mice could be considered medium responders, while C3H, SLJ, and A/J mice are low responders. Nonetheless, all the mouse strains tested exhibited predominant IFN-γ responses with a multifunctional profile (Fig. 4C). Unlike differences observed with IFN-γ secretion, there were no significant differences in the production of IL-2 between the strains. Comparisons of TNF-α expression revealed that A/J mice produced significant lower levels of TNF-α than DBA and C57BL/6 strains did ($P < 0.01$). It is noteworthy that while A/J had the lowest frequencies of PvRMC-CSP-specific CD4$^+$ T cells secreting IFN-γ, IL-2, or TNF-α alone, this strain had the highest frequency of multifunctional CD4$^+$ T cells (Fig. 4C).

After stimulation with PvRMC-CSP peptide pools, no strain showed a preferential recognition of a single peptide pool for any of the cytokines evaluated (Fig. 4B). Likewise, association between the proportion of multifunctional cells and the peptide pools was not observed (Fig. 4D). The fact that there was not a preferential response toward a single peptide pool and that the different pools were recognized by all the strains tested indicates that there is a broad recognition of the protein, likely due to the presence of several promiscuous T cell epitopes.

Frequency and functionality of CD8$^+$ T cells after immunization with PvRMC-CSP. CD8$^+$ IFN-γ production is strongly correlated with *P. falciparum* protection in humans (19). The percentages of CD8$^+$ T cells producing IFN-γ, TNF-α, or IL-2 are presented in Fig. 5A. Immunization with PvRMC-CSP is capable of inducing CD8$^+$ T cells in all mouse strains with preferential production of IFN-γ. Similar to the differences in cytokine production reported for CD4$^+$ T cells, cytokine production by CD8$^+$ T cells seems to be strain dependent. This is demonstrated in BALB/c, DBA, and C57BL/6 mice, which elicited the highest number of IFN-γ-producing CD8$^+$ T cells, and A/J mice producing the lowest number (Fig. 5A). IL-2 production was significantly higher in BALB/c mice ($P < 0.001$) and C57BL/6 mice ($P < 0.001$) than in the other strains. Additionally, A/J mice showed the lowest level of TNF-α production among the strains tested ($P < 0.05$) with the exception of C3H mice, since the difference between these two strains did not reach statistical significance. Despite the differences observed during comparisons of single cytokines, all of the strains tested produced multifunctional CD8$^+$ T cells after stimulation with PvRMC-CSP (Fig. 5C). Furthermore, as with CD4$^+$ T cells, no association between the cytokine production or the proportion of multifunctional cells and the recognition of a specific region of PvRMC-CSP was observed (Fig. 5B and D).

Recognition of PvRMC-CSP by naturally acquired antibodies from individuals exposed to malaria. To confirm that the B cell epitopes are preserved in the recombinant chimeric protein and to evaluate the antigenicity of PvRMC-CSP, plasma samples collected from individuals naturally exposed to malaria were used to evaluate the recognition of PvRMC-CSP by ELISAs. The cohort of individuals who participated in this study has been studied previously (30). These individuals were living in two areas where *P. vivax* accounts for more than 70% of the clinical cases of malaria. Samples were collected from rainforest natives, who have resided in a region where malaria is endemic (Ribeirinha) for more than 25 years, and from transmigrants originating from several areas of Brazil where malaria is not endemic who have resided in a region for 10 years or more (Colina). Recognition of PvRMC-CSP was demonstrated as 65.3% (164/251) of all the samples exhibited IgG antibodies against PvRMC-CSP, with a mean reactiv-

