Immunogenicity of a prime-boost vaccine containing the circumsporozoite proteins of *Plasmodium vivax* in rodents

Running title: *Plasmodium vivax* CS protein vaccine candidates.

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ABSTRACT (250 words)

Plasmodium vivax is the most widespread, and the second most prevalent, species causing malaria in the world. Current measures used to control the transmission of this disease would benefit from the development of an efficacious vaccine. In the case of the deadly parasite P. falciparum, the recombinant RTS,S vaccine containing the circumsporozoite (CSP) antigen consistently protects between 30-50% of human volunteers against infection and is undergoing Phase III clinical trials in Africa with similar efficacy. These findings encouraged us to develop a P. vivax vaccine containing the three circulating allelic forms of the P. vivax CSP. Toward this goal, we generated 3 recombinant bacterial proteins representing the CSP alleles as well as a hybrid polypeptide called PvCSP–All CSP epitopes. This hybrid contains the conserved N- and C-terminus of the P. vivax CSPs and the three variant repeat domains in tandem. We also generated simian and human recombinant replication-defective adenovirus vectors expressing PvCSP-All CSP epitopes. Mice immunized with the mixture of recombinant proteins in a formulation containing the adjuvant poly(I:C) developed high and long lasting serum IgG titers comparable to those elicited by proteins emulsified in complete Freund’s adjuvant. Antibody titers were similar in mice immunized with homologous (protein/protein) or heterologous (adenovirus/protein) vaccine regimens. The antibodies recognized the three allelic forms of CSP, reacted to the repeated and non-repeated regions of CSP and recognized sporozoites expressing the alleles VK210 and VK247. The vaccine formulations
described in this work should be useful for the further development of an anti-\textit{P. vivax} vaccine.

**Introduction**

Human malaria infection starts when an \textit{Anopheles} mosquito injects sporozoites of \textit{Plasmodium sp.} into the skin of a person. The sporozoites transverse the skin, enter the blood circulation and infect hepatocytes. While sporozoites are in the skin or migrating to the liver, their infectivity can be abolished by antibodies against CSP. The neutralizing antibodies are predominantly, but not exclusively, directed against the immunodominant B epitopes in the CSP repeat domain (reviewed ref. 1).

Multiple trials in experimental animals and more recently in humans provide a solid basis for the use of vaccines against CSP to prevent malaria. Thus far, the only vaccine against the deadly \textit{P. falciparum} tested in a phase III clinical trial is RTS,S, a fusion protein between portions of CSP and the hepatitis B surface antigen (S) that is administered in powerful adjuvant systems (AS), either an oil-in-water emulsion (AS02) or in a liposomal suspension (AS01). These adjuvants contain monophosphoryl lipid A (MPL, a detoxified form of LPS) and QS21 (saponin purified from \textit{Quillaja saponaria}). Clinical Phase IIa trials in naïve, vaccinated volunteers who were challenged with bites from mosquitoes infected with \textit{P. falciparum} reported protective efficacies of ranging from 32% to 50%.

Protective immunity largely correlated with the serum levels of specific IgG
antibodies against the repeats in the CSP antigen, and, to a lesser extent, to the
frequency of CD4+ T cells expressing ≥2 of the cytokines IL-2, TNF-α or IFN-γ (2).

One of these formulations (RTS,S/AS01E) is currently being tested in a
large Phase III clinical trial in African children living in malaria endemic areas. The
results reported from this trial indicate 49.5% or 30.1% protective efficacy during a
14-month-period post-vaccination in 5- to 17-month-old or 6 to 12-week-old African
children, respectively (3, 4). These trials and previous human trials have
established that the immunodominant CSP is a worthwhile candidate antigen to be
included in future vaccine formulations to combat malaria infections including
infections with *P. vivax*.

*Plasmodium vivax* is the most widespread malaria-causing species in the
world and is the second most prevalent. It is estimated that more than 2.8 billion
people are at risk of contracting *P. vivax* infection (5). Nevertheless, only three
clinical trials based on subunit *P. vivax* vaccines have been completed
(http://www.clinicaltrials.gov/). One complication of *P. vivax* vaccine development is
that, in contrast to *P. falciparum*, different allelic forms of *P. vivax* CSP have been
described. The two most common CSP alleles are VK210 and VK247 (6, 7). A third
allelic form exists at a low frequency (8-12). The main variation between these
allelic forms is in the central repeat region of CSP, which is a possible target for
neutralizing antibodies.

To generate a vaccine with universal coverage against *P. vivax* malaria
strains, we tested a prime-boost regimen using recombinant proteins and
adenovirus vectors expressing epitopes from the three CSP alleles as antigens.
We used two approaches to generate these vaccines. The first approach consisted
of mixing recombinant proteins expressing the three CSP alleles to generate a vaccine. Additionally, we generated a single recombinant fusion protein called \textit{PvCSP-All CS epitopes} that contains epitopes from the three \textit{P. vivax} CSP alleles. We primed animals with either simian or human recombinant replication-defective adenovirus vectors expressing \textit{PvCSP-All CS epitopes} in some experiments. In most experiments, recombinant antigens were administered in formulations containing the adjuvant poly(I:C). We compared the immunogenicity of homologous (protein/protein) with heterologous (adenovirus/protein) immunization regimens. We primarily measured the magnitude and the longevity of the serum IgG against the entire CSP and the CSP domains. In selected experiments, we also evaluated the cell-mediated immune response against CSP.
Materials and Methods.

Ethics statement.

This study was carried out in strict accordance with the recommendations provided by 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 Sao Paulo (Id # CEP 0307/09). Six- to eight-week-old isogenic female C57BL/6 (H-2b) mice were maintained under specific pathogen free (SPF - specific pathogen free) were purchased from the Center for Development of Experimental Models for Medicine and Biology (CEDEME) (Federal University of Sao Paulo, Brazil). C57BL/6 TLR4 knock-out mice (tlr4^-/) were kindly provided by Dr. Shizuo Akira at Osaka University, Japan (13). Experiments were approved by the Ethics Committee in Research of the Federal University of Sao Paulo and Sao Paulo Hospital (CEP 0307/09).

Generation of recombinant bacterial proteins containing sequences encoding the CSP antigen of P. vivax.

For the purpose of immunization, recombinant bacterial proteins representing the different allelic forms of the P. vivax were expressed and purified as described below. These recombinant proteins were denominated His6-PvCSP-VK210, His6-PvCSP-VK247, His6-PvCSP-Vivax-like and His6-PvCSP-All-CSP-epitopes. The deduced amino acid (AA) sequences encoding the recombinant proteins are depicted in the Fig. 1b. Codon optimized synthetic genes were
purchased from Genescript Inc. containing the sequences of nucleotides \textit{PvCSP-vk210} (GenBank Accession number: to be submitted), \textit{PvCSP-vk247} (GenBank Accession number: to be submitted), \textit{PvCSP-Vivax-like} (GenBank Accession number: to be submitted) and \textit{PvCSP-All-CSP- epitopes} (GenBank Accession number: to be submitted). These genes were subcloned into the commercial pET28a expression vector (Novagen), expressed and purified as briefly described. Recombinant \textit{E. coli} BL21 DE3 (Novagen) transformed with each recombinant plasmid was cultivated at 37°C in flasks containing Luria broth (LB) and kanamycin (30 µg/ml). Protein expression was induced at an \textit{OD}_{600} of 0.6 with 0.1 mM IPTG (Invitrogen) for 4 h. After centrifugation, bacteria were lysed on ice with the aid of an ultrasonic processor (Sonics and Materials INC Vibra Cell VCX 750) in a phosphate buffer (pH 8.0) with 1.0 mg/ml lysozyme (Sigma) and 1 mM PMSF (Sigma). Bacterial lysate was centrifuged, and the supernatant was resuspended in 8M Urea (Invitrogen). After boiling at 100°C for 15 min, the proteins were applied to a column with \textit{Ni}^{2+}–NTA–agarose resin (Quiagen). After several washes, bound proteins were eluted with 0.5 M imidazole (Sigma). The eluted protein was dialysed against 20 mM Tris–HCl (pH 8.0), and the recombinant proteins were purified by ion-exchange chromatography using a Resource Q column (GE Healthcare) coupled to an FPLC system (GE Healthcare). Fractions containing the recombinant proteins with a high degree of purity were pooled and incubated with polymyxin B agarose (Sigma) overnight. Unbound material was extensively dialysed against phosphate-buffered saline (PBS/pH=7.2). Protein concentration was determined with the Bradford assay and by SDS-PAGE analyses. LPS determination was made using the E-TOXATE Kit (Sigma). Tests were performed with different
dilutions of each sample and results demonstrated that the LPS contamination varied between 300 and 3,000 EU per mg of purified protein.

