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Preclinical assessment of the receptor-binding domain of *Plasmodium vivax* Duffy-binding protein as a vaccine candidate in rhesus macaques

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Abstract

The receptor-binding domain of *Plasmodium vivax* Duffy binding protein, region II (PvRII), is an attractive candidate for a vaccine against *P. vivax* malaria. Here, we have studied the safety and immunogenicity of recombinant PvRII in *Macaca mulatta* (rhesus monkeys). Recombinant PvRII with a C-terminal 6-histidine tag was expressed in *E. coli*, recovered from inclusion bodies, refolded into its functional conformation, purified to homogeneity and formulated with three adjuvants, namely, Alhydrogel, Montanide ISA 720 and the GSK proprietary Adjuvant System AS02A for use in immunogenicity studies. All the PvRII vaccine formulations tested were safe and highly immunogenic. The overall magnitude of the antibody response was significantly higher for both Montanide ISA 720 and AS02A formulations in comparison with Alhydrogel. Furthermore, there was a significant correlation between antibody recognition titers by ELISA and binding inhibition titers in *in vitro* binding assays. The PvRII vaccine formulations also induced IFN-γ recall responses that were identified using *ex vivo* ELISPOT assays. These results provide support for further clinical development of a vaccine for *P. vivax* malaria based on recombinant PvRII.

Keywords

malaria vaccine; Duffy Binding Protein; *Plasmodium vivax*; adjuvant formulation

1. Introduction

The annual number of clinical cases of malaria is estimated to be over 500 million with the number of fatal malaria cases being greater than one million [1]. These numbers place malaria at the top of the list of transmissible diseases with significant global health impact. The

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epidemiology of malaria is characterized by increased prevalence of insecticide-resistant vectors and drug-resistant parasites resulting in the failure of vector and parasite control measures [2]. *Plasmodium falciparum* and *P. vivax* are the two species of malaria parasites that are the primary cause of human malaria. Epidemiological evidence has indicated that following repeated exposure, clinical immunity to malaria is acquired by residents of endemic areas suggesting that the development of a malaria vaccine should be feasible [3].

Merozoite proteins that are involved in erythrocyte invasion are important candidates for the development of vaccines aimed at neutralizing blood-stage growth by modifying the kinetics of erythrocyte invasion. Extensive research has shown that the invasion of human erythrocytes by *P. vivax* and the related simian species *P. knowlesi* is completely dependent on merozoite interaction with the Duffy antigen receptor for chemokines (DARC) [4-7]. The *P. vivax* and *P. knowlesi* Duffy binding proteins (PvDBP and PkDBP), which mediate this interaction, belong to a family of erythrocyte binding proteins (EBP) that also includes the 175 kD *P. falciparum* erythrocyte binding antigen (EBA-175) [8]. The binding domains of EBPs reside in conserved, extracellular, cysteine-rich regions known as region II [9]. Antibodies raised against region II, the receptor-binding domain of PkDBP, have been shown to block erythrocyte invasion by *P. knowlesi* [10]. This result provides support for the development of a vaccine for *P. vivax* malaria based on the homologous receptor-binding domain, region II (PvRII), of PvDBP. It has been demonstrated that naturally acquired antibodies elicited against PvDBP can block the binding of PvRII to Duffy positive human erythrocytes although the binding inhibitory activity is poor [11,12]. Interestingly, structural analysis has demonstrated that clusters of polymorphic amino acid residues in PvRII from *P. vivax* field isolates lie in regions that are distant from the binding site [13]. The DARC binding site within PvRII thus does not appear to be under significant immune pressure. Although high titer binding inhibitory antibodies against PvRII do not develop upon natural exposure to *P. vivax* [14], it is possible to raise high titer binding inhibitory antibodies by immunization with recombinant PvRII [15]. Importantly, since the polymorphism clusters are distal to the binding site, anti-PvRII, binding inhibitory antibodies elicited by immunization should be effective against diverse *P. vivax* isolates [16].

We have previously described the production of recombinant PvRII in its functional, correctly folded form [17,18]. Immunization with recombinant PvRII formulated with Freund's adjuvant has been shown to provide partial protection to *Aotus lemurinus griseimembra* monkeys against *P. vivax* blood stage challenge [19]. The immunogenicity of recombinant PvRII formulated with human compatible adjuvants has also been studied in small animals [15]. Of the five adjuvants tested, namely, Montanide ISA 720, AS02A, MF59, QS21 and Alhydrogel, formulations made with Montanide ISA 720 and AS02A elicited the highest titer binding inhibitory antibodies [15]. Recombinant PvRII formulated with Alhydrogel also yielded antibodies with significant binding inhibitory activity. Based on these observations, we decided to test the safety and immunogenicity of recombinant PvRII formulated in Montanide ISA 720, AS02A and Alhydrogel in rhesus monkeys. Safety of these PvRII vaccine formulations was assessed by characterization of several clinical, haematological and biochemical parameters at different time points after immunization. The immunogenicity of PvRII in rhesus monkeys was determined by measuring end point titers for recognition of PvRII by total IgG using ELISA, measuring 50% binding inhibition titers using *in vitro* PvRII-DARC binding assays and by characterizing the prevalence of protein-specific IFN-γ secreting cells by *ex vivo* ELISPOT assays. We report that all three adjuvant formulations were found to be safe and highly immunogenic in rhesus monkeys. All three formulations tested yielded high titer antibodies with significant binding inhibitory activity. Montanide ISA 720 and AS02A formulations had higher binding inhibitory activity than the Alhydrogel formulation. These results provide support for further development of a vaccine for *P. vivax* malaria based on PvRII.

