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## Protective Efficacy Induced by Genetically Attenuated Mid-to-Late Liver-Stage Arresting *Plasmodium berghei* $\Delta mmp2$ Parasites

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**Abstract.** Whole parasite immunization strategies employing genetically attenuated parasites (GAP), which arrest during liver-stage development, have been applied successfully for induction of sterile malaria protection in rodents. Recently, we generated a *Plasmodium berghei* GAP-lacking expression of multidrug resistance-associated protein (MRP2) ( $Pb\Delta mmp2$ ) that was capable of partial schizogony in hepatocytes but showed complete growth arrest. Here, we investigated the protective efficacy after intravenous (IV) immunization of BALB/c and C57BL/6J mice with  $Pb\Delta mmp2$  sporozoites. Low-dose immunization using 400  $Pb\Delta mmp2$  sporozoites induced 100% sterile protection in BALB/c mice after IV challenge with 10,000 wild-type sporozoites. In addition, almost full protection (90%) was obtained after three immunizations with 10,000 sporozoites in C57BL/6J mice. Parasite liver loads in nonprotected  $Pb\Delta mmp2$ -challenged C57BL/6J mice were reduced by 86%  $\pm$  5% on average compared with naive control mice. The mid-to-late arresting  $Pb\Delta mmp2$  GAP was equipotent in induction of protective immunity to the early arresting  $Pb\Delta b9\Delta slarp$  GAP. The combined data support a clear basis for further exploration of *Plasmodium falciparum* parasites lacking *mmp2* as a suitable GAP vaccine candidate.

### INTRODUCTION

*Plasmodium berghei* genetically attenuated parasite (GAP) immunization strategies using whole, live parasites have been applied successfully for induction of sterile malaria protection in mice.<sup>1–3</sup> Compared with immunizations using radiation-attenuated parasites<sup>4</sup> or sporozoites under chloroquine cover,<sup>5</sup> GAPs have the advantages of consisting of homogenous populations of attenuated parasites, and circumvent the necessity of prophylactic drug use. This potentiates their use as a vaccine strategy to prevent malaria, which is a severe disease causing more than 400,000 deaths of (mostly) children under 5 years of age, annually.<sup>6</sup> Multiple rodent GAPs have been generated that abort development in the liver at different time -points after invasion of hepatocytes resulting in different levels of protection.<sup>7,8</sup> By comparing two *Plasmodium yoelii* GAPs, it was found that the late arresting *P. yoelii*  $Py\Delta fabb/f$  GAP<sup>9</sup> resulted in enhanced protective immunity as compared with the early arresting  $Py\Delta sap1$ <sup>10,11</sup> highlighting the potential benefit that late-arresting GAPs provide. Next to inducing high-level protection, a critical requirement for a vaccine consisting of GAP is complete attenuation of the sporozoites and, hence, the absence of breakthrough blood-stage infections after immunization.<sup>12</sup> In contrast to the late-arresting  $Py\Delta fabb/f$  GAP, immunization of mice with the orthologous *P. berghei*  $\Delta fabb/f$  GAP resulted in breakthrough blood infection in a number of mice, emphasizing the need to generate fully attenuated GAPs. Recently, early arresting *P. berghei* GAPs have been generated by removing genes encoding sporozoite and liver asparagine-rich protein (SLARP)<sup>13</sup> (ortholog of *P. yoelii* SAP1) or B9.<sup>14</sup> Immunization of mice with mutant sporozoites lacking B9 mutant resulted in low-grade blood-stage breakthrough infections.<sup>14</sup>

