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Immunogenicity and protection-inducing ability of recombinant *Plasmodium vivax* rhoptry-associated protein 2 in *Aotus* monkeys: A potential vaccine candidate

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ABSTRACT

Rhoptry proteins have been extensively shown to be important in invasion and parasitophorous vacuole (PV) formation. This work evaluates the immunogenicity and protective efficacy of *Plasmodium vivax* RAP2 in the non-human *Aotus* primate model, when expressed as a recombinant molecule in *E. coli* and formulated in Freund and Alum hydroxide adjuvants. Our results show that r*Pv*RAP2 is immunogenic in both formulations, finding a trend of higher cytokine levels in immunized monkeys, specially in IL-4 levels (using Freund's adjuvant) and IL-5 (using Alum hydroxide). RAP2 is suggested as a *P. vivax*-vaccine candidate since immunized monkeys exhibited lower parasitemias than control groups after being experimentally challenged with the *P. vivax* VCG-I strain.

1. Introduction

Malaria is one of the most prevalent parasitic diseases among tropical countries. Every year nearly 500 million new cases are reported worldwide, of which an estimated 1–2 million result in death [1]. Epidemiologically speaking, *Plasmodium vivax* is considered as the second most important malarial parasite species, as it accounts for more than 75 million annual cases of malaria occurring mainly in Asia, Central and South America [2,3].

Despite the great efforts in developing an effective strategy for the control of this scourging disease, malaria remains a public health threat worldwide [4]. One of the most appealing strategies for eradicating this disease is the development of a vaccine. Nevertheless, obtaining a fully effective vaccine has been hindered by the great diversity of surface proteins expressed throughout the parasite's life cycle, its polymorphism and the fact that immunity induced by a natural infection is gradually acquired and species-, stage- and strain-specific [5–7]. Aiming at developing a vaccine against *P. falciparum*, several research groups have characterized new antigens in this species, among which the rhoptry-associated protein 2 (RAP2) has long been considered a major vaccine candidate due to its ability to induce antibodies capable of inhibiting *in vitro* invasion of red blood cells (RBCs) [8], its low degree of genetic polymorphism [9], its binding ability to RBCs [10], its recognition by sera from patients living in endemic areas [11] and, more importantly, its capacity to confer protection against experimental challenge with *P. falciparum* in *Saimiri boliviensis* monkeys [12]. All this evidence has encouraged its recent characterization in *P. vivax* [13], even though its role in RBCs invasion is still not clearly elucidated in this parasite species.

Diverse components of the immune system are involved in the response against *Plasmodium*, depending on the parasite's life stage. In the liver stage, specific parasite antigens are recognized by Interferon-γ (IFN-γ)-secreting CD8+ T lymphocytes [14]. IFN-γ-activated macrophages display a direct antiparasitic effect by secreting nitric oxide (NO) through an inducible nitric oxide synthase (iNOS) [15]. Regarding the erythrocytic stage, antibodies directed against merozoite surface antigens are able to block RBC invasion and/or facilitate phagocytosis [16,17]. Both Th1 and

Abbreviations: RAP2, rhoptry-associated protein 2; r*Pv*RAP2, recombinant rhoptry-associated protein 2; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; VCG-I, Vivax Colombia Guaviare I; PHA, phytohemagglutinin; RBCs, red blood cells; iRBCs, infected red blood cells.

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Th2 responses are directly involved in conferring immune protection against malarial blood stages and the balance between both cytokine profiles is important to determine disease outcome [17].

Given the difficulties in analyzing the human immune response, various animal models have been used to determine the mechanisms underlying the induction of protection against malaria, among which the World Health Organization (WHO) has suggested *Aotus* spp. monkeys as the ideal experimental non-human primate model for evaluating malaria vaccine candidates [18]. The aim of the present study was to evaluate the immunogenicity of the recombinant *Pv*RAP2 (r*Pv*RAP2) when it was formulated in either Freund's adjuvant or Alum hydroxide, as well as the resulting cytokine profile response and its protection-inducing ability in *Aotus nancymaae* monkeys against an experimental challenge with *P. vivax*.

2. Materials and methods

2.1. P. vivax purification

The *P. vivax* (Vivax-Colombia-Guaviare I (VCG-I)) strain was used as RNA and protein source. *P. vivax*-infected RBCs (iRBCs) were isolated from a 3 mL blood sample taken from an infected monkey using a 30–50% Percoll density gradient (Amersham Biosciences, Uppsala, Sweden) following a previously described protocol [19].

