



## The use of transgenic parasites in malaria vaccine research

Ahmad Syibli Othman, Catherin Marin-Mogollon, Ahmed M. Salman, Blandine M. Franke-Fayard, Chris J. Janse & Shahid M. Khan

**To cite this article:** Ahmad Syibli Othman, Catherin Marin-Mogollon, Ahmed M. Salman, Blandine M. Franke-Fayard, Chris J. Janse & Shahid M. Khan (2017) The use of transgenic parasites in malaria vaccine research, *Expert Review of Vaccines*, 16:7, 685-697, DOI: [10.1080/14760584.2017.1333426](https://doi.org/10.1080/14760584.2017.1333426)

**To link to this article:** <https://doi.org/10.1080/14760584.2017.1333426>



© 2017 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



Published online: 30 May 2017.



Submit your article to this journal [↗](#)



Article views: 3724



View related articles [↗](#)



View Crossmark data [↗](#)




Citing articles: 10 View citing articles [↗](#)

REVIEW



## The use of transgenic parasites in malaria vaccine research

Ahmad Syibli Othman<sup>a,b\*</sup>, Catherin Marin-Mogollon<sup>a\*</sup>, Ahmed M. Salman<sup>c</sup>, Blandine M. Franke-Fayard<sup>a</sup>, Chris J. Janse<sup>a</sup> and Shahid M. Khan <sup>a</sup>

<sup>a</sup>Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, the Netherlands; <sup>b</sup>Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia; <sup>c</sup>The Jenner Institute, University of Oxford, Oxford, UK

### ABSTRACT

**Introduction:** Transgenic malaria parasites expressing foreign genes, for example fluorescent and luminescent proteins, are used extensively to interrogate parasite biology and host-parasite interactions associated with malaria pathology. Increasingly transgenic parasites are also exploited to advance malaria vaccine development.

**Areas covered:** We review how transgenic malaria parasites are used, *in vitro* and *in vivo*, to determine protective efficacy of different antigens and vaccination strategies and to determine immunological correlates of protection. We describe how chimeric rodent parasites expressing *P. falciparum* or *P. vivax* antigens are being used to directly evaluate and rank order human malaria vaccines before their advancement to clinical testing. In addition, we describe how transgenic human and rodent parasites are used to develop and evaluate live (genetically) attenuated vaccines.

**Expert commentary:** Transgenic rodent and human malaria parasites are being used to both identify vaccine candidate antigens and to evaluate both sub-unit and whole organism vaccines before they are advanced into clinical testing. Transgenic parasites combined with *in vivo* pre-clinical testing models (e.g. mice) are used to evaluate vaccine safety, potency and the durability of protection as well as to uncover critical protective immune responses and to refine vaccination strategies.

### ARTICLE HISTORY

Received 2 February 2017  
Accepted 18 May 2017

### KEYWORDS

*Plasmodium*; malaria; transgenic; vaccine; reporter; GAP; chimeric

### 1. Introduction

In the mid-nineties, genetic modification to create permanent modifications in malaria parasite genomes was first described in the rodent malaria parasite (RMP) *Plasmodium berghei* [1]. This technology was extended to other *Plasmodium* species, including the human malaria parasite *P. falciparum*, and was initially used for loss-of-function analyses to uncover the function of *Plasmodium* genes, including genes encoding potential vaccine candidate antigens (reviewed in [2,3]). In addition to gene disruption and gene mutation, methodologies have been developed to create malaria parasites that express ‘foreign’ genes from other organisms, so-called transgenic parasites. Among the first transgene mutants were RMP that expressed fluorescent and luminescent reporter proteins. These parasites have been used to visualize and analyze parasite growth and development *in vitro* and *in vivo* and have been valuable tools to analyze cellular and molecular features of malaria parasite biology (reviewed in [4–7]). Transgenic rodent parasites have also been used to provide mechanistic insights into host–parasite interactions that regulate host (immune) responses to infection or those that mediate malarial pathology [8–13].

Transgenic parasites expressing fluorescent or luminescent reporter proteins have been created in rodent malaria species, the human parasite *P. falciparum* and the primate parasite *P. cynomolgi*. These parasites have been exploited in screening assays to measure (inhibition of) parasite growth at different

points of the parasite life cycle. Fluorescent and luminescent *P. falciparum* parasites have been used *in vitro* to examine the effect of drugs and other inhibitors on blood-stage growth and on gametocytes [6,14–17] and fluorescent *P. cynomolgi* parasites have been generated to screen for compounds that target the hypnozoite stage in the liver [18]. Transgenic fluorescent and luminescent rodent parasites have been used in *in vitro* screening assays to test inhibitors that target parasite development in the blood and liver [6,19–22].

In addition to measuring growth inhibition *in vitro*, transgenic rodent parasites have been used to examine the impact of drug or vaccine interventions *in vivo*, where inhibition of parasite development is measured as the reduction of reporter signal (mostly luminescent) in organs of the treated (compared to unimmunized/untreated) rodent host [6,17,19,22,23]. As the life cycle and basic biology of rodent and human *Plasmodium* parasites are very similar and since the vast majority of genes within their genomes are conserved [24], transgenic rodent parasites are frequently used to evaluate protective immunity against candidate *Plasmodium* antigens *in vivo* and are used to assess different vaccine delivery platforms and vaccination regimens. Several of these studies have been conducted in different inbred mice strains that exhibit different, often polarized, immunological responses to infection. Transgenic rodent parasites have been used in preclinical studies to examine protective immune responses to pre-erythrocytic (sporozoite and liver stage) vaccines (see Section 2).

**CONTACT** Shahid M. Khan  [S.M.Khan@lumc.nl](mailto:S.M.Khan@lumc.nl)  Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, the Netherlands

\*These authors contributed equally to this work.

More recently, transgenic rodent parasites have been generated that express proteins of the human *Plasmodium* species *P. falciparum* and *P. vivax*. These so-called 'chimeric' parasites have been used to evaluate the (*in vivo*) action of drugs against human *Plasmodium* protein targets [25,26], to study malaria pathology during pregnancy *in vivo* [27], and to evaluate the protective efficacy of vaccines that target human *Plasmodium* antigens (reviewed in [28–30] and see Table 1). In these vaccine studies, mice are immunized with *P. falciparum* or *P. vivax* antigens and subsequently challenged with chimeric rodent parasites expressing the cognate *P. falciparum* or *P. vivax* antigens. Such chimeric parasites permit an *in vivo* immunological evaluation of novel target *Plasmodium* antigens and vaccination strategies and can indicate the magnitude and type of protective immune response induced. This knowledge can be used to down-select from candidate antigens under consideration before proceeding to clinical studies [31].

Last, genetic modification of rodent and human malaria parasites has also been used to generate parasites that arrest in the liver. These parasites can provoke strong protective immune responses in the host and are therefore being evaluated as live, attenuated vaccines [48–50]. Many gene-deletion rodent parasites have been tested in rodents for growth arrest in the liver and for their capacity to induce potent protective immune responses. These so-called genetically attenuated parasites (GAPs) have been created in transgenic reporter lines, which simplify the *in vivo* evaluation of both their safety and protective efficacy. In order to generate completely safe GAP vaccines, GAPs must be generated that completely arrests in the liver. Consequently, multiple gene deletions in the same GAP are considered necessary, each governing independent but essential processes during liver-stage development. Therefore, in order to generate and test a *P. falciparum* GAP in human test subjects, large-scale screening of single and multiple gene-deletion mutants in rodents is necessary to identify suitable genes for deletion in *P. falciparum*.

In this review, we describe the use of transgenic malaria parasites and their use as preclinical evaluation tools to measure vaccine efficacy and immune responses after vaccination. We describe: (i) transgenic rodent and human parasites that express reporter proteins that have been used to evaluate immunogenicity of vaccine antigens and vaccine efficacy; (ii) the use of transgenic chimeric rodent parasites, expressing antigens of *P. falciparum* or *P. vivax*, to compare immunogenicity of vaccines and vaccine strategies; and (iii) the use of transgenic parasites to identify and evaluate GAP vaccines and to examine immunological correlates of protection after vaccination *in vivo*.

## 2. Transgenic parasites expressing reporter proteins

Transgenic rodent and human malaria parasites that express fluorescent and luminescent reporter proteins have been used in screening assays to efficiently and rapidly measure inhibition of parasite growth at different points of the parasite life cycle [6,17,22,51]. These assays have been used to identify and characterize anti-*Plasmodium* drugs and small-molecule inhibitors, as well as vaccines targeting parasite development at different points of the life cycle. Transgenic parasites expressing fluorescent or luminescent proteins have been generated in three

RMP, *P. berghei*, *P. yoelii*, and *P. chabaudi*. For *P. berghei* and *P. yoelii*, a number of transgenic lines exist that express different reporter proteins such as GFP, mCherry, or luciferase (or fusions thereof). Most of these lines express these proteins under control of *Plasmodium* promoters of constitutively expressed *Plasmodium* genes (often housekeeping genes), which creates parasites that can be visualized and quantified throughout the complete life cycle (Figure 1(a)). Frequently used promoter regions of RMP genes include *elongation factor 1-alpha* (*eef1a*), *dihydrofolatereductase-thymidylate synthase* (*dhfr-ts*), or *heat-shock protein 70* (*hsp70*). Information on all published RMP transgenic lines can be found in the RMgm database of genetically modified rodent parasites ([www.pberghei.eu](http://www.pberghei.eu)).

Different assays have been developed to quantify parasite growth using reporter parasites. To test the effect of inhibitors on blood- and liver-stage growth, simple and rapid assays exist that can quantify parasite numbers in blood samples, infected hepatocytes, or in other tissues. For example, flow cytometric-based assays counting GFP (or mCherry)-positive parasite-infected red blood cells [20,52,53] (Figure 1(a)) or quantification of luminescence signals to determine parasite numbers or parasite loads in blood, liver, or other organs [19,21] (Figure 1(b)). Infecting mice with defined numbers of luciferase expressing parasites and subsequent quantifying parasite loads (luminescence signal) in the liver by real-time imaging of live mice is frequently used to establish the *in vivo* effect of either inhibitors and vaccines on liver-stage development [6,17,22,23]. Bioluminescence imaging is simple to execute and can be used to monitor the course of an infection without sacrificing the animal [19] (Figure 1(b)). This reduces the number of animals required for experimentation because multiple measurements can be made in the same animal over time that also minimizes the effects of biological variation. In addition, since imaged mice do not have to be sacrificed, additional features of parasite development can be established, for example, characteristics of the ensuing blood-stage infection such as the prepatent period, i.e. the duration between sporozoite infection and a microscopically detectable blood infection.

