

Generation, characterization and immunogenicity of a novel chimeric recombinant protein based on *Plasmodium vivax* AMA-1 and MSP1¹⁹

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ABSTRACT

Plasmodium vivax is the most widely distributed malaria species and the most prevalent species of malaria in America and Asia. Vaccine development against *P. vivax* is considered a priority in the global program for the eradication of malaria. Earlier studies have characterized the Apical Membrane Antigen 1 (AMA-1) ectodomain and the C-terminal region (19 kDa) of the Merozoite Surface Protein 1 (MSP-1) of *P. vivax* as immunodominant antigens. Based on this characterization, we designed a chimeric recombinant protein containing both merozoite immunodominant domains (*PvAMA1₆₆-MSP1₁₉*). The recombinant *PvAMA1₆₆-MSP1₁₉* was successfully expressed in *Pichia pastoris* and used to immunize two different mouse strains (BALB/c and C57BL/6) in the presence of the Poly (I:C) as an adjuvant. Immunization with the chimeric protein induced high antibody titers against both proteins in both strains of mice as detected by ELISA. Antisera also recognized the native proteins expressed on the merozoites of mature *P. vivax* schizonts. Moreover, this antigen was able to induce IFN-gamma-secreting cells in C57BL/6 mice. These findings indicate that this novel yeast recombinant protein containing *PvAMA1₆₆* and *PvMSP1₁₉* is advantageous, because of improved antibody titers and cellular immune response. Therefore, this formulation should be further developed for pre-clinical trials in non-human primates as a potential candidate for a *P. vivax* vaccine.

Keywords: malaria, *Plasmodium vivax*, recombinant vaccine.

Highlights:

- A novel chimeric recombinant protein was produced as a malaria immunogen
- This vaccine candidate was highly immunogenic in mice
- The protein potentially offers benefits over the use of each antigen alone or mixed

INTRODUCTION

Over the last decade, renewed efforts have been directed towards the development of an effective *P. falciparum* vaccine. The lead candidate; RTS,S/AS01, has undergone extensive clinical trials, and has shown to be protective against severe *falciparum* malaria [1]. In contrast, the advanced development of vaccines against *vivax* malaria has been neglected, with almost all candidates are still in the early stages of preclinical testing [2]. The general lack of urgency behind *vivax* malaria vaccine development was partly due to the misconception, that *vivax* malaria is relatively benign. This 'benign *vivax* malaria' view has been challenged by a number of studies showing *P. vivax* infection may result in severe clinical complications, with appreciable levels of mortality [3,4]. The increased awareness of severe infections, along with increasing reports of drug resistant *vivax* malaria [5] have spurred efforts to reinvigorate the development of vaccines targeting *P. vivax*.

Most of the *P. vivax* vaccine development effort has targeted its erythrocytic development cycle; specifically proteins on the surface of merozoites involved in the invasion of reticulocytes [6]. Perhaps one of the most promising invasion antigens being considered for further vaccine development are, Apical Membrane Antigen 1 (AMA-1) and the C-terminal of Merozoite Surface Protein 1 (MSP-1) [7,8].

Even if relatively conserved vaccine targets are identified; the complexity of the reticulocyte invasion sequence and rapid evolution of new antigenic variants warrants the development malaria vaccine containing more than one antigen. One useful method for the development of a multi-antigenic vaccine is the construction of a chimeric protein; which is particularly advantageous as it only requires manufacture of a single biotechnological product. A number of chimeric proteins have been developed as malaria vaccine candidates [6], including the *P. falciparum* MSP1-AMA1 (domain III) protein produced in *Pichia pastoris*. This chimeric antigen was highly immunogenic in rabbits and monkeys [9,10] and well tolerated in humans [11].

As *P. vivax* MSP1₁₉ and AMA1₆₆ have shown significant immunogenicity as single antigen vaccines [12–17]; our aim was to combine these promising candidates in a novel chimeric recombinant protein using the yeast *P. pastoris*.

METHODS

Ethics statement

Blood samples were previously collected with the written informed consent of all study participants enrolled in a study approved by the Ethics Committee of the Faculty of Medicine of São José do Rio Preto, Brazil (CEP No. 2438/2011 and 4599/2011), and the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2008-215). The protocol was approved by the Committee on the Ethics of Animal Experiments of the School of Pharmaceutical Sciences of University of São Paulo, Brazil (CEUA No. 279/2010).

