

Blood-Stage PvRBP2b and PvDBP-RII Merozoite Recombinant Proteins Confer Partial Immunity in a Heterologous Plasmodium vivax Aotus Challenge Model

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Abstract

Plasmodium vivax remains a major challenge to malaria control. Blood-stage vaccines targeting reticulocyte invasion ligands such as PvDBP-RII and PvRBP2b offer a promising strategy to reduce parasite replication and disease. We evaluated the immunogenicity and protective efficacy of recombinant PvDBP-RII and PvRBP2b proteins, administered alone or in combination in *Aotus l. lemurinus* monkeys.

For this purpose, sixteen male and female *Aotus* monkeys were randomized by weight and sex into four groups (n = 4 per group). Each group was immunized subcutaneously on days 0, 21, and 36 with 20 µg of recombinant protein formulated with Alhydrogel®, or with adjuvant alone. Two weeks after the final dose, all animals were challenged intravenously with 5.0×10^4 infected red blood cells of the heterologous chloroquine- and pyrimethamine-resistant *P. vivax* AMRU-1 strain. Parasitemia was monitored daily. Antibody responses were measured by ELISA.

Analysis of parasite multiplication rate (PMR) revealed reduced replication in vaccinated groups (3.7–5.3) compared to controls (7.9). This shows partial control of blood-stage infection. In contrast, no significant difference in peak parasitemia was observed between the vaccinated and control groups. Unexpectedly, vaccinated animals had shorter prepatent periods than controls ($p < 0.05$), suggesting earlier parasite detection or enhanced parasitemia in vaccinated animals.

All vaccine formulations elicited strong IgG responses. In the combination group, higher pre-challenge antibody levels correlated with earlier peak parasitemia ($r = -0.9686$; $p = 0.0314$). Suggesting enhanced parasite control. Similarly, an inverse, non-significant trend was observed between antibody levels and PMR.

These findings demonstrate that PvDBP-RII and PvRBP2b recombinant protein vaccines induce robust humoral immunity in *Aotus* monkeys, providing partial control of parasite growth as detected by PMR. This supports further development of multicomponent blood-stage vaccines against *P. vivax*.

Highlights

- The *Plasmodium vivax* blood-stage recombinant vaccine challenge model requires sensitive endpoints beyond parasitemia to detect protective effects.
- A *Plasmodium vivax* recombinant protein vaccine targeting complementary reticulocyte invasion pathways, PvRBP2b and PvDBP-RII, was tested in *Aotus* monkeys against a heterologous challenge.
- Parasite multiplication rate (PMR) and antibody correlates emerge as robust metrics for evaluating blood-stage vaccines.
- Vaccination significantly lowered parasite multiplication rate (PMR), indicating partial control of blood-stage replication.

- High pre-challenge antibody levels correlated with earlier parasite control, supporting a functional role for vaccine-induced humoral immunity.
- These findings support multicomponent vaccine strategies targeting invasion pathways to limit parasite growth rather than achieve sterilizing immunity.

Introduction

Malaria is caused by parasites of the genus *Plasmodium*, which are transmitted to humans through the bite of female *Anopheles* mosquitoes. In 2024, there were approximately 262 million cases and 0.6 million deaths attributed to malaria, with the majority caused by *P. falciparum*, the most pathogenic species, especially among children under five years old in sub-Saharan Africa. (1).

Plasmodium vivax, in contrast, is responsible for 9 to 22 million clinical cases annually, putting about 4 billion people at risk. Although it causes fewer deaths than *P. falciparum*, it is more widespread and presents a significant public health challenge for elimination in Asia, the Americas, and the Horn of Africa. (1).

Unlike *P. falciparum*, it selectively infects reticulocytes, immature red blood cells that constitute only a small proportion of circulating erythrocytes (2, 3), resulting in lower detectable parasitemia levels in the blood while ongoing transmission occurs, even before the appearance of symptoms (4).

Moreover, its ability to relapse from dormant liver hypnozoites, combined with recurrences from cryptic reservoirs in the bone marrow and spleen (5), rapid gametocyte maturation (4), and substantial genetic diversity (6) that drives pathogenicity and drug resistance (7), makes this parasite particularly challenging to control and eliminate. These characteristics underscore the urgent need for effective vaccines that block blood-stage infection and prevent clinical disease in endemic regions.

A major hurdle in vaccine development and a key limitation in *P. vivax* research is the absence of a standardized in vitro culture system (8–11). Consequently, a critical component of current pre-clinical vaccine development for *P. vivax* is evaluating efficacy in non-human primates, such as *Aotus* monkeys, which are a good model for evaluating vaccines against *Plasmodium falciparum* (12, 13) and *vivax* (14, 15). Furthermore, *Aotus* monkeys have been used as models for the development of antimalarial drugs (16) and vaccines (14), with human parasites adapted through serial passage (17–19).

Recently, we demonstrated sterile immunity in *Aotus* monkeys following two repeated homologous infections with the SAL-1 strain of *P. vivax*, while a subsequent challenge with the heterologous AMRU-1 strain offered only partial protection. (15), establishing the first benchmark for evaluating *P. vivax* blood-stage vaccines in *Aotus* monkeys. The protein microarray analysis of the *P. vivax* infections showed that repeated blood-stage exposure progressively increased both the magnitude and breadth of antibody responses, particularly during the development of sterile immunity. The most seroreactive antigens were MSP-1 fragments (PVX_099980), followed by ETRAMP (PVX_090230), PV1 (PVX_092070), and several

exported proteins, including PVX_121930, PVX_121935, and PVX_083560. MSP-1 and ETRAMP exhibited the strongest correlations with ELISA antibody titers and protective immunity. In total, 66 of 244 antigens were reactive, indicating that broad, multi-antigen or multi-strain vaccines may be required for cross-strain protection against *P. vivax*.

Blood-stage vaccines targeting reticulocyte invasion offer a promising strategy to reduce clinical disease. Among the invasion ligands expressed in *P. vivax* merozoites, two surface-exposed proteins have emerged as leading vaccine candidates: the Duffy binding protein (PvDBP) (20) and the reticulocyte binding protein 2b (PvRBP2b) (21). But antigen polymorphism (22) and strain-transcending immunity remain puzzling to researchers studying these two candidate proteins (23, 24). Consequently, the development of a highly effective, strain-transcending blood-stage vaccine to prevent clinical disease remains elusive.