As well as transgenic RMP lines, reporter parasites have been generated for the human parasite *P. falciparum*. Transgenic *P. falciparum* parasites expressing fluorescent or luminescent proteins have been used to quantify blood-stage growth *in vitro* in standard growth inhibition assays (see below), to quantify parasite development in the mosquito in standard membrane feeding assays to measure transmission-blocking (TB) activity and in high-throughput screening of TB compounds against *P. falciparum* gametocytes (see below). For the TB assays against mosquito stages and gametocytes, transgenic *P. falciparum* (NF54 strain) parasite lines have been generated that express a GFP-luciferase fusion protein under control of the strong constitutive *hsp70* [54] or the gametocyte-specific *pfs16* promoter [55].

In addition to RMP expressing fluorescent and luminescent proteins for vaccine studies, multiple transgenic RMP lines expressing the model antigen ovalbumin (OVA) as an immunological reporter have been created to study immune responses after vaccination. Transgenic *Plasmodium* parasites expressing OVA have been exploited to examine parasite-specific CD8<sup>+</sup>T cell responses during both blood and liver

Table 1. Transgenic rodent and human malaria parasites used in malaria vaccine research.

Transgenic rodent malaria parasites (RMP) expressing reporter proteins		Remarks	RMgm ID	Ref
Reporter				
Fluorescent proteins (e.g. GFP, mCherry)	A number of RMP expressing different fluorescent reporter proteins have been used to quantify parasite growth of different life cycle stages and to analyze interactions between infected cells and immune factors (see Section 2 for references) <sup>a</sup>			
Luminescent proteins (e.g. luciferase)	A number of different luminescent reporter RMP have been generated that have been used to quantify parasite growth of different life cycle stages, both <i>in vitro</i> and <i>in vivo</i> (see Sections 2 and 4 for references) <sup>a</sup>			
Ovalbumin (OVA)	Several OVA-expressing RMP have been used to analyze interactions of the parasite with the host immune system (see Sections 2 and 4 for references) <sup>a</sup>			
<b>Transgenic <i>P. falciparum</i> parasites expressing reporter proteins</b>				
Reporter	Remarks			
GFP	GFP-expressing <i>P. falciparum</i> parasites have been used in GAI assays [16]			
Luciferase	Luminescent <i>P. falciparum</i> parasites have been used to quantify inhibition of oocyst production in SMFA assays [14]			
<b>Chimeric rodent malaria parasites expressing human <i>Plasmodium</i><sup>b</sup> proteins</b>				
Protein product	<i>P. falciparum</i> / <i>P. vivax</i> gene	Remarks	RMgm ID	Ref
PLSA-1	PF3D7_1036400	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1314	[31]
PLSA-3	PF3D7_0220000	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1315	[31]
PCeITOS	PF3D7_1216600	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1310	[31]
PUIS3 (ETRAMP13)	PF3D7_1302200	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1311	[31]
PLSAP1	PF3D7_1201300	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1308	[31]
PLSAP2	PF3D7_0202100	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1312	[31]
PFETRAMP5	PF3D7_0532100	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1309	[31]
PFalstatin	PF3D7_0911900	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1313	[31]
PCSP	PF3D7_0304600	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1316	[31]
PTRAP	PF3D7_1335900	<b>Additional copy:</b> <i>Pf</i> (NF54) gene under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#1317	[31]
PUIS3/	PF3D7_1302200	<b>(2) Additional copies:</b> <i>Pf</i> (NF54) genes under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	#4076	[32]
PTRAP	PF3D7_1335900	<b>(2) Additional copies:</b> <i>Pf</i> (NF54) genes under the control of <i>Pbuis4</i> promoter; in <i>Pb</i> (ANKA)	[33]	
PCSP/	PF3D7_0304600	<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>csp</i> replaced by <i>Pf</i> (Wellcome strain) <i>csp</i> , full-length <i>Pbcs</i> p promoter & 302bp <i>Pbcs</i> p3'UTR.	#69	[34]
PCSP	PF3D7_0304600	<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>csp</i> replaced by <i>Pf</i> (NF54) <i>csp</i> under control of endogenous <i>Pbcs</i> p promoter and 3'UTR; No drug selectable marker.	#4110	
PCSP	PF3D7_0304600	Normal sporozoite production and infectivity		
PCSP	PF3D7_0304600	<b>Replacement copy:</b> <i>Pf</i> (17XNL) <i>csp</i> replaced with <i>Pf</i> (3D7) <i>csp</i> . Human DHFR selectable marker. <i>Pb</i> hsp70 3'UTR	#1442	[35]
PCSP	PF3D7_0304600	Normal sporozoite production and infectivity		
PTRAP	PF3D7_1335900	<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>trap</i> replaced by <i>Pf</i> (NF54) <i>trap</i> under control of endogenous <i>Pb</i> trap promoter and 3'UTR; No drug selectable marker	#4112	
PvTRAP	PVP01_1218700	Normal sporozoite production and infectivity		
Pv25	PVX_111175	<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>trap</i> replaced with <i>Pv</i> (Sal-1) <i>trap</i> . No selectable marker.	#1103	[36]
PZ5	PF3D7_1031000	Normal sporozoite production and infectivity		
PCeITOS	PF3D7_1216600	<b>Replacement copy:</b> <i>Pb</i> 25 and <i>Pb</i> 28 replaced with <i>Pv</i> 25; in <i>Pb</i> (ANKA)	#222	[37]
		<b>Replacement copy:</b> <i>Pb</i> 25 and <i>Pb</i> 28 replaced with <i>Pf</i> 25; in <i>Pb</i> (ANKA)	#273	[38]
		<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>celts</i> replaced by <i>Pf</i> (NF54) <i>celts</i> under control of endogenous <i>Pb</i> celts promoter and 3'UTR; No drug selectable marker	#4066	[39]
PvCSP (VK210)	PVX_119355	Normal sporozoite production and infectivity		
		<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>csp</i> replaced by PvVK210 <i>csp</i> under control of endogenous <i>Pbcs</i> p promoter and 3'UTR; No drug selectable marker	[40]	
PvCSP (VK247)	PVX_119355	Normal sporozoite production and infectivity		
		<b>Replacement copy:</b> <i>Pb</i> (ANKA) <i>csp</i> replaced by Pv VK247 <i>csp</i> under control of endogenous <i>Pbcs</i> p promoter and 3'UTR; No drug selectable marker	[40]	
		Normal sporozoite production and infectivity		

(Continued)

Table 1. (Continued).

Transgenic rodent malaria parasites (RMP) expressing reporter proteins		Replacement copy: <i>Pb</i> (ANKA) <i>celt</i> os replaced by <i>Pvcelt</i> os under control of endogenous <i>Pbcelt</i> os promoter and 3'UTR; No drug selectable marker	#4111	[41]
<i>Pv</i> CeTOS	PVX_123510	Normal sporozoite production and infectivity		
Rodent malaria parasites expressing HMP-RMP fusion proteins <sup>b</sup>				
CSP	PF3D7_0304600	The repeat region of <i>Pb</i> (NK65) <i>csp</i> is replaced with the <i>Pf</i> (7G8) <i>csp</i> repeat region.	#76	[42]
MSP1	PF3D7_0930300	The <i>Pb</i> (ANKA) <i>msp-1_19</i> C-terminal replaced with the <i>Pf</i> (D10) <i>msp-1_19</i> C-terminal	#201	[43]
MSP1	PF3D7_0930300	The <i>Pb</i> (ANKA) <i>msp-119</i> C-terminal replaced with the <i>Pf</i> (FCC1/HN) <i>msp-1_19</i> C-terminal	#330	[44]
CSP (VK210)	PVX_119355	The repeat region of <i>Pb</i> (ANKA) <i>csp</i> is replaced with the <i>Pv</i> (210) <i>csp</i> repeat region	#906	[45]
CSP (VK210)	PVX_119355	The repeat region of <i>Pb</i> (ANKA) <i>csp</i> is replaced with (part of) <i>Pv</i> (210) <i>csp</i> gene	#1104	[46]
CSP (VK247)	PVX_119355	The majority of <i>Pb</i> (ANKA) <i>csp</i> gene is replaced with <i>Pv</i> (247) <i>csp</i> ; the fusion gene retains <i>Pb</i> signal sequence (1–20aa) and <i>Pb</i> GPI anchor	#1443	[47]
P25	PVX_111175	The <i>Pb</i> (ANKA)25 and 28 genes replaced with a fusion of <i>Pv</i> 25 and <i>Pb</i> 25	#223	[37]
VAR2CSA	PF3D7_1200600	A synthetic <i>Pf</i> 3D7 DBLIX-6ε gene ( <i>var2csa</i> ) fused to <i>Pb</i> (ANKA) <i>farm-a</i>	#1436	[27]
Genetically attenuated parasites (GAPs)				

See Section 4 for details (and references) of transgenic parasites used to generate and test GAP vaccines

<sup>a</sup>For full list of transgenic reporter parasites generated in RMP, see the **RMgm Database** [www.pberghei.eu](http://www.pberghei.eu)

<sup>b</sup>*Plasmodium* species abbreviations: *Pf* – *P. falciparum*; *Pv* – *P. vivax*; *Pb* – *P. berghei*; *Py* – *P. yoelii*.

infections [9,10,56–58]. For example, intravital two-photon microscopy of livers of mice infected with *P. berghei* parasites that express OVA and GFP in their cytoplasm showed that transferred OVA-specific CD8 + T cells recognize and forms clusters around infected hepatocytes, leading to the elimination of the intra-hepatic parasites [56]. In addition, analysis of liver stage parasites expressing OVA, either in their cytoplasm or exported to the parasitophorous vacuole membrane, in conjunction with OVA-specific CD8<sup>+</sup>- and CD4<sup>+</sup>-OVA T cells demonstrated that export of parasite proteins into the infected hepatocytes enhanced immunogenicity and CD8<sup>+</sup> T cell based protection [10].