Synthesis of the *pvama1₆₆-msp1₁₉* gene

A recombinant protein comprising the ectodomain of AMA-1 (AMA1₆₆) (GenBank KJ010958) fused to the C-Terminal 19 kDa region of MSP-1, named MSP1₁₉ (GenBank M60807.1) was designed. The gene *pvama1₆₆-msp1₁₉* was synthesized by GenScript USA Inc. with codon optimization to improve expression in *P. pastoris*. Four potential N-glycosylation sites were altered; three were in the AMA-1 sequence as previously described [17] and the other one was in MSP1₁₉ sequence, where a substitution was performed to prevent unwanted glycosylation (494 N→D). A carboxyl-terminal Histidine-tag (His₆) to enable purification was added to the construction.

Cloning and yeast expression of *PvAMA1₆₆-MSP1₁₉*

The synthetic gene was inserted into the pPIC9K *P. pastoris* expression vector (Invitrogen, CA, USA). This vector contains the α -factor signal peptide for protein secretion. The plasmid pPIC9K-*pvama1₆₆-msp1₁₉* was linearized to transform the *P. pastoris* GS115 strain by electroporation according to the manufacturer's instructions. A clone His⁺ secreting high levels of *PvAMA1₆₆-MSP1₁₉* was selected.

The expression of the recombinant *PvAMA1₆₆-MSP1₁₉* was performed as previously described [17]. The protein was purified by Ni-affinity with Histrap FF and ion exchange chromatography with a Q FF resin, respectively, coupled to an ÄKTA prime plus (all purchased from GE Healthcare). The purity was confirmed by RP-HPLC using a Phenomenex Jupiter C₁₈ column (4.6 mm × 250 mm, 5 μ m particle and pore 300 Å) on a Shimadzu Prominence HPLC System Solution (Shimadzu JPN Corp., Kyoto, KY).

Immunoblotting assay for *PvAMA1*₆₆-*MSP1*₁₉ detection

For this analysis, the protein fractions were transferred from a 12% SDS-PAGE gel to a nitrocellulose membrane and incubated with a mouse monoclonal antibody (MAb) anti-His₆ (GE Healthcare) at a final dilution of 1:1,000 or with a MAb against *PvMSP1*₁₉ K₂₄ (1:1,500) [15] or with a MAb against *PvAMA-1* domain II K₂₄₃ (1:1,500) [18]. The fractions were visualized after reaction using a chemiluminescence detection assay (ECL, GE Healthcare).

Secondary structural evaluation of *PvAMA1*₆₆-*MSP1*₁₉

To evaluate the secondary structure of the chimeric protein, circular dichroism (CD) was performed using a JASCO-J720 spectropolarimeter. The methodology used was previously described [17,19] and the secondary structure was estimated by CDNN software analysis (Applied Photophysics Ltd).

ELISA for detection of human antibodies to *PvAMA1*₆₆-*MSP1*₁₉

Serum samples were collected from 111 *P. vivax*-infected individuals living in different endemic areas in the North of Brazil. The detection of total human IgG antibodies against *PvAMA-1*, *PvMSP1*₁₉ and *PvAMA1*₆₆-*MSP1*₁₉ was performed by ELISA, as previously described [12,17]. The cutoff was determined using three standard deviations above the mean OD₄₉₂ from sera of 16 healthy individuals who were never exposed to malaria [17].

Mouse immunization regimen

For all of the experiments, groups of six female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice at 6-8 weeks old were used. The immunization was performed via the subcutaneous (s.c.) route with 10 µg of chimeric protein in the presence of 50 µg of polyinosinic-polycytidylic acid adjuvant [(Poly I:C), Invivogen)]/dose, three times, 15 days apart. Mice were immunized with *PvAMA*₁₆₆ or *PvMSP*₁₁₉ individually, or they were co-administered with both recombinant proteins (10 µg of each protein). The controls received only adjuvant emulsified in PBS.

ELISA for detection of mouse antibodies to *PvAMA*₁₆₆-*MSP*₁₁₉

Total IgG antibodies against *AMA*₁₆₆ and/or *MSP*₁₁₉ in mouse sera were detected by ELISA 14 days after immunization, as described previously [17]. The anti-*PvAMA*₁₆₆-*MSP*₁₁₉ titers were determined as the highest dilutions presenting OD₄₉₂ values higher than 0.1. The results are expressed as the means of the IgG titers (log₁₀) ± SEM.