2. Materials and Methods

2.1. Animals

A group of 60 rhesus macaques of Chinese origin from the Yerkes National Primate Research Center facility were initially included in the study. The monkeys were screened for antibody reactivity against PvRII by ELISA and to simian malaria parasites by immunofluorescence using *P. cynomolgi*-infected red blood cells. From this group, thirty-six healthy, malaria naïve rhesus macaques were included in the immunization protocol (Table 1). Selected animals were matched by age, sex and weight, housed in social settings and randomly assigned to six experimental groups of 5 individuals each that received different vaccine formulations (Groups 1-6) and three control groups of two individuals each that received adjuvant alone (Groups 7-9). The procedures were approved by Emory University's Institutional Animal Care and Use Committee.

2.2. Vaccine formulation and immunization schedule

The production and characterization of recombinant PvRII has been described previously [17,20]. Briefly, a gene coding for PvRII from *P. vivax* Salvador I strain (aminoacid D_{194}-T_{521}; GenBank accession number M61095) was cloned as a *Nco*I-*Sal*I fragment in the *E. coli* expression vector pET28a(+) as described [17]. Bacterial transformation for expression of the recombinant construct was performed using *E. coli* BL21 (DE3) cells (Novagen, Madison, WI) and kanamycin selection. Protein expression of the recombinant 6-His tag PvRII was induced with 1 mM IPTG for 4 hours, purified by metal affinity chromatography under denaturing conditions, refolded by rapid dilution and purified further to homogeneity by ion exchange and gel filtration chromatography resulting in a protein of apparent molecular mass of ~39 kDa. The molecular mass determined by electron spray ionization mass spectrometry was 39,802 Da. Refolded PvRII was characterized for purity, homogeneity, identity and functional activity by SDS-PAGE, western blotting, reverse phase chromatography and erythrocyte binding assays as described previously [17]. Using reverse phase chromatography refolded PvRII elutes as a single, homogeneous product. The endotoxin content of purified PvRII was less than 25 EU per 25 μg using standard LAL assay [15,21]. Recombinant PvRII was formulated with either Alhydrogel [15], Montanide ISA 720 (Seppic, France) or AS02A Adjuvant System (GlaxoSmithKline Biologicals, Belgium) prior to immunization following manufacturers' protocols. The final formulation was adjusted to 0.5 ml containing 50μg or 10 μg of PvRII (see Table 1). Groups 1 and 2 were immunized with 50 μg and 10 μg of PvRII adsorbed to Alhydrogel, respectively. Groups 3 and 4 received 50 μg and 10 μg of PvRII emulsified in Montanide ISA 720, respectively. Groups 5 and 6 were immunized with 50 μg and 10 μg of PvRII formulated in AS02A, respectively. Six control animals were distributed in three groups (Groups 7 to 9) of two individuals each. Groups 7, 8 and 9 received saline solution adsorbed to Alhydrogel, Montanide ISA 720 and AS02A, respectively. The immunizations were given intramuscularly (IM) using the following schedule: priming into the right quadriceps femoris on day 0, first boost into the right musculus deltoideus on day 60 and the last boost into the left musculus deltoideus on day 150. Immunizations were given using a tuberculin syringe and a 22 gauge needle. Blood samples were obtained by femoral phlebotomy at seven time points before and during the study on days 0, 30, 60, 90, 120, 150 and 180. Serum and plasma were separated and either used for clinical chemistry assays immediately or frozen in aliquots at -80 °C for immunological studies. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradients and frozen in aliquots at -80 °C for use in ELISPOT assays.