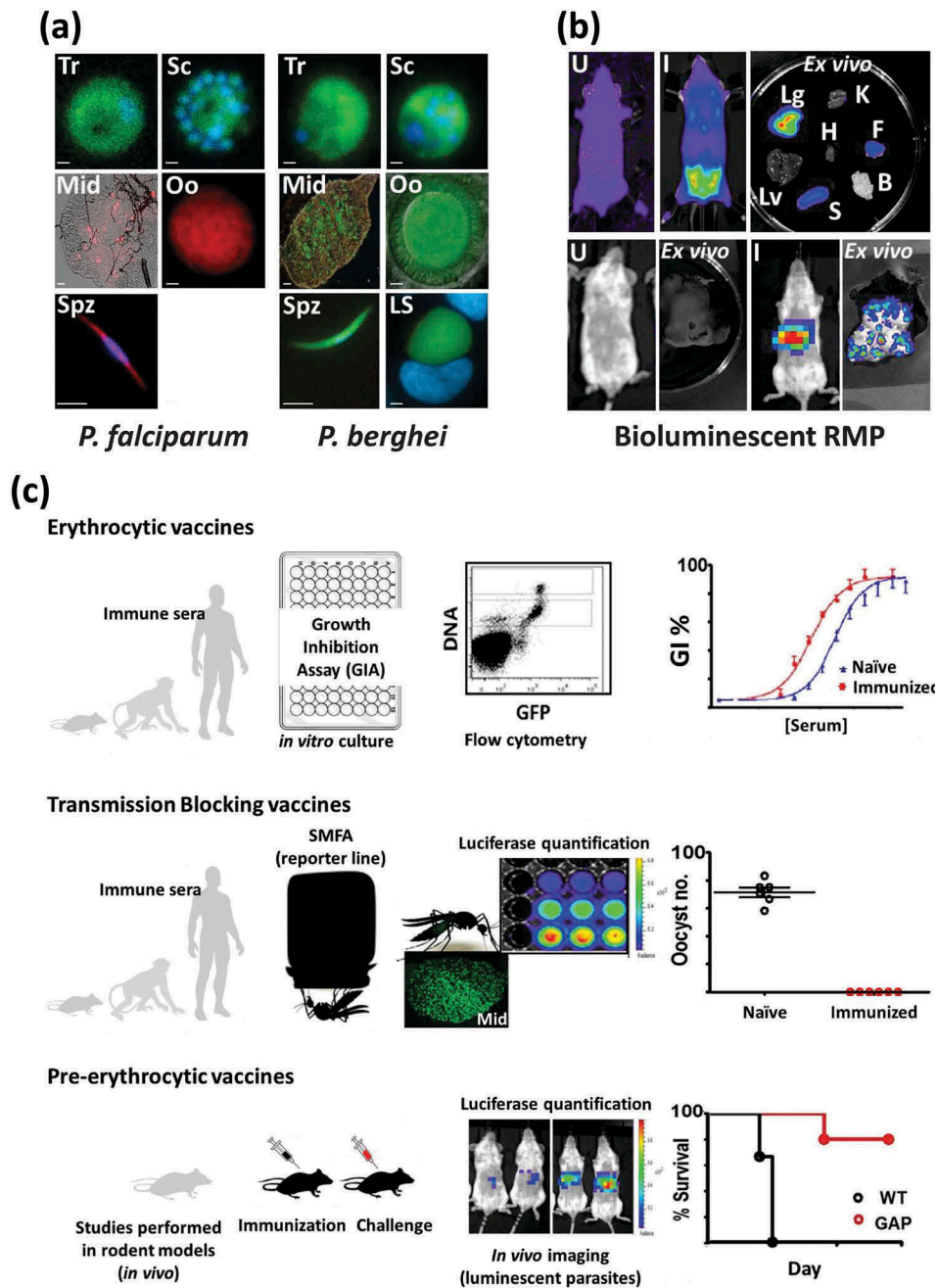
Below we describe the use of transgenic *Plasmodium* reporter parasites in preclinical assays to evaluate different *Plasmodium* vaccines and vaccination approaches that target the three major points of the parasite life cycle: erythrocytic vaccines, transmission blocking vaccines, and pre-erythrocytic vaccines.

## 2.1. Erythrocytic vaccines

Although a number of RMP transgenic reporter parasites have been used in screening assays to evaluate drugs or other inhibitors, not many studies have reported the use of these parasites in assays to assess blood-stage vaccines. The inhibitory activity of sera from (semi) immune individuals or purified immunoglobulins from vaccinated animals or people is mostly evaluated in *P. falciparum* using *in vitro* erythrocyte reinvasion and growth inhibition activity assays (GIA assays). These assays are used to quantitatively measure antibody-mediated effects on parasite invasion and growth, often in small-scale synchronized cultures of blood-stage parasites that are maintained in microtiter plates for 1–2 cycles in the presence or absence of antibodies. Determination of inhibition of invasion and growth in these assays is mainly performed by (automatic and high-throughput) microscopic, enzymatic, or flow cytometric assays using wild-type *P. falciparum* parasites [30,59–61]. In one study, a flow cytometric assay was developed that used transgenic *P. falciparum* parasites expressing GFP [16]. In this study, *P. falciparum* parasites of the D10 strain were genetically modified to express GFP under control of the constitutive *Pf**hsp86* promoter and inhibition of parasite growth by inhibitory antibodies and human serum was determined by measuring parasitemia by flow cytometry. This assay was superior to microscopy-based approaches and comparable to DNA-staining-based techniques to quantify growth inhibition (Figure 1(c)).

## 2.2. Transmission blocking vaccines

Mutant RMP are frequently used in (loss-of-function) studies that aim to identify and characterize *Plasmodium* proteins essential for parasite development in mosquitoes, which may be suitable targets for TB vaccine strategies. Often these deletion mutants have been created in transgenic RMP that express fluorescent or luminescent reporters, under control of constitutive stage-specific promoters permitting a detailed examination of parasite development in the mosquito, for example, enabling easier quantification of gametocyte development, fertilization, and oocyst or sporozoite production. While the use of transgenic RMP in TB vaccine studies is limited, chimeric RMP lines expressing the ookinete surface



**Figure 1.** The use of transgenic reporter parasites in malaria vaccine research.

(a) Representative fluorescent images of different life cycle stages of *P. falciparum* and *P. berghei* (mCherry and GFP) reporter parasites. Blood stage trophozoites (Tr); schizonts (Sc); dissected infected mosquito midguts (Mid) with mature oocysts (Oo); salivary gland sporozoites (Spz); *P. berghei* liver stage schizont (LS). Host and parasite DNA are stained with Hoechst or DAPI (blue). (b) Representative rainbow images of luminescence intensity in blood (upper panels) or liver (bottom panels) of live mice either uninfected (U) or infected (I) with luminescent reporter parasites. Parasite density (luminescence intensity) can also be determined in extracted tissue (*ex vivo*); lungs (Lg), kidney (K), adipose/fat tissue (F), liver (Lv), spleen (S), brain (B) and heart (H). Bottom panel shows luminescence in extracted livers of infected and uninfected mice, 48 h after infection with sporozoites. (c) Schematic representations showing the use of transgenic reporter parasites in assays to determine efficacy of erythrocytic, transmission blocking (TB) and pre-erythrocytic (sporozoite and liver stage) vaccines. Erythrocytic Vaccines: The inhibitory activity of sera from (semi) immune individuals or purified immunoglobulins from vaccinated animals/people on parasite invasion and growth in red blood cells are frequently determined in Growth Inhibition Assays (GIA). GFP expressing *P. falciparum* parasites have been used in GIA where inhibition of parasite growth was determined by measuring parasitemia by flow cytometry. Transmission Blocking Vaccines: The standard membrane-feeding assay (SMFA) is a well-established method to evaluate the activity of antibodies/serum against human malaria parasites in the mosquito, mainly quantified by determination of oocyst production. A transgenic reporter *P. falciparum* line expressing luciferase has been used in SMFA to quantify oocyst production in mosquitoes, thus eliminating the need for mosquito dissections. Pre-erythrocytic (sporozoite and liver stage) vaccines: Assays employing luciferase-expressing RMP have been developed to visualize and quantify liver stage development. Quantification of parasite liver loads by real time imaging has been performed in vaccinated and unvaccinated mice that have been challenged with luminescent parasites that either only express luciferase (e.g. in GAP studies; Section 4) or also express human malaria proteins (e.g. in studies on human malaria vaccines; Section 3). Full color available online.

protein P25 of *P. vivax* and *P. falciparum* have been used in direct mosquito feeding (DMF) assays for evaluation of the efficacy of vaccines targeting P25 of *P. vivax* and *P. falciparum*.

In these assays, immunized mice were challenged with the chimeric RMP parasites expressing the human antigen, followed by determination of oocyst reduction in mosquitoes

that were fed on the immunized and challenged mice [37,38,46,59,62].

The standard membrane-feeding assay (SMFA) is a well-established and recognized method to evaluate TB activity of antibodies/serum against human malaria parasites [63]. This assay has been utilized widely to assess the TB activity of purified antibodies and serum, both in preclinical and clinical vaccine studies. TB activity in the SMFA is defined by the reduction in oocyst numbers in mosquitoes that have been fed with infected blood containing gametocytes in the presence of antibodies/serum compared to no (or control) antibodies (Figure 1(b)). Often oocyst production is measured by a microscopic analysis of dissected mosquito midguts. Recently, a transgenic reporter *P. falciparum* line expressing luciferase has been used in SMFA to quantify oocyst production in mosquitoes, thus eliminating the need for mosquito dissections [54]. This transgenic line was made in parasites of the *P. falciparum* NF54 strain and expresses a fusion protein of GFP and luciferase which is under control of the constitutive *Pf*hsp70 promoter and parasites of this line do not express a drug-selectable marker. This novel dissection-free luminescence-based SMFA method, using a transgenic *P. falciparum* reporter parasite which is not resistance to known antimalarials, makes this assay much more amenable to high-throughput screening for both TB drugs and vaccines.

