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 Pf64/45 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 Pf64/45 in the rodent parasite *P. berghei*. Pf64/45 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.

**Keywords:** Plasmodium; Pf64/45; Transgene-expression; Immunization; Transmission blocking; Standard membrane feeding

**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 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.
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
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]. Pf48/45 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 Pf48/45 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 Pf48/45 with monoclonal antibodies against conformational epitopes of Pf48/45 has indicated this misfolding [19,20].

2. Results and discussion

In this study the coding sequence of the gene encoding Pf48/45 (PF3D7_1346700) was introduced into the redundant P. berghei p230p gene locus (PBANKA_0306000) [3,21,22]. The Pf48/45 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 (Pf48/45@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 Pf48/45 expression cassette in a cloned line of Pf48/45@PbMSP1 (1807c12) 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 Pf48/45@PbMSP1 parasites during the cloning period demonstrated normal growth of blood stages, comparable to wild type (WT) PBANKA 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 Pf48/45 in the transgenic schizonts by Western and immuno-fluorescence analysis using four anti-Pf48/45 monoclonal antibodies 8SRF45.1 (45.1), 8SRF45.2b (45.2b), 8SRF45.3 (45.3), and 8SRF45.5 (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 Pf48/45 [14].

In Western analysis all the antibodies recognize a protein of the expected size (48 kDa) in protein extracts from Pf48/45@PbMSP1 schizonts and WT Pf gametocytes but not in extracts from a P. falciparum expressing Pf48/45 (i.e. GIMO_{PbANKA}, line 1596c1). As a positive control, recombinant P. falciparum Pf48/45 fused to GLURP R0 domain (R0.10C) was included (expected molecular size is 150 kDa). Blots were stained with four different anti-Pf48/45 antibodies (45.1-3, 45.5) that recognize different epitopes. Anti-PyMSP1 antibody staining was used as a loading control.

(D) Immuno-fluorescence analyses of Pf48/45 expression in purified schizonts of Pf48/45@PbMSP1 (1807c12), and the reference parent P. berghei GIMO line (i.e. WT; 1596c1). Fixed parasites were stained with four different rat anti-Pf48/45 mAbs (45.1-3, 45.5) and rabbit anti-PyMSP1 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.
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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (ng)</th>
<th>OD</th>
<th>OD (µg/ml)</th>
<th>% Pfs48/45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf Gam</td>
<td>500</td>
<td>48.87</td>
<td>26.44</td>
<td>0.62</td>
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<tr>
<td>PbWT</td>
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<td>24.26</td>
<td>1.83</td>
<td>n.a</td>
</tr>
<tr>
<td>Pfs48/45@PbMSP1</td>
<td>500</td>
<td>35.08</td>
<td>12.65</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>28.09</td>
<td>5.65</td>
<td>0.12</td>
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failure to induce TR activity of IgG of mice immunized with WT schizont lysates indicates that the small proportion of anti-Pfs48/45 antibodies are mediating the TR activity after Pfs48/45@PbMSP1 schizont lysate preparations and immunization with purified schizonts of Pfs48/45@PbMSP1 and PwWT. 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 < 0.001.

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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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