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Expression of full-length *Plasmodium falciparum* P48/45 in *P. berghei* blood stages: A method to express and evaluate vaccine antigens

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ABSTRACT

The transmission-blocking vaccine candidate Pfs48/45 from the human malaria parasite *Plasmodium falciparum* is known to be difficult to express in heterologous systems, either as full-length protein or as correctly folded protein fragments that retain conformational epitopes. In this study we express full-length Pfs48/45 in the rodent parasite *P. berghei*. Pfs48/45 is expressed as a transgene under control of the strong *P. berghei* schizont-specific *msp1* gene promoter (Pfs48/45@PbMSP1). Pfs48/45@PbMSP1 schizont-infected red blood cells produced full-length Pfs48/45 and the structural integrity of Pfs48/45 was confirmed using a panel of conformation-specific monoclonal antibodies that bind to different Pfs48/45 epitopes. Sera from mice immunized with transgenic Pfs48/45@PbMSP1 schizonts showed strong transmission-reducing activity in mosquitoes infected with *P. falciparum* using standard membrane feeding. These results demonstrate that transgenic rodent malaria parasites expressing human malaria antigens may be used as means to evaluate immunogenicity and functionality of difficult to express malaria vaccine candidate antigens.

1. Introduction

Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed [1,2]. This hampers the screening of *Plasmodium* antigens in immunization studies for their suitability as vaccine candidate antigens. Preclinical evaluation of *Plasmodium* antigens often involves immunizing rodents with recombinant *Plasmodium* proteins followed by an examination of induced immune responses, either *in vivo* using rodent models of malaria or *in vitro* by performing functional assays with human malaria parasites incubated with immune sera [3]. Multiple factors contribute to inefficient expression of *Plasmodium* proteins, such as the high AT content of *Plasmodium* genes, large size and often unique protein structure (i.e. encoding repeated stretches of amino acids) and unique post-translational modifications [1,4]. This is particularly evident for cysteine-rich proteins where correct folding depends on accurate formation of disulfide bridges to form domains specific for *Plasmodium* proteins [5–7]. Transgenic rodent malaria parasites (RMP) expressing human malaria parasite (HMP) proteins are increasingly used to evaluate and rank order candidate malaria vaccines before investing in scalable manufacture to support advancement to clinical testing [3]. Such transgenic RMP have been used in preclinical assays to evaluate vaccine potential of HMP proteins, both *in vivo* where mice are immunized with HMP antigens and subsequently challenged with transgenic RMP expressing the cognate HMP or in *in vitro* assays where immune sera or antibodies are evaluated for inhibition of parasite growth or invasion. Both the functional complementation of RMP genes by the HMP orthologs [3] and analysis of HMP expression using antisera, provide evidence for correct expression

