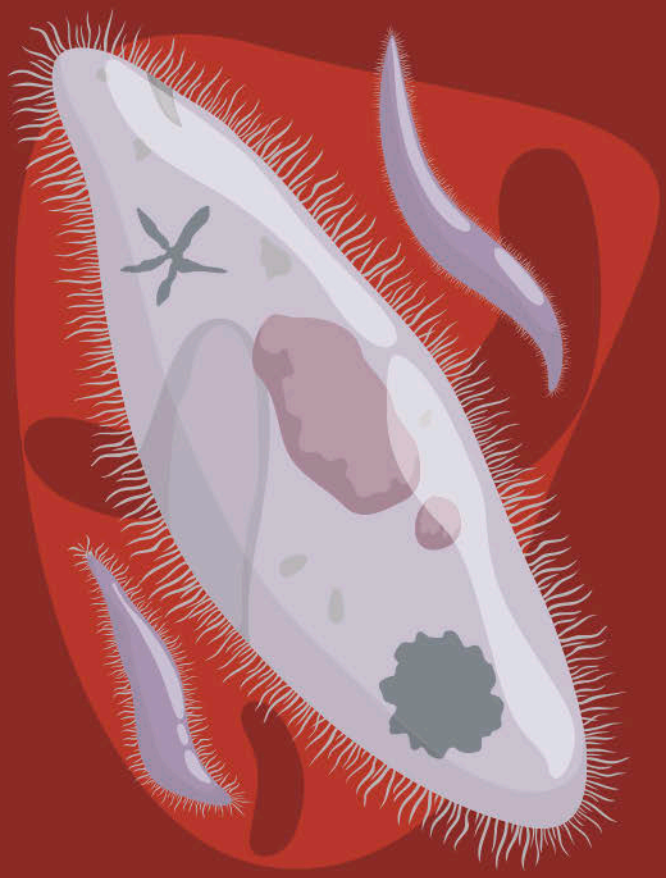
Studies using

TRANSGENIC RODENT MALARIA PARASITES



to improve

LIVE ATTENUATED MALARIA VACCINES

Ahmad Syibli Bin Othman

STUDIES USING TRANSGENIC RODENT MALARIA PARASITES TO IMPROVE LIVE ATTENUATED MALARIA VACCINES

Ahmad Syibli bin Othman

ISBN: 978-94-6182-915-3

The work presented in this thesis was performed at the Leiden Malaria Research Group, Department of Parasitology of the Leiden University Medical Center in the Netherlands.

Layout and printing production: Off Page, Amsterdam.

STUDIES USING TRANSGENIC RODENT MALARIA PARASITES TO IMPROVE LIVE ATTENUATED MALARIA VACCINES

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 25 oktober 2018
klokke 15:00 uur

door

Ahmad Syibli bin Othman
geboren te Kuala Terengganu (Malaysia) in 1986

PROMOTOR

Prof. Dr. M. Yazdanbakhsh

CO-PROMOTOR

Dr. S.M. Khan
Dr. C.J. Janse

LEDEN PROMOTIECOMMISSIE

Prof. Dr. A. Geluk
Prof. Dr. F.A. Ossendorp
Dr. M. Prudêncio (Instituto de Medicina Molecular, Portugal)
Dr. C. Kocken (Biomedical Primate Research Centre, The Netherlands)

DEDICATION

This thesis is dedicated to the memory of my father,
Othman bin Zit,
believed in and inspired me;
to my mother,
Zarina binti Abd. Lateh,
provided unconditional love and patience;
to my wife,
Nurul Alia binti Azizan,
has supported me in all endeavors;
and my children,
Aisyah and Amiru,
made it all so worthwhile.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

MALARIA, THE PARASITE AND DISEASE

Malaria is a life-threatening disease caused by *Plasmodium* parasites that are transmitted from person to person by the bites of infected *Anopheles* mosquitoes. There are five *Plasmodium* species that can cause malaria in humans; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* [1] and two of these species, *P. falciparum* and *P. vivax*, are responsible for most cases of human mortality and morbidity [2]. The World Health Organization (WHO) report that in 2016, there were an estimated 216 million cases of malaria in 91 countries, an increase of 5 million cases from 2015. Malaria deaths reached 445,000 in 2016, with a comparable number of deaths for 2015 (446,000). The WHO African Region carries a disproportionately high share of the global malaria burden. In 2016, the region was accounted for 90% of malaria cases and 91% of malaria deaths [3].

The *P. falciparum* life-cycle

The malaria parasite is transmitted to a vertebrate host when an infected female *Anopheles* mosquito takes a blood meal and simultaneously injects sporozoites into the skin. The life-cycle of the human malaria parasite *P. falciparum* is shown in **Figure 1**. Sporozoites migrate out of the skin, by locating and traversing a blood vessel whereupon they enter the blood stream. Sporozoites are carried to the liver where they actively invade hepatocytes; here, depending on the *Plasmodium* species, they grow and divide over 2-16 days and produce tens of thousands of merozoites per liver cell [4]. Merozoites exit the liver and enter the blood stream where they invade and multiply inside the red blood cells which eventually break open, allowing the parasites to infect additional red blood cells. Blood stage parasites continue their cycle of invading red blood cells, asexual replication, and then releasing newly formed merozoites repeatedly. This invasion-replication-release-reinvasion cycle of blood stages can cause an exponential increase in infected red blood cells and it is the host response to parasite molecules in combination with interactions of infected erythrocytes with host tissue that give rise to the pathological symptoms of malaria. During each cycle, a small subset of asexual blood stage parasites divert from asexual replication and instead differentiate into male or female sexual forms, known as gametocytes. In the case of the human malaria parasite *P. falciparum*, these intracellular gametocytes mature and progress through stages I–V over the course of eight to ten days (gametocytogenesis). If taken up by a mosquito in a blood meal, the mature gametocytes are capable of propagating an infection in mosquitoes. Inside the mosquito midgut, male and female gametocytes mature into gametes (gametogenesis), with the male gametocyte rapidly dividing to form eight flagellated microgametes (exflagellation) and the female gametocyte emerges from the red blood cell and develops into a single macrogamete. Fertilization of a macrogamete by a microgamete results in the development of a zygote, which undergoes meiosis and matures into an invasive ookinete that can penetrate the mosquito gut wall. The ookinete forms an oocyst within which the parasite asexually replicates, creating several thousand sporozoites (sporogony). Upon oocyst rupture,

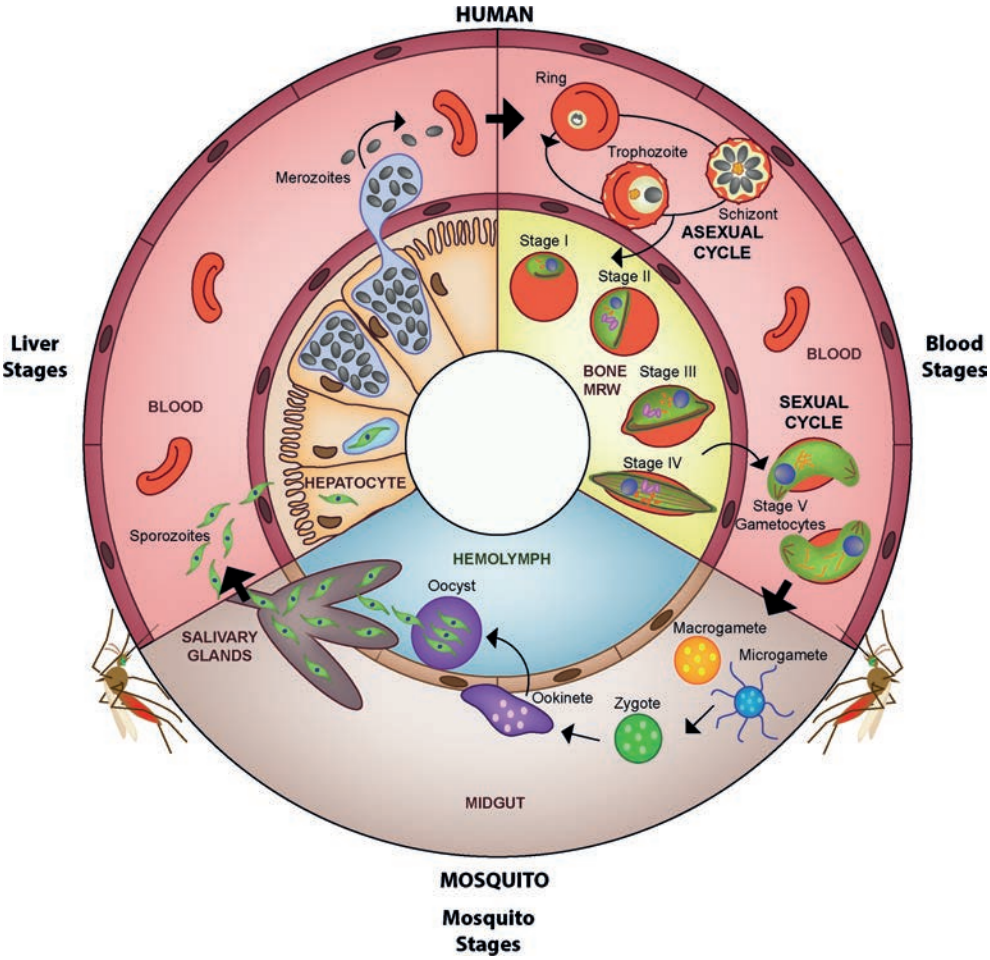


Figure 1. The life-cycle of the human malaria parasite *P. falciparum*. *P. falciparum* replication and maturation in humans (in red) and mosquitoes (in grey). This image was taken from Nilsson, S.K., et al. (2015).

these sporozoites travel to the salivary glands, where they can be transmitted back to the vertebrate host during a blood meal.

MALARIA, THE HEALTH PROBLEM

Nearly half of the world's population are at risk of malaria. Most malaria cases and deaths occur in sub-Saharan Africa [5]. However, regions of South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas are also at risk. In 2016, 91 countries had ongoing malaria transmission. Some population groups are at a considerably higher risk of contracting malaria and developing severe diseases than others. These include infants, children under 5 years of age, pregnant women and patients with HIV/AIDS, as

well as non-immune migrants, mobile populations and travelers [3]. Many prevention and control measures, such as the use of insecticide-treated mosquito nets, indoor spraying with insecticides and implementation of drug treatment programs limit both the incidence and spread of the infection, as well as limiting the severity of the disease. In addition, recent measures to improve early diagnosis and treatment of malaria has reduced disease and prevented deaths [6-8], and has also resulted in reducing malaria transmission. While these measures have contributed to a global decline in malaria, they are all under threat from the acquisition of development of resistance, either by *Plasmodium* parasites to antimalarial drugs or by mosquitoes to insecticides. In recent years, mosquito resistance to pyrethroids has emerged in many countries [9, 10] and resistance has been developed by the parasites against antimalarial drugs, specifically resistance against artemisinin which has been detected in a number of countries in South-East Asia [11, 12]. *P. falciparum* is responsible for the most severe disease and accounts for the most numbers of deaths and has, therefore, been the target of most antimalarial drugs and vaccine development efforts.

Malaria causes significant economic losses in high-burden countries. UNICEF has estimated that malaria costs Africa more than $12 billion annually in lost Gross Domestic Product (GDP), and a reduction in economic growth by more than 1% a year [13]. Malaria-endemic countries are among the world's most impoverished and a family can spend an average of over one quarter of its income on malaria treatment, as well as paying prevention costs and suffering loss of income [14, 15]. Despite the recent reduction in deaths and mortality due to malaria, it is widely believed that the most cost-effective means to prevent disease , and indeed disease elimination or eradication, is the mass administration of vaccines [16, 17].

MALARIA, VACCINE DEVELOPMENT

Despite major efforts over the past 70 years to develop a vaccine, there is currently no licensed malaria vaccine available. The most advanced malaria vaccine is the sub-unit vaccine RTS,S that is based on the immunodominant sporozoite surface antigen, circumsporozoite protein (CSP), fused to hepatitis B virus surface antigen [18]. This sub-unit vaccine was formulated with the potent liposomal adjuvant system AS01 from GlaxoSmithKline to target the sporozoite/liver stage of *P. falciparum* and has advanced to Phase IV clinical trials [19]. However in field studies, the efficacy of RTS,S against clinical malaria has been modest; between 30% and 40% in children between the ages of 5 and 17 months [20], and vaccine efficacy rapidly declined over time [21]. Nonetheless, in 2016 the WHO announced that the RTS,S vaccine would be rolled out in pilot projects in selected areas in 3 countries in sub-Saharan Africa: Ghana, Kenya and Malawi [19]. The limited success achieved in inducing sterile and long-lasting protective immunity against malaria using sub-unit vaccines has led to renewed interest in whole organism vaccination strategies [22].

Whole sporozoite (wsp) vaccine approaches

Despite three decades of testing different (recombinant) sub-unit vaccines both in the clinic and the field, only modest protection against malaria infection has been achieved [21-24] and this has renewed an interest in whole parasite-based vaccine approaches [25, 26]. Sporozoite-based vaccination strategies aim at preventing the parasite's life-cycle progression from hepatic stages to the symptomatic blood stages of infection while eliciting potent pre-erythrocytic immune responses. Such whole sporozoite malaria (wsp) vaccination strategies are unique in their potential to induce sterile protection against a new infection and have led to the development of various vaccine candidates, currently undergoing preclinical and clinical development. It was the discovery of the pre-erythrocytic stages of *Plasmodium* [7], followed by the establishment of a mouse model of malaria [8], that enabled the laboratory production of all stages of the parasite's life-cycle [9], which eventually led to the landmark demonstration that live sporozoites attenuated by X-irradiation (RAS) could be used to elicit sterile protection against a new infection [10, 11]. This discovery was soon expanded to humans with the demonstration that volunteers could be protected against homologous and heterologous strains of *P. falciparum* parasites by immunization of live, attenuated sporozoites [12–14]. The success of these studies in both animal malaria models and humans resulted in a large number of subsequent studies that were aimed at optimizing sporozoite-based immunization and to characterize the immune responses elicited by these strategies [15–20]. However, the production and administration of live sporozoites attenuated by irradiation (Irr-Spz) was considered to be a major obstacle to the development of sporozoite vaccines at the end of the 20th century and efforts focused more on the characterization of sub-unit vaccine that targeted different points of the parasite life-cycle. However, as the reduced efficacy of sub-unit vaccines became increasingly evident, at the beginning of the new century, a renewed call for the development of sporozoite-based immunization strategies took place. In the early part of this century malaria research entered the genomic era, with genome sequences of various *Plasmodium* parasite species becoming available, as well as transcriptomic and proteomic datasets from different parasite developmental stages [42–48]. This information was used to identify genes that play essential roles in distinct points of the parasite's life-cycle, and these were targeted for deletion using increasingly sophisticated methodologies for stable transfection of *Plasmodium* parasites [49–55]. This in turn, resulted in the generation of genetically attenuated parasites (GAPs), whose liver-stage development is arrested by deletion of specific gene(s). Studies in rodents demonstrated that GAPs, like irradiated sporozoites, were able to elicit a strong immune protection [56–58]. Informed by GAP studies performed in rodent models, the first *P. falciparum* GAPs were developed, with some now entering into clinical development as human vaccine candidates [59, 60].

Improving wsp vaccine approaches: the aim of the studies described in this thesis

A number of studies have shown that Irr-Spz can generate strong protective immunity in humans [27-29]. However, in order to achieve sterile immunity, multiple immunizations with high numbers of attenuated sporozoites are required [27, 30]. These high numbers of sporozoites has cost-of-goods implications and increases the burden on the complicated sporozoite production procedure, which can result in limitations in the practical mass administration of such vaccines in malaria-endemic countries.

The major challenge for sporozoite-based vaccines is to produce a highly immunogenic live-attenuated vaccine, which requires the fewest attenuated sporozoites per dose and the fewest doses in order to induce sustained sterile protection against malaria in the field. In rodent models of malaria it has been shown that immunization with sporozoites of GAPs can induce similar, or even better, levels of protective immunity compared to Irr-Spz [31-34]. Genetic attenuation of sporozoites has been achieved through the deletion of one or more genes that play a critical role during liver stage development, resulting in complete arrest of parasite growth in the liver, thereby preventing a blood stage infection after immunization with GAP sporozoites. An advantage of GAP compared to Irr-Spz vaccination, is that GAP sporozoites are genetically homogenous with defined genetic identity and attenuation phenotype, and GAPs can be further modified to induce optimal protective immunity.

GAPs have additional advantages over Irr-spz, in particular in manufacturing. GAP sporozoites do not need to be irradiated before they are vialed and their production poses little risk to the individuals who produce the vaccine as, GAP sporozoites are unable to establish a pathogenic blood stage infection [32-34].

GAP studies performed in rodent malaria models have been critical for the creation of several *P. falciparum* GAP vaccines, which are undergoing clinical evaluation [35-39]. Studies in rodents have also been used to identify the immunological basis of GAP-induced immune responses and to improve GAP immunogenicity [33, 34, 37]. While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, T cells appear to be critical for protection and in particular $CD8^+$ T cells are thought to play a major role in eliminating infected hepatocytes. Early rodent studies using Irr-Spz have demonstrated a vital role for $CD8^+$ T cells [40, 41]. Recent mechanistic investigations into protective immune responses induced by immunization with attenuated sporozoites have demonstrated diverse and robust immune responses that encompasses both $CD8^+$ and $CD4^+$ T cells, as well as a significant contribution from antibodies [42, 43]. Nonetheless, $CD8^+$ T cells are considered to be the main effector cells in eliciting protection after sporozoites immunization [44].

In this thesis we describe a set of studies performed in rodent models of malaria to improve malaria vaccines, in particular GAP vaccines. We attempted to increase GAP immunogenicity by: (i) adding adjuvants during GAP immunization; (ii) introducing genes

encoding putative immunomodulatory proteins in the GAP genome to create 'self-adjuvanting' parasites; (iii) generating GAPs that arrest late into liver-stage development (LA-GAP) to increase antigen load and diversity during immunization; and (iv) exploring possibilities to genetically modify parasite to express vaccine antigens from different life-cycle stages, in order to test the ability of parasites to induce immune responses against multiple life-cycle stages and to inform the creation of a 'multi-stage' GAP vaccine.

Outlined below is the rationale for choosing the different adjuvants, putative immunomodulatory proteins, the different approaches to generate LA-GAPs and parasites engineered to express additional vaccine candidate antigens. We have used well established rodent malaria models [31, 45, 46] in combination with standard and adapted protocols for immunization [46, 47] in order to evaluation of protective immune responses induced by the different GAPs and immunization approaches.

Transgenic parasites and malaria vaccine research (Chapter 2)

Chapter 2 reviews the use of transgenic malaria parasites in vaccine research. Genetic modification of rodent and human malaria parasites have been critical for generation of GAPs that arrest in the liver and transgenic rodent malaria parasites have been extensively used for testing the safety and immunogenicity of GAPs [33, 34, 37]. Many gene-deletion rodent parasites have been tested in mice to examine growth and arrest in the liver and for their capacity to induce potent protective immune responses. Many GAPs have been created in transgenic reporter lines that express fluorescent and/or luminescent proteins, which permits an *in vivo*, real-time, evaluation of both their arrest characteristics 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 thesis we use a variety of well-established and genetic modification technologies to create a variety of (transgenic) rodent malaria parasite mutants. Specifically, we have generated transgenic 'self-adjuvanted' GAPs (**Chapter 4**), gene-deletion late-arresting GAPs (**Chapter 5**) and transgenic parasites expressing additional *Plasmodium* vaccine antigens (**Chapter 6**). In studies where we examined if exogenous adjuvants could improve GAP immunogenicity (**Chapter 3**) as well as in the studies in **Chapters 4** & **5**, we made use of transgenic parasites that express luminescent and fluorescent reporter proteins to quantify parasite development *in vivo*.

Improving GAP immunization by the addition of immunostimulatory molecules (Chapter 3)

Protection against a malaria infection can be achieved by immunization with live-attenuated *Plasmodium* sporozoites. While the precise mechanisms of protection remain unknown, T cell responses are thought to be critical in the elimination of infected liver cells. Only a limited number of studies have been performed on the effect of adjuvants on protective immunity induced by whole sporozoite immunization. In particular, the use of the glycolipid α-galactosylceramide (α-GalCer) [48] and its analog 7DW8-5 have been analyzed [49]. Co-administration of these molecules with sporozoites resulted in enhanced recruitment and activation/maturation of dendritic cells in lymph nodes draining the site of vaccine administration and thereby enhancing parasite-specific T cell immunogenicity.

Recently, cancer immunotherapies have employed antibodies that target proteins on the surface of T cells, as treatment with these antibodies have been shown to restore, expand and enhance the function of tumour-reactive T cells. The antagonistic antibodies targeting CTLA-4 and PD-1 have been used to block inhibitory signals to T cells [50, 51], while agonistic antibodies targeting CD27, OX40 and 4-1BB on $CD4^+$ and $CD8^+$ T cells have been used to increase costimulatory signals [52-54]. These immunostimulatory antibodies have been shown to improve the control of tumors and this was associated with an increase in tumor-specific T cell function [55]. In **Chapter 3**, we describe studies that examine if agonistic OX40 monoclonal antibody (OX40 mAb) treatment improved protective immunity in mice, induced by immunization with a late-arresting GAP. We immunized BALB/c mice using sporozoites of a *P. yoelii* GAP, an established rodent model to evaluate GAP vaccination [31]. In addition, we describe the development of a GAP immunization protocol in BALB/c mice that permits a rapid screening and evaluation of different approaches to enhance GAP protective immunity in BALB/c mice.

Improving GAP immunogenicity by creating 'self-adjuvanting' parasites that also express putative immunomodulatory molecules (Chapter 4)

As described in **Chapter 3**, GAP immunization in combination with exogenous adjuvants provides useful information about mechanisms underlying protective immunity. However, the use of such adjuvants in populations where malaria is endemic may be difficult due to cost-of-goods, applicability or side-effects. Further, induction of protective immune responses by GAP immunization is dependent on sporozoites migrating to the liver and invading hepatocytes. The administration of adjuvants at the site of GAP injection will result in systemic distribution of the adjuvant which will therefore be considerably diluted at the sites where parasite antigens are taken up by antigen presenting cells (APCs), i.e. the liver, spleen or proximal lymph nodes [56]. In order to maximize the adjuvant effect, i.e. the increase of antigen uptake by APCs and providing stimulatory signals to enhance APC function, it is important to maximize the adjuvant effect at the point of antigen uptake and processing [56, 57].

Due to the limitations of co-injecting adjuvants with attenuated sporozoites, we explored in **Chapter 4** the possibility of creating GAPs that express immunomodulatory proteins in sporozoites and liver stages, so-called adjuvant GAPs [58-61]. Self-adjuvanting vaccines, in which the antigenic and adjuvanting moieties of the vaccines are present in the same molecule, have been developed for sub-unit vaccines targeting cancer cells, viruses [62, 63], nematodes [64] and bacteria [65, 66], for example by conjugation of lipopeptide-based Toll-like receptor (TLR) agonists to the target protein [61]. In vaccine development against malaria, the vaccine candidate antigen CSP has been fused to bacterial flagellin [67], a protein which is a potent TLR5 agonist [68]. However, to the best of our knowledge, no sporozoite-based vaccine has been reported that expresses additional immunomodulatory/adjuvant molecules [33, 37].

We selected four TLR agonists that can increase adaptive immune responses and have the ability to improve cross-presentation of antigens as has been demonstrated in other animal and/or human studies. The selected adjuvant proteins are: (i) nontoxic cholera toxin B sub-unit from *Vibrio cholerae* (CTB) [69, 70]; (ii) heat shock protein Gp96 of mice (Gp96) [71-73]; (iii) heat shock protein X from *Mycobacterium tuberculosis* (HspX) [74, 75]; and (iv) the TLR5 binding region of *Salmonella typhimurium* flagellin (amino acids 89–96; FliC) [68, 76, 77].

To facilitate the generation of multiple 'self-adjuvanting' lines in *P. yoelii* LA-GAP, we generated a GIMO locus in the *P. yoelii fabb/f* gene locus, thereby creating a novel *P. yoelii* GIMO GAP mother line. This line was used for the rapid introduction of the adjuvant fusion-transgenes into the *P. yoelii* genome without retention of a drug selectable marker (SM). The genes encoding the 'adjuvant' proteins were fused to a *Plasmodium* gene expressed in liver stages, *uis4* (PY17X_0502200). UIS4 is located at the parasitophorous vacuole membrane (PVM) in infected hepatocytes [78]. We fused the adjuvant proteins to a PVM protein as it has been shown that ovalbumin (OVA) fused to proteins located in the PV/PVM induce stronger T cell responses than ovalbumin expressed in the cytoplasm of transgenic parasites [79, 80]. The fusion genes were introduced by GIMO transfection [81, 82] into the novel GIMO GAP mother line. The four adjuvant GAP were analyzed for protective immunity using the *P. yoelii*-BALB/c screening model for assessing protective immunity after GAP immunization [45]. This model is described in **Chapter 3**, where we describe analyses on protective immunity induced by immunization of GAP in combination with the exogenous adjuvant OX40.

The generation and characterization of novel late-arresting GAPs (LA-GAPs) (Chapter 5)

It has been shown that immunization of mice with GAP that arrest late during liver stage development can induce higher levels of protective immunity compared to immunization with GAP that arrest early after invasion of hepatocytes. Specifically, it has been shown that late-arresting GAP (LA-GAP) induce greater numbers of a broader range of $CD8^+$ T cells, which results in increased protection against a malaria infection compared to

immunization with early-arresting GAPs [31], most probably due to a greater number and repertoire of antigens expressed by LA-GAP. This may also explain the high degree of protection observed when humans are immunized by fully infectious sporozoites under chemoprophylactic treatment with chloroquine [83, 84]. In this immunization approach, liver stage development progresses normally but the merozoites that are released from the liver and infect erythrocytes are killed by chloroquine. This whole sporozoite vaccination approach induces sterile protection against parasite challenge, but requires approximately 60-fold- fewer cumulative sporozoites than immunization with Irr-Spz that arrest early during liver stage development [85].

A prerequisite for a GAP vaccine for human use is that the GAP sporozoites are unable to establish a potentially pathogenic blood stage infection and therefore parasites must completely arrest during development in the liver. Consequently, multiple gene deletions in the same GAP are considered necessary, each governing independent but essential processes during liver-stage development. Currently, three *P. falciparum* GAPs have been developed for clinical evaluation and all are early-arresting GAPs, that arrest development soon after hepatocyte invasion. In these GAPs either two or three genes have been deleted, which encode proteins that play a vital role in early liver stage development. Three of the selected proteins, P52, P36 and B9, are all members of the so-called 6-Cys gene family and all participate in the formation/maintenance of the parasitophorous vacuole (PV) inside the infected hepatocyte [86, 87]. The fourth protein, SLARP/SAP1, is involved in regulation of parasite gene expression [88, 89]. In contrast to the creation of early arresting-GAPs, the generation of safe LA-GAPs have been challenging. Several genes have been identified that encode proteins that play an important role during late liver stage development but deletion of those genes did not result in complete growth arrest in rodent models of malaria. Examples include multiple proteins involved in type II fatty acid synthesis pathways (FAS II, i.e. Fab proteins) [90, 91], a transcription factor with AP2 domain(s) (AP2-L) [92], biotin-protein ligase 1 (HCS1) [93] and proteins involved in formation and egress of merozoites from liver schizonts, i.e. liver merozoite formation protein (PALM) [94], putative liver stage protein 1 (LISP1) [95, 96], sequestrin or liver-specific protein 2 (LISP2) [86] and ZIP domain-containing protein (ZIPCO) [97]. Only the deletion of the genes encoding FabB/F [90] and MEI2-like RNA-binding protein (PlasMei2) [98] have been reported to result in complete growth arrest in the rodent parasite *P. yoelii*. However, studies in *P. falciparum* have shown that parasites lacking FabB/F expression are unable to complete mosquito stage development [99].

In order to create an LA-GAP that completely arrests late into liver stage development and cannot establish a blood infection, we describe in **Chapter 5** studies where we create double gene deletion mutants using combinations of different genes that have a role in late liver stage development and could synergize to create fully arrested GAPs. We describe attenuation evaluation studies as well as immunogenicity testing of LA-GAPs to identify the ones with the best profile to advance into *P. falciparum* studies.

Generation of transgenic parasites expressing antigens from other life-cycle stages (Chapter 6)

The creation of GAPs expressing vaccine antigens from either different parasite life-cycle stages or strains could improve GAP vaccine potency by providing stage and strain transcending immunity, respectively. In order to establish if transgenic parasites can express additional proteins and if these antigens are able to provoke immune responses, we examined if *P. berghei* parasite could be used to express the *P. falciparum* transmission blocking vaccine candidate antigen, Pfs48/45. We expressed Pfs48/45 in *P. berghei* blood stages, as they are easier to produce than sporozoites, and next we examined if these blood stage parasites could be used to provoke antibody responses against Pfs48/45.

Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed [100, 101]. This hampers the screening of *Plasmodium* antigens in immunization studies for their suitability as vaccine candidate antigens. Preclinical evaluation of *Plasmodium* antigens often involves immunizing rodents with recombinant *Plasmodium* proteins followed by an examination of induced immune responses, either *in vivo* using rodent models of malaria or *in vitro* by performing functional assays with human malaria parasites incubated with immune sera [46]. Transgenic rodent malaria parasites (RMP) expressing human malaria parasite (HMP) antigens are increasingly used to evaluate and rank the order of candidate malaria vaccines before investing in scalable manufacture to support advancement to clinical testing [46].

We reasoned that the use of transgenic RMP expressing HMP proteins for production of HMP proteins would circumvent the above-mentioned problems associated with expression in heterologous expression systems including peculiarities of post-translational modifications and *Plasmodium*-specific domains involved in protein trafficking and cellular location. As a proof of concept we explored in **Chapter 6** the possibility of expressing an antigen, Pfs48/45 of gametocytes of the human malaria parasite *P. falciparum* in blood stages of the rodent parasite *P. berghei*. Expression of Pfs48/45 for transmission blocking immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein [102, 103].

The creation of transgenic parasites that express antigens from multiple life-cycles that can induce potent immune responses is also of interest to the development of whole organism vaccines [32]. GAPs could be further modified to induce immune responses against multiple life-cycle stages by expression in GAPs blood- or transmission-stage antigens to produce a multi-stage GAP vaccine. In **Chapter 6** we describe studies analyzing expression of Pfs48/45 in *P. berghei* blood stages and the immunogenicity of *P. berghei* expressed Pfs48/45 by performing assays to measure transmission-reducing activity of sera/IgG of mice immunized with lysates of blood stage parasite that express the introduced antigen.

In **Chapter 7** the results of the studies described in **Chapters 2-6** are summarized and discussed, including a discussion on the composition of the 'next generation' GAP vaccine and challenges of creating a GAP vaccine that needs to induce strong, sustained protective immune responses against malaria parasites in the field.

REFERENCES

1. Cowman, A.F., et al., *Malaria: Biology and Disease.* Cell, 2016. **167**(3): p. 610-624.
2. Zuck, M., et al., *The Promise of Systems Biology Approaches for Revealing Host Pathogen Interactions in Malaria.* Front Microbiol, 2017. **8**: p. 2183.
3. WHO. *WHO Malaria Report 2017*. 2017; Available from: http://www.who.int/malaria/publications/world-malaria-report-2017/report/en/.
4. Nilsson, S.K., et al., *Targeting Human Transmission Biology for Malaria Elimination.* Plos Pathogens, 2015. **11**(6).
5. Cibulskis, R.E., et al., *Malaria: Global progress 2000 - 2015 and future challenges.* Infect Dis Poverty, 2016. **5**(1): p. 61.
6. Bhatt, S., et al., *The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015.* Nature, 2015. **526**(7572): p. 207-+.
7. White, N.J., et al., *Malaria.* Lancet, 2014. **383**(9918): p. 723-35.
8. Birkett, A.J., *Status of vaccine research and development of vaccines for malaria.* Vaccine, 2016. **34**(26): p. 2915-2920.
9. Trape, J.F., et al., *Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study.* Lancet Infect Dis, 2011. **11**(12): p. 925-32.
10. Ranson, H. and N. Lissenden, *Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control.* Trends Parasitol, 2016. **32**(3): p. 187-196.
11. Woodrow, C.J. and N.J. White, *The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread.* FEMS Microbiol Rev, 2017. **41**(1): p. 34-48.
12. Blasco, B., D. Leroy, and D.A. Fidock, *Antimalarial drug resistance: linking Plasmodium falciparum parasite biology to the clinic.* Nature Medicine, 2017. **23**(8): p. 917-928.
13. Gallup, J.L. and J.D. Sachs, *The economic burden of malaria.* Am J Trop Med Hyg, 2001. **64**(1-2 Suppl): p. 85-96.
14. CDC. *Malaria's Impact Worldwide*. 2018 May 3, 2018; Available from: https://www.cdc.gov/malaria/malaria_worldwide/impact.html.
15. Sicuri, E., et al., *The economic costs of malaria in children in three sub-Saharan countries: Ghana, Tanzania and Kenya.* Malar J, 2013. **12**: p. 307.
16. Ozawa, S., et al., *Cost-effectiveness and economic benefits of vaccines in low- and middle-income countries: a systematic review.* Vaccine, 2012. **31**(1): p. 96-108.
17. Greenwood, B., *The contribution of vaccination to global health: past, present and future.* Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1645): p. 20130433.
18. Draper, S.J., et al., *Malaria Vaccines: Recent Advances and New Horizons.* Cell Host Microbe, 2018. **24**(1): p. 43-56.
19. Coelho, C.H., et al., *Advances in malaria vaccine development: report from the 2017 malaria vaccine symposium.* npj Vaccines, 2017. **2**(1): p. 34.
20. Duncan, C.J. and A.V. Hill, *What is the efficacy of the RTS,S malaria vaccine?* BMJ, 2011. **343**: p. d7728.
21. Tinto, H., et al., *Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial.* Lancet, 2015. **386**(9988): p. 31-45.
22. Hoffman, S.L., et al., *The March Toward Malaria Vaccines.* American Journal of Preventive Medicine, 2015. **49**(6): p. S319-S333.
23. White, M.T., et al., *Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial.* Lancet Infectious Diseases, 2015. **15**(12): p. 1450-1458.

24. Mahmoudi, S. and H. Keshavarz, *Efficacy of phase 3 trial of RTS, S/AS01 malaria vaccine: The need for an alternative development plan.* Human Vaccines & Immunotherapeutics, 2017. **13**(9): p. 2098-2101.

25. Pinzon-Charry, A. and M.F. Good, *Malaria vaccines: the case for a whole-organism approach.* Expert Opinion on Biological Therapy, 2008. **8**(4): p. 441-448.

26. Hollingdale, M.R. and M. Sedegah, *Development of whole sporozoite malaria vaccines.* Expert Review of Vaccines, 2017. **16**(1): p. 45-54.

27. Sissoko, M.S., et al., *Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.* Lancet Infect Dis, 2017. **17**(5): p. 498-509.

28. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination.* Nature Medicine, 2016. **22**(6): p. 614-+.

29. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(10): p. 2711-2716.

30. Seder, R.A., et al., *Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine.* Science, 2013. **341**(6152): p. 1359-65.

31. Butler, N.S., et al., *Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites.* Cell Host Microbe, 2011. **9**(6): p. 451-62.

32. Bijker, E.M., et al., *Novel approaches to whole sporozoite vaccination against malaria.* Vaccine, 2015. **33**(52): p. 7462-7468.

33. Kreutzfeld, O., K. Muller, and K. Matuschewski, *Engineering of Genetically Arrested Parasites (GAPs) For a Precision Malaria Vaccine.* Front Cell Infect Microbiol, 2017. **7**: p. 198.

34. Singer, M. and F. Frischknecht, *Time for Genome Editing: Next-Generation Attenuated Malaria Parasites.* Trends Parasitol, 2017. **33**(3): p. 202-213.

35. van Schaijk, B.C.L., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.

36. Mikolajczak, S.A., et al., *A Next-generation Genetically Attenuated Plasmodium falciparum Parasite Created by Triple Gene Deletion.* Molecular Therapy, 2014. **22**(9): p. 1707-1715.

37. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Current Opinion in Biotechnology, 2012. **23**(6): p. 908-916.

38. Kublin, J.G., et al., *Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects.* Sci Transl Med, 2017. **9**(371).

39. Spring, M., et al., *First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers.* Vaccine, 2013. **31**(43): p. 4975-83.

40. Schofield, L., et al., *Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites.* Nature, 1987. **330**(6149): p. 664-6.

41. Weiss, W.R., et al., *CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites.* Proc Natl Acad Sci U S A, 1988. **85**(2): p. 573-6.

42. Doll, K.L. and J.T. Harty, *Correlates of protective immunity following whole sporozoite vaccination against malaria.* Immunol Res, 2014. **59**(1-3): p. 166-76.

43. Van Braeckel-Budimir, N., S.P. Kurup, and J.T. Harty, *Regulatory issues in immunity to liver and blood-stage malaria.* Curr Opin Immunol, 2016. **42**: p. 91-97.

44. Silvie, O., R. Amino, and J.C. Hafalla, *Tissue-specific cellular immune responses to malaria pre-erythrocytic stages.* Curr Opin Microbiol, 2017. **40**: p. 160-167.

45. Haeberlein, S., et al., *Protective immunity differs between routes of administration of*

attenuated malaria parasites independent of parasite liver load. Scientific Reports, 2017. **7**.

46. Othman, A.S., et al., *The use of transgenic parasites in malaria vaccine research.* Expert Review of Vaccines, 2017. **16**(7): p. 685-697.
47. van der Velden, M., et al., *Protective Efficacy Induced by Genetically Attenuated Mid-to-Late Liver-Stage Arresting Plasmodium berghei Deltamrp2 Parasites.* Am J Trop Med Hyg, 2016. **95**(2): p. 378-82.
48. Gonzalez-Aseguinolaza, G., et al., *Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines.* J Exp Med, 2002. **195**(5): p. 617-24.
49. Li, X., et al., *Colocalization of a CD1d-Binding Glycolipid with a Radiation-Attenuated Sporozoite Vaccine in Lymph Node-Resident Dendritic Cells for a Robust Adjuvant Effect.* J Immunol, 2015. **195**(6): p. 2710-21.
50. Curran, M.A., et al., *PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(9): p. 4275-4280.
51. Wolchok, J.D., et al., *Nivolumab plus Ipilimumab in Advanced Melanoma.* New England Journal of Medicine, 2013. **369**(2): p. 122-133.
52. Croft, M., *Costimulation of T cells by OX40, 4-1BB, and CD27.* Cytokine Growth Factor Rev, 2003. **14**(3-4): p. 265-73.
53. Dawicki, W., et al., *4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses.* J Immunol, 2004. **173**(10): p. 5944-51.
54. Melero, I., et al., *Immunostimulatory monoclonal antibodies for cancer therapy.* Nat Rev Cancer, 2007. **7**(2): p. 95-106.
55. Schaer, D.A., D. Hirschhorn-Cymerman, and J.D. Wolchok, *Targeting tumor-necrosis factor receptor pathways for tumor immunotherapy.* J Immunother Cancer, 2014. **2**: p. 7.
56. Anderson, R.J., et al., *A self-adjuvanting vaccine induces cytotoxic T lymphocytes that suppress allergy.* Nature Chemical Biology, 2014. **10**(11): p. 943-949.
57. Brown, L.E. and D.C. Jackson, *Lipid-based self-adjuvanting vaccines.* Curr Drug Deliv, 2005. **2**(4): p. 383-93.
58. Tiptiri-Kourpeti, A., et al., *DNA vaccines to attack cancer: Strategies for improving immunogenicity and efficacy.* Pharmacol Ther, 2016. **165**: p. 32-49.
59. Moyle, P.M., *Biotechnology approaches to produce potent, self-adjuvanting antigen-adjuvant fusion protein sub-unit vaccines.* Biotechnol Adv, 2017. **35**(3): p. 375-389.
60. Chauhan, N., et al., *An overview of adjuvants utilized in prophylactic vaccine formulation as immunomodulators.* Expert Review of Vaccines, 2017. **16**(5): p. 491-502.
61. McDonald, D.M., S.N. Byrne, and R.J. Payne, *Synthetic self-adjuvanting glycopeptide cancer vaccines.* Frontiers in Chemistry, 2015. **3**.
62. Kalnin, K., et al., *Incorporation of RG1 epitope concatemers into a self-adjuvanting Flagellin-L2 vaccine broaden durable protection against cutaneous challenge with diverse human papillomavirus genotypes.* Vaccine, 2017. **35**(37): p. 4942-4951.
63. Chua, B.Y., et al., *A self-adjuvanting lipopeptide-based vaccine candidate for the treatment of hepatitis C virus infection.* Vaccine, 2008. **26**(37): p. 4866-4875.
64. Gomez-Samblas, M., et al., *Self-adjuvanting C18 lipid vinil sulfone-PP2A vaccine: study of the induced immunomodulation against Trichuris muris infection.* Open Biology, 2017. **7**(4).
65. Azmi, F., et al., *Self-adjuvanting vaccine against group A streptococcus: Application of fibrillized peptide and immunostimulatory lipid as adjuvant.* Bioorganic & Medicinal Chemistry, 2014. **22**(22): p. 6401-6408.
66. Moyle, P.M. and I. Toth, *Self-adjuvanting lipopeptide vaccines.* Current Medicinal Chemistry, 2008. **15**(5): p. 506-516.

67. Carapau, D., et al., *Protective Humoral Immunity Elicited by a Needle-Free Malaria Vaccine Comprised of a Chimeric Plasmodium falciparum Circumsporozoite Protein and a Toll-Like Receptor 5 Agonist, Flagellin.* Infection and Immunity, 2013. **81**(12): p. 4350-4362.

68. Cui, B.F., et al., *Flagellin as a vaccine adjuvant.* Expert Review of Vaccines, 2018. **17**(4): p. 335-349.

69. Plant, A. and N.A. Williams, *Modulation of the immune response by the cholera-like enterotoxins.* Current Topics in Medicinal Chemistry, 2004. **4**(5): p. 509-519.

70. Stratmann, T., *Cholera Toxin Sub-unit B as Adjuvant-An Accelerator in Protective Immunity and a Break in Autoimmunity.* Vaccines, 2015. **3**(3): p. 579-596.

71. Bolhassani, A. and S. Rafati, *Heat-shock proteins as powerful weapons in vaccine development.* Expert Review of Vaccines, 2008. **7**(8): p. 1185-1199.

72. Strbo, N., et al., *Secreted heat shock protein gp96-Ig: next-generation vaccines for cancer and infectious diseases.* Immunologic Research, 2013. **57**(1-3): p. 311-325.

73. Ding, Y., et al., *Heat-Shock Protein gp96 Enhances T Cell Responses and Protective Potential to Bacillus Calmette-Guerin Vaccine.* Scand J Immunol, 2016. **84**(4): p. 222-8.

74. Jung, I.D., et al., *Enhancement of Tumor-Specific T Cell-Mediated Immunity in Dendritic Cell-Based Vaccines by Mycobacterium tuberculosis Heat Shock Protein X.* Journal of Immunology, 2014. **193**(3): p. 1233-1245.

75. Kim, H.Y., et al., *Heat shock protein X purified from Mycobacterium tuberculosis enhances the efficacy of dendritic cells-based immunotherapy for the treatment of allergic asthma.* Bmb Reports, 2015. **48**(3): p. 178-183.

76. Andersen-Nissen, E., et al., *Evasion of Toll-like receptor 5 by flagellated bacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(26): p. 9247-9252.

77. Kang, X.L., Z.M. Pan, and X.N. Jiao, *Amino acids 89-96 of Salmonella flagellin: a key site for its adjuvant effect independent of the TLR5 signaling pathway.* Cellular & Molecular Immunology, 2017. **14**(12): p. 1023-1025.

78. Mueller, A.K., et al., *Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(8): p. 3022-3027.

79. Lin, J.W., et al., *The Subcellular Location of Ovalbumin in Plasmodium berghei Blood Stages Influences the Magnitude of T-Cell Responses.* Infection and Immunity, 2014. **82**(11): p. 4654-4665.

80. Montagna, G.N., et al., *Antigen Export during Liver Infection of the Malaria Parasite Augments Protective Immunity.* Mbio, 2014. **5**(4).

81. Lin, J.W., et al., *A Novel ' Gene Insertion/Marker Out' (GIMO) Method for Transgene Expression and Gene Complementation in Rodent Malaria Parasites.* Plos One, 2011. **6**(12).

82. Salman, A.M., et al., *Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins.* Methods Mol Biol, 2015. **1325**: p. 257-86.

83. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.

84. Mordmuller, B., et al., *Sterile protection against human malaria by chemoattenuated PfSPZ vaccine.* Nature, 2017. **542**(7642): p. 445-449.

85. Bijker, E.M., et al., *Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(19): p. 7862-7867.

86. Annoura, T., et al., *Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development.* Faseb Journal, 2014. **28**(5): p. 2158-2170.

87. Arredondo, S.A. and S.H.I. Kappe, *The s48/45 six-cysteine proteins: mediators*

of interaction throughout the Plasmodium life cycle. International Journal for Parasitology, 2017. **47**(7): p. 409-423.

88. Silvie, O., K. Goetz, and K. Matuschewski, *A sporozoite asparagine-rich protein controls initiation of Plasmodium liver stage development.* Plos Pathogens, 2008. **4**(6).

89. Aly, A.S.I., et al., *SAP1 is a critical post-transcriptional regulator of infectivity in malaria parasite sporozoite stages.* Molecular Microbiology, 2011. **79**(4): p. 929-939.

90. Vaughan, A.M., et al., *Type II fatty acid synthesis is essential only for malaria parasite late liver stage development.* Cellular Microbiology, 2009. **11**(3): p. 506-520.

91. Yu, M., et al., *The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites.* Cell Host Microbe, 2008. **4**(6): p. 567-78.

92. Iwanaga, S., et al., *Identification of an AP2-family Protein That Is Critical for Malaria Liver Stage Development.* Plos One, 2012. **7**(11).

93. Dellibovi-Ragheb, T.A., et al., *Host biotin is required for liver stage development in malaria parasites.* Proceedings of the National Academy of Sciences, 2018.

94. Haussig, J.M., K. Matuschewski, and T.W. Kooij, *Inactivation of a Plasmodium apicoplast protein attenuates formation of liver merozoites.* Mol Microbiol, 2011. **81**(6): p. 1511-25.

95. Ishino, T., et al., *LISP1 is important for the egress of Plasmodium berghei parasites from liver cells.* Cell Microbiol, 2009. **11**(9): p. 1329-39.

96. Kumar, H., et al., *Protective efficacy and safety of liver stage attenuated malaria parasites.* Sci Rep, 2016. **6**: p. 26824.

97. Sahu, T., et al., *ZIPCO, a putative metal ion transporter, is crucial for Plasmodium liver-stage development.* EMBO Mol Med, 2014. **6**(11): p. 1387-97.

98. Dankwa, D.A., et al., *A Plasmodium yoelii Mei2-Like RNA Binding Protein Is Essential for Completion of Liver Stage Schizogony.* Infect Immun, 2016. **84**(5): p. 1336-1345.

99. van Schaijk, B.C., et al., *Type II fatty acid biosynthesis is essential for Plasmodium falciparum sporozoite development in the midgut of Anopheles mosquitoes.* Eukaryot Cell, 2014. **13**(5): p. 550-9.

100. Flick, K., et al., *Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli.* Malar J, 2004. **3**: p. 50.

101. Tuju, J., et al., *Vaccine candidate discovery for the next generation of malaria vaccines.* Immunology, 2017. **152**(2): p. 195-206.

102. 102. Milek, R.L., et al., *Plasmodium falciparum: heterologous synthesis of the transmission-blocking vaccine candidate Pfs48/45 in recombinant vaccinia virus-infected cells.* Exp Parasitol, 1998. **90**(2): p. 165-74.

103. Milek, R.L., H.G. Stunnenberg, and R.N. Konings, *Assembly and expression of a synthetic gene encoding the antigen Pfs48/45 of the human malaria parasite Plasmodium falciparum in yeast.* Vaccine, 2000. **18**(14): p. 1402-11.

CHAPTER 2

THE USE OF TRANSGENIC PARASITES IN MALARIA VACCINE RESEARCH

Ahmad Syibli Othman[1,2*], Catherin Marin-Mogollon[1*], Ahmed M. Salman[3], Blandine M Franke-Fayard[1], Chris J. Janse[1], Shahid M. Khan[1#]

[1] Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, the Netherlands
[2] Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[3] The Jenner Institute, University of Oxford, ORCRB, Roosevelt Drive, Oxford, United Kingdom
* These authors contributed equally to this review
[#] Corresponding author: Shahid Khan

Expert Review of Vaccines, 2017, 16(7): p. 685-697

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.

INTRODUCTION

In the mid-nineties genetic modification to create permanent modifications in malaria parasite genomes was first described in the rodent malaria parasite *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. Amongst the first transgene mutants were rodent malaria parasites 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, 5, 6, 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, 9, 10, 11, 12, 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, 15, 16, 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, 20, 21, 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 regiments. 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**).

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, 29, 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].

Lastly, 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 [32, 33, 34].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 simplifies 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 genetically attenuated parasite(GAP) vaccines and to examine immunological correlates of protection after vaccination *in vivo*.

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, 35].

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

Transgenic rodent malaria parasites (RMP) expressing reporter proteins

Reporter	Remarks
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)[a]
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 *in vitro* and *in vivo* (see **Sections2**and **4** for references)[a]
Ovalbumin (OVA)	Several OVA-expressing RMP have been used to analyze interactions of the parasite with the host immune system (see **Sections2**and **4** for references)[a]

Transgenic *P. falciparum* parasites expressing reporter proteins

Reporter	Remarks
GFP	GFP-expressing *P. falciparum* parasites have been used in GAI assays [16]
Luciferase	Luminescent *P. falciparum* parasites have been used to quantify inhibition of oocyst production in SMFA assays [14]

Chimeric rodent malaria parasites expressing human *Plasmodium*[b] proteins

Protein product	*P. falciparum*/ *P. vivax* gene	Remarks	RMgm ID	Ref
*Pf*LSA-1	PF3D7_1036400	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#1314	[31]
*Pf*LSA-3	PF3D7_0220000	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#1315	[31]
*Pf*CelTOS	PF3D7_1216600	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1310	[31]
*Pf*UIS3 (ETRAMP13)	PF3D7_1302200	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1311	[31]
*Pf*LSAP1	PF3D7_1201300	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1308	[31]
*Pf*LSAP2	PF3D7_0202100	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1312	[31]
PfETRAMP5	PF3D7_0532100	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1309	[31]
*Pf*Falstatin	PF3D7_0911900	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1313	[31]
*Pf*CSP	PF3D7_0304600	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1316	[31]
*Pf*TRAP	PF3D7_1335900	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1317	[31]
*Pf*UIS3/ *Pf*TRAP	PF3D7_1302200 PF3D7_1335900	**(2) Additional copies**; *Pf* (NF54) genes under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#4076	[76]
*Pf*CSP/ *Pf*TRAP	PF3D7_0304600 PF3D7_1335900	**(2) Additional copies**; *Pf* (NF54) genes under the control of *Pbuis4* promoter; in*Pb* (ANKA)		[95]

Table 1. (continued)

Protein product	*P. falciparum*/ *P. vivax* gene	Remarks	RMgm ID	Ref
*Pf*CSP	PF3D7_0304600	**Replacement copy**; *Pb* (ANKA)*csp* replaced by *Pf*(Wellcome strain) *csp*, full-length *Pbcsp* promoter & 302bp *Pbcsp*3'UTR. Reduced sporozoite production	#69	[73]
*Pf*CSP	PF3D7_0304600	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pf*(NF54) *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker. Normal sporozoite production and infectivity	#4110	
*Pf*CSP	PF3D7_0304600	**Replacement copy;***Py* (17XNL) *csp* replaced with *Pf* (3D7) *csp*. Human DHFR selectable marker. *Pbhsp70* 3'UTR Normal sporozoite production and infectivity	#1442	[96]
*Pf*TRAP	PF3D7_1335900	**Replacement copy;***Pb* (ANKA)*trap* replaced by *Pf*(NF54) *trap* under control of endogenous *Pbtrap* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4112	
*Pv*TRAP	PVP01_1218700	**Replacement copy;***Pb* (ANKA) *trap* replaced with *Pv* (Sal-1) *trap*. No selectable marker. Normal sporozoite production and infectivity	#1103	[97]
*Pv*25	PVX_111175	**Replacement copy;** *Pb25* and *Pb28* replaced with *Pv 25; in Pb (ANKA)*	#222	[49]
*Pf*25	PF3D7_1031000	**Replacement copy;** *Pb25* and *Pb28* replaced with *Pf25; in Pb (ANKA)*	#273	[50]
*Pf*CelTOS	PF3D7_1216600	**Replacement copy;** *Pb* (ANKA) *celtos* replaced by *Pf* (NF54) *celtos* under control of endogenous *Pbceltos* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4066	[74]
*Pv*CSP (VK210)	PVX_119355	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pv*VK210 *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity		[77]
*Pv*CSP (VK247)	PVX_119355	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pv* VK247 *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity		[77]
*Pv*CelTOS	PVX_123510	**Replacement copy;** *Pb* (ANKA) *celtos* replaced by *Pvceltos* under control of endogenous *Pbceltos* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4111	[75]
Rodent malaria parasites expressing HMP-RMP fusion proteins[b]				
CSP	PF3D7_0304600	The repeat region of *Pb*(NK65) *csp* is replaced with the *Pf* (7G8) *csp* repeat region.	#76	[98]

Table 1. (continued)

Protein product	*P. falciparum*/ *P. vivax* gene	Remarks	RMgm ID	Ref
MSP1	PF3D7_0930300	The *Pb* (ANKA) *msp-1*_19 C-terminal replaced with the *Pf* (D10) *msp-1*_19 C-terminal	#201	[78]
MSP1	PF3D7_0930300	The*Pb*(ANKA) *msp-1*19 C-terminal replaced with the *Pf* (FCC1/HN) *msp-1*_19 C-terminal	#330	[99]
CSP (VK210)	PVX_119355	The repeat region of *Pb* (ANKA) *csp* is replaced with the *Pv* (210) csp repeat region.	#906	[100]
CSP (VK210)	PVX_119355	The repeat region of*Pb* (ANKA) *csp* is replaced with (part of) *Pv* (210) *csp* gene	#1104	[47]
CSP (VK247)	PVX_119355	The majority of *Pb* (ANKA) *csp* gene is replaced with *Pv* (247) *csp;* the fusion gene retains *Pb* signal sequence (1-20aa) and *Pb* GPI anchor sequence (372-395aa)	#1443	[101]
P25	PVX_111175	The *Pb* (ANKA)*25* and *28* genes replaced with a fusion of *Pv*25 and *Pb* 25	#223	[49]
VAR2CSA	PF3D7_1200600	A synthetic *Pf* 3D7 DBL1X-6ε gene (*var2csa*) fused to *Pb* (ANKA) *fam-a*	#1436	[27]
Genetically Attenuated Parasites (GAPs)				
See **Section 4** for details (and references) of transgenic parasites used to generate and test GAP vaccines				

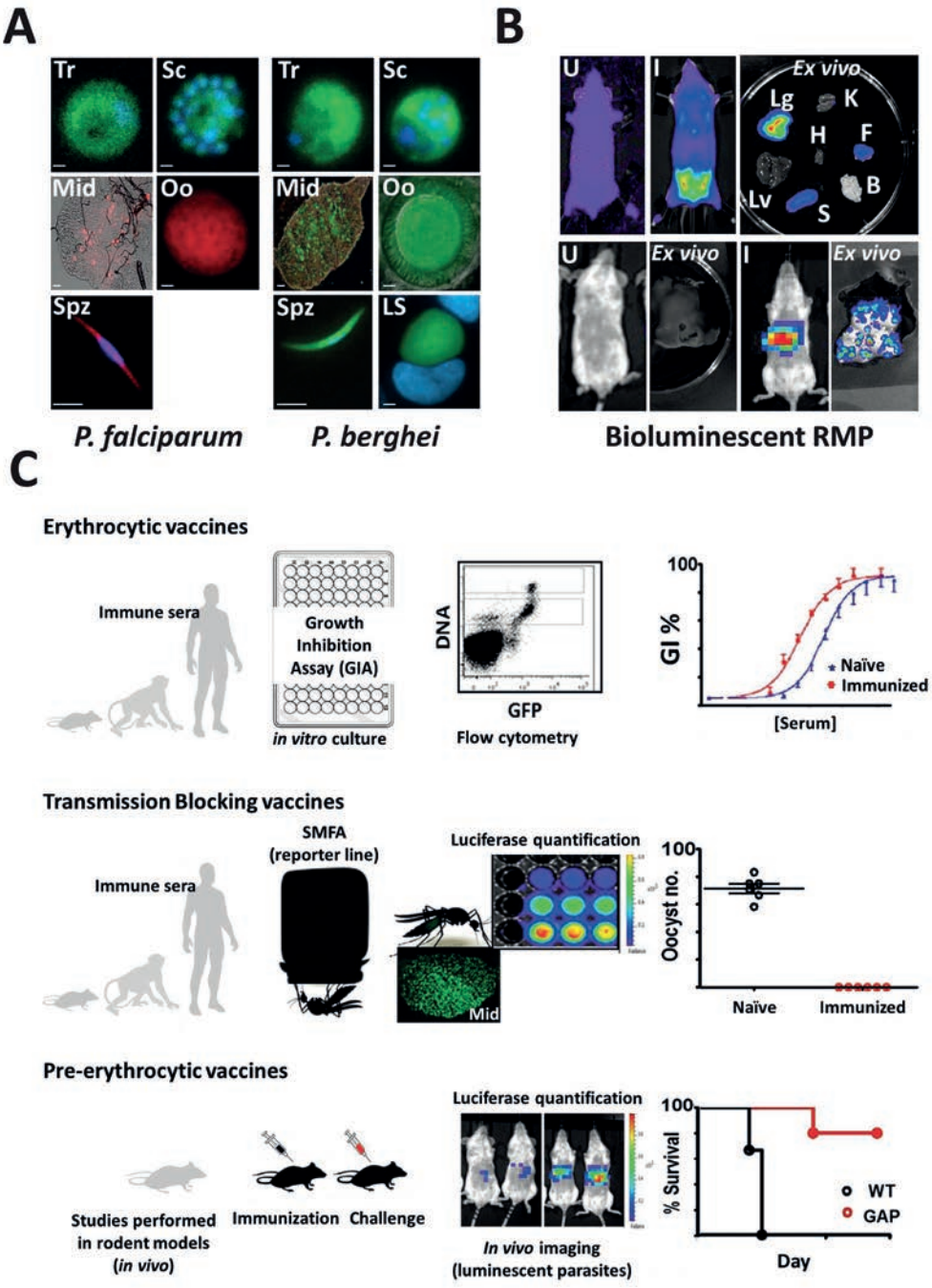
[a]For full list of transgenic reporter parasites generated in RMP see the **RMgm Database** www.pberghei.eu
[b]*Plasmodium* species abbreviations: *Pf - P. falciparum*; *Pv- P. vivax*; *Pb- P. berghei*; *Py- P. yoelii*

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 rodent malaria parasites (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 1A,B**). Frequently used promoter regions of RMP genes include *elongation factor 1-a*pha (*eef1α*), *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).