### 2.3. Pre-erythrocytic vaccines

Transgenic RMP are frequently used in preclinical sporozoite and liver-stage vaccine studies. Simple and sensitive *in vitro* and *in vivo* assays employing luciferase-expressing *P. berghei* and *P. yoelii* parasites have been developed to visualize and quantify liver-stage development [19,22]. In these assays, parasite hepatic development is determined by bioluminescence measurement of cultured liver stages or by real-time imaging of luminescence emanating from the liver of live mice. These measurements correlate well with established (but more laborious) quantitative RT-PCR methods [64,65]. Both *in vitro* and *in vivo* luminescence imaging assays have been used to screen inhibitors and vaccines against liver stages (Figure 1(c); [23,29,31,66,67]). The simplicity and speed of quantitative analysis of parasite liver loads by real-time imaging and the possibility to analyze parasite development in live mice without surgery greatly enhances the analysis of the effect of individual vaccines or vaccine strategies that target pre-erythrocytic stages. Quantification of parasite liver loads by real-time imaging has been performed in mice that have been first vaccinated with human *Plasmodium* subunit vaccines and then challenged with luminescent chimeric RMP that express human parasite antigens (see Section 3) or in mice that have been immunized with GAPs and subsequently challenged with luminescent RMP (see Section 4). In addition, imaging of luminescent parasites in mice has been successfully used to examining host factors regulating liver infections [68] and to analyze the impact of immune responses on inhibition of liver-stage development [23,69–71]. Such studies have revealed the importance of adaptive and innate immune responses in protective immunity after vaccination. In these studies, passively or actively immunized mice (including immunological compromised mice) were challenged with luciferase-expressing

parasites to monitor reduction in parasite liver loads. In addition to the use of luminescent RMP, transgenic RMP expressing fluorescent proteins have been used to provide insight into interactions of sporozoites with cells in lymph nodes and with dermal tissue and blood vessels, and their interactions specifically with cells of the innate and adaptive immune system [72–77]. Using fluorescent *P. berghei* sporozoites, it was demonstrated that fewer sporozoites enter the blood and reach the liver in sporozoite-immunized mice than naïve mice. Specifically, high circumsporozoite protein (CSP) antibody titers were shown to affect sporozoite motility in the skin, preventing immobilized sporozoites of entering dermal blood vessels [78].

No assays have yet been reported to analyze *P. falciparum* liver-stage development *in vitro* with fluorescent or luminescent parasites. Most studies on *P. falciparum* liver stages, either cultured in hepatocytes (primary human or HC-O4 hepatocytes) or in chimeric mice with human liver tissue, have used wild-type parasites that were analyzed by RT-PCR or by microscopy of fixed and stained cells. One study reported the use of transgenic *P. falciparum* parasites that express luciferase to study liver infection in immune compromised mice engrafted with human liver tissue [70]. This FRG huHep mouse is susceptible to a *P. falciparum* sporozoite infection and supports complete liver-stage development. The reporter *P. falciparum* (NF54) parasites express a *gfp-luciferase* fusion gene under the constitutive *Pf*ee1a promoter and the reporter expression cassette is introduced into the *pf47* locus [79]. In this study [70], a clear effect could be detected on infection of livers of FRG huHep mice by passively transferred antibodies against CSP and parasite liver loads in these mice were analyzed using bioluminescence imaging 6 days after infection with sporozoites (i.e. at the peak of liver-stage luciferase activity).

### 3. Chimeric rodent parasites expressing human plasmodium proteins

In addition to transgenic reporter parasites, rodent parasites expressing human malaria parasite proteins (HMP; *P. falciparum* and *P. vivax*) have been used in vaccine studies. These ‘chimeric’ RMP are used both to analyze immune responses against HMP antigens and to evaluate *in vivo* protective efficacy of vaccines that target HMP antigens (reviewed in [28,29] and see Table 1). The preclinical evaluation of protective immunity involves mice being immunized with vaccines targeting different *P. falciparum* or *P. vivax* antigens followed by challenge with chimeric rodent parasites that express the corresponding HMP antigen. Mainly chimeric RMP expressing pre-erythrocytic HMP antigens have been generated (Table 1). Chimeric parasites have also been used to study immunogenicity and protective efficacy of transmission blocking HMP vaccine antigens, i.e. *P. falciparum* and *P. vivax* P25 [37,38,46] and blood-stage vaccine antigens, i.e. *P. falciparum* MSP1 (Table 1).

Generation of chimeric parasites have been performed using standard methods of RMP transfection [80] by introducing HMP genes into the RMP genome, either as additional gene copies or by replacing the complete RMP with its HMP ortholog [29]. In addition, chimeric parasites have been generated that express fusions of the RMP and HMP orthologous

genes (Table 1). The recently described gene insertion-marker out (GIMO) transfection method [81] greatly simplifies and speeds up the generation of transgenic parasites expressing heterologous proteins, which are free of drug-selection marker genes. Using this method, two principle types of chimeric RMP expressing HMP proteins have been created ([29]; Figure 2(a)). The first type are ‘additional copy mutants;’ here, the HMP gene is introduced as an additional gene copy into a silent/neutral locus of the GIMO mother-line and the HMP gene is under the control of a constitutive or stage-specific RMP gene promoter. This strategy is often used when an ortholog of the HMP gene is absent from the RMP genome. The second type of chimeric parasites are ‘replacement mutants;’ here, the coding sequence (CDS) of the RMP gene is replaced with the CDS of the orthologous HMP gene. This method creates chimeric parasites expressing the HMP gene under control of the endogenous RMP gene promoter and transcriptional terminator. The absence of a drug-selectable marker in both the additional copy and replacement mutants makes it possible to rapidly introduce additional genetic modifications in these chimeric parasites, e.g. introduction of additional HMP genes or fluorescent/luminescent reporter genes.

Chimeric parasites have been used in vaccine studies for a number of reasons. While a high level of genetic orthology exists between genes of RMP and HMP, critical differences often exist in the sequence and structure of the encoded proteins [24]. In addition, HMP express a number of genes encoding vaccine candidates that are absent from RMP [24,31]. These differences complicate the analysis of immunogenicity and protective efficacy of HMP antigens in rodent models and compromise the effective translation of findings into a human malaria vaccine. Therefore, ‘humanizing’ RMP by introducing HMP genes into rodent parasite genomes can help to circumvent some of these problems. HMP cannot readily infect small animals and testing of *P. falciparum* parasites in rodents is expensive as it is largely restricted to immune-deficient mice (i.e. DRAG or FRG) transplanted with human hematopoietic stem cells and/or liver tissue [82,83]. While it is possible to test both pre-erythrocytic and blood-stage *P. falciparum* vaccine candidates directly in human subjects, these studies are expensive and laborious to perform and therefore less suitable for larger screening studies [84]. Preclinical screening studies using chimeric RMP make it possible to rapidly evaluate and compare the protective efficacy of novel target antigens and vaccination strategies in order to down-select candidate antigens and strategies that can proceed into clinical studies.

Recently, 10 pre-erythrocytic *P. falciparum* vaccine candidate antigens were tested for their protective efficacy using chimeric parasites [31]. The antigens were selected based on published literature, immuno-profiling, and expression studies. Mice, immunized with viral-vectored vaccines expressing the HMP antigens, were challenged with chimeric parasites for evaluation of protective immune responses and characterization of the immune responses (see Figure 2(b) for the immunization/challenge protocol). In this study, two antigens, *Pf*LSA1 and *Pf*LAP2, generated better protective efficacy than two leading pre-erythrocytic *P. falciparum* vaccine antigens, *Pf*CSP or *Pf*TRAP, in both inbred BALB/c and outbred CD-1 mice. The chimeric parasites used in this study had the HMP gene introduced as an additional gene copy as a number of

the selected genes did not have an ortholog in the *P. berghei* genome, thereby excluding the possibility to make replacement mutants. A number of other chimeric RMP have been used, which express a HMP ortholog in place of their own RMP gene (Table 1), for example chimeric parasites expressing pre-erythrocytic vaccine candidates such as *P. vivax* and *P. falciparum* CSP and CeTOS ([34,39,41,85]; Table 1).