In addition, for the purpose of epitope mapping, we used His\textsubscript{6}-FliC, His\textsubscript{6}-FliC-PvCSP-VK210, His\textsubscript{6}-FliC-PvCSP-VK247, His\textsubscript{6}-FliC-PvCSP-Vivax-like. These proteins were generated as described in detail in ref. 14. Finally, a recombinant protein devoid of the repeat regions expressing both the N- and C-terminal region of the CSP was generated denominated His\textsubscript{6}-PvCSP-No repeats. These recombinant proteins were expressed and purified as described previously (15).

**Generation of recombinant replication-defective adenovirus vectors expressing sequences encoding the CSP antigens from *P. vivax***.

Recombinant replication-defective human adenovirus serotype 5 (AdHu5) and chimpanzee adenovirus serotype 68 (AdC68) vectors were generated, purified, quality controlled and titrated as previously described (16) using the sequence ad-PvCSP (GenBank Accession number: to be submitted). The deduced amino acid sequences encoding the CS antigen are shown in Fig. 6b.

**Immunization regimens**.

Mice were given three immunizations three weeks apart subcutaneously (s.c.) in the two hind footpads using a final volume of 50 µl in each footpad (first dose) and a final volume of 100 µl at the base of the tail (second and third dose). For each dose, the indicated amount of protein was used. Complete (CFA) and incomplete (IFA) Freund’s adjuvants (Sigma) were emulsified with the protein...
antigens (1:1, v/v) prior to injection. For CFA/IFA immunization regimens, CFA was used for the first dose and IFA was used for subsequent doses. As additional adjuvants, 50 µg of high molecular weight poly(I:C) (Invivogen) or Hiltonol® (PolyICL, Oncovir Inc., kindly provided by Dr. Salazar) was admixed with the protein antigens in a final volume of 100 µL.

Recombinant adenovirus vectors were injected intra-muscularly (i.m.) in the tibialis anterioris using a final volume of 50 µl in each leg.

**Peptide synthesis**

Synthetic peptides were purchased from Genscript (Piscataway, New Jersey). Peptide purity was higher than 90%. Peptide identities were confirmed by a Q-TOF Micro equipped with an electrospray ionization source (Micromass, UK).

**Immunological assays.**

Serum IgG anti-P. vivax CSP antibodies were detected by ELISA as previously described (14, 15). The recombinant proteins (100-200 ng/well) were employed as the solid-phase bound antigen. A peroxidase-conjugated goat anti-mouse IgG (Sigma) was applied at a final dilution of 1:1,000, while the sera from mice were tested at serial dilutions starting with 1:200. Specific antibody titers were determined as the highest dilution yielding an OD$_{492}$ higher than 0.1. In parallel, we performed a standard curve of purified mouse IgG (Sigma) using the capture antibody anti-Fab (Sigma) according to the protocol suggested in ref. 17.
Detection of IgG subclass responses was performed as described above except that the secondary antibody was specific for mouse IgG1, IgG2b and IgG2c (Southern Technologies). The results are presented as the mean ± SD.

Monoclonal antibodies (MAbs) against the *P. vivax* CS proteins representing the allelic forms VK210 (2F2) or VK247 (2E10.E9, Dr. Alan Cochrane, unpublished results) were generated after immunization with radiation-attenuated sporozoites (18). The hybridomas were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). Polyclonal antibodies against the vivax-like epitopes were obtained from C57BL/6 mice immunized with the His<sub>6</sub>-FliC-PvCSP-Vivax-like antigen as described (14). Monoclonal antibodies to the His tag were purchased from GE Health Systems.

**Cell-mediated immunity assays.**

Splenocytes collected from immunized C57BL/6 mice were used for ELISPOT assays, assays to measure the secretion of IFN-γ, and in vivo cytotoxicity assays. These assays were performed as in our previous studies [19, 20].

For the intracellular expression of cytokines (IFN-γ and TNF-α, ICS), splenocytes collected from C57BL/6 mice were treated with ACK buffer (NH₄Cl, 0.15 M; KHCO₃, 10 mM; Na₂-EDTA 0.1 mM; pH = 7.4) for lysing the erythrocytes. ICS were evaluated after in vitro culture of splenocytes in the presence or absence of the indicated antigenic stimulus. Cells were washed in cell culture medium consisting of RPMI 1640 medium, pH 7.4, supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 59 mg/L of penicillin, 133 mg/L of streptomycin, and 10%
Hyclone fetal bovine sera (Hyclone, Logan, Utah). The viability of the cells was evaluated using 0.2% trypan blue. Cell concentration was adjusted to $5 \times 10^6$ cells/mL in cell culture medium containing anti-CD28 (2 µg/mL), brefeldin A (10 µg/mL), monensin (5 µg/mL, BD Pharmingen). In half of the cultures, a final concentration of 10 µg/mL of the indicated peptides, recombinant proteins or 2 µg/mL of Concanavalin A (ConA, Sigma) were added. The cells were cultivated in V-bottom 96-well plates (Corning) in a final volume of 200 µL in duplicate, at 37°C in a humid environment containing 5% CO₂. After 12-h incubation, cells were stained for surface markers with PerCP-Cy5.5-labeled anti-CD4 (clone RM4-5, or PECy7-labeled CD8 (clone 53-6.7, on ice for 20 min. To detect IFN-γ and TNF-α by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA, fixed in 4% PBS-paraformaldehyde solution for 10 minutes, and permeabilized for 15 minutes in a PBS, 0.1% BSA, and 0.1% saponin solution. After being washed twice, cells were stained for intracellular markers using FITC-Labeled anti-IL-2 (JES6-5H4), APC- labeled anti-IFN-γ (Clone XMG1.2) and PE-labeled anti-TNF-α (clone MP6-XT22), for 20 minutes on ice. Finally, cells were washed twice and fixed in 1% PBS-paraformaldehyde. At least 300,000 cells were acquired on a BD FacsCanto flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

**P. vivax slide preparation and indirect immunofluorescence assay (IIA).**

Sera from animals immunized simultaneously with the three recombinant proteins (protein mix of His₆-PvCSP-VK210+His₆-PvCSP-VK247+His₆-PvCSP-Vivax-like) in
a formulation containing the adjuvant poly(I:C) or from mice that received a heterologous prime-boost vaccination regimen (adenovirus/protein mix) were selected for the immunofluorescence assay. *P. vivax* sporozoites expressing *PvCSP* VK210 or VK247 were obtained as previously described (21, 22). Slides containing sporozoites fixed in air were blocked with phosphate-buffered saline containing 3% bovine serum albumin (PBS + 3% BSA) for 30 minutes at room temperature. Slides were incubated in a humid chamber for 1 hour at room temperature with 1 μg/mL of the monoclonal antibody 2F2 (Mab anti-PvCSP-VK210) as a positive control or with sera from mice were immunized with adjuvant only diluted 1:1,000 as negative control. Additionally, we used sera from immunized animals that had been diluted 1:100, 1:1,000, 1:10,000 and 1:100,000.