2.2. Cloning and sequencing

One microgram of total parasite RNA extracted by the Trizol method [20] and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA) was used as template for cDNA synthesis, using the SuperScript III enzyme (Invitrogen, California, USA) in 20 μL RT-PCR reactions carried out at 50 °C for 60 min, according to manufacturer's recommendations. The *Pv*RAP2 gene was then amplified in 35 PCR-cycles as follows: 15 s at 94 °C, 30 s at 56 °C, 80 s at 68 °C and a final 5 min extension at 68 °C; using Platinum *Pfx* DNA polymerase enzyme (Invitrogen, California, USA) and primers specifically designed to cover the entire *Pv*RAP2 transcript (5′-ATGCATACTTGCACAAAAGAATTG-3′ forward primer and 5′-TGACTCCATACCTTTCTCC-3′ reverse primer), according to the reported gene sequence [13]. PCR products were purified with the Wizard PCR preps kit (Promega, Wisconsin, USA) and cloned into pEXP5-CT/TOPO vector (Invitrogen, California, U SA), which adds a six-histidine tag at the C-terminus of the recombinant protein to allow its detection by anti-polyhistidine monoclonal antibodies and further purification by affinity chromatography. The resulting recombinant DNA plasmid was purified using a Miniprep purification system kit (Promega, Wisconsin, USA) and then sequenced in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA) to verify the cloned insert integrity.

2.3. rPvRAP2 expression and purification

E. coli BL21 cells harboring the pEXP5-CT/TOPO recombinant construct were grown in 50 mL of Terrific broth (TB) medium (12 g/L Tryptone, 24 g/L Yeast extract, 4 mL/L Glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4, 0.1 mg/mL Ampicillin and 0.1% (w/v) D-glucose) for 12 h at 37 °C, under constant shaking. This culture was then used for inoculating 950 mL of TB medium, which was kept at 37 °C until reaching an optical density (OD_{600}) of 0.6–0.8. Once achieving this optimum cell concentration (after to 2 h approximately), L-arabinose was added at a 0.2% (w/v) final concentration to induce expression of the recombinant protein. Cells were further incubated at 37 °C for 5 h and then harvested by centrifugation at 12,000 × *g* for 30 min at 4 °C.

Protein solubilization was achieved using high concentrations of denaturing agents (6 M Urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 15 mM Imidazole), 1 mg/mL lysozyme and further sonication of the cellular pellet. The resulting recombinant solubilized protein was recovered from the supernatant by centrifugation at 12,000 × *g* for 30 min at 4 °C and its expression was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-polyhistidine monoclonal antibodies. A Ni^{+2}-NTA Agarose resin was then used to purify r*Pv*RAP2 from the clear supernatant by affinity chromatography (Qiagen, CA). Briefly, the resin's pH was first adjusted with extraction buffer (6 M Urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 15 mM Imidazole) to pH 8 and the protein extract was then allowed to pass through the column. Non-retained proteins were eluted using the same buffer solution, while a lysis buffer containing 500 mM Imidazole was used to elute the recombinant protein. All fractions individually collected were analyzed by SDS-PAGE and Western blotting.

2.4. Ascertaining the expression of rPvRAP2 by Western blotting

The affinity chromatography fractions were separated by electrophoresis on 12% polyacrylamide gels in the presence of SDS (sodium-dodecyl sulphate) under reducing conditions and then transferred to a nitrocellulose membrane; using a 5% skimmed milk in PBS–0.05% Tween to block membranes for 1 h. After washing membranes thrice with PBS–0.05% Tween, they were incubated with peroxidase-coupled anti-polyhistidine monoclonal antibodies diluted 1:4500 in PBS–0.05% Tween containing 5% skimmed milk, for 2 h at room temperature; then washed thrice with PBS–0.05% Tween and revealed using the VIP Peroxidase substrate kit (Vector Laboratories, Burlingame, CAN) according to the manufacturer's recommendations. Pure fractions were pooled and dialyzed thoroughly against a step Urea gradient (6–1 M) to avoid protein precipitation. In brief, the protein was first dialyzed against 4 M Urea–PBS for 6 h, 2 M Urea–PBS for another 6 h, followed by dialysis against 1 M Urea–PBS and finally, thorough dialyzed against 1× PBS, pH 7.2. The protein was then ultrafiltrated and its concentration was determined by the bicinchoninic acid assay using BSA as standard [21].

2.5. Immunization of A. nancymaae monkeys with rPvRAP2

The immunization trials were carried out in *A. nancymaae* monkeys native to the Colombian Amazon region and kept in our Institute's primate station according to the guidelines stipulated by the Colombian Ministry of Health (law 84/1989) and the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA), under the supervision of a primatologist. A total of 22 monkeys presenting no evidence of previous *Plasmodium* spp. infection, as determined by indirect immunofluorescence assay (IFA), were randomly distributed into immunization groups as follows. Two groups containing seven monkeys each, were immunized with 50 μg of recombinant antigen, the first one formulated in Freund's adjuvant and the second one in Alum hydroxide. Two groups of four monkeys were inoculated at the same immunization schedule with PBS 1× formulated in Freund's adjuvant and Alum hydroxide, respectively, and served as adjuvant controls. All animals received booster immunizations with their respective adjuvants on days 20 and 40; and were bled on days 0, 20, 40 and 60. The so obtained sera samples were used for evaluating the antibody titers.

