Immunogenicity of Recombinant Proteins Consisting of *Plasmodium vivax* Circumsporozoite Protein Allelic Variant-Derived Epitopes Fused with *Salmonella enterica* Serovar Typhimurium Flagellin

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A *Plasmodium falciparum* circumsporozoite protein (CSP)-based recombinant fusion vaccine is the first malaria vaccine to reach phase III clinical trials. Resistance to infection correlated with the production of antibodies to the immunodominant central repeat region of the CSP. In contrast to *P. falciparum*, vaccine development against the CSP of *Plasmodium vivax* malaria is far behind. Based on this gap in our knowledge, we generated a recombinant chimeric protein containing the immunodominant central repeat regions of the *P. vivax* CSP fused to *Salmonella enterica* serovar Typhimurium-derived flagellin (FliC) to activate the innate immune system. The recombinant proteins that were generated contained repeat regions derived from each of the 3 allelic variants of the *P. vivax* CSP or a fusion of regions derived from each of the 3 allelic forms. Mice were subcutaneously immunized with the fusion proteins alone or in combination with the Toll-like receptor 3 (TLR-3) agonist poly(I:C), and the anti-CSP serum IgG response was measured. Immunization with a mixture of the 3 recombinant proteins, each containing immunodominant epitopes derived from a single allelic variant, rather than a single recombinant protein carrying a fusion of regions derived from each of 3 allelic forms elicited a stronger immune response. This response was independent of TLR-4 but required TLR-5/MyD88 activation. Antibody titers significantly increased when poly(I:C) was used as an adjuvant with a mixture of the 3 recombinant proteins. These recombinant fusion proteins are novel candidates for the development of an effective malaria vaccine against *P. vivax*.

For the past 20 years, a *Plasmodium falciparum* circumsporozoite protein (CSP)-based recombinant malaria vaccine has been at the forefront of malaria vaccine research (reviewed in reference 1). This vaccine comprises a recombinant fusion protein of the C-terminal portion of the *P. falciparum* CSP (RT) and the hepatitis B surface antigen (S). The fusion protein (RTS) when expressed together with the hepatitis B antigen (S) in *Saccharomyces cerevisiae* naturally assembles into a virus-like particle termed RTS,S. This particle is part of the vaccine formulation RTS,S/AS01E that also includes monophosphoryl lipid A and QS21 in a liposomal suspension (1).

This vaccine formulation is the first of the malaria vaccines to reach phase III clinical trials. The vaccination efficiency, which was measured for 14 months after the administration of the first dose, was 50.4%, and it was determined by examining the retardation of the first clinical malaria episode in 5- to 17-month-old African children (2). A parallel study in 6- to 12-week-old infants vaccinated with RTS,S/AS01E revealed a vaccine efficacy of 30.1% (3). In spite of the initial success in the 5- to 17-month-old children, the efficacy of RTS,S/AS01E declined to 16.8% over a 4-year period (4).

An examination of the mechanism of vaccine action revealed that an increase in anti-CSP antibody titers correlated with protection against clinical malaria episodes (5). This field observation is in agreement with the results obtained from the phase IIb clinical trials in adults, which showed that anti-CSP antibodies played a prominent role in conferring resistance to malaria challenge (6). These putative protective antibodies mainly target the immunodominant repeat region of the *P. falciparum* CSP (6).

There still remains a tremendous need to develop new malaria vaccine candidates, especially those targeting *Plasmodium vivax*, the second-most-prevalent cause of malaria worldwide (7). On the basis of the results obtained with the *P. falciparum* CSP-derived vaccine, we generated recombinant polypeptides containing various immunodominant regions of the *P. vivax* CSP fused with a strong agonist of the innate immune system, the flagellin (FliC) protein of *Salmonella enterica* serovar Typhimurium. The enhanced immunogenicity of FliC-containing fusion proteins has been previously demonstrated in studies of *P. vivax* and *P. falciparum* merozoite-derived vaccines (8–10).