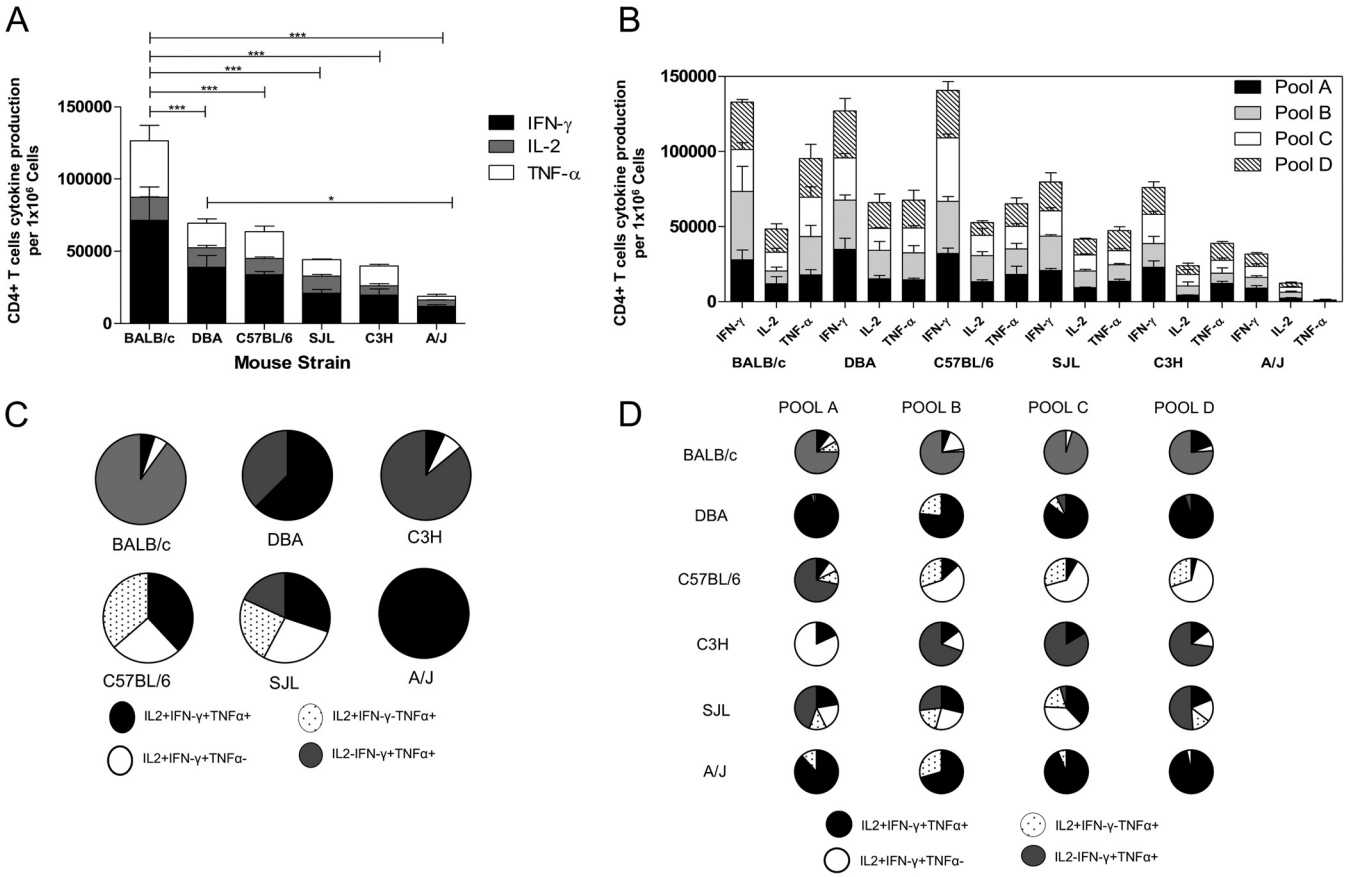

FIG 4 $CD4^+$ T cell responses following immunization with the PvRMC-CSP protein. Antigen-specific cytokine production was assessed after the final immunization. The magnitude and quality of PvRMC-CSP-specific $CD4^+$ T cells were determined using flow cytometry multiparametric analysis. Numbers of $CD4^+$ T cells producing IL-2, IFN-γ, or TNF-α upon stimulation with the protein (A) or pools of synthetic peptides (B) are presented after background subtraction. Bars represent mean responses ± SD for three mice per group. Statistical analyses are presented for IFN-γ: *, $P < 0.05$; ***, $P < 0.001$ by one-way ANOVA with Bonferroni's multiple-comparison posttest. The analyses for IL-2 and TNF-α are presented in the text. To determine the proportions of multifunctional $CD4^+$ T cells, Boolean gate analysis was used to identify and quantify the fraction of the total response of cells that produced two or three cytokines in response to PvRMC-CSP (C) or the peptide pools (D). The pie charts summarize the fractions of double or triple cytokine producers as indicated.

ity index of 1.61. The results for the different populations are presented in Table 2.

DISCUSSION

Clinical and epidemiological data have shown that effective malaria vaccines must be able to induce balanced humoral and cellular responses. We previously designed and expressed chimeric recombinant proteins based on well-characterized vaccine candidates that contain several cognate T cell epitopes arrayed in tandem conformation and genetically linked to immunodominant B cell epitopes. Proof of concept studies conducted in mice using *P. yoelii* sequences have shown enhanced immunogenicity and efficacy of this type of construct (23, 29). Here we report the design, expression, and characterization of a *P. vivax* CSP chimeric protein (PvRMC-CSP) expressed in *E. coli* that incorporates similar features. Previously, the immune response in mice to a recombinant *P. vivax* CSP has been reported to be genetically restricted (16). We initially tested whether the topology changes introduced in the PvCSP would overcome the reported genetic restriction in mice. We found that PvRMC-CSP was able to induce robust antibody responses in six different inbred mouse strains. Antibodies elicited by immunization with PvRMC-CSP recognized not only the chimeric protein but also synthetic peptides representing the allelic variants of the *P. vivax* CSP repeat domain. This is in sharp contrast with a previous publication that showed that only mice of the $H\text{-}2^k$ and $H\text{-}2^a$ haplotypes produced antibodies able to recognize the *P. vivax* CSP (16). VMP001, a vaccine candidate based on *P. vivax* CSP, was able to induce responses in BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice. Interestingly, in contrast to our findings with PvRMC-CSP, BALB/c mice were low responders to VMP001, with antibody titers 1 log unit lower than the $H\text{-}2^k$ strain tested (B10.BR) showing that it only partially overcame the genetic restriction (32). Evidence of genetic restriction has also been reported in humans. In a Brazilian area where *P. vivax* malaria is endemic, the HLA-DR7 allele is associated with a lack of response against the VK210 allele (17). An effective *P. vivax* vaccine based on CSP requires the induction of universal immune responses. Here we presented evidence that the chimeric PvRMC-CSP protein tailored to improve the immunogenicity of the native PvCSP widens the breadth of the immune responses.

Antibody titers against the VK247 variant sequence induced by immunization with PvRMC-CSP were low in five of the six strains of mice tested here. However, the antibody titers were as high as 1:81,920 in A/J mice. Our results are similar to the findings re-