2.3. Clinical characterization and safety assays

Animals were observed for general behavior and visible side effects during the entire study by two veterinary clinicians and their staff. Observations included analysis of skin for warmth,

erythema and edema/swelling, and for muscle induration or necrosis. The clinical veterinary staff examining the animals and injection sites was blinded to which vaccine formulation had been given. At the specified time points, the animals were sedated for bleedings and examination of the injection sites by the veterinary clinician. The total observation period of the animals was 240 days. Analysis of hematology and clinical chemistry of the different animals was performed on days 0, 30, 60, 90, 120, 150 and 180. Hematological analysis consisted of determination of number of erythrocytes (RBC), leukocytes (WBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV) and platelets (PLT). Analysis of clinical chemistry consisted of glucose, blood urea nitrogen (BUN), creatinine, protein, albumin, alkaline phosphatase, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), amylase and creatine phosphokinase (CPK) determinations.

2.4. ELISA

Sera were tested for recognition of PvRII by ELISA using Immulon-2 plates (Dynatech Laboratories, Chantilly, VA) coated with 1 μg/ml of the recombinant protein. Antigen specificity was confirmed by testing sera samples with a *P. yoelii* his-tag recombinant protein and synthetic peptides containing a $(His)_5$ sequence (VDKLAAALEHHHHH and HHHHHLESTSLYKKAG). After blocking with 5% bovine serum albumin (BSA), the plates were incubated with individual sera diluted in PBS with 2.5% BSA for 1 h at 37°C. Bound antibodies were detected using peroxidase-labeled goat anti-monkey IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and H_2O_2/2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Optical densities (OD) were determined using a VERSAmax ELISA reader (Molecular Device Corporation, Sunnyvale, CA) with a 405 nm filter. The reciprocal end point antibody titers, defined as the last serum dilution that yielded an OD greater than mean OD plus two standard deviations (SD) obtained with malaria naïve rhesus macaques at 1:200 dilution was considered as the endpoint ELISA titer.

2.5 Blocking of binding of PvRII to DARC-Fc by rhesus sera

Inhibition of binding of PvRII to DARC by sera from immunized rhesus macaques was determined using an ELISA-based binding assay. The N-terminal extracellular region of DARC was expressed as a fusion to Fc of human IgG (DARC-Fc) in a mammalian cell culture system as previously described [22]. ELISA plates were coated with 1 μg/ml DARC-Fc using sodium bicarbonate buffer pH 9.6 and the plates incubated overnight at 4°C. The plates were then washed three times with PBS pH 7.4 containing 0.05% Tween 20. Plates were then blocked with 1% skimmed milk in PBS for 2 hours at 37°C. Pooled rhesus sera from groups 1 to 6 collected on days 30, 90, 150 or 180 were used as test sera. Pre-immune sera and adjuvant alone sera from each group were used as controls. PvRII (0.1μg/ml) was pre-incubated with pooled sera at final dilutions of 1:10, 1:50, 1:250, 1:1,250 and 1:6,250 for 1 hour at room temperature prior to addition to DARC-Fc coated plates. Sera from pre-immune and adjuvant control groups were used at a dilution of 1:10. PvRII (0.1 μg/ml) without sera was used as positive control for binding to DARC-Fc. PvRII with/without sera was allowed to bind DARC-Fc coated wells for 1 hour at 37°C. Bound PvRII was then detected with anti-PvRII rabbit polyclonal sera (1:1,500), followed by mouse anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Aldrich Corp., St Louis, MO) at a dilution of 1:2,500. Plates were developed using o-phenelenediamine (Sigma Aldrich Corp., St Louis, MO). Mean OD (A_{490} nm) values from test and control groups were determined. Mean OD values of binding in presence of sera were normalized as %OD compared to OD values of PvRII binding in absence of sera. A standard curve for PvRII binding to DARC was used to estimate bound PvRII in presence of sera using the %OD values. The results are expressed as the dilution of sera at which 50% binding inhibition was observed.

2.6. ELISPOT

The frequency of peptide-specific T lymphocytes was determined by IFN-γ–specific ELISPOT as described [23]. Briefly, 96-well nitrocellulose-bottom plates (Multiscreen-HA, Millipore, Molsheim, France) were coated with 100 μl/well of GZ-4 monoclonal antibody (mAb) at 15 μg/ml (Mabtech Inc., Mariemont, OH) and incubated overnight at 4 °C. The following day, plates were washed five times in PBS and blocked with RPMI medium containing 10% FCS. A cytokine cocktail composed of 25 ng/ml each of IL-7 and IL-15 (R&D Systems, Minneapolis, MN, USA) was added in 100 μl followed by 50 μl of PvRII or negative control recombinant protein to a final concentration of 10 μg/ml. Cryopreserved PBMC were thawed, washed twice with RPMI and the cell viability evaluated by Trypan blue exclusion. 2×10^5 PBMCs were then added in a volume of 50 μl, bringing the total volume in each well to 200 μl. Incubation was continued for 24 hours at 37 °C, 5% CO_2. After incubation the plates were washed and incubated with biotinylated mAb 7-B6-1 (Mabtech Inc., Mariemont, OH) followed by incubation with Streptavidin-HRP. The reaction was developed using 3-amino-9-ethylcarbazole (AEC) (BD Biosciences Pharmingen, San Jose, CA) and evaluated in an Immunospot Analyzer (Cellular Technology-Becton Dickinson, San Diego, CA). Staphylococcal enterotoxin B (SEB) (Sigma Aldrich Corp., St Louis, MO) was used as positive control for activation. The results are expressed as total number of spot forming cells (SFC) per 10^6 splenocytes. Total number for duplicate wells were averaged and normalized to numbers of IFN-γ spot forming cells per 1×10^6 PBMC. Average values for negative medium control wells in the presence of cytokines were subtracted from the average values from antigen-stimulated wells. To take in to consideration individual variability, recall PvRII-reactive T cells were calculated by subtracting the spot forming cells obtained with pre-immune samples.

