SUPPLEMENTARY

Material and Methods

Experimental animals and parasites

Female OF1 and C57Bl/6 mice (6 to 8 weeks old; Charles River/Janvier) and Wistar rats (HsdCpb:WU; 175-199 gr, Harlan Netherlands BV) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042, 12043). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All experiments were performed in accordance with relevant guidelines and regulations. The following reference lines of the ANKA strain of *P. berghei* (*Pb*) were used in this study: line cl15cy1 (Janse *et al.*, 2006) and line $GIMO_{pbANKA}$ (1596cl1; RMgm-687 in www.pberghei.eu; referred to as *Pb*WT). The $GIMO_{pbANKA}$ (1596cl1) was generated in the cl15cy1 parent line and this line expresses a fusion of a drug resistance gene *hdhfr (human dihydrofolate reductase*) and a drug sensitivity gene *yfcu* (*yeast cytosine deaminase and uridyl phosphoribosyl transferase*), the so called positive-negative selectable marker (SM), constitutively expressed by the *P. berghei eef1α* promoter stably integrated into the *230p* locus [1].

Generation and genotyping of the transgenic parasite line, Pfs48/45@PbMSP1

To introduce the *Pfs48/45* gene (PF3D7_1346700) into the redundant *p230p* gene locus (PBANKA_0306000) of the *Pb* genome, we generated DNA construct pL1706. The basic gene insertion construct pL0046 was used, which contains the 5' and 3' *230p* targeting regions, the *tgdhfr/ts* selectable marker (SM) cassette and an mCherry expression cassette under the control of the *eef1α* promoter with 3' terminal sequence of *pbdhfr/ts*. The *eef1α* promoter was replaced by the *msp1* promoter (PBANKA_0831000) using *Afl*II and *Bam*HI digestion. The *msp1* promoter was amplified from genomic *Pb* ANKA DNA using primers 6145 and 6146. In addition the mCherry coding sequence (CDS) was replaced by the *Pfs48/45* CDS using *Bam*HI and *Sgr*AI digestion. The *Pfs48/45* CDS was amplified from genomic DNA of the *Pf* NF54 strain using primers 5583 and 5584. This resulted in construct pL1706. In order to introduce the expression construct in the genome of the parent GIMO *Pb*ANKA line (1596cl1) , we next removed the *tgdhfr/ts* SM by digestion of the plasmid with *Sb*fI and *Afl*II. The ends of the linearized constructs were then rendered blunt using Klenow enzyme treatment, and re-ligated. This final construct (pL1707) were analyzed via restriction

digestions to confirm correct assembly. Before transfection, the construct pL1707 was linearized by digesting the plasmid with *Ksp*I.

Parasites of line 1596cl1 were transfected with this construct (exp. 1807) using standard transfection technologies and transformed parasites selected by negative selection with 5-fluorocytosine (5-FC) [1, 2]. Selected parasites were cloned by limiting dilution. Three independent clones have been obtained after the cloning and correct integration of the construct was confirmed by Southern Analysis of PFG-separated chromosomes (data not shown). Mutant 1807cl2 was used for further genotype and phenotype analysis. Correct integration of the construct into the *p230p* gene locus was performed by diagnostic PCR-analysis and Southern analysis of pulsed field gel (PFG) separated chromosomes as described previously [3]. For Southern analysis, PFG-separated chromosomes were hybridized to a mixture of two probes, one recognizing *hdhfr* and one control probe, recognizing the *p25* gene on chromosome 5 [2].

Western and IFA analyses of Pfs48/45 expression

Transgenic schizonts were obtained from short-term overnight cultures of infected blood obtained by cardiac puncture from rats or mice as previously described [4]. Leucocytes were removed from the infected blood using Plasmodipur filters before the parasites were put into short-term overnight culture. Schizonts from the short-term cultures were purified using Nycodenz gradient centrifugation, resulting in parasite populations consisting of >90% schizonts [4] .

For Western analysis, purified schizont preparations were extracted in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation at 13,000 g for 5 min at room temperature (RT) and the supernatant was used for Western analysis [5]. Parasite proteins were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) for 2 h at 200 mAh. Membranes were blocked for non-specific binding in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) containing 3% skim milk (Elk, Campina, The Netherlands) overnight at 4°C. Blots were hybridized with 4 anti-*Pf*s48/45 monoclonal antibodies that recognize *Pf*s48/45 epitopes I, IIb, III and V (antibodies 85RF45.1, 85RF45.2b, 85RF45.3, 85RF45.5) [6]. One microgram of protein was loaded in each lane and for reduced reaction, the DTT was added at final concentration of 10 mM [7]. After incubation with the monoclonal antibodies the membranes were washed with PBST and incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG secondary antibody (Sigma-Aldrich) and developed in Amersham ECL Western Blotting Detection Kit according to the manufacturer's instructions (GE Healthcare). As a loading control, the membranes

were also incubated with rabbit anti-*P. yoelii* MSP1 antibody [8], followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (GE Healthcare).

