Analysis of Pre-Erythrocytic Immunity During Plasmodium Vivax Infection Reveals a Diversity of Responses That is Partially Due to Blood Stage Cross-Reactivity

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Research

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Abstract

Background

Plasmodium vivax (*Pv*) represents the most geographically widespread human malaria parasite. Targeting the pre-erythrocytic (PE) stage of the parasite life cycle is especially appealing for *Pv* vaccines as it would prevent disease and transmission. Here, we explore naturally acquired immunity to a panel of *Pv* PE antigens as a first step to enable vaccine development and to better understand naturally-acquired PE immunity.

Methods

Humoral and cellular immunity were evaluated by ELISA and ELISpot, using samples from *Pv* infected individuals from a low endemic malaria region in the Peruvian Amazon Basin. In addition, we utilized experimental infection of *Aotus* non-human primates with *Pv* or *P. falciparum (Pf)* in order to evaluate the contribution of blood stage infection to the humoral response observed in human samples.

Results

In our clinical samples, twelve PE antigens showed positive antibody reactivity with variable prevalence of 58–99%. The magnitude of the IgG antibody response against PE antigens was lower compared with blood stage antigens MSP1 and DBP-II although titers persisted better for PE antigens, six months later after infection (average decrease of 6% for PE antigens and 43% for MSP1) in general. A significant correlation between IgG antibodies and number of previous malaria episodes was observed only for blood stage antigens. High IgG responders across PE and blood stage antigens showed a significantly lower parasitemia compared to low responders (median 1873 vs 4663 par/μl). We observed a positive T cell response in 35% vs 9–35% of total volunteers against blood stage antigen MSP1 and PE antigens, respectively, and saw no correlation with IgG responses. *Aotus* monkeys infected with *Pv* blood stage showed positive reactivity against the seven PE antigens tested. In contrast, only 2 of 10 monkeys infected with *Pf* showed low positive IgG cross-reactivity against *Pv* MSP1 and none of which cross-reacted to *Pv* CSP.

Conclusions

Our results demonstrate clear humoral and T cell responses against *Pv* PE antigens in individuals naturally infected with *P. vivax*. In addition, these results are largely replicated in a novel *Aotus nancymaae Pv* blood stage challenge model which suggest a contribution from blood stages to PE cross-reactivity. Together, these data clearly identify novel attractive PE antigens suitable for use in the development of new malaria vaccine candidates.

Background

Plasmodium spp. is the causative agent of malaria and one of the world's deadliest infectious diseases. In 2018 about 228 million cases of malaria were reported by WHO with approximately 405,000 deaths, particularly in young children and pregnant women in sub-Saharan Africa[1]. While *Plasmodium falciparum* (*Pf*) is the most prevalent malaria parasite on the African continent and responsible for most observed infections and deaths, *P. vivax* (*Pv*) represents the most geographically widely distributed human malaria parasite worldwide. Outside of Africa, *P. vivax* is the dominant cause of malaria with over 3 billion people living within its transmission limits[1]. Globally, several million clinical cases of *P. vivax* malaria are detected each year with a relevant portion in South East Asia and Latin America, where *Pv* is responsible for 50% and 70–85% of all malaria cases, respectively[1]. Traditionally, *P. vivax* has been considered to cause a "benign" form of malaria but is now recognized as a significant cause of morbidity and mortality due to increasing evidence of severe cases with a possible fatal outcome[2].

Malaria elimination efforts have resulted in a substantial decline in the global malaria burden in the past two decades. It is estimated that these efforts have resulted in a reduction of global malaria infections by 29% and mortality caused by malaria by 60% between 2000 and 2018[1]. However, from 2014 to 2018 this downward trend has slowed considerably and even reversed in some regions[1, 3]. *P. vivax* represents a special challenge to control efforts due to its unique biological features, including low-density blood-stage infection, asymptomatic infections and the formation of hypnozoites, which are dormant forms residing in hepatocytes that can cause relapses months or years after the initial infection. Hypnozoites are believed to be responsible for approximately 80% of infections and there is currently no diagnostic tool available for their detection[4]. Further progress towards malaria elimination will require additional tools including the development of an effective vaccine or intervention which can prevent or eliminate these dormant stages. Targeting the pre-erythrocytic (PE) stage—the asymptomatic stages from the skin to liver which precede the symptomatic blood (erythrocytic) stage—of the parasite is especially appealing for *P. vivax* vaccine development as it would prevent disease arising from both primary and relapse infection.

For many years, the efforts to develop a PE malaria vaccine have mainly been focused on *P. falciparum* while only two *P. vivax* vaccine candidates have reached clinical trials for *P. vivax* (targeting blood stage proteins DBPII and MSP1)[5]. In contrast, several vaccine candidates against *P. falciparum* are under development. The most advanced of these is the subunit vaccine RTS,S-AS01 which targets the Circumsporozoite protein (CSP) via neutralizing antibodies and has shown consistent but short-lived efficacy in children in phase 3 clinical trials[6, 7]. In addition to CSP, other proteins have been under investigation as vaccine targets, most notably the PE antigens thrombospondin-related adhesive protein (TRAP) and the highly promising blood stage protein reticulocyte-binding protein Homolog 5 (RH5)[8]. The latter was chosen based on association with protection in naturally infected persons and is progressing into clinical trials after encouraging preclinical results in non-human primates[9, 10].

Only a few such studies of naturally acquired immunity to *P. vivax* have been conducted, largely with a focus on antibodies to erythrocytic antigens, which has limited our selection of PE antibody targets mostly to orthologs of *Pf* candidates. In these studies, reticulocyte-binding proteins critical for merozoite

invasion have been associated with protective immunity (e.g. *Pv*DBPII and *Pv*RBP2b)[11–14]. Large-scale screenings have identified antibodies to other blood-stage antigens associated with protection particularly when seen in combinations rather than with any single antigen alone[15, 16]. However, even fewer studies have explored the immunogenicity of *Pv* PE antigens[17] despite the fact that they can be targeted potentially with both antibodies and T cells[18, 19]. Furthermore, targeting this stage to prevent or eliminate hypnozoites would have an outsized effect on elimination given that relapse is the main driver of disease and transmission[4].

Taken together, evidence from *Pf* and *Pv* indicate that antibodies and T cells targeting multiple PE *Pv* proteins could be an integral part of the first effective *Pv* vaccine. The almost exclusive focus on the blood stage has resulted in a general lack of *Pv* PE candidate antigens and an even more opaque understanding of their role in natural immunity or even simply immunogenicity. This paucity of data around PE immunity in *Pv* limits our ability to take the first steps towards novel vaccine candidates. Here, we explore naturally acquired immunity to a panel of *Pv* PE antigens as a key step to enable vaccine development and to better understand naturally-acquired PE *Pv* immunity.

Methods

Human plasma and lymphocyte samples

Human samples were obtained from three protocols approved by the Institutional Review Board of the U.S. Naval Medical Research Unit No.6 (NAMRU-6) in compliance with all applicable federal regulations governing the protection of human subjects (NMRCD.2007.0004, NMRCD.2010.0002 and NAMRU6.2012.0006). These studies were conducted at peri-urban health centers of Hospital Regional de Loreto (HRL, n = 41) and Hospital de Apoyo de Iquitos (HAI, n = 35) between 2012 and 2017. Subjects were enrolled by passive malaria surveillance of patients presenting malaria symptoms at both health centers located in Iquitos, the largest city in the Peruvian Amazon Basin. This area is described as low *P. vivax* endemic with annual average cases of 42,164 ± 7,779 during 2012–2017, although heterogeneous and sustained local *P. vivax* transmission has been reported[20], resulting in underestimated malaria prevalence due to low-parasitemia and/or asymptomatic cases. All subjects enrolled were diagnosed by microscopy and confirmed by PCR[21] for detection of *Pv* mono-infection. Additionally, we used twenty-four samples of *P. vivax* infected patients for long-term analysis with 6 months follow-up enrolled at NMRCD.2010.0002 and NAMRU6.2012.0006 protocols. A set of twenty human *P. vivax* negative control blood samples were obtained from individuals living in the department of Piura, located in the North Coast of Peru (NMRCD.2010.0002), which has reported very low incidence of *P. vivax* malaria (0.01 per 1000 inhabitants) during 2014.