2.6. Challenge and parasitemia assessment

Twenty days after the third immunization, all *A. nancymaae* monkeys were intravenously challenged with 2.5×10^6 of *P. vivax* VCG-I strain–iRBCs taken from previously infected *A. nancymaae* monkey donors. Beginning on day 4, monkeys were followed-

up daily for the development of parasitemia by quantitative Giemsa-stained slides and Acridine Orange staining determined by fluorescence microscopy. All monkeys were treated with oral doses of 30 mg/kg/day quinine sulphate during 5 days, 30 mg clindamycin (intramuscular), 25 mg/kg sulfadoxine with 1 mg/kg pyrimethamine administered orally and an oral dose of 0.75 mg primaquine following the conclusion of the study (day 18) or before, whenever one or more of the following criteria were met: parasitemia levels ≥1.5% during three consecutive days, parasitemia levels ≥5%, or clinical deterioration of the animal. Treated animals were quarantined until ensuring their cure and subsequently returned to the jungle.

2.7. Detection of anti-rPvRAP2 antibodies in immunized monkeys by immunoblotting

The recombinant protein was size-separated on 12% polyacrylamide gel in the presence of SDS and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS–0.05% Tween for 1 h at room temperature and then washed thrice with PBS–0.05% Tween before being cut into strips. Sera samples from each monkey, both pre-immune and immune, diluted 1:100 in 5% skimmed milk-PBS–0.05% Tween were incubated independently with a membrane strip for 1 h at room temperature. All strips were then washed thrice with PBS–0.05% Tween and subsequently incubated with a 1:4000 dilution of peroxidase-coupled goat anti-*Aotus* IgG antibody for 1 h at room temperature. Blots were then revealed using the VIP Peroxidase substrate kit (Vector Laboratories) according to the manufacturer's recommendations.

2.8. Determining anti-rPvRAP2 antibody titers by ELISA

One microgram of purified r*Pv*RAP2 per well was used to coat the surface of an ELISA plate by incubation at 37 °C for 1 h, then at 4 °C overnight and again at 37 °C for 1 h. Each plate was washed thrice with PBS–0.05% Tween and then blocked with 200 μL per well of 5% skimmed milk in PBS–0.05% Tween for 1 h. Antibody titers were determined by loading an initial 1:100 serum sample (in duplicate) and successively diluting this sample 1:1 in 5% skimmed milk in PBS–0.05% Tween. Plates were incubated for 1 h at 37 °C and then washed thrice with PBS–0.05% Tween. Plates were incubated with 100 μL of peroxidase conjugated anti-*Aotus* IgG secondary antibody diluted 1:8000 at 37 °C for 1 h. Wells were then washed thrice with PBS–0.05% Tween. The immunoreaction was detected by adding TMB Microwell Peroxidase Substrate solution (KPL Laboratories, WA, USA) according to the manufacturer's recommendations, taking absorbance measures at 620 nm (A_{620}) with a Labsystems Multiskan MJ ELISA reader. Antibody titers were determined by successive 1:1 primary antibody dilutions until reaching an A_{620} value equal to control value ±2 S.D.

2.9. Indirect immunofluorescence assay (IFA)

IFA experiments were carried out using air-dried samples of *P. vivax* (VCG-I) infected RBCs loaded into multiwell glass slides. In brief, fixed slides were blocked for 10 min with 1% skimmed milk and incubated for 30 min with each monkey serum sample (PI, PII and PIII) diluted 1:40 in PBS 1×. Immunoreactivity was examined by fluorescence microscopy using a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated anti-*Aotus* IgG. Slides were incubated at 37 °C for additional 30 min and then washed with PBS. Autologous pre-immune sera were used as negative controls and a hyperimmune serum was used as a positive control.