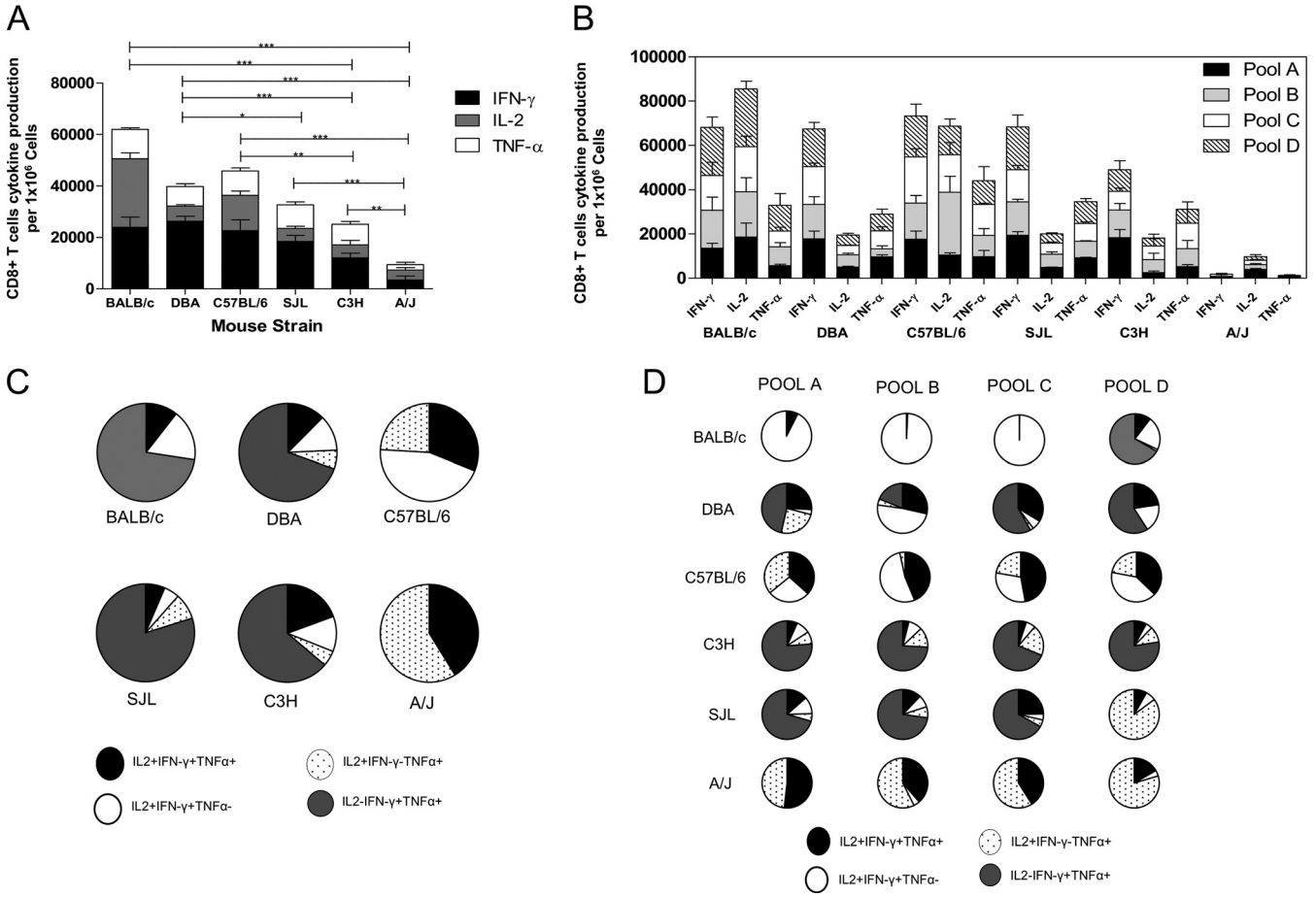

FIG 5 CD8$^+$ T cell responses following immunization with the PvRMC-CSP protein. Antigen-specific cytokine production and the magnitudes and quality of PvRMC-CSP specific cells were determined as described. (A and B) Numbers of CD8$^+$ T cells producing IL-2, IFN-γ, or TNF-α upon stimulation with PvRMC-CSP (A) or pools of synthetic peptides comprising its sequence after background subtraction (B). Bars represent mean responses ± SD for three mice per group. Statistical analyses are presented for IFN-γ: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ by one-way ANOVA with Bonferroni's multiple comparison posttest. Statistical analyses for IL-2 and TNF-α are presented in the text. (C and D) To determine the proportions of multifunctional CD8$^+$ T cells, Boolean gate analysis was used to identify and quantify the fraction of the total response of cells that produced two or three cytokines in response to PvRMC-CSP (C) or the peptide pools (D). The pie charts summarize the proportions of double or triple cytokine producers as indicated.

ported for the vaccine candidates VMP001 and CVS-S,S in nonhuman primates that also showed the induction of low antibody responses to the VK247 peptide (46). It has been suggested that the VK247 allele could be less immunogenic than the VK210 allele, since antibody response against VK210 is higher than VK247 in a population living in an area with a high prevalence of the VK247 variant (47). Nonetheless, seroepidemiological studies conducted in Myanmar and Brazil have not shown a predominant antibody response toward a specific CSP allele (17, 48). The low antibody response to VK247 after immunization with PvRMC-CSP could also be explained by the low copy number of repeats included in PvRMC-CSP.

TABLE 2 Prevalence of antibodies against PvRMC-CSP in Brazilian donors by IgG ELISA

Region in Brazil	No. (%) of positive samples	No. (%) of negative samples	Total no. of samples
Colina	39 (45.3)	47 (54.7)	86
Ribeirinha	125 (75.8)	40 (24.2)	165
Total	164 (65.3)	87 (34.7)	251

In both rodent and human *Plasmodium* infections, antibodies directed against the conserved regions of CSP are also related to protection. Both immune individuals immunized with sporozoites and prophylactically treated with chloroquine and semi-immune patients from an area where malaria is endemic are able to recognize CSP, showing that anti-CSP immunity is necessary for protection (49). CSP binds to highly sulfated heparan sulfate proteoglycans (HSPG) on the surfaces of hepatocytes; this interaction is critical for cell invasion, arresting circulating sporozoites in the liver (45, 50, 51). Our heparan sulfate binding assay showed that the structural domains involved in such interactions are conserved in PvRMC-CSP.

One of the conserved structural domains present in PvRMC-CSP is region I, a cell adhesive motif exposed by the proteolytic cleavage of CSP after the sporozoite-hepatocyte interaction (52, 53). It has been demonstrated that antibodies directed against this region are able to inhibit the invasion of HepG2 cells by different *Plasmodium* species (54). We showed that immunization with

PvRMC-CSP induced antibodies against region I in all mouse strains tested. However, antibody titers against this epitope were 1 log unit lower than titers against the repeat regions. Similar findings have been reported in comparative seroepidemiological studies with the N-terminal and C-terminal regions (32, 55). Interestingly, not all the synthetic peptides containing region I that we tested were recognized by antibodies elicited by PvRMC-CSP immunization. Our results suggest that amino acids located upstream of the conserved KLKQP sequence are required for effective recognition. The data are consistent with the fine mapping of B cell epitopes reported by Cespedes et al. that showed that additional N-terminal amino acids are essential for the recognition of the KLKQP fragment by sera from humans in areas where malaria is endemic (56) and for their capacity to inhibit sporozoite liver invasion (57).