PvDBP and PvRBP2b are essential invasion ligands from distinct protein families that interact with separate host receptors (25). Incorporating both ligands into a bivalent vaccine is expected to enhance and extend protective efficacy by simultaneously blocking two critical invasion pathways, thereby reducing immune evasion and broadening protection across diverse erythrocyte subsets. For instance, PvDBP facilitates parasite entry by binding to the Duffy antigen/receptor for chemokines (DARC) on human reticulocytes. The receptor-binding domain, known as region II (PvDBP-RII or DBPII), establishes a distinct interface with DARC that is structurally and immunologically accessible (26, 27).

PvDBP-RII is a well-characterized ligand essential for reticulocyte invasion via the Duffy antigen receptor for chemokines (DARC), and antibodies targeting this interaction have been shown to inhibit invasion in vitro and correlate with reduced parasitemia in endemic populations (28). Similarly, PvRBP2b plays a critical role in reticulocyte recognition through transferrin receptor 1 (CD71), and recent studies have demonstrated potent invasion-inhibitory activity of PvRBP2b-specific antibodies, supporting its candidacy as a blood-stage vaccine antigen (29).

Antibodies targeting PvDBP-RII can prevent DARC binding and stop erythrocyte invasion in vitro. However, significant variability in PvDBP-RII across global parasite populations presents a major challenge, as many naturally occurring amino acid changes at key antibody-binding sites may weaken strain-specific immune responses. PvDBP-RII is currently being tested in clinical trials. (20) (30).

PvRBP2b has been identified as a complementary vaccine target that functions through a distinct invasion pathway (31, 32). PvRBP2b binds to transferrin receptor 1 (TfR1, CD71), a surface marker highly enriched on reticulocytes, and this interaction facilitates parasite tropism for these immature erythrocytes, although it is not essential (33). Structural studies have delineated specific receptor-binding domains within PvRBP2b, and both naturally acquired and vaccine-induced antibodies have been shown to inhibit ligand–receptor interactions. Notably, because the PvRBP2b–TfR1 pathway operates independently of DARC, it may provide an alternative invasion route in populations with Duffy-negative individuals or parasites that exhibit altered DBP usage (34).

PvDBP and PvRBP2b constitute rational, complementary targets for a multivalent blood-stage vaccines that block *P. vivax* invasion through both DARC-dependent and DARC-independent mechanisms. Comprehensive characterization of their structural features, polymorphism patterns

(22, 23), immunogenicity, and roles in host–parasite interactions are critical for optimizing antigen design and advancing next-generation vaccine formulations. The integration of structural biology, functional assays, and field-based immunoepidemiology will guide the development of vaccine strategies with sufficient breadth and potency to reduce the global burden of *P. vivax* malaria.

This study shows that recombinant PvDBP-R11 and PvRBP2b vaccines, when given individually or together to Aotus monkeys, confer partial immunity under heterologous AMRU-1 challenge. This is shown by lower parasite multiplication rates and slower infection progression. However, these vaccines do not prevent parasite emergence. These results underscore the value of the parasite multiplication rate as a sensitive endpoint for assessing functional vaccine effects in the *Plasmodium vivax* non-human primate Aotus model under heterologous challenge conditions.

Materials and Methods

Ethical Statement.

The experimental protocol was approved and registered with the ICGES – Institutional Animal Care and Use Committee (CIUCAL) under accession number CIUCAL-03/2021. It was conducted in accordance with the Animal Welfare Act, the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Resources, National Research Council, National Academy Press, 1996), and the laws and regulations of the Republic of Panama.

Study design.

Animals. Sixteen spleen-intact, laboratory-bred male and female *Aotus l. lemurinus* monkeys with karyotypes VIII and IX (35), weighing 624–1040 g (**Supplementary Table 1**), were randomly assigned by weight and sex into four groups of four animals each. Additionally, two Aotus were used as donors of infected blood for the parasite challenge. In total, eighteen animals were used in the experiment. The laboratory-bred monkeys were cared for and treated as described (36).

Parasites. The chloroquine and pyrimethamine-resistant *Plasmodium vivax* AMRU-1 strain, originally isolated from a patient infected in Papua New Guinea (37), was used for the challenge. This strain was subsequently adapted to spleen-intact Aotus by serial passage (7) (38).

Recombinant Protein Synthesis and Purification. Recombinant proteins were synthesized in HEK293-6E mammalian cells transfected with plasmid constructs in a *P. vivax* SAL-1 allele, as described in (39). Protein purification was performed using Ni²⁺ affinity chromatography (40). (**Supplementary Figures S1 and S2**).

Immunogen preparation. One antigen concentration (20 µg/mL) for each of the recombinant *P. vivax* proteins was emulsified with Aluminum Hydroxide Al(OH)₃ (Alhydrogel® adjuvant, 2% InvivoGen, San Diego, CA), a Th2-specific adjuvant, as described (41).

Immunization. Each animal received three subcutaneous injections in the lower abdomen on days 0, 21, and 36, with 20 µg of recombinant PvRBP2b or PvDBP-RII protein alone or in combination, formulated with Alhydrogel® adjuvant (**Figure 1**).

Two weeks after the last immunization, the animals were challenged with 50×10^3 infected red blood cells (iRBCs)/mL of the *P. vivax* AMRU-1 strain obtained from a donor monkey as described (42). Parasitemia was monitored daily with thick blood smears and qPCR, and ELISA was used to measure antibody levels. In summary, Group 1 received the adjuvant only; Group 2 received PvDBP-RII; Group 3 received PvRBP2b; and Group 4 received the combined PvDBP-RII / PvRBP2b formulation. Standardized clinical observations, hematological evaluations, and clinical chemistry analyses were performed at predefined intervals throughout the study (**Figure 1**).

Post-challenge monitoring

Parasitemia. During the post-challenge (PoC) period, infected monkeys were observed twice daily for signs of illness, such as loss of appetite or depression. Parasite counts were performed using the Earle and Perez method (1932) (43) with daily Giemsa-stained thick blood smears obtained by pricking the marginal ear vein with a lancet.

Hematology and Blood Chemistry. Blood samples (0.5 mL) were collected in EDTA microtubes on day -1, 14, 34, 56, 72 (15 PoC), and 85 (28 PoC) from the femoral vein to perform a complete blood count (CBC), and conduct blood chemistry analysis (liver and renal panels) using a veterinary hematology analyzer (VetScan HM5, Abaxis, Inc., Union City, CA) and a chemistry analyzer (VetScan 2, Abaxis, Inc., Union City, CA).