The amount of *Pfs48/45* protein in total schizont extract was estimated by quantitative Western blot analysis. Protein extracts of the schizont and gametocyte lysates (see above) and R0.10C recombinant protein were quantified using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Protein extracts (500 ng) were loaded on the SDS-PAGE gel and a serial dilution series (50, 25, 12.5, 6 and 3 ng) of the recombinant *P. falciparum* P48/45 fused to GLURP R0 domain (R0.10C) was loaded on the gel. Proteins were separated by electrophoresis and transferred to nitrocellulose membrane as described above and the blot was probed with antibody 85RF45.1 (1:2000 dilution) as the primary antibody. The X-ray film was exposed to the membrane for 30 sec and developed using HQ 350XT X-ray Film Processor. The optical intensity (or Optical Densitometry (OD) values) of the signals were quantified with a BioRad GS-800™ Calibrated Densitometer using Quantity One software (Bio-Rad).

For immunofluorescence analyses (IFA), schizonts-infected red blood cells (RBC) were collected from short-term overnight cultures of infected mice blood described above [4]. The schizont-infected RBC were washed 3 times in PBS and 5 µl of packed cells resuspended in 1 ml PBS. 15-20 µl of this suspension was placed in a well of a 10-well black cell-line diagnostic microscope slide (Thermo Scientific) and allowed to air dry. The slides were fixed with 4% paraformaldehyde in PBS for 30 min and cells were permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The slides were incubated overnight at 4°C with the four different rat anti-*Pf*s48/45 antibodies and rabbit anti-MSP1 antibody (described above) in 10% fetal calf serum in PBS, washed 3 times with PBS at RT, followed by incubation for 1 h with secondary conjugated antibodies anti-rabbit IgG Alexa Fluor®488 (Invitrogen) or anti-rat IgG Alexa Fluor®594 (Invitrogen). Nuclei were stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10 µM (Sigma, The Netherlands) for 30 min at RT. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed onto of the cells and sealed with nail polish. Stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa: 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain).

Immunization with schizont-extracts and purification of IgG from immunized mice

For generation of the schizont-extracts for immunization, 10 Wistar rats were infected with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 parasites and at a parasitemia of 1-3% infected blood was collected by cardiac puncture. Leucocyte removal, short-term overnight culture of infected RBC and Nycodenz gradient purification of schizonts was performed as described above. Purified schizont-

infected RBC were divided into samples containing 1.1×10^9 schizont-infected RBC cells. The cells were pelleted by centrifugation (450 *g*, 8 minutes) and stored at −80°C after removal of the supernatant.

Groups of 10 C57BL/6 mice were immunized with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 schizonts by intravenous injection of 1×10^8 schizont-infected red blood cells (in 200 µl RPMI). Mice were immunized a total of four times, at 2 week intervals. Before injection, schizonts were subjected to 3 freeze (dry ice) - thaw (RT) cycles, to ensure parasites were killed. Fourteen days after the last immunization blood was collected from all mice by cardiac puncture and serum collected after centrifugation (1500×g for 10 min). Serum was stored at −20°C until further analysis. IgG was purified from the pooled serum from 10 mice, by protein G affinity chromatography (Pierce, Rockford, IL) according to the manufacturer's instructions and adjusted to a final concentration of 4 mg/ml in phosphate-buffered saline (PBS).

Standard Membrane Feeding Assay (SMFA)

IgG purified from sera obtained from immunized mice was assessed for transmission reducing (TR) activity in SMFA as previously described [9, 10] using *P. falciparum* (*Pf*) gametocytes. Briefly, *Pf* gametocyte cultures (16 to 18 days old) of *P. falciparum* NF54 (originally provided by Steve Hoffman, Sanaria, Rockville, MD) were adjusted to 0.15 to 0.2% stage V gametocytemia at 50% hematocrit. Sixty microliters of a test sample (with a defined concentration of purified mouse IgG) in 1x PBS was mixed with 100 µl of the gametocyte mixture, and the final mixture was immediately fed to 50 female *Anopheles stephensi* (Nijmegen strain, 3 to 6 days old) mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected (20 per sample) to count the number of oocysts. As assay controls both malaria-naïve human sera and an anti-Pf25 monoclonal antibody (4B7; [11]) were used to establish background and complete inhibition of oocyst formation, respectively. Significance of inhibition (% inhibition in oocyst intensity) was determined by the zero-inflated negative binomial model described previously [10].

References

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