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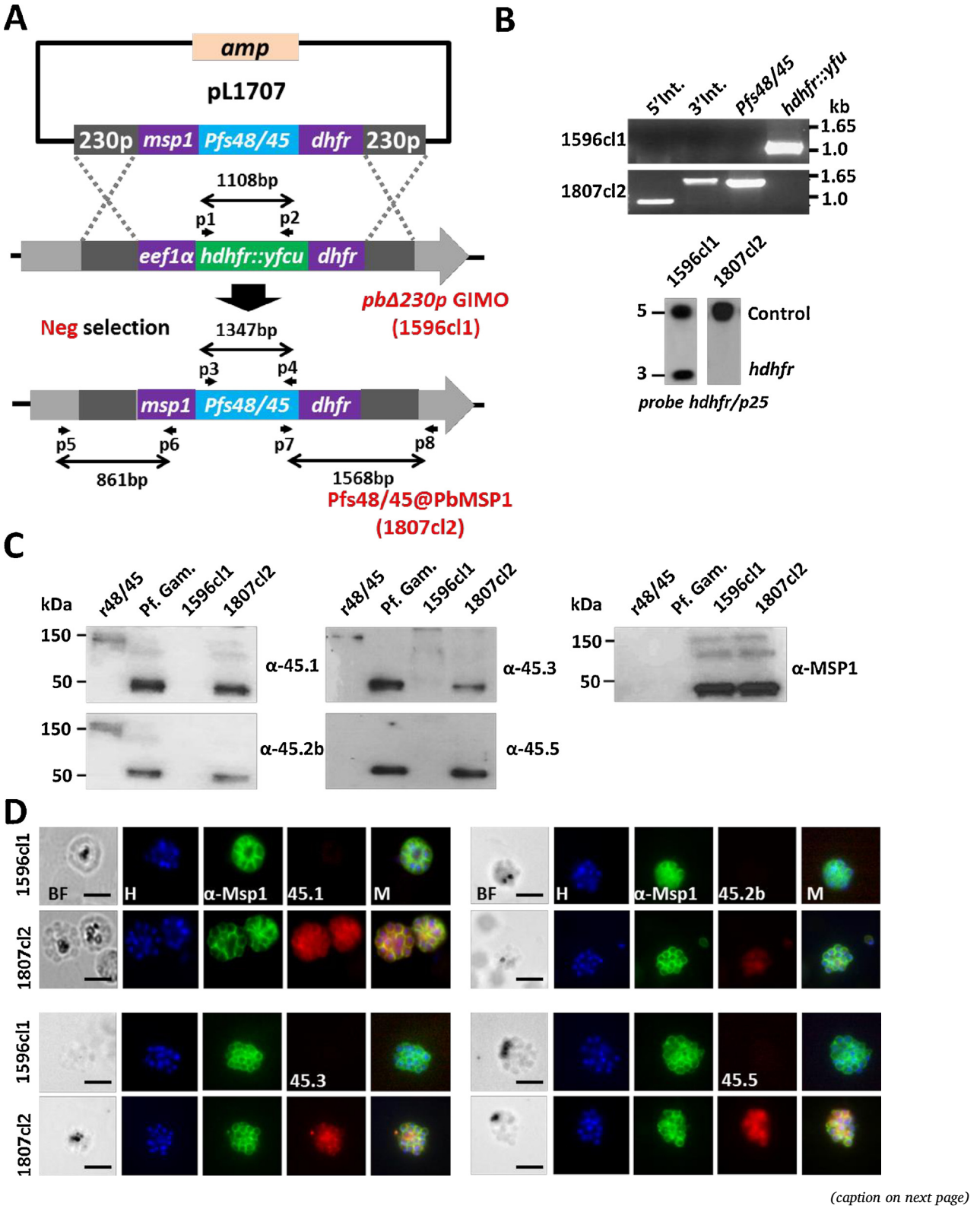

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of functional HMP proteins in transgenic RMP [8]. Based on these studies, we reasoned that transgenic RMP can be used as expression systems to more efficiently express, screen, validate and down-select HMP antigens as potential novel malaria vaccine candidates [2,9]. Further, the expression of conformationally-accurate *Plasmodium* proteins could be used to generate epitope-specific monoclonal antibodies, which in turn can be used to better characterize the vaccine antigen. The use of RMP would circumvent many of the above-mentioned problems associated with expression in heterologous expression systems including, but not limited to, peculiarities of post-translational modifications and *Plasmodium*-specific domains involved in protein trafficking and cellular location. As a proof of concept, we generated transgenic *P. berghei* (*Pb*) parasites that express full length Pfs48/45 from *P. falciparum* (*Pf*). The Pfs48/45 protein is expressed in