Quantitative Polymerase Chain Reaction (qPCR). Whole blood was preserved on filter paper (903TM Five Spot Blood Card (Eastern Business Forms, 530 Greenville, SC, USA) for quantitative PCR-based determination of parasitemia as described (4). Extracted DNA was quantified by qPCR using primers for the *P. vivax* 18S rRNA gene. Cycle threshold (Ct) values were converted to parasite equivalents per reaction using the standard curve, then normalized to parasite equivalents per microliter of blood based on extraction input, elution, and PCR template volumes. *P. vivax* 18S rRNA primers F 5'-GTTAAGGGAGTGAAGACGATCAGA-3' R 5'-AACCCAAAGACTTTGATTTCTCATAA-3'

Rescue treatment. Animals were rescue-treated with a single oral dose of mefloquine (MQ) to terminate the experiment if: 1) parasitemia exceeded $150 \times 10^3/\mu\text{L}$ of blood; 2) hematocrit fell below 50% of the baseline value, or 3) platelets fell to levels below $50 \times 10^3/\mu\text{L}$. Treated animals were followed up until negative for three consecutive days. On day 28 PoC, any previously untreated animals were treated with MQ at 25 mg/Kg and returned to the breeding colony.

The Parasite Multiplication Rate (PMR) was calculated during the exponential phase of parasitemia between days 10-15 PoC (before treatment or clinical cutoff) from the slope of a linear model fitted to log₁₀-transformed data, using the formula $PMR = 10^{(\beta \times 2)}$ where β = slope, with a replication cycle of 2 days for *P. vivax* (44).

Serology. Blood samples were drawn from the femoral vein on days -1, 14, 34, 56, 72 (15 PoC), and 85 (28 PoC) of immunization to measure antibody responses by ELISA.

ELISA. An indirect ELISA was used to measure total IgG antibody levels, as described (7). Nunc MaxiSorb® ELISA plates were coated with 50 µL of PvRBP2b-161–813-N (30 µg/mL) or 50 µL of PvDBP H6-d (5 µg/mL) per well. MSP1-42 protein was used as a control antigen. Data are shown as mean OD ± SEM at 492 nm.

Statistical analysis. The Prism-10® plotting and statistical software package (GraphPad Software Inc., La Jolla, CA) was used to determine differences in parasitemia means among immunized groups, using the unpaired t-test. Furthermore, we calculated the mean daily cumulative parasitemia for each treatment group. Finally, we measured the change in pre-challenge antibody levels relative to parasitemia outcomes for each immunized group using a simple Pearson correlation in JMP (SAS Institute, Cary, NC).

Data availability.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Results

Subcutaneous immunization with PvRBP2b alone or in combination with PvDBP-RII formulated with Alhydrogel® confers partial protection against a heterologous *P. vivax* blood-stage challenge.

To evaluate the protection provided by PvDBP-RII and PvRBP2b recombinant proteins, administered alone or in combination by subcutaneous injection, sixteen Aotus monkeys were divided into four groups. Each group was infected intravenously with 50.0×10^3 *P. vivax* AMRU-1-infected red blood cells (iRBCs) and monitored for parasitemia using thick blood smears stained with Giemsa and qPCR.

Outcomes included PMR, prepatent period, peak parasitemia, and daily mean cumulative parasitemia.

Individual parasitemias in monkeys immunized with PvRBP2b alone, PvDBP-RII alone, or the PvDBP-RII / PvRBP2b combination are shown in **Figures 2 and 3** and **Supplementary Table 1**.

The qPCR dataset reveals clear differences in parasite dynamics following challenge between vaccinated and control animals (**Supplementary Table 2**). Control monkeys (Al(OH)₃) exhibit earlier parasite detection and a more rapid increase in parasitemia, characterized by consistently rising genome copy numbers and higher peak values over time. Parasitemia rapidly expands following initial detection,

indicating limited control of the blood-stage infection. Although inter-individual variability is observed, the predominant trend is a progressive and sustained increase in parasite burden.

Monkeys vaccinated with PvDBP-R11, PvRBP2b, or the combination (PvDBP-R11 / PvRBP2b) show reduced parasitemia. These animals exhibit delayed parasite emergence, lower peak parasitemia, and slower parasite expansion than controls. The combination vaccine produces the greatest effect, improving parasite control beyond that achieved by single-antigen vaccines. However, individual responses differ, resulting in partial and inconsistent protection rather than complete immunity. Overall, the qPCR results indicate that vaccination, particularly with the combined formulation, reduces parasite burden and slows infection kinetics. These findings are consistent with functional but incomplete protective efficacy in the *Aotus* model.

qPCR provides substantially greater sensitivity than thick blood smear microscopy, enabling detection of low-density parasitemia and earlier parasite emergence, whereas microscopy remains essential for confirming patent infection and parasite species identification (45).

The vaccine formulations did not reduce the peak or cumulative parasitemia, nor delay the prepatent period (**Figure 3**); peak parasitemia occurred 15–20 days PoC. During this period, the median peak parasitemia in the control group (AlOH₃) was $258.5 \times 10^3 \times \text{iRBCs}/\mu\text{L}$, while in the PvRBP2b group (Group 2) it was $250.5 \times 10^3 \times \text{iRBCs}/\mu\text{L}$ ($p = 0.4672$; t test). The PvDBP-R11 group (Group 3) had a median peak of $301.5 \times 10^3 \times \text{iRBCs}/\mu\text{L}$ ($p = 0.4672$; t test), and the PvRBP2b/PvDBP-R11 group (Group 3) showed a median peak of $330.0 \times 10^3 \times \text{iRBCs}$ ($p = 0.2007$; t test). These results show no statistically significant differences, with no evidence of a delay to peak parasitemia, a reduction in peak level, or a change in daily mean cumulative parasitemia in the immunized groups (**Figure 3**). Because each group had only 4 animals, the study lacked sufficient power to detect small differences in parasitemia.

Similarly, the prepatent period in the control group (AlOH₃) ranged from 8 to 12 days (mean of 10.5 ± 1.7) post-challenge (PoC). In contrast, animals in Groups 2, 3, and 4, immunized with PvRBP2b, PvDBP-R11, and PvDBP-R11 / PvRBP2b, respectively, developed parasitemia between days 6 and 8 PoC, with mean prepatent periods of 7.3 ± 1.0 , 6.5 ± 1.0 , and 7.5 ± 1.3 days, respectively.

Notably, all recombinant protein vaccinated groups showed significantly shorter prepatent periods than the control group (**Figure 3D**).