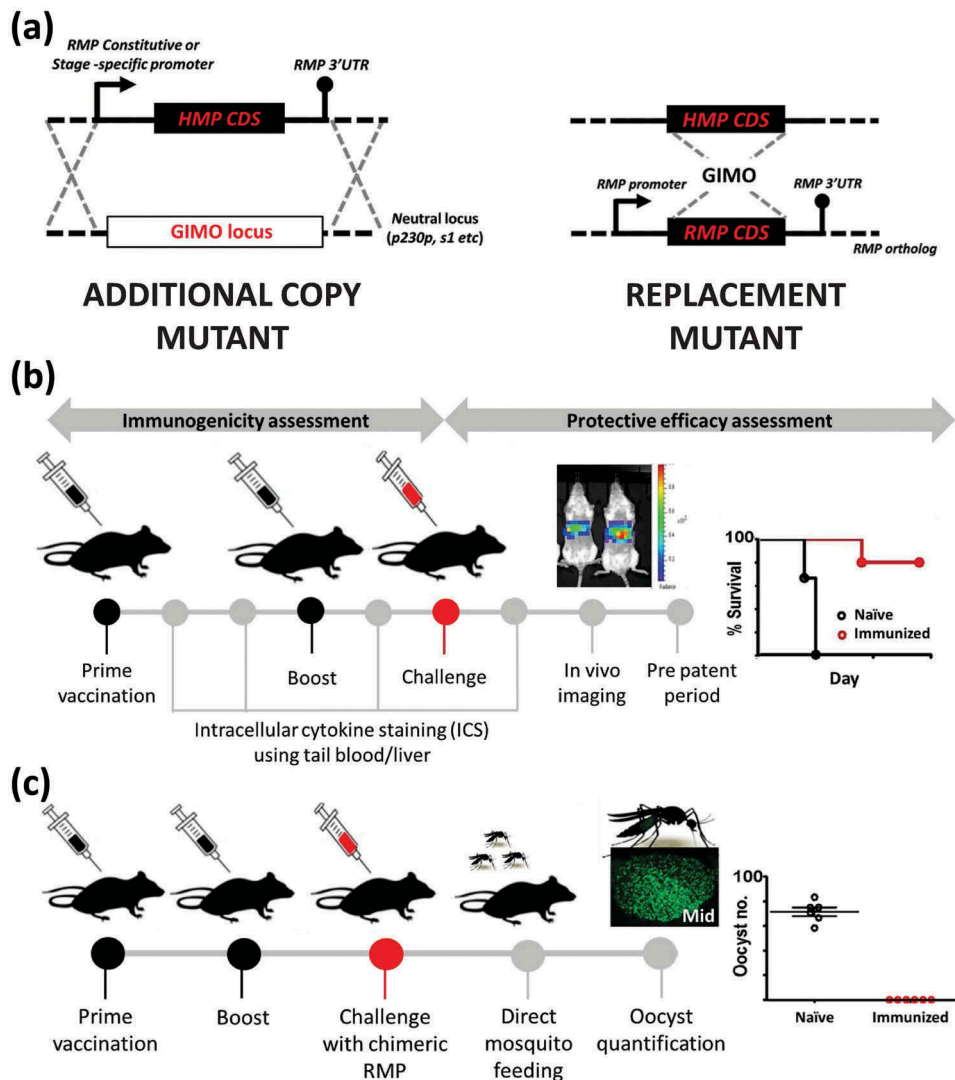
Chimeric parasites have also been used to evaluate immunogenicity of antigens against other lifecycle stages (i.e. TB vaccines see Figure 2(c)) as well as being used to evaluate different vaccine delivery platforms and to optimize the vaccination strategy and schedule. For example, the use of a single chimeric parasite expressing two HMP genes, TRAP and UIS3, showed that combination of two vaccines expressing these antigens could protect 100% of immunized mice, despite these antigens demonstrating only modest protective immunity when administered as a single antigen formulation [32]. This synergistic effect was only evident when the two vaccines were mixed and administered into two legs. Another study, testing different vaccine delivery platforms targeting *P. vivax* CSP using chimeric RMP that expressed *P. vivax* CSP, demonstrated that superior immunogenicity was generated by virus-like particles (VLP) expressing *P. vivax* CSP compared to other formulations, including viral-vectored vaccines or protein plus adjuvant [40].

Chimeric parasites expressing either full-length HMP proteins or fusions of HMP-RMP proteins can be instructive in determining critical immunological determinants of the protective immune responses after vaccination, for example, in GIA using material obtained from immunized humans or animals [43,86,87]. However, the mechanisms of protection after vaccination can be lost in *in vitro* assays if only individual components of the adaptive immune response are examined in isolation. For example, responses that require both antibody and cell-mediated responses, either acting independently or when they work in concert such as in antibody-dependent cell-mediated cytotoxicity responses [88]. Ultimately, however, even positive results generated using chimeric parasites in rodents or *in vitro* assays will need to be validated in human vaccine trials.

#### 4. Attenuated parasite vaccines

Transgenic parasites have not only been used for development and evaluation of immunogenicity of antigens and protective immunity of subunit vaccines, they have also been used to develop and evaluate whole-organism vaccines consisting of (genetically) attenuated parasites. Vaccination with live, attenuated, sporozoites has been shown to induce strong protective immune responses both in rodents and in humans (reviewed in [48]). Sporozoite attenuation has been performed by radiation or by genetic modification of parasites (reviewed in [48–50,89,90]). A prerequisite for induction of protective immunity is that the attenuated sporozoites enter the liver, since heat-killed or over-irradiated sporozoites that do not invade hepatocytes do not efficiently confer protection [49,91]. These so-called GAPs have genes encoding proteins essential for parasite development in the liver removed, thereby producing parasites that arrest in the liver. For both GAPs and radiation-attenuated parasites, immunogenicity (protective efficacy) and safety are critical factors for





**Figure 2.** The use of chimeric RMP expressing human malaria parasite (HMP) proteins in malaria vaccine research.

(a) Additional Copy Mutants have the HMP gene (e.g. the *P. falciparum* gene coding sequence; Pf CDS) introduced as an additional gene copy into a silent/neutral locus of the RMP; the HMP gene is under the control of a constitutive or stage-specific RMP gene promoter. Replacement Mutants have the RMP coding sequence (Pb CDS) replaced by the orthologous HMP CDS. This often 2 step replacement method, employing the methods of GIMO transfection, creates chimeric parasites expressing the HMP gene under control of the endogenous RMP gene promoter and transcriptional terminator. (b) Vaccine immunogenicity and protective efficacy measured in mice immunized with HMP liver stage sub-unit vaccines or rodent GAPs. Immunized (and naïve) mice are challenged either with luminescent chimeric RMP expressing the cognate HMP antigen or with luminescent 'wild-type' RMP. Protective efficacy, relative to unvaccinated mice, is quantified by measuring the parasite load by real time imaging of the liver of live mice at 44–48 h after challenge with sporozoites (in vivo imaging of luminescence) and/or measuring the time to establish a detectable blood stage infection (pre-patent period; % survival). (c) Vaccine efficacy of HMP transmission blocking vaccines determined in a direct mosquito feeding assay (DMFA) in mosquitoes. In these assays mice are immunized with the HMP transmission blocking vaccine. Immunized and naïve mice are then infected with chimeric RMP parasites expressing the cognate HMP antigen. The infected mice are used to feed mosquitoes and (reduction in) oocyst production in mosquitoes is quantified 8–10 days after feeding in order to measure of the transmission blocking potential of the HMP vaccine.

further clinical development as whole-organism vaccines. Transgenic rodent parasites have been used extensively in pre-clinical evaluation studies to establish the safety profile of GAPs, i.e. absence of a blood-stage infection in mice after inoculation with high numbers of GAPs [50]. A number of different GAP vaccine candidates have been generated in rodent parasites, by deletion of either single or multiple genes. These have been analyzed in mice to ensure they completely arrest in the liver and therefore meet the necessary safety profile for translation into human GAP. Introducing genes encoding fluorescent and luminescent genes into the genomes of GAPs has permitted a detailed analysis on the timing and magnitude of arrest in the liver [92,93] (Figure 1(b)). Based on studies on growth arrest and safety of rodent GAPs, three multiple gene-deletion *P. falciparum*

GAPs have been developed that have advanced into clinical evaluation [94–96].

In addition to examining the safety profile of a GAP, transgenic RMP have also been used to evaluate the protective immunity induced by attenuated sporozoites, both radiation-attenuated sporozoites and GAPs. In multiple studies, mice immunized with attenuated parasites have been challenged with fully infectious sporozoites that express luciferase to determine liver loads by real-time imaging, similar to what has been described above for evaluation of protective immunity of subunit vaccines (Sections 2 and 3; Figure 2(b)). Quantification of parasite liver loads and the pre-patent period provide a direct measurement of protective immunity induced by different immunization regimens.

Rodent GAPs expressing luciferase have also been used to investigate different attenuated sporozoite administration strategies [97,98]. These studies demonstrated that the route and dose of administration of attenuated sporozoites are critical factors in inducing protective immunity. Intradermal, subcutaneous, and intramuscular administration of attenuated sporozoites resulted in reduced parasite liver loads when compared to the same number of sporozoites introduced intravenously. Lower parasite liver loads after intradermal delivery was associated with reduced protective efficacy compared to intravenous immunization. Transgenic fluorescent rodent GAPs have been used to analyze direct interactions of lymphocytes with infected hepatocytes using intravital imaging of mice that had previously been immunized with attenuated sporozoites [13,56,99,100]. These studies have revealed the importance of CD8<sup>+</sup> T-mediated killing and elimination of infected hepatocytes in mice immunized with attenuated sporozoites. Further, using transgenic RMP expressing the immunological reporter protein ovalbumin, it has been possible to analyze direct interactions and effects of antigen specific CD8<sup>+</sup> T cell-mediated immune responses in the liver of mice immunized with attenuated sporozoites ([10,56]; see also Section 2).

## 5. Expert commentary

The ability to genetically manipulate the malaria parasite by deleting, mutating genes, or introducing transgenes in the parasite genome has advanced our understanding of the molecular and cellular biology of malaria parasites for the last 20 years. Genetic modification has been central to the functional characterization of genes including genes encoding putative vaccine candidate antigens. The generation of reporter parasites with additional genes in their genome has resulted in the increased use of transgenic parasites in translation-oriented research, for example, in preclinical studies evaluating immunogenicity and protective efficacy of novel antigens and vaccines. These studies involve transgenic parasites of both rodent and human malaria species. Two examples of transgenic human parasites are luminescent *P. falciparum* parasites that have been used in high-throughput assays to quantify transmission blocking activity and the use of luminescent *P. falciparum* parasites to analyze the effects of (passively transferred) immune sera on liver infection in mice engrafted with human liver tissue (Section 2). These assays are used to generate insights into the immunogenicity of putative vaccine candidate antigens, knowledge which in turn can be used to improve vaccine strategies that target transmission blocking stages and pre-erythrocytic stages, respectively.

Compared to transgenic *P. falciparum* parasites, transgenic RMP have been more widely applied in experimental vaccine studies, especially in the evaluation of pre-erythrocytic antigens and to assess different pre-erythrocytic vaccination strategies. For example, luminescent parasites are frequently used to challenge immunized mice in standard assays that measure the reduction in parasite liver load as a consequence of the protective immune responses induced by different antigens or vaccine strategies. Another example is the application of intravital imaging using fluorescent parasites in immunized mice,

which has revealed critical insights into the immune response targeting sporozoites and infected liver cells (Section 2). Such *in vivo* assays to analyze crucial protective immune responses after vaccination and to evaluate protective immunity are valuable tools to improve pre-erythrocytic vaccines.