After immersion in PBS to wash the slides, slides were incubated for 1 h at room temperature with PBS + 3% BSA containing FITC-conjugated goat antibody reactive against murine IgG (Kirkegaard & Perry Laboratories, Inc.) at a concentration of 0.02 mg/mL. The slides were washed in PBS containing 50 mM glycine, slightly dried and mounted with buffered glycerol. We analyzed the slides under an optical microscope with fluorescence at a magnification of 100X.

**Statistical analyses.**

One-way ANOVA and Tukey HSD tests were used to compare the differences between the values of the serum IgG titers of the different groups.
Results

Production and purification of the *P. vivax* CSP recombinant proteins and recognition by MAbs.

Fig. 1A shows a schematic representation of the *P. vivax* CSP antigen and the sequences of the repeated regions present in each allelic form of CSP (VK210, VK247 and Vivax-like). Codon-optimized genes were synthesized that contained the same coding regions for the N- and C-terminus. The central domain encoded the different allelic forms of the *P. vivax* CSP protein. These genes were expressed as recombinant fusion proteins representing each individual allelic form of the *P. vivax* CSP antigen linked to a hexahistidine tag (His$_6$). These antigens were named His$_6$-PvCSP-VK210, His$_6$-PvCSP-VK247 and His$_6$-PvCSP-Vivax-like. The predicted AA sequence of each recombinant fusion protein is shown in Fig. 1B. In addition, a fourth synthetic gene was constructed encoding the three distinct allelic forms expressed as a single fusion polypeptide called His$_6$-PvCSP-All-CSP-epitopes (Fig. 1B).

Recombinant proteins were purified as described in the Methods Section and separated by SDS-PAGE under reducing conditions. Although the predicted MW of the recombinant proteins were 35,207.42, 33,633.48, 33,878.94 and 34,346.40 Da, the apparent MW as determined by SDS-PAGE varied from approximately 50,000 to 55,000 Da (Fig. 1C).

To determine whether the polypeptides retained the epitopes recognized by specific MAbs generated against radiation-attenuated *P. vivax* sporozoites, we performed ELISA with each of the four recombinant proteins bound to the plates.
As depicted in Fig. 2A, all four recombinant proteins were recognized well by a MAb against the His tag. The MAb against the allelic variant VK210 (2F2) recognized the recombinant proteins His$_6$-PvCSP-VK210 and His$_6$-PvCSP-All-CSP- epitopes equally well. This MAb extensively cross-reacted with the recombinant protein His$_6$-PvCSP-Vivax-like and reacted to a minor extent with His$_6$-PvCSP-VK247 (Fig. 2B).

The MAb against the allele VK247 (2E10.E9) reacted with the recombinant proteins His$_6$-PvCSP-VK247 and His$_6$-PvCSP-All-CSP- epitopes equally well. No cross-reactivity was detected with the recombinant proteins His$_6$-PvCSP-VK210 or His$_6$-PvCSP-Vivax-like (Fig. 2C). Finally, polyclonal antibodies to the Vivax-like repeat region reacted equally well with the recombinant proteins His$_6$-PvCSP- Vivax-like and His$_6$-PvCSP-All CS epitopes. No cross-reactivity was detected with the recombinant proteins His$_6$-PvCSP-VK210 or His$_6$-PvCSP-VK247 (Fig. 2D).

Based on these results, we concluded that the repeat epitopes encoded by the different allelic variants of *P. vivax* were preserved in the bacterial recombinant proteins. Most relevant, the recombinant His$_6$-PvCSP-All-CSP-epitopes were equally recognized by all three different MAbs and polyclonal sera used, indicating the presence of the three distinct epitopes in this fusion polypeptide.

**Induction of specific antibody responses in mice immunized with recombinant *P. vivax* CSP antigens.**

The serum IgG responses to *P. vivax* CSP antigens were determined in C57BL/6 mice immunized s.c. with purified proteins in the presence of the adjuvant.
poly(I:C) or emulsified in complete/incomplete Freund’s adjuvant (CFA/IFA). Mice were immunized with three doses 21 days apart, and the antibody titers were analyzed according to the timeline described in Fig. 3A. Twelve mouse groups were immunized with: a) poly(I:C); b) poly(I:C) plus His$_{6}$-PvCSP-VK210 (10 $\mu$g/dose/mouse); c) poly(I:C) plus His$_{6}$-PvCSP-VK247 (10 $\mu$g/dose/mouse); d) poly(I:C) plus His$_{6}$-PvCSP-Vivax-like (10 $\mu$g/dose/mouse); e) poly(I:C) plus a protein mix (His$_{6}$-PvCSP-VK210/His$_{6}$-PvCSP-VK247/His$_{6}$-PvCSP-Vivax-like- 30 $\mu$g/dose/mouse); f) poly(I:C) plus His$_{6}$-PvCSP-All-CSP-epitopes (30 $\mu$g/dose/mouse); g) CFA/IFA; h) CFA/IFA plus His$_{6}$-PvCSP-VK210 (10 $\mu$g/dose/mouse); i) CFA/IFA plus His$_{6}$-PvCSP-VK247 (10 $\mu$g/dose/mouse); j) CFA/IFA plus His$_{6}$-PvCSP-Vivax-like (10 $\mu$g/dose/mouse); k) CFA/IFA plus a protein mix (His$_{6}$-PvCSP-VK210/His$_{6}$-PvCSP-VK247/His$_{6}$-PvCSP-Vivax-like- 30 $\mu$g/dose/mouse); and l) CFA/IFA plus His$_{6}$-PvCSP-All-CSP-epitopes (30 $\mu$g/dose/mouse).

At the indicated days, the antibody titers were determined by ELISAs to each individual allelic form of the *P. vivax* CSP. In all groups of mice immunized with a formulation containing recombinant CSP antigen and adjuvant, antibody titers were above $10^5$ after the second dose. According to our standard curve of mouse IgG, this antibody titer represents approximately 50 $\mu$g/mL of specific IgG. After the third dose, in some cases we observed an enhancement of these titers to $\geq 10^6$. High antibody titers lasted for a period of 183 days after the first immunization. At that time, titers were still in the range of $10^5$ to $10^6$. 


By comparing the antibody titers of groups of mice immunized with the recombinant antigens in the presence of the adjuvant poly(I:C) (Fig. 3B to D), we found that immunization with the protein mix generated higher antibody titers than immunization with each individual protein or with the protein His$_6$-PvCSP-All-CSP-epitopes (p<0.01 in all cases for the period of 183 days). In contrast, all groups of mice immunized with the recombinant CSP antigens emulsified in CFA/IFA produced similar antibody titers (Fig. 3E to G, p= NS in all cases for the period of 183 days).

We then compared groups of mice injected with the protein mix or His$_6$-PvCSP-All-CSP-epitopes in formulations containing the adjuvants poly(I:C) or CFA/IFA. We found that immunization with the protein mix generated similar antibody titers when emulsified in CFA/IFA or admixed with the adjuvant poly(I:C) (p= NS). In contrast, mice immunized with His$_6$-PvCSP-All-CSP-epitopes emulsified in CFA/IFA displayed significantly higher antibody titers than animals injected with the antigen in the presence of poly(I:C). These experiments allowed us to conclude that immunization with the protein mix was superior when compared to immunization with His$_6$-PvCSP-All-CSP-epitopes when poly(I:C) was used as adjuvant. Additionally, we concluded that when using the protein mix in the presence of poly(I:C), the antibody titers could be as high and long lasting as the titers generated by this same protein mix emulsified in CFA/IFA.