Fig. 1. Generation, genotype and phenotype analyses of Pfs48/45@PbMSP1, a transgenic *P. berghei* parasite expressing *P. falciparum* P48/45 in schizonts.
(A) Schematic representation of the introduction of the *Pfs48/45*-expression cassette into the $GIMO_{pbANKA}$ parasite (line 1596cl1). Construct pL1707 contains the *Pfs48/45* gene flanked by the *msp1* promoter region and the 3′ *pbdhfr UTR*. This construct is integrated into the modified *P. berghei 230p* locus of $GIMO_{pbANKA}$ that contains the *hdhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination at the homology regions (*230p*; grey boxes). Negative selection with 5-FC selects for parasites that have the *SM* cassette replaced by the *Pfs48/45* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown.
(B) Diagnostic PCR (upper panel) and Southern analysis of PFG-separated chromosomes (lower panel) confirm correct integration of construct pL1707 in line 1807cl2 parasites. PCR shows the absence of the *hdhfr::yfcu* marker and the presence of the *Pfs48/45*. 5′ integration PCR (5′ int; primers p5/p6), 3′ integration PCR (3′ int; primers p7/p8), *hdhfr::yfcu* (primers p1/p2), *Pfs48/45* (primers p3/p4). Primer locations and product sizes are shown in **A** and primer sequences in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker in the *230p* locus on chromosome 3 in 1807cl2 parasites.
(C) Western analysis of Pfs48/45 expression in protein extracts of purified gametocytes of *P. falciparum* (Pf Gam), purified schizonts of wild type *P. berghei* (1596cl1) and purified schizonts of Pfs48/45@PbMSP1 (1807cl2). As a positive control, recombinant *P. falciparum* P48/45 fragment fused to GLURP R0 domain (R0.10C) was included (expected molecular size is 150 kDa). Blots were stained with 4 different anti-*Pf*s48/45 antibodies (45.1-3, 45.5) that recognize different epitopes. Anti-*Py*MSP1 antibody staining was used as a loading control.
(D) Immuno-fluorescence analyses of Pfs48/45 expression in purified schizonts of Pfs48/45@PbMSP1 (1807cl2), and the reference parent *P. berghei* GIMO line (*i.e.* WT; 1596cl1). Fixed parasites were stained with four different rat anti-*Pf*s48/45 mAbs (45.1-3, 45.5) and rabbit anti-*Py*MSP1 antibody followed by secondary conjugated antibodies anti-rabbit IgG Alexa Fluor ® 488 (green) or anti-rat IgG Alexa Fluor ® 594 (red). Nuclei stained with the DNA-specific dye Hoechst 33,342 (H). All pictures were recorded with the same exposure/gain times; anti-rabbit IgG Alexa Fluor ® 488 (green) 0.7 s; anti-rat IgG Alexa Fluor ® 594 (red) 0.6 s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). BF: bright field; M: merged. Scale bar: 2 µm.

Plasmodium gametocytes and gametes [10,11] and contains multiple cysteine-rich domains with multiple disulfide bonds [12–14]. These constitute distinct conformational B cell epitopes that can be recognized by several monoclonal antibodies some of which have transmission-blocking (TB) activity [15]. Pfs48/45 becomes exposed on the surface of gametes once the parasite is taken up in blood meal by a mosquito and here the antigen can be targeted by antibodies and other components of the blood meal [16]. Expression of Pfs48/45 for TB immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein [17,18]. The limited reactivity of recombinant Pfs48/45 with monoclonal antibodies against conformational epitopes of Pfs48/45 has indicated this misfolding [19,20].

2. Results and discussion

In this study the coding sequence of the gene encoding *Pfs48/45* (PF3D7_1346700) was introduced into the redundant *P. berghei p230p* gene locus (PBANKA_0306000) [3,21,22]. The *Pfs48/45* gene was placed under control of 1.3 kb of the promoter region of the schizont-specific *Pb msp1* gene (PBANKA_0831000). This promoter was chosen since *msp1* is one of the highest transcribed genes in developing *Pb* schizonts [23] and the *Pb* schizont stage can be easily produced and purified in large quantities [24]. The transgenic parasite (Pfs48/45@PbMSP1) was generated by the method of GIMO transfection and selection [21]. Using this method transgenes can be rapidly introduced into the *p230p* gene locus in a $GIMO_{pbANKA}$ parent line by replacing the positive-negative selectable marker expression cassette by the transgene expression cassette (**Supplementary M&M** and Fig. 1A). Correct replacement of the selectable marker cassette and insertion of the Pfs48/45 expression cassette in a cloned line of Pfs48/45@PbMSP1 (1807cl2) was confirmed by diagnostic PCR and Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (Fig. 1B). Analysis of the growth rate of transgenic Pfs48/45@PbMSP1 parasites during the cloning period demonstrated normal growth of blood stages, comparable to wild type (WT) *Pb*ANKA parasites (*i.e.* all mice (n = 3) achieved a 0.5–2% parasitemia on day 8, after inoculation with a single infected red blood cell). To obtain transgenic schizonts, parasites were cultured overnight using standard methods to produce and purify *Pb* schizonts (**Supplementary M&M**).