2.7. Statistical analysis

Antibody levels were log-transformed and multiple comparisons were conducted using Student's *t*-test. To evaluate the correlation between antibody titers and inhibition of binding of PvRII to DARC-Fc by anti-PvRII rhesus sera, a Spearman rank correlation was used. For hematology and clinical laboratory values repeated measures of analysis of variance (ANOVA) were conducted to evaluate overall differences at a particular time point. For the ELISPOT assays, we tested the significance of the differences between doses using the one-tailed paired Student's *t*-test after subtracting the background spots and using data log transformation. *P*-values of 0.05 or less were considered significant.

3. Results

3.1 Safety assessment

Animals were evaluated daily for the presence of clinical abnormalities and general behavior. They were also examined closely under general anesthesia at days 30, 60, 90, 120, 150 and 180 to identify signs of local reactogenicity. No local abnormalities were identified in the inoculation sites upon close examination. Hematology and clinical chemistry values determined at days 0, 30, 60, 90, 120, 150 and 180 remained within the normal range. In some animals, in both experimental or control groups at different time points, high levels of CPK were detected randomly without clinical expression of tissue damage. These changes were considered within a normal range for rhesus macaques housed in social settings where minor sub-clinical traumas are expected.

3.2 Antibody mediated immune response

We have evaluated six formulations of PvRII using three different clinically accepted adjuvants. Figure 1 summarizes the kinetics of the anti-rPvRII antibody response determined

by ELISA. Pre-immune geometric mean antibody titers ranged between 1:200 and 1:3200. Thirty days after the first immunization all rhesus macaques seroconverted (defined by fourfold or greater increase over baseline) after receiving PvRII with either formulation with five to eight-fold increase in titers from the pre-immune values. After a single immunization, differences were significant only between Montanide and Alhydrogel groups for the 10 μg protein concentration (P=0.004). This is in contrast with antibody titers evaluated 60 days after the second immunization where both Montanide and AS02A groups were significantly greater than Alhydrogel groups (P values: Montanide 50 -Alhydrogel 50 = 0.01; AS02A 50-Alhydrogel 50 = 0.0009; Montanide 10-Alhydrogel 10 = 0.000277 and AS02A 10-Alhydrogel 10 = 0.015). Differences in antibody titers between animals that received PvRII formulated in Montanide or AS02A were not significant. These trends were maintained after second boosting conducted 150 days after the first immunization. Differences in antibody titers obtained with 10 and 50 μg immunization regimes at different time points were only statistically significant for the PvRII formulated with Alhydrogel (P-values ranged from 0.005 to 0.019). There were no differences in antibody titers in rhesus macaques that received placebo in comparison to the pre-immune levels.

3.3. Functional characterization of antibodies elicited by immunization

The ability of the antibodies to block binding of PvRII to DARC was characterized by binding inhibition assays. Figure 2 summarizes the kinetics of functional antibodies determined at four different time points expressed as the dilution of sera at which 50% binding inhibition was observed. Functional antibodies were detected in pools of sera obtained from animals 60 days after the second immunization with each formulation. When the inhibition of binding profiles were compared between groups that received 10 or 50 μg with the same adjuvant, differences were only significant for Alhydrogel (P=0.05). Significant correlations were found between the antibody titers determined by ELISA and inhibition of binding assays. For statistical analysis antibody titers determined on days 0, 90, 150 and 180 were used to rank antibody levels for both protein concentrations and adjuvants used for immunization, and the data were compared with inhibition of binding using a Spearman rank correlation test. The correlation between ELISA and inhibition of binding ranked from 0.9048 to 0.9461, all with P values of <0.05. For overall data the Spearman rank-order correlation between ELISA titers obtained with adjuvants Alhydrogel, Montanide and AS02A and inhibition of binding coefficient was 0.9097 (P<0.000001).