However, using a double gene deletion mutant lacking both the *b9* and the *slarp* gene ( $\Delta b9\Delta slarp$ ), a complete arrest was obtained at early liver stage with absence of both nuclear division of parasites and exported protein 1 expression, a marker for the parasitophorous vacuole membrane (PVM).<sup>15</sup> High protective efficacy was obtained after immunization of C57BL/6J mice with an immunization scheme of 3  $\times$  10,000  $Pb\Delta b9\Delta slarp$  sporozoites.<sup>15</sup> More recently, we generated *P. berghei* mutants lacking expression of the adenosine triphosphate-binding cassette transport protein MRP2 ( $\Delta mmp2$ ) that showed a severely compromised phenotype during liver-stage development resulting in complete arrest at mid-to-late liver stage.<sup>16</sup> Although  $Pb\Delta mmp2$  liver-stage parasites show nuclear division and are still present at 52 hours postinfection, breakthrough blood infections were never observed, even after administration of very high doses of sporozoites.<sup>16</sup> Here, our main objective was to address the protective efficacy induced by immunization with  $Pb\Delta mmp2$  sporozoites in two different mouse strains, BALB/c and C57BL/6J. Furthermore, we compared the induced protection to early arresting  $Pb\Delta b9\Delta slarp$  GAP immunizations performed in parallel.

### METHODS

**Experimental mice and *P. berghei* lines.** Animal experiments were performed in female C57BL/6J mice (from 8 weeks; Janvier, Le Genest-Saint-Isle, France) and approved by the Radboud University Experimental Animal Ethical Committee (RUDEC 2014-170). In animal experiments performed at the Leiden University Medical Center, female BALB/c (4–6 weeks old) from Charles River were used, as approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12111). The Dutch Experiments on Animal Act is established under European guidelines on protection of animals used for scientific purposes (EU directive no. 2010/63/EU). Two previously generated *P. berghei* ANKA mutant parasite lines were used to

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immunize mice:  $Pb\Delta b9\Delta slarp$  (1844 c11)<sup>15</sup> and  $Pb\Delta mpr2$  (1025 c12).<sup>16</sup> For challenge of the immunized mice with wild-type (WT) parasites, we used the reference *P. berghei* ANKA line 676m1c11 ( $PbGFP-Luc_{con}$ )<sup>17</sup> that expresses the reporter fusion protein green fluorescent protein (GFP)-luciferase.

**Immunizations and challenge.** Sporozoites of the three above mentioned *P. berghei* lines were produced by feeding infected mice to *Anopheles stephensi* mosquitoes. After 3–4 weeks of parasite development, mosquitoes were hand dissected, and salivary glands were homogenized in Dulbecco's Modified Eagle Medium (+4.5 g/L D-glucose, +NEAA, -L-glutamine, -pyruvate; Life Technologies, Bleiswijk, The Netherlands) supplemented with 1% human albumin (Albuman; Sanquin, Nijmegen, The Netherlands) using a homemade glass grinder. Sporozoites were then quantified using a Bürker-Türk counting chamber using phase contrast microscopy to prepare immunization or challenge solutions. After anesthetizing using isoflurane, female BALB/c and C57BL/6J mice were immunized and challenged by intravenous (IV) injection of 200  $\mu$ L sporozoite solution in the tail vein according to the schedule presented in Figure 1A and B. Thirty-one BALB/c mice were divided into groups of 16 and 15, receiving either  $Pb\Delta b9\Delta slarp$  or  $Pb\Delta mpr2$  immunizations. These groups were subdivided in three groups of five (six in the high  $Pb\Delta b9\Delta slarp$  dose group) mice receiving immunization doses of  $1 \times 400$ ,  $1 \times 800$ , or  $1 \times 1,200$  sporozo-

ites. Immunized mice and five age-matched naive mice were challenged after 21 days by 10,000 wild type  $PbGFP-Luc_{con}$  sporozoites, which express the GFP-luciferase reporter protein. For the C57BL/6J strain, 60 mice were divided into two equal groups, receiving either  $Pb\Delta b9\Delta slarp$  or  $Pb\Delta mpr2$  immunizations. These groups were subdivided in three groups of 10 mice receiving immunization doses of  $3 \times 10,000$ ,  $3 \times 1,000$ , or  $3 \times 200$  sporozoites with a 1-week interval between each immunization. Immunized mice and three age-matched naive mice were challenged after 14 days by 10,000 wild type  $PbGFP-Luc_{con}$  sporozoites. Subsequently, liver loads were determined by measuring luciferase activity by bioluminescence imaging (see section In vivo liver-stage bioluminescence imaging) 48 hours after injection of sporozoites, and tail blood was analyzed by Giemsa-stained films for blood-stage positivity from 5 to 21 days post challenge.