Recognition of native protein by indirect immunofluorescence assay

Immunofluorescence assays were performed as previously described [20]. The blood samples were collected from malaria patients infected by *P. vivax* (Mae Sot region, in Northwestern Thailand). These *ex vivo* samples were processed using the methods outlined in Russell et al 2012 [21]. Binding was visualized using an Olympus BX60 after the slides were rinsed in distilled water and mounted in Fluor save (Calbiochem).

CFSE labeling, expansion and surface staining

Mice splenocytes (50×10^6 cells) were labeled for 10 min with 1.25 mM succinimidyl ester carboxy fluorescein diacetate [(CFSE), Invitrogen] in PBS (37°C). CFSE-labeled cells (3×10^5 cells/well) were expanded with antigen-specific (recombinant proteins MSP1₁₉ or AMA1₆₆, 10 µg/mL) or mitogen-concavalin A (ConA, 2.5 µg/mL, Sigma) for 5 days at 37°C in 5% CO₂. Expanded cells were stained with antibodies coupled to fluorochromes [CD3/APC-CY7, CD4/PerCP-CY5.5, CD8/PE-CY7 (BD Biosciences)] diluted in MACS buffer [BSA (0.5%) (m/v), EDTA (2 mM) PBS, (pH= 7.4)]. Flow-cytometric acquisition was performed with a FACSCanto II (BD, Biosciences) and analyses were done using FlowJo® software (version 9.0.6; Tree Star). A minimum of 200,000 events were acquired, and CD4⁺ and CD8⁺ T cells were selected from lymphocyte population gates. The proliferating cells were identified as CFSE^{low}.

IFN-γ ELISPOT

Mouse IFN-γ capture antibody (BD Biosciences) was used to coat flat-bottom Multiscreen HTS plates (Millipore) overnight at 4°C. After 3 washes with PBS-T the plates were blocked with R10 [(fetal calf serum (10%) (v/v), RPMI 1640), Gibco], for 2 hours at 37°C and 5×10^5 cells/well mice splenocytes were stimulated overnight at 37°C, 5% CO₂, in the presence of antigen-specific (MSP1₁₉ or AMA1₆₆, 10 µg/mL) or ConA (2.5 µg/mL) diluted in RPMI 1640-IL2 [IL-2 0.03% (v/v)]. Plates were then washed 3 times with PBS, and mouse IFN-γ detection antibody biotinylated [XMG 1.2, (1:200), Pharmingen] was added to the plates overnight at 4°C. Streptavidin-labeled peroxidase [(1:500), BD, Biosciences] was added to the plates, after 6 PBS-T washes, for 2 hours and spots were visualized with 4',6-diamidino-2-phenylindole, dihydrochloride (20 mg).

Cytometric bead array (CBA) analyses of cytokines

Cytokine levels secreted on cell culture supernatant stimulated for 24 hours with the recombinant proteins MSP1₁₉ or AMA1₆₆ (10 µg/mL) or ConA (2.5 µg/mL), were determined using a mouse Th1/Th2/Th17 cytometric bead array kit (CBA; BD Biosciences) on a FACSCanto II flow cytometer in accordance with manufacturer's instructions and analysis was performed by FCAP Array™ software.

Statistical analysis

Differences between the proportions of responder individuals were analyzed using a Chi-square test with Yates correction. Comparison of antibody levels (IR) in independent samples was performed by one-way analysis of variance (ANOVA), and correlations were determined by the nonparametric Spearman correlation coefficient. One-way ANOVA was also used to compare normally distributed log-transformed means for the different animal groups. Multiple comparisons were assessed by the Tukey test, with the level of significance set at $p < 0.05$.

RESULTS

Design, expression and characterization of yeast *PvAMA1₆₆-MSP1₁₉*

We successfully developed a novel chimeric recombinant protein based on previously described AMA1₆₆ [17] and MSP1₁₉ sequences [22]. The protein *PvAMA1₆₆-MSP1₁₉* was expressed and secreted to the supernatant as a soluble protein, and the yield was approximately 8.6 mg/L of culture. The final protein purity was >80% according to SDS-PAGE and Coomassie blue staining, which revealed a major band that migrated at approximately 65 kDa under reducing conditions (Fig 1A). Immunoblotting was performed with MAbs anti-*PvAMA*-1 and anti-*PvMSP1₁₉*. The results showed that all of the MAbs reacted strongly with *PvAMA1₆₆-MSP1₁₉*, suggesting that its integrity was maintained during the expression and purification process (Fig 1B). From the deconvolution of far-UV CD spectroscopy data, we observed that the *PvAMA1₆₆-MSP1₁₉* CD spectrum is consistent with a folded protein and that the prediction shows the predominance of α -helix structures (99.8%) with a smaller number of beta-turn structures (10.9%) (Fig 1C). The purity of the recombinant protein was confirmed by HPLC, and only a peak was detected (Fig 1D).

