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Supplemental Information

A Humanized Mouse Model for *Plasmodium vivax* to Test Interventions that Block Liver Stage to Blood Stage Transition and Blood Stage Infection

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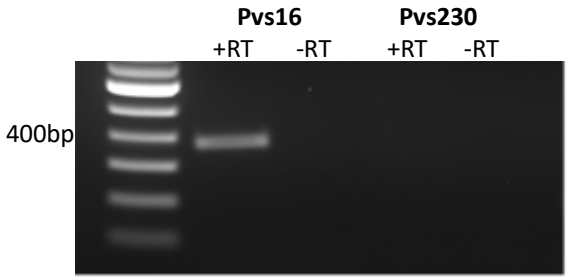

Figure S1. Detection of Pvs16 expression by RT-PCR, Related to Figure 3

RNA was isolated from *P. vivax* infected FRG KO huHep livers eight days post sporozoite infection. Following reverse transcription, Pvs16 transcript was successfully detected by PCR, confirming the expression of Pvs16 by *P. vivax* liver stages, whereas we were not able to detect expression of the mature gametocyte marker Pvs230. +RT=with reverse transcriptase;

-RT=without reverse transcriptase.

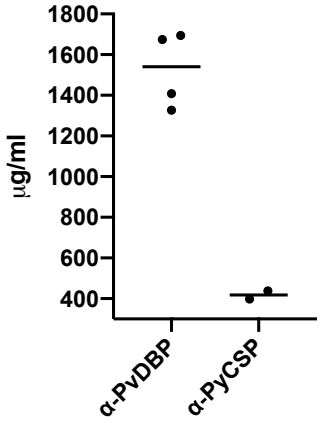

Figure S2. High serum antibody levels were detected before reticulocyte injection, Related to Figure 4

Serum antibody levels were measured by ELISA on day 9 post infection, directly before the first reticulocyte injection and revealed high antibody concentrations. Each dot represents one mouse and median is shown.

Transparent Methods

Ethics statement

The Human Subjects Protocol for this study was approved by the Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University.

All animal experiments were carried out in accordance with the recommendations of the NIH Office of Laboratory Animal Welfare standards. Mice were maintained under specific pathogen-free conditions at the Center for Global Infectious Disease Research, Seattle Children's Research Institute. The protocol was approved by the Center for Infectious Disease Research Institutional Animal Care and Use Committee (IACUC) under protocol 00480.

Mice

Female FRGN KO huHep mice were purchased from Yecuris Inc. Serum albumin levels of all mice used exceeded 4mg/ml, corresponding to >70% human hepatocyte repopulation. Mice were maintained on drinking water containing 3% Dextrose and were cycled on 8 mg/L NTBC once a month for 4 days to maintain hepatocyte chimerism.

Infection of FRGN KO huHep mice with *P. vivax* sporozoites and mAb passive transfer

All sporozoites were obtained by microdissection of salivary glands from mosquitoes that had been fed on *P. vivax* infected blood obtained from patients seeking treatment for *P. vivax* malaria at malaria clinics along the Thai–Myanmar border. 14–16 days after the blood meal, mosquito salivary glands were dissected and sporozoites were collected into cold Schneider's insect media (Sigma Aldrich). Salivary glands were crushed and sporozoites were enumerated using a hemocytometer. 0.6 – 0.9 million sporozoites were injected intravenously into FRGN KO huHep mice. To prevent bacterial coinfections, mice were injected intraperitoneally with 1000 Units Penicillin and 1mg Streptomycin (Sigma Aldrich) every other day after the infection.

We tested a modified version of the reported human DB9 monoclonal antibody, targeting the essential PvDBP-DARC interaction (Rawlinson et al., 2019), which is referred to in the text as "anti-PvDBP". 100 mg/kg of this antibody, an antibody against PyCSP as off-target control or 100ul PBS were injected intraperitoneally 24 hours prior to the first reticulocyte injection.