**In vivo liver-stage bioluminescence imaging.** Forty-eight hours after challenge with  $PbGFP-Luc_{con}$  parasites, the parasite liver load was determined in abdomen-shaved mice by measuring luciferase activity by real-time in vivo imaging using the IVIS Lumina II system (Caliper Life Sciences, Hopkinton, MA) as described previously<sup>18,19</sup> with minor adaptations. In brief, BALB/c mice were injected subcutaneously in the neck with 60  $\mu$ L (C57BL/6J: 200  $\mu$ L) D-luciferin (120 mg/kg [C57BL/6J: 150 mg/kg]; PerkinElmer, Groningen, The Netherlands) dissolved in PBS (Life Technologies,

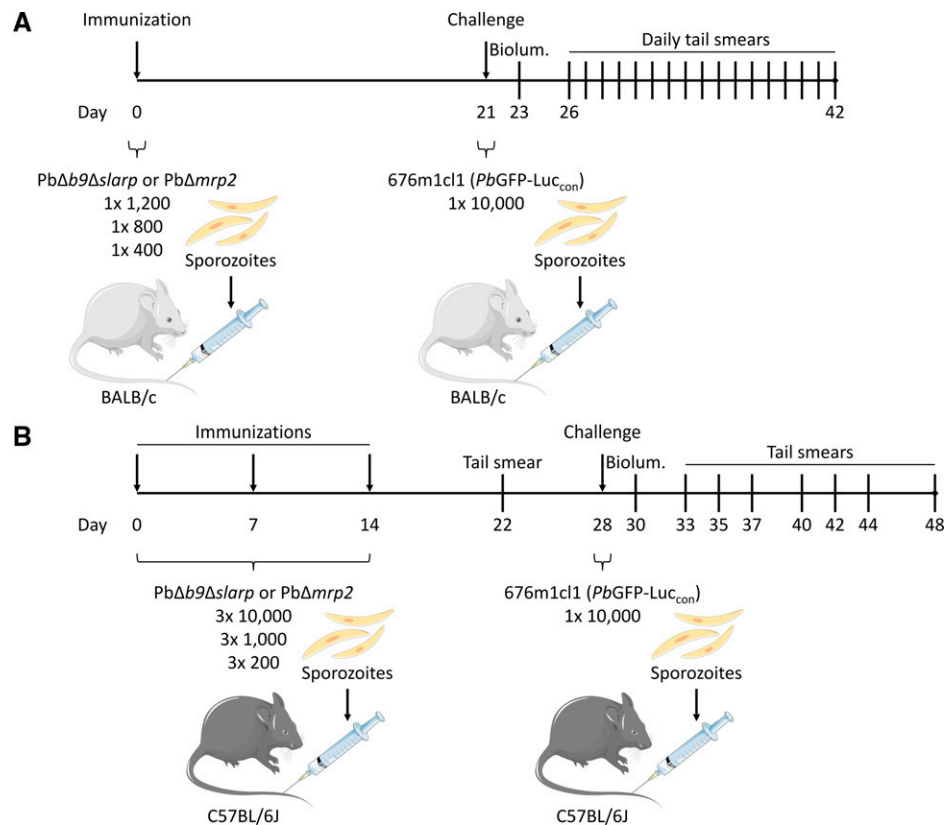


FIGURE 1. Schematic setup of mouse immunizations, challenge, and follow-up. (A) Thirty-one BALB/c mice were separated in groups of five (and one group of six), and (B) 60 C57BL/6J mice were separated in groups of 10, receiving either  $Pb\Delta b9\Delta slarp$  or  $Pb\Delta mpr2$  genetically attenuated parasites immunization doses (BALB/c high:  $1 \times 1,200$ , medium:  $1 \times 800$ , low:  $1 \times 400$  sporozoites; C57BL/6J high:  $3 \times 10,000$ , medium:  $3 \times 1,000$ , low:  $3 \times 200$  sporozoites) on the indicated days. All immunized mice and five (BALB/c) or three (C57BL/6J) naive controls were challenged with 10,000 WT sporozoites 2 weeks succeeding the last immunization. Liver-stage parasite development was monitored by bioluminescence (Biolum.) 2 days after challenge. Finally, tail smears were prepared to screen for malaria blood-stage positivity from daum 5 to 20 (BALB/c) or 21 (C57BL/6J) (with varying intervals) after challenge.