Although PvRBP2b vaccination was associated with a slight reduction in median peak parasitemia compared with the AlOH₃ control, it did not delay parasite emergence. In fact, all vaccinated groups had significantly shorter prepatent periods than the control group. Neither PvDBP-R11 alone nor the PvDBP-R11 / PvRBP2b combination reduced peak parasitemia or delayed the prepatent period. Taken together, the peak parasitemia and the prepatent period endpoints did not demonstrate a protective effect of the recombinant protein vaccines against blood-stage *P. vivax* infection. Overall, these findings indicate limited efficacy of the tested formulations in modifying key parasitological outcomes under the conditions of this study.

Immunization with PvRBP2b, alone or in combination with PvDBP-R11, was associated with reduced PMR. The mean PMRs of the immunized groups, PvRBP2b, PvDBP-R11, and PvRBP2b / PvDBP-R11, at 3.7, 5.3, and 4.0, respectively, indicate partial control of parasite replication. In contrast, the AIOH₃ adjuvant control group, with a mean PMR of 7.9, suggests uncontrolled parasitemia. (**Figure 2**).

These results suggest partial blood-stage immunity detectable by PMR analysis, underscoring PMR as a sensitive measure of vaccine effects in this type of study, as reported by others in control human malaria infection experiments (CHMI) (20).

All vaccinated monkey groups showed shorter prepatent periods than the control monkeys, indicating that these blood-stage vaccine candidates did not delay parasite emergence as expected but may have enhanced it instead (46). Previous studies evaluating candidate blood stage antigens such as MSP1, AMA1, and RH5 have reported no significant differences in prepatent period between vaccinated and control groups, despite measurable immunogenicity and, in some cases, reduced PMR (47-49). These results confirmed the limited utility of the prepatent period as a primary endpoint, particularly when using ultrasensitive detection methods such as qPCR.

Regarding immunogenicity, **Figure 4** shows ELISA total IgG titer plots for PvDBP-R11 and PvRBP2b recombinant proteins, alone or in combination. In **Figure 5A panel d**, the combination vaccine group (PvDBP-R11 / PvRBP2b) showed a strong statistically significant negative correlation ($r = -0.9686$; $p = 0.0314$, Pearson) between pre-challenge antibody titers (OD) and the day of peak parasitemia, suggesting that higher antibody levels were associated with an earlier peak and faster control of parasite growth. This finding supports the idea that vaccine-induced antibodies may have a protective effect, as increased humoral responses could help restrain parasite expansion after challenge

Similarly, a moderate negative correlation was observed between pre-challenge antibody titers and the PMR ($r = -0.4479$; $p = 0.521$, Pearson), but this relationship was not statistically significant. Although the trend suggests that higher antibody levels might reduce parasite growth, the lack of significance indicates that this observation should be interpreted with caution and requires confirmation in larger studies (**Figure 5C, panel i**).

Clinical chemistry, hematologic, and general health parameters were comparable across all groups prior to challenge (**Supplementary Figure S3**). Blood urea nitrogen, creatinine, alanine aminotransferase, albumin, hemoglobin, hematocrit, platelets, and body weight values were closely grouped, with no consistent differences observed between vaccinated (PvDBP-R11, PvRBP2b, alone or in combination) and control groups. These results suggest an absence of vaccine-associated toxicity at baseline.

After parasite challenge, hematologic and clinical measures showed increased variability, including consistent declines in hemoglobin and hematocrit as expected (50), along with individual fluctuations in platelet counts and body weight. These patterns demonstrate heterogeneous infection dynamics and immune responses. In contrast, renal and hepatic markers remained stable, with only mild and transient

changes observed. Overall, these findings suggest that infection, not vaccination, primarily drives the observed variability.

Discussion

In this study, we assessed the immunogenicity and protective efficacy of two *P. vivax* blood-stage recombinant proteins, PvDBP-R11 and PvRBP2b, administered with aluminum hydroxide adjuvant alone or combined, in the Aotus monkey model following intravenous challenge with the heterologous *P. vivax* AMRU-1 strain. Protection was evaluated using PMR, prepatent period, and peak parasitemia, enabling a detailed analysis of vaccine effects on parasite dynamics. This study shows that PMR detects functional vaccine effects in a non-human primate (NHP) model using recombinant *P. vivax* PvDBP-R11 and PvRBP2b proteins under heterologous challenge conditions.

Analysis of PMR during the exponential phase of parasitemia revealed a clearer vaccine-associated effect. For instance, monkeys immunized with PvRBP2b and PvDBP-R11 recombinant proteins alone or in combination exhibited group mean PMR values below 4, indicating partial protection. In contrast, the adjuvant-only control group had a mean PMR of 7.9, consistent with uncontrolled parasite replication. Reduced PMR reflects a measurable slowing of Intraerythrocytic parasite growth is increasingly recognized as a sensitive indicator of functional blood-stage immunity. (49) (51).

The use of the PMR (44) as an outcome measure has gained prominence in malaria vaccine research, especially in CHMI models, where it has demonstrated a strong ability to distinguish vaccine effects on growth inhibition, even without reductions in peak parasitemia. (45, 52, 53). Our findings reinforce the usefulness of PMR in the Aotus model and suggest that immune responses induced by PvRBP2b and PvDBP-R11 exert meaningful biological pressure on parasite replication.

In contrast, analysis of the prepatent period showed that all vaccinated groups became parasitemic earlier than the AlOH₃ control group (Figure 3). This finding suggests that vaccination did not delay the appearance of parasites in the blood and may indicate a lack of sterilizing immunity against a heterologous strain challenge, as reported in Aotus monkeys repeatedly infected with *P. vivax* SAL-1 and challenged with the heterologous AMRU-1 strain. (15).

Longer prepatent periods have been associated with protection in some vaccine regimens, but not consistently across all groups or endpoints. In this study, we cannot rule out that the prolonged prepatent period observed in the control animals may have been caused by the inoculation dose, the inoculation order, adjuvant effects, (54) innate immunity, or antibody-mediated enhancement (46, 55).

PMR better captures growth kinetics than first detection (prepatent period). These observations align with previous blood-stage vaccine studies in both *P. vivax* and *P. falciparum*, where immune responses target merozoite invasion or intraerythrocytic development but do not prevent initial parasite establishment (49) (45).

On the other hand, earlier detection of parasitemia by ultrasensitive qPCR indicates that parasitemia appears earlier in vaccinated animals, showing small differences in early parasite dynamics. This can create an apparent shortening of the prepatent period without worsening the underlying biology, but not necessarily indicate increased pathogenicity, highlighting the limitations of using the prepatent period as a primary endpoint for blood-stage vaccine assessment (56, 57).