3.4. T cell recall response

PvRII-specific IFN-γ producing T cells were identified by ELISPOT at different time points. Broad individual variability was observed in the magnitude of IFN-γ ELISPOT responses. Normalized results were expressed as IFN-γ Spot Forming Cells (SFC) per million PBMC after subtracting pre-immune values (Figure 3). For statistical analysis, comparisons between groups that received 10 μg or 50 μg of PvRII formulated with the same adjuvant were conducted. The differences observed between 10 μg or 50 μg doses were significant for Montanide ISA 720 (P=0.0018) and Alhydrogel (P<0.0001).

4. Discussion

The Duffy binding protein is a leading vaccine candidate for protection against *P. vivax* malaria. The functional DARC-binding domain is located in region II (PvRII) of *P. vivax* DBP [9]. PvRII is the prototypical Duffy-binding-like (DBL) domain, which shares structural features with other receptor-binding domains belonging to the DBL family [8,24,25]. Antibodies directed against the homologous DBL domains of *P. knowlesi* DBP and *P. falciparum* EBA-175 block red cell invasion by *P. knowlesi* and *P. falciparum* respectively providing a rationale for development of a vaccine for *P. vivax* malaria based on PvRII [6,10,26,27]. Rhesus

macaques were used to demonstrate safety and immunogenicity of recombinant PvRII. We have shown that recombinant PvRII induced comparable levels of antibodies when it is formulated either with Montanide 720 or AS02A. Titers of antibodies significantly correlate with their ability to inhibit binding of PvRII to DARC. Antibody levels induced by immunization with PvRII adsorbed to Alhydrogel were lower than the titers induced by immunization with PvRII formulated in Montanide ISA 720 and AS02A. Despite these low antibody titers, sera samples obtained from animals immunized with the Alhydrogel formulation also had an effect on neutralizing the binding of PvRII to DARC-Fc *in vitro*. Functional antibodies that block PvRII-DARC-Fc interaction are likely to inhibit erythrocyte invasion by *P. vivax* merozoites. The binding inhibitory activity of such antibodies may serve as an important *in vitro* correlate for protection in efficacy trials of a PvRII-based malaria vaccine [28].

All three PvRII formulations tested in rhesus monkeys were well tolerated. To facilitate the identification of local reactivity, immunization sites were alternated between right quadriceps femoris and right and left musculus deltoideus. Systemic reactogenicity was evaluated by periodic evaluation of hematological and clinical chemistry parameters. Although we found individual variability, clinical laboratory values remained within normal reference ranges. These results are comparable to recent reports testing a malaria vaccine candidate in rhesus macaques using similar adjuvant formulations [29]. The use of rhesus macaques in preclinical trials of malaria vaccine candidates has recently gained attention [29,30]. Rhesus macaques are phylogenetically closely related to humans. The broad use of this animal model in biomedical research has stimulated and facilitated the development of species-specific reagents [31]. The substantial body size of rhesus macaques, compared with small New World monkeys [32], also facilitates the routine assessment of clinical laboratory chemistry and hematological parameters using standard phlebotomy procedures in the course of the trial. Future investigations using this model can evaluate the efficacy of vaccine candidates based on *P. cynomolgi* antigens for proof of principle, with the challenge of different strains of *P. cynomolgi* parasites. Animals immunized with *P. vivax* antigens, as reported here, can also be challenged with *P. cynomolgi* parasites as a rigorous test for efficacy, to show and predict the likelihood of achieving heterologous protection as would be desired in field trials [33].

The prevalence of naturally acquired antibodies to *P. vivax* DBP increases with age in endemic areas. This age-dependent pattern of immune recognition also correlates with acquisition of protection against *P. vivax* malaria [12,14,34,35]. Acquired anti-DBP antibodies are also correlated with levels of exposure [36,37]. Relevant for malaria vaccine development, anti-DBP antibodies are higher in asymptomatic individuals suggesting that the prevalence of *P. vivax* DBP antibodies may modify the clinical outcome, reducing the severity of the disease state [14,36]. The immunological relevance of PvRII has been confirmed by genotypic characterization of wild isolates, which indicates a decline in the proportion of individuals infected with multiple PvRII haplotypes with increasing age [38]. We show here that immunization of rhesus macaques with a refolded PvRII induced functional antibodies with the potential to inhibit parasite invasion. These results are consistent with the high immunogenicity and partial protection induced in *Aotus* monkeys in previously described experiments [19].

PvRII also contains a cluster of T cell epitopes that are recognized by individuals living in endemic areas of *P. vivax* malaria [14,39]. PvRII-specific IFN-γ secreting cells were identified here at different time points after immunization. Surprisingly, statistically significant differences were defined in animals immunized with Alhydrogel and Montanide ISA 720 formulations but not with AS02A. Recent clinical trials of malaria vaccine candidates have defined the complexity of the T cell reactivity and the relevance of using cultured ELISPOT assays to characterize protective T cells [40]. In contrast with *ex vivo* ELISPOT assays, as used

here, cultured ELISPOT assays involve the *in vitro* culture of PBMC in the presence of relevant antigens and IL-2 for several days to identify resting memory cells. This T cell subset persists for several months after immunization and correlates with protection [41]. We have evaluated T cell reactivity using ELISPOT after *ex vivo* stimulation with PvRII. The evidence that PvRII formulated with different adjuvants induces heterogeneous T cell reactivity in rhesus macaques requires further characterization and the use of a panel of synthetic peptides for *ex vivo* stimulation. It has been described that several assays to test T cell reactivity are required to have a clear picture of memory cells in clinical trials of malaria vaccine candidates [42].