A possible caveat when using chimeric recombinant proteins is the potential expression of neoantigens when the structure of the natural protein is modified. Naturally acquired antibodies in a population with differences in exposure and immunity (31) were able to recognize PvRMC-CSP when tested by ELISAs, confirming that the antigenic domains of CSP are conserved in our construct. The recognition of *P. vivax* sporozoites by mice immunized with PvRMC-CSP further confirms that antibody responses induced by this vaccine candidate are directed toward the *P. vivax* CSP native sequences. Differences in the recognition of PvRMC-CSP by plasma samples from individuals with heterogeneous exposure to malaria support the use of this protein in seroepidemiological studies. The seroprevalence of anti-PvRMC-CSP antibodies in a cohort of individuals living in a western Amazon region of Brazil was related to endemicity and was not limited by the population genetic background, demonstrated by the higher anti-PvRMC-CSP responses in an area where malaria is prevalent (75.8%) compared to the low seroprevalence (45.3%) in an area with a lower incidence of malaria. Previous seroepidemiological studies obtained in a Brazilian population with a similar epidemiological setting found that the CSP seroprevalence ranged from 24 to 34.2% when using synthetic peptides and that these responses depended on the population HLA alleles (17). Our results are comparable to those reported by Cespedes et al. using two vaccine candidates, PvCS-NRC (56) and PvNR1R2 (57), which showed a seroprevalence ranging between 69 and 83% in Papua New Guinea, an area where malaria is endemic, and a seroprevalence between 24 and 58% in an area of Colombia with a lower incidence of malaria.

Clinical trials with the *P. falciparum* CSP-based fusion protein RTS,S have shown that protection against an infectious challenge although associated with antibodies (58) is also correlated with IFN-γ production by $CD4^+$ and $CD8^+$ T cells (20), as *Plasmodium* liver stage development is hampered by IFN-γ (59, 60). Long-lasting protection has also been associated with IFN-γ production (20, 61). Subjects that exhibited a high level of antibody titers and a high $CD4^+$ T cell response were more likely to be protected than those subjects with high antibody titers but poor $CD4^+$ T cell response (62). ELISPOT assays revealed that PvRMC-CSP induced high frequencies of IFN-γ-secreting cells (more than 300 IFN-γ-secreting SFC/10^6 splenocytes) in all strains of mice tested. These responses are higher than those reported for the rPvCSP-ME vaccine candidate that induced more than 50 IFN-γ-secreting SFC/10^6 splenocytes in a single strain of mice (BALB/c) (63). The high IFN-γ/IL-4 ratios obtained after *ex vivo* stimulation with PvRMC-CSP allow us to infer that PvRMC-CSP elicits a Th1-biased immune response. Additionally, the ability of PvRMC-CSP to produce multifunctional $CD4^+$ T cells is also encouraging, since the high frequency of multifunctional $CD4^+$ T cells is strongly related to protection in humans immunized with RTS,S (20, 21) and has also been observed to be associated with immunity and protection against *Leishmania major* (22).

The IFN-γ production induced by PvRMC-CSP is also mediated by $CD8^+$ T cells. More strikingly, the proportions of triple- and double-cytokine-producing cells were high for all strains of mice tested. The breadth of the cellular immune response induced by the chimeric protein resembles that reported for irradiated sporozoites, considered the standard for exoerythrocytic vaccine development. The high frequency of multifunctional $CD8^+$ T cells induced after immunization with PvRMC-CSP, ranging between 6 and 41%, is similar to that associated with long-lasting protection in a *Plasmodium berghei* model (64). This result is in sharp contrast with data reported for mice in response to immunization with VMP001 (65), where the protein was unable to induce $CD8^+$ T cells even when the vaccine candidate was formulated in an oil-in-water emulsion supplemented with Toll-like receptor 4 (TLR4) and TLR7/8 agonists (65). When the same formulation was tested in nonhuman primates, IFN-γ-secreting cells were not produced, an effect that was attributed to differences in TLR4 signaling in the species (66). Formulation of PvRMC-CSP in a water-in-oil emulsion as reported here induced very robust cellular responses; it is possible to speculate that such responses could be further enhanced by the use of more-complex adjuvant systems.

The $CD8^+$ induction could be explained by the presence of undetermined cytotoxic T cell epitopes in PvRMC-CSP that can be cross-presented. We have previously shown that positively charged polymeric synthetic peptide constructs (PvRMC-CSP exhibits an isoelectric point of 9.39) are internalized by antigen-presenting cells via endocytosis (67). In this work, peptide uptake was enhanced by the interaction of positively charged residues with negatively charged membranes similar to the mechanism used by a family of compounds called cell-penetrating peptides. The endocytosis of these peptides upregulated cytokine production and expression of cell surface maturation markers by DCs and ultimately was related to protection in mice against a *P. yoelii* challenge (67).

The cellular responses in all mouse strains tested showed different patterns of recognition of the PvRMC-CSP peptide pools, with a profile depending on the strain, rather than the pool. However, no preferential recognition of a protein segment was detected. This suggests a broad response recognizing different epitopes within PvRMC-CSP, rather than the recognition of a single immunodominant epitope. The central repeat domain of *P. vivax* CSP is under immune selection inducing its expansion, contraction, and rearrangement (68), and this variability could lead to immune escape. Therefore, an *P. vivax* vaccine that increases the depth and breadth of the immune response as we have shown here with PvRMC-CSP is highly desirable.

The inclusion of promiscuous T helper epitopes in subunit vaccines is aimed at broadening the frequency of responders in a randomly chosen population (69). Different strategies have been used to associate T cell epitopes to poorly immunogenic *Plasmodium* antigens. The use of parasite T cell epitopes in malaria vaccine constructs has an advantage over the use of exogenous T cell

epitopes, as clonal expansion of T cells elicited by immunization provides not only cognate help to B cells but potential antiparasite immunity. PvRMC-CSP includes cognate T cell epitopes arrayed in tandem at the N-terminal region of the chimeric protein. The unresponsiveness of previously reported constructs based on *P. vivax* CSP is therefore not related to the absence of T helper epitopes in the native sequence. Previous evidence suggests that the tandem array of promiscuous T cell epitopes could impact antigen processing, modifying immunodominance (70, 71). In fact, it has been suggested that the position of T helper epitopes within a chimeric protein impacts peptide cleavage, association with MHC, or T cell receptor (TCR) recognition (72). Therefore, we cannot rule out the possibility that neoepitopes could have been generated by the introduction of linkers in PvRMC-CSP. The neoepitopes could have contributed to the overall T cell response, including help for antibody production. Although we did not evaluate responses to these joining sequences, we found that the IEDB server (41) predicts $CD4^+$ and $CD8^+$ T cell neoepitopes in H-2^d (BALB/c) and H-2^b (C57BL/10) mice, respectively. Interestingly, the GPGPG spacer sequence provides the preferred G and P amino acids in positions 2 and 3 for binding to MHC class I D^d molecules (73) and positions 3 and 4 for binding to MHC class II IA^b molecules (74). Further studies are required to determine the mechanism involved in the immune enhancement and the relative contribution of these neoepitopes to the response elicited by PvRMC-CSP.