Enrichment of reticulocytes from whole blood and reticulocyte repopulation of FRGN KO huHep mice

Blood was collected either from the umbilical cord directly after delivery of a child, or by phlebotomy of healthy donors or patients with hemochromatosis. All blood collections were performed at Bloodworks NW. CPDA was used as anticoagulant. The blood was leuko-reduced using a Leukocyte reduction filter (Haemonetics Corp. MA, USA) and subsequently washed three times in RPMI 1640 (25 mM HEPES, 2 mM L-glutamine; Sigma Aldrich) to deplete any remaining white blood cells. Packed RBCs were resuspended in RPMI1640 to 20% hematocrit. 4ml of this RBC preparation was layered on top of 4ml 25% OptiPrep^{TM} in PBS (Stemcell Technologies Inc., USA) and centrifuged at 3000xg for 30 mins without brake. The resulting layer of enriched reticulocytes was washed three times in RPMI1640 and stored in McCoy's 5A media (Thermo Fisher Scientific, USA) at 4°C for a maximum of 5 days. Reticulocyte content was determined by New Methylene Blue staining and reticulocyte preparations contained 30 - 60% reticulocytes. On days 3, 7 and 10 post infection, mice received 150ul or 100ul respectively of Clodronate liposomes (Chlophosome®-A; FormuMax) and 150mg/kg Cyclophosphamide (Sigma Aldrich) intraperitoneally. After two reticulocyte injections on days 9 and 10 post infection, mice reached about 30% red blood cell chimerism. Assuming that 30 - 60% of the human cells injected are reticulocytes, mice had between 9 and 18% human reticulocytes of the total red blood cells available for invasion by the parasite. The number of invaded cells is shown as % parasitemia.

Notably, this parasitemia is measured as a percentage of total red blood cells, including mouse cells.

18S rRNA qRT-PCR quantification of parasite load

On day 10 post infection, 5 hours after the second reticulocyte injection, 50ul blood was drawn, immediately added to 1ml NucliSENS Lysis buffer (bioMérieux, Marcy-l'Étoile, France) and frozen at -80°C. The samples were processed and analyzed for presence of *Plasmodium* 18S rRNA using a pan-*Plasmodium* probe as previously described (Seilie et al., 2019).

Immunofluorescence assays of liver sections

After harvest, liver samples were fixed in 4% Paraformaldehyde over night at room temperature. After two washes in 1xTBS they were sliced to 50µm sections using a vibratome. Subsequent incubations were performed on a shaker set to 80rpm. Liver sections were permeabilized in 10% H_2O_2 and 0.25% Triton X-100 in 1xTBS for 30 minutes at room temperature. After one wash in 1xTBS sections were blocked in 5% skim milk in 1xTBS for one hour at room temperature before incubating overnight at 4°C with anti-Pvs16 or anti-Pvs230 (rabbit polyclonal serum, kindly provided by Tomoko Ishino, Ehime University, Japan) diluted 1:200 in 5% skim milk and anti-UIS4 mouse monoclonal (Schafer et al., 2018) diluted 1:500 in 5% skim milk. Liver sections were subjected to three five-minute washes before addition of 2µg/ml Alexa Fluor-488 labeled goat anti-mouse IgG and 2µg/ml Alexa Fluor-594 labeled goat anti-rabbit IgG (Thermo Fisher) and incubation for two hours at room temperature. After one five-minute wash in 1xTBS, sections were transferred to 1xTBS containing 2µg/ml DAPI and incubated for 10 minutes at room temperature, before washing again in 1xTBS. To reduce background fluorescence, sections were incubated for ~20 seconds in 0.06% KMnO4 in 1xTBS before washing one more time in 1xTBS and mounting on a poly-Lysine coated slide using ProLong™ Gold Antifade mounting solution (ThermoFisher)

to preserve fluorescence before analysis and image acquisition using Olympus 1x70 DeltaVision deconvolution microscopy.

RT-PCR to detect Pvs16 and Pvs230 transcript in mouse liver samples

FRG KO huHep mice were infected with one million *P. vivax* sporozoites. On day 8 post infection, livers were harvested, each liver lobe was cut in half and all lobes from each mouse were pooled together in TRIzol reagent (Invitrogen). Livers were homogenized and RNA was extracted using the RNeasy purification kit (Qiagen). RNA was reverse transcribed to cDNA using the Primescript RT reagent kit (Takara) prior to PCR using BioMix (Bioline) and primers specific to Pvs16 (primer sequences: fwd: TCCTCTTGTGTGTCCTGCTAA rev: CCCCTTTTGGGCTTCTGGA Product size:374 bp) or Pvs230 (Primer sequences: fwd: 5'- CGCCAAGCAGACGGATGATA -3'; rev: 5'- GTCCTCCGTGGATTGCTCATC -3'; Product size: 526 bp).