Although the antibody titers of anti-PvCSP-specific IgG after immunization with the protein mix in formulations containing CFA/IFA or poly(I:C) were equally high, these immune responses differed significantly in terms of the sub-classes of
IgG that were elicited. Responses generated in the presence of CFA/IFA resulted in predominately IgG1 compared to IgG2c (ratio of 19.1), while responses generated in the presence of poly(I:C) resulted in an IgG1 to IgG2c subclass ratio of 0.95.

To compare the potency of the poly(I:C) with the commercially available Hiltonol (Poly (ICLC)) that is being used in human trials (23), we performed side by side comparison experiments immunizing mice with: a) poly(I:C); b) poly(I:C) plus a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like- 30 µg/dose/mouse); and c) Hiltonol plus this same protein mix. We observed that the antibody titers of mice immunized with the protein mix in the presence of either adjuvant elicited similarly high antibody titers that lasted until at least day 160 (p = NS, data not shown).

We then determined whether lower doses of the recombinant protein mix could be as effective in eliciting high and sustained antibody immune responses. For this purpose, mice were immunized side by side with doses of 10 or 1 µg of each of the three recombinant proteins in the presence of the adjuvant poly(I:C). Mice immunized with both 30 and 3 µg of the total protein mix responded equally well. Antibody titers above $10^5$ lasted for more than 200 days (Fig. 4A to C, p= NS).

Purified bacterial recombinant proteins often contain traces of contaminating LPS that can interfere with immunogenicity. To show that contaminating LPS was not influencing the immunogenicity results, we performed a side by side comparison of the antibody immune responses to the protein mix when administered to $\text{tlr}4^{+/+}$ or $\text{tlr}4^{-/-}$ mice in the presence of poly(I:C). After immunization, both mouse strains responded equally well for the entire 177 day observation.
period (Fig. 5A to C, p= NS). These observations ruled out any possible interference from LPS contamination in our samples.

**Induction of specific antibody responses in mice immunized with recombinant adenovirus vectors expressing the P. vivax CSP as part of a heterologous prime-boost regimen for vaccination.**

In addition to the recombinant proteins, we generated two recombinant replication-defective adenovirus vectors expressing the P. vivax CSP gene encoding the PvCSP-All-CSP-epitopes antigen. Schematic representations of the deduced amino acid sequences of the proteins expressed by these recombinant adenovirus vectors are depicted in Fig. 6A and B. Antigen expression was confirmed by infection of HEK-293 cells and immunofluorescence analyses (data not shown). The recombinant adenoviruses were designated AdC68-PvCSP and AdHu5-PvCSP. Immunization of C57BL/6 mice with 2x10⁷ pfu either of these recombinant adenovirus vectors elicited specific IgG antibodies against each individual allelic form of PvCSP. This dose was determined experimentally as the minimum dose to elicit antibody titers higher that 10³. After 21 days, antibody titers to all three different alleles of recombinant PvCSP were in the range of 10⁴. No statistically significant difference was observed in the serum IgG titers between the groups of mice vaccinated with AdC68-PvCSP or AdHu5-PvCSP (p=NS, Fig. 6C).

As expected, control mice injected with the same dose of AdHu5β-gal did not generate any P. vivax-specific antibodies. However, at a later time point, antibody
titers of mice immunized with AdC68-PvCSP were higher than animals immunized with AdHu5-PvCSP (p<0.05, Fig. 6D).

To test whether recombinant adenovirus vectors could be used as priming immunogens in a heterologous prime-boost regimen, mice primed with the adenovirus vector were boosted s.c. 42 days after priming with a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like 3 µg/dose/mouse) in a formulation containing poly(I:C). Serum IgG antibody titers against all three allelic forms of PvCSP at day 63 in mice primed with AdC68-PvCSP (G5) or AdHu5-PvCSP (G6) and boosted with the protein mix were significantly higher than the titers in control groups (G1, G2, G3 and G4) (p<0.05, Fig. 6D). No statistically significant differences were scored between mouse groups G5 and G6. These results provide evidence that priming with AdC68-PvCSP and AdHu5-PvCSP improves antibody responses upon subsequent immunization with the recombinant protein mix.

These same mice described above received a second boost with protein mix in a formulation containing poly(I:C) or with only adjuvant (control) as depicted in Fig. 7A. We followed the titers of specific serum IgGs against the different allelic forms of PvCSP for 178 days (Fig. 7, B to D). Antibody titers in the different mouse groups immunized (boosted) twice with the recombinant protein mix were equally high, and we did not detect statistically significant differences between groups G4, G5 and G6 (p=NS in all cases). Not only were the titers of anti-PvCSP-specific IgG sustained, with high antibody titers persisting until day 178. Additionally, these
responses did not differ significantly in terms of the sub-classes of IgG that were elicited. The IgG1/IgG2c ratios of groups G4 and G5 were 0.95 and 1.125, respectively.

These experiments allow us to conclude that recombinant adenoviruses expressing the PvCSP protein can successfully be used in a heterologous prime-boost protocol for vaccination. However, no significant impact on the serum IgG antibody titers was observed when we used either the homologous (protein/protein) or heterologous (adenovirus/protein) regimen.

Specificity of the antibodies elicited during homologous (protein/protein) or heterologous (adenoviruses/protein) regimens.

We then studied the specificity of the antibodies against PvCSP. We selected for the sera from mice that had been immunized with the homologous (protein/protein) or heterologous (adenovirus/protein) regimens. To aid in the determination of the specificity of anti-PvCSP serum IgG antibodies, we used a number of recombinant proteins containing different portions of the antigen. Schematic representations of each recombinant protein used, and the antibody titers are depicted in Fig. 8A.

We found that antibodies from mice immunized with either the homologous or heterologous regimen reacted with all of the recombinant proteins containing different portions of the three distinct allelic forms of the PvCSP antigen. These antibodies reacted with each repeat domain expressed as a part of a fusion protein.
at the C-terminal region of FltC. The mice also had significant antibody titers to a recombinant protein expressing the N- and C-terminal regions but not the repeat region of the PvCSP antigen (His$_6$-PvCSP No repeats). As expected however, the maximal reactivity was directed against the recombinant proteins containing the entire PvCSP (Fig. 8B). We concluded that both the repeated and non-repeated regions of PvCSP are targets for antibodies.

Finally, we determined whether sera from mice immunized with the homologous or heterologous immunization regimens reacted with *P. vivax* sporozoites in immunofluorescence assays. We observed that sera from both groups reacted to sporozoites of the *P. vivax* VK247 strain at titers of 1:1,000 and in the case of the homologous immunization also in 1:10,000 (Fig. 9A). Antibody recognition was specific, as control sera from mice immunized either with adjuvant poly(I:C) or AdHu5β-gal/poly(I:C) did not react, even at a 1:100 dilution (Fig. 9B). We also confirmed that sera from both groups of mice that were immunized with a formulation containing the protein mix and poly(I:C) reacted to sporozoites of the VK210 strain at dilutions of 1:10,000 (Fig. 9C).

**Cell–mediated immune responses.**

To determine whether the vaccination protocols elicited T cell-mediated immune responses, we employed several methods of analyses. Spleen cells from mice immunized with either the homologous or heterologous regimen were stimulated *in vitro* with recombinant proteins or overlapping synthetic peptides (15-mers) covering the entire PvCSP. We measured IFN-γ secretion by ELIspot, ICS
and by detection of the cytokine in the supernatants. We also attempted to detect IL-2 and TNF by ICS. Finally, we also used an in vivo cytotoxicity assay to estimate the presence of CTL in vaccinated mice (24). The results were negative in most cases independent of the timing used for analyses. Positive controls performed in parallel with experimental samples were consistently successful (data not shown).