In summary, the chimeric recombinant protein PvRMC-CSP, designed to optimize the immune response by genetic linkage of cognate T cell epitopes to conformational B cell epitopes based on the *P. vivax* CSP, was able to induce broad cellular and antibody responses in six different inbred strains of mice regardless of their haplotype. The antibodies induced were able to recognize the recombinant PvRMC-CSP, synthetic peptides representing the PvCSP repeats, the conserved region I, and native CSP on sporozoites. PvRMC-CSP was also broadly recognized by antibodies obtained from individuals naturally exposed to *P. vivax* transmission. More importantly, both $CD4^+$ and $CD8^+$ IFN-γ-secreting T cells and at least one other cytokine (IL-2 or TNF-α) were induced in all the strains of mice tested. To our knowledge, this is the first evidence that a multifunctional $CD8^+$ T cell response is elicited by immunization with a protein vaccine based on *P. vivax* CSP. This is also the first vaccine candidate able to induce a balanced cellular and humoral response necessary for protection against malaria, which highlights that our results warrant further preclinical studies in nonhuman primates to evaluate its potential for clinical development.

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REFERENCES

1. **World Health Organization.** 2014. World malaria report 2014. World Health Organization, Geneva, Switzerland.
2. **Battle KE, Gething PW, Elyazar IR, Moyes CL, Sinka ME, Howes RE, Guerra CA, Price RN, Baird KJ, Hay SI.** 2012. The global public health significance of Plasmodium vivax. Adv Parasitol **80:**1–111. http://dx.doi.org/10.1016/B978-0-12-397900-1.00001-3.
3. **Baird JK.** 2013. Evidence and implications of mortality associated with acute Plasmodium vivax malaria. Clin Microbiol Rev **26:**36–57. http://dx.doi.org/10.1128/CMR.00074-12.
4. **Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, George DB, Horby P, Wertheim HF, Price RN, Mueller I, Baird JK, Hay SI.** 2012. A long neglected world malaria map: Plasmodium vivax endemicity in 2010. PLoS Negl Trop Dis **6:**e1814. http://dx.doi.org/10.1371/journal.pntd.0001814.
5. **Hulden L, Hulden L.** 2011. Activation of the hypnozoite: a part of Plasmodium vivax life cycle and survival. Malar J **10:**90. http://dx.doi.org/10.1186/1475-2875-10-90.
6. **Millet P, Collins WE, Broderson JR, Bathurst I, Nardin EH, Nussenzweig RS.** 1991. Inhibitory activity against Plasmodium vivax sporozoites induced by plasma from Saimiri monkeys immunized with circumsporozoite recombinant proteins or irradiated sporozoites. Am J Trop Med Hyg **45:**44–48.
7. **Aley SB, Barnwell JW, Bates MD, Collins WE, Hollingdale MR.** 1987. Plasmodium vivax: exoerythrocytic schizonts recognized by monoclonal antibodies against blood-stage schizonts. Exp Parasitol **64:**188–194. http://dx.doi.org/10.1016/0014-4894(87)90142-1.
8. **Hollingdale MR, Collins WE, Campbell CC, Schwartz AL.** 1985. In vitro culture of two populations (dividing and nondividing) of exoerythrocytic parasites of Plasmodium vivax. Am J Trop Med Hyg **34:**216–222.
9. **Herrera S, Corradin G, Arevalo-Herrera M.** 2007. An update on the search for a Plasmodium vivax vaccine. Trends Parasitol **23:**122–128. http://dx.doi.org/10.1016/j.pt.2007.01.008.
10. **Coppi A, Pinzon-Ortiz C, Hutter C, Sinnis P.** 2005. The Plasmodium circumsporozoite protein is proteolytically processed during cell invasion. J Exp Med **201:**27–33. http://dx.doi.org/10.1084/jem.20040989.
11. **Bilsborough J, Baumgart K, Bathurst I, Barr P, Good MF.** 1997. Fine epitope specificity of antibodies to region II of the Plasmodium vivax circumsporozoite protein correlates with ability to bind recombinant protein and sporozoites. Acta Trop **65:**59–80. http://dx.doi.org/10.1016/S0001-706X(97)00648-7.
12. **Gordon DM, Cosgriff TM, Schneider I, Wasserman GF, Majarian WR, Hollingdale MR, Chulay JD.** 1990. Safety and immunogenicity of a Plasmodium vivax sporozoite vaccine. Am J Trop Med Hyg **42:**527–531.
13. **Herrington DA, Nardin EH, Losonsky G, Bathurst IC, Barr PJ, Hollingdale MR, Edelman R, Levine MM.** 1991. Safety and immunogenicity of a recombinant sporozoite malaria vaccine against Plasmodium vivax. Am J Trop Med Hyg **45:**695–701.
14. **Herrera S, Bonelo A, Perlaza BL, Fernandez OL, Victoria L, Lenis AM, Soto L, Hurtado H, Acuna LM, Velez JD, Palacios R, Chen-Mok M, Corradin G, Arevalo-Herrera M.** 2005. Safety and elicitation of humoral and cellular responses in Colombian malaria-naive volunteers by a Plasmodium vivax circumsporozoite protein-derived synthetic vaccine. Am J Trop Med Hyg **73:**3–9.
15. **Herrera S, Fernandez OL, Vera O, Cardenas W, Ramirez O, Palacios R, Chen-Mok M, Corradin G, Arevalo-Herrera M.** 2011. Phase I safety and immunogenicity trial of Plasmodium vivax CS derived long synthetic peptides adjuvanted with montanide ISA 720 or montanide ISA 51. Am J Trop Med Hyg **84:**12–20. http://dx.doi.org/10.4269/ajtmh.2011.09-0516.
16. **Nardin EH, Barr PJ, Heimer E, Etlinger HM.** 1988. Genetic restriction of the murine humoral response to a recombinant Plasmodium vivax circumsporozoite protein. Eur J Immunol **18:**1119–1122. http://dx.doi.org/10.1002/eji.1830180722.
17. **Oliveira-Ferreira J, Pratt-Riccio LR, Arruda M, Santos F, Ribeiro CT, Goldberg AC, Banic DM.** 2004. HLA class II and antibody responses to circumsporozoite protein repeats of P. vivax (VK210, VK247 and P vivax-like) in individuals naturally exposed to malaria. Acta Trop **92:**63–69. http://dx.doi.org/10.1016/j.actatropica.2004.02.011.
18. **Doolan DL, Hoffman SL.** 2000. The complexity of protective immunity against liver-stage malaria. J Immunol **165:**1453–1462. http://dx.doi.org/10.4049/jimmunol.165.3.1453.
19. **Ewer KJ, O'Hara GA, Duncan CJ, Collins KA, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD, Longley RJ, Rowland R, Poulton ID, Draper SJ, Blagborough AM, Berrie E, Moyle S, Williams N, Siani L, Folgori A, Colloca S, Sinden RE, Lawrie**