Consistent with findings during the prepatent period, peak parasitemia occurred within a similar timeframe across all groups, and none of the vaccine formulations caused a significant delay in reaching peak parasitemia. Median peak parasitemia levels in the PvDBP-R11 and PvRBP2b / PvDBP-R11 immunized groups were similar to or higher than those in the control group, indicating no effect on the maximum parasite burden. However, vaccination with PvRBP2b alone resulted in a modest decrease in median peak parasitemia; although this reduction was not accompanied by changes in the timing of peak parasitemia, limiting its biological significance. Similar results have been observed in studies with non-human primates and controlled human malaria infection (CHMI), where peak parasitemia alone was insufficient to demonstrate partial blood-stage immunity (42, 43). The observed reduction in PMR in PvRBP2b-immunized animals is consistent with these mechanistic insights.

The absence of additive or synergistic effects in the PvDBP-R11 / PvRBP2b combination group suggests that this antigen combination alone may be insufficient to enhance protective efficacy. This may reflect overlapping functional mechanisms, suboptimal antigen dosing, immune interference, or the need for improved adjuvant systems that can elicit higher-quality or more durable antibody responses. Similar challenges have been reported with multicomponent blood-stage vaccines for *P. falciparum*, underscoring the complexity of achieving synergistic protection (58).

The recombinant proteins used in this study were not of pharmaceutical grade for human use, because the His₆-tags were not removed. However, His₆-tags are generally considered minimally immunogenic (59) and have been incorporated into several malaria candidate vaccines, including those that have advanced to Phase IIb clinical trials in African children without apparent safety concerns (40). Still, the possibility that the tags may alter antigen conformation or provoke off-target antibody responses cannot be entirely excluded (60, 61). Studies have shown that both tagged and tag-free forms of candidate malaria vaccine antigens can induce distinct cellular and humoral immune responses (60). On the other hand, some studies have shown no significant changes in protein structure or biological function caused by the affinity tags (62, 63). Thus, comparative future studies of tagged and tag-free PvDBP and PvRBP2b constructs are warranted to assess whether the His₆-tag influences the functional immunogenicity of PvDBP and PvRBP2b vaccine formulations in the Aotus monkey model.

Recently, we observed sterile immunity in Aotus monkeys after two repeated homologous exposures to the *P. vivax* SAL-1 strain, resulting in complete protection with no detectable parasitemia (15). This likely indicates highly strain-specific immune responses that fully prevent blood-stage infection. In contrast, the present study, using recombinant PvDBP-R11 and PvRBP2b vaccines, shows only partial immunity under heterologous AMRU-1 challenge. This partial protection is characterized by decreased PMR and

slower infection kinetics, but it does not stop parasite emergence. The difference highlights the challenge of achieving strain-transcending protection against *P. vivax*, due to antigenic variation and alternative invasion pathways. Although natural homologous immunity can achieve sterilizing levels, vaccine-induced responses are more limited but still biologically meaningful, as shown by their ability to control parasite growth. These findings emphasize the importance of targeting multiple antigens and invasion pathways to better replicate the breadth and effectiveness of natural immunity (15).

In conclusion, although vaccines based on PvDBP-R11 and PvRBP2b did not delay the prepatent period or reduce peak parasitemia after a heterologous *P. vivax* blood-stage challenge, they were associated with lower PMR, indicating partial blood-stage immunity. These findings underscore the importance of including dynamic growth metrics, such as PMR, in vaccine efficacy assessments and support the further development of PvRBP2b- and PvDBP-R11-based vaccine strategies that aim to control parasite replication rather than achieve sterilizing immunity.

This study shows that subcutaneous immunization with PvDBP-R11 and PvRBP2b recombinant proteins, administered alone or in combination with Alhydrogel®, elicited strong total IgG responses in *Aotus* monkeys after 2 doses. This outcome reflects the high immunogenicity of reticulocyte invasion ligands and supports further exploration as blood-stage vaccine candidates. Importantly, the elevated antibody levels observed after just 2 immunizations indicate that this formulation can rapidly induce humoral responses. Such rapid induction is beneficial for vaccines targeting pathogens that multiply rapidly in erythrocytes. Although high antibody titers may not ensure complete protection, these responses form a foundation for functional activity, such as blocking parasite invasion. The comparable immunogenicity observed with both individual and combined antigen formulations indicates that co-administration does not diminish antigen-specific responses. These findings reinforce the potential of multicomponent vaccine strategies targeting different invasion pathways in *P. vivax*.

Developing effective blood-stage vaccines against *Plasmodium vivax* will probably require a change in expectations. Instead of aiming for sterilizing immunity (15), efforts should focus on achievable control of parasite replication and disease severity. Our findings support adopting a new framework. In this approach, dynamic metrics, such as the PMR, combined with functional antibody correlates, provide endpoints that are more sensitive and biologically relevant than traditional measures, such as peak parasitemia (64).

The partial control observed after heterologous challenge shows both the potential and the limitations of current antigen designs targeting reticulocyte invasion pathways (25). Progress depends on iterative improvement of multicomponent vaccines. These vaccines should include complementary invasion ligands, improved structural antigen designs to better target epitopes, and next-generation adjuvants to induce lasting, high-quality antibody responses (65). At the same time, standardizing PMR and immunological correlates across preclinical and controlled human malaria infection studies is crucial (66). Such standardization will enable comparisons across platforms and speed up translational progress. Overall, these strategies could help redefine success for *P. vivax* blood-stage vaccines, with

success measured by sustainable suppression of parasite growth and clinical impact, rather than complete sterilizing immunity.

Limitations of the study.

Several limitations should be recognized. The small sample size (n = 4 per group) limits the statistical power to detect modest differences in parasitological endpoints. Using only a single adjuvant (Alhydrogel®) may have restricted both the strength and functionality of vaccine-induced immune responses. Additionally, inherent variability in the Aotus model and the use of a single heterologous challenge strain may restrict the generalizability of the results. The absence of functional antibody assays also hampers the mechanistic understanding of the observed immune responses.

Conclusion

Subcutaneous immunization with Alhydrogel® containing recombinant proteins PvRBP2b + PvDBP-R11 elicited strong antibody responses and partial protection, supporting the development of multistage or multivalent *P. vivax* vaccines.

Declarations

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Author Contributions

NOIII, UK, MN, MM, and MD planned and performed the experiments; NOIII, SM, MN, and AM analyzed the data, and NIII wrote the paper.