In contrast to the *P. falciparum* CSP, 3 different allelic forms of the *P. vivax* CSP have been identified to date. The 2 most common alleles are VK210 and VK247 (11, 12). A third allelic form (called *P. vivax*-like) may exist but is still a matter of debate. Several reports have described the existence of this allelic form in different parts of the world (13–16). However, one study that analyzed 126 samples from *P. vivax*-infected individuals from Central America, South America, Africa, southeast Asia, and India failed to detect...
this allelic form of the CSP (17). The variations between the allelic forms mainly occur in the central immunodominant repeat region. Distinct nonapeptides have been identified for each of the allelic forms, GDRA(D/A)GQA in the VK210 CSP, ANGA(G/D)(N/D)QPG in the VK247 CSP, and APGANQ(E/G)GAA in the P. vivax-like CSP. The existence of 3 allelic forms requires a vaccine to elicit antibodies against all 3 immunodominant epitopes of the P. vivax CSP in order to have universal coverage. To achieve this, 2 approaches were used in this study. The first approach involved mixing 3 recombinant fusion proteins, each of which expressed 1 of the 3 allelic forms. Alternatively, a single recombinant fusion protein that contained representative epitopes from the 3 allelic forms (All-CS-epitopes) was generated. The immunogenicity of the recombinant fusion proteins was assessed by immunizing mice with the recombinant proteins individually, the mixture of the 3 allelic forms, or the protein containing a fusion of all 3 immunodominant epitopes (All-CS-epitopes). The serum IgG immune response was compared following the immunization of mice with each of the different preparations, given alone or in combination with an adjuvant, the TLR-3 agonist poly(I-C).

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations provided by the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of São Paulo (no. CEP 042609).

Mice. Wild-type (WT) female 6- to 8-week-old C57BL/6 (H2b) mice were obtained from the animal facility of the Federal University of São Paulo, Brazil.

Generation of recombinant fusion proteins. Details on the generation of the His-Flic-VK210 fusion protein have been previously described (21). To generate the other recombinant fusion proteins, we followed the same strategy, using the following recombinant gene sequences: for VK247, 5′-CTCGAG-AAGCTT-3′ (18-20). The regions in bold type represent the target sites for HindIII and XhoI. The recombinant protein was expressed and purified as previously described (8, 21). Protein concentrations were determined by the Bradford assay and SDS-PAGE analysis.

Flic purification. Native Salmonella Typhimurium Flic was purified from the attenuated Salmonella Typhimurium SL3201 strain, which expresses Flic but not HlyB (8). The purity of the preparations was verified by SDS-PAGE.

Immunization regimen. Mice were subcutaneously (s.c.) immunized thrice with 10 μg of the fusion protein or the purified Flic, with immunizations being administered 3 weeks apart. In the first immunization, a final volume of 50 μl was injected into each hind footpad, while for the second and third immunizations, a final volume of 100 μl was injected at the base of the tail. In some experiments, the recombinant fusion proteins were administered with an adjuvant. The TLR-3 agonist poly(I-C) (50 μg/dose/mouse) (InvivoGen, San Diego, CA) or incomplete (IFA) Freund’s adjuvant (Sigma-Aldrich Co., St. Louis, MO), administered as an emulsion (1:1 [vol/vol]) with the immunogen, were used.

Immunological assays. Serum anti-P. vivax CSP (PvCSP) antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described previously (8, 21). Briefly, the recombinant proteins hexahistidine (His6)-PvCSP-VK210, His6-PvCSP-VK247, and His6-PvCSP-P. vivax-like (100 to 400 ng/well) were employed as capture agents. A detailed description of these proteins, which contain the entire PvCSP protein, including the N- and C-terminal portions and the repeats (VK210, VK247, or P. vivax-like), will be published elsewhere. Serum samples from immunized and nonimmunized mice were tested at serial dilutions from 1:200 to 1:819,200. A peroxidase-conjugated goat anti-mouse IgG (Sigma) was used as the detection antibody at a final dilution of 1:1,000. The highest dilution yielding an optical density at 492 nm (OD492) of >0.1 was considered the anti-PvCSP antibody titer. We performed, in parallel, a standard curve of purified mouse IgG (Sigma) using the capture anti-Fab (fragment, antigen binding) antibody (Sigma) according to the protocol suggested in reference 22. According to our standard curve of mouse IgG titers, antibody titers of 104 represent ~5 μg/ml of specific IgG. The results are presented as means ± the standard deviation (SD).

Statistical analyses. One-way analysis of variance (ANOVA), the Student t test, the Tukey honestly significant difference (HSD) test, and the Kruskal-Wallis one-way analysis of variance were used to compare the differences between the mean values of the antibody titers in the immunization groups. Differences were considered significant when the P value was <.05.