Antigenicity of *PvAMA1₆₆-MSP1₁₉*

Sera from *P. vivax*-infected individuals from the northern region of Brazil were tested for recognition of the *PvAMA1₆₆-MSP1₁₉* protein by ELISA. The results showed that 73.0%, 91.9%, and 91.0% of the sera recognized *PvAMA*-1, *PvMSP1₁₉* and *PvAMA1₆₆-MSP1₁₉*, respectively (Fig 2A). There was a significant difference between *PvAMA1₆₆* and

PvMSP1₁₉ ($p=0.0008$) and *PvAMA1₆₆* and *PvAMA1₆₆-MSP1₁₉* ($p=0.0018$). A higher correlation coefficient was obtained when we compared the reactivity of human antibodies to *PvAMA1₆₆-MSP1₁₉* and *PvMSP1₁₉* ($r=0.9696$; $p<0.0001$) than the coefficients obtained when *PvAMA1₆₆* and *PvAMA1₆₆-MSP1₁₉* ($r=0.8257$; $p<0.0001$) or *PvMSP1₁₉* and *PvAMA1₆₆* ($r=0.7906$; $p<0.0001$) were compared (Fig 2B in panels i, ii and iii, respectively).

Immunogenicity of *PvAMA1₆₆-MSP1₁₉*

Magnitude of the induced antibody response. Immunogenicity in each group was determined by the mean of the total serum IgG antibody titers of each individual mouse against each protein estimated by ELISA. After the third dose in BALB/c mice with different formulations, we observed that there was no significant difference in the antibody titers against *PvAMA1₆₆* between the groups that received *PvAMA1₆₆*, *PvAMA1₆₆-MSP1₁₉* and the co-administration of *PvAMA1₆₆* and *PvMSP1₁₉* ($p>0.05$). We also observed that the protein *PvAMA1₆₆-MSP1₁₉* induced higher antibody titers against *PvMSP1₁₉* when compared to *PvMSP1₁₉* alone or in combination with *PvAMA1₆₆* (4.41 ± 0.16 , 3.30 ± 0.40 and 2.30 ± 0.15 , respectively, $p<0.001$) (Fig 3A). Therefore, we conclude that the administration of recombinant protein *PvAMA1₆₆-MSP1₁₉* is capable of generating antibody titers as efficiently as the recombinant protein *PvAMA1₆₆* alone and further improves the antibody titers against *PvMSP1₁₉*.

After the third immunization dose in C57BL/6 mice, we observed that there were no significant differences in antibody titers against *PvAMA1₆₆*, *PvMSP1₁₉* and *PvAMA1₆₆-MSP1₁₉* between the groups that received each recombinant protein individually, the combination of *PvAMA1₆₆* and *PvMSP1₁₉* and the fusion protein *PvAMA1₆₆-MSP1₁₉* (Fig

3B). In both experiments, the control groups that received only PBS in the presence of adjuvant did not present any antibody titers against any of the recombinant proteins.

Next, we aimed to demonstrate that serum samples, regardless the mice strain, were able to recognize the native proteins expressed on *P. vivax* merozoites (mature schizonts). First, the pooled serum samples from BALB/c were tested (Fig 4). We observed that all the mouse serum samples reacted with the native protein present in mature schizonts of *P. vivax* that were isolated from an infected individual. Finally, the pooled serum samples from C57BL/6 mice were also tested and the same pattern of recognition presented by serum samples from BALB/c was observed (Fig 5).