Aotus nancymaae **infection and plasma samples**

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of

Laboratory Animals Resources, National Research Council, National Academy Press, 2011. All animals were bred in captivity and included in NAMRU-6 IACUC approved protocols (NAMRU-6 13 – 04, NAMRU-6 11–12 and NAMRU-6 14 – 06). In addition, these animal studies were approved by the Peruvian government agency of "Servicio Nacional Forestal y de Fauna Silvestre" (SERFOR) with permit codes: RDG: 096-2014-SERFOR-DGGSPFFS, RDG: 184-2012-AG-DGFFFS and RDG: 050-2017-SRFOR-DGGSPFFS.

We obtained plasma samples from *Aotus nancymaae* monkeys (n = 19) between days 3 to 39 post blood stage infection with *P. vivax* or *P. falciparum* to evaluate immunogenicity against *P. vivax* PE antigens.

We used plasma samples from *A. nancymaae* monkeys (n = 10) infected with *P. falciparum* belonged to control groups used in *P. falciparum* pre-clinical trials of RH5 vaccine efficacy performed previously at NAMRU-6 (NAMRU-6 11–12 and NAMRU-6 14 – 06)[9, 10]. For these, one vial of cryopreserved infected red blood cells (iRBCs) of *P. falciparum* FVO strain was inoculated to a donor monkey and once parasitemia was between 2,000–10,000 par/µl, this blood was used to infect study animals with a dose of $1x10^4$ par/animal. These monkeys were followed up by microscopy detection of parasitemia for 39 days post-infection. This group did not receive any RH5 vaccine and thus developed natural parasite kinetics requiring treatment with Mefloquine (MQ) when parasite density reached > 200,000 par/µl in peripheral blood or when hematocrit fell below 25%.

Plasma samples from monkeys infected with *P. vivax* were collected from our current work in the development of a *P. vivax* blood stage infection *Aotus nancymaae* model. Briefly, cryopreserved iRBCs from *P. vivax* Vietnam IV line were used to infect a donor monkey and once parasitemia at peripheral blood was between 2,000–10,000 par/µl this blood was used to infect spleen-intact monkeys with dose of $2.5x10^6$ par/animal (n = 3) or $1.0x10^6$ par/animal (n = 6). Parasitemia and plasma samples were assessed for 39 days post- infection by microscopy. Treatment with Chloroquine (CQ) was initiated when parasite density reached > 250,000 par/µl in peripheral blood or when hematocrit fell below 25%.

Selection of P. vivax PE antigens for immunological assessments

The 13 *Pv* PE antigens are orthologs to a panel of *P. falciparum* antigens previously identified as PE vaccine targets[22]. These *Pf* antigens were selected from a larger set of 131 *Pf* recombinant proteins based on their reactivity against sera samples from sterilely protected subjects who underwent immunization with radiation attenuated sporozoites (RAS) followed by challenge with the bites of viable *Pf*-infected *Anopheles* mosquitoes[23, 24]. This approach has been partially validated for three *P. yoelii* orthologs from this list of candidates: a putative cysteine protease inhibitor (Falstatin), the gamete egress and sporozoite traversal (GEST) and the early transcribed membrane protein (ETRAMP) which induced up to 60% protective efficacy when used in combination using the DNA and vaccinia virus prime-boost immunization approach[25]. In addition, our subset of *Pv* proteins have been verified as expressed in a published in *P. vivax* proteome[26].

These proteins were successfully expressed by the cell-free wheat germ expression system[27] using the bi-layer method (small-scale) with a yield between ~ 300–1200 ug of protein per reaction.

ELISA - human samples

Briefly, thirteen *Pv* PE antigens and two blood stage antigens were used to determine antibody prevalence and relative immunogenicity. ELISA plates were coated with recombinant proteins at concentration of 2–4 ug/ml to high-binding 96-well microplates (Nunc Maxisorp) overnight at room temperature and then washed with T-PBS1X (PBS1X/0.05%Tween20) five times. Microplates were blocked for 1 hour with TBS1X-SM (TBS1X/Skim milk 5% buffer). After five washes with T-PBS1X, human plasma samples were added in duplicate at a 1:200 dilution in TBS1X-SM and incubated for 2 hours at room temperature. After five washes with T-PBS1X, antibodies were detected using peroxidase-conjugated anti-human IgG monoclonal antibodies (Jackson Immunoresearch Cat#: 309-035-033, 1:6,000 dilution) and incubated for one hour at room temperature. After five washes with T-PBS1X, we used o-phenylenediamine (Sigma Aldrich Cat#: P3804) with hydrogen peroxide as a substrate and the reaction was stopped after 1 hour with 50 ul of 3N HCl. Plates were read at 492 nm to determine optical density OD.

A two-fold plasma serial dilution of a positive pool of 30 individuals with more than 10 *P. vivax* confirmed events were used as standard curve and included on each plate run. In addition, for positivity cut-off calculation we used 20 plasma samples from individuals from malaria area with low incidence to calculate a cut-off value for each protein using the average OD value plus 3 standard deviations.

ELISA - Aotus nancymaae **samples**

The immunogenicity of *Pv* PE antigens following blood stage infection of *A. nancymaae* monkeys was assessed for seven *Pv* PE antigens one Blood stage antigen similar to our human ELISA. Briefly, 96-well microplates (Nunc Maxisorp) were coated with 4 µg/ml of each *Pv* protein and incubated overnight at room temperature. After washing, plates were blocked for 1 hour with TBS1X-SM and monkey plasma samples were then added at 1:100 in TBS1X-SM and incubated for 2 hours at room temperature. Antibodies were detected using peroxidase-conjugated anti-monkey IgG (Sigma Aldrich Cat#:A2054) with an incubation of 1 hour. O-phenylenediamine (Sigma Aldrich Cat#: P3804) with hydrogen peroxide was used as substrate and the reaction was stopped after 1 hour with 3N HCl, then plates were read at 492 nm.

ELISpot

A library of overlapping 15-mer synthetic peptides was obtained from Mimotopes Pty Ltd. The peptides overlapped by 9 aminoacids spanning the entire protein. PvMSP-1 was used as positive control of blood stage antigens, and seven pre-erythrocytic antigens (CSP, CelTOS, Falstatin, ETRAMP, PVX_119755, GEST and HSP PVX_089585). These were resuspended according to the manufacturer instructions and each peptide pool used at 10 µg/ml. These peptides were used to determine the frequency of T-cell response producing IFN-γ to Pv PE proteins using Human IFN-γ ELISpot PRO (Cat.# 3420-2APW-10. Mabtech AB, Sweden) according to the manufacturer's instructions.

Peripheral blood mononuclear cells (PBMCs) from *P. vivax* patients were isolated from whole blood by density centrifugation using a Percoll gradient, counted and cryopreserved for storage in liquid nitrogen. Briefly, PBMCs were thawed in media with 10% FBS (SIGMA, F4135), incubated overnight at 37°C and then plated at 0.2 x 10^6 cells/well in duplicates. Peptides were added at 10 ug/ml to stimulate T cell response for 18 hours with PMA/ION used as positive stimulation control. Spots were counted using the CTL IMUNOSPOT Analyzer. Subjects were defined as positive when the number of spots was higher than the negative control by 20%.

Statistical analysis

Analysis was performed using STATA v16.0 statistical software (Stata Corp., College Station, TX, USA) and GraphPad Prism v9 (GraphPad Software, LLC). Differences among the frequency of epidemiological and immunological variables were analyzed using Chi-square test for categorical variables and Mann-Whitney or Kruskal-Wallis test for numerical continuous variables to compare the median values. Spearman rank correlation coefficient test was used to evaluate the correlation between epidemiological and immunological variables. To measure differences of antibodies level at different time points between groups (related samples) per each antigen we used Wilcoxon signed-rank test, the significance level for all statistical analysis performed was set at $p < 0.05$ or $p < 0.001$.

Results

Epidemiological variables

Epidemiological variables by site of enrollment showed significant differences only for weight and number of previous *P. vivax* episodes (Table 1). Both variables were higher for patients enrolled at Hospital Regional de Loreto (HRL).

Human plasma samples fromP. vivaxinfected population and humoral response

In order to determine if antibodies recognizing *Pv* PE antigens are induced during naturally occurring *Pv* infections, we tested plasma samples from up to 76 *Pv* positive patients enrolled at health centers of HRL and HAI during 2012–2017 by ELISA.