RESULTS

Recombinant flagellin fusion proteins. We generated 3 recombinant fusion proteins containing a hexahistidine tag (His6) and the allelic variants of the CSP immunodominant regions (VK210, VK247, or P. vivax-like) fused to the C-terminal end of Flic. In addition, we generated a fourth recombinant protein expressing all 3 CSP immunodominant regions as a fusion polypeptide linked to the C-terminal end of Flic (schematically represented in Fig. 1). This recombinant protein contained fewer repeats of each allelic form of the CSP due to the constraint of the number of amino acids that we can add to the C-terminal region. We observed that recombinant proteins with larger amino acid insertions were not stable (M. T. A. Leal and M. M. Rodrigues, unpublished data).

Under denaturing (reducing) conditions, the purified proteins yielded 2 bands, a predominant band of ~60 to 70 kDa and a larger band of ~130 kDa (Fig. 2A). The recombinant protein in the bands was identified using a monoclonal antibody (MAB) to the His6 tag (Fig. 2B). The larger band most likely represents a dimer of the recombinant protein, which remains in this form even under denaturing conditions.
In previous studies, we determined that the fusion polypeptide His₆-FliC-VK210 retained the ability to be recognized by MAb (2F2) specific for the immunodominant VK210 epitope (21, 23). ELISA and an immunoblot assay performed with MAb (2F2) showed that this epitope is available for immune recognition in the fusion protein His₆-FliC-All-CS-epitope (data not shown). The anti-Vk210 MAb (2E10.E9) (A. Cochrane, unpublished data) recognized the His₆-FliC-VK247 and His₆-FliC-All-CS-epitope recombinant proteins (Fig. 2C).

CSP-specific antibody responses in mice immunized with the recombinant fusion proteins. The anti-CSP serum IgG response was determined in 6 groups of C57BL/6 mice immunized with either the purified fusion proteins or native FliC. Each mouse received 10 μg of protein per immunization, in the absence of any adjuvants. The vaccination timeline is depicted in Fig. 3A.

Mice immunized with the recombinant proteins His₆-FliC-VK210 (G2), His₆-FliC-VK247 (G3), or His₆-FliC-P. vivax-like (G4) developed significant anti-PvCSP IgG titers, with the antibodies recognizing the homologous allelic forms (Fig. 3B, C, and D). After 2 or 3 immunizations, the antibody titers to the CSP allelic variants VK210 and VK247 were in the range of 10⁴ (equivalent to 5 μg/ml of specific IgG). In contrast, the antibody titers to the P. vivax-like CSP allelic form were significantly higher (in the range of 10⁵) (Fig. 3D).

The sera of mice immunized with the individual proteins (G2, G3, and G4) were tested for the presence of cross-reactive antibodies using the His₆-PvCSP-VK210, His₆-PvCSP-VK247, or His₆-PvCSP-P. vivax-like protein on the plates. We found that mice immunized with His₆-FliC-VK247 (G3) or His₆-FliC-P. vivax-like (G4) developed cross-reactive anti-PvCSP IgG titers to His₆-PvCSP-VK210. However, these titers were significantly lower than those of mice immunized with G2 (P < 0.01 in both cases, data not shown). In addition, mice immunized with His₆-FliC-VK210 (G2) or His₆-FliC-P. vivax-like (G4) developed negligible IgG titers to His₆-PvCSP-VK247 (data not shown). Finally, mice immunized with His₆-FliC-VK210 (G2) or His₆-FliC-P. vivax-like protein (data not shown). We concluded that the cross-reactive IgG antibodies are very limited and it is not possible to use a single variant as a universal vaccine.

Mice immunized with the mixture of His₆-FliC-VK210, His₆-FliC-VK247, and His₆-PvCSP-P. vivax-like (G5) proteins produced immune responses targeting all 3 allelic forms. The immune response in mice injected with this mixture was as high as or slightly lower than (Fig. 3B, C, and D) that in mice receiving single-fusion-protein immunizations. In one case only (VK210), there was a statistically significant difference between groups 2 and 5 (P < 0.05, one-way ANOVA). In contrast, mice immunized with the recombinant fusion protein His₆-FliC-All-CS-epitopes (G6) failed to generate a significant antibody response to the VK210 or VK247 CSP variants (Fig. 3B and C). Hence, we concluded that immunization with a mixture of the 3 different allelic forms is the most suitable strategy to elicit an effective immune response.