Examples of these studies are depicted in Fig. 10 and 11. Splenic cells of mice immunized with the heterologous or homologous prime and boost regimen (described in Fig. 10A) were re-stimulated in culture with the recombinant proteins or synthetic peptides spanning the entire N or C-terminal of the CSP of P. vivax (Fig 10B). Significant differences in the frequencies of IFN-γ+, IL-2+ or TNF-α+ in CD4+ or CD8+ were not detected between the experimental and control groups (Fig. 10C and D).

In parallel, these cells were assayed for ELIspot. Likewise in the experiments of ICS, significant differences in the frequencies of IFN-γ− producing cells were not detected between the experimental and control groups (Fig. 11). One peptide only representing the repeat region of PvCSP of the vivax-like allele elicited modest number of specific SFC. This peptide possibly represents a T-cells epitope for the C57Bl/6 mice. We were able to obtain higher immune response using IFA for immunization (data not shown). We concluded that although we were able to obtain high levels of antibody production following immunization, this response was not paralleled by high levels of T cell-mediated immunity.
Discussion

In the present study, we tested whether recombinant proteins and adenovirus vectors could be used for the development of a universal vaccine against *P. vivax* malaria. Using homologous or heterologous prime-boost regimens of immunization, we were able to successfully elicit high titers of antibodies recognizing the three allelic forms of the PvCSP antigen. This malaria vaccine formulation could be used either as a protein mix containing His<sub>6</sub>-PvCSP-VK210, His<sub>6</sub>-PvCSP-VK247 and His<sub>6</sub>-PvCSP-Vivax-like or a single recombinant protein containing epitopes of all three different allelic forms in a single fusion polypeptide (His<sub>6</sub>-PvCSP-All-CSP-epitopes). However, in the presence of poly(I:C), we found the protein mix to be more immunogenic. This improved immunogenicity was not observed when the antigens were emulsified in CFA/IFA. The precise reasons for these differences are not clear. Nevertheless, these data highlight the importance of testing different formulations for the purpose of vaccine selection.

Both homologous and heterologous vaccine protocols elicited specific antibody titers that were as high as those elicited following immunization with recombinant proteins emulsified in CFA/IFA. In addition, the antibody responses induced by both protocols were long lasting, and significant titers were observed during the ~180 day period of the study. We have focused our efforts on inducing persistently high titers of specific antibodies against the CSP antigen. Antibodies against CSP have been described previously as a critical mechanism of anti-sporozoite immunity in diverse malaria models (reviewed ref. 1). Based on Phase II human trials performed with the RTS,S/AS candidate vaccine, antibodies continue
to be the factor that strongly correlates with sterile immunity. In RTS,S/AS vaccinated individuals who are experimentally challenged with *P. falciparum* sporozoites by mosquito bite, vaccine efficacy was correlated with higher antibody titers against CSP and sporozoites (2). Similarly, Phase II vaccine trials performed with African children reported a non-linear relationship between the antibody titers against CSP and a lower incidence of malaria (25).

Several anti-parasitic mechanisms have been described that are mediated by antibodies against CSP. First, antibodies reduce the sporozoite inoculum and parasite viability during a mosquito bite. Using an experimental rodent model, it has been shown that an immune complex formed during the mosquito bite reduces the number of sporozoites deposited during feeding (26). The parasites injected into the immune mice were immobilized and not capable of invading dermal blood vessels (26). When the sporozoites are inoculated i.v., therefore bypassing the skin, anti-CSP antibodies efficiently block infection of hepatocytes (26). The inhibition of the hepatocyte infection may be due to reduction of the sporozoites’ ability to move or attach to hepatocytes (27-29).

The specificity of the inhibitory antibodies for the malaria CSP antigen was initially assigned to the central repeat domain (30). In the case of *P. vivax*, this postulate was recently confirmed by the use of a transgenic *P. berghei* sporozoite expressing the repeat domain from the VK210 strain. *In vivo* parasite infectivity in mice was drastically inhibited by passive transfer of a MAb against the VK210 repeats (31). These observations further confirm similar data from other parasites, which showed that the repeat domain of CSP is a target of neutralizing antibodies.
In addition to the repeat domain, in the case of the *P. vivax* CSP protein, humans immunized with long synthetic peptides representing the N- and C-terminus of CSP generated antibodies that can inhibit sporozoite invasion *in vitro* (32). These results indicate that vaccines that elicit high antibody titers against different regions of PvCSP should be pursued. In the vaccination protocols described here, we generated high antibody titers against all three allelic forms of the PvCSP protein that reacted with different domains of the protein.

IIA analyses using sporozoites from the *P. vivax* strains VK210 and VK247 demonstrated that these vaccine-elicited antibodies can recognize the native CSP on sporozoites. However, the ability of these antibodies to neutralize sporozoites *in vivo* has yet to be evaluated using transgenic parasites (31).

In addition to antibodies, CD4+ and CD8+ T cells have been described as powerful mediators of pre-erythrocytic stages elimination in rodent experimental models (reviewed ref. 32-38). In fact, a number of T cell epitopes have been described by us and others in CSP (reviewed ref. 32-38). The results in the mouse model have fueled a number of human trials using plasmid DNA, recombinant viruses and, more recently, radiation-attenuated sporozoites as vaccine formulations. Unfortunately, in spite of the enthusiasm generated by the mouse model, T cell-based vaccines against pre-erythrocytic stages of malaria have only provided sterile immunity against infection with *P. falciparum* to few vaccinated individuals to date (39-44). The precise reason for this failure is not clear. Perhaps the frequency of T cells generated by the different vaccine protocols was not sufficient, as these T cells have to patrol the entire human liver. In fact, since the
first reports of adoptive T cell transfer more than 20 years ago, it has been known that large numbers of multifunctional effector CD8+ T cells must be transferred to a mouse to provide sterile immunity (45-47). More recently, elegant studies in the mouse model have confirmed that a large number of multifunctional effector CD8+ T cells must be generated to provide sterile protection (48).

The development of vaccine candidates against pre-erythrocytic stages of *P. vivax* has been performed mostly in pre-clinical immunization models (mice and non-human primates, 49-51). Only three phase I clinical trial against this parasite has been reported using recombinant proteins or long synthetic peptides (52-54).

The reagents and protocols we described in the present study, when used in homologous or heterologous regimens, can be immediately transferred to humans. Poly(ICLC) is currently being tested for human use (23). Similarly, recombinant human replication-defective adenoviruses of serotype 5 are being used in multiple human trials (55-57).

The fact that we did not detect significant differences in the antibody titers or cell-mediated immunity using either the homologous or heterologous vaccination regimens should not be considered as a failure for the second. Considering that our ultimate goal is human trials, the use of recombinant adenoviruses vector may still improve the immune response by the presence of human T-cell epitopes. Based on that, we consider that the formulations described here provide new *P. vivax* vaccine candidates with universal coverage that warrant further clinical testings.
Acknowledgments

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Conflict of interest: LHT, CAT, MOL, OBR, ISS, RSN, HE, VN and MMR are named inventors on patent applications entitled “PLASMODIUM VIVAX VACCINE COMPOSITIONS” File reference no.:27522-0201WO1.

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Figure legends

Figure 1. Production and purification of the *P. vivax* CSP recombinant proteins.

(A) Schematic representation of the *P. vivax* CSP antigen and the sequences of the repeat regions present in each allelic form (PvCSP-VK210, PvCSP-VK247 and PvCSP-Vivax-like).

(B) The predicted AA sequence of each recombinant protein.