In addition to reporter rodent parasites, chimeric rodent parasites expressing proteins of the human malaria parasites *P. falciparum* and *P. vivax* are now being increasingly used in vaccine studies. Chimeric RMP expressing HMP proteins are used to determine protective efficacy in mice immunized with different subunit vaccines expressing *P. falciparum* and *P. vivax* antigens (Section 3). These studies have been used to select novel vaccine candidate antigens for advancement into clinical trials. Chimeric RMP can not only support identification of novel antigens, but also contribute to the *in vivo* evaluation of novel delivery platforms and vaccine strategies, both for vaccines targeting pre-erythrocytic parasites and transmission blocking vaccines (Section 3). The use of chimeric rodent parasites to evaluate protective immunity or transmission blocking immunity is not without its limitations. First, the use of chimeric RMP still relies on a murine model, often inbred mice strains, and encounter issues related to restriction of MHC epitopes and marked immune dominance of certain epitopes [101]. Outbred mice can possibly be used to more accurately reflect what may be seen in humans but it is possible that some antigens identified as poorly immunogenic in these studies may in fact be immunogenic in humans. Second, when using 'additional copy' chimeric parasites, the HMP gene expression is dependent on the RMP promoter used, which is unlikely to exactly mimic the timing and magnitude of the expression of the HMP protein in the HMP. In studies where multiple vaccine antigens are examined, the chimeric parasites will express the different HMP antigens at the same level, which is unlikely to be the case in wild-type HMP. Therefore, where possible, it would be useful to also compare protective vaccine efficacy in mice using a chimeric RMP parasite where the HMP antigen expression matches its expression in the HMP, both in timing and magnitude. Despite these limitations, chimeric RMP allow for rapid vaccine (rank-order) screening *in vivo* and can provide critical insights into both the importance of the vaccine target and the mechanism of protection. Indeed data from chimeric RMP are being used to justify the selection of novel HMP antigen vaccines (and delivery platforms) to advance into clinical testing.

In addition to the role of transgenic parasites in the development of subunit vaccines, transgenic parasites have played a central role in the development and evaluation of whole-organism vaccines consisting of attenuated sporozoites. Studies in rodent malaria models on the safety and immunogenicity of GAPs have formed the basis of the development of different (multiple gene deletion) *P. falciparum* GAPs that have now advanced into clinical trials (see Section 4). Given the data from rodents studies with both GAPs and irradiated sporozoites and from data emerging from irradiated sporozoite vaccine research in humans, it is anticipated that further improvements can be made to increase GAP potency. Here again, transgenic RMP can play an important role, for example, to optimize the routes of attenuated parasite vaccine administration (e.g. studies with devices to improve intradermal or

intramuscular delivery, use of adjuvants, etc.) and in development of the so-called 'next-generation' GAP vaccines with increased potency requiring fewer sporozoites per dose and fewer vaccination doses to achieve sustained sterile protection (e.g. GAPs which arrest late into liver-stage development).

Transgenic parasites used in conjunction with 'humanized' animal models or in sophisticated *in vitro* assays are designed to aid and speed up malaria vaccine design, specifically to suggest potential priorities for expensive and time-consuming clinical trials. As mentioned above, however, the predictive power of these assays can only be determined after human trials have been performed and lessons learnt from the success and discrepancies that will arise. In addition, over-reliance on a single experimental model may result in putative valid vaccine targets not being advanced further, as they did not generate sufficient immunity in the testing platform (e.g. in mice).

## 6. Five-year view

Despite considerable effort, over decades, a highly effective vaccine against malaria still does not exist. This is in part due to the limited number of antigens and methods of immunization that have advanced into clinical testing. Most vaccine studies have focused on a limited number of antigens but for a broad acting, highly durable, and potent malaria vaccine, this is likely to be too restrictive and insufficient to provide the protection required. Therefore, in order to create multi-antigen and multi-stage vaccines, many more antigens and improved vaccine delivery platforms will need to be investigated and evaluated as a priority in the next 5 years. In addition, the critical host and parasite factors mediating protective immunity and those that are necessary for maintaining durable protection need also further investigation in the upcoming years. The use of transgenic parasites in conjunction with other enabling technologies (e.g. genetic modification of mice or human cell lines, advances in imaging, etc.) has opened up new possibilities and will be used to contribute to a more rapid preclinical evaluation of vaccines, vaccination strategies, and identification of critical factors of protective immune responses. Transgenic *P. falciparum* parasites expressing luminescent reporter proteins are currently valuable tools to assess drugs and inhibitors against the parasite in high-throughput assays and are now also being used to test the immunogenicity of (novel) transmission blocking antigens and will continue to be used to evaluate novel transmission blocking vaccine strategies. In addition, the recent availability of luminescent *P. falciparum* parasites that express luciferase under strong promoters (i.e. constitutive, sporozoite or liver-stage specific) will act as a bridge between rodent and clinical studies. They will be increasingly used in assays to evaluate the effects of (human) immune serum, cells, and factors on *P. falciparum* blood and liver cell infection, both in cultured cells and in humanized mice with human hematopoietic and human liver cells. Such assays will contribute to generate essential insights into the immunogenicity of (in particular pre-erythrocytic) antigens and vaccination strategies. Both reporter RMP expressing fluorescent and luminescent proteins as well as chimeric RMP expressing HMP antigens will contribute to these studies examining protective immune responses

in particular of vaccine strategies targeting pre-erythrocytic vaccines. The use of transgenic parasites may not only help to rank order existing candidates but also help to reveal novel vaccine candidate antigens and vaccination strategies. Loss of function and protein-tagging mutants often reveal parasite proteins that have critical roles in parasite development or, for example, are located on the surface of extracellular forms of the parasite and may therefore be vulnerable to antibody-based vaccines. Uncovering critical protective immune responses and efforts to establish correlates of protection after vaccination may be greatly aided by the use of both transgenic parasites and humanized mice, which could be used to examine both the induction and recall of immune responses in different organs. Transgenic RMP will continue to play an important role in preclinical evaluation of novel attenuated sporozoite vaccines both in studies to develop GAPs that are more immunogenic and in studies to improve vaccination strategies (e.g. optimizing the route of administration). In particular, next-generation *P. falciparum* GAPs that have been further modified to express multistage and antigens from multiple strains.

## Key issues

- Most vaccine studies have focused on a limited number of antigens but for a broad acting, highly durable and potent malaria vaccine this is likely to be too restrictive and insufficient to provide the protection required. Multi-stage, multiple-antigen sub-unit or genetically attenuated parasite vaccines may provide a solution.
- Transgenic (human and rodent) malaria parasites expressing 'foreign' proteins, for example fluorescent and luminescent proteins, have been used to determine the protective efficacy of different antigens and to evaluate vaccination platforms/strategies.
- Transgenic parasites (e.g. expressing OVA) are being used to understand the critical determinants of protection after vaccination; specifically to examine the induction and recall of protective immune responses in the blood and the liver
- Luminescent rodent parasites are now increasingly used to challenge vaccinated mice, and non-invasive measurements of parasite liver load permits examination of both the protective responses generated by different antigens and to evaluate novel vaccine strategies.
- Luminescent *P. falciparum* parasites are being used both in high-throughput assays to quantify transmission blocking activity and to analyze the effects of human immune sera/immunoglobulins on parasite development in the liver of humanized mice.
- Chimeric rodent parasites, expressing *P. falciparum* or *P. vivax* antigens, are being used to directly evaluate and rank-order human malaria vaccine candidates and determination of the most suitable for clinical testing.
- Chimeric rodent parasites permit an *in vivo* comparison of different *P. falciparum/vivax* vaccine delivery platforms and vaccination strategies; they are being used to determine the best combination of antigens, delivery system and immunization protocol to move forward into clinical testing.
- Transgenic parasites play a central role in the development and evaluation of whole organism vaccines consisting of

attenuated sporozoites. Both in evaluation of safety and in assessing protective efficacy. Improvements in genetically attenuated parasite vaccines and strategies for vaccination (i.e. optimizing the route of administration) will continue to require the use of transgenic parasites

## Funding

The manuscript was not funded.

## Declaration of interest

A.S Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C Marin-Mogollon is supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). A. M Salman is supported by Prof. Adrian Hill's Senior Investigator Award from the Wellcome Trust (095540/Z/11/Z). S Khan, B.M Franke-Fayard and C.J Janse are full time employees of the Leiden University Medical Center (LUMC).

## ORCID

Shahid M. Khan  <http://orcid.org/0000-0002-0533-4670>

## References

Papers of special note have been highlighted as either of interest (\*) or of considerable interest (\*\*\*) to readers.