Gibco) 3 minutes (C57BL/6J: 4 minutes) before imaging. After anesthetization by isoflurane, mice were imaged using a field of view of 12.5 cm, medium binning factor and an exposure time of 25–120 seconds (C57BL/6J: 300 sec.). Luminescence intensity was quantified and visualized using the Living Image 4.5 software (Caliper Life Sciences), shown as rainbow plots with automatic scale bars per measurement and represented as radiance units (photons/second/cm<sup>2</sup>/steradian). The region of interest (ROI) was set to include the liver, quantified the total flux of photons (photons/second) per mouse and per dose group and is represented as the mean  $\pm$  standard error of the mean (SEM).

**Statistical analysis.** The different immunization groups were scored on a binominal distribution (protected, blood smear negative 20 or 21 days post challenge, or nonprotected, blood smear positive) and significant differences between these groups were determined using the Fisher's exact test. The ROIs of these immunization groups and naive controls, calculated by the Living Image 4.5 software, were statistically compared with a one-way analysis of variance using the Tukey's posttest, and the overall percent reduction in C57BL/6J liver load was depicted as mean (%)  $\pm$  SEM. All tests were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).

## RESULTS AND DISCUSSION

To study the protective efficacy induced by immunization of mice with sporozoites of the mid-to-late arresting *PbΔmrp2* GAP,<sup>16</sup> we followed an immunization scheme employing single doses of 1,200 (high), 800 (medium), or 400 (low) sporozoites in BALB/c mice followed by a challenge after 3 weeks (Figure 1A). Immunized mice were challenged with 10,000 GFP-luciferase expressing WT sporozoites showing sterile protection in all mice immunized with the low (5/5) and high dose (5/5), whereas three of five mice were protected with the medium dose (Supplemental Figure 1). Immunization of BALB/c mice with sporozoites of the early arrester *PbΔb9Δslarp* induced a similar level of protection (Supplemental Figure 1), as all mice immunized with the low (5/5) and high (6/6) doses were protected against a subsequent challenge, whereas four of five mice were protected with the medium dose. As expected, challenging naive BALB/c mice with 10,000 WT sporozoites resulted in all five mice becoming liver- and blood-stage positive (Supplemental Figure 1). As summarized in Table 1, there was no significant difference in protective efficacy induced by immunization of BALB/c mice with *PbΔb9Δslarp* or *PbΔmrp2* sporozoites.

Next, we immunized C57BL/6J mice, known as a more stringent model for inducing protective immunity against *P. berghei*,<sup>20</sup> according to the scheme shown in Figure 1B. Here, three weekly immunizations of 10,000 (high), 1,000 (medium), or 200 (low) were followed by challenge with 10,000 GFP-luciferase expressing WT sporozoites 2 weeks after the last immunization. Eight days after the last immunization, before challenge, we analyzed tail blood for the presence of possible breakthrough blood infections. Neither immunization with *PbΔmrp2* nor *PbΔb9Δslarp* GAP parasites caused breakthrough infections, confirming previously reported complete attenuation of these parasites.<sup>15,16</sup> Nine of 10 mice immunized with the high dose of *PbΔmrp2* were completely protected against a WT challenge (Supplemental

TABLE 1

Protection in BALB/c or C57BL/6J mice immunized with *PbΔmrp2* or *PbΔb9Δslarp* sporozoites