AM, Cortese R, Gilbert SC, Nicosia A, Hill AV. 2013. Protective $CD8^+$ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. Nat Commun **4:**2836. http://dx.doi.org/10.1038/ncomms3836.

20. **Sun P, Schwenk R, White K, Stoute JA, Cohen J, Ballou WR, Voss G, Kester KE, Heppner DG, Krzych U.** 2003. Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific $CD4^+$ and $CD8^+$ T cells producing IFN-gamma. J Immunol **171:**6961–6967. http://dx.doi.org/10.4049/jimmunol.171.12.6961.
21. **Lumsden JM, Schwenk RJ, Rein LE, Moris P, Janssens M, Ofori-Anyinam O, Cohen J, Kester KE, Heppner DG, Krzych U.** 2011. Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells. PLoS One **6:**e20775. http://dx.doi.org/10.1371/journal.pone.0020775.
22. **Seder RA, Darrah PA, Roederer M.** 2008. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol **8:**247–258. http://dx.doi.org/10.1038/nri2274.
23. **Singh B, Cabrera-Mora M, Jiang J, Galinski M, Moreno A.** 2010. Genetic linkage of autologous T cell epitopes in a chimeric recombinant construct improves anti-parasite and anti-disease protective effect of a malaria vaccine candidate. Vaccine **28:**2580–2592. http://dx.doi.org/10.1016/j.vaccine.2010.01.019.
24. **Silva-Flannery LM, Cabrera-Mora M, Jiang JL, Moreno A.** 2009. Recombinant peptide replicates immunogenicity of synthetic linear peptide chimera for use as pre-erythrocytic stage malaria vaccine. Microbes Infect **11:**83–91. http://dx.doi.org/10.1016/j.micinf.2008.10.009.
25. **Arevalo-Herrera M, Herrera S.** 2001. Plasmodium vivax malaria vaccine development. Mol Immunol **38:**443–455. http://dx.doi.org/10.1016/S0161-5890(01)00080-3.
26. **Arnot DE, Barnwell JW, Stewart MJ.** 1988. Does biased gene conversion influence polymorphism in the circumsporozoite protein-encoding gene of Plasmodium vivax? Proc Natl Acad Sci U S A **85:**8102–8106. http://dx.doi.org/10.1073/pnas.85.21.8102.
27. **Rosenberg R, Wirtz RA, Lanar DE, Sattabongkot J, Hall T, Waters AP, Prasittisuk C.** 1989. Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax*. Science **245:**973–976. http://dx.doi.org/10.1126/science.2672336.
28. **Livingston BD, Newman M, Crimi C, McKinney D, Chesnut R, Sette A.** 2001. Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. Vaccine **19:**4652–4660. http://dx.doi.org/10.1016/S0264-410X(01)00233-X.
29. **Singh B, Cabrera-Mora M, Jiang J, Moreno A.** 2012. A hybrid multistage protein vaccine induces protective immunity against murine malaria. Infect Immun **80:**1491–1501. http://dx.doi.org/10.1128/IAI.05980-11.
30. **Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-Silva GM, Porto LC, Meyer EV, Moreno A, Rodrigues MM, Barnwell JW, Galinski MR, de Oliveira-Ferreira J.** 2012. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody response to the P. vivax MSP-1, MSP-3alpha and MSP-9 in individuals from Brazilian endemic area. PLoS One **7:**e36419. http://dx.doi.org/10.1371/journal.pone.0036419.
31. **Ferreira AR, Singh B, Cabrera-Mora M, Magri De Souza AC, Queiroz Marques MT, Porto LC, Santos F, Banic DM, Calvo-Calle JM, Oliveira-Ferreira J, Moreno A, Lima-Junior JDC.** 2014. Evaluation of naturally acquired IgG antibodies to a chimeric and non-chimeric recombinant species of Plasmodium vivax reticulocyte binding protein-1: lack of association with HLA-DRB1*/DQB1* in malaria exposed individuals from the Brazilian Amazon. PLoS One **9:**e105828. http://dx.doi.org/10.1371/journal.pone.0105828.
32. **Yadava A, Sattabongkot J, Washington MA, Ware LA, Majam V, Zheng H, Kumar S, Ockenhouse CF.** 2007. A novel chimeric Plasmodium vivax circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. Infect Immun **75:**1177–1185. http://dx.doi.org/10.1128/IAI.01667-06.
33. **Nardin E, Zavala F, Nussenzweig V, Nussenzweig RS.** 1999. Pre-erythrocytic malaria vaccine: mechanisms of protective immunity and human vaccine trials. Parassitologia **41:**397–402.
34. **Kumar KA, Sano G-I, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, Nussenzweig V.** 2006. The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. Nature **444:**937–940. http://dx.doi.org/10.1038/nature05361.
35. **Singh H, Raghava GP.** 2001. ProPred: prediction of HLA-DR binding sites. Bioinformatics **17:**1236–1237. http://dx.doi.org/10.1093/bioinformatics/17.12.1236.
