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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 infected livers without causing overt symptoms. 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...
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 (PI-ME-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 seem 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 Sall regions predicted to contain promiscuous T cell epitopes (GenBank accession no. AAA29535.1, F265-L310 and A325-N343); (iii) the segment containing amino acids G35-G62 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 Arnott et al. (26) interspaced between two copies of the nonapeptide GD RADGQPA, followed by three copies of the nonapeptide ANGAGNQPG representing the type 2 repeat sequence (VK247) (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 Xhol and cloned in pET24d (+), which expresses the protein with a C-terminal His6 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 (FFLC) (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 for 4 to 20% polyacrylamide gels (Lonza, Allendale, NJ). For Western blots, following electrophoresis, the proteins were blotted onto nitrocellulose 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 [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 rabbit 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 E265-L310; PvRMC-CSP pool B included 14 peptides representing a region downstream of E265-L310 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 synthesized by RS Synthesis (Louisville, KY): VK210AD (GDRADGQPA)3, VK210AA (GDRAGQQP)3, North Korean (GDRAGQQA)3, and VK247 (ANGAGNQPG)3 (Table 1).

Mice. Female A/J (H-2b), DBA/1 (H-2b), and SJL/J (H-2s) mice, 6 to 8 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Female BALB/c (H-2d), C3H (H-2a), and C57BL/6 (H-2b) 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 specificity of the antibodies elicited by immunization with PvRMC-CSP in mice was determined by ELISA using Immunol 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-
ping peptides representing the N-terminal region G37-G62, 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 end-point dilution.

(ii) Human ELISAs. To evaluate reactivity of naturally acquired antibodies against PvRM-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 rain-forest 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 and washed as described above. Finally, o-phenylenediamine and hydrogen peroxide were used to reveal bound antibodies. The absorbance was read

FIG 1 (A) Schematic representation of the recombinant protein reported here. PvRM-CSP includes the regions of the native CSP that contain predicted promiscuous T cell epitopes E265-L310 and A325-N343 interspaced with a GPGPG spacer and genetically fused to sequences that are the target of protective antibodies. These sequences include the following: (i) G37-G62 from the N-terminal PvCSP including the native region I; (ii) two copies of the octapeptide GDRAGQPA, flanked by one copy of the nonapeptide GDRAGQPA followed by three copies of the DRAGQPA 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 (AAA29353.1) (26). GPGPG spacers were also inserted between the three B cell domains (striped boxes). (B) Sequence of the PvRM-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 PvRM-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. PvRM-CSP, but not PvMSP1, binds to heparan sulfate in a dose-dependent manner.
TABLE 1 Peptides used for immunological assays

<table>
<thead>
<tr>
<th>Assay and peptide used</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ELISA</td>
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<tr>
<td>VK210AD</td>
<td>(GDRADGQPA)</td>
</tr>
<tr>
<td>VK210AA</td>
<td>(GDRAAQGPA)</td>
</tr>
<tr>
<td>VK247</td>
<td>(ANGAGNQPQ)</td>
</tr>
<tr>
<td>North Korean</td>
<td>(GDRAGQPA)</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td></td>
</tr>
<tr>
<td>Pool A overlapping E265-L310</td>
<td>MAVDEYLDKVRATVG</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool B overlapping A325-N343 and G37-G62</td>
<td>GPAG1F1N7V5NSSLG</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool C VK210 repeats</td>
<td>GPAGPGDPADROAQPA</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool D VK247 repeats</td>
<td>PGDPRAAQPGAPDGDA</td>
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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′-azino-bis(3-ethylbenzthiazolinesulfonic 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; TreeStar, 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+ cells were analyzed.
CD8<sup>+</sup> 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-producing cells was determined by subtracting the percentage of cytokine-producing T cells after incubation with medium alone from 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 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<sub>265</sub>-L<sub>310</sub> and A<sub>325</sub>–N<sub>343</sub>) 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 was 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 [<i>P</i> < 0.01]) and the SJL mice (ranging between 1:320 and 1:81,920 [<i>P</i> < 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 (<i>P</i> < 0.01) and DBA (<i>P</i> < 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 (<i>P</i> < 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<sub>37</sub>–G<sub>62</sub>. As predicted, most antibodies recognized the conserved region I (KLKQP).
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-
Based on these results, different levels of CD4+ considered high responders, as they produce significantly higher amounts on the mouse strain can be inferred. BALB/c mice could be 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 significantly lower levels of TNF-α than DBA and C57BL/6 strains (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 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, SJL, 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 significantly lower levels of TNF-α than DBA and C57BL/6 strains (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 the samples exhibited IgG antibodies against PVRMC-CSP, with a mean reactiv-
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 \textit{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 \textit{P. vivax} CSP chimeric protein (PvRMC-CSP) expressed in \textit{E. coli} that incorporates similar features. Previously, the immune response in mice to a recombinant \textit{P. vivax} CSP has been reported to be genetically restricted (16). We initially tested whether the topology changes introduced in the \textit{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 \textit{P. vivax} CSP repeat domain. This is in sharp contrast with a previous publication that showed that only mice of the H-2\textsuperscript{k} and H-2\textsuperscript{a} haplotypes produced antibodies able to recognize the \textit{P. vivax} CSP (16). VMP001, a vaccine candidate based on \textit{P. vivax} CSP, was able to induce responses in BALB/c (H-2\textsuperscript{d}) and C57BL/6 (H-2\textsuperscript{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-2\textsuperscript{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 \textit{P. vivax} malaria is endemic, the HLA-DR7 allele is associated with a lack of response against the VK210 allele (17). An effective \textit{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-
ported for the vaccine candidates VMP001 and CVS-S,S in non-human 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.

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

<table>
<thead>
<tr>
<th>Region in Brazil</th>
<th>No. (%) of positive samples</th>
<th>No. (%) of negative samples</th>
<th>Total no. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colina</td>
<td>39 (45.3)</td>
<td>47 (54.7)</td>
<td>86</td>
</tr>
<tr>
<td>Ribeirinha</td>
<td>125 (75.8)</td>
<td>40 (24.2)</td>
<td>165</td>
</tr>
<tr>
<td>Total</td>
<td>164 (65.3)</td>
<td>87 (34.7)</td>
<td>251</td>
</tr>
</tbody>
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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/10⁶ 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/10⁶ 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-2Kd (BALB/c) and H-2Dd (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 molecules (73) and positions 3 and 4 for binding to MHC class II Aα 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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