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Figures

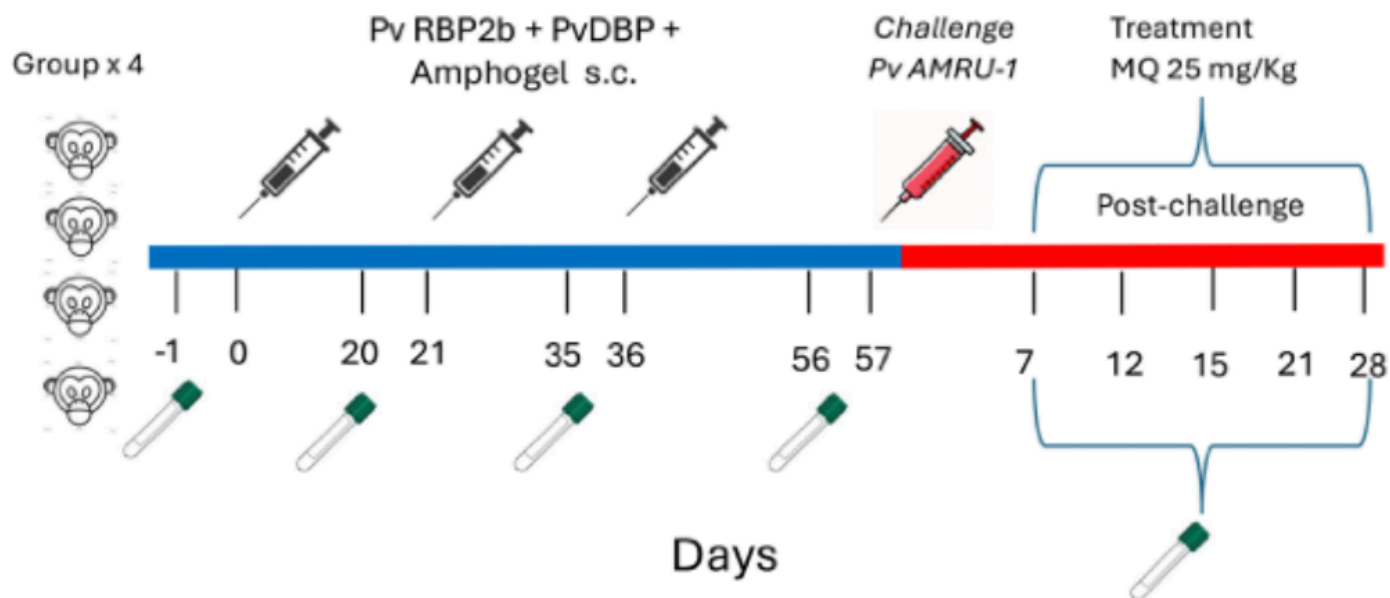


Figure 1

Immunization and bleeding Schedule.

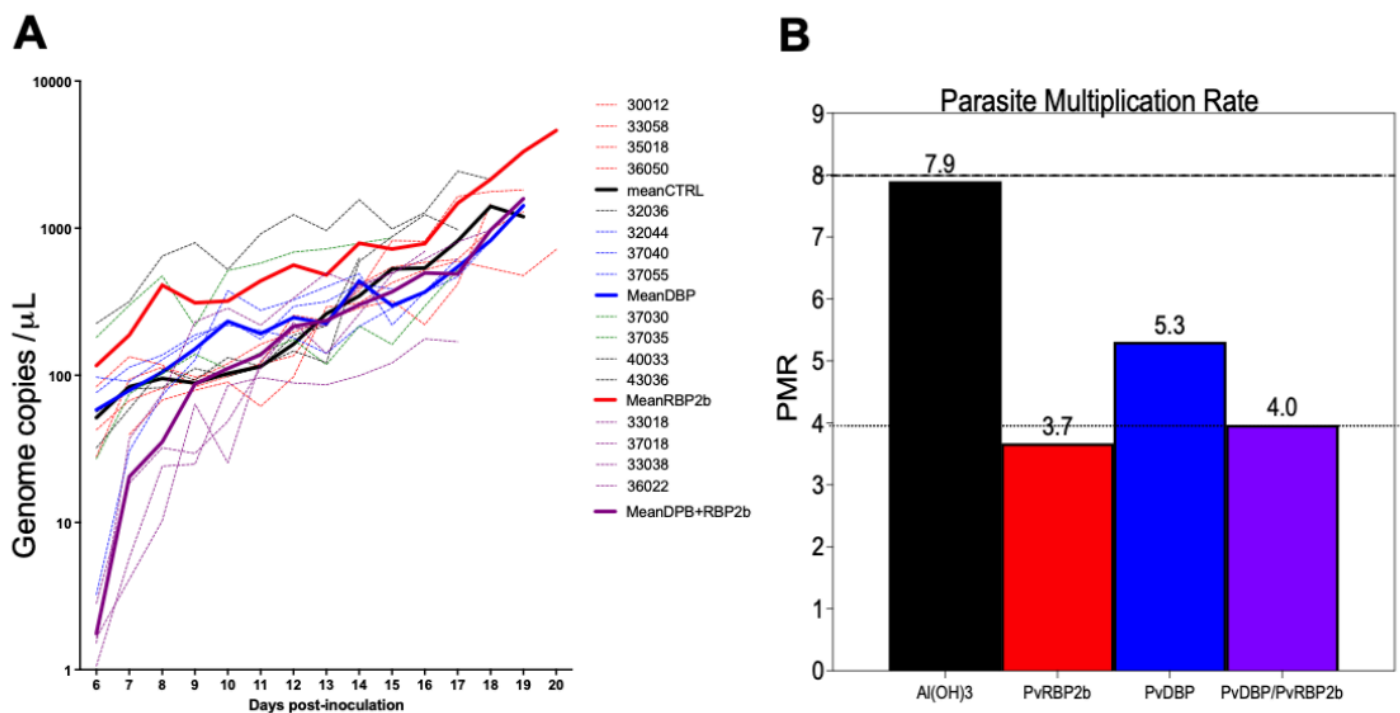


Figure 2

qPCR parasitemia and parasite multiplication rate. A) Individual and group mean qPCR parasitemia curves related to the outcome (rescue treatment) for Aotus monkeys immunized with recombinant proteins PvRBP2b and PvDBP-II alone or in combination after challenge with PvAMRU-1 strain. **B)** Average Parasite Multiplication Rate (PMR) by group (n = 4). The mean PMR was calculated during the exponential growth phase between days 10-15 post-infection. (Parasite multiplication rate (PMR) was determined by fitting a linear regression model to $\log_{10}$ -transformed parasitemia data during the exponential growth phase and converting the slope to fold change per 48-hour erythrocytic cycle, as described (44). i.e., typically, *P. falciparum* PMR in malaria challenge studies is 8–12-fold per 48 hours in naïve volunteers. Lower PMRs may indicate partial immunity, either natural or vaccine-induced ($PMR = 10^{(\text{period} \times \text{slope})}$)).