2.10. Isolating PBMCs and assessing of cytokine production

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 mL blood samples by gradient centrifugation in Ficoll-Hypaque (1.077 density) (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Cells were isolated, pulsed with 10 μg/mL r*Pv*RAP2 and then cultured in triplicate in 100 μL complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 25 U/mL penicillin, 50 mg/mL streptomycin, 5×10^{-5} M 2-mercapto-ethanol and 10% autologous plasma). Positive control cells were pulsed with 5 μg/mL phytohemagglutinin (Difco, Michigan, USA). After culturing cells for 72 h at 37 °C with 5% CO_2, the supernatant was harvested and used to quantify the levels of IL-2, IL-4, IL-5, IL-6, TNF and IFN-γ by using a non-human primate cytometric bead array kit (CBA) (BD Biosciences). Briefly, 50 μL PBMCs sample taken from each monkey's culture supernatant were mixed with 50 μL capturing beads and 50 μL Th1/Th2 human II-Phycoerythrin (PE)-conjugated detection reagent, individually. All samples (those pulsed with 10 μg/mL of r*Pv*RAP2 together with their respective controls) as well as standards for each cytokine, were incubated away from light for 3 h at room temperature. Once non-attached detection antibody had been removed by washing and centrifuging at 200 × *g* for 5 min, samples were placed on 200 μL buffer and acquired on a Flow cytometer

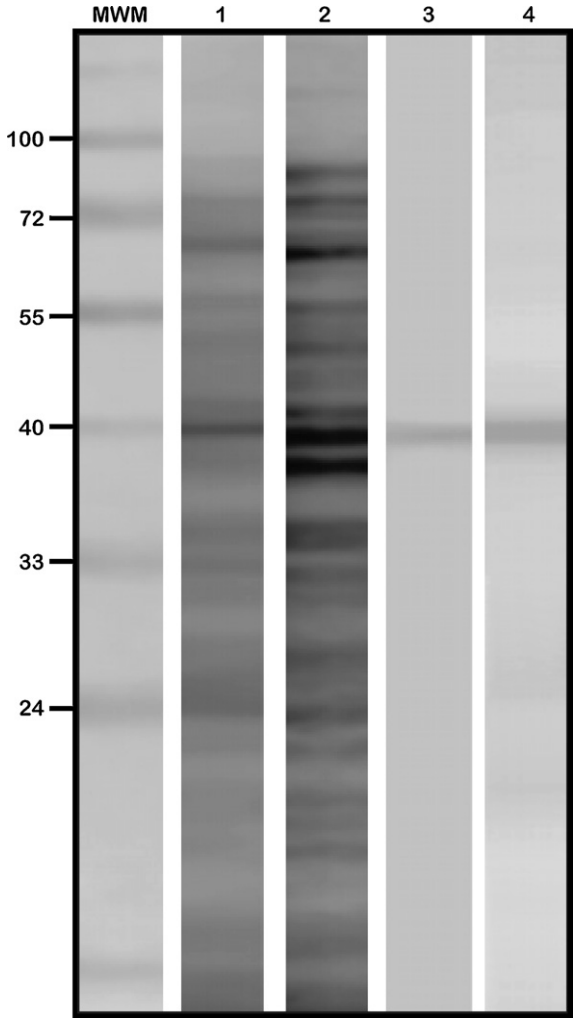

Fig. 1. Western blotting and Coomassie staining analysis of r*Pv*RAP2 expression. MWM: molecular weight marker; lane 1: non-induced bacterial lysate; lane 2: L-arabinose induced bacterial lysate; lane 3: Western blot analysis of the purified protein; lane 4: Coomassie staining of the purified protein.

(FACScan, Becton Dickinson, CA, USA) coupled to a CBA specialized software for determining each cytokine's concentration.

3. Results

3.1. Expression and purification of rPvRAP2

The use of an expression vector adding a six-histidine tag at the protein's C-terminus allowed detecting the recombinant protein through the use of anti-polyhistidine monoclonal antibodies and subsequently purifying it by affinity chromatography. This led to detecting r*Pv*RAP2 as an insoluble aggregate forming inclusion bodies, thus requiring high concentrations of denaturing agents (6 M urea) to solubilize it. Protein expression from a 1 L L-arabinose-induced culture was first verified by Western blot using anti-polyhistidine monoclonal antibodies. The ~43 kDa band in lysate from induced bacteria shows that r*Pv*RAP2 is among the most abundantly expressed proteins (Fig. 1, lane 2). The recombinant protein was then purified by affinity chromatography using a Ni^{+2}-NTA resin and the so collected fractions were individually analyzed by SDS-PAGE and Western blot. Those fractions presenting a single band at the expected molecular weight for r*Pv*RAP2 were pooled and concentrated by ultrafiltration. The recombinant protein was then thoroughly dialyzed against a step urea gradient and finally against PBS 1×, pH 7.2 to ensure the removal of all denaturing agents and also to allow the protein to acquire a similar conformation to that of the native one. The amount of protein was then determined by BCA assay [21]. The yield of final product was 1.6% and 83% of this product was recovered after thorough dialysis. The protein's purity, as assessed by densitometry, showed purity values above 90%.