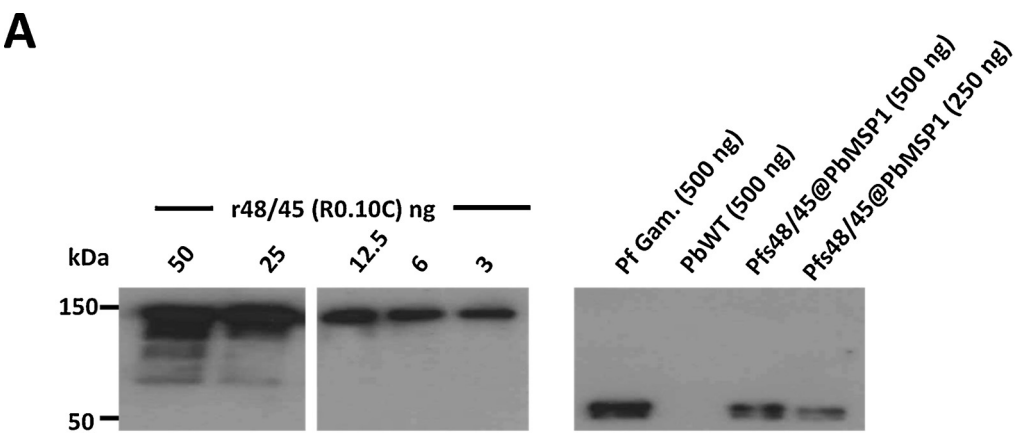
We confirmed expression of Pfs48/45 in the transgenic schizonts by Western and immuno-fluorescence analysis using four anti-Pfs48/45 monoclonal antibodies 85RF45.1 (45.1), 85RF45.2b (45.2b), 85RF45.3 (45.3), and 85RF45.5 (45.5). Three of these (45.1, 45.2b and 45.3) recognize conformational epitopes (epitopes I, IIb and III respectively) in the C terminal region of Pfs48/45 [14].

In Western analysis all the antibodies recognize a protein of the expected size (48 kDa) in

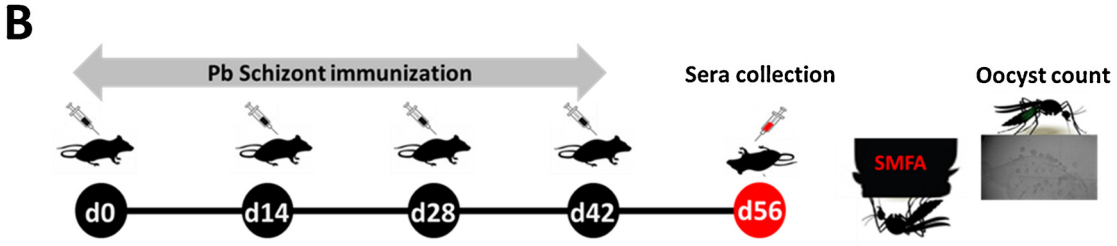
protein extracts from Pfs48/45@PbMSP1 schizonts and WT *Pf* gametocytes but not in extracts from a *Pb* line that does not express Pfs48/45 (*i.e.* $GIMO_{pbANKA}$ line 1596cl1). As a positive control, recombinant protein that contains a fragment of *P. falciparum* Pfs48/45 fused to the GLURP R0 domain (R0.10C) was included and as expected a 150 kDa band was present after probing with monoclonal antibodies 45.1, 45.2b and 45.3 but was not present after probing with 45.5 (Fig. 1C) [25]. We next examined the presence of Pfs48/45 epitopes using the anti-*Pf*s48/45 antibodies by immuno-fluorescence assay (IFA) (Fig. 1D). All antibodies recognized Pfs48/45 produced in the Pfs48/45@PbMSP1 schizonts and did not react with proteins of WT *Pb* schizonts (Fig. 1**C and 1D**). These results demonstrate that transgenic *Pb* schizonts can effectively express full length Pfs48/45, which retains a number of conformational epitopes. Pfs48/45, like MSP1, contains a GPI anchor and is present at the plasma membrane of *Pf* gametocytes/gametes [26]. The immuno-fluorescence analyses indicate that Pfs48/45 was located in the cytoplasm of the transgenic merozoites as the fluorescence signals did not completely overlap with fluorescence signals obtained with anti-MSP1 antibodies, which stain MSP1 at the merozoite plasma membrane in mature schizonts (Fig. 1D). Possible reasons for this observation is that GPI attachment may be different between rodent and human *Plasmodium* parasites, or that the attachment of Pfs48/45 onto *P. berghei* merozoites would require the presence of other *Plasmodium* proteins normally present in gametocytes/gametes.