T cell response. CFSE, ELISPOT and CBA analyses methods were used to measure the cellular response induced by immunization. T cell populations were separated into CD4⁺ and CD8⁺, and their proliferation percentages obtained by CFSE fluorescence decay. In BALB/c, we observed 4% of CD4⁺ proliferation in the group vaccinated with *PvAMA1₆₆* protein and 0.9% in the other groups (Fig 6A). The percentages for C57BL/6 CD4⁺ cells proliferation were 17.5% in the animals that received *PvAMA1₆₆* alone and approximately 12% in the other groups (Fig 6B). Overall, specific proliferation of CD8⁺ cells was lower compared to CD4⁺ against *PvAMA1₆₆* stimulus, independent of the animal strain (Fig 6C and 6D). Immunized mice displayed no significant proliferative responses by CD4⁺ and CD8⁺ T cells after *in vitro* stimulation with the protein *PvMSP1₁₉*.

The ELISPOT results demonstrated that the number of IFN- γ secretion spot forming cells observed in C57BL/6 mice in response to *PvAMA1₆₆* re-stimulation was also notably superior. In BALB/c, the animals vaccinated with *PvAMA1₆₆* alone, mix of proteins

and the fusion protein formed 80, 19 and 17 spots, respectively (Fig 7A). For the other hand, in C57BL/6 these numbers achieved 1627, 282 and 325 spots in the same groups (Fig 7B). IFN- γ production was significantly higher when we used the protein *PvAMA1₆₆* alone to immunization, when compared with the groups of mice that received the co-administration of two antigens or the fusion protein [$p < 0.05$, in BALB/c and $p < 0.001$ in C57BL/6].

To verify the secretion of other cytokines and evaluate reproducibility of the ELISPOT assay, CBA Th1/Th2/Th17 was conducted. In BALB/c cell supernatant culture, IL-4, IL-6, IFN- γ , TNF- α and IL-10 were detected in response to *PvAMA1₆₆* re-stimulation (Fig 8A). Among the animals vaccinated with *PvAMA1₆₆* alone the IL-10 were predominant, followed by TNF- α . The predominance of IL-10 and TNF- α was also observed in the groups vaccinated with the fusion protein and co-administration of both proteins (Fig 8A). Corroborating with the ELISPOT data, we found increased levels of IFN- γ in the C57BL/6 mice groups immunized with *PvAMA1₆₆* alone, mixed or fused (Fig 8B). In the groups vaccinated with the mix and fusion proteins a significant increase of IL-10 was observed (Fig 8B). No IL-17A was detected in any of groups. In both strains, we were not able to detect any specificity of cytokine secretion in response to *PvMSP1₁₉* stimulation (data not shown).

DISCUSSION

We successfully developed a chimeric recombinant protein *PvAMA1₆₆*-MSP1₁₉ in yeast *P. pastoris* with high purity and yield, comparable to *PvAMA-1* ectodomain previously obtained [17]. Initially, this new protein was evaluated for its ability to bind to

IgG antibodies in the serum of individuals exposed to *P. vivax* malaria and compared to *PvAMA*₁₆₆ and *PvMSP*₁₁₉ individually. We observed that these sera contained high levels of antibodies to this chimeric protein, with a response comparable to the protein *MSP*₁₁₉ and even higher than the one observed in response to *PvAMA*₁₆₆. The reactivity of each protein individually, and the positive correlation that we observed, support the idea that important epitopes are maintained in the chimeric protein.

Next, we administered the chimeric protein to BALB/c and C57BL/6 mice in the presence of adjuvant Poly (I:C) and, as a comparison, the proteins *PvAMA*₁₆₆ and *PvMSP*₁₁₉, either individually or in combination. Poly (I:C) is a synthetic analog of double-stranded RNA; it can activate the immune response through TLR-3 and has already been tested in humans [23]. The *PvAMA*₁₆₆-*MSP*₁₁₉ was able to generate antibody titers as efficiently as the protein *PvAMA*₁₆₆ alone and further improves the antibody titers against *PvMSP*₁₁₉ in BALB/c. In this strain of mouse, we observed that *PvMSP*₁₁₉ alone or in combination with *PvAMA*₁₆₆ presented lower antibody titers against *PvMSP*₁₁₉ than the chimeric protein, results also shown in previous studies that fused AMA-1 (domain I and II) and *MSP*₁₁₉ of *P. falciparum* [24]. The decrease in antibody titers against *PvMSP*₁₁₉ when the combination of antigens was administered could be explained by a competition in the epitopes presented to the immune system, resulting in impaired *PvMSP*₁₁₉ presentation. This effect was probably influenced by the genetic background of the mouse, since does not exist in C57BL/6.