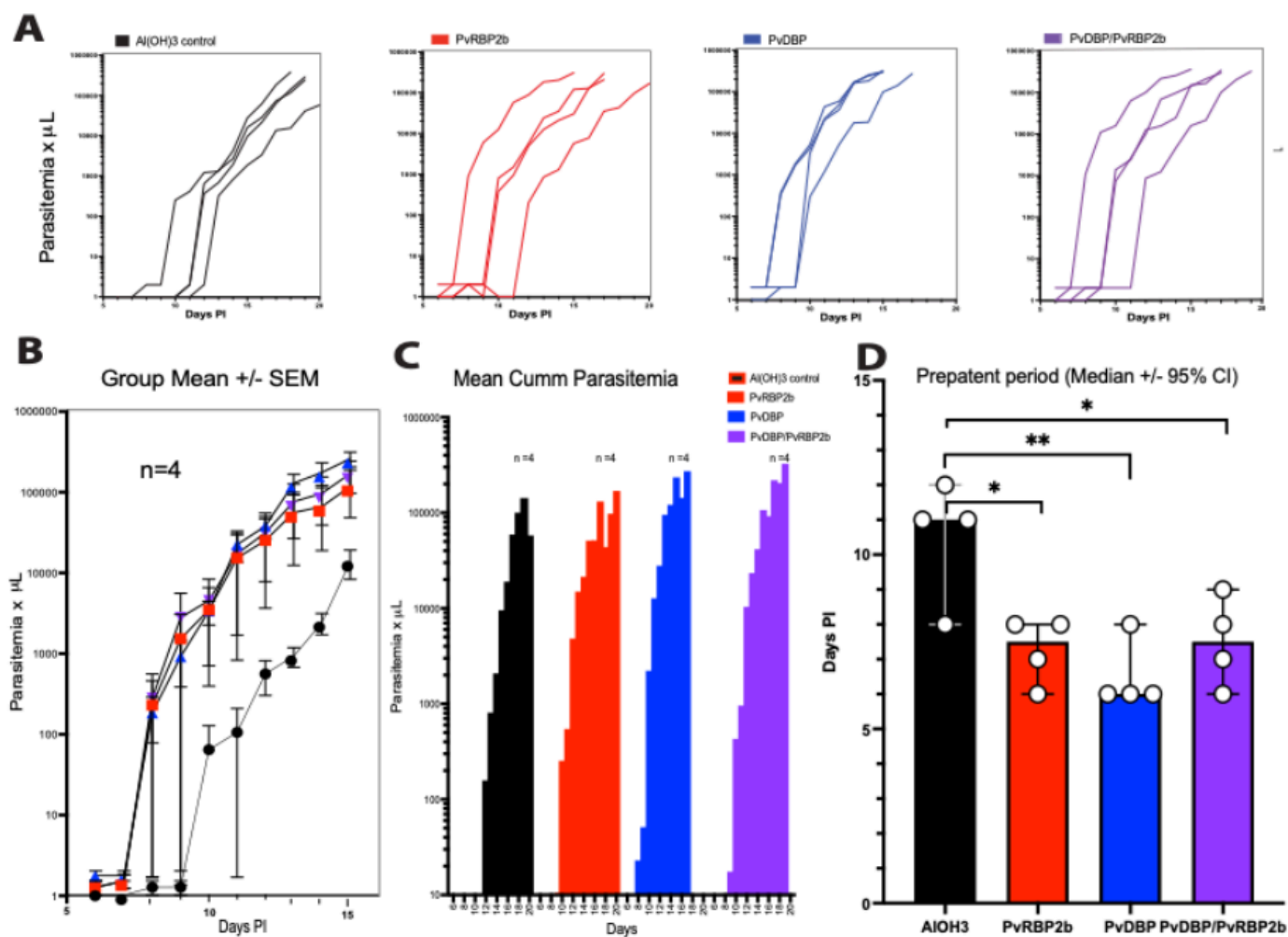


Figure 3

Thick blood smear parasitemia. A) Individual parasitemia curves determined by thick blood smear of Aotus immunized with recombinant proteins PvRBP2b and PvDBP-II alone or in combination after challenge with PvAMRU-1. **B)** Group mean parasitemia curves. **C)** Longitudinal mean daily cumulative parasitemia relative to outcome (rescue treatment) in immunized Aotus groups. **D)** Individual and mean

prepatent periods. Columns represent the median prepatent period in days for each experimental group, with 95% confidence intervals. * $P < 0.05$; ** $P < 0.01$, unpaired t-test.