This group of 26–76 *Pv* patients showed positive antibody reactivity with high prevalence for blood stage antigens MSP1 (92%), DBP-II (79%) and the PE stage antigen CSP (99%) (Fig. 1A). Twelve other *Pv* PE antigens showed positive antibody reactivity with variable prevalence in the range of 58–99%. Prevalence for each antigen was: TRAP PVX_082735 (99%), HSP PVX_089585 (93%), GAP40 PVX_08046 (93%), Hypothetical protein PVX_119755 (93%), CELTOS PVX_123510 (91%), GEST PVX_121950 (89%), SPECT1 PVX_083025 (89%), FALSTATIN PVX_09903 (88%), Hypothetical protein PVX_094725 (75%), Hypothetical protein PVX_111090 (75%), ETRAMP (UIS3 ortholog) PVX_121950 (72%), and Hypothetical protein PVX_093660 (58%) (Fig. 1).

The magnitude of the antibody response as measured by OD values was higher for the blood stage MSP1 antigen with an OD average of 1.8 compared to the canonical PE antigen CSP with an OD average of 0.8. The other 12 *Pv* PE antigens showed variable intensity with a mean OD range of 0.3–0.7. (Fig. 1). Overall, the antibody magnitude did not correlate with parasitemia levels at the time of sampling except for a modest negative correlation in 5 PE antigens (Spearman RHO: -0.23 to -0.39, $p < 0.05$) (**Table S1**).

To determine if antibody magnitude correlated with previous malaria exposure, we used a subgroup of 59 patients who self-reported previous malaria episodes, which we stratified by no previous (n = 26), one previous (n = 16) and two or more previous (n = 17) *P. vivax* episodes. There was a significant increase of IgG antibodies against only the blood stage antigens MSP1 and DBP in groups with more than one previous episodes as compared to the group with no previous *P. vivax* infection (Fig. 2). IgG antibodies against all *Pv* PE antigens showed similar IgG levels independent of the number of self-reported previous malaria episodes (Fig. 2**)**. Together, these data reveal a broad and variable seropositivity to multiple *Pv* PE antigens during acute *P. vivax* infection that, unlike blood stage antigens, appear not to be boosted by multiple previous blood stage infections.

Antibody magnitude also showed substantial variability between volunteers, especially amongst pre-erythrocytic antibodies (Fig. 3**)**. Some volunteers responded broadly to nearly every antigen while others appeared to have weak antibody responses in terms of both breadth and magnitude. Indeed, a common predictor of antibody response to one PE antigen was a response to another **(Fig.S2)** suggesting that some volunteers naturally respond to infection with a greater antibody response. Those “high responders”, defined simply as those in the top 50% of total IgG magnitude across all antigens were evaluated against epidemiological variables. We did not observe any significant differences between high and low responders by place of sample collection, sex, age, weight, temperature and number of previous *P. vivax* episodes. However, high responders showed a significantly lower parasitemia compared to low responders (median par/µl 1873 vs 4663, $p < 0.05$) at time of enrollment **(Table 2)**. Interestingly, high and low antibody responders did not differ in parasitemia levels after stratification by number of previous episodes **(Fig.S3 A and B)**.

Long-term analysis of a sub-group of 24 *P. vivax* patients at 6 months after enrollment showed a significant decrease of OD values only for MSP1 (OD average decrease of -43%, Day 0: 1.71 vs Day 180: 0.97, $p < 0.05$) and CSP (OD average decrease of -28%, Day 0: 0.83 vs Day 180: 0.60, $p < 0.001$). Nine PE antigens showed more stable IgG levels with an average decrease of between antigens of 6% (range: -2 to -21 and SD ± 6%) **(**Fig. 4**)**, indicating that some PE antibodies are maintained for a significant period after infection.

Human T cell responses

We next evaluated the T cell PE immune response in a small set of individuals (n = 17) for which we had PMBCs. *Ex vivo* IFN-gamma response was detected in at least one subject for all of the antigens tested. Positivity was the highest for the blood stage protein MSP1 and the pre-erythrocytic stage protein CSP, both at 35.3% or 6/17 volunteers positive. From the remaining antigens tested, the highest percentage of

positive responses was for ETRAMP at 33.3% (5/15), followed by Falstatin (25%, 4/16), CelTOS (23.5%, 4/17), the Hypothetical protein PVX_119755 (23.1%, 3/13) and HSP (18.2%, 2/11).

Across individuals, we observed broad and variable T cell responses to PE antigens (Fig. 5) with 2 subjects positive to CSP only, and 6 subjects positive to 2–4 PE antigens (in addition to or besides CSP). Variability between subjects in the number of SFU (spot forming units) per million cells was also seen, with two subjects highly reactive to multiple antigens. Additionally, two subjects were positive to only MSP1 and no T cell response was found for seven subjects.

We were able to assess the antibody response in seven of the subjects for which we had both plasma and PBMCs. In this limited set, there was no apparent correlation between positive IgG response and *ex vivo* IFN-gamma response (Table 3). Together, these data indicate that T cell responses to both blood stage and PE antigens are also present but variable during acute *Pv* infection.

Aotus nancymaaeas a model for studyingP. vivaxblood stage infection and the humoral immune response

The study of *P. vivax* immunology is severely limited by the lack of appropriate animal or human challenge models in which to perform controlled infections. This complicates analysis of exact infection and immune kinetics especially in the context of discerning the relative contribution of primary vs. relapse infection. Existing animal models in *Aotus* and *Saimiri* new world monkeys typically require splenectomy to observe consistent infection which precludes study of the subsequent immune response. To address this, we tested a strain of *P. vivax* previously reported to elicit robust infection in *Aotus* new world monkeys for its ability to produce reliable infection in spleen-intact animals. *A. nancymaae* monkeys were inoculated with $2.5x10^6$ (Experiment 1) or $1x10^6$ (Experiment 2) infected red blood cells (iRBC) /animal of *P. vivax* Vietnam-IV strain (Fig. 6A). These animals showed low variation between experiments 1 and 2 for day of first parasitemia or “patency” (5 ± 1 vs 6 ± 1), and day of maximum parasitemia (13 ± 2 vs 14 ± 2). We observed a numerically higher average of maximum parasitemia in experiment 2 (41,747 ± 68,672 par/ul) compared with experiment 1(16,400 ± 3735 par/ul) but this was not statistically significant (p = 0.714) and was driven largely by a single animal which had a peak of 194,000 parasites/μL compared to a range of 2,100 – 29,200 parasites/μL for the remaining animals (Fig. 6A **and** Table 4). Monkeys from both experiments had an average of 14 days of detectable parasitemia and all self-cured parasitemia on average at day 24 ± 3 post-infection. In summary, infection of spleen-intact *A. nancymaae* new world monkeys with *P. vivax* Vietnam IV demonstrates 100% infection with variable parasitemia in the ranges as seen during human infection.

Plasma samples from these animals were used to measure IgG to a subset of our *P. vivax* antigens during the course of *P. vivax* blood stage infection.

All *P. vivax* Vietnam-IV blood stage infected monkeys showed positive reactivity against the seven *Pv* PE antigens tested. We observed antibody peak between days 21–28 post-infection which was approximately 1–2 weeks post-peak parasitemia (Fig. 6B).

To evaluate the relationship between parasitemia and IgG antibody response against *Pv* PE antigens we calculated Area Under the Curve (AUC) for both variables. Comparison of AUC of parasitemia or peak parasitemia with antibodies during the follow-up period showed variable response with no clear correlation between either parasitemia or IgG response (Fig. 6C-D). Individual IgG responses and parasitemia for each animal are detailed in **Fig S4.** Together, these data indicate that the *Pv* blood stage infection induces antibodies to both erythrocytic and PE antigens which vary between individual animals in a manner that does not correlate with parasitemia but rather with the incidence of other anti-parasite antibodies.

The IgG reactivity to PE antigens following blood stage infection showed a correlation with MSP1 antigen (Pearson: 0.86–0.94, $p < 0.05$) as well as with all other antigens tested (Fig. 6E). Together, this clearly demonstrates the expression or cross-reactivity of PE antigens during blood stage infection where, like our human cohort, an antibody response to one antigen correlates with the response to others (Fig. 6B **and C-D**).

Cross-reactivity of IgG antibodies between P. vivax **and** P. falciparum **infections.**

Given that *A. nancymaae* monkeys can also be infected with *P. falciparum* blood stages, this offers the unique opportunity to investigate the cross-reactivity of *Pf* and *Pv* antigens in a controlled animal model. To this end, we used plasma samples from *A. nancymaae* monkeys used as infectivity controls in previously published *Pf* infection studies[9, 10].