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)

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 have 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**).

positive parasite-infected red blood cells [20, 36, 37] (**Figure 1A**) or quantification of luminescence signals to determine parasite numbers or parasite loads in blood, liver or other organs [19, 21](**Figure 1B**). 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 1B**). 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. Bio-luminescence imaging is a proven and sensitive method to measure parasite liver loads in mice, even after infection with low sporozoite doses. It has been shown that parasite liver loads can still be determined even after inoculation of 1-10 sporozoites and that *in vivo* imaging quantification of parasite loads correlates very



well with qPCR quantification methods [38]. The sporozoite doses used in different studies vary according to the vaccines being tested. Specifically, when examining potential GAP vaccines (see below) high doses of the GAP sporozoites are used to infect mice in order

to establish if these parasites completely arrest in the liver, an essential and critical safety requirement of a live-attenuated vaccine. In addition, mice immunized with GAP parasites (see below) are often challenged with relatively high doses of WT parasites (i.e. $1x10^4$), in order to test the protective efficacy of different GAP vaccines and vaccination regiments. In sub-unit vaccine studies mice are usually challenged with lower doses of sporozoites ($1\text{-}3 \times 10^3$), a dose reflective of 1-5 mosquito bites, after which parasite liver loads are established.

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* [39] or the gametocyte specific *pfs16* promoter [40].

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 $CD8^+$ T cell responses during both blood and liver infections [9, 10, 41, 42, 43]. 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 [41]. 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 $CD8^+$ and $CD4^+$ OVA T cells demonstrated that export of parasite proteins into the infected hepatocytes enhanced immunogenicity and $CD8^+$ 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 the3 major points of the parasite life-cycle: erythrocytic vaccines, transmission blocking vaccines and pre-erythrocytic vaccines.

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, 44, 45, 46].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 *Pfhsp86* 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 1C**).

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 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 [44, 47, 48, 49, 50].

The standard membrane-feeding assay (SMFA) is a well-established and recognized method to evaluate TB activity of antibodies/serum against human malaria parasites [51]. 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 1B**).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[39]. 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 *Pfhsp70* 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.

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 [38, 52]. Both *in vitro* and *in vivo* luminescence imaging assays have been used to screen inhibitors and vaccines against liver stages (**Figure 1C**; [23, 29, 31, 53, 54]).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* sub-unit vaccines and then challenged with luminescent chimeric RMP that express human parasite antigens (see **Section 3**) or in mice that have been immunized with genetically attenuated parasites 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[55]and to analyze the impact of immune responses on inhibition of liver stage development[23, 56, 57, 58]. 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 [59, 60, 61, 62, 63, 64]. 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 affect sporozoite motility in the skin, preventing immobilized sporozoites of entering dermal blood vessels [65].

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 [57]. 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 theconstitutive*Pfeef1a* promoter and the reporter expression cassette is introduced into the *pf47* locus [66]. In this study [57]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).

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 [47, 49, 50]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 [67]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 GIMO (Gene Insertion-Marker Out) transfection method [68] 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 2A**). 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 [69, 70]. 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[71]. 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 2B** for the immunization/challenge protocol). In this study two antigens, *Pf*LSA1 and *Pf*LSAP2, 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 CelTOS ([72, 73, 74, 75]; **Table 1**).

Chimeric parasites have also been used to evaluate immunogenicity of antigens against other lifecycle stages (*i.e.* TB vaccines see **Figure 2C**) 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

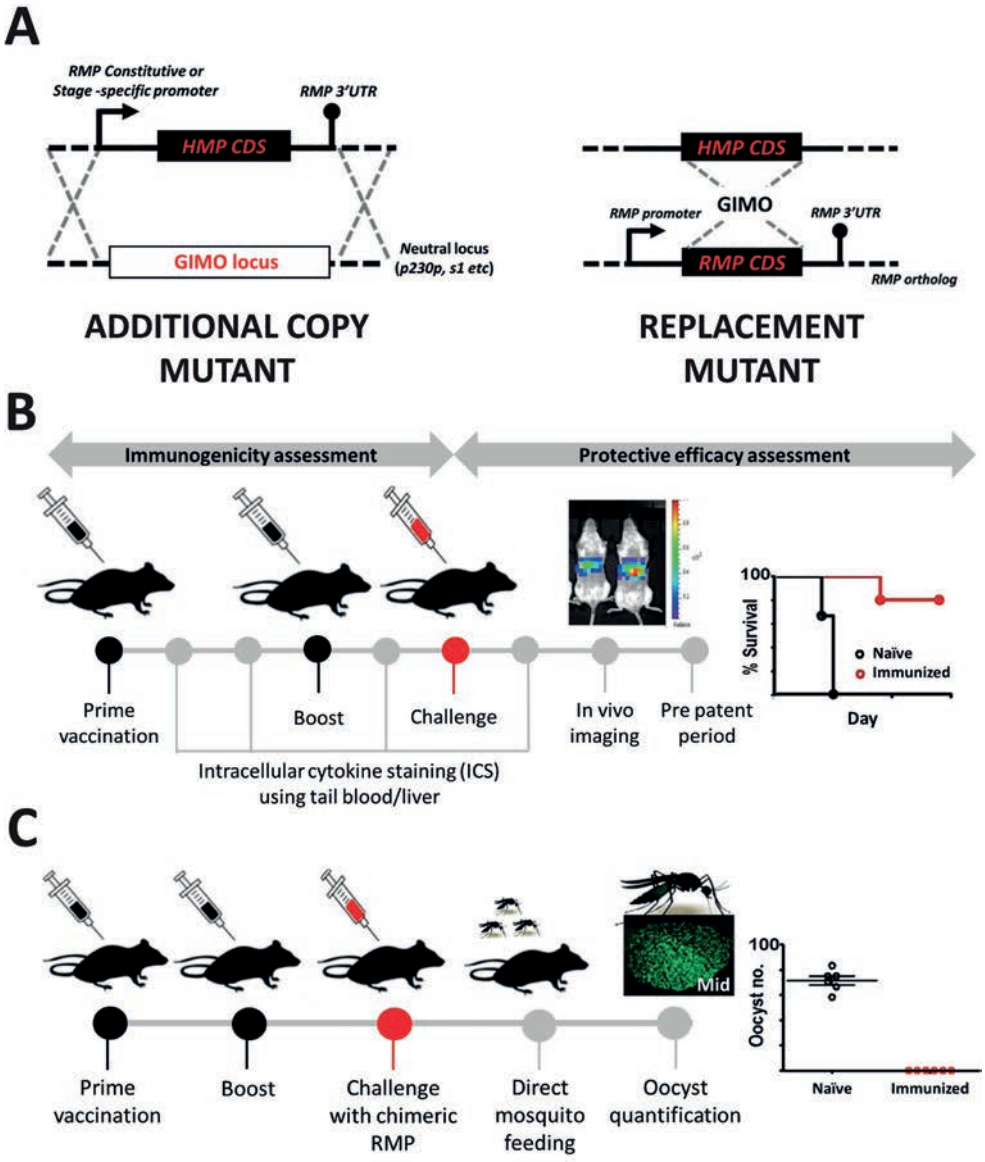


Figure 2. The use of chimeric RMP expressing human malaria parasite (HMP) proteins in malaria vaccine research. (A) Additional Copy Mutants have the HMP gene (e.g. the *P. falciparum* gene coding sequence; *Pf CDS*) introduced as an additional gene copy into a silent/neutral locus of the RMP; the HMP 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.

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 [76]. 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 [77].

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 [78, 79, 80]. 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 [81]. Ultimately, however, even positive results generated using chimeric parasites in rodents or in vitro assays will need to be validated in human vaccine trials.

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 [32]). Sporozoite attenuation has been performed by radiation or by genetic modification of parasites (reviewed in [32, 33, 34, 82, 83]). 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 [33, 84]. These so-called genetically attenuated parasites (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 further clinical development as whole organism vaccines. Transgenic rodent parasites have been used extensively in preclinical 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[34]. 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 [85, 86] (**Figure 1B**). 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 [87, 88, 89].

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 sub-unit vaccines (**Section 2** and **3; Figure 2B**). 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 [90, 91]. 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, 41, 92, 93]. These studies have revealed the importance of $CD8^+$ T cell 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 $CD8^+$ T cell mediated immune responses in the liver of mice immunized with attenuated sporozoites ([10, 41]; see also **Section 2**).

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 rodent malaria parasites 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 sub-unit 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 [94]. 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 is 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 has 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).

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 multi-stage 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).

REFERENCES

1. van Dijk MR, Janse CJ, Waters AP. Expression of a Plasmodium Gene Introduced into Subtelomeric Regions of Plasmodium berghei Chromosomes. Science. 1996;271:662-5.

2. Carvalho TG, Menard R. Manipulating the Plasmodium genome. Curr Issues Mol Biol. 2005;7:39-55.

3. de Koning-Ward TF, Gilson PR, Crabb BS. Advances in molecular genetic systems in malaria. Nat Rev Microbiol. 2015;13:373-87.

4. Amino R, Menard R, Frischknecht F. In vivo imaging of malaria parasites--recent advances and future directions. Curr Opin Microbiol. 2005;8:407-14.

5. Heussler V, Doerig C. In vivo imaging enters parasitology. Trends Parasitol. 2006;22:192-5; discussion 5-6.

6. Siciliano G, Alano P. Enlightening the malaria parasite life cycle: bioluminescent Plasmodium in fundamental and applied research. Front Microbiol. 2015;6:391.

7. De Niz M, Burda PC, Kaiser G, Del Portillo HA, Spielmann T, Frischknecht F, Heussler VT. Progress in imaging methods: insights gained into Plasmodium biology. Nat Rev Microbiol. 2017;15:37-54.

8. Franke-Fayard B, Fonager J, Braks A, Khan SM, Janse CJ. Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria? PLoS Pathog. 2010;6:e1001032.

9. Lin JW, Shaw TN, Annoura T, Fougere A, Bouchier P, Chevalley-Maurel S, Kroeze H, Franke-Fayard B, Janse CJ, Couper KN, Khan SM. The subcellular location of ovalbumin in Plasmodium berghei blood stages influences the magnitude of T cell responses. Infect Immun. 2014;82:4654-65.

10. Montagna GN, Beigier-Bompadre M, Becker M, Kroczek RA, Kaufmann SH, Matuschewski K. Antigen export during liver infection of the malaria parasite augments protective immunity. MBio. 2014;5:e01321-14.

11. Fernandez-Ruiz D, Ng WY, Holz LE, Ma JZ, Zaid A, Wong YC, Lau LS, Mollard V, Cozijnsen A, Collins N, Li J, Davey GM, Kato Y, Devi S, Skandari R, Pauley M, Manton JH, Godfrey DI, Braun A, Tay SS, Tan PS, Bowen DG, Koch-Nolte F, Rissiek B, Carbone FR, Crabb BS, Lahoud M, Cockburn IA, Mueller SN, Bertolino P, McFadden GI, Caminschi I, Heath WR. Liver-Resident Memory CD8+ T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. Immunity. 2016;45:889-902.

12. Holz LE, Fernandez-Ruiz D, Heath WR. Protective immunity to liver-stage malaria. Clin Transl Immunology. 2016;5:e105.

13. Frevert U, Nacer A, Cabrera M, Movila A, Leberl M. Imaging Plasmodium immunobiology in the liver, brain, and lung. Parasitol Int. 2014;63:171-86.

14. Stone WJ, Churcher TS, Graumans W, van Gemert GJ, Vos MW, Lanke KH, van de Vegte-Bolmer MG, Siebelink-Stoter R, Dechering KJ, Vaughan AM, Camargo N, Kappe SH, Sauerwein RW, Bousema T. 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;210:1456-63.

15. Wang Z, Liu M, Liang X, Siriwat S, Li X, Chen X, Parker DM, Miao J, Cui L. A flow cytometry-based quantitative drug sensitivity assay for all Plasmodium falciparum gametocyte stages. PLoS One. 2014;9:e93825.

16. Wilson DW, Crabb BS, Beeson JG. Development of fluorescent Plasmodium falciparum for in vitro growth inhibition assays. Malaria journal. 2010;9:152.

17. Swann J, Corey V, Scherer CA, Kato N, Comer E, Maetani M, Antonova-Koch Y, Reimer C, Gagaring K, Ibanez M, Plouffe D, Zeeman AM, Kocken CH, McNamara CW, Schreiber SL, Campo B,

Winzeler EA, Meister S. High-Throughput Luciferase-Based Assay for the Discovery of Therapeutics That Prevent Malaria. ACS Infect Dis. 2016;2:281-93.

18. Voorberg-van der Wel A, Zeeman AM, van Amsterdam SM, van den Berg A, Klooster EJ, Iwanaga S, Janse CJ, van Gemert GJ, Sauerwein R, Beenhakker N, Koopman G, Thomas AW, Kocken CH. Transgenic fluorescent Plasmodium cynomolgi liver stages enable live imaging and purification of Malaria hypnozoite-forms. PLoS One. 2013;8:e54888.

19. Annoura T, Chevalley S, Janse CJ, Franke-Fayard B, Khan SM. Quantitative analysis of Plasmodium berghei liver stages by bioluminescence imaging. Methods Mol Biol. 2013;923:429-43.

20. Le Bihan A, de Kanter R, Angulo-Barturen I, Binkert C, Boss C, Brun R, Brunner R, Buchmann S, Burrows J, Dechering KJ, Delves M, Ewerling S, Ferrer S, Fischli C, Gamo-Benito FJ, Gnadig NF, Heidmann B, Jimenez-Diaz MB, Leroy D, Martinez MS, Meyer S, Moehrle JJ, Ng CL, Noviyanti R, Ruecker A, Sanz LM, Sauerwein RW, Scheurer C, Schleiferboeck S, Sinden R, Snyder C, Straimer J, Wirjanata G, Marfurt J, Price RN, Weller T, Fischli W, Fidock DA, Clozel M, Wittlin S. Characterization of Novel Antimalarial Compound ACT-451840: Preclinical Assessment of Activity and Dose-Efficacy Modeling. PLoS Med. 2016;13:e1002138.

21. Lin JW, Sajid M, Ramesar J, Khan SM, Janse CJ, Franke-Fayard B. Screening inhibitors of P. berghei blood stages using bioluminescent reporter parasites. Methods Mol Biol. 2013;923:507-22.

22. Prudencio M, Mota MM, Mendes AM. A toolbox to study liver stage malaria. Trends Parasitol. 2011;27:565-74.

23. Sack BK, Miller JL, Vaughan AM, Kappe SH. Measurement of Antibody-Mediated Reduction of Plasmodium yoelii Liver Burden by Bioluminescent Imaging. Methods Mol Biol. 2015;1325:69-80.

24. Otto TD, Bohme U, Jackson AP, Hunt M, Franke-Fayard B, Hoeijmakers WA, Religa AA, Robertson L, Sanders M, Ogun SA, Cunningham D, Erhart A, Billker O, Khan SM, Stunnenberg HG, Langhorne J, Holder AA, Waters AP, Newbold CI, Pain A, Berriman M, Janse CJ. A comprehensive evaluation of rodent malaria parasite genomes and gene expression. BMC Biol. 2014;12:86.

25. Tewari R, Patzewitz EM, Poulin B, Stewart L, Baker DA. 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.

26. Blume M, Hliscs M, Rodriguez-Contreras D, Sanchez M, Landfear S, Lucius R, Matuschewski K, Gupta N. A constitutive pan-hexose permease for the Plasmodium life cycle and transgenic models for screening of antimalarial sugar analogs. FASEB J. 2011;25:1218-29.

27. de Moraes LV, Dechavanne S, Sousa PM, Barateiro A, Cunha SF, Nunes-Silva S, Lima FA, Murillo O, Marinho CR, Gangnard S, Srivastava A, Braks JA, Janse CJ, Gamain B, Franke-Fayard B, Penha-Goncalves C. Murine Model for Preclinical Studies of Var2CSA-Mediated Pathology Associated with Malaria in Pregnancy. Infect Immun. 2016;84:1761-74.

28. Cockburn I. Chimeric parasites as tools to study Plasmodium immunology and assess malaria vaccines. Methods Mol Biol. 2013;923:465-79.

29. Salman AM, Mogollon CM, Lin JW, van Pul FJ, Janse CJ, Khan SM. Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins. Methods Mol Biol. 2015;1325:257-86.

30. 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;7:1875-9.

31. Longley RJ, Salman AM, Cottingham MG, Ewer K, Janse CJ, Khan SM, Spencer AJ,

Hill AV. Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates. Sci Rep. 2015;5:11820.

32. Bijker EM, Borrmann S, Kappe SH, Mordmuller B, Sack BK, Khan SM. Novel approaches to whole sporozoite vaccination against malaria. Vaccine. 2015;33:7462-8.

33. Hollingdale MR, Sedegah M. Development of whole sporozoite malaria vaccines. Expert Rev Vaccines. 2017;16:45-54.

34. Khan SM, Janse CJ, Kappe SH, Mikolajczak SA. Genetic engineering of attenuated malaria parasites for vaccination. Curr Opin Biotechnol. 2012;23:908-16.

35. Dube A, Gupta R, Singh N. Reporter genes facilitating discovery of drugs targeting protozoan parasites. Trends Parasitol. 2009;25:432-9.

36. Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, van der Linden R, Sinden RE, Waters AP, Janse CJ. 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.

37. Hopp CS, Chiou K, Ragheb DR, Salman A, Khan SM, Liu AJ, Sinnis P. Longitudinal analysis of Plasmodium sporozoite motility in the dermis reveals component of blood vessel recognition. Elife. 2015;4.

38. Ploemen IH, Prudencio M, Douradinha BG, Ramesar J, Fonager J, van Gemert GJ, Luty AJ, Hermsen CC, Sauerwein RW, Baptista FG, Mota MM, Waters AP, Que I, Lowik CW, Khan SM, Janse CJ, Franke-Fayard BM. Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. PLoS One. 2009;4:e7881.

39. Vos MW, Stone WJ, Koolen KM, van Gemert GJ, van Schaijk B, Leroy D, Sauerwein RW, Bousema T, Dechering KJ. 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.

40. 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;60:2097-107.

41. Kimura K, Kimura D, Matsushima Y, Miyakoda M, Honma K, Yuda M, Yui K. 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;81:3825-34.

42. Lundie RJ, de Koning-Ward TF, Davey GM, Nie CQ, Hansen DS, Lau LS, Mintern JD, Belz GT, Schofield L, Carbone FR, Villadangos JA, Crabb BS, Heath WR. 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;105:14509-14.

43. Miyakoda M, Kimura D, Yuda M, Chinzei Y, Shibata Y, Honma K, Yui K. Malaria-specific and nonspecific activation of CD8+ T cells during blood stage of Plasmodium berghei infection. J Immunol. 2008;181:1420-8.

44. Blagborough AM, Musiychuk K, Bi H, Jones RM, Chichester JA, Streatfield S, Sala KA, Zakutansky SE, Upton LM, Sinden RE, Brian I, Biswas S, Sattabonkot J, Yusibov V. Transmission blocking potency and immunogenicity of a plant-produced Pvs25-based subunit vaccine against Plasmodium vivax. Vaccine. 2016;34:3252-9.

45. Bergmann-Leitner ES, Duncan EH, Mullen GE, Burge JR, Khan F, Long CA, Angov E, Lyon JA. Critical evaluation of different methods for measuring the functional activity of antibodies against malaria blood stage antigens. Am J Trop Med Hyg. 2006;75:437-42.

46. Duncan EH, Bergmann-Leitner ES. Miniaturized Growth Inhibition Assay to Assess the Anti-blood Stage Activity of Antibodies. Methods Mol Biol. 2015;1325:153-65.

47. Mizutani M, Iyori M, Blagborough AM, Fukumoto S, Funatsu T, Sinden RE,

Yoshida S. Baculovirus-vectored multistage Plasmodium vivax vaccine induces both protective and transmission-blocking immunities against transgenic rodent malaria parasites. Infect Immun. 2014;82:4348-57.

48. Blagborough AM, Yoshida S, Sattabongkot J, Tsuboi T, Sinden RE. Intranasal and intramuscular immunization with Baculovirus Dual Expression System-based Pvs25 vaccine substantially blocks Plasmodium vivax transmission. Vaccine. 2010;28:6014-20.

49. Ramjanee S, Robertson JS, Franke-Fayard B, Sinha R, Waters AP, Janse CJ, Wu Y, Blagborough AM, Saul A, Sinden RE. 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;25:886-94.

50. 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;76:2018-24.

51. Miura K, Deng B, Tullo G, Diouf A, Moretz SE, Locke E, Morin M, Fay MP, Long CA. Qualification of standard membrane-feeding assay with Plasmodium falciparum malaria and potential improvements for future assays. PLoS One. 2013;8:e57909.

52. Miller JL, Murray S, Vaughan AM, Harupa A, Sack B, Baldwin M, Crispe IN, Kappe SH. Quantitative bioluminescent imaging of pre-erythrocytic malaria parasite infection using luciferase-expressing Plasmodium yoelii. PLoS One. 2013;8:e60820.

53. Meister S, Plouffe DM, Kuhen KL, Bonamy GM, Wu T, Barnes SW, Bopp SE, Borboa R, Bright AT, Che J, Cohen S, Dharia NV, Gagaring K, Gettayacamin M, Gordon P, Groessl T, Kato N, Lee MC, McNamara CW, Fidock DA, Nagle A, Nam TG, Richmond W, Roland J, Rottmann M, Zhou B, Froissard P, Glynne RJ, Mazier D, Sattabongkot J, Schultz PG, Tuntland T, Walker JR, Zhou Y, Chatterjee A, Diagana TT, Winzeler EA. Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery. Science. 2011;334:1372-7.

54. Mwakingwe A, Ting LM, Hochman S, Chen J, Sinnis P, Kim K. Noninvasive real-time monitoring of liver-stage development of bioluminescent Plasmodium parasites. J Infect Dis. 2009;200:1470-8.

55. Portugal S, Carret C, Recker M, Armitage AE, Goncalves LA, Epiphanio S, Sullivan D, Roy C, Newbold CI, Drakesmith H, Mota MM. Host-mediated regulation of superinfection in malaria. Nat Med. 2011;17:732-7.

56. Keitany GJ, Sack B, Smithers H, Chen L, Jang IK, Sebastian L, Gupta M, Sather DN, Vignali M, Vaughan AM, Kappe SH, Wang R. 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;82:5143-53.

57. Sack BK, Miller JL, Vaughan AM, Douglass A, Kaushansky A, Mikolajczak S, Coppi A, Gonzalez-Aseguinolaza G, Tsuji M, Zavala F, Sinnis P, Kappe SH. Model for in vivo assessment of humoral protection against malaria sporozoite challenge by passive transfer of monoclonal antibodies and immune serum. Infect Immun. 2014;82:808-17.

58. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH. Interferon-mediated innate immune responses against malaria parasite liver stages. Cell Rep. 2014;7:436-47.

59. 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.

60. Cockburn IA, Tse SW, Radtke AJ, Srinivasan P, Chen YC, Sinnis P, Zavala F. Dendritic cells and hepatocytes use distinct pathways to process protective antigen from plasmodium in vivo. PLoS Pathog. 2011;7:e1001318.

61. Vanderberg JP. Imaging mosquito transmission of Plasmodium sporozoites into the mammalian host: immunological implications. Parasitol Int. 2014;63:150-64.

62. Dups JN, Pepper M, Cockburn IA. Antibody and B cell responses to Plasmodium sporozoites. Front Microbiol. 2014;5:625.

63. Menard R, Tavares J, Cockburn I, Markus M, Zavala F, Amino R. Looking under the skin: the first steps in malarial infection and immunity. Nat Rev Microbiol. 2013;11:701-12.

64. Radtke AJ, Kastenmuller W, Espinosa DA, Gerner MY, Tse SW, Sinnis P, Germain RN, Zavala FP, Cockburn IA. Lymph-node resident CD8alpha+ dendritic cells capture antigens from migratory malaria sporozoites and induce CD8+ T cell responses. PLoS Pathog. 2015;11:e1004637.

65. 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.

66. Vaughan AM, Mikolajczak SA, Camargo N, Lakshmanan V, Kennedy M, Lindner SE, Miller JL, Hume JC, Kappe SH. A transgenic Plasmodium falciparum NF54 strain that expresses GFP-luciferase throughout the parasite life cycle. Mol Biochem Parasitol. 2012;186:143-7.

67. 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;1:346-56.

68. Lin JW, Annoura T, Sajid M, Chevalley-Maurel S, Ramesar J, Klop O, Franke-Fayard BM, Janse CJ, Khan SM. A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS One. 2011;6:e29289.

69. Wijayalath W, Majji S, Villasante EF, Brumeanu TD, Richie TL, Casares S. Humanized HLA-DR4.RagKO. IL2RgammacKO.NOD (DRAG) mice sustain the complex vertebrate life cycle of Plasmodium falciparum malaria. Malaria journal. 2014;13:386.

70. Vaughan AM, Mikolajczak SA, Wilson EM, Grompe M, Kaushansky A, Camargo N, Bial J, Ploss A, Kappe SH. Complete Plasmodium falciparum liver-stage development in liver-chimeric mice. J Clin Invest. 2012;122:3618-28.

71. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev Immunol. 2011;11:57-64.

72. 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.

73. Tewari R, Spaccapelo R, Bistoni F, Holder AA, Crisanti A. Function of region I and II adhesive motifs of Plasmodium falciparum circumsporozoite protein in sporozoite motility and infectivity. J Biol Chem. 2002;277:47613-8.

74. Espinosa DA, Vega-Rodriguez J, Flores-Garcia Y, Noe AR, Munoz C, Coleman R, Bruck T, Haney K, Stevens A, Retallack D, Allen J, Vedvick TS, Fox CB, Reed SG, Howard RF, Salman AM, Janse CJ, Khan SM, Zavala F, Gutierrez GM. The P. falciparum Cell-Traversal Protein for Ookinetes and Sporozoites as a Candidate for Pre-Erythrocytic and Transmission-Blocking Vaccines. Infect Immun. 2016.

75. Alves E, Salman AM, Leoratti F, Lopez-Camacho C, Viveros-Sandoval ME, Lall A, El-Turabi A, Bachmann MF, Hill AV, Janse CJ, Khan SM, Reyes-Sandoval A. Evaluation of PvCelTOS as a pre-erythrocytic P. vivax vaccine. Clin Vaccine Immunol. 2017.

76. Longley RJ, Halbroth BR, Salman AM, Ewer KJ, Hodgson SH, Janse CJ, Khan SM, Hill AV, Spencer AJ. Assessment of the Plasmodium falciparum pre-erythrocytic antigen UIS3 as a potential candidate for a malaria vaccine. Infect Immun. 2016.

77. Salman AM, Montoya-Diaz E, Lall A, Atcheson E, Lopez-Camacho C, Ramesar J, Bauza K, Collins K, Reis F, Pappas L, González-

Cerón L, Janse CJ, Hill AV, Khan SM, Reyes-Sandoval A. Rational development of a highly protective P. vivax vaccine evaluated using transgenic rodent parasite challenge models. Sci Rep. 2017;7:46482

78. de Koning-Ward TF, O'Donnell RA, Drew DR, Thomson R, Speed TP, Crabb BS. 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;198:869-75.