To understand the nature of the humoral immune response, we examined the subclass distribution of IgG anti-CSP response in mice from groups 2, 3, and 4. All mice immunized with the recombinant fusion proteins developed higher IgG1 levels, with IgG1/IgG2c ratios ranging from 10.66 to 512 (Fig. 4).

The PvCSP-specific antibody response in mice immunized with His₆-FliC-PvCSP-VK210 is dependent on TLR-5 and MyD88 activation. The innate immune agonist FliC activates different pattern recognition receptors (24–26). In our study, we investigated the activation of TLR-4, TLR-5, and MyD88 by immunizing wild-type (WT), as well as TLR-4, TLR-5, and MyD88 KO mice with His₆-FliC-VK210. We estimated the serum antibody titers in these animals after each immunization. The antibody titers in TLR-5 or MyD88 KO mice were significantly lower than those in WT mice (Fig. 5). In contrast, TLR-4 KO mice mounted a humoral immune response similar to that observed in WT mice. These results demonstrated that TLR-5 and MyD88 but not TLR-4 signaling were required for the immunogenicity of the recombinant fusion proteins. In addition to the antibody titers, we estimated the IgG1/IgG2c ratio in these animals. We found that in WT, TLR-5, MyD88, or TLR-4 KO mice, the ratios were 217.6, 38.4, 65.6, and 64, respectively (data not shown).

Use of poly(I·C) as an adjuvant improved antibody response to the admixed proteins. To increase the antibody titers to all 3 allelic variants of CSP, we employed new vaccine formulations that contained a mixture of the recombinant fusion protein and a...
strong adjuvant. Initially, mice were immunized with the recombinant fusion protein His6-FliC-All-CS-epitopes alone or in combination with either CFA/IFA or poly(I·C). Following the administration of 3 doses, we found that the antibody titers to all 3 allelic forms were similar, regardless of whether the protein was used alone or in combination with either adjuvant (Fig. 6).

Subsequently, mice were immunized with the mixture of recombinant fusion proteins either alone or in combination with poly(I·C). After the second dose, the antibody titers to all 3 different allelic forms of CSP were higher in mice immunized with the protein mixture in combination with poly(I·C) than in mice im-

FIG 3 Immunogenicity of recombinant fusion proteins. (A) C57BL/6 mice (n = 6) were immunized s.c. with 3 doses (10 µg/animal/dose) of the indicated proteins (G1 to G6) at the indicated times. The serum antibody levels measured 74 days after the administration of the first dose were determined by ELISA using plates coated with the capture agents His6-PvCSP-VK210 (B), His6-PvCSP-VK247 (C), and His6-PvCSP-P. vivax-like (D). The results are expressed as means ± SD. *, the values are significantly higher than those in controls immunized with FliC alone (G1) (P < 0.05, one-way ANOVA). +, the value of G2 is significantly higher than those of all other groups (G1, G5, and G6) (P < 0.05, one-way ANOVA).

FIG 4 Determination of IgG subclasses in C57BL/6 mice (n = 4) immunized with 3 doses of the indicated proteins. The serum antibody titers, measured 74 days after the first dose, were determined by ELISA using plates coated with the proteins representing the homologous alleles (His6-PvCSP-VK210 [G2], His6-PvCSP-VK247 [G3], or His6-PvCSP-P. vivax-like [G4] protein). The results are expressed as means ± SD.

FIG 5 TLR-5/MyD88 dependence of the serum IgG response in mice immunized with the recombinant fusion protein His6-FliC-VK210. (A) C57BL/6 mice (n = 6) were immunized s.c. with 3 doses (10 µg/animal/dose) of recombinant protein at the indicated time points. Serum samples were collected at the indicated time points following the first dose, and serum antibody titers were determined by ELISA by using plates coated with His6-PvCSP-VK210. (B) The results are expressed as means ± SD. *, the values are significantly lower in TLR-5 or MyD88 KO mice than in immunized WT controls (P < 0.001).
FIG 6 Presence of strong adjuvants did not increase the immunogenicity of the recombinant fusion protein His6-FliC-All-CS-epitopes. (A) C57BL/6 mice (n = 6) were immunized s.c. with 3 doses (10 μg/animal/dose) of recombinant protein according to the timeline in the presence or absence of the indicated adjuvants. The serum antibody titer, 53 days after administration of the first dose, was quantified by ELISA by using plates coated with His6-PvCSP-VK210, His6-PvCSP-VK247, or His6-PvCSP-P. vivax-like proteins. (B) Results are expressed as means ± SD. *p < 0.001, one-way ANOVA. No statistically significant differences were found when we compared the three formulations used with or without adjuvants (one-way ANOVA).