In conclusion we report that PvRII formulated in the human compatible adjuvants Alhydrogel, Montanide ISA 720 and AS02A is safe and highly immunogenic in rhesus monkeys. Each formulation tested elicited high titer binding inhibitory antibodies. These results support further clinical development of this promising candidate as a vaccine for *P. vivax*.

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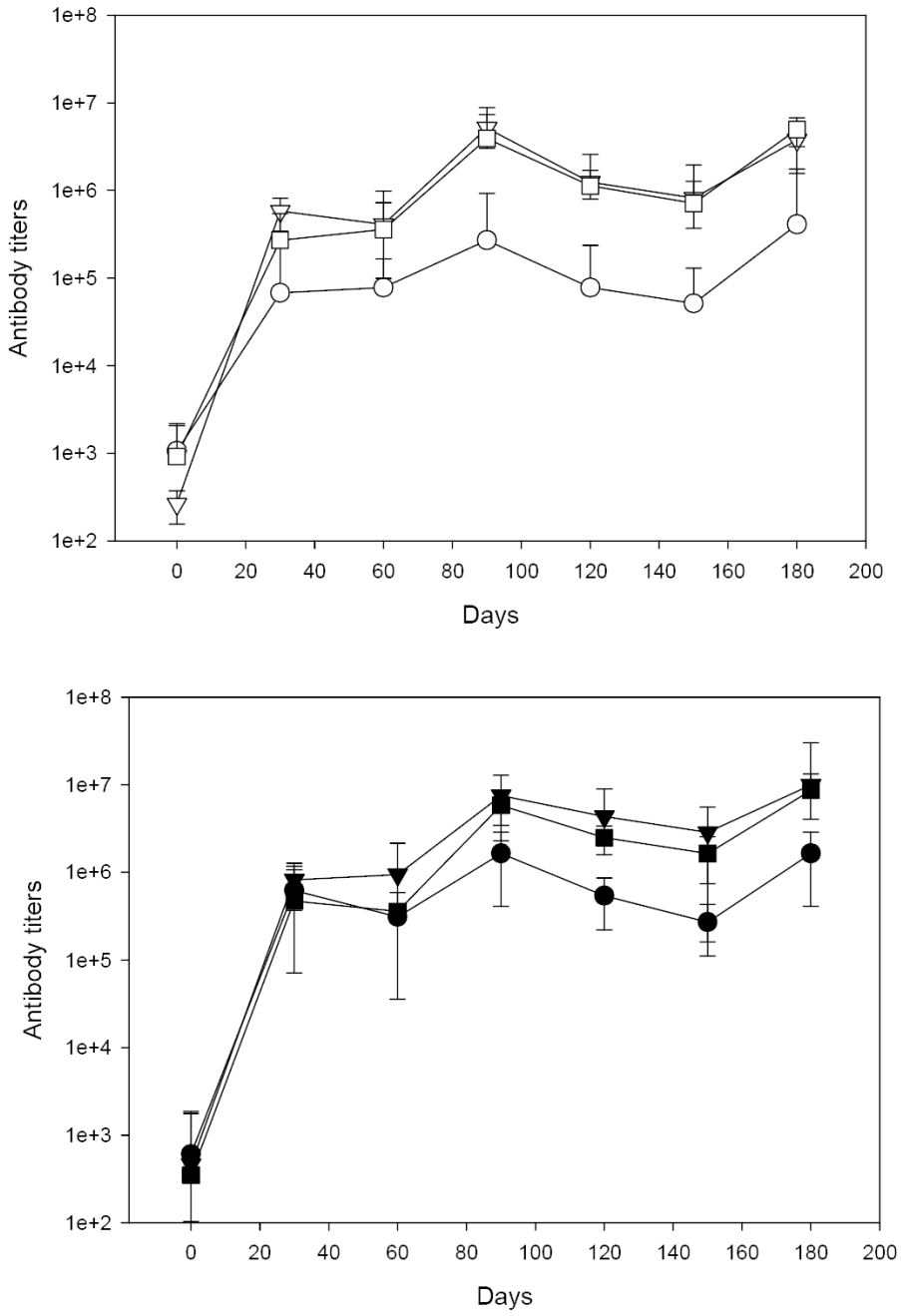

Figure 1.