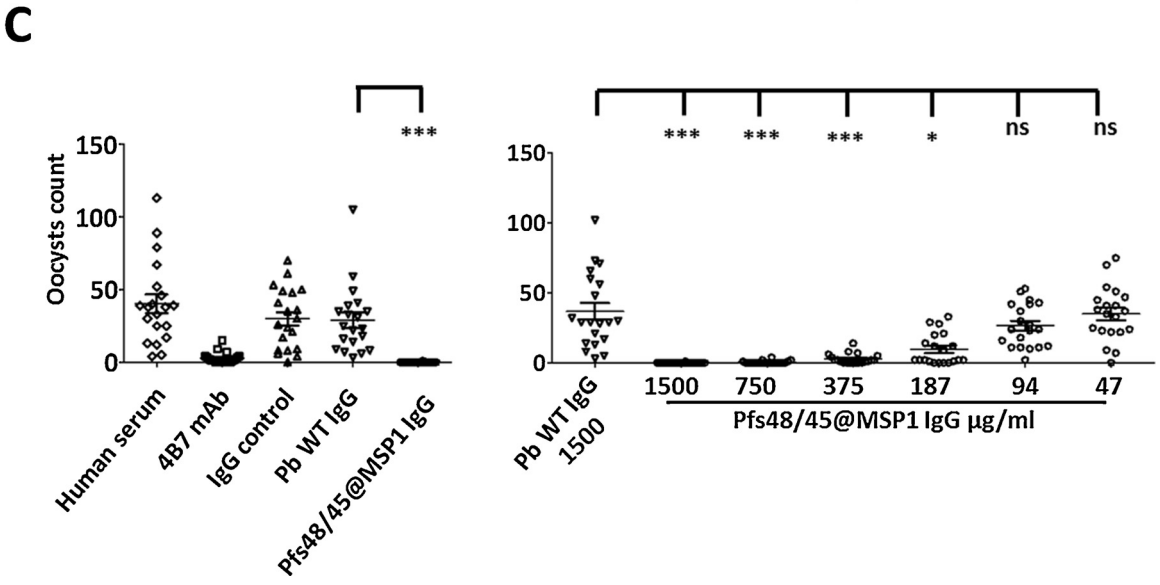
In order to estimate the proportion of Pfs48/45 present in the protein lysates of Pfs48/45@PbMSP1 schizonts we performed a quantitative Western Blot analysis. Densitometry analysis of signals obtained after probing known amounts of Pfs48/45@PbMSP1 protein schizont lysates and a dilution series of recombinant Pfs48/45 with anti-*Pf*s48/45 monoclonal 45.1, revealed that the intensity of the schizont lysate signals corresponds to less than 1 ng of recombinant Pfs48/45 (**Supplementary Fig. S1)**, indicating that is between 0.25 - 0.12% of the total schizont lysate is Pfs48/45 (Fig. 2A).

Next, we examined if Pfs48/45@PbMSP1 transgenic schizont lysate could be used to raise sera that could block *Pf* transmission in mosquitoes, presumably by Pfs48/45 specific antibodies. Two groups of 10 C57BL/6 mice were immunized 4 times (2 week interval) with lysates of 1×10^8 schizonts of either Pfs48/45@PbMSP1 or WT (c15cy1) parasites (Fig. 2B). Purified schizonts were inactivated by three rounds of freezing on dry ice followed by thawing at room temperature before immunization and schizont lysates were injected intravenously. Two



Sample	Total protein (ng)	OD	OD (-*b/g)	Pfs48/45 (ng)	% Pfs48/45
Pf Gam	500	48.87	26.44	3.12	**0.62**
PbWT	500	24.26	1.83	n.a	n.a
Pfs48/45@PbMSP1	500	35.08	12.65	1.25	**0.25**
	250	28.09	5.65	0.29	**0.12**





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weeks after the final immunization, serum was collected from all animals and a serum pool made for each group. Total IgG was isolated from the pooled sera and tested for transmission-reducing activity (TR activity) in standard membrane feeding assays (SMFA) using *Pf* gametocytes (**Supplementary M&M**). *Pf* gametocytes were fed to *A. stephensi* mosquitoes in the presence of IgG obtained from mice immunized with schizonts of either Pfs48/45@PbMSP1 or WT. In the first experiment, IgG (1500 µg/ml) from Pfs48/45@PbMSP1-immunized mice showed 99.8% inhibition in oocyst density ($p = 0.001$) compared to the IgG obtained from WT immunized (Fig. 2C). Next, TR activity was determined in SMFA using a dilution series of the IgG obtained from Pfs48/45@PbMSP1-immunized mice. Significant TR activity with IgG from Pfs48/45@PbMSP1-immunized mice was still observed at a concentration of 187 µg/ml ($p = 0.014$) compared to the control IgG (Fig. 2C). The quantitative Western blot analysis (Fig. 2A) indicated that is between 0.25-0.12% of the total Pfs48/45@PbMSP1 schizont lysate was Pfs48/45 and therefore it is likely that the majority of the IgG from the immunized mice is not directed against Pfs48/45. The