3.2. rPvRAP2 immunogenicity in the A. nancymaae animal model

3.2.1. Humoral immune response

The purified r*Pv*RAP2 was formulated in Freund's adjuvant and Alum hydroxide as described in materials and methods. Although a trend towards an increase in anti-r*Pv*RAP2 antibody production following each immunization with both adjuvant formulations was observed by the ELISA-determined antibody titers (Fig. 2a and b), when the non-parametric analysis of Mann–Whitney–Wilcoxon ranks was applied to evaluate differences in data distribution [22,23], no significant differences were found in the post-third dose (PIII) response between the two r*Pv*RAP2 groups immunized with

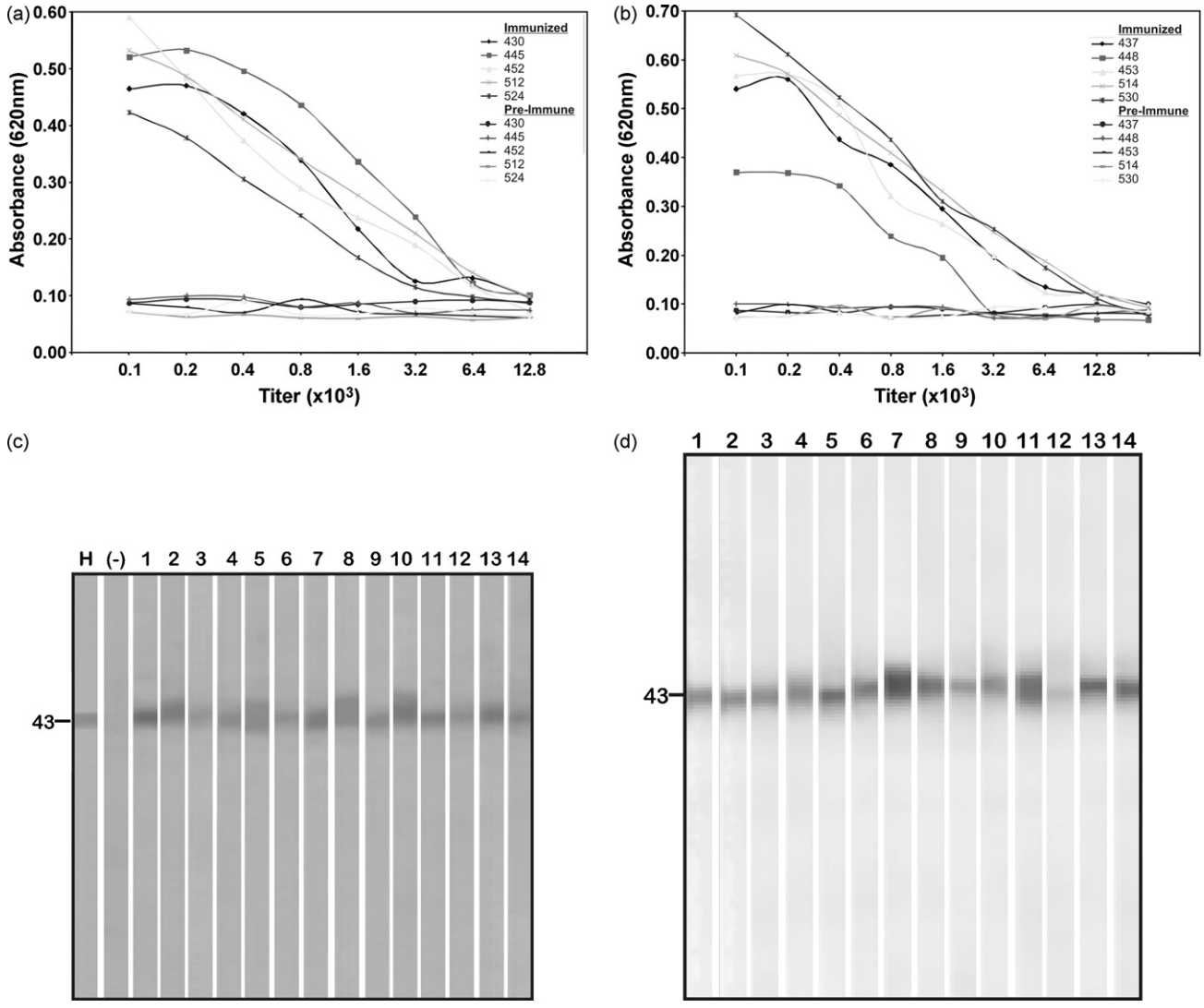

Fig. 2. Monkey anti-r*Pv*RAP2 antibody detection assessed by ELISA and Western blot. (a) Group vaccinated with r*Pv*RAP2 in Alum hydroxide. (b) Group vaccinated with r*Pv*RAP2 in Freund's adjuvant. Antibody titers were determined by successive 1:1 primary antibody dilutions until reaching an A_{620} value equal to the control value ±2 S.D. (c) Western blot analysis showing antibody recognition of the purified r*Pv*RAP2 and (d) the protein in the *P. vivax* lysate.

different adjuvants, neither between these two groups and their respective adjuvant control groups.