The increased antibody titers against *PvMSP*₁₁₉ observed with administration of *PvAMA*₁₆₆-*MSP*₁₁₉ is likely due to the presence of T helper epitopes present in *PvAMA*-1 sequence, similarly to what has been shown in *P. falciparum* when only *PvMSP*₁₁₉ is

administered in comparison to *PfMSP1*₃₃, which helps the antibody respond to the C-terminal region [25].

In addition to the high antibody titers obtained with *PvAMA1*₆₆-*MSP1*₁₉ immunization, the immunofluorescence results confirmed the recognition of the native protein on *P. vivax* merozoites. The slightly lower immunofluorescence signal for *PvAMA1*₆₆ (relative to *MSP1*₁₉) on the Thai merozoites may be due to polymorphisms in this protein, which was originally based on an Amazonian isolate of *P. vivax* [26].

Given the importance of the T cell-mediated response in malaria, the specific T cell proliferation and cytokine secretion were analyzed using the vaccinated mice splenocytes. CD4⁺ T cells from immunized mice with the formulations containing *PvAMA1*₆₆ proliferated by after *in vitro* stimulation with the recombinant protein. The *PvAMA1*₆₆ alone was more efficient inducing CD4⁺ T cells proliferative response to chimeric protein stimulus. In C57BL/6, significant levels of the proliferative responses, after *PvAMA1*₆₆ stimulus was also observed using vaccination regimens with mixed or chimeric proteins. These results are particularly important, since this response was obtained with a homologous protein/protein protocol and the high rate of cell proliferation was related mainly to viral vectors on heterologous protocols [16]. In parallel, these cells were assayed for ELISPOT. We were able to obtain higher IFN- γ responses using C57BL/6 mice for immunization, suggesting the presence of *PvAMA1* T-cells epitope for this strain of mice. Therefore, CBA revealed that the BALB/c mice cells secreted preferably IL-10 and TNF- α , while C57BL/6, IFN- γ . The relative rates between the cytokines simultaneously analyzed changed when the *PvAMA1*₆₆ alone, fused or co-

administered groups are compared. Higher IL-10 values were found when the both proteins were administered (chimeric or mixed) in C57BL/6.

In conclusion, the chimeric recombinant protein based on *P. vivax* AMA-1 and MSP1₁₉ potentially offers benefits over the use of each antigen alone or mixed. Further studies are necessary to evaluate the functionality of these antibodies in the inhibition of reticulocyte invasion.

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Contributors

Designed the study and associated protocols: MVR, KSF, MMR, ISS; performed research work: MVR, KSF, LCL, TMC, BR; analyzed data: MVR, KSF, LCL, TMC, FTMC, LR, BR, MMR, and ISS; Contributed reagents and materials: RLDM, FTMC, LR, BR, and MMR; Wrote the manuscript: MVR, KSF, LCL, and ISS. All authors read and approved the final version of the manuscript.

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Figure legends

Fig 1. Analysis of recombinant protein *PvAMA1*₆₆-*MSP1*₁₉. A novel chimeric recombinant protein was expressed in *P. pastoris* with a hexa histidine tag and purified from the supernatant by affinity chromatography, followed by anion-exchange chromatography, as described in the Materials and Methods section. (A) SDS-PAGE analysis of purified recombinant *PvAMA1*₆₆-*MSP1*₁₉ stained with Coomassie blue. (B) Immunoblotting analysis of recombinant *PvAMA1*₆₆-*MSP1*₁₉ using an anti-histidine tagged antibody, anti-*PvMSP1*₁₉ and anti-*PvAMA-1* (domain II) monoclonal antibodies, respectively. (C) Circular dichroism spectrum of recombinant *PvAMA1*₆₆-*MSP1*₁₉. The spectrum was recorded from 200 to 260 nm using a JASCO-J720 spectropolarimeter. The plot represents the mean residue ellipticity of the recombinant protein. (D) Purified *PvAMA1*₆₆-*MSP1*₁₉ was performed by HPLC using C₁₈ column. The HPLC procedure was performed using acetonitrile gradient 90% in 0.1% trifluoroacetic acid at room temperature ($\approx 22^{\circ}\text{C}$) at 1 mL/min for 30 min. The detection system used was UV – visible absorbance detector (DAD: diode array detector/ Shimadzu SPD M20A) at 214 nm.