(C) Recombinant proteins were purified by affinity purification followed by anion exchange and separated by SDS-PAGE under reducing conditions. Lanes: a - His$_6$-PvCSP-VK210; b - His$_6$-PvCSP-VK247; c - His$_6$-PvCSP-Vivax-like; d - His$_6$-PvCSP-All-CSP-epitopes. Each lane was loaded with 1 µg of protein.

Figure 2. Antibody recognition of the *P. vivax* CSP recombinant proteins.

ELISAs were performed using each of the four PvCSP recombinant proteins bound to the plates to determine whether the polypeptides retained the epitopes recognized by specific MAbs generated against radiation-attenuated *P. vivax* sporozoites. (A) MAb against the His tag, (B) MAb 2F2 specific to the allelic form PvCSP-VK210; (C) MAb 2E10.E9 specific to the allelic form PvCSP-VK247; or (D) polyclonal antibodies to the PvCSP-Vivax-like repeat region.

Figure 3. Induction of specific antibody responses in mice immunized with recombinant *P. vivax* CSP antigens.

(A) Mice were immunized with three doses 21 days apart, and the antibody titers were analyzed according to the timeline shown. The serum IgG responses to...
P. vivax CS antigens were determined in C57BL/6 mice immunized s.c. with the purified proteins in a formulation containing the adjuvant poly(I:C) (B – D) or emulsified in complete or incomplete Freund’s adjuvant (CFA/IFA) (E – G). Mice were immunized with His$_6$-PvCSP-VK210 (10 µg/dose/mouse), His$_6$-PvCSP-VK247 (10 µg/dose/mouse), His$_6$-PvCSP-Vivax-like (10 µg/dose/mouse), a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like- 30 µg/dose/mouse), or His$_6$-PvCSP-All-CS-epitopes (30 µg/dose/mouse). Serum IgG antibody titers against recombinant proteins representing each allelic form of PvCSP were measured as indicated. The results are expressed as the means ± SD (n=6). Statistical comparisons using a One-Way ANOVA of the antibody titers from mice immunized with formulations containing the adjuvant poly(I:C) (B to D) show significantly higher antibody levels in animals immunized with the protein mix (p<0.01, asterisks). Comparisons of the antibody titers from mice immunized with formulations containing the adjuvant CFA/IFA and recombinant proteins did not show significant differences (p=NS).

Figure 4. Induction of specific antibody responses in mice immunized with recombinant P. vivax CSP antigens.

C57BL/6 mice were immunized with doses of 30 or 3 µg/dose/mouse of a formulation containing a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like) in the presence of adjuvant poly(I:C). The immunizations were s.c. with three doses 21 days apart according to the timeline described in the legend of Fig. 3A. Serum IgG antibody titers against recombinant proteins
representing each allelic form of PvCSP were measured as indicated. Mice immunized with 30 (inverted triangles) and 3 µg/dose/mouse (squares) of the protein mix responded equally well according to statistical comparisons (One-Way ANOVA, p=NS). The results as expressed as the means ± S.D. (n=6).

**Figure 5.** Induction of specific antibody responses in tlr4⁻/⁻ mice immunized with recombinant *P. vivax* CS antigens. Mice were immunized with three doses 21 days apart according to the protocol and timeline described in the legend of Fig. 3A. The serum IgG responses against *P. vivax* CSP recombinant proteins were determined in *Tlr4*⁺/⁺ and *Tlr4*⁻/⁻ mice immunized with formulations containing poly(I:C) and PvCSP mix (His₆-PvCSP-VK210 + His₆-PvCSP-VK247 + His₆-PvCSP-Vivax-like - 3 µg/dose/mouse) or control recombinant FliC (10 µg/dose/mouse). *tlr4*⁻/⁻ (inverted triangles) and *tlr4*⁺/⁺ (diamonds) mice immunized with either PvCSP protein mix responded equally well according to statistical comparisons (One-Way ANOVA, p=NS). The results are expressed as the means ± S.D. (n=6).

**Figure 6.** Induction of specific antibody responses in mice immunized with recombinant adenoviruses expressing the *P. vivax* CSP as part of a heterologous prime-boost regimen of vaccination. (A and B) Schematic representations and the deduced AA sequences of the PvCSP-All-CSP-epitopes expressed by recombinant replication-defective simian or human adenoviruses.
C) C57BL/6 mice were immunized i.m. with $2 \times 10^7$ pfu/mouse of AdHu5-βgal (control), AdC68-PvCSP or AdHu5-PvCSP. Serum IgG titers were determined 21 days later against recombinant proteins of each allelic form of PvCSP as indicated. Mice immunized with AdC68-PvCS and AdHu5-PvCS responded equally well according to statistical comparisons (One-Way ANOVA, $p=NS$). The results are expressed as the means ± S.D. (n=5).

D) Mice were boosted s.c. 42 days after priming with a formulation containing a protein mix (His$_6$-PvCSP-VK210 + His$_6$-PvCSP-VK247 + His$_6$-PvCSP-Vivax-like - 3 µg/dose/mouse) in the presence of poly(I:C). The titers of specific antibodies to the different allelic forms of PvCSP were measured at day 63. According to statistical comparisons (One-Way ANOVA, Tukey HSD), mice immunized with AdC68-PvCS and AdHu5-PvCS and boosted with a protein mix (G5 and G6) had serum IgG antibody titers higher than the other groups as denoted in the figure.

Figure 7. Induction of specific antibody responses in mice immunized with heterologous (adenovirus/protein) or homologous (protein/protein) regimens of vaccination.

(A) C57BL/6 mice were immunized with three doses of the indicated immunogens, and the antibody titers were analyzed according to the timeline. The first dose consisted of $2 \times 10^7$ pfu/mouse of AdHu5-βgal (control), AdC68-PvCSP or AdHu5-PvCSP administered i.m. The first and second boosts consisted of a formulation containing a protein mix (His$_6$-
PvCSP-VK210 + His\textsubscript{6}-PvCSP-VK247 + His\textsubscript{6}-PvCSP-Vivax-like - 3

µg/dose/mouse) in the presence of poly (I:C).

(B) Serum IgG titers against His\textsubscript{6}-PvCSP-VK210 were determined.

(C) Serum IgG titers against His\textsubscript{6}-PvCSP-VK247 were determined.

(D) Serum IgG titers against His\textsubscript{6}-PvCSP-Vivax-like were determined.

Mice immunized with AdHu5-βgal, AdC68-PvCS and AdHu5-PvCS and boosted twice with a formulation containing a protein mix in the presence of poly (I:C) (G4, G5 and G6) responded equally well according to statistical comparisons (One-Way ANOVA, Tukey HSD, p=NS). The results are expressed as the means ± S.D. (n=5).

**Figure 8. Specificity of the antibodies elicited during homologous (protein/protein) or heterologous (adenovirus/protein) regimens.**

(A) Schematic representation of each recombinant protein used as a substrate bound to the plates in the ELISA.

(B) Serum IgG antibodies from mice immunized with the homologous (protein/protein with poly(I:C) or heterologous (AdC68-PvCSP/protein mix) regimens. The detailed immunization protocol is described in the legends of Fig. 3 (homologous) and Fig. 7 (heterologous). Sera were collected two weeks after the third dose. The results are expressed as the means ± S.D (n=5).
Figure 9. *Indirect immunofluorescence assay for the recognition of the native protein PvCSP.*

Pools of sera from mice immunized with the homologous (protein/protein with poly(I:C)) or heterologous (AdC68-PvCSP/protein mix) regimens were used. The detailed immunization protocol is described in the legends of Fig. 3 (homologous) or Fig. 7 (heterologous).