36. **Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, Lundegaard C, Sette A, Lund O, Bourne PE, Nielsen M, Peters B.** 2012. Immune epitope database analysis resource. Nucleic Acids Res **40:**W525–W530. http://dx.doi.org/10.1093/nar/gks438.
37. **Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O.** 2003. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci **12:**1007–1017. http://dx.doi.org/10.1110/ps.0239403.
38. **Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M.** 2008. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. Nucleic Acids Res **36:**W509–W512. http://dx.doi.org/10.1093/nar/gkn202.
39. **Peters B, Sette A.** 2005. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. BMC Bioinformatics **6:**132. http://dx.doi.org/10.1186/1471-2105-6-132.
40. **Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, Peters B.** 2008. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. Immunome Res **4:**2. http://dx.doi.org/10.1186/1745-7580-4-2.
41. **Wang P, Sidney J, Dow C, Mothe B, Sette A, Peters B.** 2008. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol **4:**e1000048. http://dx.doi.org/10.1371/journal.pcbi.1000048.
42. **Wang P, Sidney J, Kim Y, Sette A, Lund O, Nielsen M, Peters B.** 2010. Peptide binding predictions for HLA DR, DP and DQ molecules. BMC Bioinformatics **11:**568. http://dx.doi.org/10.1186/1471-2105-11-568.
43. **Hoof I, Peters B, Sidney J, Pedersen L, Sette A, Lund O, Buus S, Nielsen M.** 2009. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics **61:**1–13. http://dx.doi.org/10.1007/s00251-008-0341-z.
44. **Nielsen M, Lundegaard C, Blicher T, Lamberth K, Harndahl M, Justesen S, Røder G, Peters B, Sette A, Lund O, Buus S.** 2007. *NetMHCpan*, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS One **2:**e796. http://dx.doi.org/10.1371/journal.pone.0000796.
45. **Pinzon-Ortiz C, Friedman J, Esko J, Sinnis P.** 2001. The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for plasmodium sporozoite attachment to target cells. J Biol Chem **276:**26784–26791. http://dx.doi.org/10.1074/jbc.M104038200.
46. **Vanloubbeeck Y, Pichyangkul S, Bayat B, Yongvanitchit K, Bennett JW, Sattabongkot J, Schaecher K, Ockenhouse CF, Cohen J, Yadava A, P. vivax Vaccine Study Group.** 2013. Comparison of the immune responses induced by soluble and particulate Plasmodium vivax circumsporozoite vaccine candidates formulated in AS01 in rhesus macaques. Vaccine **31:**6216–6224. http://dx.doi.org/10.1016/j.vaccine.2013.10.041.
47. **Gonzalez JM, Hurtado S, Arevalo-Herrera M, Herrera S.** 2001. Variants of the Plasmodium vivax circumsporozoite protein (VK210 and VK247) in Colombian isolates. Mem Inst Oswaldo Cruz **96:**709–712. http://dx.doi.org/10.1590/S0074-02762001000500023.
48. **Kim TS, Kim HH, Lee SS, Na BK, Lin K, Cho SH, Kang YJ, Kim DK, Sohn Y, Kim H, Lee HW.** 2010. Prevalence of Plasmodium vivax VK210 and VK247 subtype in Myanmar. Malar J **9:**195. http://dx.doi.org/10.1186/1475-2875-9-195.
49. **Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J, Nakajima-Sasaki R, Molina D, Teelen K, Hermsen CC, Sauerwein R.** 2013. Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. Sci Rep **3:**3549. http://dx.doi.org/10.1038/srep03549.
50. **Sinnis P, Coppi A.** 2007. A long and winding road: the Plasmodium sporozoite's journey in the mammalian host. Parasitol Int **56:**171–178. http://dx.doi.org/10.1016/j.parint.2007.04.002.
51. **Pradel G, Garapaty S, Frevert U.** 2002. Proteoglycans mediate malaria sporozoite targeting to the liver. Mol Microbiol **45:**637–651. http://dx.doi.org/10.1046/j.1365-2958.2002.03057.x.
52. **Ejigiri I, Sinnis P.** 2009. Plasmodium sporozoite-host interactions from the dermis to the hepatocyte. Curr Opin Microbiol **12:**401–407. http://dx.doi.org/10.1016/j.mib.2009.06.006.
53. **Coppi A, Natarajan R, Pradel G, Bennett BL, James ER, Roggero MA, Corradin G, Persson C, Tewari R, Sinnis P.** 2011. The malaria circumsporozoite protein has two functional domains, each with distinct roles as

sporozoites journey from mosquito to mammalian host. J Exp Med **208:** 341–356. http://dx.doi.org/10.1084/jem.20101488.