- Van Dijk MR, Janse CJ, Waters AP. Expression of a plasmodium gene introduced into subtelomeric regions of plasmodium berghei chromosomes. *Science*. 1996;271:662–665.
- Carvalho TG, Ménard R. Manipulating the Plasmodium genome. *Curr Issues Mol Biol*. 2005;7:39–55.
- De Koning-Ward TF, Gilson PR, Crabb BS. Advances in molecular genetic systems in malaria. *Nat Rev Microbiol*. 2015;13:373–387.
- Amino R, Ménard R, Frischknecht F. In vivo imaging of malaria parasites—recent advances and future directions. *Curr Opin Microbiol*. 2005;8:407–414.
- Heussler V, Doerig C. In vivo imaging enters parasitology. *Trends Parasitol*. 2006;22:192–196; discussion 5–6.
- Siciliano G, Alano P. Enlightening the malaria parasite life cycle: bioluminescent Plasmodium in fundamental and applied research. *Front Microbiol*. 2015;6:391.
- De Niz M, Burda PC, Kaiser G, et al. Progress in imaging methods: insights gained into Plasmodium biology. *Nat Rev Microbiol*. 2017;15:37–54.
- Franke-Fayard B, Fonager J, Braks A, et al. Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria? *Plos Pathog*. 2010;6:e1001032.
- Lin J-W, Shaw TN, Annoura T, et al. The subcellular location of ovalbumin in Plasmodium berghei blood stages influences the magnitude of T-cell responses. *Infect Immun*. 2014;82:4654–4665.
- Montagna GN, Beigier-Bompadre M, Becker M, et al. Antigen export during liver infection of the malaria parasite augments protective immunity. *MBio*. 2014;5:e01321–14.
- Fernandez-Ruiz D, Ng WY, Holz LE, et al. Liver-resident memory CD8(+) T cells form a front-line defense against Malaria liver-stage infection. *Immunity*. 2016;45:889–902.
- Holz LE, Fernandez-Ruiz D, Heath WR. Protective immunity to liver-stage malaria. *Clin Transl Immunol*. 2016;5:e105.
- Frevert U, Nacer A, Cabrera M, et al. Imaging Plasmodium immunobiology in the liver, brain, and lung. *Parasitol Int*. 2014;63:171–186.
- Stone WJ, Churcher TS, Graumans W, et al. A scalable assessment of Plasmodium falciparum transmission in the standard membrane-feeding assay, using transgenic parasites expressing green fluorescent protein-luciferase. *J Infect Dis*. 2014;210:1456–1463.
- Wang Z, Liu M, Liang X, et al. A flow cytometry-based quantitative drug sensitivity assay for all Plasmodium falciparum gametocyte stages. *Plos One*. 2014;9:e93825.
- Wilson DW, Crabb BS, Beeson JG. Development of fluorescent Plasmodium falciparum for in vitro growth inhibition assays. *Malar J*. 2010;9:152.
- Swann J, Corey V, Scherer CA, et al. High-throughput luciferase-based assay for the discovery of therapeutics that prevent Malaria. *ACS Infect Dis*. 2016;2:281–293.
- Voorberg-van der Wel A, Zeeman AM, Van Amsterdam SM, et al. Transgenic fluorescent Plasmodium cynomolgi liver stages enable live imaging and purification of Malaria hypnozoite-forms. *Plos One*. 2013;8:e54888.
- Annoura T, Chevalley S, Janse CJ, et al. Quantitative analysis of Plasmodium berghei liver stages by bioluminescence imaging. *Methods Mol Biol*. 2013;923:429–443.
- Le Bihan A, De Kanter R, Angulo-Barturen I, et al. Characterization of novel antimalarial compound ACT-451840: preclinical assessment of activity and dose-efficacy modeling. *PLoS Med*. 2016;13:e1002138.
- Lin J-W, Sajid M, Ramesar J, et al. Screening inhibitors of P. berghei blood stages using bioluminescent reporter parasites. *Methods Mol Biol*. 2013;923:507–522.
- Prudêncio M, Mota MM, Mendes AM. A toolbox to study liver stage malaria. *Trends Parasitol*. 2011;27:565–574.
- Sack BK, Miller JL, Vaughan AM, et al. Measurement of antibody-mediated reduction of plasmodium yoelii liver burden by bioluminescent imaging. *Methods Mol Biol*. 2015;1325:69–80.
- Otto TD, Böhme U, Jackson AP, et al. A comprehensive evaluation of rodent malaria parasite genomes and gene expression. *BMC Biol*. 2014;12:86.
- Tewari R, Patzewitz EM, Poulin B, et al. Development of a transgenic Plasmodium berghei line (Pb pfpkg) expressing the P. falciparum cGMP-dependent protein kinase, a novel antimalarial drug target. *Plos One*. 2014;9:e96923.
- Blume M, Hliscs M, Rodriguez-Contreras D, et al. A constitutive pan-hexose permease for the Plasmodium life cycle and transgenic models for screening of antimalarial sugar analogs. *Faseb J*. 2011;25:1218–1229.
- De Moraes LV, Dechavanne S, Sousa PM, et al. Murine model for preclinical studies of Var2CSA-mediated pathology associated with Malaria in pregnancy. *Infect Immun*. 2016;84:1761–1774.
- Cockburn I. Chimeric parasites as tools to study Plasmodium immunology and assess malaria vaccines. *Methods Mol Biol*. 2013;923:465–479.
- Salman AM, Mogollon CM, Lin JW, et al. Generation of transgenic rodent malaria parasites expressing human malaria parasite proteins. *Methods Mol Biol*. 2015;1325:257–286.
- Description of methods to rapidly generate different chimeric RMP (additional copy and replacement mutants) expressing HMP genes.**
- Mlambo G, Kumar N. Transgenic rodent Plasmodium berghei parasites as tools for assessment of functional immunogenicity and optimization of human malaria vaccines. *Eukaryot Cell*. 2008;7:1875–1879.
- Longley RJ, Salman AM, Cottingham MG, et al. Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates. *Sci Rep*. 2015;5:11820.
- Selection of novel pre-erythrocytic P. falciparum vaccine candidates for their protective efficacy using chimeric RMP expressing P. falciparum antigens.**
- Longley RJ, Halbroth BR, Salman AM, et al. Assessment of the Plasmodium falciparum pre-erythrocytic antigen UIS3 as a potential candidate for a malaria vaccine. *Infect Immun*. 2016;85(3):e00641-16.
- Ewer KJ, Sierra-Davidson K, Salman AM, et al. Progress with viral vectored malaria vaccines: a multi-stage approach involving “unnatural immunity”. *Vaccine*. 2015;33:7444–7451.

34. Tewari R, Spaccapelo R, Bistoni F, et al. Function of region I and II adhesive motifs of *Plasmodium falciparum* circumsporozoite protein in sporozoite motility and infectivity. *J Biol Chem*. 2002;277:47613–47618.
35. Zhang M, Kaneko I, Tsao T, et al. A highly infectious *Plasmodium yoelii* parasite, bearing *Plasmodium falciparum* circumsporozoite protein. *Malar J*. 2016;15:201.
36. Bauza K, Malinauskas T, Pfander C, et al. Efficacy of a *Plasmodium vivax* malaria vaccine using ChAd63 and modified vaccinia Ankara expressing thrombospondin-related anonymous protein as assessed with transgenic *Plasmodium berghei* parasites. *Infect Immun*. 2014;82:1277–1286.
37. Ramjane S, Robertson JS, Franke-Fayard B, et al. The use of transgenic *Plasmodium berghei* expressing the *Plasmodium vivax* antigen P25 to determine the transmission-blocking activity of sera from malaria vaccine trials. *Vaccine*. 2007;25:886–894.
38. Mlambo G, Maciel J, Kumar N. Murine model for assessment of *Plasmodium falciparum* transmission-blocking vaccine using transgenic *Plasmodium berghei* parasites expressing the target antigen Pfs25. *Infect Immun*. 2008;76:2018–2024.
39. Espinosa DA, Vega-Rodríguez J, Flores-García Y, et al. The *P. falciparum* cell-traversal protein for ookinetes and sporozoites as a candidate for pre-erythrocytic and transmission-blocking vaccines. *Infect Immun*. 2017;85(2):e00498–516.
40. Salman AM, Montoya-Díaz E, Lall A, et al. Rational development of a highly protective *P. vivax* vaccine evaluated using transgenic rodent parasite challenge models. *Sci Rep*;Forthcoming 2017.
- **A study describing the evaluation of different vaccine platforms, all targeting the same *P. vivax* antigen, in mice using chimeric RMP expressing the *P. vivax* antigen.**
41. Alves E, Salman AM, Leoratti F, et al. Evaluation of PvCelTOS as a pre-erythrocytic *P. vivax* vaccine. *Clin Vaccine Immunol*. 2017;24(4):e00501–16.
42. Persson C, Oliveira GA, Sultan AA, et al. Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid *Plasmodium falciparum* circumsporozoite protein. *J Immunol*. 2002;169:6681–6685.
43. De Koning-Ward TF, O'Donnell RA, Drew DR, et al. A new rodent model to assess blood stage immunity to the *Plasmodium falciparum* antigen merozoite surface protein 119 reveals a protective role for invasion inhibitory antibodies. *J Exp Med*. 2003;198:869–875.
44. Cao Y, Zhang D, Pan W. Construction of transgenic *Plasmodium berghei* as a model for evaluation of blood-stage vaccine candidate of *Plasmodium falciparum* chimeric protein 2.9. *Plos One*. 2009;4:e6894.
45. Espinosa DA, Yadava A, Angov E, et al. Development of a chimeric *Plasmodium berghei* strain expressing the repeat region of the *P. vivax* circumsporozoite protein for in vivo evaluation of vaccine efficacy. *Infect Immun*. 2013;81:2882–2887.
46. Mizutani M, Iyori M, Blagborough AM, et al. Baculovirus-vectored multistage *Plasmodium vivax* vaccine induces both protective and transmission-blocking immunities against transgenic rodent malaria parasites. *Infect Immun*. 2014;82:4348–4357.
47. Mizutani M, Fukumoto S, Soubeiga AP, et al. Development of a *Plasmodium berghei* transgenic parasite expressing the full-length *Plasmodium vivax* circumsporozoite VK247 protein for testing vaccine efficacy in a murine model. *Malar J*. 2016;15:251.
48. Bijker EM, Borrmann S, Kappe SH, et al. Novel approaches to whole sporozoite vaccination against malaria. *Vaccine*. 2015;33:7462–7468.
49. Hollingdale MR, Sedegah M. Development of whole sporozoite malaria vaccines. *Expert Rev Vaccines*. 2017;16:45–54.
50. Khan SM, Janse CJ, Kappe SH, et al. Genetic engineering of attenuated malaria parasites for vaccination. *Curr Opin Biotechnol*. 2012;23:908–916.
- **A review on genetically attenuated malaria vaccines and how genetically modified RMP are used in their characterization and further improvement.**
51. Dube A, Gupta R, Singh N. Reporter genes facilitating discovery of drugs targeting protozoan parasites. *Trends Parasitol*. 2009;25:432–439.
52. Franke-Fayard B, Trueman H, Ramesar J, et al. A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol*. 2004;137:23–33.
53. Hopp CS, Chiou K, Ragheb DR, et al. Longitudinal analysis of *Plasmodium* sporozoite motility in the dermis reveals component of blood vessel recognition. *Elife*. 2015;4:e07789.
54. Vos MW, Stone WJ, Koolen KM, et al. A semi-automated luminescence based standard membrane feeding assay identifies novel small molecules that inhibit transmission of malaria parasites by mosquitoes. *Sci Rep*. 2015;5:18704.
- **The development of a high-throughput assay to test transmission blocking vaccines using a transgenic *P. falciparum* line expressing luciferase in a SMFA.**
55. Lucantoni L, Fidock DA, Avery VM. Luciferase-based, high-throughput assay for screening and profiling transmission-blocking compounds against *Plasmodium falciparum* gametocytes. *Antimicrob Agents Chemother*. 2016;60:2097–2107.
56. Kimura K, Kimura D, Matsushima Y, et al. CD8+ T cells specific for a malaria cytoplasmic antigen form clusters around infected hepatocytes and are protective at the liver stage of infection. *Infect Immun*. 2013;81:3825–3834.
- **Analysis of mechanisms of protective immune responses against pre-erythrocytic malaria parasites using transgenic RMP expressing the immunological reporter protein, ovalbumin (OVA).**
57. Lundie RJ, De Koning-Ward TF, Davey GM, et al. Blood-stage *Plasmodium* infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. *Proc Natl Acad Sci U S A*. 2008;105:14509–14514.
58. Miyakoda M, Kimura D, Yuda M, et al. Malaria-specific and non-specific activation of CD8+ T cells during blood stage of *Plasmodium berghei* infection. *J Immunol*. 2008;181:1420–1428.
59. Blagborough AM, Musychuk K, Bi H, et al. Transmission blocking potency and immunogenicity of a plant-produced Pvs25-based subunit vaccine against *Plasmodium vivax*. *Vaccine*. 2016;34:3252–3259.
- **Testing immunogenicity of transmission blocking vaccines, in vivo, using chimeric RMP expressing the *P. vivax* surface ookinete antigen P25.**
60. Bergmann-Leitner ES, Duncan EH, Mullen GE, et al. Critical evaluation of different methods for measuring the functional activity of antibodies against malaria blood stage antigens. *Am J Trop Med Hyg*. 2006;75:437–442.
61. Duncan EH, Bergmann-Leitner ES. Miniaturized growth inhibition assay to assess the anti-blood stage activity of antibodies. *Methods Mol Biol*. 2015;1325:153–165.
62. Blagborough AM, Yoshida S, Sattabongkot J, et al. Intranasal and intramuscular immunization with Baculovirus Dual Expression System-based Pvs25 vaccine substantially blocks *Plasmodium vivax* transmission. *Vaccine*. 2010;28:6014–6020.
63. Miura K, Deng B, Tullo G, et al. Qualification of standard membrane-feeding assay with *Plasmodium falciparum* malaria and potential improvements for future assays. *PLoS One*. 2013;8:e57909.
64. Ploemen IH, Prudencio M, Douradinha BG, et al. Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. *Plos One*. 2009;4:e7881.
- **A study describing the use of luciferase expressing transgenic RMP in simple and quantitative assays to determine parasite liver loads in mice.**
65. Miller JL, Murray S, Vaughan AM, et al. Quantitative bioluminescent imaging of pre-erythrocytic malaria parasite infection using luciferase-expressing *Plasmodium yoelii*. *Plos One*. 2013;8:e60820.
66. Meister S, Plouffe DM, Kuhlen KL, et al. Imaging of *Plasmodium* liver stages to drive next-generation antimalarial drug discovery. *Science*. 2011;334:1372–1377.
67. Mwakwingwe A, Ting L-M, Hochman S, et al. Noninvasive real-time monitoring of liver-stage development of bioluminescent *Plasmodium* parasites. *J Infect Dis*. 2009;200:1470–1478.
68. Portugal S, Carret C, Recker M, et al. Host-mediated regulation of superinfection in malaria. *Nat Med*. 2011;17:732–737.