These *A. nancymaae* monkeys infected with *P. falciparum* FVO showed consistent infection kinetics between experiments for prepatency day (6 ± 1 vs 6 ± 1) and day of maximum parasitemia (12 ± 1 vs 11 ± 1) (Fig. 7A **and** Table 5). Monkeys from both experiments were treated after counting a parasitemia over 200,000 par/ul (day of maximum parasitemia).

All animals had positive reactivity against *P. falciparum* MSP1 antigen with peak antibody day of 21, while only 2 of 10 monkeys had IgG which cross-reacted with *Pv* MSP1 and none of which cross-reacted to *Pv* CSP. In summary, in a controlled *Pf* mono-infection of spleen intact *A. nancymaae* new world monkeys, we saw minimal cross-reactivity between blood stage antigen MSP1 and none to CSP between *Pf* and *Pv*, indicating that PE antigens may be better at distinguishing species-specific infections in serological surveys.

Discussion

Few *P. vivax* PE candidates have been fully evaluated and those that have are still at the pre-clinical or early clinical stages. New *Pv* PE candidates and in particular new antigens will be necessary to obtain a better understanding of *Pv* PE immunity and to provide insight into their role in the prevention of both liver and blood stage infection. In the absence of well-established animal models of *P. vivax*, studying the acquisition of PE antibodies during natural infection is a logical starting point with the long-term goal of

studying the role of PE antibodies in functional immunity to justify integration into novel vaccine candidates. However, to date, few studies of this kind have focused on PE antibodies specifically.

A *P. vivax* cohort study in a low-transmission *P. vivax* area in western Thailand during 2013 showed prevalence of 86%, 21% and 0% for CSP, CELTOS and TRAP antigens, respectively[28]. However, a cross-sectional study of naturally acquired immunity performed in Brazil during 2016 against whole and synthetic peptides of PE TRAP showed prevalence of 46% and 25–32%, respectively[29]. Our results showed naturally acquired immunity against all 12 PE antigens tested in *P. vivax* patients with generally higher proportions of individuals responding to all PE antigens (range of 58–99%). This variation could be related to the inherent characteristics of different circulating *P. vivax* parasites, differences in transmission dynamics or simply differences in protein production platforms as these previous studies used HEK-293T cells compared to our wheat germ Cell-free system. Compared to the magnitude of blood stage responses, the IgG response to PE antigens was lower. This is likely due to the fact we did not see a boosting effect of anti-PE IgG with repeated infection.

Interestingly, we found IgG responses to one antigen often correlated simply with a response to another antigen within individuals—leading to high and low antibody responders. Comparative analysis of high and low antibody responders against *Pv* PE and blood stage antigens vs. parasitemia showed that high responders had lower parasitemia levels ($p < 0.05$). In addition, we observed that the low parasitemia in high responders was similar even through multiple of previous *P. vivax* episodes. Whether this is a result of a diverse antibody response better controlling multiple infections, from a low parasitemia exerting less suppressive effects on the antibody response[30] or is due to inherent individual differences between individuals which dictate broad reactivity[31] are questions which warrant further investigation in well-designed longitudinal cohort studies.

Stability analysis of IgG antibodies six months after *P. vivax* infection showed a significant decrease of antibodies against blood stage MSP-1(-43%) and PE CSP (-28%) antigens. However, the other nine PE antigens showed relative stability (− 6% STD ± 6) as did the blood stage DBP antigen. This characteristic could be useful in defining serological markers of recent vs. historical exposure.

Even less well-studied than PE *Pv* antibodies are T cell responses to natural *Pv* infection. A few studies have assessed vaccine candidates eliciting CD8 + and/or CD4 + T cells that correlate with protection from liver-stage infection in mice[32, 33] and one clinical trial[34], but the mechanism that leads to T cell protection remains to be elucidated. Here, in a limited sample of volunteers, we observed a positive T cell response in 35% vs 9–35% of total individuals against blood stage antigen MSP1 and PE antigens, respectively. From patients with a positive T cell response we observed that 40% showed reactivity for both blood stage and PE antigens which did not appear to correlate with IgG responses. While the role of T cell-mediated protection against rodent malaria liver stages is robust[35, 36], the absence of appropriate animal models for *Pv* and in particular hypnozoites leaves this possibility unconfirmed. However, recent evidence demonstrated that CD8 T cells can eliminate infected reticulocytes[37] opening the possibility of multi-stage CD8 T cell vaccines to prevent or limit *Pv* infection. Our limited data

demonstrate that natural PE T cell immunity does exist and will need to be included in future studies investigating the relationship between T cells and protection from infection at multiple stages.

Importantly, our *A. nancymaae P. vivax* model showed a predictable and consistent blood stage infection that can be immediately useful for testing new blood stage vaccine candidates or drugs against *Pv* blood stage infection. In addition to demonstrating an infection course similar in variation and magnitude observed in humans, the IgG antibody response also appeared to largely reflect our observations in humans. Specifically, a positive IgG response to one antigen was highly predictive of an IgG response to others. However, there were no animals which were negative for PE antigens as seen in our human samples perhaps due to more homogenous genetics or baseline states of our animals. Unlike humans, we saw no correlation between parasitemia and IgG response in our monkeys which could indicate a difference between humans and monkeys or simply reflect differences in sampling time points and the effect of previous exposure.

Our results in monkeys also demonstrate that blood stage infection on its own elicits antibodies that are reactive to PE antigens. Given the lack of evidence that these proteins are actually expressed at the blood stage, this cross-reactivity raises interesting considerations for the design of multi-stage *P. vivax* vaccines and interpretation of natural infection serology. In contrast to this cross-stage reactivity, monkeys infected with *P. vivax* or *P. falciparum* showed low cross-species reactivity against blood stage antigen MSP1 and no cross-reactivity against the PE antigen CSP. This also warrants further investigation as it could prove useful for serological surveys of malaria prevalence especially in co-endemic areas.

Conclusion

In conclusion, we present the most in-depth survey of PE immunity acquired during natural infection in the Peruvian Amazon. Our results demonstrate broad but variable reactivity across *Pv* PE antigens and between individuals. In addition, we find these results are largely replicated in a novel *Aotus* non-human primate *Pv* blood stage model which closely-reproduces human infection in terms of parasitology and IgG responses to blood stage and PE antigens. Together, these data clearly demonstrate a previously underappreciated prevalence of natural immunity to PE antigens which warrants specifically designed longitudinal cohorts where better correlations to transmission and protection from infection can be assessed. Such studies can add to a growing understanding of immunity to *P. vivax* infection in terms of both antigens and immune effector mechanisms with the goal of designing vaccines capable of preventing or reducing infection.

Abbreviations

Pv: Plasmodium vivax ; PE: pre-erythrocytic; ELISA: enzyme-linked immunosorbent assay; ***Pf: P falciparum***; MSP1: merozoite surface protein-1; DBP: Duffy Binding Protein; CSP: Circumsporozoite protein; WHO: World Health Organization; TRAP: thrombospondin-related adhesive protein; RH5: reticulocyte-binding protein Homolog 5; iRBCs: infected red blood cells; MQ: Mefloquine; CQ: Chloroquine;

RAS: radiation attenuated sporozoites; GEST :egress and sporozoite traversal; ETRAMP: early transcribed membrane protein; AUC: Area Under the Curve; STD: standard deviation

Declarations

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Disclaimers

“The views expressed in this article reflect the results of research conducted by the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the United States Government”

Authors’ contributions

GCB, JCA and BKW conceived, designed, and supervised the project studies. JVA, LLT, RP, AF and ML performed experiments of protein production and/or evaluation of immunogenicity by ELISA or ELISPOT. JVA, LLT and BKW performed the data and statistical analysis. JVA, LLT, LS and BKW wrote the first draft of the manuscript. JVA, LLT and BKW wrote the final version of the paper. All authors reviewed, edited and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The human study protocols NMRCD.2007.0004, NMRCD.2010.0002 and NAMRU6.2012.0006 were approved by the U.S. Naval Medical Research Unit N°6 Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects

The animal experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011.

Consent for publication

Not applicable. No personal identifiable data was used in the study. All authors have provided consent to publish the findings in this study.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Demographic information of *P. vivax* infected patients by site of sample collection. Differences of epidemiological variables between groups of sample collection were assessed using *Mann-Whitney U* test and *Chi-square* test with significant values for $p < 0.05$ and $p<0.001$.