Monkey antibodies' specificity was demonstrated by Western blot using purified r*Pv*RAP2 as antigen (Fig. 2c). All PIII sera of monkeys immunized with r*Pv*RAP2 recognized a band at the expected r*Pv*RAP2 molecular weight. A monoclonal anti-polyhistidine antibody was used to detect the recombinant protein as positive control, while a random pre-immune serum served as negative control. None of the immunized animals' pre-immune (PRE) sera recognized the recombinant antigen (data not shown). The recognition of the parasite protein was also demonstrated in a Western blot with *P. vivax* lysate (Fig. 2d), where a ~43 kDa band was detected. Such recognition suggests that the recombinant protein shares epitopes with the RAP2 protein expressed by the parasite.

However, when monkey sera recognition of the native RAP2 was assessed by IFA using *P. vivax*-infected RBCs and sera samples obtained 20 days after administering each immunization dose (PI, PII and PIII), it can be clearly seen that the parasite's RAP2 is not recognized by antibodies in these sera, which lead us to conclude that the recombinant protein used in immunization trials was not in a completely native form. Therefore, we tested the antibody recognition of the denatured recombinant protein and the thoroughly dialyzed protein by ELISA and find out that antibodies raised in monkeys against the recombinant protein were predominantly recognizing the protein in its denaturated form (data not shown), agreeing with the Western blot, and not in its native form in parasitized RBCs.

3.2.2. Cellular immune response

Three cytokines typical of the Th1 response (IFN-γ, TNF-α and IL-2) and three from the Th2 response (IL-4, IL-5 and IL-6) were quantified in each of the 14 monkeys being immunized with r*Pv*RAP2 emulsified in the different adjuvants, as well as in the 8 control monkeys. When PIII and PRE cytokine levels generated in each vaccinated group were compared, PIII cytokine levels tended to be higher than PRE cytokine levels, in particular, higher IL-4 levels were observed in the group receiving r*Pv*RAP2 in Freund's adjuvant as well as IL-5 levels in the group being vaccinated with r*Pv*RAP2 in Alum hydroxide. Nevertheless, this was only a trend, since no significant statistical differences were found when applying the Mann–Whitney–Wilcoxon test (data not shown).

3.3. Protection in rPvRAP2-immunized A. nancymaae monkeys against experimental challenge with P. vivax

Following experimental challenge with the *P. vivax* VCG-I strain, percentages of parasitemia developed by each monkey was monitored daily. Fig. 3 shows daily parasitemias in the four groups of monkeys from day 4 until day 18 post-challenge. A non-parametric analysis of Mann–Whitney–Wilcoxon ranks was done, in order to evaluate whether there were differences between the percentage of parasitemia developed by those monkeys vaccinated with r*Pv*RAP2 and those receiving placebo injections [22,23] using the mean parasitemia values recorded between days 9 and 12, since a considerable number of monkeys was withdrawn from the study in the following days, thus hampering comparisons between groups from then on. A comparison of parasitemia percentages in all r*Pv*RAP2-immunized monkeys against all controls was initially made, finding statistically significant higher parasitemias in controls (p-value = 0.049). Each vaccinated group was then evaluated against its corresponding control, finding that the distribution of parasitemia was not significantly different in the group receiving r*Pv*RAP2 emulsified in Freund's adjuvant (Fig. 3a). The same analysis in the group of monkeys vaccinated in Alum hydroxide and its control with r*Pv*RAP2 showed higher parasitemias in the control group (Fig. 3b). It should be noted, however, that the statistical analysis was affected due to the lack of a sufficiently large control group of monkeys.