Fig 2. Comparative recognition of the recombinant proteins *PvAMA-1*, *PvMSP1*₁₉ and *PvAMA1*₆₆-*MSP1*₁₉ during patent *P. vivax* infection. (A) The bars express the percentage response for each of the analyzed proteins, as determined by ELISA. Serum samples from 111 individuals with patent *P. vivax* malaria from endemic areas of northern Brazil were tested at dilutions of 1:100. The cutoff was determined using three standard deviations above the mean OD₄₉₂ from the serum samples of 16 healthy individuals who were never exposed to malaria. The statistical analysis was evaluated using the Chi-

square test. (B) Each panel represents the reactivity index of the serum samples against the indicated recombinant proteins. (i) AMA1₆₆-MSP1₁₉ and MSP1₁₉ (ii) MSP1₁₉ and AMA1₆₆ (iii) AMA1₆₆-MSP1₁₉ and AMA1₆₆. The trend lines and the values for the Spearman correlation coefficient (r) are illustrated in the three figures.

Fig 3. IgG antibody response in mice immunized with the formulations containing PvMSP1₁₉ and PvAMA1₆₆ emulsified in Poly (I:C) adjuvant. Groups of six mice BALB/c (A) or C57BL/6 (B) mice were immunized s.c. with 10 µg of the recombinant protein in the presence of Poly I:C adjuvant (50 µg/dose), as described in the Materials and Methods section. The co-administered group received 10 µg of each recombinant protein. The results are expressed as the means ± SEM of antibody titers (log₁₀). Significant differences between groups are denoted on the graph: *p<0.05, **p <0.01, ***p<0.001 (One-way ANOVA followed by the Tukey test for multiple comparisons). Non-significant (n.s.) differences are indicated (p>0.05).

Fig 4. Indirect Immunofluorescence analysis using serum samples from BALB/c mice. The smears were incubated with pooled serum samples (1:100) from mice immunized with: (A) PBS, (B) PvAMA1₆₆, (C) PvMSP1₁₉, (D) PvAMA1₆₆ and PvMSP1₁₉ in the presence of Poly I:C. Antibody binding was detected using a secondary Alexa 568-labeled antibody (red), and nuclei were visualized by DAPI staining (blue).

Fig 5. Indirect immunofluorescence analysis using sera from C57BL/6 mice. The smears were incubated with pooled serum samples (1:100) from mice immunized with:

(A) PBS emulsified in Poly I:C (B) *PvAMA1*₆₆, (C) *PvMSP1*₁₉, (D) *PvAMA1*₆₆ and *PvMSP1*₁₉ in the presence of Poly I:C. Antibody binding was detected with a secondary Alexa 568-labeled antibody (red), and nuclei were visualized by DAPI staining (blue).

Fig 6. T cell proliferation assay. Fifteen days after the last immunization with recombinant proteins administered alone or in combination in BALB/c (A and C) and C57BL/6 (B and D) mice, the proliferative T cell responses were determined by CFSE dilution assay. Pooled splenocytes (n=3 per group) were labeled with CFSE and cultured for 5 days in the presence of 10 µg/mL of *PvAMA1*₆₆, *PvMSP1*₁₉ or 2.5 µg/mL of ConA. After staining with fluorochrome-labeled anti-CD3, -CD4 and -CD8 monoclonal antibodies, cells were analyzed by flow cytometry. The percentage of proliferating CD3⁺CD4⁺ T cells (CFSE^{low}) are represented in the graph. The percentage obtained after stimulation with the mitogen ConA for CD4⁺ and CD8⁺ T cells was above 90% (data not shown).

Fig 7. Ex-vivo ELISPOT detection of IFN-γ secretion by immunized mice splenocytes, following stimulation with *PvAMA1*. Splenocytes were collected, individually, from BALB/c (A) or C57BL/6 (B) immunized mice. The cells were plated and stimulated, during 24 h, with 10 µg/mL of *PvAMA1* protein or 2.5 µg/mL of ConA (data not shown). ELISPOT data are expressed in spot forming units (SFU), which is the number of specific T cells producing IFN-γ per 10⁶ splenocytes.

Fig 8. Analysis of cytokine secretion by cytometric bead array (CBA). Pooled splenocytes (n=3 per group) were collected from BALB/c (A) and C57BL/6 mice (B) vaccinated with the recombinant protein *PvAMA1₆₆* or combination of proteins *PvAMA1* and *MSP1₁₉*. Aliquots of 5 days of culture supernatant were used for the IL-2, IL-4, IL-6, IFN- γ , TNF- α , IL-17A and IL-10 determination, using specific beads by flow cytometry. The results were expressed as pg/mL.