79. 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;80:633-42.

80. Sachdeva S, Mohmmed A, Dasaradhi PV, Crabb BS, Katyal A, Malhotra P, Chauhan VS. Immunogenicity and protective efficacy of Escherichia coli expressed Plasmodium falciparum merozoite surface protein-1(42) using human compatible adjuvants. Vaccine. 2006;24:2007-16.

81. Bouharoun-Tayoun H, Druilhe P. Antibody-Dependent Cell-Mediated Inhibition (ADCI) of Plasmodium falciparum: One- and Two-Step ADCI Assays. Methods Mol Biol. 2015;1325:131-44.

82. Epstein JE, Richie TL. The whole parasite, pre-erythrocytic stage approach to malaria vaccine development: a review. Curr Opin Infect Dis. 2013;26:420-8.

83. Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, Chakravarty S, Gunasekera A, Chattopadhyay R, Li M, Stafford R, Ahumada A, Epstein JE, Sedegah M, Reyes S, Richie TL, Lyke KE, Edelman R, Laurens MB, Plowe CV, Sim BK. Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. Hum Vaccin. 2010;6:97-106.

84. Hafalla JC, Rai U, Morrot A, Bernal-Rubio D, Zavala F, Rodriguez A. Priming of CD8+ T cell responses following immunization with heat-killed Plasmodium sporozoites. Eur J Immunol. 2006;36:1179-86.

85. Annoura T, Ploemen IH, van Schaijk BC, Sajid M, Vos MW, van Gemert GJ, Chevalley-Maurel S, Franke-Fayard BM, Hermsen CC, Gego A, Franetich JF, Mazier D, Hoffman SL, Janse CJ, Sauerwein RW, Khan SM. Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. Vaccine. 2012;30:2662-70.

86. Labaied M, Harupa A, Dumpit RF, Coppens I, Mikolajczak SA, Kappe SH. Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. Infect Immun. 2007;75:3758-68.

87. Kublin JG, Mikolajczak SA, Sack BK, Fishbaugher ME, Seilie A, Shelton L, VonGoedert T, Firat M, Magee S, Fritzen E, Betz W, Kain HS, Dankwa DA, Steel RW, Vaughan AM, Noah Sather D, Murphy SC, Kappe SH. Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects. Sci Transl Med. 2017;9.

88. Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, Williams J, de la Vega P, Ware L, Komisar J, Polhemus M, Richie TL, Epstein J, Tamminga C, Chuang I, Richie N, O'Neil M, Heppner DG, Healer J, O'Neill M, Smithers H, Finney OC, Mikolajczak SA, Wang R, Cowman A, Ockenhouse C, Krzych U, Kappe SH. First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. Vaccine. 2013;31:4975-83.

89. van Schaijk BC, Ploemen IH, Annoura T, Vos MW, Foquet L, van Gemert GJ, Chevalley-Maurel S, van de Vegte-Bolmer M, Sajid M, Franetich JF, Lorthiois A, Leroux-Roels G, Meuleman P, Hermsen CC, Mazier D, Hoffman SL, Janse CJ, Khan SM, Sauerwein RW. A genetically attenuated malaria vaccine

candidate based on P. falciparum b9/slarp gene-deficient sporozoites. Elife. 2014;3.

90. Nganou-Makamdop K, Ploemen I, Behet M, Van Gemert GJ, Hermsen C, Roestenberg M, Sauerwein RW. Reduced Plasmodium berghei sporozoite liver load associates with low protective efficacy after intradermal immunization. Parasite Immunol. 2012;34:562-9.

91. Ploemen I, Behet M, Nganou-Makamdop K, van Gemert GJ, Bijker E, Hermsen C, Sauerwein R. Evaluation of immunity against malaria using luciferase-expressing Plasmodium berghei parasites. Malaria journal. 2011;10:350.

92. Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, Mac-Daniel L, Ganusov VV, Zavala F, Menard R. In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. Proc Natl Acad Sci U S A. 2013;110:9090-5.

93. Trimnell A, Takagi A, Gupta M, Richie TL, Kappe SH, Wang R. Genetically attenuated parasite vaccines induce contact-dependent CD8+ T cell killing of Plasmodium yoelii liver stage-infected hepatocytes. J Immunol. 2009;183:5870-8.

94. Yewdell JW. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. Immunity. 2006;25:533-43.

95. Ewer KJ, Sierra-Davidson K, Salman AM, Illingworth JJ, Draper SJ, Biswas S, Hill AV. Progress with viral vectored malaria vaccines: A multi-stage approach involving "unnatural immunity". Vaccine. 2015;33:7444-51.

96. Zhang M, Kaneko I, Tsao T, Mitchell R, Nardin EH, Iwanaga S, Yuda M, Tsuji M. A highly infectious Plasmodium yoelii parasite, bearing Plasmodium falciparum circumsporozoite protein. Malaria journal. 2016;15:201.

97. Bauza K, Malinauskas T, Pfander C, Anar B, Jones EY, Billker O, Hill AV, Reyes-Sandoval A. 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;82:1277-86.

98. Persson C, Oliveira GA, Sultan AA, Bhanot P, Nussenzweig V, Nardin E. Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid Plasmodium falciparum circumsporozoite protein. J Immunol. 2002;169:6681-5.

99. 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.

100. Espinosa DA, Yadava A, Angov E, Maurizio PL, Ockenhouse CF, Zavala F. 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;81:2882-7.

101. Mizutani M, Fukumoto S, Soubeiga AP, Soga A, Iyori M, Yoshida S. Development of a Plasmodium berghei transgenic parasite expressing the full-length Plasmodium vivax circumsporozoite VK247 protein for testing vaccine efficacy in a murine model. Malaria journal. 2016;15:251.

CHAPTER 3

OX40 STIMULATION ENHANCES PROTECTIVE IMMUNE RESPONSES INDUCED AFTER VACCINATION WITH ATTENUATED MALARIA PARASITES

Ahmad Syibli Othman[1,3], Blandine M Franke-Fayard[1], Takashi Imai[1], Esmé T. I. van der Gracht[2], Anke Redeker[2], Ahmed M. Salman[1,4], Catherin Marin-Mogollon[1], Jai Ramesar[1], Séverine Chevalley-Maurel[1], Chris J. Janse[1], Ramon Arens[2]* and Shahid M. Khan[1]*

[1]Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[3]Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[4]The Jenner Institute, University of Oxford, ORCRB, Roosevelt Drive, Oxford, United Kingdom
* The corresponding authors

Frontiers in Cellular and Infection Microbiology, 2018, 8: 247

ABSTRACT

Protection against a malaria infection can be achieved by immunization with live-attenuated *Plasmodium* sporozoites and while the precise mechanisms of protection remain unknown, T cell responses are thought to be critical in the elimination of infected liver cells. In cancer immunotherapies, agonistic antibodies that target T cell surface proteins, such as CD27, OX40 (CD134) and 4-1BB (CD137), have been used to enhance T cell function by increasing co-stimulation. In this study, we have analyzed the effect of agonistic OX40 monoclonal antibody treatment on protective immunity induced in mice immunized with genetically attenuated parasites (GAPs). OX40 stimulation enhanced protective immunity after vaccination as shown by an increase in the number of protected mice and delay to blood-stage infection after challenge with wild-type sporozoites. Consistent with the enhanced protective immunity enforced OX40 stimulation resulted in an increased expansion of antigen-experienced effector ($CD11a^{hi}CD44^{hi}$) $CD8^+$ and $CD4^+$ T cells in the liver and spleen and also increased IFN-γ and TNF producing $CD4^+$ T cells in the liver and spleen. In addition, GAP immunization plus α-OX40 treatment significantly increased sporozoite-specific IgG responses. Thus, we demonstrate that targeting T cell costimulatory receptors can improve sporozoite-based vaccine efficacy.

INTRODUCTION

Malaria remains a major threat to the lives of more than 3 billion people world-wide and there remains a pressing but unmet need for an effective vaccine, which can provide sustained protection against either infection or disease. Despite three decades of clinical testing different (recombinant) sub-unit vaccines, only modest protection has been reported so far [1-4] and this has renewed an interest in whole parasite-based vaccine approaches [5, 6]. It was first shown in rodent models of malaria that complete protection against infection can be obtained by vaccination using live attenuated sporozoites [7, 8]. Sterile protection against a malaria infection was also demonstrated in humans after immunization with *Plasmodium falciparum* sporozoites, either attenuated by radiation or administered under chemoprophylaxis [9-11]. A prerequisite for induction of protective immunity using sporozoite-based vaccines is that sporozoites retain their capacity to invade liver cells after their administration. The most advanced live-attenuated vaccine is based on radiation-attenuated sporozoites (*Pf*SPZ-Vaccine), which is currently being evaluated both in the clinic and in field trials [12, 13]. In rodent models, immunization with sporozoites of genetically-attenuated parasites (GAP) can induce similar or even better levels of protective immunity compared to irradiated sporozoites (Irr-Spz) [14, 15] . Rodent GAP studies have been critical in the creation of two *P. falciparum* GAP-based vaccines that are currently undergoing clinical evaluation [16-18].

A number of studies from both the clinic and the field have shown that Irr-Spz can generate strong protective immunity in humans [13, 19, 20]. However, in order to achieve high level protective immunity multiple immunizations with high doses of attenuated sporozoites are required [9, 13]. The high numbers of sporozoites required for vaccination increases the costs of sporozoite-based vaccines and complicates the production and application of such vaccines for mass administration in malaria-endemic countries. The major challenge is to produce a highly immunogenic live-attenuated vaccine, which requires the fewest attenuated sporozoites per dose and the fewest doses to induce sustained sterile protection against a malaria infection.

While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, T cells appear to be critical for protection and in particular $CD8^+$ T cells are thought to play a major role in eliminating infected hepatocytes. Early rodent studies using Irr-Spz have demonstrated a vital role for $CD8^+$ T cells [21, 22]. Recent mechanistic investigations into protective immune responses induced by immunization with attenuated sporozoites have demonstrated diverse and robust immune responses that encompasses both $CD8^+$ and $CD4^+$ T cells, as well as a significant contribution from antibodies [23, 24]. Nonetheless, $CD8^+$ T cells are considered to be the main effector cells in eliciting protection after sporozoites immunization [25].

Recently, cancer immunotherapies have employed antibodies that target proteins on the surface of T cells, as treatment with these antibodies have been shown to restore, expand and enhance the function of tumor-reactive T cells. The antagonistic antibodies

targeting CTLA-4 and PD-1 have been used to block inhibitory signals to T cells [26, 27], while agonistic antibodies targeting CD27, OX40 and 4-1BB on $CD4^+$ and $CD8^+$ T cells have been used to increase costimulatory signals [28-30]. These immunostimulatory antibodies have been shown to improve the control of tumors and this was associated with an increase in tumor-specific T cell function [31].In this study, we have analyzed the effect of agonistic OX40 monoclonal antibody (OX40 mAb) treatment on protective immunity induced in mice by immunization with GAP sporozoites. We immunized BALB/c mice using sporozoites of a *P. yoelii* GAP, an established rodent model to evaluate GAP vaccination [14]. We found that OX40 mAb (α-OX40) treatment enhanced protective immunity, which was correlated with an expansion effector $CD4^+$ and $CD8^+$ T cell subsets, in both the liver and the spleen. In addition α-OX40 treatment induced the production of effector cytokine-producing T cells in the liver and spleen. Our results indicate that targeting costimulatory receptors on T cells can be used to improve sporozoite-based vaccine potency and in turn could be used to improve GAP vaccine implementation by reducing the numbers of sporozoites required to induce protective immunity.

MATERIALS AND METHODS

Experimental animals and parasites

Female BALB/cByJ mice (6-7 weeks; Charles River, NL and Harlan, Bicester, UK) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 13132 and 14307). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All experiments were performed in accordance with relevant guidelines and regulations. Two *P. yoelii* (*Py*) lines were used: i) the reference 'wild type' *Py*17XNL parasite line 1971cl1 (*Py*WT; *Py*GFP-luc_{con}; line RMgm-689; www.pberghei.eu [32]; which contains the fusion gene *gfp-luc* gene under control of the constitutive *eef1*α promoter integrated into the silent *230p* gene locus (PY17X_0306600) and does not contain a drug-selectable marker and ii) the 'genetically attenuated parasite' *Py*17XNL mutant that lacks the gene *fabb/f* (3-oxoacyl-acyl-carrier protein synthase; PY17X_1126500). This mutant (Δ*Py*FabBF-GFP-Luc_{con}; *Py*Δ*fabb/f*; mutant RMgm-4109; *www.pberghei.eu)* was generated in the reference line 1971cl1 [33] by standard methods of transfection using a DNA construct that targets the *fabb/f* gene containing *hdhfr/fcu* selectable marker cassette by double cross-over integration.

Mosquito infection, analysis of oocysts and preparation and injection of sporozoites

Sporozoites were obtained by manual dissection of the salivary glands of infected female *Anopheles stephensi* mosquitoes 14 days after feeding on infected mice. Mosquitoes were kept at a temperature of 24.5°C and 80% humidity. Salivary glands were collected in RPMI medium, homogenized and filtered (40μm Falcon, Corning, Amsterdam, NL). The free

sporozoites were counted in a Bürker counting chamber using phase-contrast microscopy. For intravenous (IV) administration sporozoites were suspended in RPMI medium and per mouse 200 µl was injected into the tail vein. Oocyst numbers in dissected midguts from infected mosquitoes were established 8 days after feeding using light-microscopy.

Determination of parasite liver load by real time *in vivo* imaging

Parasite liver loads in live mice after immunization and after challenge were quantified by real time *in vivo* imaging as previously described [34]. Liver stages were visualized and liver loads quantified by measuring luciferase activity of parasites in whole bodies of mice at 44 h after injection of sporozoites using the IVIS Lumina II Imaging System (Perkin Elmer Life Sciences, Waltham, USA). During measurements mice were anesthetized using the isofluorane-anesthesia system (XGI-8, Caliper Life Sciences, Hopkinton, USA). D-luciferin was dissolved in PBS (100 mg/kg; Caliper Life Sciences, USA) and injected subcutaneously in the neck. Measurements were performed within 8 min after the injection of D-luciferin. Quantitative analysis of bioluminescence of whole bodies was performed by measuring the luminescence signal intensity using the ROI (region of interest) settings of the Living Image® 4.4 software.

Immunization protocol and determination of prepatent period after challenge

For the immunization experiments mice were immunized using isolated *PyΔfabb/f* sporozoites according to the immunization protocols described in the Results section. Blood of immunized mice was analyzed for possible breakthrough blood infections by Giemsa-stained blood smears one day before challenge with *Py*WT sporozoites. Immunized mice and naïve controls were challenged 14 days after the last immunization with 3000 (i.e. 3×10^3) *Py*WT sporozoites. Challenged mice were monitored for blood-stage infections by Giemsa-stained blood smears made at day 4 to 14 after challenge. The prepatent period (measured in days after sporozoites challenge) is defined as the day when a blood stage infection with a parasitemia of 0.5–2% is observed [35]. Organs (and serum) used for immunological analysis were collected from the mice at day 7 after immunization or at 7 days after challenge.

OX40 monoclonal antibody (mAb) treatment

Mice were treated with 200 µg of OX40 mAb (clone RM134L; Bio X Cell, West Lebanon, NH, United States) in 200 µl PBS and administrated by intraperitoneal injection (IP) either at day 0 or one day after prime or boost immunization.

Treatment with ARTC2-blocking nanobodies

Immunized mice and naïve controls were treated with 50 µg ARTC2-blocking nanobodies (Biolegend) in 200 µl PBS administered by IP injection 30 min before sacrificing mice for collection of the organs for the immunological assays.

Liver perfusion and purification of liver and spleen cells

Mice were perfused under anesthesia by intracardiac injection of 20 ml PBS (B. Braun, Oss, NL). Perfused livers were minced in small pieces and digested for 30 min at 37°C in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Breda, NL) containing 250 U/ml collagenase and 20 µg/ml DNase. Hepatic leukocytes was obtained by passing the digested tissue through a 70 µM cell-strainer (BD Biosciences. San Diego, CA) and Percoll gradient. For spleens, splenocytes were harvested by mincing the tissue through a 70 µm cell strainer.

Cell surface staining, intracellular staining, and flow cytometry

For cell surface staining, hepatic leukocytes and splenocytes were resuspended in staining buffer (PBS, 2% FCS. 0.05% sodium azide) and incubated with fluorescent conjugated Abs for 30 min at 4°C. For intracellular cytokine staining, hepatic leukocytes and splenocytes were re-stimulated *in vitro* with medium containing 5×10^4 *Py*WT sporozoites for 24 h in 96-well flat-bottom plates (1.5×10^6 hepatic leukocytes and splenocytes per well) as described [36]. In order to improve re-stimulation and to increase the number of antigen-presenting cells, 1.5×10^5 of splenocytes were added at the start of the cultures to all wells. Twenty hours after incubation 1 µg/ml brefeldin A (Golgiplug; BD Pharmingen) was added to all wells. After re-stimulation, cells were transferred to U-bottom 96-well plates, and the cell surface stained with fluorescent conjugated Abs at 4°C for 30 min in staining buffer. After washing, cells were fixed with 0.5% paraformaldehyde at 4°C for 30 min, followed by intracellular staining for cytokines at 4°C for 30 min in Perm/Wash buffer (BD Biosciences). After washing and resuspending in staining buffer, cells were acquired using a BD LSRII flow cytometer and data were analyzed using FlowJo software (Tree Star). Fluorochrome-conjugated mAbs specific for CD3, CD4, CD8, CD44, CD11a, KLRG1, CD134, IFN-γ, IL-2, and TNF were purchased from BD Biosciences or eBioscience (San Diego, CA).

ELISA

Enzyme-linked immunosorbent assay (ELISA) plates (Corning,Inc.) were coated overnight at 4°C by adding 1×10^4 *PyΔfabb/f* sporozoite lysate diluted in 100 µl $NaHCO_3$ buffer (pH 9,6) per well. Plates were washed three times with PBS-T (0.05% Tween 20 in 1×PBS) prior to blocking for 2 h in blocking buffer (1% BSA in PBS-T). Next, sera was diluted in blocking buffer at 1:100 for sporozoite lysate per well. Plates were incubated for 3 h at room temperature before washing as described above. Next, 100 µl of a 1:5000 dilution of horseradish peroxidase (HRP) conjugated anti-mouse IgG (Jackson Immuno-Research) was added and incubated for an additional 1 h at room temperature. Finally, plates were washed again and 100 µl of TMB Substrate solution (Thermo Scientific) was added for 5 min. The reaction was stopped by addition of 50 µl of 0.5 N sulfuric acid prior to measurement of absorbance at 450 nm using a Multiskan FC (Thermo Scientific) microplate reader.

Statistics

All data are calculated using the GraphPad Prism software package 5.04 (GraphPad Software, Inc). For ELISA, cell surface and intracellular staining analysis, statistical analysis was performed using the unpaired Student's *t*-test. For the survival analysis, statistical analyzes to determine differences in protection after challenge were performed using a Kaplan–Meier survival plot, and survival curves were compared using the log-rank (Mantel-Cox) test. Survival was considered as the complete absence of parasites in blood. The significance threshold were 0.05 in all analysis.

RESULTS

Establishing a GAP Sporozoite-BALB/c immunization-challenge protocol to investigate strategies to improve GAP immunization

The *P. yoelii*-BALB/c parasite-mouse combination is a well-established model used to analyze vaccines that target sporozoites or liver-stage parasites. To analyze protective immunity induced after GAP immunization, we used sporozoites of *P. yoelli* Δ*fabb/f* GAP parasites. The *Py*Δ*fabb/f* parasites (GAP) lacks the *fabb/f* gene (PY17X_1126500) and arrest late into liver stage development [33, 37]. This GAP produces oocysts and salivary gland sporozoites comparable to the wild-type parent *P. yoelii* 17XNL *Py*GFP-luc$_{con}$ line (*Py*WT) (**Figure S1A**). Both GAP and *Py*WT sporozoites express the fusion protein GFP-Luciferase under control of the constitutive *eef1a* promoter, permitting the determination of parasite liver loads in live mice by real time bioluminescence imaging [33]. The GAP sporozoites exhibit levels of *in vivo* liver infection that are comparable to *Py*WT sporozoites (**Figure S1B**), however, these GAP sporozoites are unable to initiate a blood infection (**Table S1**).

Protective immunity in immunized mice after challenge with WT sporozoites is defined either by the number of mice that are completely protected from infection or by the delay in time taken to establish a blood stage infection, i.e. the prepatent period (time-to-event analysis) [38]. In this study the prepatent period is defined as the number of days to reach a 0.5-2% parasitemia after *Py*WT sporozoites challenge as previously described [17]. Previously we had established that a primary immunization followed by boost immunization with 1×10^4 GAP sporozoites induced sterile protection in more than 90% of BALB/c mice against challenge with 1×10^4 *Py*WT sporozoites [33]. To examine putative enhancing protective immunity of treatment with adjuvants/immunomodulatory molecules we attempted to identify a 'sub-saturating' immunization regiment by immunizing mice with only a single dose of GAP parasites. Mice were immunized with either 1, 2.5 or 5×10^4 GAP sporozoites and then challenged 14 days later with 3×10^3 *Py*WT sporozoites (**Figure 1A**). A single immunization with all three doses resulted in none of the mice being completely protected. We observed a maximum of one day delay in prepatent period in immunized mice compared to naïve mice. Since the blood stage multiplication rate is 10× per 24 h, a 1 day delay in the prepatent period of blood stage infection represents 90%

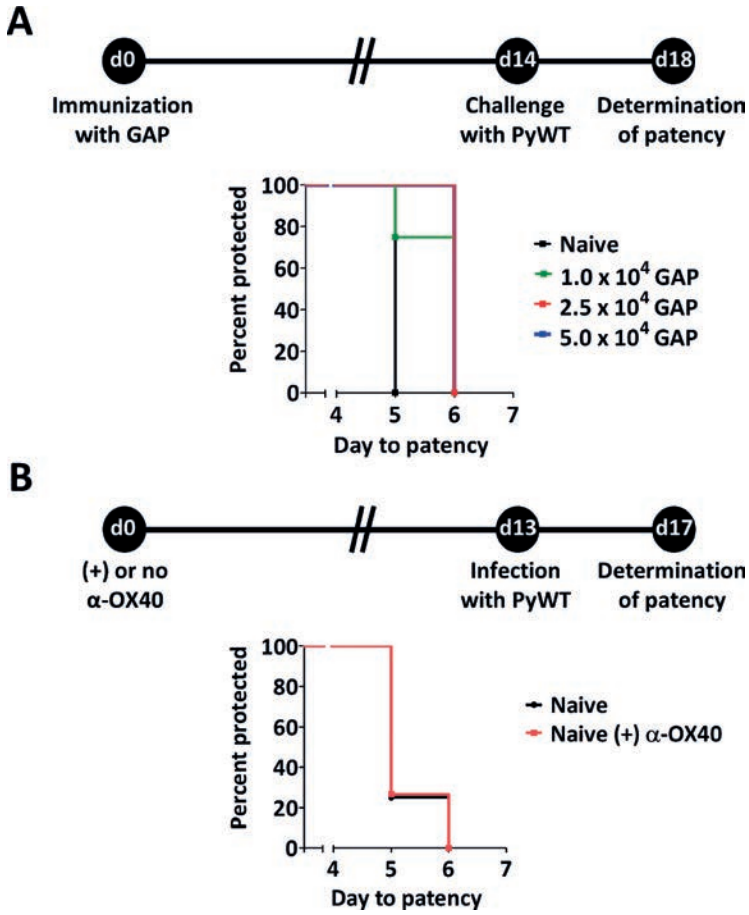


Figure 1. Suboptimal protection after GAP immunization and effect of α-OX40 treatment on prepatent period after infecting mice with wild type (*Py*WT) sporozoites. (A) Protection assays performed in groups of BALB/c mice (n=5 per group) immunized with as single dose of 1, 2.5 or 5 × 10^4 GAP sporozoites and challenged 14 days later with 3 × 10^3 *Py*WT sporozoites. Challenged mice were monitored for blood-stage infections. The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of 0.5–2% is observed). Immunization with 2.5 and 5 × 10^4 GAP sporozoites resulted in a significant longer prepatent period compared to control, non-immunized mice (Log-Rank (Mantel-Cox) test; $p = 0.014$ and $p = 0.025$; respectively). (B) Effect of α-OX40 treatment on parasite development in liver and blood. BALB/c mice were treated with α-OX40 on day 0 and infected with 3.0 × 10^3 *Py*WT sporozoites 13 days later. Infected mice were monitored for blood-stage infections. The Kaplan-Meier curves illustrate that there was no significant differences were observed in prepatent period between α-OX40 treated mice (n=15) and control, non-treated mice (n=8) in two experiments.

reduction in the infection in the liver [39]. The immunization with 2.5 and 5 × 10^4 GAP sporozoites resulted in a significance longer prepatent period ('survival'; $p = 0.014$ and $p = 0.025$, respectively) compared to naïve mice after challenge with *Py*WT sporozoites. Since we did not observe a major difference between the dose of 2.5 and 5 × 10^4, we

choose the protocol of 2.5 × 10^4 GAP immunization followed by 3 × 10^3 *Py*WT sporozoites challenge, to analyze the effect of α-OX40 treatment on protective immunity.

In addition, we performed an experiment to analyze the possible effect of treatment with α-OX40 in naive mice on liver and/or blood stage infection. Naïve mice treated with 200 µg of α-OX40 in 200 µl PBS by intraperitoneal injection (IP) and non-treated mice were challenged with *Py*WT sporozoites 14 days after treatment. No differences in prepatent period were observed between treated and untreated mice (**Figure 1B**), indicating that α-OX40 treatment has no effect on growth/multiplication of *Py*WT parasites in both the liver and blood in non-immunized mice.

α-OX40 treatment increases the protective immunity in mice immunized with a single GAP immunization

To determine whether OX40 is expressed on activated T cells in mice immunized with 2.5 × 10^4 *P. yoelii fabb/f* GAP sporozoites, we determined the OX40 cell surface expression at day 3 post-immunization. OX40 expression was clearly detected on activated ($CD44^{hi}$) $CD4^+$ and $CD8^+$ T cells in the liver (**Figure 2A**). In the spleen, the expression of OX40 on activated $CD4^+$ T cells was also observed, albeit at lower levels compared to activated liver $CD4^+$ T cells whereas OX40 expression on activated splenic $CD8^+$ T cells was not detected (**Figure 2A**). These data show that GAP immunization is associated with the upregulation of co-stimulatory OX40 receptor on $CD4^+$ and $CD8^+$ T cells. Therefore to examine if α-OX40 treatment enhances protective immune responses after GAP vaccination, we treated mice with α-OX40 one day after they were immunized with a single dose of 2.5 × 10^4 GAP sporozoites as described above (**Figure 2B**). As expression of OX40 is upregulated after antigen recognition, we scheduled α-OX40 administration 1 day after the immunization [40]. Immunized mice were injected intraperitoneally with 200 µg α-OX40 in 200 µl PBS by IP injection. In two experiments we observed an increase in protective immunity in GAP-immunized plus α-OX40 mice (**Figure 2C**). In the control groups of mice, naïve and GAP-immunized but not OX40 treated, none of the mice were protected against *Py*WT sporozoites challenge and all mice became patent at day 5 or 6 in two experiments. In contrast, in the two groups of GAP-immunized plus α-OX40 treated mice, a total of 4 out of 15 (26.7%) mice were completely protected and in 9 of the remaining 11 mice (60%) *Py*WT parasites emerged in the blood one day later than GAP-immunized mice and naive mice (**Figure 2C**). The vaccination of GAP with α-OX40 induced a significant increase in protection compared to immunization with only GAP parasites in both independent experiments (*p = 0.011 and **p = 0.0017).