Mouse strain	Parasite line	Immunization dose(s) (sporozoites)	No. protected/ no. challenged
BALB/c	<i>PbΔmrp2</i>	1 $\times$ 1,200	5/5*
		1 $\times$ 800	3/5†
		1 $\times$ 400	5/5
	<i>PbΔb9Δslarp</i>	1 $\times$ 1,200	6/6‡
		1 $\times$ 800	4/5
		1 $\times$ 400	5/5
	Naive control	–	0/5
C57BL/6	<i>PbΔmrp2</i>	3 $\times$ 10,000	9/10§
		3 $\times$ 1,000	0/10
		3 $\times$ 200	0/10
	<i>PbΔb9Δslarp</i>	3 $\times$ 10,000	10/10¶**
		3 $\times$ 1,000	3/10
		3 $\times$ 200	0/10
		Naive control	–

\**P* = 0.44 (Fisher's exact test) for *PbΔmrp2* 1  $\times$  1,200 vs. 1  $\times$  800.

†*P* = 1 (Fisher's exact test) for *PbΔmrp2* 1  $\times$  800 vs. *PbΔb9Δslarp* 1  $\times$  800.

‡*P* = 0.45 (Fisher's exact test) for *PbΔb9Δslarp* 1  $\times$  1,200 vs. 1  $\times$  800.

§*P* = 0.0001 (Fisher's exact test) for *PbΔmrp2* 3  $\times$  10,000 vs. 3  $\times$  1,000 or 3  $\times$  200.

||*P* = 0.21 (Fisher's exact test) for *PbΔmrp2* 3  $\times$  1,000 vs. *PbΔb9Δslarp* 3  $\times$  1,000.

¶*P* = 0.0031 (Fisher's exact test) for *PbΔb9Δslarp* 3  $\times$  10,000 vs. 3  $\times$  1,000.

\*\**P* < 0.0001 (Fisher's exact test) for *PbΔb9Δslarp* 3  $\times$  10,000 vs. 3  $\times$  200.

Figure 1), which was clearly superior to the medium- and low-dose immunization groups where none of the mice were protected (*P* < 0.0001) (Table 1). Previously, it had been shown that after three weekly immunizations of 10,000 sporozoites of the early arresting *PbΔb9Δslarp*, sterile protection was obtained against a WT challenge.<sup>15</sup> Here, three *PbΔb9Δslarp* GAP immunizations (Supplemental Figure 1) also resulted in sterile protection when using the high dose. Protection declined dose-dependently, resulting in 3/10 protected mice in the medium dose group (versus 6/10<sup>15</sup>) and 0/10 protected mice in the low-dose group. As summarized in Table 1, the high dose provided significant better protection than the medium- (*P* = 0.0031) and low-dose groups (*P* < 0.0001). As expected, all three naive mice became liver- and blood-stage positive after challenge with 10,000 WT sporozoites (Supplemental Figure 1). Although most C57BL/6J mice in the medium- and low-dose groups became blood smear positive after immunization using both GAPs, parasite liver loads in all immunized groups were strongly reduced (*P* < 0.0001) compared with control mice (Figure 2). The nonprotected mice had an average reduction in liver load of 86%  $\pm$  5% and 90%  $\pm$  2% upon immunization with *PbΔmrp2* and *PbΔb9Δslarp*, respectively, highlighting the immune potency of both GAPs.

It has been suggested that GAPs arresting late during liver stage provide superior protection presumably due to expression of additional parasite proteins during late liver-stage development and formation of daughter merozoites by schizogony.<sup>7,8,11</sup> *PbΔmrp2* parasites enter schizogony as shown by nuclear division, and develop to an average size of ~50% compared with fully developed liver-stage schizonts of WT parasites.<sup>16</sup> In contrast, *PbΔb9Δslarp* abort development soon after invasion of the hepatocytes. These parasites remain very small, have a compromised PVM, and show no features of schizogony.<sup>14</sup> Apparently, this difference in degree of development between the two GAPs is not sufficient to translate into a difference in induction of protective immune responses.