Table 1. Demographic information of *P. vivax* infected patients by site of sample collection

Characteristics	Hospital Apoyo Iquitos (HAI) n=35	Hospital regional Loreto (HRL) n=41	*p-value*
Age (Median, IQR)	27 (9 - 68)	35 (8 - 88)	0.391
Male sex (%)	54	60	0.309
Asexual par/ul (Median, IQR)	2,830 (36 - 14,130)	3,045 (24 - 24,180)	0.692
Temperature C° (Median, IQR)	37.8 (36.0 - 41.5)	37.0 (35.9 - 39.6)	0.198
Weight Kg (Median, IQR)	56.0 (16 - 98)	65.0 (24 - 90)	0.037
Number of previous *P. vivax* episodes (Median, IQR)	0 (0 - 6)	1 (0 - 15)	0.028

Table 2. Demographic information of *P. vivax* infected patients by low and high antibody responders.
Epidemiological variables were compared between low and high responder group. Categorical variables were tested by Chi2 and numerical variables by *Mann-Whitney U test*, significance was reported by $p < 0.05$, and $p<0.001$.

Table 2. Demographic information of *P. vivax* infected patients by low and high antibody responders

Characteristics	low Ab responders n=38	high Ab responders n=38	*p-value*
Place-HRL (%)	44	56	0.250
Place-HAI (%)	57	43	0.250
Male sex (%)	49	51	0.817
Age (Median, IQR)	30	30	0.790
Asexual par/ul (Median, IQR)	4,663 (36 - 24,180)	1,873 (24 - 12,572)	**0.008**
Temperature C° (Median, IQR)	37.0 (36.0- 41.5)	37.0 (35.9 - 40.0)	0.468
Weight Kg (Median, IQR)	60.0 (16-98)	66.5 (29 - 90)	0.433
Number of previous *P. vivax* episodes (Median, IQR)	0 (0 - 8)	1 (0 - 15)	0.172

Table 3. Comparative analysis of antibody and T cell responders against blood stage and PE antigens.
Prevalence of ELISA and ELISOPT was measured by IgG and Interferon gamma positivity in *P. vivax* infected individuals.

Table 3. Paired ELISA and ELISPOT results of up to 7 subjects for each protein tested by both assays

Protein ID	PVX Code	Parasite stage	ELISA +	ELISPOT +	IgG+ / IFN+	IgG+ / IFN-	IgG- / IFN+	IgG- / IFN-
MSP1	PVX_099980	Blood	100%	29%	2/7	5/7	0/7	0/7
CSP	PVX_119355	PE	86%	29%	2/7	4/7	0/7	1/7
CelTOS	PVX_123510	PE	57%	29%	2/7	2/7	0/7	3/7
ETRAMP	PVX_121950	PE	50%	33%	1/6	1/3	1/6	1/3
FALSTATIN	PVX_09903	PE	57%	14%	1/7	3/7	0/7	3/7
Hypothetical	PVX_119755	PE	80%	20%	1/5	3/5	0/5	1/5
HSP	PVX_089585	PE	100%	0%	0/1	1/1	0/1	0/1
GEST	PVX_121950	PE	100%	0%	0/1	1/1	0/1	0/1

Table 4. Parasitemia kinetics of *A. nancymaae* monkeys infected with *P. vivax* Vietnam-IV strain.
Parasitemia was measured by microscopy and followed up from day 4 to 37 post infection with *P. vivax* Vietnam-IV. Monkeys were infected with dose of 2.5 x 10^6 par/animal (experiment 1) or 1.0 x 10^6 par/animal (experiment 2). Prepatency day (day to first parasitemia), day of maximum parasitemia and maximum parasitemia were determined by microscopy.

Table 4. *Aotus nancymaae* and *P. vivax* parasitemia information

Monkey ID	Experiment	Prepatency Day	Day of maximun parasitemia	Maximun parasitemia
T3044	1	4	14	14600
T3122	1	6	14	21600
T3212	1	4	10	13000
T3242	2	5	11	2100
T3251	2	7	11	29200
T3215	2	7	15	13500
T3237	2	7	13	7920
T3244	2	5	15	3760
T3308	2	5	17	194000
Exp 1 average		**5**	**13**	**16400**
STD		1	2	3735
Exp 2 average		**6**	**14**	**41747**
STD		1	2	68672

Table 5. Parasitemia kinetics of *A. nancymaae* monkeys infected with *P. falciparum* FVO strain.
Parasitemia was measured by microscopy and followed up from day 3 to 30 post infection with *P. falciparum* FVO. Monkeys were infected with dose of 1.0 x 10^4 par/animal for experiment 1 and 2. Prepatency day, day of maximum parasitemia and maximum parasitemia were determined by microscopy results.

Table 5. ***Aotus nancymaae*** **and** ***P. falciparum*** **parasitemia information**

Monkey ID	Experiment	Prepatency Day	Day of maximun parasitemia	Maximun parasitemia
T2736	1	7	12	365444
T2758	1	5	12	382500
T2815	1	7	12	321879
T2825	1	7	14	231250
T2844	1	7	10	214700
T2875	1	5	12	530462
T3409	2	5	10	223600
T3427	2	7	11	283050
T3473	2	6	11	769500
T3496	2	6	11	385400
Exp 1 average		**6**	**12**	**341039**
STD		1	1	105339
Exp 2 average		**6**	**11**	**415388**
STD		1	1	212480

Supplementary

Figure S1 is not available with this version

Figures

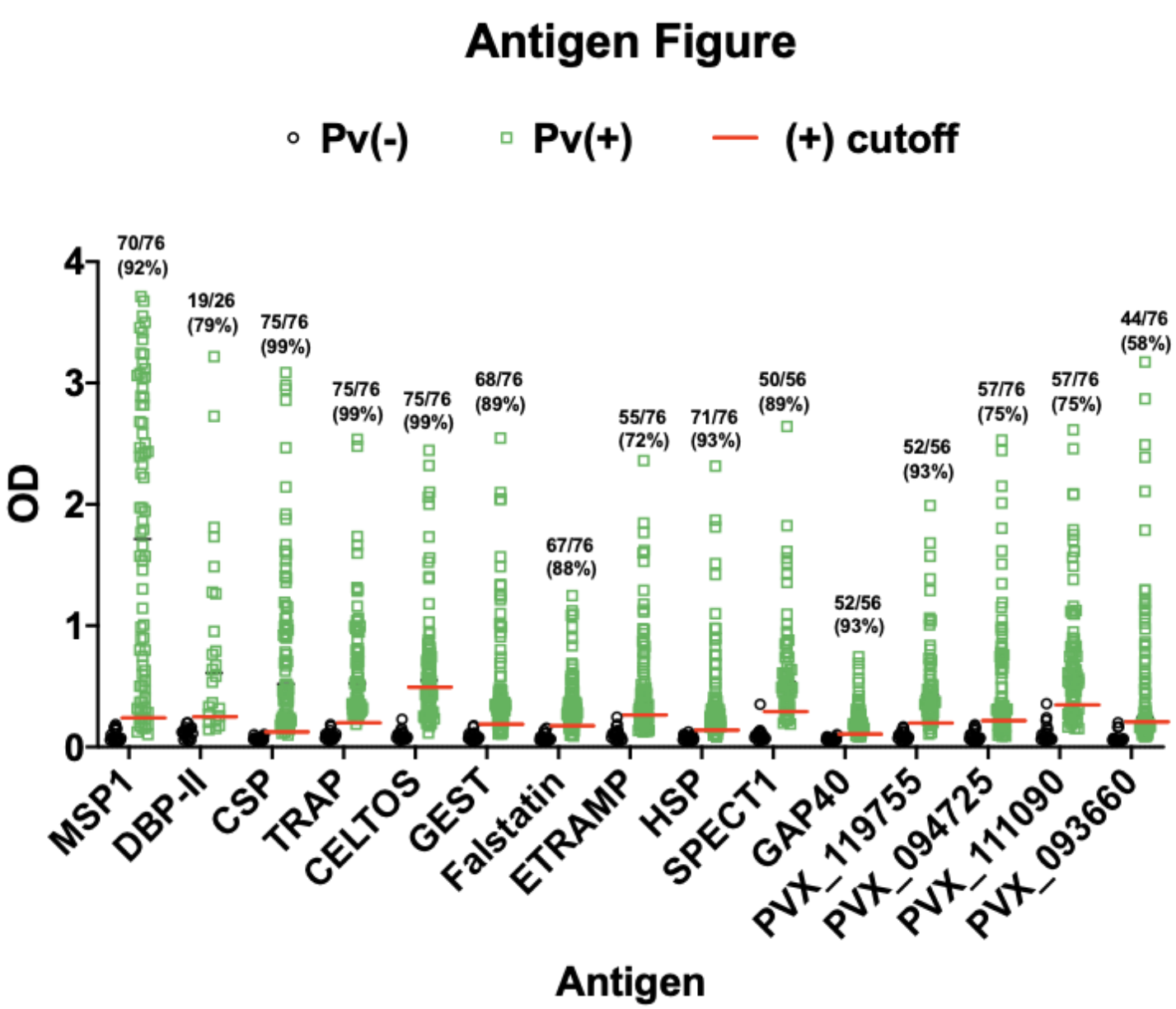

Figure 1

Prevalence against P. vivax PE antigens in P. vivax infected Peruvian population. Plasma samples from healthy controls (black circles) and P. vivax patients (green squares) were tested against 15 P. vivax antigens to determine seroprevalence by ELISA. Data shows individual values of IgG antibodies against each antigen measured by OD values. OD positivity cut-off (red line) value was defined as the average of low endemic control samples plus three standard deviations per each antigen.