Figure 10. *Cell-mediated immunity of mice immunized with recombinant *P. vivax* CSP antigens.*

A) C57BL/6 mice were immunized with three doses, 14 days apart, and the ICS analyses were performed according to the timeline shown.

B) Twenty six overlapping peptides spanning the entire sequence of the N or C-terminal of PvCSP were used to stimulate splenocytes.

C and D) G1 mice (control) were primed i.m. with $2 \times 10^8$ pfu/mouse of AdHu5-b-gal and boosted s.c. twice with Poly(I:C). G2 mice were primed i.m. with $2 \times 10^8$ pfu/mouse of AdC68-PvCSP and boosted twice with a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like- 30 µg/dose/mouse), in the presence of Poly(I:C). G3 mice were primed and boosted with a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like- 30 µg/dose/mouse).

Fourteen days later, the splenic cells of these mice were cultured in the presence of anti-CD28, monensin and brefeldin-A with or without the peptides or recombinant proteins or ConA as indicated. Peptides PvCS-VK210, PvCS-VK247 or PvCS-Vivax-like represent the repeat regions of each allelic variant. After 12 h,
cells were stained with anti-CD4, anti-CD8, anti-IL-2, anti-IFN-γ, and anti-TNF-α. The results are expressed as the total frequency (%) of CD4⁺ or CD8⁺ cells stained for each molecule. Results were obtained from pooled cells from 5 mice.

Figure 11. Cell-mediated immunity of mice immunized with recombinant P. vivax CSP antigens. C57BL/6 mice were immunized as described in the legend of Fig. 10. Splenic cells of these mice were cultured in the presence or absence of the peptides or ConA as indicated. Peptides PvCS-VK210, PvCS-VK247 or PvCS-Vivax-like represent the repeat regions of each allelic variant. After 48 h, IFN-γ secreting cells (spot forming cells, SFC) were estimated by ELISpot assay. The results are expressed as frequency of SFC per 10⁶ spleen cells obtained from pooled cells from 5 mice. Asterisk denotes statistically significant difference (P<0.01).
Figure 1. **Production and purification of the *P. vivax* CSP recombinant proteins.**

(A) Schematic representation of the *P. vivax* CS antigen and the sequences of the repeat regions present in each allelic form (PvCSP-VK210, PvCSP-VK247 and PvCSP-Vivax-like).

(B) The predicted AA sequence of each recombinant protein.

(C) Recombinant proteins were purified by affinity purification followed by anion exchange and separated by SDS-PAGE under reducing conditions. Lanes: a - His<sub>6</sub>-PvCSP-VK210; b - His<sub>6</sub>-PvCSP-VK247; c - His<sub>6</sub>-PvCSP-Vivax-like; d - His<sub>6</sub>-PvCSP-All-CSP-epitopes. Each lane was loaded with 1 µg of protein.
Figure 2

Figure 2. Antibody recognition of the *P. vivax* CS recombinant proteins. ELISAs were performed using each of the four PvCSP recombinant proteins bound to the plates to determine whether the polypeptides retained the epitopes recognized by specific MABs generated against radiation-attenuated *P. vivax* sporozoites. (A) MAb against the His tag, (B) MAb 2F2 specific to the allelic form PvCSP-VK210; (C) MAb 2E10.E9 specific to the allelic form PvCSP-VK247; or (D) polyclonal antibodies to the PvCSP-Vivax-like repeat region.
Figure 3

Primming First Boosting Second boosting
Analysis Analysis Analysis

Days 0 21 42 63 93 123 183

B

Serum IgG titers

Antibodies to His_6[PvCSP-VK210] 10^3 10^4 10^5 10^6

Days 50 100 150 200

C

Serum IgG titers

Antibodies to His_6[PvCSP-VK247] 10^3 10^4 10^5 10^6

Days 50 100 150 200

D

Serum IgG titers

Antibodies to His_6[PvCSP-Vivax-like] 10^3 10^4 10^5 10^6

Days 50 100 150 200

E

Serum IgG titers

Antibodies to His_6[PvCSP-VK210] 10^3 10^4 10^5 10^6

Days 50 100 150 200

F

Serum IgG titers

Antibodies to His_6[PvCSP-VK247] 10^3 10^4 10^5 10^6

Days 50 100 150 200

G

Serum IgG titers

Antibodies to His_6[PvCSP-Vivax-like] 10^3 10^4 10^5 10^6

Days 50 100 150 200

Antigens used for immunizations
- Adjuvant only
- Single Protein
- Protein Mix
- His_6[PvCSP-All CS epitopes]

Adjuvants used for immunizations:
- B – D poly (I:C)
- E – G CFA/IFA
Figure 3. Induction of specific antibody responses in mice immunized with recombinant P. vivax CSP antigens.

(A) Mice were immunized with three doses 21 days apart, and the antibody titers were analyzed according to the timeline shown. The serum IgG responses to P. vivax CS antigens were determined in C57BL/6 mice immunized s.c. with the purified proteins in a formulation containing the adjuvant poly(I:C) (B – D) or emulsified in complete or incomplete Freund’s adjuvant (CFA/IFA) (E – G). Mice were immunized with His$_6$-PvCSP-VK210 (10 µg/dose/mouse), His$_6$-PvCSP-VK247 (10 µg/dose/mouse), His$_6$-PvCSP-Vivax-like (10 µg/dose/mouse), a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like- 30 µg/dose/mouse), or His$_6$-PvCSP-All-CS-epitopes (30 µg/dose/mouse). Serum IgG antibody titers against recombinant proteins representing each allelic form of PvCSP were measured as indicated. The results are expressed as the means ± SD (n=6). Statistical comparisons using a One-Way ANOVA of the antibody titers from mice immunized with formulations containing the adjuvant poly(I:C) (B to D) show significantly higher antibody levels in animals immunized with the protein mix (p<0.01, asterisks). Comparisons of the antibody titers from mice immunized with formulations containing the adjuvant CFA/IFA and recombinant proteins did not show significant differences (p=NS).
Figure 4. *Induction of specific antibody responses in mice immunized with recombinant *P. vivax* CSP antigens.*

C57BL/6 mice were immunized with doses of 30 or 3 µg/dose/mouse of a formulation containing a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like) in the presence of adjuvant poly(I:C). The immunizations were s.c. with three doses 21 days apart according to the timeline described in the legend of Fig 3A. Serum IgG antibody titers against recombinant proteins representing each allelic form of PvCSP were measured as indicated. Mice immunized with 30 (inverted triangles) and 3 µg/dose/mouse (squares) of the protein mix responded equally well according to statistical comparisons (One-Way ANOVA, p=NS). The results as expressed as the means ± S.D. (n=6).
Figure 5. Induction of specific antibody responses in tlr4−/− mice immunized with recombinant P. vivax CSP antigens.

Mice were immunized with three doses 21 days apart according to the protocol and timeline described in the legend of Fig. 3A. The serum IgG responses against P. vivax CSP recombinant proteins were determined in tlr4+/+ and tlr4−/− mice immunized with formulations containing poly(I:C) and PvCSP mix (His6-PvCSP-VK210 + His6-PvCSP-VK247 + His6-PvCSP-Vivax-like - 3 µg/dose/mouse) or control recombinant FliC (10 µg/dose/mouse). tlr4+/+ (inverted triangles) and tlr4−/− (diamonds) mice immunized with either PvCSP protein mix responded equally well according to statistical comparisons (One-Way ANOVA, p=NS). The results are expressed as the means ± S.D. (n=6).
Figure 6

A

B

C

D

**Figure 6.** Induction of specific antibody responses in mice immunized with recombinant adenoviruses expressing the P. vivax CSP as part of a heterologous prime-boost regimen of vaccination.

(A and B) Schematic representations and the deduced AA sequences of the PvCSP-All-CS-epitopes expressed by recombinant replication-defective simian or human adenoviruses.