α-OX40 treatment after a single GAP immunization results in an increase of effector ($CD44^{hi}$ $CD11a^{hi}$) $CD4^+$ T cells in both liver and spleen

One week after immunization, organs and blood were collected from mice that were immunized with a single dose of 2.5 × 10^4 GAP sporozoites either with or without α-OX40

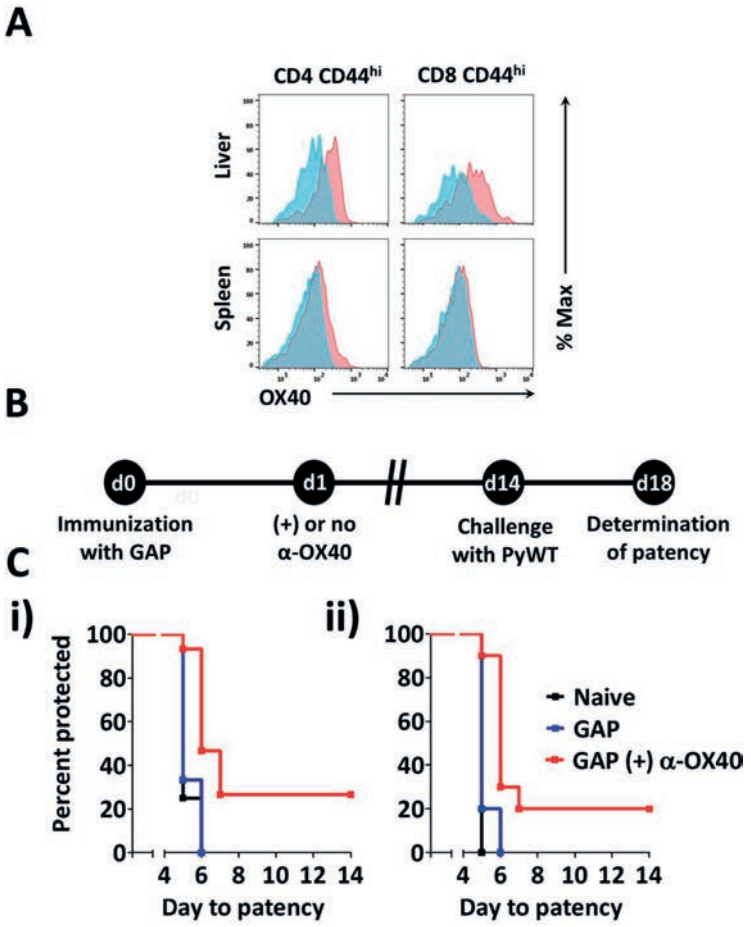


Figure 2. α-OX40 treatment increases the protective immunity in mice that received a single GAP immunization. (A) OX40 expression on activated CD44^{hi} CD4^{+} and CD44^{hi} CD8^{+} T cells in the spleen and liver at day 3 after immunization. Flow cytometric histograms indicate OX40 expression (red) and fluorescence minus-one (FMO) controls (blue). (B) The time line shows immunization of BALB/c mice with GAP sporozoites (2.5 × 10^{4}), α-OX40 treatment and challenge with wild type (*Py*WT) sporozoites (3 × 10^{3}). Challenged mice were monitored for blood-stage infections from day 18 onwards to determine the prepatent period. (C) The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of 0.5–2% is observed). Data show representative from 2 independent experiments with (i) 5 and (ii) 10 mice per group: Naïve vs GAP not significant (n.s.); GAP + α-OX40 vs GAP p = 0.011 and p = 0.0017; GAP + α-OX40 vs Naïve, p = 0.008 and 0.004, respectively in both experiments.

treatment (Figure 3A). In all immunized mice we observed a significant and strong increase in total white blood cells (WBCs) and CD4^{+}/CD8^{+} T cells compared to the naïve control mice. Strikingly, we observed only in the GAP-immunized plus α-OX40 treated mice a significant increase (**p =0.0023) in CD4^{+} T cell numbers in the liver compared to GAP-immunized mice (Figure 3B). We analyzed the phenotype of the antigen-experienced

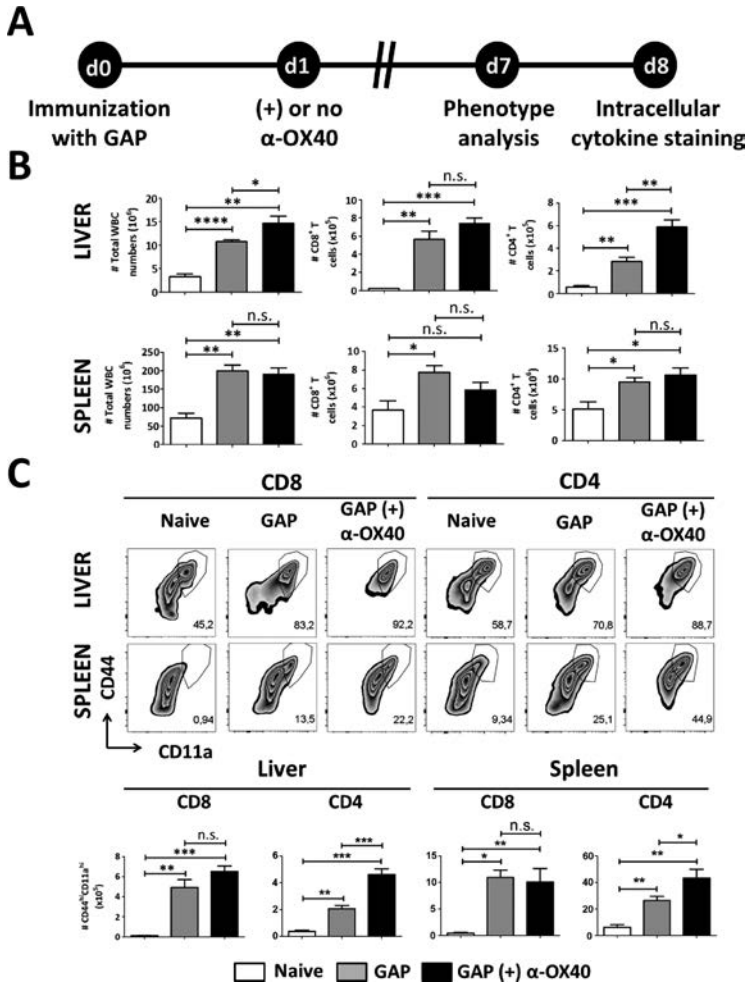


Figure 3. α-OX40 treatment after a single GAP immunization results in an increase of effector ($CD44^{hi}CD11a^{hi}$) $CD4^+$ T cells in both liver and spleen. (A) The time line showing immunization of 2 groups of BALB/c mice with GAP sporozoites (2.5×10^4) that were treated or not treated with α-OX40 one day after immunization. T cells were collected from the liver and spleen at day 7 and analyzed for phenotype analysis at day 7 or for cytokine expression at day 8 after *in vitro* re-stimulation with whole sporozoites. (B) The total number of WBC, $CD8^+$ and $CD4^+$ T cells in liver and spleen of different groups of mice. Significant differences in total WBC (*p = 0.03) and $CD4^+$ T cells (**p = 0.0023) were observed between the livers of α-OX40 treated and non-treated mice. Representative data is shown from 2 independent experiments with 6 mice per group. (C) The upper panel shows the percentages of ($CD44^{hi}CD11a^{hi}$) T cells of total $CD8^+$ and $CD4^+$ T cells in liver and spleen in the different groups of mice. The lower panel shows the total number of ($CD44^{hi}CD11a^{hi}$) $CD8^+$ and ($CD44^{hi}CD11a^{hi}$) $CD4^+$ T cells in liver and spleen. A significant increase of ($CD44^{hi}CD11a^{hi}$) $CD4^+$ cells was observed in both liver and spleen of mice immunized with GAP plus α-OX40 compared to only GAP-immunized mice (***p =0.0004 and *p =0.044, respectively). Representative data is shown from 2 independent experiments with 6 mice per group. Significant difference by unpaired *t*-test is indicated by not significant (n.s.) and significant; *p<0.05 or ** p<0.01, ***p<0.001, ****p<0.0001.

effector T cells using CD44 and CD11a as markers [41-43]. When we compared effector ($CD44^{hi}CD11a^{hi}$) $CD8^+$ and $CD4^+$ T cells we found that in both the spleen and the liver of GAP-immunized plus α-OX40 treated mice the number of ($CD44^{hi}CD11a^{hi}$) $CD4^+$ T cells were significantly increased (*p = 0.044 and ***p = 0.0004; respectively), compared to GAP-immunized mice. No significant differences were observed in ($CD44^{hi}CD11a^{hi}$) $CD8^+$ T cells in α-OX40 treated or untreated GAP-immunized mice, either in the liver or spleen (**Figure 3C**). Combined these results show that the administration of α-OX40 after a priming GAP immunization enhances the number of antigen-experienced effector $CD4^+$ T cells in both the liver and spleen.

Increased effector T cell formation by α-OX40 treatment after prime-boost GAP immunization

In order to examine the effect of α-OX40 treatment on both the formation and recall of the adaptive immune response after GAP vaccination, we adopted a prime-boost immunization strategy. Specifically, we immunized mice initially with a 2.5 × 10^4 GAP sporozoites followed 2 weeks later by a boost with 2.5 × 10^4 GAP sporozoites (**Figure 4**). This GAP immunization schedule provides 90-100% sterile protective immunity in BALB/c mice [14]. α-OX40 treatment, as described above, was performed 1 day after the boost immunization as described for a vaccination protocol against mouse cytomegalovirus infection [44], and organs and blood collected one week after the boost immunization. In GAP-immunized plus α-OX40 treated mice we observed a strong significant increase in total WBCs in both the spleen (**p = 0.001) and liver (*p = 0.035) compared to GAP-immunized mice (**Figure 4**). We also observed an increase in total $CD4^+$ and $CD8^+$ T cells in GAP-immunized plus α-OX40 mice in the liver (p = 0.0625, *p = 0.043 respectively). In the spleen we only observed a significant increase in total $CD4^+$ T cells (*p = 0.019) but not in the $CD8^+$ T cells in GAP-immunized plus α-OX40 treated mice compared to GAP-immunized mice. In both the spleen and the liver of GAP-immunized plus α-OX40 treated mice the number of ($CD44^{hi}CD11a^{hi}$) $CD4^+$ (**p = 0.0014 and **p = 0.0045; respectively) and $CD8^+$ (**p = 0.0073 and *p = 0.0357; respectively) T cells were significantly increased compared to GAP-immunized mice (**Figure 5A**). Also, when we compared activated effector-type ($CD44^{hi}KLRG1^{hi}$) $CD4^+$ and $CD8^+$ T cells, we found that in the spleens of GAP-immunized plus α-OX40 mice the number of ($CD44^{hi}KLRG1^{hi}$) $CD4^+$ T cells were significantly increased (*p = 0.043) compared to GAP-immunized mice (**Figure 5B**). Combined these results suggest that enforced OX40 stimulation after a prime-boost immunization does not only impact the expansion of antigen-experienced effector $CD4^+$ T cells, as was shown after a single immunization, but also expands the pool of antigen-experienced effector $CD8^+$ T cells.

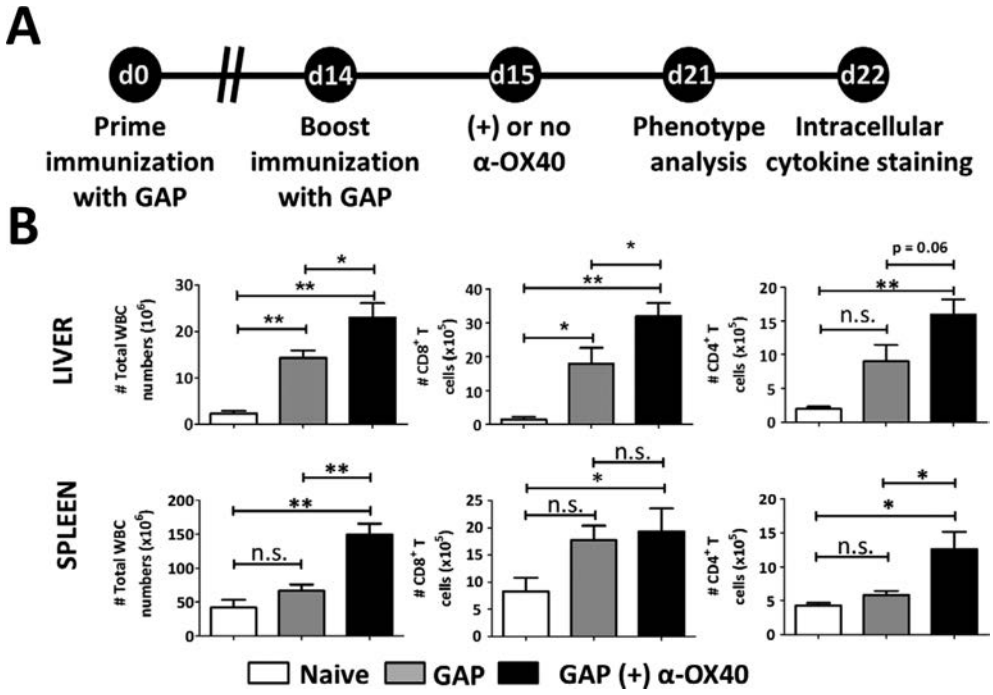


Figure 4. Prime-boost GAP immunization plus α-OX40 during the boost provokes the expansion of total WBC, $CD8^+$ and $CD4^+$ T cell numbers in the liver and spleen. (A) Time line showing immunization of 2 groups of BALB/c mice with GAP sporozoites (2.5×10^4). Both groups received a prime (day 0) and boost (day 14) immunization, and were either treated or not treated with α-OX40 one day after the boost immunization. T cells were collected from the liver and spleen at day 7 for phenotype analysis at day 7 or for cytokine expression at day 8 after *in vitro* re-stimulation with whole sporozoites. (B) The total number of WBC, $CD8^+$ and $CD4^+$ T cells in liver and spleen of different groups of mice. Significant differences were observed between total WBC (*p = 0.035 and **p = 0.001) and $CD4^+$ T cells (*p = 0.0625 and *p = 0.019) collected from the liver and spleen of treated and untreated mice. In addition, a significant difference in total $CD8^+$ T cells (*p = 0.019) was observed between livers of treated and untreated mice. Representative data is shown from 2 independent experiments with 6 mice per group. Significant difference by unpaired *t*-test is indicated as not significant (n.s.) or significant; * p<0.05 or ** p<0.01.

α-OX40 treatment increases IFN-γ and TNF producing $CD4^+$ T cells in both liver and spleen and increases the amount of sporozoite-specific antibodies after prime-boost GAP immunization

In order to study the impact of the α-OX40 treatment on the cytokine production of the $CD4^+$ and $CD8^+$ T cells after prime-boost immunization, we performed intracellular staining for IFN-γ and TNF of hepatic leucocytes and splenocytes isolated 7 days after the final immunization. Before staining cells were stimulated *in vitro* for a period of 24 h with sporozoites. It has been reported that treatment with anti-ARTC2 antibodies can improve T cell survival and recovery after *in vitro* stimulation consequently mice were

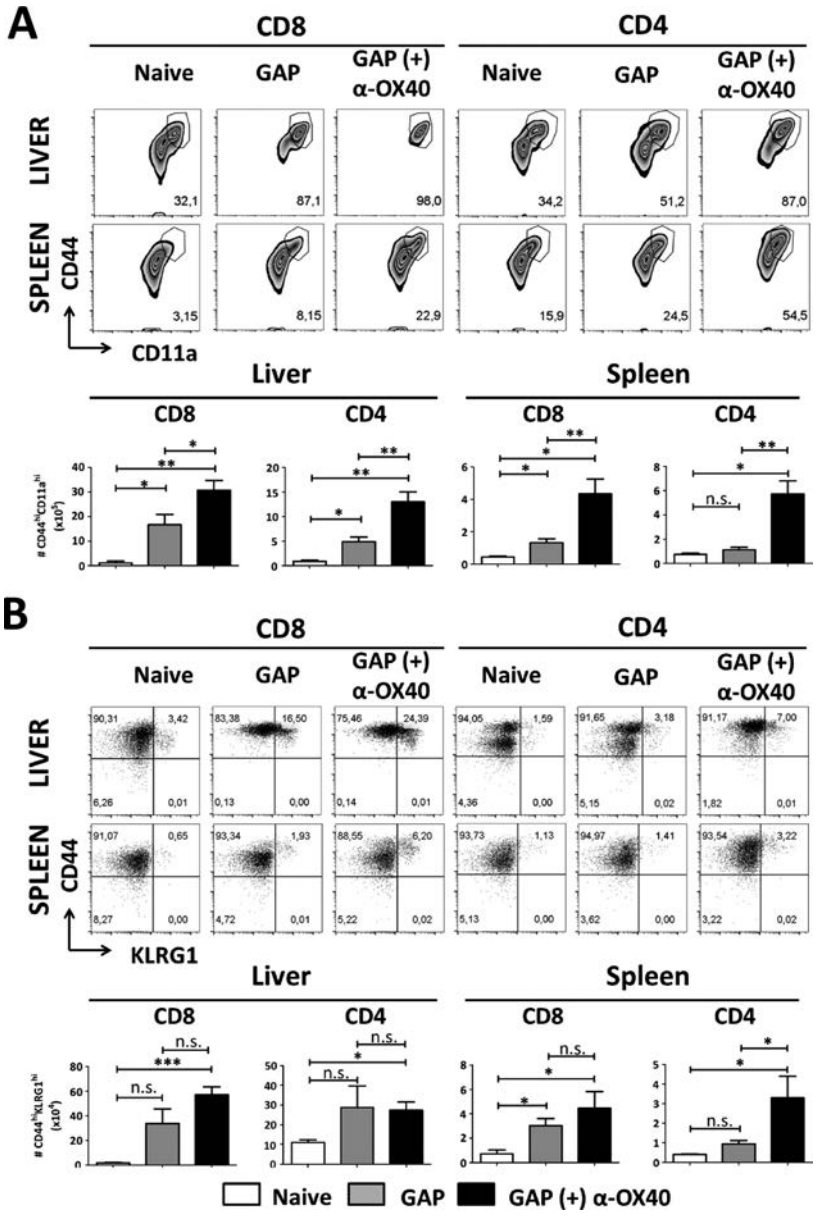
Figure 5. Increased effector T cell formation by α-OX40 treatment after prime-boost GAP immunization. **(A)** See **Figure 4** for the time line of immunization and collection of T cells. The upper panel shows the percentages of ($CD44^{hi}CD11a^{hi}$) $CD8^+$ and $CD4^+$ T cells in liver and spleen. The lower panel shows the total number of ($CD44^{hi}CD11a^{hi}$) $CD8^+$ and ($CD44^{hi}CD11a^{hi}$) $CD4^+$ T cells in liver and spleen. A significant increase of ($CD44^{hi}CD11a^{hi}$) $CD8^+$ cells (*p = 0.0357 and **p = 0.0073) and ($CD44^{hi}CD11a^{hi}$) $CD4^+$ (**p = 0.0045 and **p = 0.0014) was observed in both liver and spleen, in mice immunized with GAP plus α-OX40 compared to only GAP-immunized mice. Representative data is shown from 2 independent experiments with 6 mice per group. Significant difference by unpaired *t*-test is indicated by not significant (n.s.) and significant; * p<0.05 or ** p<0.01, ***p<0.001. **(B)** The upper panel shows percentages of ($CD44^{hi}KLRG1^{hi}$) $CD8^+$ and $CD4^+$ T cells in liver and spleen in the different groups of mice. The lower panel shows the total number of ($CD44^{hi}KLRG1^{hi}$) $CD8^+$ and ($CD44^{hi}KLRG1^{hi}$) $CD4^+$ T cells. A significant increase of ($CD44^{hi}KLRG1^{hi}$) $CD4^+$ T cells was observed in spleens of mice immunized with GAP plus α-OX40 compared to only GAP-immunized mice (*p =0.043, respectively). Representative data is shown from 2 independent experiments with 6 mice per group. Significant difference by unpaired *t*-test is indicated by not significant (n.s.) and significant; * p<0.05 or ** p<0.01, ***p<0.001.

treated with ARTC2 nanobodies 30 min before collection of the organs [45]. We observed a significant increase in IFN-γ producing $CD4^+$ (**p = 0.0065) and $CD8^+$ (**p = 0.0018) T cells in the spleens of GAP-immunized plus α-OX40 treated mice compared to GAP-immunized mice. In the liver of GAP-immunized plus α-OX40 treated mice, there was an increase of IFN-γ producing $CD4^+$ T cells (p = 0.0506) but not of $CD8^+$ T cells. Further, we observed a significant increase in TNF producing $CD4^+$ T cells in both liver (*p = 0.0398) and spleen (**p = 0.0068) of GAP-immunized plus α-OX40 treated mice compared to GAP-immunized mice but TNF production in $CD8^+$ T cells was not significantly different in either the liver or spleen (**Figure 6A**). Taken together, these results show that α-OX40 treatment after a prime-boost GAP immunization elicits a significant increase in IFN-γ and TNF producing $CD4^+$ T cells in both liver and spleen of GAP-immunized plus α-OX40 treated mice compared to GAP-immunized mice.

In addition to collecting organs at day 7 after the final immunization, we also collected serum from these mice to perform ELISA analysis with *P. yoelii* sporozoite lysate to quantify parasite-specific IgG responses. This analysis revealed that mice immunized with GAP sporozoites generate sporozoite-specific antibody responses and that there is a significant (*p=0.02, Student's *t*-test) increase in the total IgG produced in mice immunized with GAP plus α-OX40 treatment compared to mice immunized with only GAP (**Figure 6B**).

DISCUSSION

Vaccination with live attenuated sporozoites can induce protective immunity in humans but induction of sterile protection requires immunization with multiple doses and each dose consisting of relatively high numbers of sporozoites [1, 6]. Enhancing the immunogenicity of whole sporozoite (wsp) vaccines, for example by adding adjuvants, can be used to both reduce the number of sporozoites per dose and the number of vaccine doses, as well as directing the adaptive immune response. We show in this study that treatment of mice



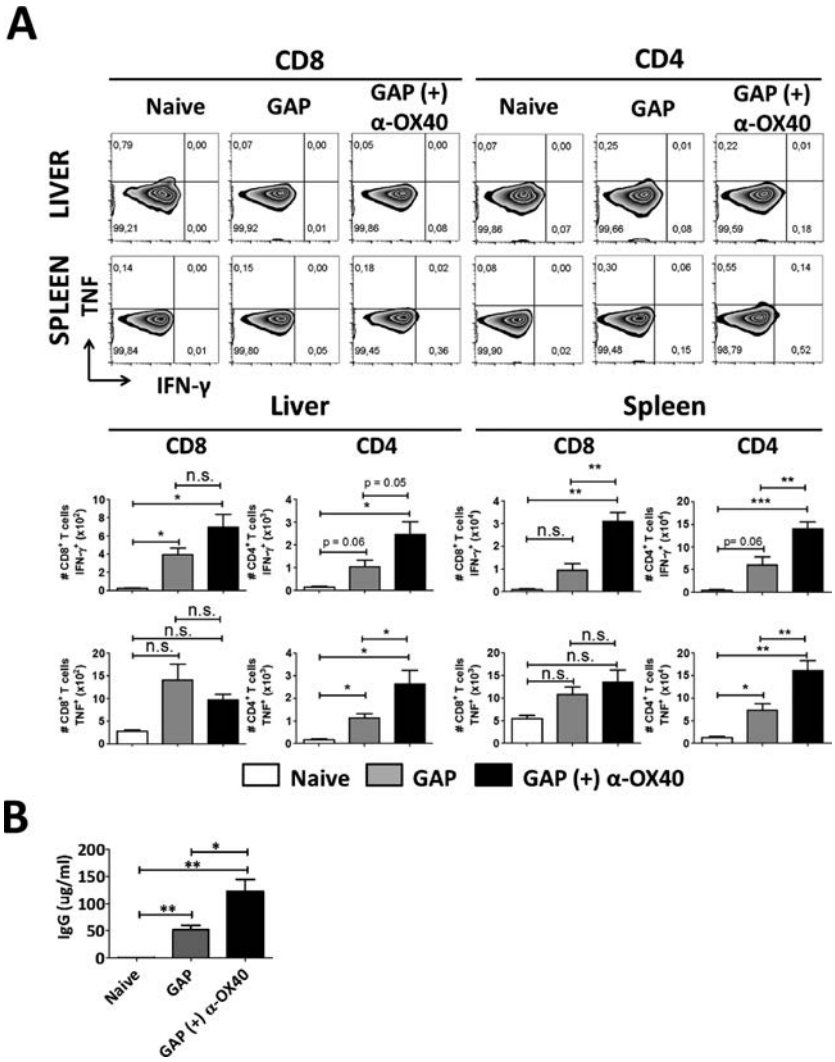
with an agonistic antibody against the T cell costimulatory molecule OX40, a member of the tumor necrosis factor receptor (TNFR) superfamily [46], enhances protective immunity after immunization with GAP sporozoites.

Previously it has been shown that targeting OX40 increases the magnitude of T cell responses and improves T cell functionality [47, 48]. OX40 is transiently expressed on T cells following cognate interactions between T cell receptors (TCRs) and antigen-major histocompatibility (MHC) complexes on antigen presenting cells (APCs) [48]. While OX40

Figure 6. α-OX40 treatment increases IFN-γ and TNF producing $CD4^+$ T cells in both liver and spleen and increases the amount of sporozoite-specific antibodies after prime-boost GAP immunization. (A) See Figure 4 for the time line of immunization and collection of T cells. The upper panel shows percentage of IFN-γ and TNF cytokine producer $CD8^+$ and $CD4^+$ T cells in liver and spleen after *in vitro* sporozoite re-stimulation. The second panel shown the total number of IFN-γ and TNF cytokine producer $CD8^+$ and $CD4^+$ T cells in liver and spleen. Significant differences in both IFN-γ cytokine producer $CD8^+$ and $CD4^+$ T cells (**p = 0.0018 and **p = 0.0065, respectively) were observed between spleens of α-OX40 treated and untreated mice. In addition, an increase in IFN-γ cytokine producer $CD4^+$ T cells (p = 0.0506) was observed in livers of treated mice compared to livers of untreated mice. Further, a significant differences of TNF producing $CD4^+$ T cells in both liver and spleen (*p = 0.0398 and **p = 0.0068, respectively) were observed between α-OX40 treated and untreated mice. Representative data is shown from 2 independent experiments with 6 mice per group. Significant difference by unpaired *t*-test is indicated by not significant (n.s.) and significant; * p<0.05 or ** p<0.01, ***p<0.001. (B) Quantification by ELISA of *P. yoelii* WT sporozoite-specific IgG obtained from naïve and prime-boost immunized mice with *P. yoelii* GAP (with or without α-OX40 treatment). The concentration of the total IgG in the ELISA was quantified using the values for each sample based on the standard curve obtained with defined concentrations of polyclonal antibodies against *Py*CSP (Bioss Antibodies Inc., USA).

is expressed on both activated $CD4^+$ and $CD8^+$ T cells, OX40 expression on $CD4^+$ T cells is greater than $CD8^+$ T cells and consequently α-OX40 treatment is expected to exert its greatest effect on $CD4^+$ T cells [46, 48, 49]. OX40 signaling promotes T cell proliferation and survival, influences $CD4^+$ T cell differentiation into T helper subsets [50-53] and is reported to reverse $CD4^+$ T cell hypo-responsiveness [54]. While it has been previously described that OX40 is expressed on activated human and rodent CD4 T cells after a malaria blood stage infection [55, 56] no data had been reported on the expression of OX40 on T cells after a sporozoite/liver stage *Plasmodium* infection/immunization. We demonstrate in this study that after GAP-sporozoite immunization OX40 expression was observed on activated ($CD44^{hi}$) $CD4^+$ and $CD8^+$ T cells in the liver. Similarly OX40 expression was upregulated on activated $CD4^+$ T cells in the spleen but not observed on activated $CD8^+$ T cells. We therefore hypothesized that therapeutic ligation of OX40 during immunization with attenuated *Plasmodium* sporozoites would increase parasite-specific $CD4^+$ and $CD8^+$ T cell activity, limit the degree of T cell exhaustion and improve T cell effector-memory formation, all resulting in increased clearance of *Py*WT sporozoites/liver stages.

To analyze the effect of adjuvants on wsp vaccination approaches we first developed a model with a sub-saturating immunization regiment, which we could use to measure enhancement of protective immunity through the application of adjuvants. In this study we demonstrate that a single immunization with 2.5 and 5 × 10^4 sporozoites induces partial protection as determined by an absence of sterile protection after *Py*WT sporozoites challenge but a 1 day delay in the emergence of parasites in the blood (prepatent period). A 1 day delay in prepatent period has been correlated with a 10× reduction in parasites released from the liver [39], indicating a 10× increase in protective immunity compared to unimmunized mice.



In cytomegalovirus (CMV) vaccination studies it was found that the increase in vaccine potency can be achieved by α-OX40 treatment through the expansion of both antigen-specific $CD4^+$ and $CD8^+$ T cells [44]. A marked upregulation of OX40 is observed on *Plasmodium* specific $CD4^+$ T cells that are generated in both human and rodent malaria blood stage infections and, in rodent studies, α-OX40 treatment was shown to increase parasite-specific memory $CD4^+$ T cells resulting in a reduced blood-stage infection [55-57]. However, prior to this study the effects of OX40 treatment on immune responses induced by wsp vaccination have not been described.