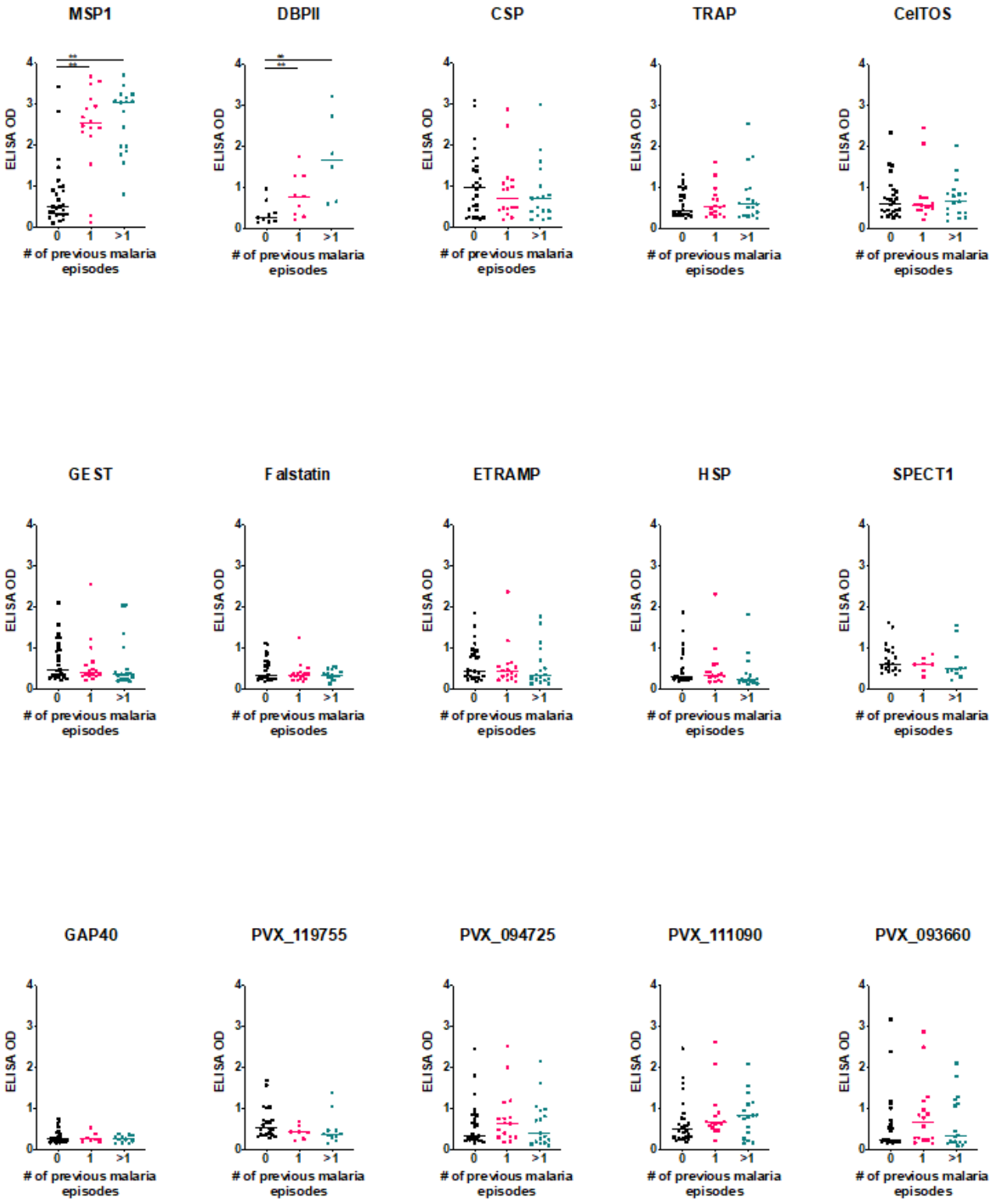

Figure 2

Boosting effect of blood and pre-erythrocytic P. vivax antigens in P. vivax infected Peruvian population. Plasma samples from zero previous (n=9-26), one previous (n=7-16) and two or more previous (n=6-17) P. vivax episodes were used to determine boosting effect against each P. vivax antigen. Dot plots represent P. vivax episode groups with OD values of IgG antibodies against each antigen. Differences between

groups per each antigen were assessed using the Kruskal-Wallis test with Mann-Whitney U post-test. * p < 0.05, and ** p<0.001.

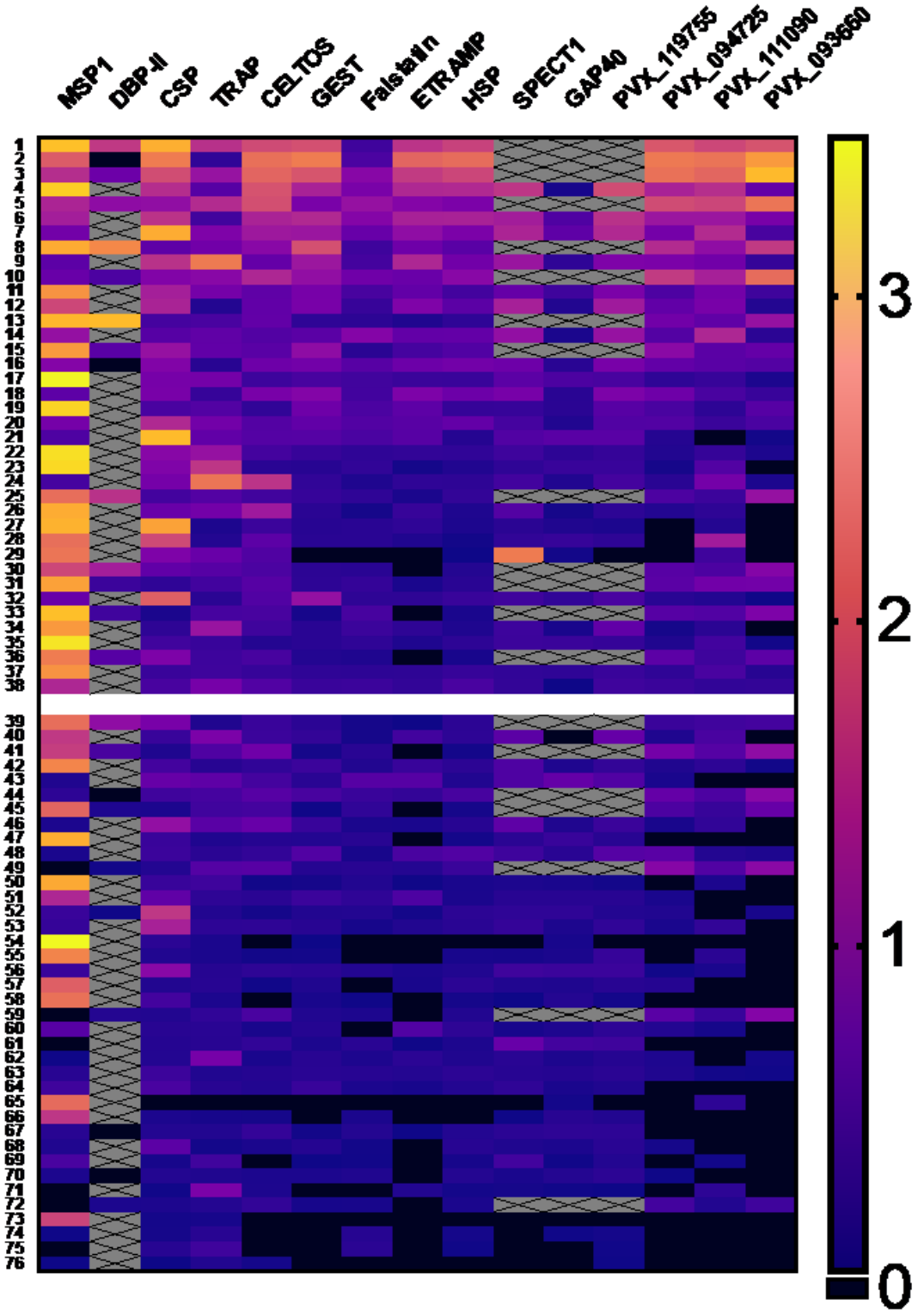

Figure 3

Reactivity of pre-erythrocytic P. vivax antigens in P. vivax infected Peruvian population. Individual reactivity against each P. vivax antigen is shown by OD. Negative reactivity is shown as 0 (dark blue) and positive reactivity with OD values between 0.2-3.5 (blue to yellow). Volunteers are arranged in order of

descending total antibody response across all antigens with the gap showing the division between “high responders” and “low responders”. Boxes with “X” indicate samples not measured due to either limited sample availability or inability to finish analysis due to the COVID-19 pandemic.