69. Keitany GJ, Sack B, Smithers H, et al. Immunization of mice with live-attenuated late liver stage-arresting *Plasmodium yoelii* parasites generates protective antibody responses to preerythrocytic stages of malaria. *Infect Immun.* 2014;82:5143–5153.
70. Sack BK, Miller JL, Vaughan AM, et al. Model for in vivo assessment of humoral protection against malaria sporozoite challenge by passive transfer of monoclonal antibodies and immune serum. *Infect Immun.* 2014;82:808–817.
- **A study describing a transgenic *P. falciparum* line expressing luciferase, to examine liver infections in the presence of *P. falciparum* sporozoite antibodies using in immune-compromised mice (FRG huHep) engrafted with human liver tissue.**
71. Miller JL, Sack BK, Baldwin M, et al. Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Rep.* 2014;7:436–447.
72. Hopp CS, Sinnis P. The innate and adaptive response to mosquito saliva and *Plasmodium* sporozoites in the skin. *Ann N Y Acad Sci.* 2015;1342:37–43.
73. Cockburn IA, Tse S-W, Radtke AJ, et al. Dendritic cells and hepatocytes use distinct pathways to process protective antigen from *Plasmodium* in vivo. *Plos Pathog.* 2011;7:e1001318.
74. Vanderberg JP. Imaging mosquito transmission of *Plasmodium* sporozoites into the mammalian host: immunological implications. *Parasitol Int.* 2014;63:150–164.
75. Dups JN, Pepper M, Cockburn IA. Antibody and B cell responses to *Plasmodium* sporozoites. *Front Microbiol.* 2014;5:625.
76. Ménard R, Tavares J, Cockburn I, et al. Looking under the skin: the first steps in malarial infection and immunity. *Nat Rev Microbiol.* 2013;11:701–712.
- **In this review, studies are highlighted on immune responses against sporozoites in the skin, including studies with transgenic RMP expressing fluorescent reporter proteins.**
77. Radtke AJ, Kastenmuller W, Espinosa DA, et al. Lymph-node resident CD8alpha+ dendritic cells capture antigens from migratory malaria sporozoites and induce CD8+ T cell responses. *PLoS Pathog.* 2015;11:e1004637.
78. Kebaier C, Voza T, Vanderberg J. Kinetics of mosquito-injected *Plasmodium* sporozoites in mice: fewer sporozoites are injected into sporozoite-immunized mice. *Plos Pathog.* 2009;5:e1000399.
- **A study describing immune responses against sporozoites quantified using transgenic RMP expressing fluorescent proteins.**
79. Vaughan AM, Mikolajczak SA, Camargo N, et al. A transgenic *Plasmodium falciparum* NF54 strain that expresses GFP-luciferase throughout the parasite life cycle. *Mol Biochem Parasitol.* 2012;186:143–147.
80. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc.* 2006;1:346–356.
81. Lin JW, Annoura T, Sajid M, et al. A novel ‘gene insertion/marker out’ (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. *PLoS One.* 2011;6:e29289.
- **The description of a simple and rapid method to introduce transgenes into RMP genome, free of drug-selectable markers.**
82. Wijayalath W, Majji S, Villasante EF, et al. Humanized HLA-DR4.RagKO. IL2RgammackO.NOD (DRAG) mice sustain the complex vertebrate life cycle of *Plasmodium falciparum* malaria. *Malar J.* 2014;13:386.
83. Vaughan AM, Mikolajczak SA, Wilson EM, et al. Complete *Plasmodium falciparum* liver-stage development in liver-chimeric mice. *J Clin Invest.* 2012;122:3618–3628.
84. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. *Nat Rev Immunol.* 2011;11:57–64.
85. Espinosa DA, Radtke AJ, Zavala F. Development and assessment of transgenic rodent parasites for the preclinical evaluation of Malaria vaccines. *Methods Mol Biol.* 2016;1403:583–601.
86. Kafuye-Mlwilo MY, Mukherjee P, Chauhan VS. Kinetics of humoral and memory B cell response induced by the *Plasmodium falciparum* 19-kilodalton merozoite surface protein 1 in mice. *Infect Immun.* 2012;80:633–642.
87. Sachdeva S, Mohammed A, Dasaradhi PV, et al. Immunogenicity and protective efficacy of *Escherichia coli* expressed *Plasmodium falciparum* merozoite surface protein-1(42) using human compatible adjuvants. *Vaccine.* 2006;24:2007–2016.
88. Bouharoun-Tayoun H, Druilhe P. Antibody-dependent cell-mediated inhibition (ADCI) of *Plasmodium falciparum*: one- and two-step ADCI assays. *Methods Mol Biol.* 2015;1325:131–144.
89. Epstein JE, Richie TL. The whole parasite, pre-erythrocytic stage approach to malaria vaccine development: a review. *Curr Opin Infect Dis.* 2013;26:420–428.
90. Hoffman SL, Billingsley PF, James E, et al. Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. *Hum Vaccin.* 2010;6:97–106.
91. Hafalla JC, Rai U, Morrot A, et al. Priming of CD8+ T cell responses following immunization with heat-killed *Plasmodium* sporozoites. *Eur J Immunol.* 2006;36:1179–1186.
92. Annoura T, Ploemen IH, Van Schaijk BC, et al. Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. *Vaccine.* 2012;30:2662–2670.
93. Labaied M, Harupa A, Dumpit RF, et al. *Plasmodium yoelii* sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. *Infect Immun.* 2007;75:3758–3768.
94. Kublin JG, Mikolajczak SA, Sack BK, et al. Complete attenuation of genetically engineered *Plasmodium falciparum* sporozoites in human subjects. *Sci Transl Med.* 2017;9(371). pii: eaad9099.
95. Spring M, Murphy J, Nielsen R, et al. First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of *Anopheles* mosquitoes to adult volunteers. *Vaccine.* 2013;31:4975–4983.
96. Van Schaijk BC, Ploemen IHJ, Annoura T, et al. A genetically attenuated malaria vaccine candidate based on *P. falciparum* b9/sIarp gene-deficient sporozoites. *Elife.* 2014;3.
97. Nganou-Makamdop K, Ploemen I, Behet M, et al. Reduced *Plasmodium berghei* sporozoite liver load associates with low protective efficacy after intradermal immunization. *Parasite Immunol.* 2012;34:562–569.
- **A study comparing protective efficacy induced by attenuated sporozoites delivered by different routes of administration using luciferase expressing chimeric RMP to determine parasite liver loads.**
98. Ploemen I, Behet M, Nganou-Makamdop K, et al. Evaluation of immunity against malaria using luciferase-expressing *Plasmodium berghei* parasites. *Malar J.* 2011;10:350.
99. Cockburn IA, Amino R, Kelemen RK, et al. In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proc Natl Acad Sci U S A.* 2013;110:9090–9095.
- **A study showing the importance of CD8+ T cells in protective immunity after immunization with attenuated sporozoites using fluorescent RMP and intravital imaging.**
100. Trimmell A, Takagi A, Gupta M, et al. Genetically attenuated parasite vaccines induce contact-dependent CD8+ T cell killing of *Plasmodium yoelii* liver stage-infected hepatocytes. *J Immunol.* 2009;183:5870–5878.
101. Yewdell JW. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity.* 2006;25:533–543.