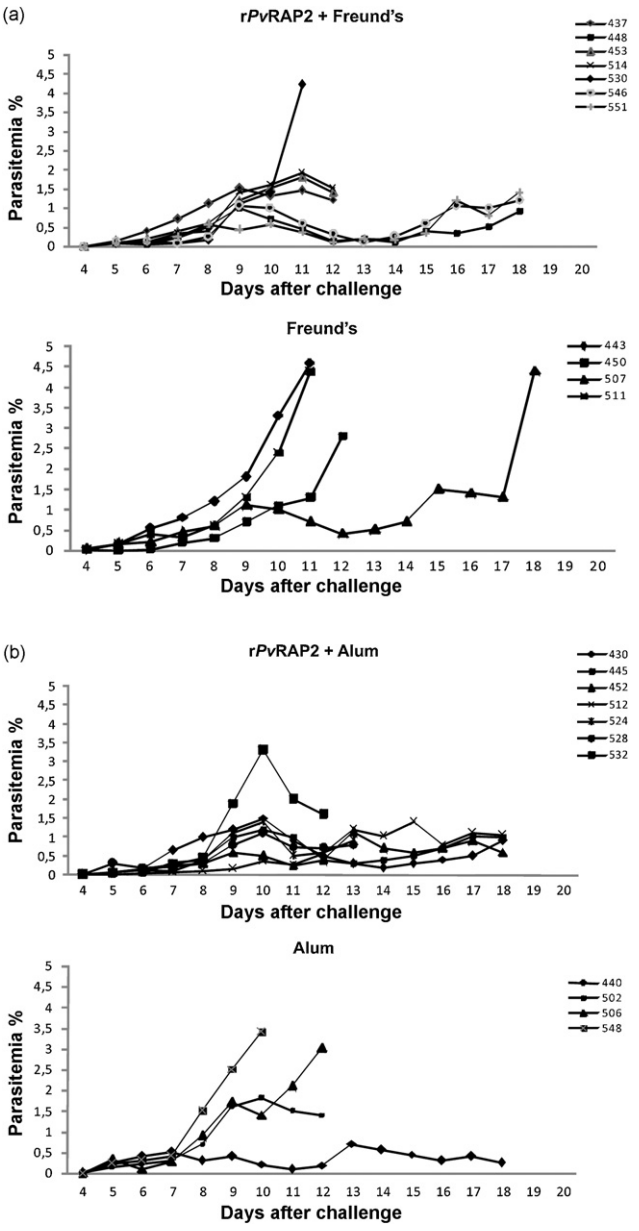

Fig. 3. Development of parasitemia in r*Pv*RAP2-immunized monkeys after being challenged with the *P. vivax* VCG-I strain. (a) Monkeys immunized with r*Pv*RAP2 emulsified in Freund's adjuvant and their respective controls. (b) Monkeys immunized with r*Pv*RAP2 emulsified in Alum hydroxide and their respective controls.

4. Discussion

The members of the low-molecular-mass rhoptry protein complex have been proposed as potential malaria vaccine candidate antigens due to their relevant role in parasite invasion to RBCs [10,24,25] and ability to confer partial protection against *P. falciparum* challenge in both *Saimiri sciureus* [26,27] and *S. boliviensis* monkeys [12]. Based on these promising protection-inducing results and the identification of a *P. vivax* homologue to *P. falciparum* RAP2 [13], we decided to evaluate its immunogenicity and protective efficacy in the non-human primate model of the *A. nancymaae* monkey using for such purpose two adjuvant formulations: Alum hydroxide and Freund's adjuvant.

The immunogenicity of r*Pv*RAP2 was observed by the antibody titers (1:12,800) raised in monkeys as a result of immunizations with r*Pv*RAP2 compared to controls, independently of the adjuvant formulation being used (Fig. 2a and b). It is worth noting that anti-r*Pv*RAP2 PIII antibody titers raised with the human-approved Alum hydroxide were comparable to those raised by a more potent adjuvant such as Freund's. Additionally, all sera from immunized monkeys recognized the recombinant protein in the Western blot analysis as well as the protein in the parasite's lysate (Fig. 2c and d), indicating that the recombinant protein shares epitopes with the protein expressed by the parasite, which are being recognized by antibodies raised against the recombinant molecule. However, since IFA results showed that native RAP2 expressed by the parasite is not recognized by monkeys' sera and the results of r*Pv*RAP2 recognition in an ELISA, both under denaturing conditions as well as after being exhaustively dialyzed, showed that the polyclonal antibodies generated in *Aotus* monkeys preferentially recognized the denatured form of the protein, we conclude that the recombinant protein used for immunizing *Aotus* monkeys was not in a completely native form, which would explain why antibodies raised against r*Pv*RAP2 did not recognize *Pv*RAP2 by IFA but did recognize the protein in the parasite lysate by Western blot.

In humans, the degree of protective immunity is correlated to IgG levels raised against various asexual stage-related antigens. In populations living in endemic areas, IgG responses against RESA, MSP-1 and MSP-2 have been seen to be partially determined by genetic factors and to be age-dependent, being the IgG1 and IgG3 subclasses the most important ones regarding protection against *P. falciparum* in humans. An increase in *P. falciparum*-specific IgG1 and IgG3 levels in individuals frequently exposed to malaria is highly associated to low parasitemias and a reduced risk of developing malaria-associated pathologies [17], thus highlighting the importance of antibody responses in protection against malaria.