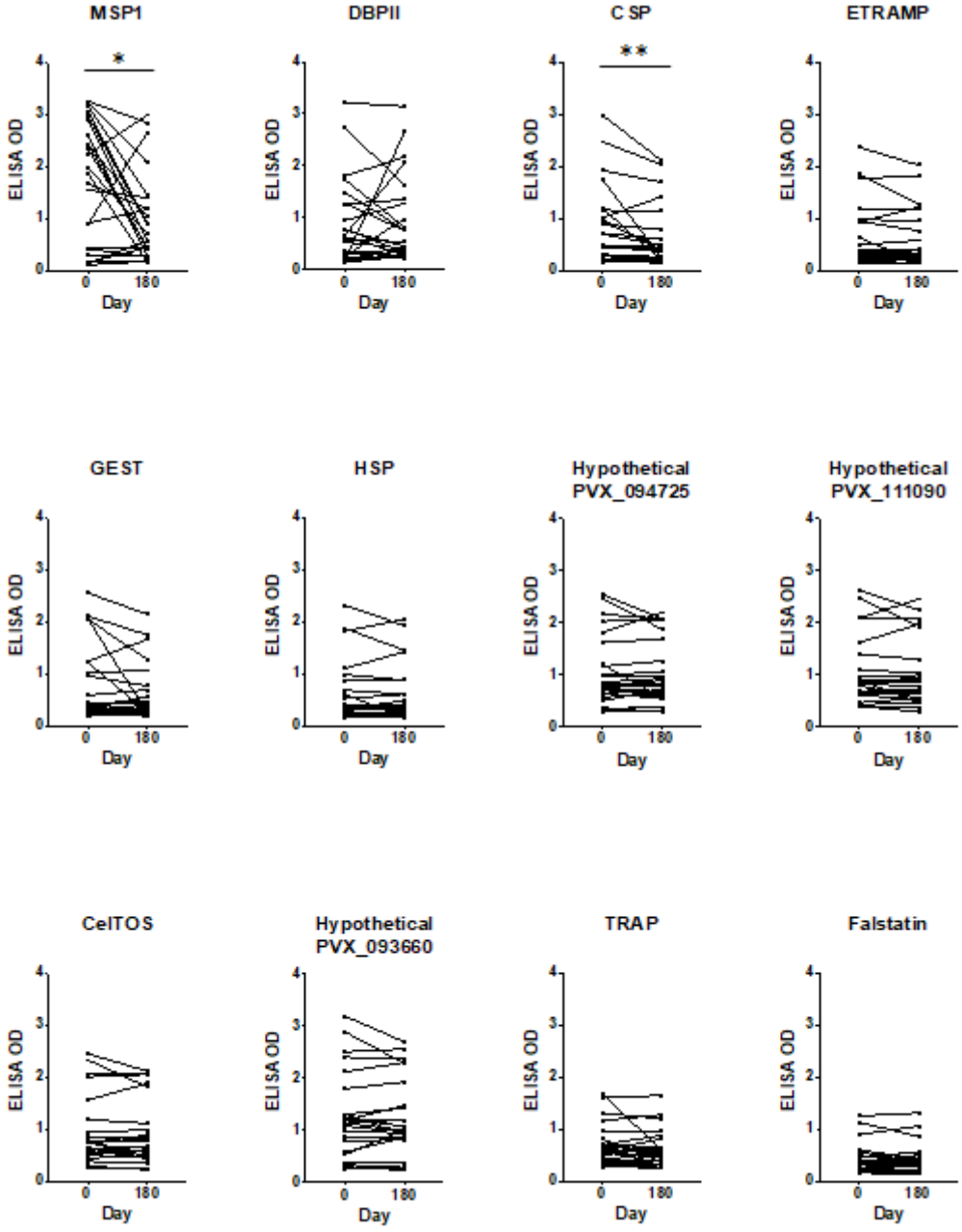

Figure 4

Long-term antibody analysis of blood and PE P. vivax antigens in P. vivax infected Peruvian population. Plasma samples from P. vivax infected volunteers (n=24) were used to represent individual antibody variation after 6 months of P. vivax infection against each P. vivax antigen. Lines connect individual antibody variation between P. vivax infection Day 0 vs Day 180. Differences between time points per each antigen were assessed using Wilcoxon signed-rank test, * $p < 0.05$, and ** $p<0.001$.

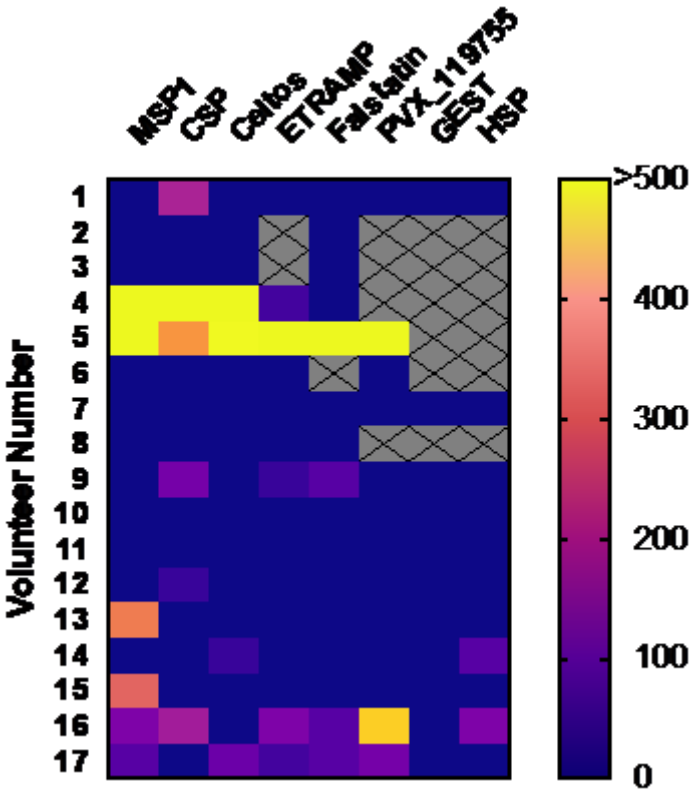

Figure 5

IFN-γ response to seven PE P. vivax antigens evaluated in 17 malaria patients from Iquitos. Shown as SFU/106 PBMCs for samples which were >20% above negative controls after subtracting background. Boxes with “X” indicate samples not measured due limited sample availability.