Fig. 2. Quantification of Pfs48/45 protein in Pfs48/45@PbMSP1 schizont lysate and transmission reducing (TR) activity of IgG isolated from mice immunized with Pfs48/45@PbMSP1 schizont lysates.
A. Pfs48/45@PbMSP1 schizont lysates (500 & 250 ng), *P. falciparum* gametocytes (Pf Gam.; 500 ng) and *P. berghei* WT schizont lysate (*Pb*WT; 500 ng) were analyzed in Western blot analysis using anti-*Pfs*48/45 monoclonal 85RF45.1 (1:2000). Densitometry analysis was performed on signals after probing Pfs48/45@PbMSP1 schizont lysate (500 ng) and a dilution series (50, 25, 12.5, 6 and 3 ng) of recombinant Pfs48/45 (r48/45; R0.10C) with antibody 45.1. The Table shows the calculated Pfs48/45 protein content (ng) and the percentage of Pfs48/45 protein in parasite samples; see **Supplementary Fig. S1** for determination of Pfs48/45 in samples. *quantification performed after subtraction of background (b/g) Optical Densitometry (OD) values and **quantitation based on regression curve calculations (see **Supplementary Fig. S1**).
B. Timeline showing the immunization of mice with extracts of Pfs48/45@PbMSP1 and *Pb*WT schizont lysates and collection of sera for isolation of IgG that is tested for TR activity in standard membrane feeding assays (SMFA) of *P. falciparum* gametocytes to *Anopheles stephensi* mosquitoes (see **C**).
C. Left panel: First SMFA with IgGs from mice immunized with purified schizonts of Pfs48/45@PbMSP1 and *Pb*WT. TR activity was determined by the mean number of oocysts 8 days after feeding, and significance of inhibition was determined by the zero-inflated negative binomial model described previously [29]. Right panel: Second SMFA with serially diluted IgGs. IgG from mice immunized with purified schizonts of Pfs48/45@PbMSP1 was titrated resulting in the concentrations shown in the Figure. Significant TR activity was detected until a concentration of 187 μg/ml (*p = 0.014). Significant; *p < 0.05, ***p < 0.001.

failure to induce TR activity of IgG of mice immunized with WT schizont lysate indicates that the small proportion of anti-*Pfs*48/45 antibodies are mediating the TR activity after Pfs48/45@PbMSP1 schizont lysate immunization. The strong TR activity mediated by the total IgG isolated from Pfs48/45@PbMSP1 immunized mice (Fig. 2C), indicates that Pfs48/45 expressed in *P. berghei* can induce antibodies with potent TR activity.

Combined, our proof-of concept studies demonstrate that transgenic *Pb* schizonts can be used as a system to produce a difficult to express HMP protein that is correctly folded and retains conformational epitopes of the native protein. This opens possibilities to use this expression system to evaluate the immunogenicity of other difficult to express antigens or specific domains of these parasites. Studies using sera obtained from mice immunized with *Pf* proteins expressed by transgenic *Pb* parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using blood stage growth inhibition assays (GIA) or sporozoite-antigens using inhibition of sporozoite invasion (ISI) assays [3]. Moreover, the expression in transgenic schizonts of HMP proteins with affinity tags will allow for the purification of these HMP proteins from whole parasite lysate preparations and immunization with purified protein will mean that all of the raised immune response is due to the target antigen and will permit a more detailed analyses of antigen immunogenicity, for example to examine and clone potent inhibitory and cross-reactive B-cells/antibodies after rodent immunization [27]. The creation of transgenic parasites that express antigens from multiple life-cycles that can induce potent immune responses is also of interest to the development of whole organism vaccines [28]. For example, genetically attenuated sporozoite vaccines could be further modified to induce immune responses against multiple life cycle stages by expression in sporozoites and liver stages antigens of blood- or transmission–stages to produce a multi stage-vaccine.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2018.07.009.

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