mumized with the recombinant proteins alone (Fig. 7B, C, and D). The increase in IgG titers persisted for 180 days after the first immunization (138 days after the last dose). The IgG1/IgG2c ratios in mice immunized with the recombinant protein mixture in the presence of poly(I·C) were 0.4 (His6-PvCSP-VK210), 1.4 (His6-PvCSP-VK247), and 0.8 (His6-PvCSP-P. vivax-like) (data not shown). In contrast, for the animals immunized with the recombinant proteins in the absence of poly(I·C), the IgG1/IgG2c ratios were 129 (His6-PvCSP-VK210), 78 (His6-PvCSP-VK247), and 69 (His6-PvCSP-P. vivax-like) (data not shown). These results indicate that the immunogenicity of the recombinant fusion protein mixture can be improved by coimmunization with the TLR-3 agonist poly(I·C). Furthermore, we observed that these mice developed a balanced IgG1/IgG2c immune response in the presence of this adjuvant, and this finding was very much in contrast to that observed when the proteins were administered alone (Fig. 4).

**DISCUSSION**

In the present study, we evaluated the immunogenicity of recombinant proteins consisting of the immunodominant epitope of *P. vivax* sporozoites fused to an innate immune agonist, *Salmonella Typhimurium*-derived FliC. Using a mixture of 3 recombinant proteins, IgG antibody responses to all 3 *P. vivax* CSP allelic forms were successfully elicited in mice. The immune response was specific and long lasting. These are characteristics of T-cell-depen-
correlation between protective immunity and the presence of antibodies to the repeat domain of the *P. falciparum* CSP (5, 6).

Although we have clearly demonstrated the presence of *P. vivax* CSP-specific antibodies targeting the repeat domain of the different allelic variants, their function remains elusive. Preliminary immunofluorescence analyses of the VK210 and VK247 strains showed that these antibodies recognized *P. vivax* sporozoites. However, detailed analyses need to be performed. Moreover, the ability of these antibodies to neutralize sporozoites in vitro has yet to be evaluated.

In contrast to *P. falciparum*, the development of a vaccine against the preerythrocytic forms of *P. vivax* has been limited to preclinical immunization models developed mostly in mice. A single phase I clinical trial has been performed. Studies in mice and a nonhuman primate examined the recombinant VMP001 protein generated in *Escherichia coli*. This protein contains repeat regions derived from the VK210 allelic variant of the *P. vivax* CSP. A repeat sequence derived from the VK247 allele was also introduced into the C-terminal region. This protein has been shown to be immunogenic in different vaccine formulations and is capable of inducing an immune response mediated by antibodies and CD4⁺ T cells (32–36). The specificities of the antibodies induced by the vaccine VMP001 were not investigated by binding inhibition experiments. While in our case the immune responses are focused on the repeat region, animals immunized with VMP001 reacted strongly with the C-terminal region (32, 36). Interestingly, VMP001 induced partial cross-protective immunity in mice challenged with *P. berghei* sporozoites (37). The region(s) of the PvCSP that elicited this cross-protective immune response remains unknown.

Studies performed in parallel used long recombinant *P. vivax* CSP-derived peptides to develop a synthetic malaria vaccine (38). Peptides representing CSP N- or C-terminal portions or the amino acid repetitions (allele VK210) have been tested in phase I clinical trials. This vaccine formulation, which contains the adjuvant Montanide ISA 720, was safe and well tolerated following administration by the intramuscular route. Vaccinated individuals developed antibodies that targeted 3 different epitopes of the *P. vivax* CSP, and gamma interferon (IFN-γ)-producing cells were observed (39).

In summary, our findings extend the understanding of the use of *Salmonella* flagellin as an adjuvant and antigen carrier in vaccine formulations in general. More specifically, we generated recombinant fusion proteins consisting of fusions of different *P. vivax* CSP allelic variants with flagellin and showed that a formulation consisting of the mixture of these 3 allelic variant-flagellin fusions, administered in the presence of the adjuvant poly(I-C), is sufficient to induce strong and long-lasting anti-CSP immunity in mice. As both flagellin and poly(I-C) have been considered to be promising adjuvants in human trials, this formulation might be a potentially new *P. vivax* vaccine candidate with broad coverage.

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