Our analyzes of T cell responses in mice immunized with a single dose of GAP parasites, showed an increase in total WBC numbers in the livers and an increase in $CD4^+$ effector ($CD44^{hi}CD11a^{hi}$) T cells in both liver and spleen of α-OX40 treated mice compared to

untreated mice. It has been reported by Cooney *et al*. that T cells with a $CD44^{hi}CD11a^{hi}$ phenotype are indicative of antigen-experienced effector cells in GAP-immunized BALB/c mice [42]. While protective immunity after wsp immunization is thought to largely dependent on the killing infected hepatocytes by $CD8^+$ T cells and IFN-γ [16, 58, 59], adoptive transfer of $CD4^+$ T cells from GAP-immunized C57BL/6 mice was able to provide sterile protection to 50% of naïve animals against a WT infection, indicating an important role for $CD4^+$ T cells in GAP induced immunity [58]. In addition, protective immunity induced by sporozoites of a *P. yoelii* GAP, similar to the one used in our study, was dependent not only on $CD8^+$ T cells but also $CD4^+$ T cells [60]. Immune responses induced by *P. yoelii* GAPs that arrest late into liver development [14] are reported to involve both the cellular and humoral arm of the adaptive immune response. Indeed, Keitany *et al*. showed that functional antibodies are induced after immunization with *P. yoelii* GAPs, which can inhibit sporozoite invasion of liver cells and reduce intrahepatic parasite development [61-63]. Since enhancement of $CD4^+$ T cell responses by OX40 stimulation may lead to an increase in humoral immunity we examined total IgG responses generated in mice after prime-boost GAP immunization, either with or without α-OX40 treatment. These studies revealed that anti-sporozoite antibodies were generated after GAP immunization and significantly more IgG was generated in mice immunized with GAP plus α-OX40 treatment compared to mice immunized with only GAP. This observation indicates that the increase in $CD4^+$ T cells after α-OX40 treatment may be directly contributing to B cell maturation/activation.

We further examined the effect of α-OX40 treatment on adaptive immune responses by analyzing immune responses in mice that had received a boost immunization after the prime immunization. After this prime-boost strategy we observed an increase in total WBC numbers in livers and spleens of both GAP-immunized plus α-OX40 and GAP-only immunized mice. However, we observed a significant increase in effector ($CD44^{hi}CD11a^{hi}$) $CD4^+$ and $CD8^+$ T cells in liver and spleen of GAP-immunized plus α-OX40 treated mice compared to GAP-only immunized mice. This is in contrast to the single prime strategy where we only observed a significant in increase only in effector ($CD44^{hi}CD11a^{hi}$) splenic and liver $CD4^+$ T cells in GAP-immunized plus α-OX40 treated mice. When we examined the activation phenotype ($CD44^{hi}KLRG1^{hi}$) of these T cells in liver and spleen, α-OX40 treatment significantly increased only the number of activated $CD4^+$ T cells and only those present in the spleen. Additionally, we observed a significant increase in IFN-γ producing $CD4^+$ and $CD8^+$ T cells in the spleen but not in the liver [60]. We also observed a significant increase in TNF producing $CD4^+$ T cells, but not $CD8^+$ T cells, in the liver and spleens of GAP-immunized plus α-OX40 treated mice. Therefore $CD4^+$ T cells in the spleen may contribute to protective immunity either by enhancing humoral responses targeting sporozoites invasion [62] or by enhancing $CD8^+$ T cell responses that target infected hepatocytes. Recently it was reported, in mice that liver resident $CD8^+$ T cells induced by wsp vaccination may be primed in the spleen and their conversion occurring after reencountering parasite antigen in the liver [64]. Our results indicate that the increased protective immunity observed in GAP-immunized plus α-OX40 treated mice acts primarily

via enhanced $CD4^+$ T cell responses in the spleen. It is known that $CD4^+$ T cell help is necessary for an effective $CD8^+$ T cell memory response against non-inflammatory antigens, such as tumor cells and certain pathogens that may not carry sufficient danger signals [65]. Mice depleted of $CD4^+$ T cells during immunization with sporozoites failed to exhibit a robust $CD8^+$ T cell expansion and were not protected against challenge [66, 67]. Murray *et al.* found that $CD4^+$ T cell help was also necessary to induce protection after immunization with GAP sporozoites [60]. OX40, in addition to being a costimulatory receptor that potentiates proliferation, survival, memory formation, and effector function of $CD4^+$ and $CD8^+$ T cells, can also overcome the suppressive activity of regulatory T cells (Tregs) [68]. Overcoming immune suppression effects could also benefit the generation of protective immunity after wsp vaccination as it has been recently shown that wsp immunization, in particular after GAP administered via the skin, can induce regulatory responses [33]. Together, our results indicate that improving $CD4^+$ T cell activation enhances protective immunity against malaria. Whether this $CD4^+$ T cell stimulation acts primarily by improving humoral responses targeting sporozoites or by increasing $CD8^+$ T cell responses against infected liver cells and how these responses may contribute to formation of immunological memory and duration of protection requires further investigation.

A limited number of other studies have been performed on the effect of adjuvants on protective immunity induced by wsp immunization. In particular the use of the glycolipid α-galactosylceramide (α-GalCer) [69] and its analog 7DW8-5 have been analyzed [70]. Co-administration of these molecules with sporozoites resulted in enhanced recruitment and activation/maturation of dendritic cells in lymph nodes draining the site of vaccine administration and thereby enhancing parasite-specific T cell immunogenicity. Although the possible use of certain adjuvants in human vaccination studies may be difficult due to costs, applicability or side-effects, these pre-clinical studies provide useful information of the largely unknown mechanisms underlying protective immunity. Although α-OX40 treatment is currently in clinical trials for cancer immunotherapy, the use of antibody-based α-OX40 treatment may, for vaccines for the developing world, be unrealistic as they are likely to be too expensive. Other (protein based) agents that can stimulate costimulatory responses, including agonists of OX40 are being developed as potential adjuvants in vaccine development. For example, combination therapy using the protein ligand of OX40, OX40L, fused to a cancer vaccine have been shown to reduce breast cancer metastasis, by enhancing antigen specific $CD4^+$ and $CD8^+$ T cell responses and inhibiting immunosuppressive Treg responses [71]. The co-administration of proteins like OX40L which are likely to be cheaper and easier to produce, may therefore be more practical and feasible approaches to pursue. In conclusion, this study demonstrates how specific immune response to vaccination coupled with activation of costimulatory molecules on the surface of T cells, can enhance protective immunity after wsp immunization and merits further investigation to see if such approaches not only increase the magnitude but also the breadth of an immune responses after vaccination.

AUTHOR CONTRIBUTIONS

ASO designed and performed most of the experiments and data analysis, and wrote the manuscript. BF performed the experiments and reviewed the manuscript. TI, EG, AR and SC conducted experiments and assisted with flow cytometry. JR, AMS and CM generated the GAP mutant. CJ, RA and SK designed and supervised the study and wrote the manuscript.

REFERENCES

1. Hoffman, S.L., et al., *The march toward malaria vaccines.* Vaccine, 2015. **33**: p. D13-D23.
2. White, M.T., et al., *Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial.* Lancet Infectious Diseases, 2015. **15**(12): p. 1450-1458.
3. Tinto, H., et al., *Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial.* Lancet, 2015. **386**(9988): p. 31-45.
4. Mahmoudi, S. and H. Keshavarz, *Efficacy of phase 3 trial of RTS, S/AS01 malaria vaccine: The need for an alternative development plan.* Human Vaccines & Immunotherapeutics, 2017. **13**(9): p. 2098-2101.
5. Pinzon-Charry, A. and M.F. Good, *Malaria vaccines: the case for a whole-organism approach.* Expert Opinion on Biological Therapy, 2008. **8**(4): p. 441-448.
6. Hollingdale, M.R. and M. Sedegah, *Development of whole sporozoite malaria vaccines.* Expert Review of Vaccines, 2017. **16**(1): p. 45-54.
7. Nussenzweig, R.S., et al., *Protective Immunity produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei.* Nature, 1967. **216**(5111): p. 160-162.
8. Nussenzweig, R.S., et al., *Specificity of Protective Immunity Produced by X-Irradiated Plasmodium Berghei Sporozoites.* Nature, 1969. **222**(5192): p. 488-+.
9. Seder, R.A., et al., *Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine.* Science, 2013. **341**(6152): p. 1359-1365.
10. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites.* Journal of Infectious Diseases, 2002. **185**(8): p. 1155-1164.
11. Roestenberg, M., et al., *Protection against a Malaria Challenge by Sporozoite Inoculation.* New England Journal of Medicine, 2009. **361**(5): p. 468-477.
12. Richie, T.L., et al., *Progress with Plasmodium falciparum sporozoite (PfSPZ)-based malaria vaccines.* Vaccine, 2015. **33**(52): p. 7452-7461.
13. Sissoko, M.S., et al., *Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.* Lancet Infectious Diseases, 2017. **17**(5): p. 498-509.
14. Butler, N.S., et al., *Superior Antimalarial Immunity after Vaccination with Late Liver Stage-Arresting Genetically Attenuated Parasites.* Cell Host & Microbe, 2011. **9**(6): p. 451-462.
15. Othman, A.S., et al., *The use of transgenic parasites in malaria vaccine research.* Expert Review of Vaccines, 2017. **16**(7): p. 685-697.
16. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Current Opinion in Biotechnology, 2012. **23**(6): p. 908-916.
17. van Schaijk, B.C.L., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.
18. Mikolajczak, S.A., et al., *A Next-generation Genetically Attenuated Plasmodium falciparum Parasite Created by Triple Gene Deletion.* Molecular Therapy, 2014. **22**(9): p. 1707-1715.
19. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination (vol 22, pg 614, 2016).* Nature Medicine, 2016. **22**(6): p. 692-692.
20. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proceedings of

the National Academy of Sciences of the United States of America, 2017. **114**(10): p. 2711-2716.

21. Schofield, L., et al., *Gamma-Interferon, Cd8+ T-Cells and Antibodies Required for Immunity to Malaria Sporozoites.* Nature, 1987. **330**(6149): p. 664-666.
22. Weiss, W.R., et al., *Cd8+ T-Cells (Cytotoxic/Suppressors) Are Required for Protection in Mice Immunized with Malaria Sporozoites.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(2): p. 573-576.
23. Doll, K.L. and J.T. Harty, *Correlates of protective immunity following whole sporozoite vaccination against malaria.* Immunologic Research, 2014. **59**(1-3): p. 166-176.
24. Van Braeckel-Budimir, N., S.P. Kurup, and J.T. Harty, *Regulatory issues in immunity to liver and blood-stage malaria.* Current Opinion in Immunology, 2016. **42**: p. 91-97.
25. Silvie, O., R. Amino, and J.C. Hafalla, *Tissue-specific cellular immune responses to malaria pre-erythrocytic stages.* Current opinion in microbiology, 2017. **40**: p. 160-167.
26. Curran, M.A., et al., *PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(9): p. 4275-4280.
27. Wolchok, J.D., et al., *Nivolumab plus Ipilimumab in Advanced Melanoma.* New England Journal of Medicine, 2013. **369**(2): p. 122-133.
28. Croft, M., *Costimulation of T cells by OX40, 4-1BB, and CD27.* Cytokine & Growth Factor Reviews, 2003. **14**(3-4): p. 265-273.
29. Dawicki, W., et al., *4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses.* Journal of Immunology, 2004. **173**(10): p. 5944-5951.
30. Melero, I., et al., *Immunostimulatory monoclonal antibodies for cancer therapy.* Nature Reviews Cancer, 2007. **7**(2): p. 95-106.
31. Schaer, D.A., D. Hirschhorn-Cymerman, and J.D. Wolchok, *Targeting tumor-necrosis factor receptor pathways for tumor immunotherapy.* Journal for ImmunoTherapy of Cancer, 2014. **2**(1): p. 7.
32. Lin, J.W., et al., *A Novel ' Gene Insertion/Marker Out' (GIMO) Method for Transgene Expression and Gene Complementation in Rodent Malaria Parasites.* Plos One, 2011. **6**(12).
33. Haeberlein, S., et al., *Protective immunity differs between routes of administration of attenuated malaria parasites independent of parasite liver load.* Scientific Reports, 2017. **7**(1): p. 10372.
34. Annoura, T., et al., *Quantitative Analysis of Plasmodium berghei Liver Stages by Bioluminescence Imaging*, in *Malaria: Methods and Protocols*, R. Ménard, Editor. 2013, Humana Press: Totowa, NJ. p. 429-443.
35. van Schaijk, B.C.L., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**(03582).
36. Arens, R., et al., *Differential B7–CD28 Costimulatory Requirements for Stable and Inflationary Mouse Cytomegalovirus-Specific Memory CD8 T Cell Populations.* The Journal of Immunology, 2011. **186**(7): p. 3874.
37. Vaughan, A.M., et al., *Type II fatty acid synthesis is essential only for malaria parasite late liver stage development.* Cellular Microbiology, 2009. **11**(3): p. 506-520.
38. O'Meara, W.P., B.F. Hall, and F.E. McKenzie, *Malaria vaccine efficacy: the difficulty of detecting and diagnosing malaria.* Malaria Journal, 2007. **6**.
39. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nature Protocols, 2006. **1**(1): p. 346-356.
40. Aspeslagh, S., et al., *Rationale for anti-OX40 cancer immunotherapy.* European Journal of Cancer, 2016. **52**: p. 50-66.

41. Rai, D., et al., *Tracking the Total CD8 T Cell Response to Infection Reveals Substantial Discordance in Magnitude and Kinetics between Inbred and Outbred Hosts.* Journal of Immunology, 2009. **183**(12): p. 7672-7681.

42. Cooney, L.A., et al., *Short-Lived Effector CD8 T Cells Induced by Genetically Attenuated Malaria Parasite Vaccination Express CD11c.* Infection and Immunity, 2013. **81**(11): p. 4171-4181.

43. Schmidt, N.W., et al., *Extreme CD8 T Cell Requirements for Anti-Malarial Liver-Stage Immunity following Immunization with Radiation Attenuated Sporozoites.* Plos Pathogens, 2010. **6**(7).

44. Panagioti, E., et al., *Enforced OX40 Stimulation Empowers Booster Vaccines to Induce Effective CD4(+) and CD8(+) T Cell Responses against Mouse Cytomegalovirus Infection.* Frontiers in Immunology, 2017. **8**.

45. Rissiek, B., et al., *Technical Advance: A new cell preparation strategy that greatly improves the yield of vital and functional Tregs and NKT cells.* Journal of Leukocyte Biology, 2014. **95**(3): p. 543-549.

46. AlShamkhani, A., et al., *OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand.* European Journal of Immunology, 1996. **26**(8): p. 1695-1699.

47. Sugamura, K., N. Ishii, and A.D. Weinberg, *Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40.* Nature Reviews Immunology, 2004. **4**(6): p. 420-431.

48. Croft, M., *Control of Immunity by the TNFR-Related Molecule OX40 (CD134).* Annual Review of Immunology, Vol 28, 2010. **28**: p. 57-78.

49. Baum, P.R., et al., *Molecular Characterization of Murine and Human Ox40/Ox40 Ligand Systems - Identification of a Human Ox40 Ligand as the Htlv-1-Regulated Protein Gp34.* Embo Journal, 1994. **13**(17): p. 3992-4001.

50. Walker, L.S.K., et al., *Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers.* Journal of Experimental Medicine, 1999. **190**(8): p. 1115-1122.

51. Murata, K., et al., *Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand.* Journal of Experimental Medicine, 2000. **191**(2): p. 365-374.

52. Song, J., T. So, and M. Croft, *Activation of NF-κB1 by OX40 Contributes to Antigen-Driven T Cell Expansion and Survival.* The Journal of Immunology, 2008. **180**(11): p. 7240.

53. Soroosh, P., et al., *Differential requirements for OX40 signals on generation of effector and central memory CD4(+) T cells.* Journal of Immunology, 2007. **179**(8): p. 5014-5023.

54. Bansal-Pakala, P., A.G.H. Jember, and M. Croft, *Signaling through OX40 (CD134) breaks peripheral T-cell tolerance.* Nature Medicine, 2001. **7**(8): p. 907-912.

55. Zander, R.A., et al., *PD-1 Co-inhibitory and OX40 Co-stimulatory Crosstalk Regulates Helper T Cell Differentiation and Anti-Plasmodium Humoral Immunity.* Cell Host & Microbe, 2015. **17**(5): p. 628-641.

56. Goncalves-Lopes, R.M., et al., *Surface expression of inhibitory (CTLA-4) and stimulatory (OX40) receptors by CD4(+) regulatory T cell subsets circulating in human malaria.* Microbes and Infection, 2016. **18**(10): p. 639-648.

57. Zander, R.A., et al., *Th1-like Plasmodium-Specific Memory CD4(+) T Cells Support Humoral Immunity.* Cell Reports, 2017. **21**(7): p. 1839-1852.

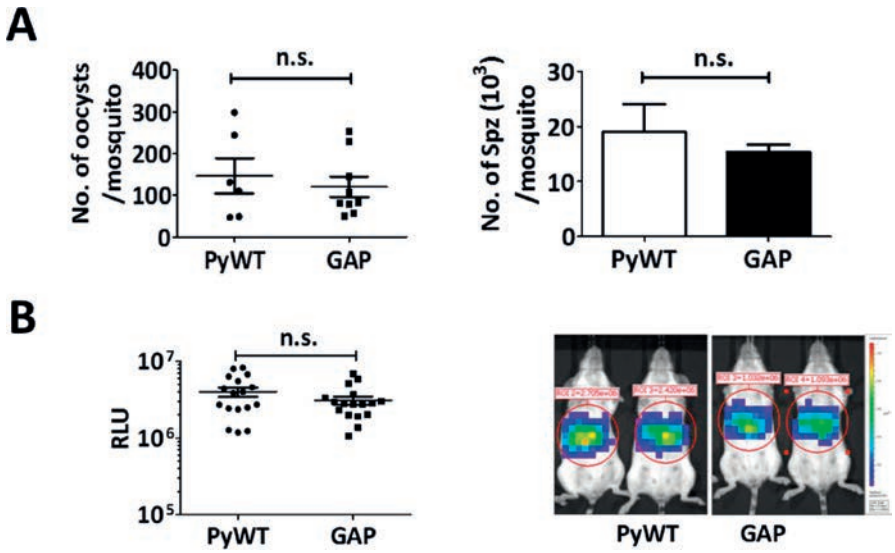
58. Tarun, A.S., et al., *Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8(+) T cells.* Journal of Infectious Diseases, 2007. **196**(4): p. 608-616.

59. Douradinha, B. and D.L. Doolan, *Harnessing immune responses against Plasmodium*

for rational vaccine design. Trends in Parasitology, 2011. **27**(6): p. 273-282.

60. Murray, S.A., et al., *CD40 Is Required for Protective Immunity against Liver Stage Plasmodium Infection*. Journal of Immunology, 2015. **194**(5): p. 2268-2279.
61. Keitany, G.J., et al., *Immunization of Mice with Live-Attenuated Late Liver Stage-Arresting Plasmodium yoelii Parasites Generates Protective Antibody Responses to Preerythrocytic Stages of Malaria*. Infection and Immunity, 2014. **82**(12): p. 5143-5153.
62. Sack, B.K., et al., *Model for In Vivo Assessment of Humoral Protection against Malaria Sporozoite Challenge by Passive Transfer of Monoclonal Antibodies and Immune Serum*. Infection and Immunity, 2014. **82**(2): p. 808-817.
63. Vanderberg, J.P. and U. Frevert, *Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes*. International Journal for Parasitology, 2004. **34**(9): p. 991-996.
64. Fernandez-Ruiz, D., et al., *Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection*. Immunity, 2016. **45**(4): p. 889-902.
65. Sun, J.C., M.A. Williams, and M.J. Bevan, *CD4(+) T cells are required for the maintenance, not programming, of memory CD8(+) T cells after acute infection*. Nature immunology, 2004. **5**(9): p. 927-933.
66. Weiss, W.R., et al., *The Role of Cd4(+) T-Cells in Immunity to Malaria Sporozoites*. Journal of Immunology, 1993. **151**(5): p. 2690-2698.
67. Overstreet, M.G., et al., *CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites*. PLoS One, 2011. **6**(1): p. e15948.
68. Croft, M., et al., *The significance of OX40 and OX40L to T-cell biology and immune disease*. Immunological Reviews, 2009. **229**: p. 173-191.
69. Gonzalez-Aseguinolaza, G., et al., *Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines*. Journal of Experimental Medicine, 2002. **195**(5): p. 617-624.
70. Li, X.M., et al., *Colocalization of a CD1d-Binding Glycolipid with a Radiation-Attenuated Sporozoite Vaccine in Lymph Node-Resident Dendritic Cells for a Robust Adjuvant Effect*. Journal of Immunology, 2015. **195**(6): p. 2710-2721.
71. Malamas, A.S., et al., *Combination therapy with an OX40L fusion protein and a vaccine targeting the transcription factor twist inhibits metastasis in a murine model of breast cancer*. Oncotarget, 2017. **8**(53): p. 90825-90841.

SUPPLEMENTARY DATA



Supplementary Figure S1. Phenotype features of *P. yoelii* GAP (GAP) parasites compared to *P. yoelii* wildtype (*Py*WT) parasites. (**A**) No significant differences in oocyst and sporozoite production in *Anopheles stephensi* mosquitoes between *Py*WT and GAP were observed. Oocysts and sporozoites were counted at day 8 and day 14 after the mosquito feeding, respectively. (**B**) No significant differences in parasite liver load between mice infected with 1 × 10^4 GAP sporozoites and mice infected with 1 × 10^4 *Py*WT sporozoites IV at 44 h post infection, were observed. Parasite liver load in mice was determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). The right panel shows representative images of real time in vivo imaging of luciferase expressing liver stage parasites in mice at 44 h after injection of *Py*WT and GAP sporozoites.

Supplementary Table S1. Breakthrough blood infections and prepatent period in mice after intravenous injection of different doses of *Py*WT and GAP sporozoites.

Parasites	Dose	Breakthrough/Infected animals[a]	Prepatency (days)
*Py*WT	3 × 10^3	6/6	5
GAP	1 × 10^4	0/4	-
	2.5 × 10^4	0/40	-
	5 × 10^4	0/4	-

[a] Number of mice showing breakthrough infections of the total number of infected mice

CHAPTER 4

GENERATION AND PROTECTIVE EFFICACY TESTING OF SELF-ADJUVANTING GENETICALLY ATTENUATED RODENT MALARIA PARASITES

Ahmad Syibli Othman[1,2], Blandine M Franke-Fayard[1], Séverine Chevalley-Maurel[1], Catherin Marin-Mogollon[1], Antonio M. Mendes[3], Helena Nunes-Cabaço[3], Hans Kroeze[1], Jai Ramesar[1], Miguel Prudêncio[3], Chris J. Janse[1] and Shahid M. Khan[1]*

[1] Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[2] Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[3] Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal
* The corresponding author

Manuscript in submission

ABSTRACT

In this study we created genetically attenuated rodent malaria parasites (GAPs) that express putative immunomodulatory proteins to increase GAP immunogenicity. Four different proteins were selected with known adjuvant activity: nontoxic cholera toxin B (CTB) subunit, mouse heat shock protein Gp96, *Mycobacterium* heat shock protein X (HspX) and *Salmonella* flagellin (FliC). These proteins were C- terminally tagged to UIS4, a protein expressed in liver stages where it is located on the parasitophorous vacuole membrane (PVM). The genes encoding the fusion proteins, were introduced into the genome of a *Plasmodium yoelii* GAP and were expressed under control of the *P. yoelii uis4* promoter. To create the adjuvant GAPs, we first developed a *P. yoelii* GAP GIMO parent line ($GIMO_{PyGAP\text{-}fabb/f}$) for rapid introduction of the adjuvant fusion-transgenes into the genome without retention of a drug selectable marker (SM). Specifically, in this GIMO parent line the *hdhfr::yfcu* positive::negative SM is introduced into the *fabb/f* gene, creating a GAP that arrests during late liver-stage development. The four adjuvant-expression cassettes were introduced into the *fabb/f* locus of $GIMO_{PyGAP\text{-}fabb/f}$ by performing GIMO transfection and negative selection. Adjuvant GAP-immunogenicity was determined by analysis of protective immunity induced by sporozoite immunization of BALB/c mice. When compared to immunization performed with non-adjuvanted *P. yoelii fabb/f* GAP, we were unable to observe a significant enhancement in protection (>10×) against wild type *P. yoelii* sporozoite challenge after immunization with the four adjuvant GAPs.

INTRODUCTION

Complete protection against a malaria infection can be obtained after immunization with live attenuated sporozoites, both in rodent models of malaria and in humans [1-3]. Sterile protection against a malaria infection has been achieved in humans after immunization with *Plasmodium falciparum* sporozoites, that have either been attenuated by radiation or administered under chemoprophylaxis [4-6]. A prerequisite for induction of protective immunity by sporozoite-based vaccines is that sporozoites retain their capacity to invade liver cells after their administration. The most advanced live-attenuated vaccine is based on radiation-attenuated sporozoites (*Pf*SPZ-Vaccine), which is currently being evaluated both in the clinic and in field trials [2, 7]. In rodent models of malaria, immunization with sporozoites of genetically-attenuated parasites (GAP) can induce similar, or even better, levels of protective immunity compared to irradiated sporozoites (Irr-Spz) [1, 8-10]. These rodent GAP studies have been critical in the creation of two *P. falciparum* GAP vaccines, which are currently undergoing clinical evaluation [11-13].

A number of studies from both the clinic and the field have shown that Irr-Spz can generate strong protective immunity in humans [7, 14, 15]. However, in order to achieve high levels of protection, multiple immunizations with high doses of attenuated sporozoites are required [4, 7]. Immunization with high sporozoite doses increases the costs of sporozoite-based vaccines and complicates their production, compromising mass administration in malaria-endemic countries. A major challenge is to produce a highly immunogenic live-attenuated vaccine, which requires the fewest attenuated sporozoites per dose and the fewest doses to induce sustained sterile protection against a malaria infection.

While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, T cells, in particular $CD8^+$ T cells, appear to be critical for protective immunity as they are thought to play a major role in eliminating infected hepatocytes [16, 17]. Recent mechanistic investigations into immune responses induced by sporozoite-based immunization have shown that protective immune responses encompass diverse and robust immune responses that include not only $CD8^+$ but also $CD4^+$ T cells, and a significant contribution from antibodies [17, 18].

Rodent models of malaria have been used to explore different approaches to enhance immunogenicity of vaccines consisting of attenuated sporozoites [9, 10, 19]. For example, it has been shown that immunization of mice with GAP that arrest growth late during liver stage development induce higher levels of protective immunity than GAP that arrest early after invasion of hepatocytes [8]. In a limited number of studies, adjuvants have been co-administered with attenuated sporozoites to enhance protective immune responses after immunization. It has been shown that the co-administration of the glycolipid α-galactosylceramide (α-GalCer) and its analog 7DW8-5 with sporozoites can enhance the recruitment and activation/maturation of dendritic cells in draining lymph nodes at the site of sporozoite administration, thereby enhancing parasite-specific T cell immunogenicity [20, 21]. We recently demonstrated that immunization

with attenuated sporozoites in combination with treatment with agonistic antibodies, targeting the costimulatory receptor on activated T–cells, OX40, can enhance protective immunity [22].

Although these preclinical studies provide useful information about the mechanisms underlying protective immunity, the use of adjuvants in human vaccination studies may be hampered by cost, applicability or side-effects. Further, induction of protective immune responses by GAP immunization is dependent on sporozoites migrating to the liver and invading hepatocytes. The administration of adjuvants at the site of GAP injection will result in systemic distribution of the adjuvant which will therefore be considerably diluted at the sites where parasite antigens are taken up by antigen presenting cells (APCs), i.e. the liver, spleen or proximal lymph nodes [23]. In order to maximize the adjuvant effect (i.e. increase antigen uptake by APCs and/or provide stimulatory signals to enhance APC function) it is important to maximize the adjuvant effect at the point of antigen uptake and processing [23, 24].

Due to the limitations of co-injecting adjuvants with attenuated sporozoites, we explored the possibility of creating GAPs that express immunomodulatory proteins in sporozoites and/or liver stages, so called adjuvant GAPs [25-28] . Self-adjuvanting vaccines, in which the antigenic and adjuvanting moieties of the vaccines are present in the same molecule, have been developed for subunit vaccines targeting cancer cells, viruses [29, 30], nematodes [31] and bacteria [32, 33], for example by conjugation of lipopeptide-based Toll-like receptor (TLR) agonists to the target protein [28]. In vaccine development against malaria, the vaccine candidate antigen CSP has been fused to bacterial flagellin [34], a protein which is a potent TLR5 agonist [35]. However, to the best of our knowledge, no sporozoite-based vaccine has been reported that expresses additional immunomodulatory/adjuvant molecules [9, 10, 19].