According to literature reports, the immunopotentiators used in this study exhibit different immune response profiles. Alum hydroxide induces preferentially Th2 responses, with secretion of IL-4 and IL-5 and proliferation of immunoglobulin-producing B-cells of both IgG and IgE isotypes [28,29], which agrees with the raised in IL-5 levels seen in r*Pv*RAP2-immunized monkeys after the third dose with this adjuvant compared to their initial cytokine levels. It has been reported that this adjuvant has the ability of delivering the antigen to immune effector cells, mainly via antigen presenting cells (APCs), when the antigen is properly adsorbed [30]. According to its effect, the Alum hydroxide was recently classified within the group of adjuvants facilitating antigen's capture, transport and efficient presentation via APCs, especially dendritic cells capable of migrating towards lymph nodes where they interact with T-cells, thus eliciting an effector-immune response [31].

Given the antibody production induced by r*Pv*RAP2 together with the specificity of raised anti-r*Pv*RAP2 antibodies shown by Western blot, it is logical to suppose that the antigen is being presented through the above-mentioned pathway, therefore highlighting the advantage of using this adjuvant. Nevertheless, this response is not necessarily maintained over time, since Alum hydroxide is a short-term depot, *i.e.*, it does not hold the antigen at the injection site for a prolonged time nor induces follicular dendritic cells capable of maintaining the antigen within the draining lymph node in order to stimulate a continuous antibody production [31].

On the other hand, Freund's complete adjuvant is known to induce a Th1-type cytokine profile [32,33], while the incomplete component induces a Th2-polarized response [34]. Freund's complete adjuvant combines *Mycobacterium tuberculosis* immunomodulating properties with the short-term depot effect of water-in-oil emulsions [30]. r*Pv*RAP2 inoculated in Freund's adjuvant induced higher IL-4 production compared to pre-immune controls.

Even though the differential cytokine profile induced by each adjuvant could account for these results, there were no significant differences between cytokine levels of r*Pv*RAP2-vaccinated monkeys and their controls. Additionally, a comparison of PIII immunization cytokine levels between protected and non-protected monkeys, independently of the adjuvant used, showed no significant differences, which suggests that cytokine results did not allow predicting the protective effect of the recombinant protein.

Regarding protection results, previous studies in *Saimiri* spp. monkeys have shown a partial protection effect conferred by r*Pf*RAP1 and r*Pf*RAP2 emulsified in Freund's complete adjuvant and Montanide ISA720 against *P. falciparum* experimental challenge [12], which is why in this study the protective efficacy of r*Pv*RAP2 was assessed using one of the above mentioned adjuvants. Protection results conferred by r*Pv*RAP2 showed a parasitemia reduction in monkeys receiving r*Pv*RAP2 emulsified in Freund's adjuvant (Fig. 3a) as well as in Alum hydroxide (Fig. 3b), compared to parasitemia developed by their respective control group; although such difference was not statistically significant for the later adjuvant as group.

Notwithstanding the state of protein used in immune assays, the results of protection trials are important and suggest r*Pv*RAP2 as a good candidate for a multi-component, multi-stage vaccine and support the development of further studies aimed at obtaining this protein in a native form in order to evaluate whether its protective efficacy increases.

In the search for possible vaccine candidates against *P. vivax* blood stages, some studies have focused mainly on AMA-1, DBP, MSP-9 and MSP-1 [35–39]. Vaccine development against *P. vivax* has been largely hampered by the vast genetic polymorphism existing among several of these proteins and the large number of parasite proteins expressed by the different stages involved in target-cell invasion. For this reason, it is necessary to develop a multi-antigenic and multi-stage vaccine capable of raising an effective immunity against *P. vivax*.

Following the same methodological approach used in this study, our group has studied the protection-inducing ability of two fragments derived from the 33 kDa cleavage product of *Pv*MSP-1. These fragments have shown to have low variability and high binding activity to reticulocytes [40]; as well as protection-inducing ability in *A. nancymaae* challenged with *P. vivax*-infected erythrocytes when the antigens were formulated in Freund's adjuvant [41,42].

Even though there was no concordance between the cytokine values obtained with each adjuvant and the expected profiles of secreted cytokines, r*Pv*RAP2 proved being a good vaccine candidate as it conferred partial protection to a group of monkeys when they were immunized with this antigen emulsified in Freund's adjuvant. Unfortunately, the production of undesirable local reactions at the site of injection, hampers the use of this adjuvant in humans [43]. A possible alternative would be emulsifying r*Pv*RAP2 in new adjuvants not only capable of inducing a good adaptative immune response, but also of potentiating innate immunity, as for instance by coupling it to Poly C, monophospholipid A, CpG, etc. [44], some of which have been recently authorized for use in humans. Further protection assays are needed in order to assess mixtures containing different proteins that have been proved to be protective when assessed individually in *Aotus* monkeys and hence potentiate the protective immune response.

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