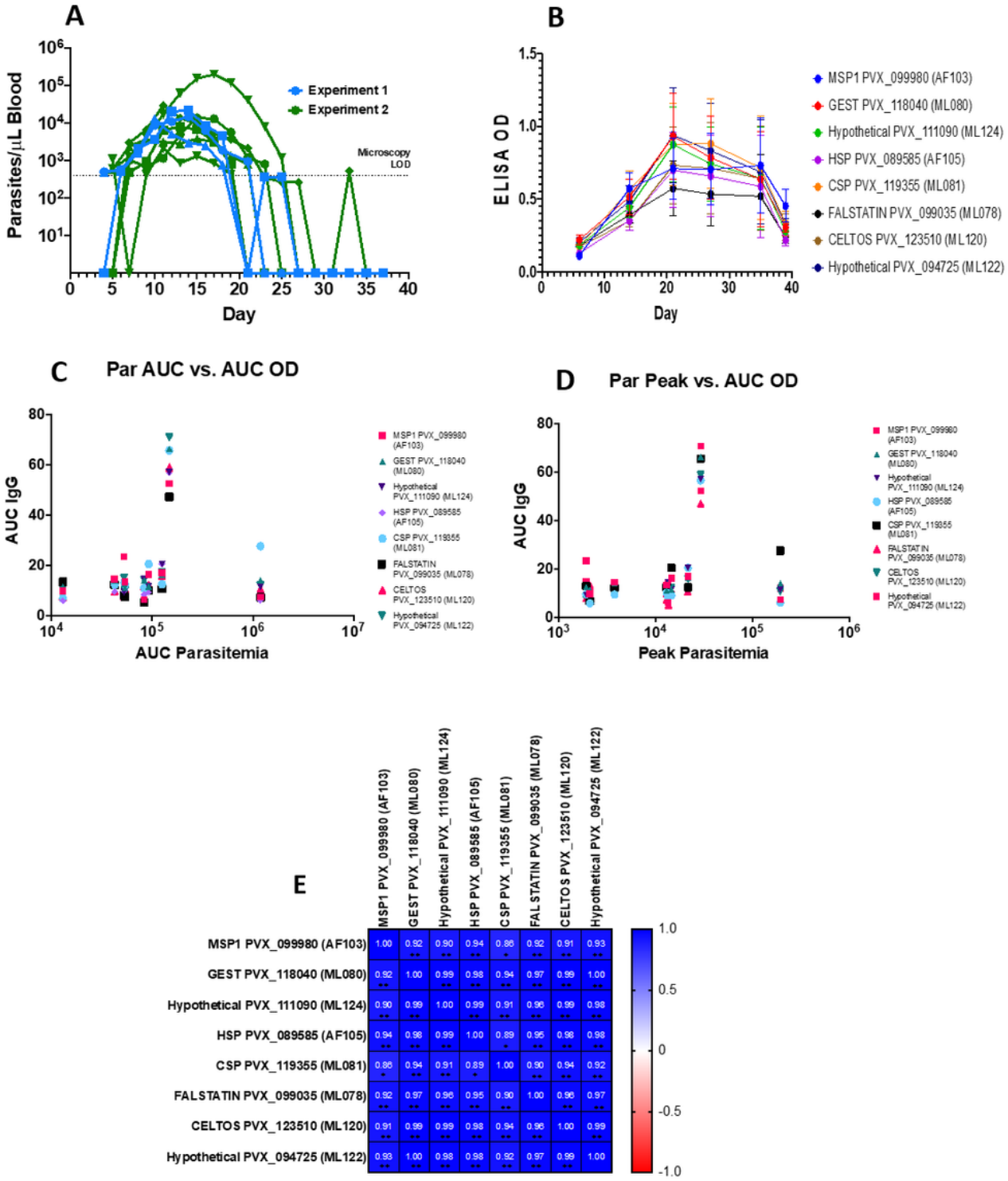

Figure 6

Parasitemia kinetics and humoral immune response against P. vivax Blood stage and PE antigens of A. nancymaae monkeys infected with P. vivax Vietnam-IV strain. (A) Parasitemia kinetics of monkeys infected with 2.5 x 106 par/animal (experiment 1, blue lines) or 1.0 x 10^6 par/animal (experiment 2, green lines). (B) Mean IgG antibodies for each antigen over the course of infection as measured by ELISA. Parasitemia levels vs. IgG antibodies were compared using the area under the curve during the entire

infection period in (C) or the day of peak parasitemia in (D) for each antigen. (E) Correlation of IgG antibody response between proteins were measured by Pearson's correlation. Coefficients were represented with values of -1.0 to 1.0, with colors red for negative correlation and blue for positive correlation. Significant correlations were represented by *p < 0.05 and **p<0.001.

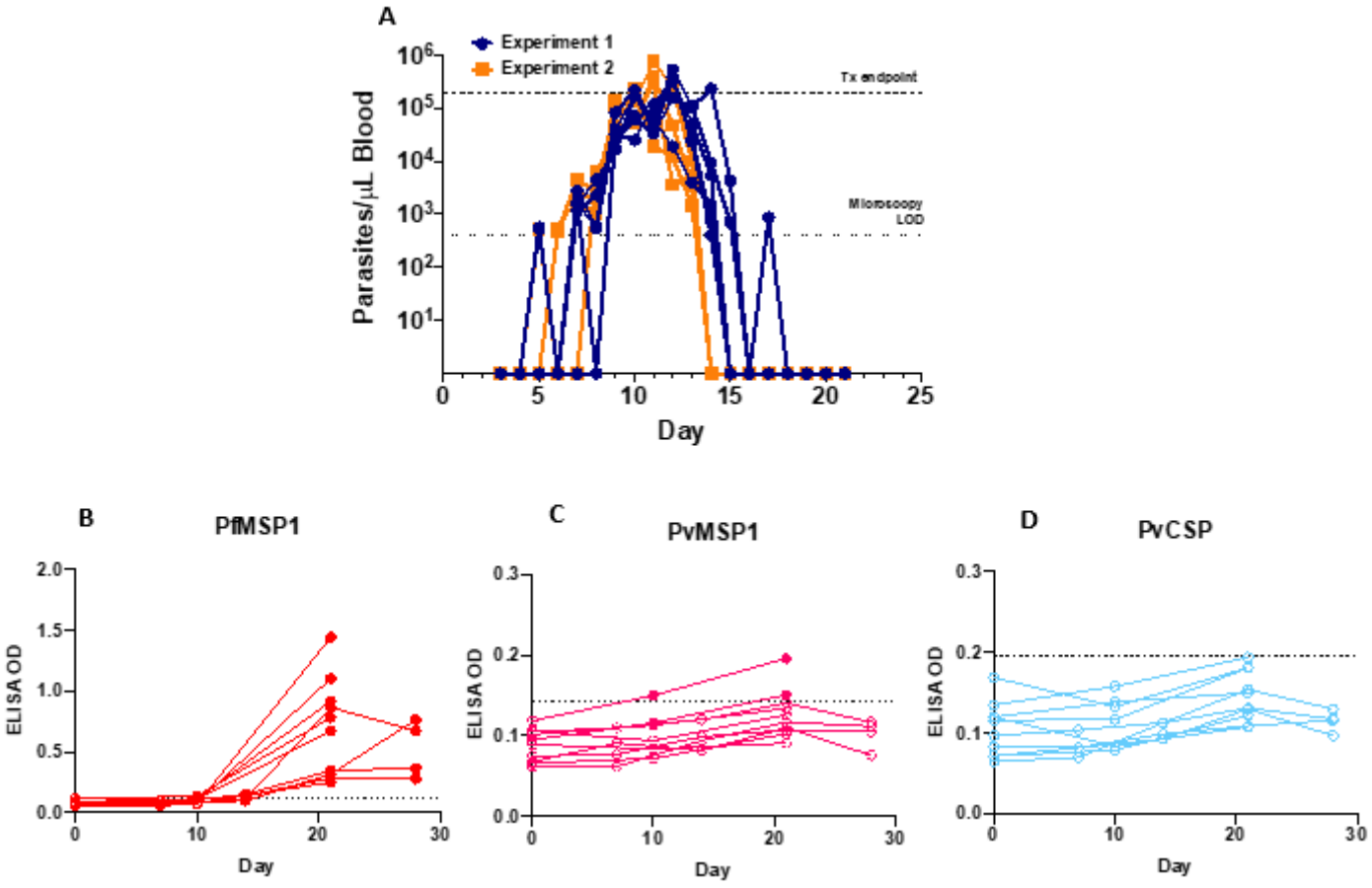

Figure 7

Parasitemia kinetics and humoral immune response against P. vivax and P. falciparum antigens of A. nancymaae monkeys infected with P. falciparum FVO strain. (A) Monkeys were infected with 1.0 x 104 iRBC/animal for experiment 1 (Blue lines) and experiment 2 (Orange lines). Parasitemia was measured by microscopy and followed up from day 3 to 30 post infection. IgG antibody responses against P. falciparum MSP1 antigen (B), P. vivax MSP1 antigen (C) and P. vivax CSP antigen (D) were measured by ELISA with individual animals shown with connecting lines. Dashed lines represent cutoff values for each antigen calculated by the average of OD values of uninfected animal plasma plus 3 standard deviations. Solid data points represent any values above this cutoff.

Supplementary Files

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