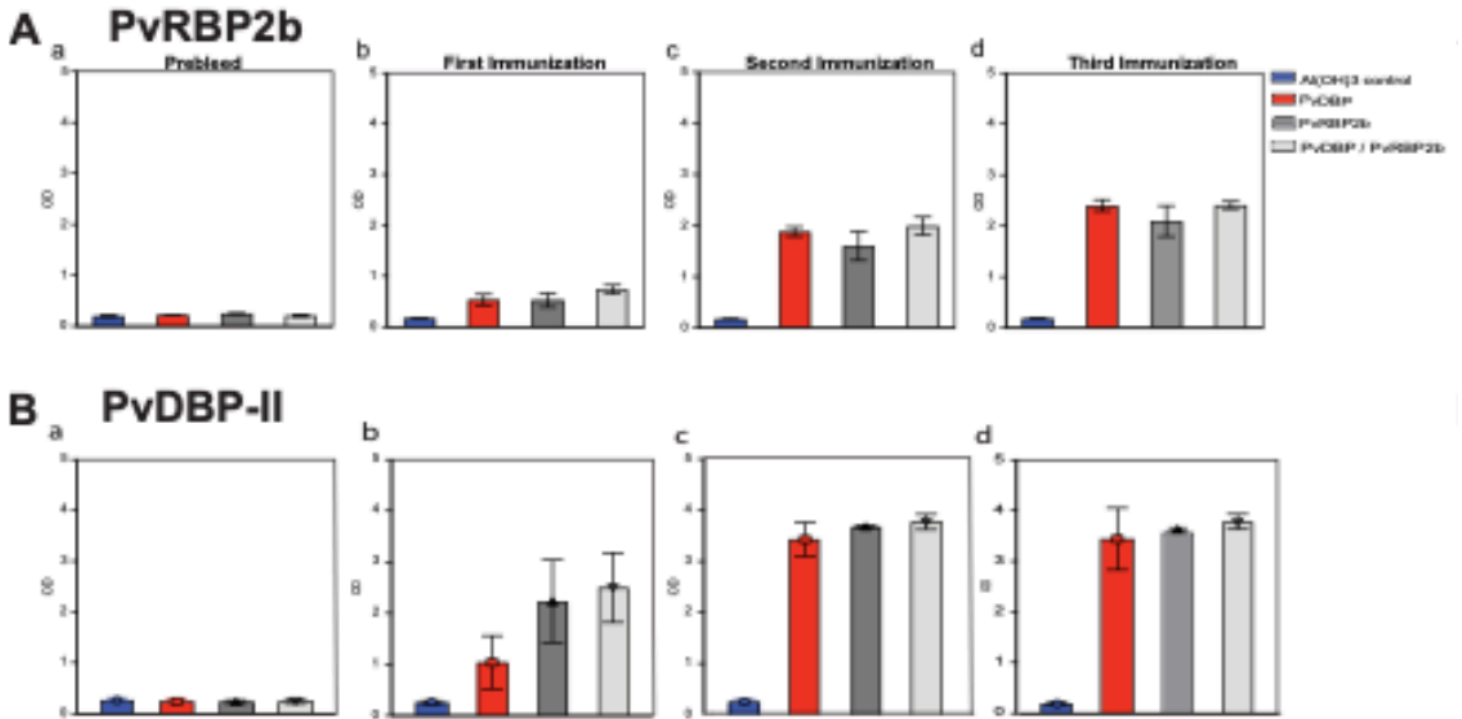


Figure 4

ELISA total IgG titer plots. A) Panels a-d; plates were coated with 50 μ L of PvRBP2b-161–813-N (30 μ g/mL). Columns in panels a-d show the group means $\pm$ SEM OD titers for each group of 4 monkeys immunized with the recombinant protein PvRBP2b alone or combined with PvDBP-II. **B)** Panels a-d; plates were coated with 50 μ L of PvDBP-II H6-d (5 μ g/mL) per well. Columns in panels a-d display the group mean $\pm$ SEM OD titers. Data are shown as mean OD $\pm$ SEM at 492 nm.

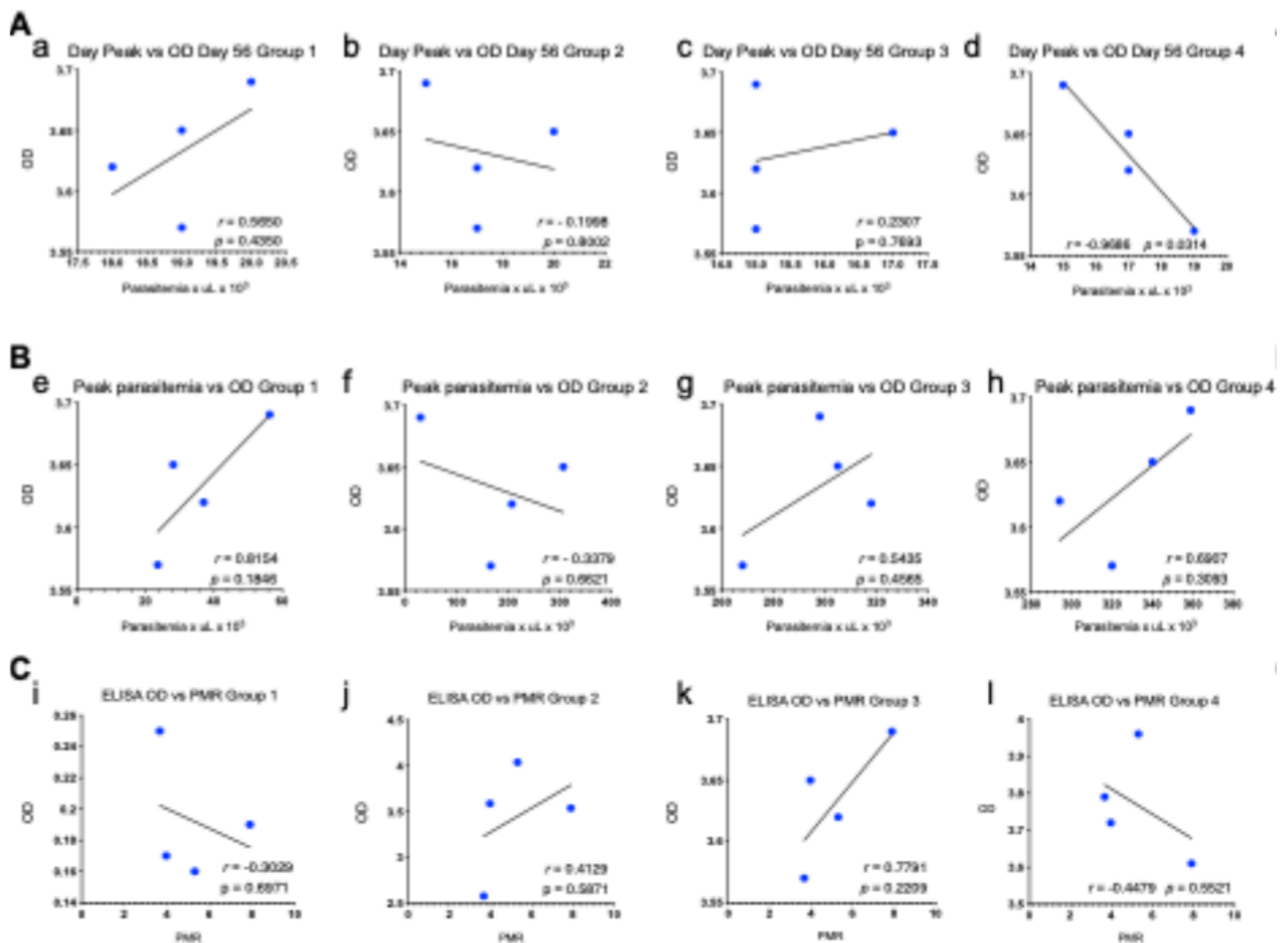


Figure 5

Correlation of pre-challenge OD ELISA antibody titers. **A)** Day of Peak Parasitemia. **B)** Peak parasitemia. **C)** PMR. Immunized monkeys: Group 1 Al(OH)₃

control; Group 2 PvRBP2b, Group 3 PvDBP-RII; Group 4 PvRBP2b / PvDBP-RII. r = Pearson correlation coefficient. p = probability.

Supplementary Files

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