Immunofluorescence assays of thin blood smears

Thin blood smears were obtained from infected mice and fixed in pre-chilled Methanol for four minutes. Slides were washed once in PBS before blocking with 4% BSA in PBS for one hour at room temperature. Incubation with primary antibodies was performed in 1% BSA in PBS at 4°C overnight after which slides were washed three times in PBS. Anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or 594 were applied at 1:500 dilution in 1% BSA in PBS together with DAPI (1:1000) to visualize DNA. Slides were incubated for 1 hour at RT after which they were washed three times in 1xPBS. One drop of ProLong™ Gold Antifade mounting solution (ThermoFisher) was added to preserve fluorescence before applying the cover slip.

Primary antibodies used are: rabbit polyclonals against Pvs16 at 1:200 dilution (kindly provided by Tomoko Ishino, Ehime University, Japan); mouse monoclonal against MSP1 at 1:100 dilution

(kindly provided by John Adams, University of South Florida, USA); mouse anti-CD235a at 1:100 dilution (Biorbyt, USA); rabbit anti-*Plasmodium* BiP at 1:200 dilution.

All images were acquired using Olympus 1x70 DeltaVision deconvolution microscopy.

Quantification of serum antibody levels by ELISA

All serum samples were heat-inactivated for 30 minutes at 56 °C prior to any assays, run with duplicate wells and confirmed in duplicate assays. To determine serum anti-PvDBP antibody concentrations, Immulon 2HB 96-well plates (Thermo Scientific, 3455) were coated with 200 ng per well of rabbit anti-human IgG antibody (Invitrogen, SA510223) overnight in 0.1M $NaHCO_3$, pH 9.5. In all assays, plates were washed between each ELISA step with PBS, 0.2% Tween-20 and incubations were 1 hour at 37°C. Coated plates were blocked with PBS, 10% non-fat milk, and 0.3% Tween-20. Following blocking, serum was serially diluted over a range of 1:200 to 1:11,809,800 and purified recombinant anti-PvDBP over a range of 1 to 0.000017 µg/ml in PBS, 10% non-fat milk, and 0.03% Tween-20. Bound antibodies were detected using goat anti-human IgG (H+L)-HRP (Invitrogen, A18811) at 1:1000 dilution in PBS, 10% non-fat milk, and 0.03% Tween-20. To determine serum anti-PyCSP antibody concentrations, plates were coated with 50 ng per well of Streptavidin (New England Biolabs, N7021S) in 0.1M $NaHCO_3$, pH 9.5. Coated plates were blocked with PBS, 3% BSA, then coated with 200 ng per well of biotinylated PyCSP in PBS, 0.2% BSA. Plates were then blocked with PBS, 10% non-fat milk, and 0.3% Tween-20. Following blocking, serum was serially diluted over a range of 1:200 to 1:11,809,800 and purified anti-PyCSP antibody over a range of 0.5 to 0.000008 µg/ml in PBS, 0.2% BSA. Bound antibodies were detected using goat anti-mouse IgG Fc-HRP (Southern Biotech, 1013-05) at 1:2000 dilution in PBS and 0.2% BSA. All plates were developed with 50 µl of TMB Peroxidase Substrate (SeraCare Life Sciences Inc, 5120-0083) equilibrated to room temperature and stopped after 3

minutes with 50 µl of 1N H_2SO_4. Absorbance at 450 nm was detected on a BioTek ELx800 microplate reader. Standard curves for anti-PvDBP or anti-PyCSP controls were generated by nonlinear regression (log[agonist] vs response[three parameters]) in GraphPad Prism V8 (San Diego, CA). Serum antibody concentrations were quantified by interpolating the average values from three different dilutions along the corresponding standard curves and multiplying by the dilution factor to determine the final concentration.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 8.3.1. The specific statistical tests performed for each experiment are included in the corresponding figure legend.

Supplemental References

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