C) C57BL/6 mice were immunized i.m. with 2x10^7 pfu/mouse of AdHu5-βgal (control), AdCh68-PvCSP or AdHu5-PvCSP. Serum IgG titers were determined 21 days later against recombinant proteins of each allelic form of PvCSP as indicated. Mice immunized with AdC68-PvCS and AdHu5-PvCS responded equally well according to statistical comparisons (One-Way ANOVA, p=NS). The results are expressed as the means ± S.D. (n=5).

D) Mice were boosted s.c. 42 days after priming with a formulation containing a protein mix (His_p-PvCSP-VK210 + His_p-PvCSP-VK247 + His_p-PvCSP-Vivax-like - 3 μg/dose/mouse) in the presence of poly (I:C). The titers of specific antibodies to the different allelic forms of PvCSP were measured at day 63. According to statistical comparisons (One-Way ANOVA, Tukey HSD), mice immunized with AdCh68-PvCS and AdHu5-PvCS and boosted with a protein mix (G5 and G6) had serum IgG antibody titers higher than the other groups as denoted in the figure.
Figure 7

A

Days

0  22  42  64  85  114  174

Priming   Analysis  First Boosting  Analysis  Second boosting  Analysis  Analysis  Analysis

B

Antibodies to His₆PvCSP-VK210

Predicted IgG titers

Days

0  20  40  60  80  100  120  140  160  180  200

10²  10³  10⁴

C

Antibodies to His₆PvCSP-VK247

Predicted IgG titers

Days

0  20  40  60  80  100  120  140  160  180  200

10²  10³  10⁴

D

Antibodies to His₆PvCSP-Vivax-like

Predicted IgG titers

Days

0  20  40  60  80  100  120  140  160  180  200

10²  10³  10⁴

Priming / Boosting

- G1: AdHu5-βgal / Adjuvant
- G2: AdC68-PvCSP / Adjuvant
- G3: AdHu5-PvCSP / Adjuvant
- G4: AdHu5-βgal / Protein Mix
- G5: AdC68-PvCSP / Protein Mix
Figure 7. Induction of specific antibody responses in mice immunized with heterologous (adenovirus/protein) or homologous (protein/protein) regimens of vaccination.

A) C57BL/6 mice were immunized with three doses of the indicated immunogens, and the antibody titers were analyzed according to the timeline. The first dose consisted of $2 \times 10^7$ pfu/mouse of AdHu5-βgal (control), AdC68-PvCSP or AdHu5-PvCSP administered i.m. The first and second boosts consisted of a formulation containing a protein mix (His$_6$-PvCSP-VK210 + His$_6$-PvCSP-VK247 + His$_6$-PvCSP-Vivax-like - 3 μg/dose/mouse) in the presence of poly (I:C).

B) Serum IgG titers against His$_6$-PvCSP-VK210 were determined.

C) Serum IgG titers against His$_6$-PvCSP-VK247 were determined.

D) Serum IgG titers against His$_6$-PvCSP-Vivax-like were determined.

Mice immunized with AdHu5-βgal, AdC68-PvCS and AdHu5-PvCS and boosted twice with a formulation containing a protein mix in the presence of poly (I:C) (G4, G5 and G6) responded equally well according to statistical comparisons (One-Way ANOVA, Tukey HSD, p=NS). The results are expressed as the means ± S.D. (n=5).
Figure 8

(A) Schematic representation of each recombinant protein used as a substrate bound to the plates in the ELISA.

(B) Serum IgG antibodies from mice immunized with the homologous (protein-protein with poly(I:C) or heterologous (AdC68-PvCSP/protein mix) regimens. The detailed immunization protocol is described in the legends of Fig. 3 (homologous) and Fig. 7 (heterologous). Sera were collected two weeks after the third dose. The results are expressed as the means ± S.D (n=5).

Figure 8. Specificity of the antibodies elicited during homologous (protein-protein) or heterologous (adenovirus-protein) regimens.

(A) Schematic representation of each recombinant protein used as a substrate bound to the plates in the ELISA.

(B) Serum IgG antibodies from mice immunized with the homologous (protein-protein with poly(I:C) or heterologous (AdC68-PvCSP/protein mix) regimens. The detailed immunization protocol is described in the legends of Fig. 3 (homologous) and Fig. 7 (heterologous). Sera were collected two weeks after the third dose. The results are expressed as the means ± S.D (n=5).
Figure 9. *Indirect immunofluorescence assay for the recognition of the native protein PvCSP.*

Pools of sera from mice immunized with the homologous (protein-protein with poly(I:C)) or heterologous (AdC68-PvCSP/protein mix) regimens were used. The detailed immunization protocol is described in the legends of Fig. 3 (homologous) or Fig. 7 (heterologous).
Figure 10

A

Priming First
First Boosting
Second Boosting
Analysis
Days 0 14 28 42

B

AA Repeats
G1 G2 G3

CD
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13

TKTSSLVLKLQLQVQINMQKGAIGDGLSLGFDIVIAIASAIKQG<br>THCGHNVDLSKAINLNGVNFNNVDASSLGAAHVGQSASRGRGLGENPD<br>DEEGDAKKKKDGKKAEPKNPRENKLPQ


NH₂

G0 G1 G2 G3 G4 G5

P14 G0 G1 G2 G3 G4 G5

COOH

C

CD4+ cells
IFN-γ+

TNF+

IL-2+

% of total CD4+ cells

D

CD8+ cells
IFN-γ+

TNF+

IL-2+

% of total CD8+ cells

protein repeats
protein repeats
Figure 10. Cell-mediated immunity of mice immunized with recombinant *P. vivax* CSP antigens. A) C57BL/6 mice were immunized with three doses, 14 days apart, and the ICS analyses were performed according to the timeline shown. B) Twenty six overlapping peptides spanning the entire sequence of the N or C-terminal of PvCSP were used to stimulate splenocytes. C and D) G1 mice (control) were primed i.m. with $2 \times 10^8$ pfu/mouse of AdHu5-b-gal and boosted s.c. twice with Poly(I:C). G2 mice were primed i.m. with $2 \times 10^8$ pfu/mouse of AdC68-PvCSP and boosted twice with a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like $30 \mu$g/dose/mouse), in the presence of Poly(I:C). G3 mice were primed and boosted with a protein mix (His$_6$-PvCSP-VK210/His$_6$-PvCSP-VK247/His$_6$-PvCSP-Vivax-like $30 \mu$g/dose/mouse). Fourteen days later, the splenic cells of these mice were cultured in the presence of anti-CD28, monensin and brefeldin-A with or without the peptides or recombinant proteins or ConA as indicated. Peptides PvCS-VK210, PvCS-VK247 or PvCS-Vivax-like represent the repeat regions of each allelic variant. After 12 h, cells were stained with anti-CD4, anti-CD8, anti-IL-2, anti-IFN-γ, and anti-TNF-α. The results are expressed as the total frequency (%) of CD4$^+$ or CD8$^+$ cells stained for each molecule. Results were obtained from pooled cells from 5 mice.
Figure 11. Cell-mediated immunity of mice immunized with recombinant P. vivax CSP antigens. C57BL/6 mice were immunized as described in the legend of Fig. 10. Splenic cells of these mice were cultured in the presence or absence of the peptides or ConA as indicated. Peptides PvCS-VK210, PvCS-VK247 or PvCS-Vivax-like represent the repeat regions of each allelic variant. After 48 h, IFN-γ secreting cells (spot forming cells, SFC) were estimated by ELispot assay. The results are expressed as frequency of SFC per 10^6 spleen cells obtained from pooled cells from 5 mice. Asterisk denotes statistically significant difference (P<0.01).