We selected four TLR agonists that can increase adaptive immune responses and have the ability to improve cross-presentation of antigens, as has been demonstrated in other animal and/or human studies. The selected adjuvant proteins are: (i) nontoxic cholera toxin B subunit from *Vibrio cholerae* (CTB)[36, 37]; (ii) heat shock protein Gp96 of mice (Gp96)[38-40]; (iii) heat shock protein X from *Mycobacterium tuberculosis* (HspX) [41, 42]; and (iv) the TLR5 binding region of *Salmonella typhimurium* flagellin (amino acids 89–96; FliC) [35, 43, 44]. The genes encoding these proteins were fused to a *Plasmodium* protein expressed in liver stages, UIS4 (PY17X_0502200), which is located at the parasitophorous vacuole membrane (PVM) in infected hepatocytes. We fused these proteins to a PVM protein as it has been shown that ovalbumin (OVA) fused to proteins located in the PV/PVM induce stronger T cell responses than ovalbumin expressed in the cytoplasm of transgenic parasites [45, 46]. The fusion genes were introduced by GIMO transfection [47, 48] into a novel GIMO GAP parasite line ($GIMO_{PyGAP\text{-}fabb/f}$) whose growth is arrested late during liver stage development. The four adjuvant GAP were analyzed using the *P. yoelli*-BALB/c screening model for assessing protective immunity after GAP immunization [49]. We describe the immunogenicity studies and compared the protective immunity

induced by immunization with these adjuvant GAPs compared to the non-adjuvanted *P. yoelii* Δ*fabb/f* GAP.

MATERIALS AND METHODS

Experimental animals and wild type and transgenic *P. yoelii* lines

Female OF1 and BALB/cByJ mice (6-7 weeks; Charles River) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042 and 14207). All experiments were performed in accordance with relevant guidelines and regulations. Two *P. yoelii* (*Py*) lines were used: (i) the reference 'wild type' *Py*17X parasite line 1971cl1 (*Py*-GFP-Luc$_{con}$; line RMgm-689; www.pberghei.eu) which contains the fusion gene *gfp-luc* gene under control of the *eef1*α promoter integrated into the silent *230p* gene locus (PY17X_0306600) and does not contain a drug-selectable marker and (ii) a *Py*17X mutant that lacks the gene *fabb/f* (3-oxoacyl-acyl-carrier protein synthase; PY17X_1126500). This mutant (2251cl3; *Py*GAP; Δ*Py*Fabb/f-GFP-Luc$_{con}$; mutant RMgm-4109; www.pberghei.eu) was generated in the reference line 1971cl1 by replacing the *fabb/f* gene by the *hdhfr::fcu* selectable marker (SM) cassette.

Generation of transgenic *P. yoelii* parasite lines

i) Generation of a P. yoelii GAP GIMO parent line (GIMO$_{PyGAP\text{-}fabb/f}$) for introduction of transgenes

The GIMO GAP parent line (GIMO$_{PyGAP\text{-}fabb/f}$; 2668cl1) was generated in line 1971cl1 by standard methods of transfection [50] using a DNA construct (pL2138) that targets the *fabb/f* gene (PY17X_1126500) by double cross-over integration and contains the *hdhfr::yfcu* SM driven by the *P. yoelii hsp70* promoter (PY17X_0712100). To generate GIMO$_{PyGAP\text{-}fabb/f}$ we constructed DNA plasmid pL2138 using the basic gene insertion construct pL0034, which contains the *hdhfr::yfcu* selectable marker (SM) cassette under the control of the *P. berghei eef1*α promoter with 3' terminal sequence of *pbdhfr/ts*. The *P. berghei eef1*α promoter was replaced by the *P. yoelii hsp70* promoter (PY17X_0712100) using *PstI* and *Nco*I digestion resulting in construct pL2137. The *P. yoelii hsp70* promoter was amplified from genomic *P. yoelii* DNA using primers 8080 and 8081 (1078 bp)(see **Table S1** for all primer sequences). Next, we used an existing construct (pL1980) that have been used to generate *P. yoelii* GAP GIMO (pL1980) [49], which contains 5' and 3' *fabb/f* targeting regions, the *hdhfr::yfcu* selectable marker (SM) cassette under the control of the *eef1*α promoter with 3' terminal sequence of *pbdhfr/ts*. We replaced the *eef1*α*-hdhfr::yfcu* selectable marker (SM) cassette from pL1980 with the *hsp70-hdhfr::yfcu* cassette from pL2137 by digestion of the plasmids with PstI and AgeI. This final construct (pL2138) was analyzed by restriction digestion to confirm correct assembly. Before transfection, the construct pL2138 was linearized by digesting with HindIII/EcoRI. Parasites of line 1971cl1 were transfected with construct pL2138 (exp. 2668) using standard transfection

technologies and transformed parasites selected by positive selection with pyrimethamine [50]. Selected parasites were cloned by limiting dilution and mutant 2668cl1 was used for genotype and phenotype analysis. Correct integration of the *hdhfr::yfcu* SM in the *fabb/f* gene in 2668cl1 of gene was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis [50]. PFG-separated chromosomes were hybridized with a mixture of two probes: a probe of the *hdhfr* gene and a ~800 bp fragment of the 5'UTR of PBANKA_0508000 located on chromosome 5 [48]. PCR primers used to confirm correct integration of the construct are listed in **Table S1**.

ii) Introducing an mCherry::uis4 expression cassette in GIMO$_{PyGAP\text{-}fabb/f}$

Parasites of GIMO$_{PyGAP\text{-}fabb/f}$ were transfected with a construct that contains a *mCherry::uis4* expression cassette. This construct (pL2154) aims at replacing the *hdhfr::yfcu* SM in the *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$. The pL2154 plasmid contains the *P. yoelii uis4* (PY17X_0502200) CDS fused to an mCherry cassette under the control of the *P. yoelii uis4* promoter with 3' terminal sequence of the *P. yoelii uis4*.

Plasmid pL2154 was generated by three cloning steps. In the first step, we amplified the *uis4* coding sequence (CDS) together with its 5'-UTR promoter region (1764 bp) from *P. yoelii* genomic DNA using 8130 and 8131 primers. Next, we replaced the *hdhfr::yfcu* selectable marker cassette (SM) of plasmid pL1980 ([49] and described above) with the PCR amplified *uis4* CDS and 5'-UTR using PstI and KpnI digestion. In the second step, the 3'-UTR (938 bp)of *P. yoelii uis4* was amplified from genomic DNA using primers 8132 and 8133 primers. This PCR-amplified cassette was cloned into the intermediate plasmid which contains the 5' and 3' *fabb/f* targeting regions and the *uis4* CDS and 5'-UTR cassette using XhoI and KpnI, resulting in construct pL2148. Next, the mCherry CDS was amplified from plasmid pL1628 [47] using primers 8148 and 7739 and cloned into pL2148 using XhoI and SpeI. This final construct (pL2154) was analyzed was analyzed by restriction digestion to confirm correct assembly. Before transfection, the construct pL2138 was linearized by digesting with HindIII/EcoRI.

Transfection (exp. 2696), negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard methods [47, 50]. Correct integration of the *mCherry::uis4* expression cassette in the *fabb/f* gene in 2696cl1 was verified was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis as described above.

iii) Generation of four transgenic GAP expressing putative immunomodulatory proteins fused to UIS4

Parasites of GIMO$_{PyGAP\text{-}fabb/f}$ were transfected with four different constructs that contain an expression cassette with *uis4* fused to the the following genes:) nontoxic cholera toxin B subunit from *Vibrio cholerae* (CTB) [36, 37]; (ii) heat shock protein Gp96 of mice (Gp96) [38-40]; (iii) Heat shock protein X from *Mycobacterium tuberculosis* (HspX) [41, 42] and (iv) the TLR5 binding region of *Salmonella typhimurium* flagellin (amino acids 89–96; FliC)

[35, 43, 44]. These constructs aims at replacing the *hdhfr::yfcu* SM in the *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$. The complete CDS of the *ctb* gene was amplified from plasmid pUC57-CTB (synthesized by GenScript HK Limited) using primers 8189 and 8190 (394 bp) and cloned into pL2148 (see above) using XhoI and SpeI, resulting in construct 2165. The complete CDS of the *gp96* gene was amplified from plasmid pCMV-mouse Hsp90b1 cDNA purchased from Dharmacon (Catalog number: MMM4769-202763350) using primers 8146 and 8147 (2428 bp) and cloned into pL2148 using SacI and MfeI, resulting in construct 2156. The complete CDS of the *hspx* gene was amplified from *Mycobacterium tuberculosis* H37Rv DNA (kindly provided by K. Franken, Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands) using primers 8144 and 8145 (460 bp) and cloned into pL2148 using XhoI and SpeI, resulting in construct 2152. The *flic* sequence (89 to 96 aa) gene was amplified from cDNA of *Salmonella enteritidis* (ATCC line 13076; kindly provided by Dr. J. J. Verweij, Leiden University Medical Center, Leiden, The Netherlands) using primers 8152 and 8153 (145 bp) and cloned into pL2148 using XhoI and SpeI, resulting in construct 2155. All amplified sequences are fused the to the *P. yoelii uis4* gene and these fusion genes are under control of the *uis4* 5'-UTR regulatory sequences. The final DNA constructs were linearized with HindIII/EcoRI before transfection. Transfections (exp 2690 with pL2165; 2692 with pL2156; 2694 with pL2152; 2698 with pL2155), negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard methods [47, 50]. Correct integration of the constructs in the *fabb/f* gene in the transgenic GAP lines, CTB::UIS4 (2690cl1); Gp96::UIS4 (2692cl2), HspX::UIS4 (2694cl1), FliC::UIS4 (2698cl2) was verified was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis as described above.

iv) Generation of the GIMO P. yoelii 17X line GIMO$_{Pys1}$

The GIMO$_{Pys1}$ (2828cl2) was generated in line 1971cl1 (see above) by standard methods of transfection [50] using a DNA construct (pL2203) that targets the neutral *s1* gene locus (PY17X_1210000) by double cross-over integration and contains the *hdhfr::yfcu* SM driven by the *P. yoelii hsp70* promoter (PY17X_0712100). We used plasmid pL2200 that contains the *hdhfr::yfcu* selectable marker (SM) cassette under the control of the *P. yoelii hsp70* 5'-UTR promoter and the 3' terminal sequence of *pbdhfr/ts*. In this plasmid we replaced the existing targeting 5'-UTR and 3'-UTR regions of gene PBANKA_1122300 with the 5'-UTR and 3'-UTR regions of *s1* gene. These regions were amplified from *P. yoelii* genomic DNA using primer sets 8450/8451 (1040 bp) and 8452/8453 (937 bp), respectively. First, the 5'UTR-targeting region (1040 bp) of *s1* was cloned into pL2200 plasmid using HindIII and PstI. Next, the 3'-UTR targeting region (937 bp) of *s1* was cloned into the intermediate plasmid using KpnI and NotI, resulting in the final GIMO *s1* plasmid (pL2203). This construct was linearized with HindIII/EcoRI before transfection. Transfection (exp 2828), positive selection with pyrimethamine, cloning and genotyping of transformed parasites was performed using standard technologies [50]. Correct integration

of the *hdhfr::yfcu* SM in the *fabb/f* gene in 2828cl2 of gene was verified was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis as described above.

v) Introducing an gp96@lisp2 expression cassette in GIMO$_{Pys1}$

Parasites of GIMO$_{Pys1}$ were transfected with a pL2221 construct that contains an expression cassette where the *gp96* gene (see above) is under control of the 5'-UTR promoter region of *lisp2* (PY17X_1004400). This construct (pL2221) aims at replacing the *hdhfr::yfcu* SM in the *s1* locus of GIMO$_{Pys1}$. First we generated an intermediate plasmid which contains an expression cassette where the *gp96* gene (see above) is under control of the 5'-UTR promoter region (1060 bp) of *lisp2* (PY17X_1004400). First, we amplified the *lisp2* promoter region from genomic DNA using primers 8507 and 8508. The amplified fragment was cloned into the plasmid pL2156 (described above) to replace the 5'*fabb/f* targeting region, *uis4* CDS and and 5'-UTR cassette using PspOMI and SacI. Next, the 3'-UTR (883 bp) of *lisp2* was amplified by PCR from genomic DNA using primers 8509 and 8510 primers and cloned using MfeI and NotI restriction enzymes into the intermediate plasmid to replace 3'-UTR *uis4* and 3'-UTR *fabb/f* targeting region. This resulted in a construct where the *gp96* gene is under control of the *lisp2* promoter region and the 3'-UTR of the *lisp2*. Next, by using the GIMO *s1* plasmid (pL2203; described above), the *hdhfr::yfcu* SM cassette was replaced with the *gp96* gene and the 5'-UTR and 3'-UTRl sequence using XmaI enzyme. The final DNA construct (pL2221) was linearized with PspOMI and NotI before transfection. Transfection (exp 2866), negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard methods [47, 50]. Correct integration of the *gp96@lisp2* expression cassette in the *s1* gene in 2866cl1 was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis as described above.

vi) Generation of transgenic GAP expressing Gp96 and FliC fused to HEP17/EXP1

We generated two constructs that target the *fabb/f* gene and that contains an expression cassette with the *gp96* gene (see above) or the *flic* sequence (see above) fused to HEP17/EXP1 (PY17X_0928700). These constructs aims at replacing the *hdhfr::yfcu* SM in the *fabb/f* locus. First, the 5'UTR promoter region of *hep17* (1380 kb upstream of the start codon) and the signal peptide (SP) sequence of *hep17* (bp 1 to 81) were amplified from wild-type *P. yoelii* DNA using primers 7838 and 7839 (1481 bp) and this fragment was subcloned into plasmid pL1980 ([49]; described above). Second, the remaining sequence of the *hep17* CDS after the SP (bp 82 to 785), along with the 3'-UTR region (806 bp) was amplified using primers 7840 and 7841 (1539 bp) and cloned into this vector. Third, we cloned into this vector the mCherry CDS, resulting in plasmid pL2100. This vector was used to replace the mCherry CDS with the *flic* and *gp96* sequence by cloning into MfeI/BglII sites

the *flic* sequence (amplified from pL2155 using 7947 and 7948 primers) and the *gp96* sequence (amplified from pL2156 using 7955 and 7956 primers). The resulting final vectors with *flic* and *gp96* fused to *hep17* were pL2114 and pL2115, respectively. The final DNA constructs were linearized with PspOMI/NotI before transfection. Transfection, negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard methods [47, 50]. Correct integration of the construct in the *fabb/f* gene in the transgenic GAP line FliC::*hep17/exp1* (2587m1cl1) was verified was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis as described above.

Mosquito infection, analysis of oocysts and preparation and injection of sporozoites

Sporozoites were obtained by manual dissection of the salivary glands of infected *A. stephensi* mosquitoes 14 days after feeding on infected mice as described [22]. For intravenous (IV) administration sporozoites were suspended in RPMI1640 medium and per mouse 200 µl was injected into the tail vein. Oocyst numbers in dissected midguts from infected mosquitoes were established 8 days after feeding using light-microscopy.

Determination of parasite liver load and prepatent period after infection, immunization or challenge

Parasite liver loads in live mice quantified by real time *in vivo* imaging as previously described [51]. Mice were monitored for blood-stage infections by Giemsa-stained blood smears made at day 4-14 after infection or challenge. The prepatent period is defined as the day when a blood stage infection with a parasitemia of 0.5–2% is observed [48].

In vitro hepatocyte cultures and analysis of parasite development

The human hepatocyte cell line HepG2-CD81 was used for *in vitro* cultures of liver stages as described [52]. Isolated sporozoites (5×10^4) were added to monolayers of HepG2-CD81 cells on coverslips in 24 well plates. At 40 hours after infection, nuclei were stained with Hoechst 33342 at a final concentration of 10 µM and live imaging of parasites was performed using a DM RA Leica fluorescence microscope (40×). Images analysis was done with the Leica LAS X software.

Statistics

All data were analyzed using the GraphPad Prism software package 5.04 (GraphPad Software, Inc). For oocysts/sporozoite number and *in-vivo* imaging (RLU) analysis, statistical analysis was performed using the unpaired Student's *t*-test. Survival analysis were performed using Kaplan–Meier survival plots and survival curves were compared using the log-rank (Mantel-Cox) test. Survival was considered as the complete absence of parasites in blood. The significance threshold was 0.05 in all analysis.

RESULTS

Generation of a *P. yoelii* GAP GIMO parent line ($GIMO_{PyGAP\text{-}fabb/f}$) to introduce transgenes

In order to introduce adjuvant transgenes into the genome of a non-lethal *P. yoelii* (17X) GAP, we created a 'GIMO GAP motherline'. This line was created for the following reasons: (i) *P. yoelii* 17X in combination with BALB/c mice is a standard combination used to analyse protective immune responses induced by GAP immunization [53]; (ii) the insertion of the positive/negative selectable marker (i.e. creation of the GIMO locus) into *P. yoelii fabb/f* gene (PY17X_1126500) creates a late-arresting *P. yoelii* GAP, therefore making it possible for adjuvant transgene expression to be maintained late into liver stage development [8, 49]; and (iii) the GIMO-locus in a *P. yoelii* GAP can be used to insert adjuvant-expression cassettes rapidly using GIMO transfection, yielding drug-resistance marker free adjuvant-GAPs [47, 48].

In the GIMO-transfection protocol, transgene-expression cassettes replace the positive-negative *hdhfr::yfcu* selection marker (SM), which is present in the genome of a standard GIMO mother line, resulting in SM-free transgenic parasites [47]. Recently, a *P. yoelii* 17X *fabb/f* GAP (line 2251cl3) has been created in a line that constitutively expresses GFP and luciferase (1971cl1) by introducing the *hdhfr::yfcu* SM into the *fabb/f* locus [49]. The presence of the fusion gene *gfp-luciferase* under control of the constitutive *eef1a* promoter permits quantification of parasite liver loads by *in vivo* imaging after sporozoite administration [48, 54]. We initially used this line as a GAP GIMO parent line for introduction of transgene-expression cassettes. However we encountered problems selecting for the desired transgenic GAP. After transfection and the application of negative drug selection in mice, we repeatedly selected for parasite populations in which the majority of the parasites still had the genotype of the GAP GIMO parental line without the transgene-expression cassettes (data not shown). Since these parasites still contained the *hdhfr::yfcu* SM we reasoned that expression of the negative marker *yfcu* in parasites of line 2251cl3 was not sufficient to kill parasites by the 5-FC treatment. In this parental GIMO line the *hdhfr::yfcu* SM expression is controlled by the *eef1a P. berghei* promoter which may be less effective in driving transgene expression in *P. yoelii* than in *P. berghei*. We therefore decided to generate a new GIMO *P. yoelii* 17X *fabb/f* GAP parent line, where the *hdhfr::yfcu* SM is under control of the strong *hsp70* promoter of *P. yoelii* [55].

This new GIMO GAP parent line ($GIMO_{PyGAP\text{-}fabb/f}$; 2668cl1) was also generated in the parent 1971cl1 line by standard methods of transfection. A DNA construct was used that targets the *fabb/f* gene to introduce the *hdhfr::yfcu* SM driven by the *P. yoelii hsp70* promoter by double cross-over integration (**Figure 1A**). Transfection followed by positive selection with pyrimethamine, cloning and genotyping of transformed parasites was performed using standard technologies [50]. Correct integration of the *hdhfr::yfcu* SM in the *fabb/f* gene was verified by Southern blot analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis (**Figure 1B**). This line (2668cl1) showed wild

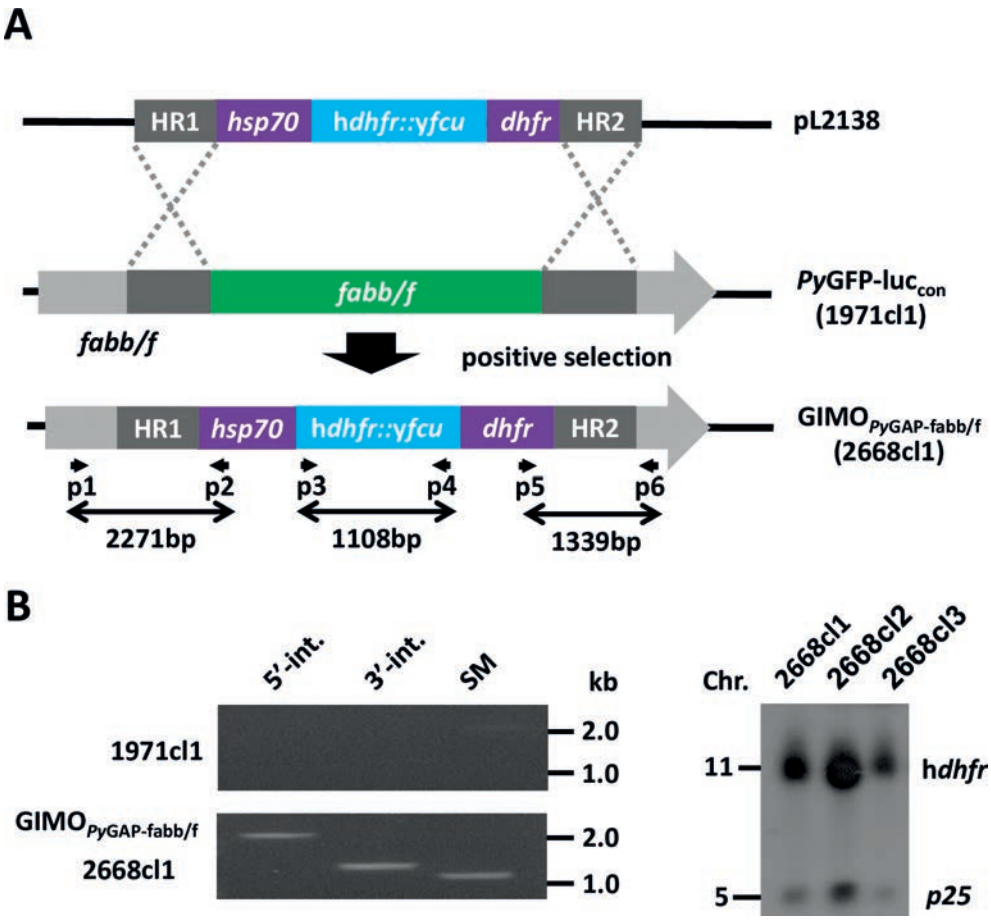


Figure 1. Generation and genotype analysis of the *P. yoelii* GAP GIMO parent line (GIMO$_{PyGAP\text{-}fabb/f}$) *for introduction of adjuvant-expression cassettes.* (A) Schematic representation of the introduction of the *hdhfr-yfcu* selectable marker (SM) cassette into the *fabb/f* gene locus of the parent *P. yoelii* parasite (line 1971cl1). Construct pL2138 contains the *hdhfr-yfcu* SM flanked by the *hsp70* promoter region and the *3' pbdhfr UTR*. This construct is integrated into the *fabb/f* locus by double cross-over homologous recombination at the *fabb/f* homology regions (HR1, HR2). Positive selection with pyrimethamine selects for parasites that have the *fabb/f* coding sequence replaced by the SM cassette, thereby creating a GAP GIMO line (2668cl1) for introduction of adjuvant expression cassettes. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2138 in parasites of line 2668cl1. PCR shows the presence of the *hdhfr::yfcu* marker. 5' integration PCR (5'-int; primers p1/p2), 3' integration PCR (3'-int; primers p5/p6), *hdhfr::yfcu* (primers p3/p4). Primer locations and product sizes are shown in A and primer sequences in Table S1). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette marker in the *fabb/f*-locus on chromosome 11 in three clones of the 2668 line.

type blood stage growth as determined during the cloning procedure (data not shown). This GIMO$_{PyGAP\text{-}fabb/f}$ line was used as the parental GIMO GAP line to introduce the different adjuvant transgenes as described in the next sections.

Introduction of the *mCherry::uis4* fusion gene into GIMO$_{PyGAP\text{-}fabb/f}$ and analysis of mCherry::UIS4 expression in sporozoites and liver stages

To express putative adjuvants, we fused the adjuvant proteins to *P. yoelii* UIS4 (PY17X_0502200; up-regulated in infective sporozoites, ETRAMP10.3) and placed the fusion genes under control of the 5'-promoter and 3'-transcriptional terminator sequences of *P. yoelii uis4*. UIS4 is a protein of the parasitophorous vacuole membrane (PVM) that surrounds the parasites in infected hepatocytes [56]. We fuses the adjuvant proteins to a PVM protein as it has been previously shown that ovalbumin (OVA) fused to PVM proteins induces stronger T cell responses than OVA located in the cytoplasm of transgenic parasites [45, 46].

To analyze whether the C-terminal fusion of a heterologous protein to UIS4 affects either UIS4 expression or its PVM location, we first generated a transgene reporter line that expresses mCherry fused to UIS4. We transfected GIMO$_{PyGAP\text{-}fabb/f}$ parasites with a construct containing a *mCherry::uis4* expression cassette (exp. 2696). This construct targets and replaces the *hdhfr::yfcu* SM in the *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$ with the *mCherry::uis4* cassette (**Figure 2A**). Transfection, negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard technologies [47, 50]. Correct integration of the *mCherry::uis4* expression cassette in the *fabb/f* gene in the cloned line 2696cl1 was verified by Southern analyses of PFG-separated chromosomes and diagnostic PCR analysis (**Figure 2B**). We next analyzed mCherry::UIS4 expression during the parasite's life cycle by fluorescence microscopy. No mCherry signal was detected in blood stages or developing oocysts (**Figure 2C**), in agreement with the absence of UIS4 expression in these stages. In sporozoites, *uis4* is transcribed but transcripts are translationally repressed [57, 58]. We could indeed detect no mCherry signals in >80% of live, freshly isolated sporozoites. However, in sporozoites kept for longer periods in RPMI1640 culture medium, weak mCherry signals were visible, with >50% of the sporozoites weakly mCherry-positive as early as 1 hour after isolation (**Figure 2C**). mCherry expression was clearly detected in cultured liver stage parasites, predominantly located at the periphery of the parasites (**Figure 2D**), consistent with UIS4 localization in the PV/PVM [57]. Although we did not further analyze the exact location of mCherry::UIS4 in the PV, our results demonstrate that the fusion protein mCherry::UIS4 is only expressed in liver stages and is mainly located at the periphery of the parasite during liver stage development. In addition, our results indicate that the fusion of UIS4 to mCherry does not affect the development of sporozoites into mature liver stages. Parasites lacking expression of UIS4 display a strongly retarded liver stage maturation [56, 59]; in contrast, mCherry::UIS4-expressing parasites display normal liver stage development, as shown both *in vitro* analysis of liver stage maturation in hepatocyte cultures (**Figure 2D**) and by *in vivo* imaging of parasite liver loads in mice