PvRII formulated with different adjuvants induces high antibody titers in rhesus macaques. The figure represents the kinetics of the antibody response to PvRII determined by ELISA using geometric mean antibody titers for individual formulations (± standard deviation). Open symbols, rhesus macaques immunized with 10 μg PvRII. Closed symbols, rhesus macaques immunized with 50 μg PvRII. Alhydrogel group (○ ●), Montanide ISA 720 group (▽ ▼) and AS02A group (□ ■).

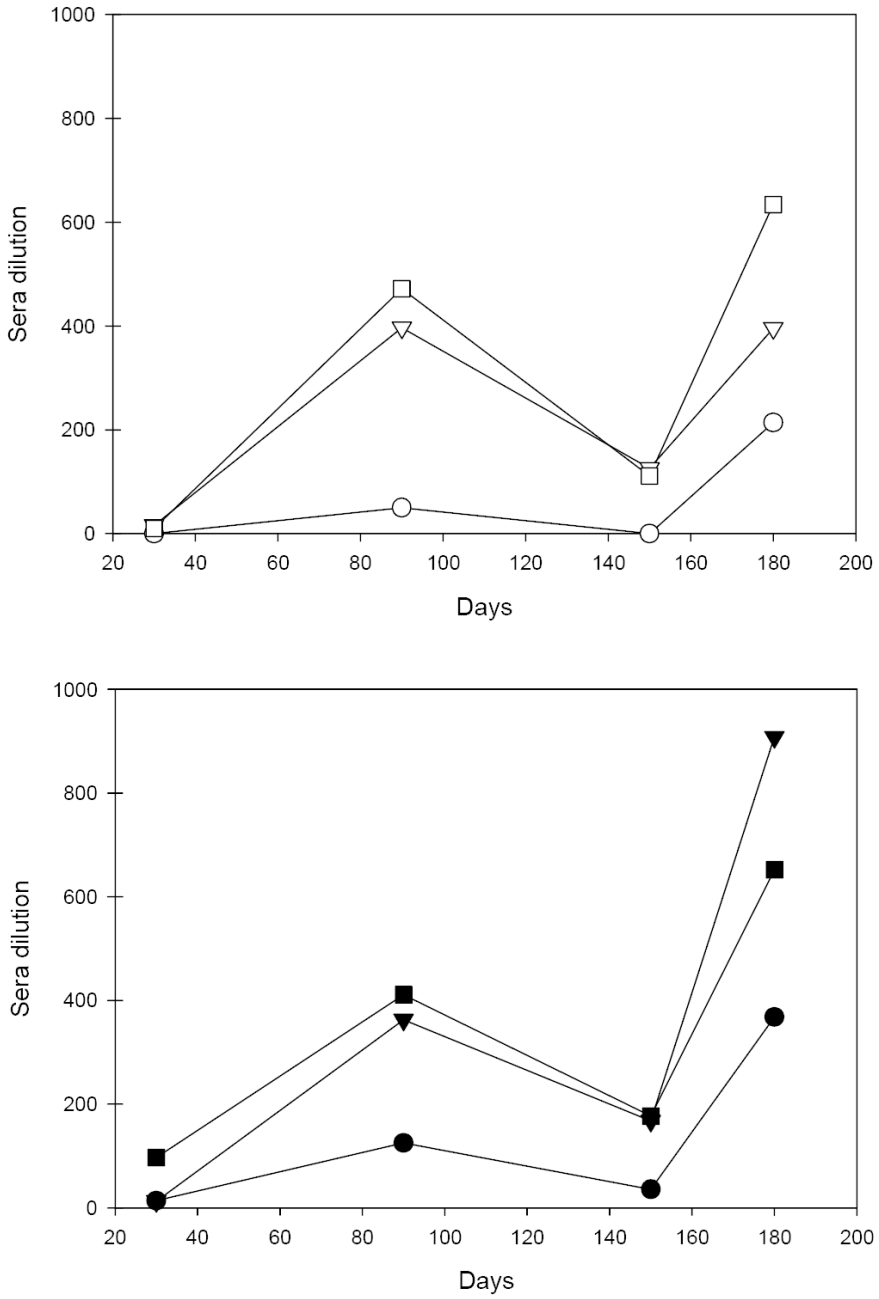

Figure 2.

Binding inhibitory activity of anti-PvRII sera induced by immunization of rhesus macaques with PvRII formulated with different adjuvants. Sera samples were obtained at different time points after immunization and tested for inhibition of PvRII binding to DARC-Fc as described [22]. Functional activity is expressed as sera dilution at which 50% binding inhibition was observed. Open symbols, rhesus macaques immunized with 10 μg rPvRII. Closed symbols, rhesus macaques immunized with 50 μg *P. vivax* DBP RII. Alhydrogel group (○ ●), Montanide ISA 720 group (▽ ▼) and AS02A group (□ ■).