*PbΔmrp2* parasites do produce a PVM in contrast to *PbΔb9Δslarp* parasites. As the PVM is considered to function

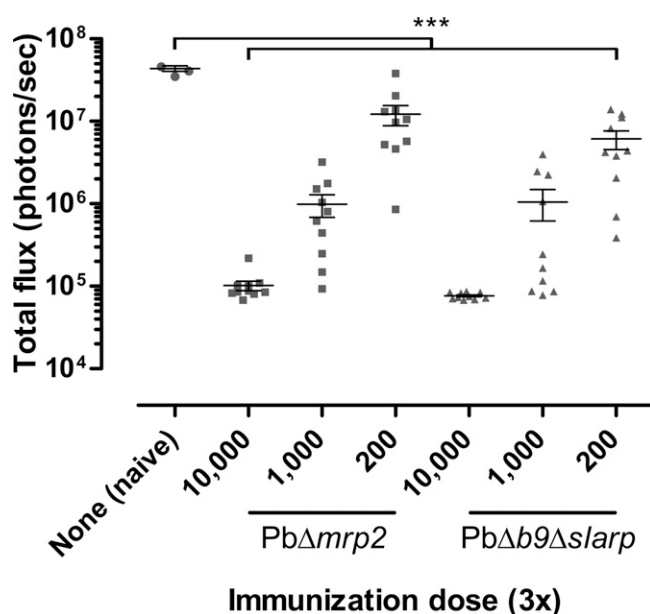


FIGURE 2. Parasite liver-stage development after challenge. The parasite liver load of entire C57BL/6J livers was quantified by measuring the total flux of photons within each region of interest (red circles in Supplemental Figure 1) and is depicted as mean (photons/second)  $\pm$  standard error of the mean. \*\*\* $P < 0.0001$  as determined with a one-way analysis of variance using the Tukey's posttest.

as decoy for host defense mechanisms including autophagy,<sup>21</sup> it is possible that  $Pb\Delta b9\Delta slarp$  parasites might be more prone to intracellular degradation, resulting in relatively effective antigen presentation. This might explain why  $Pb\Delta b9\Delta slarp$  parasites, despite expressing fewer antigens, induce comparable protective immune responses as  $Pb\Delta mrp2$  parasites. While the late arresting *P. yoelii*  $\Delta fabb/f$  GAP<sup>9</sup> forms a PVM and lacks expression of MSP1, a similar phenotype as compared with the mid-to-late arresting *P. berghei*  $\Delta mrp2$  GAP,<sup>16</sup> the  $Py\Delta fabb/f$  GAP induces stronger protective immune responses compared with the early arresting  $Py\Delta sap1$  GAP.<sup>11</sup> Whether this is caused by parasite species (*P. yoelii* versus *P. berghei*) or difference in antigen presentation between the  $Py\Delta fabb/f$  GAP and the  $Pb\Delta mrp2$  GAP remains to be investigated in comparable immunization/challenge protocols.

While potency is important, it is absolutely essential that GAP immunizations will be safe and do not result in breakthrough blood infections. Several *P. berghei* GAPs do generate protective immunity, but most produce breakthrough blood infections at high doses.<sup>12</sup> The only safe *P. berghei* GAPs based on single gene deletions are *slarp*<sup>10,13,15</sup> or *mrp2* gene knock-out parasites.<sup>16</sup> As we have reported here, both studied GAPs are capable of generating highly potent protective immunity in different strains of mice. To further reduce the risks for breakthrough infections, it has been recommended to delete multiple genes targeting vital but independent functions during liver-stage development.<sup>22</sup> Currently, two multiple gene deletion GAPs have been generated in *P. falciparum*, and in both lines the *slarp* gene has been removed.<sup>15,23</sup> These early arresting *P. falciparum* GAPs have undergone preclinical evaluation for safety and are now being advanced into Phase 1 clinical trials.<sup>15,23,24</sup> Combining  $Pf\Delta slarp$  with  $Pf\Delta mrp2$ , active in unrelated pathways, would remove two genes both of which independently result

in complete liver-stage arrest (increases the safety profile of the GAP). Moreover, as  $Pb\Delta mrp2$  is the first mid-to-late arresting GAP that shows both protective efficacy and no evidence for breakthrough blood infections, we believe that *P. falciparum* GAPs lacking the *mrp2* gene merit investigation to further develop a GAP vaccine that is suitable and potent for human use.

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