54. **Ying P, Shakibaei M, Patankar MS, Clavijo P, Beavis RC, Clark GF, Frevert U.** 1997. The malaria circumsporozoite protein: interaction of the conserved regions I and II-plus with heparin-like oligosaccharides in heparan sulfate. Exp Parasitol **85:**168–182. http://dx.doi.org/10.1006/expr.1996.4134.
55. **Arevalo-Herrera M, Roggero MA, Gonzalez JM, Vergara J, Corradin G, Lopez JA, Herrera S.** 1998. Mapping and comparison of the B-cell epitopes recognized on the Plasmodium vivax circumsporozoite protein by immune Colombians and immunized Aotus monkeys. Ann Trop Med Parasitol **92:**539–551. http://dx.doi.org/10.1080/00034989859230.
56. **Cespedes N, Arevalo-Herrera M, Felger I, Reed S, Kajava AV, Corradin G, Herrera S.** 2013. Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the P. vivax circumsporozoite protein. Vaccine **31:**4923–4930. http://dx.doi.org/10.1016/j.vaccine.2013.05.082.
57. **Cespedes N, Jimenez E, Lopez-Perez M, Rubiano K, Felger I, Alonso P, Arevalo-Herrera M, Corradin G, Herrera S.** 2014. Antigenicity and immunogenicity of a novel Plasmodium vivax circumsporozoite derived synthetic vaccine construct. Vaccine **32:**3179–3186. http://dx.doi.org/10.1016/j.vaccine.2014.04.007.
58. **Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, Lang T, Gould J, Dubois MC, Jongert E, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S, Mturi N, Drakeley CJ, Savarese B, Villafana T, Lapierre D, Ballou WR, Cohen J, Lemnge MM, Peshu N, Marsh K, Riley EM, von Seidlein L, Bejon P.** 2011. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. Lancet Infect Dis **11:**102–109. http://dx.doi.org/10.1016/S1473-3099(10)70262-0.
59. **Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V.** 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature **330:**664–666. http://dx.doi.org/10.1038/330664a0.
60. **Mellouk S, Maheshwari RK, Rhodes-Feuillette A, Beaudoin RL, Berbiguier N, Matile H, Miltgen F, Landau I, Pied S, Chigot JP, Friedman RM, Mazier D.** 1987. Inhibitory activity of interferons and interleukin 1 on the development of Plasmodium falciparum in human hepatocyte cultures. J Immunol **139:**4192–4195.
61. **Olotu A, Moris P, Mwacharo J, Vekemans J, Kimani D, Janssens M, Kai O, Jongert E, Lievens M, Leach A, Villafana T, Savarese B, Marsh K, Cohen J, Bejon P.** 2011. Circumsporozoite-specific T cell responses in children vaccinated with RTS,S/AS01E and protection against P. falciparum clinical malaria. PLoS One **6:**e25786. http://dx.doi.org/10.1371/journal.pone.0025786.
62. **Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, Schwenk R, Nielsen RA, Debebe Z, Pinelis E, Juompan L, Williams J, Dowler M, Stewart VA, Wirtz RA, Dubois MC, Lievens M, Cohen J, Ballou WR, Heppner DG, Jr, RTS,S Vaccine Evaluation Group.** 2009. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis **200:**337–346. http://dx.doi.org/10.1086/600120.
63. **Almeida AP, Dias MO, Vieira CDA, Chavez-Olortegui C, Gazzineli RT, Rodrigues MM, Fujiwara RT, Bruna-Romero O.** 2014. Long-lasting humoral and cellular immune responses elicited by immunization with recombinant chimeras of the Plasmodium vivax circumsporozoite protein. Vaccine **32:**2181–2187. http://dx.doi.org/10.1016/j.vaccine.2014.02.053.
64. **Reyes-Sandoval A, Berthoud T, Alder N, Siani L, Gilbert SC, Nicosia A, Colloca S, Cortese R, Hill AV.** 2010. Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria $CD8^+$ T-cell responses. Infect Immun **78:**145–153. http://dx.doi.org/10.1128/IAI.00740-09.
65. **Lumsden JM, Nurmukhambetova S, Klein JH, Sattabongkot J, Bennett JW, Bertholet S, Fox CB, Reed SG, Ockenhouse CF, Howard RF, Polhemus ME, Yadava A.** 2012. Evaluation of immune responses to a Plasmodium vivax CSP-based recombinant protein vaccine candidate in combination with second-generation adjuvants in mice. Vaccine **30:**3311–3319. http://dx.doi.org/10.1016/j.vaccine.2012.03.004.
66. **Lumsden JM, Pichyangkul S, Srichairatanakul U, Yongvanitchit K, Limsalakpetch A, Nurmukhambetova S, Klein J, Bertholet S, Vedvick TS, Reed SG, Sattabongkot J, Bennett JW, Polhemus ME, Ockenhouse CF, Howard RF, Yadava A.** 2011. Evaluation of the safety and immunogenicity in rhesus monkeys of a recombinant malaria vaccine for *Plasmodium vivax* with a synthetic Toll-like receptor 4 agonist formulated in an emulsion. Infect Immun **79:**3492–3500. http://dx.doi.org/10.1128/IAI.05257-11.
67. **Silva-Flannery LM, Cabrera-Mora M, Dickherber M, Moreno A.** 2009. Polymeric linear peptide chimeric vaccine-induced antimalaria immunity is associated with enhanced in vitro antigen loading. Infect Immun **77:**1798–1806. http://dx.doi.org/10.1128/IAI.00470-08.
68. **Parobek CM, Bailey JA, Hathaway NJ, Socheat D, Rogers WO, Juliano JJ.** 2014. Differing patterns of selection and geospatial genetic diversity within two leading Plasmodium vivax candidate vaccine antigens. PLoS Negl Trop Dis **8:**e2796. http://dx.doi.org/10.1371/journal.pntd.0002796.
69. **Calvo-Calle JM, Hammer J, Sinigaglia F, Clavijo P, Moya-Castro ZR, Nardin EH.** 1997. Binding of malaria T cell epitopes to DR and DQ molecules in vitro correlates with immunogenicity in vivo: identification of a universal T cell epitope in the *Plasmodium falciparum* circumsporozoite protein. J Immunol **159:**1362–1373.
70. **Ghosh M, Solanki AK, Roy K, Dhoke RR, Ashish, Roy S.** 2013. Carrier protein influences immunodominance of a known epitope: implication in peptide vaccine design. Vaccine **31:**4682–4688. http://dx.doi.org/10.1016/j.vaccine.2013.06.110.
71. **Kjerrulf M, Löwenadler B, Svanholm C, Lycke N.** 1997. Tandem repeats of T helper epitopes enhance immunogenicity of fusion proteins by promoting processing and presentation. Mol Immunol **34:**599–608. http://dx.doi.org/10.1016/S0161-5890(97)00078-3.
72. **De Berardinis P, Guardiola J, Manca F.** 1997. Epitope context and reshaping of activated T helper cell repertoire. Hum Immunol **54:**189–193. http://dx.doi.org/10.1016/S0198-8859(97)00075-X.
73. **Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, Peters B.** 2008. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. Immunome Res **4:**2. http://dx.doi.org/10.1186/1745-7580-4-2.
74. **Liu X, Dai S, Crawford F, Frugé R, Marrack P, Kappler J.** 2002. Alternate interactions define the binding of peptides to the MHC molecule IAb. Proc Natl Acad Sci U S A **99:**8820–8825. http://dx.doi.org/10.1073/pnas.132272099.