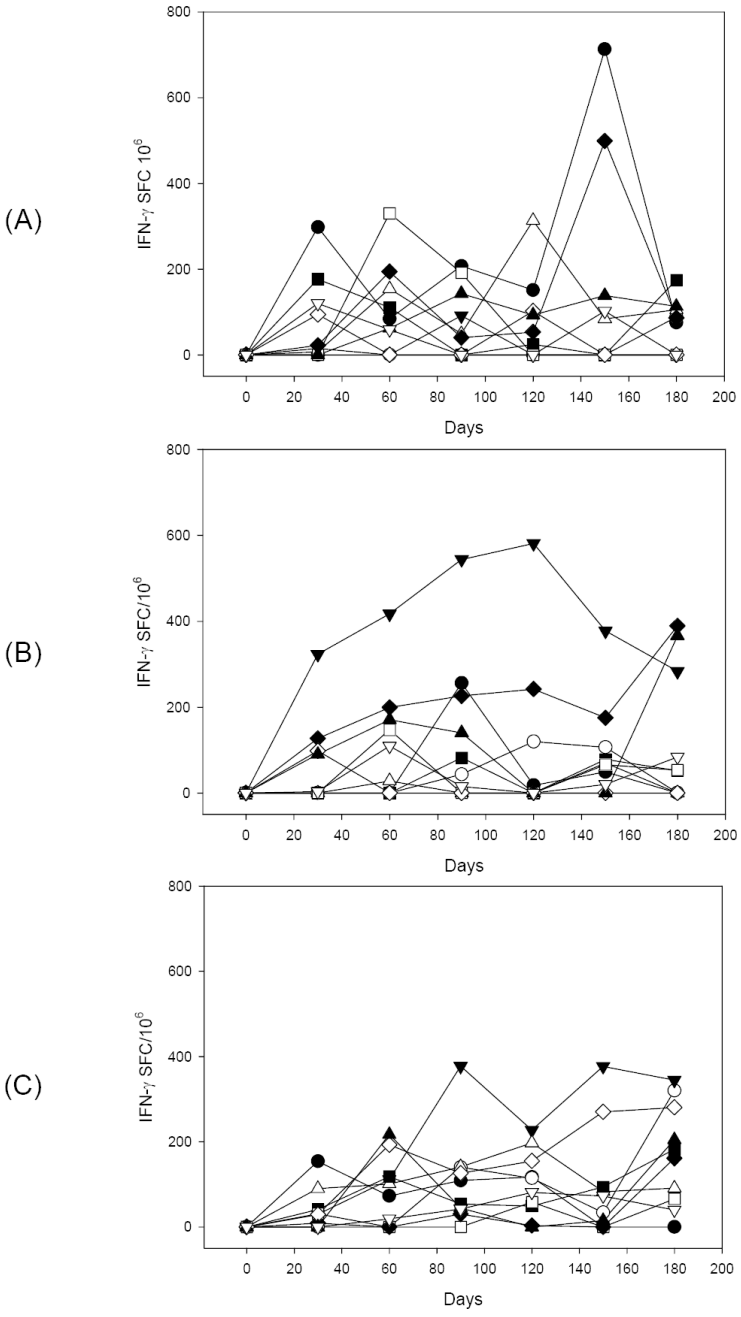

Figure 3.

Kinetics of the prevalence of IFN-γ secreting cells determined by ELISPOT in PBMC obtained from animals immunized with different formulations of rPvRII after *ex vivo* stimulation with the recombinant protein. Data are presented for individual animal at different time points and expressed as IFN-γ spot forming cells per 1×10^6 PBMC. Average values for medium control wells in the presence of cytokines were initially subtracted from the average values obtained from antigen-stimulated wells. To take in consideration individual variability, recall PvRII-reactive T cells were calculated by subtracting the spot forming cells obtained with pre-immune samples. Rhesus macaques were immunized with 50 μg *P. vivax* DBP RII (closed symbols) or

10 μg *P. vivax* DBP RII (open symbols) formulated with Alhydrogel (A), Montanide ISA 720 (B) or AS02A (C).

Table 1

Groups of rhesus macaques immunized with rPvRII using several adjuvant formulations.

Group code	rPvRII dose[a]	Adjuvant formulation[b]
1	50 μg	Alhydrogel
2	10 μg	Alhydrogel
3	50 μg	Montanide ISA 720
4	10 μg	Montanide ISA 720
5	50 μg	AS02A
6	10 μg	AS02A
7	Saline	Alhydrogel
8	Saline	Montanide ISA 720
9	Saline	AS02A

[a] Administered intramuscularly in a final volume of 500 μl.

[b] Rhesus macaques per experimental groups *N*=5 and placebo groups *N*=2.