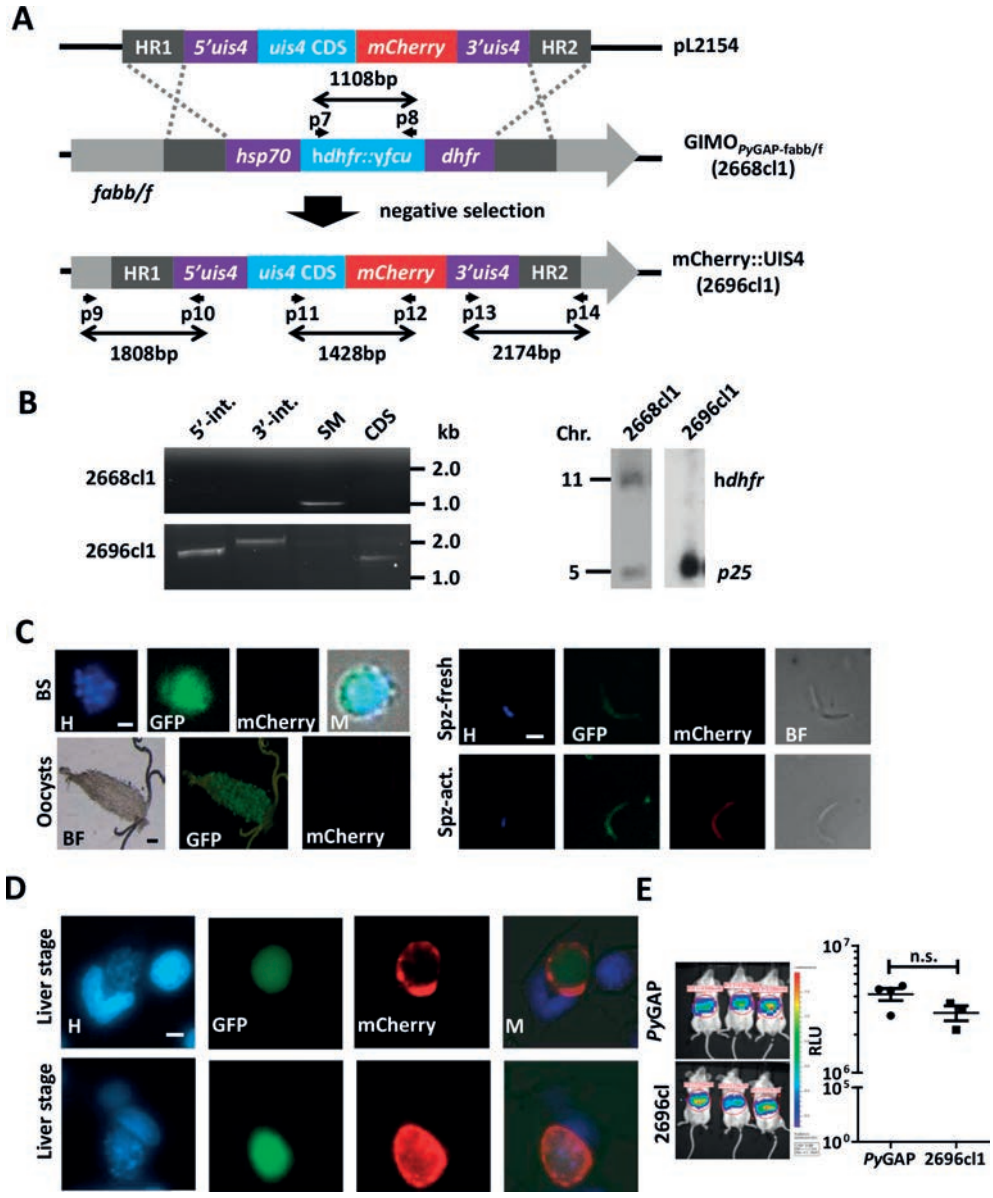


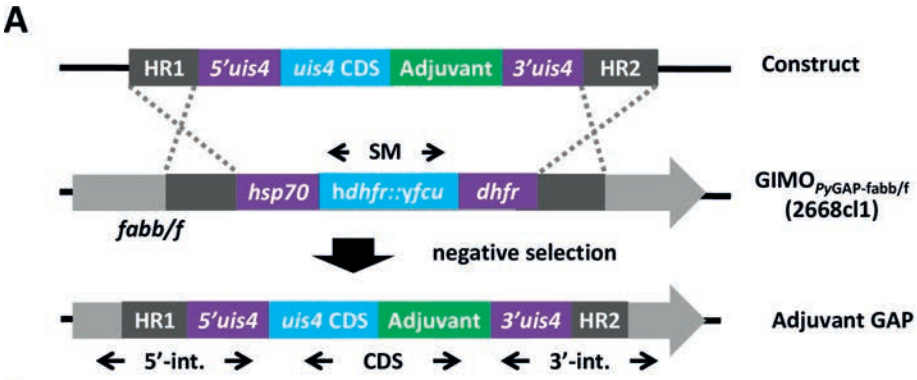
Figure 2. Introduction of the *mCherry::uis4* fusion gene into GIMO$_{PyGAP\text{-}fabb/f}$ and analysis of mCherry::UIS4 expression in sporozoites and liver stages. (A) Schematic representation of the introduction of the *mCherry::uis4* expression cassette into the genome of GIMO$_{PyGAP\text{-}fabb/f}$ (line 2868cl1). Construct pL2154 contains the *uis4* coding DNA sequence (CDS) fused to mCherry which is flanked by the *uis4* promoter and *3'-UTR* regions. This construct is integrated into the modified *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$, that contains the *hdhfr::yfcu* selectable marker (SM) cassette, by double cross-over homologous recombination at the homology regions (HR1, HR2). Negative selection with 5-FC selects for parasites that have the SM cassette replaced by the *mCherry::uis4* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration

of construct pL2154 in in parasites of line 2696cl1. PCR shows the absence of the *hdhfr::yfcu* SM marker and the presence of the *mCherry::uis4* fusion gene. 5' integration PCR (5'-int; primers p9/p10), 3' integration PCR (3'-int; primers p13/p14), *hdhfr::yfcu* (SM; primers p7/p8), *mCherry::uis4* (CDS; primers p11/p12). Primer locations and product sizes are shown in **A** and primer sequences in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker from the *fabb/f* locus on chromosome 11 in 2696cl1 parasites. (**C**) Analysis of mCherry::UIS4 expression in live blood and mosquito parasite stages by fluorescence microscopy. No mCherry signal was detected in blood stages and oocysts. Left panel: a representative schizont of the negative blood stages is shown and an oocyst-containing midgut of an *A. stephensi* mosquito. Right panel: no signal was detected in >80% of sporozoites that were analyzed directly after isolation (Spz-fresh; upper panel). Incubation of sporozoites in RPMI 1640 for longer periods (>1 hour) resulted in weak mCherry signals in >50% of the 'activated' sporozoites (Spz-act; lower panel). H: Hoechst; BF: bright field; M: merged. BS and Spz; Scale bar: 2 µm. (**D**) Analysis of mCherry::UIS4 expression in live, cultured liver stages. Representative images of maturing liver schizonts, showing mCherry signals, mainly located at the periphery of the parasite. H: Hoechst; BF: bright field; M: merged. Scale bar: 10 µm. (**E**) Parasite liver loads in mice at 44 hour after infection with 1 × 10^4 sporozoites of a parent *Py*GAP (line 2251cl3) and the *Py*GAP expressing UIS4::mCherry (line 2696cl1). Parasite liver loads in mice were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). The left panel shows images of real time *in vivo* imaging of luciferase expressing liver stage parasites in mice at 44 h after injection of sporozoites.

infected with *mCherry::uis4* sporozoites (**Figure 2E**). All mice infected with *mCherry::uis4* sporozoites did not develop a blood stage infection as expected based on the introduction of the transgene expression cassette into the *fabb/f* gene locus, resulting in the absence of FabB/F expression and late liver stage developmental arrest.

Generation of four adjuvant GAPs expressing putative immunomodulatory proteins fused to UIS4

To generate adjuvant GAP that express putative immunomodulatory proteins we selected the following four adjuvants: (i) nontoxic cholera toxin B subunit from *Vibrio cholerae* (CTB), (ii) heat shock protein Gp96 of mice (Gp96), (iii) heat shock protein X from *Mycobacterium tuberculosis* (HspX) and (iv) the TLR5 binding region of *Salmonella typhimurium* flagellin (amino acids 89–96; FliC). We fused the coding sequence of these genes to the *uis4* gene of *P. yoelii* under control of the 5'-promoter and 3'-transcriptional terminator sequences of *uis4* and the expression cassettes were introduced into the *fabb/f* gene locus of $GIMO_{PyGAP\text{-}fabb/f}$ parasites by GIMO transfection. We transfected $GIMO_{PyGAP\text{-}fabb/f}$ parasites with the four adjuvant constructs that are designed to replace the *hdhfr::yfcu* SM in the *fabb/f* locus of $GIMO_{PyGAP\text{-}fabb/f}$ (**Figure 3A, B**) with the adjuvant transgene. Transfection, negative selection with 5-FC, cloning and genotyping of transformed parasites was performed using standard technologies [47, 50]. Correct integration of the transgene expression cassette into the *fabb/f* gene was verified by Southern analyses of PFG-separated chromosomes and diagnostic PCR analysis for the following transgenic GAP lines, CTB::UIS4 (2690cl1), Gp96::UIS4 (2692cl2), HspX::UIS4 (2694cl1), FliC::UIS4 (2698cl2) (**Figure 3C**). These



B

Adjuvants	Constructs	Adjuvant GAP name	Line number
CTB	pL2165	CTB::UIS4@uis4	2690cl1
Gp96	pL2156	Gp96::UIS4@uis4	2692cl2
HspX	pL2152	HspX::UIS4@uis4	2694cl1
FliC	pL2155	FliC::UIS4@uis4	2698cl2

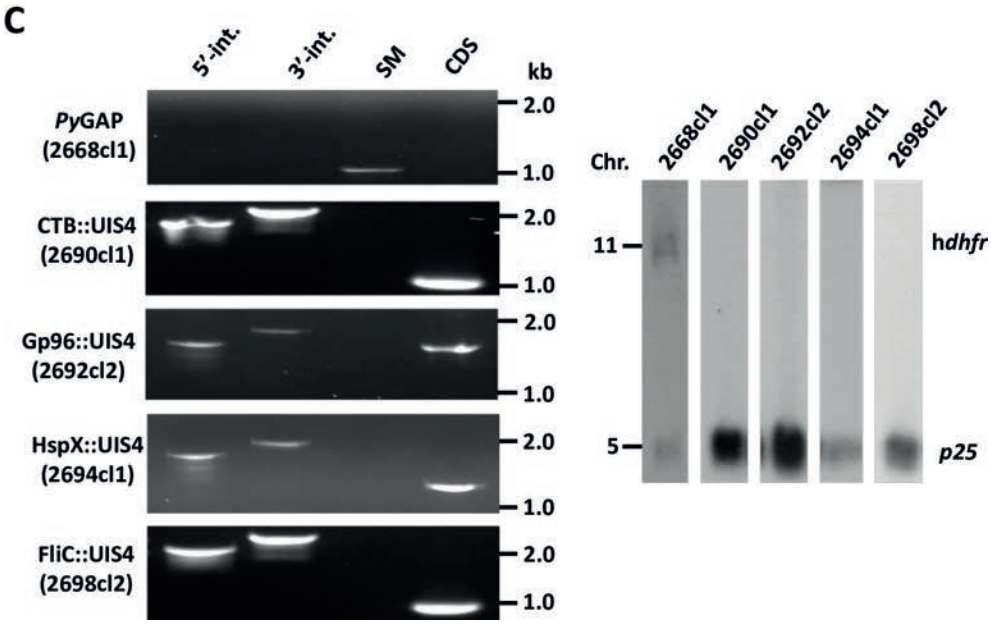


Figure 3. Introduction of four adjuvant-expression cassettes into GIMO$_{PyGAP\text{-}fabb/f}$ and genotype analysis of the adjuvant GAPs. (A) Schematic representation of the introduction of the adjuvant-expression cassette into the genome GIMO$_{PyGAP\text{-}fabb/f}$ (line 2868cl1). The construct contains the *uis4* coding DNA sequence (CDS) fused to the adjuvant which is flanked by the *uis4* promoter and *3' UTR* regions. This construct is integrated into the modified *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$, that contains the *hdhfr::yfcu* selectable marker (SM) cassette, by double cross-over homologous recombination at the homology regions (HR1, HR2). Negative selection with 5-FC selects for parasites that have the SM cassette replaced by the adjuvant expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) The four adjuvant GAP that were generated and contain

- CTB, Gp96, HspX or FliC fused to *uis4* under control of the promoter and 3'-UTR regions of *uis4* as shown in **A**. (**C**) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of the constructs in parasites of the four different adjuvant GAP and the parent *Py*GAP (line 2668cl1). PCR shows the absence of the *hdhfr::yfcu* SM marker and the presence of the coding DNA sequence (CDS) of the fusion gene. 5' integration PCR (5'-int.), 3' integration PCR (3'-int.). Primer locations are shown in **A** and primer sequences in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) confirms the removal of the SM cassette marker from the *fabb/f* locus on chromosome 11 in the different adjuvant GAP parasites.

adjuvant GAP lines showed wild type blood stage growth as determined during the cloning procedure (data not shown).

Sporozoites of 3 adjuvant GAPs, CTB::UIS4, HspX::UIS4 and FliC::UIS4, are infectious to mice and develop into maturing liver stages

The four adjuvant GAP lines were infectious to mosquitoes and produced wild type-like numbers of oocysts. All lines produced salivary gland sporozoites, with three lines (Gp96::UIS4; HspX::UIS4, FliC::UIS4) producing sporozoite numbers that were comparable to sporozoite numbers produced by the parent *Py*GAP, whereas CTB::UIS4 produced lower numbers of sporozoites (**Figure 4A**). Sporozoites of three of the four adjuvant GAP (CTB::UIS4; HspX::UIS4, FliC::UIS4) were infective to mice, as shown by *in vivo* imaging of parasite liver loads after infection of mice with adjuvant GAP sporozoites (**Figure 4B**). However, no hepatic infection was detected in mice infected with Gp96::UIS4 sporozoites (**Figure 4B** and **Figure 5B**). The normal liver parasite liver loads of CTB::UIS4; HspX::UIS4 and FliC::UIS4 parasites, as visualised by *in vivo* imaging of the liver 44h after infection and shown by the presence of developing schizonts in *in vitro* cultures of infected hepatocytes (**Figure 4C**) demonstrate that the expression of these adjuvants do not disrupt intrahepatic development of the late arresting *P. yoelii fabb/f* GAP.

Fusion of Gp96 to PVM proteins affects parasite development

The absence of detectable parasites in the liver of mice infected with Gp96::UIS4 sporozoites may be due to different reasons. Gp96::UIS4 sporozoites may not be able to invade hepatocytes, or they may be able to invade but arrest growth soon after invasion. Growth arrest may result from either toxicity of Gp96 for the parasite or from a perturbation of the correct function of UIS4 (i.e. the formation maintenance of PV/PVM) upon fusion to GP96. Another possibility is that expression of the Gp96::UIS4 results in efficient detection and clearance of infected hepatocytes by the immune system. To examine the possible toxicity of Gp96 for developing liver stage parasites, we generated a transgenic *P. yoelii* 17XNL line (2866cl1) that expressed Gp96 in the cytoplasm of the parasite and was not fused to a *Plasmodium* protein. Gp96 was placed under control of the promoter of the liver stage specific gene, *lisp2* (**Figure S1A**) which has been previously used to drive transgene expression in the cytoplasm of liver stages [60-62]. The *gp96@lisp2* expression cassette

A

Adjuvant GAP	Oocyst no.[a] Mean ± sd	Sporozoite no. (x10^3)[b] Mean ± sd
*Py*GAP	358 ± 138.8	15.7 ± 6.1
CTB::UIS4	410 ± 18.4	5.6 ± 3.4
Gp96::UIS4	406 ± 103.7	10.3 ± 8.4
HspX::UIS4	303 ± 101.1	10.9 ± 2.3
FliC::UIS4	349 ± 133.8	16.5 ± 1.9

[a] Mean number of oocysts per mosquito

[b] Mean number of sporozoites per mosquito

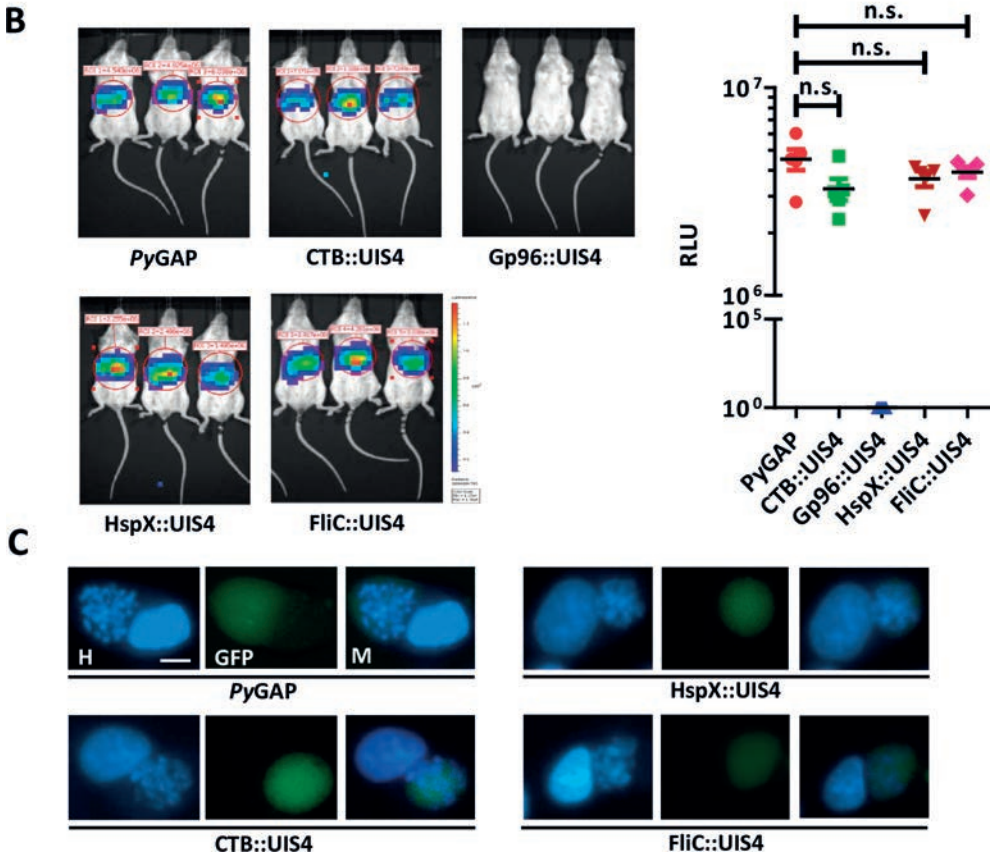


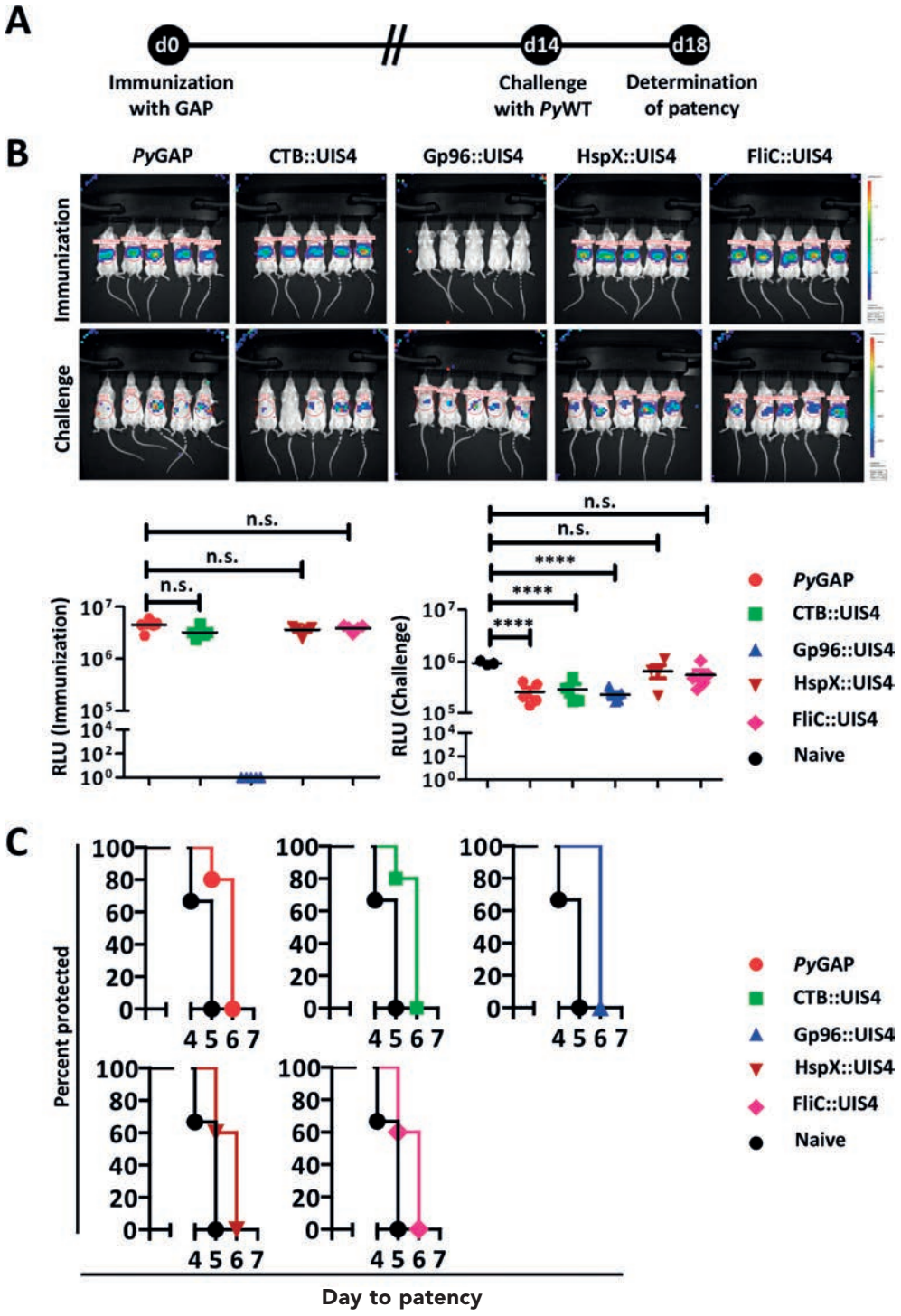
Figure 4. Analysis of mosquito and liver stage development of the four adjuvant GAP. (A) Oocyst and sporozoite production in *A. stephensi* of the four adjuvant GAP and the parent *Py*GAP (line 2251cl3). Oocyst and sporozoite numbers were determined at day 8 and day 14, respectively, after mosquito infection. (B) Parasite liver loads in mice 44 hour after infection with 1 × 10^4 sporozoites of a parent *Py*GAP (line 2251cl3) and the four adjuvant GAP. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). The left panel shows images of real time *in vivo* imaging of luciferase-expressing liver stage parasites in mice at 44 h after injection of sporozoites. (C) Analysis of development of liver stages in in vitro cultures of infected hepatocytes. Representative images of maturing GFP-expressing liver schizonts of three adjuvant GAP are shown. H: Hoechst; M: merged. Scale bar: 10 µm.

Figure 5. Analysis of protective immunity induced by immunization of mice with sporozoites of the adjuvant GAP. (A) The time line shows immunization of BALB/c mice with adjuvant GAP sporozoites (a single dose of 2.5 × 10^4) and challenge 14 days later with 3 × 10^3 wild type (*Py*WT; line 1971cl1) sporozoites. Challenged mice were monitored for blood-stage infections from day 18 onwards to determine the prepatent period. (B) Parasite liver loads in mice at 44 hour after immunization with 2.5 × 10^4 sporozoites of a parent *Py*GAP (line 2251cl3) and the four adjuvant GAP and after challenge of the immunized mice with 3 × 10^3 wild type sporozoites (*Py*WT; line 1971cl1). Parasite liver load were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). The upper panel shows images of real time *in vivo* imaging of luciferase expressing liver stage parasites in mice at 44 h after sporozoite injection. (C) Survival curves (Kaplan-Meier analysis) illustrating the prepatent period (day at which a parasitemia of 0.5–2% is observed) and the Log-Rank (Mantel-Cox) Test was used to compare groups of mice. Data shown correspond to groups of 5 mice. Significance values: naïve vs *Py*GAP *p = 0.027; naïve vs CTB::GAP *p = 0.027; naïve vs gp96::GAP **p = 0.007; naïve vs HspX::GAP p = 0.065; naïve vs FliC::GAP p = 0.065).

was introduced into a novel *P. yoelii* 17XNL GIMO line (GIMO$_{Pys1}$; 2828cl2), which has a *hdhfr::yfcu* SM under control of the *P. yoelii hsp70*, introduced into the silent *s1* locus of the 1971cl1 line (Figure S2). The *gp96@lisp2* parasites (line 2866cl1) exhibit normal development throughout the parasite's life cycle, including its liver stage development and the time to patency in the blood after sporozoite inoculation is comparable to that of WT parasites (Figure S1C). These observations indicate that expression of Gp96 in the cytoplasm is not toxic for the parasite. This suggests that fusion of Gp69 to UIS4 compromises the essential function of UIS4 at the PVM, resulting in the absence of liver stage development, comparable to mutants lacking expression of UIS4 [56, 59]. Previously we have fused transgenes, such as ovalbumin and mCherry, to another PVM protein HEP17/EXP1 [45, 63], which is expressed on the PVM of both blood- and liver-stage parasites and is essential for blood stage development. We therefore attempted to generate a transgenic line that express Gp69 fused to HEP17/EXP1 and, as a control, a transgenic line where FliC was fused to HEP17/EXP1. In seven independent transfection experiments we were unable to select for parasites that contained a *gp96::hep17/exp1* expression cassette integrated into the *fabb/f* locus, whereas we were able to select parasites where the FliC*::hep17/exp1* expression cassette had integrated into the *fabb/f* locus (line 2587m1cl1) of the parental GIMO GAP line 2567cl1 (Figure S3). Combined, our observations indicate that fusion of Gp96 to *Plasmodium* PVM proteins is lethal to the parasite.

Analysis of protective immunity induced by immunization of mice with sporozoites of the adjuvant GAP

To analyze protective immunity induced by immunization with adjuvant GAP sporozoites, we used a recently developed 'sub-saturating' immunization regiment in BALB/c mice using GAP-sporozoites (Δ*Py*FabBF-GFP-Luc$_{con}$) [22]. This 'sub-optimal' immunization regiment results in mice that are only partially protected against a challenge with a fixed dose of


A
d0
d14
d18
Immunization with GAP
Challenge with PyWT
Determination of patency
B
PyGAP
CTB::UIS4
Gp96::UIS4
HspX::UIS4
FliC::UIS4
Immunization
Challenge
RLU (Immunization)
RLU (Challenge)
n.s.
****
PyGAP
CTB::UIS4
Gp96::UIS4
HspX::UIS4
FliC::UIS4
Naive
C
Percent protected
Day to patency

WT parasites as determined by a delay of blood-stage prepatency. In this protocol (**Figure 5A**) mice are immunized with a single dose of 2.5 × 10^4 GAP sporozoites and challenged 14 days later with 3 × 10^3 wild type *P. yoelii* sporozoite (*Py*-GFP-Luc_{con}; *Py*WT). A single immunization with this parasite dose results in a maximum of one day delay in prepatent period in immunized mice compared to naïve mice and none of the mice are completely protected. Since the blood stage multiplication rate is approximately 10× per 24 h, a one day delay in the prepatent period represents a ~90% reduction of liver infection, and this protocol was used to determine 10-fold increase in GAP vaccine potency after the addition of an adjuvant [22].

We used this protocol to analyze protective immunity of the four adjuvant GAP, CTB::UIS4; Gp96::UIS4, HspX::UIS4, FliC::UIS4. Groups of 5 mice were immunized with a single dose of 2.5 × 10^4 adjuvant GAP sporozoites and challenged 14 days later with 3 × 10^3 WT sporozoites. Parasite liver loads were determined by *in vivo* imaging at 44 hours after injection of sporozoites, both after immunization and after challenge. In addition, the time to blood stage patency (prepatent period) after challenge was determined to be 4-7 days after WT sporozoite challenge. As a control we immunized mice with sporozoites of the non-adjuvanted *P. yoelii fabb/f* GAP (line 2251cl3).

Parasite liver loads of all groups of mice immunized with the adjuvant-GAPs, except for the Gp96::UIS4, were comparable to liver loads of mice immunized with the parent *P. yoelii fabb/f* GAP (**Figure 5B**). The absence of Gp96::UIS4 parasites in the liver at 44h after immunization was expected based on the absence of liver stage development Gp96::UIS4-expressing parasites (see previous section above). After challenge with WT sporozoites 14 days after immunization, the parasite liver load of mice immunized with CTB::UIS4, Gp96::UIS4 and the control GAP were significantly reduced compared to naïve mice infected with the same parasite dose (**Figure 5C**). Although parasite liver loads of HspX::UIS4 and FliC::UIS4 immunized mice were reduced compared to those of naïve mice, this reduction was not significant. Despite the variation in the parasite liver loads after WT sporozoite challenge all adjuvant GAP immunized mice became patent with a 1-2 day delay compared to the naïve mice, but all groups of adjuvant GAP-immunized mice had a prepatent period that was similar to that of *P. yoelii fabb/f* GAP-immunized mice (at day 5-6). These observations indicate that the four adjuvant GAP had a similar or lower than 10-fold increased potency when compared to the parent, non-adjuvanted GAP.

DISCUSSION

In this study we generated four different transgenic GAPs engineered to express the potential immunomodulatory proteins CTB, Gp96, HspX and FliC, shown to act as adjuvants in other vaccine studies [35-43]. These proteins have been shown to interact with innate immune pattern recognition receptors on antigen presenting cells (APC) that can both trigger cytokine production to limit infection and directing adaptive immune responses against pathogen as well as tumor antigens. The selected adjuvant molecules

are thought to stimulate different Toll-like receptors (TLRs), which can not only improve antibody and $CD4^+$ T cell responses but also promote the cross-presentation of vaccine antigens directing the immune response towards the formation of cytotoxic ($CD8^+$) T cells. Parasite antigen-specific $CD8^+$ T cells are considered of particular importance in detection and clearance of *Plasmodium*-infected hepatocytes [59, 64]. However, immunization with none of the four adjuvant GAP developed in the present study resulted in a significant increase in protective efficacy (more than 10-fold) compared to the unmodified *Py*GAP in the *P. yoelli*-BALB/c model employed in this study to measure enhanced protective immunity.

This inability to achieve significantly higher protective immunity with the adjuvant GAPs could be due to a number of factors, but is unlikely to be due to a poor expression of the adjuvant proteins. These proteins were fused to UIS4, a protein associated with the PVM, which surrounds the parasites inside a hepatocyte [57]. UIS4 is strongly expressed during *Plasmodium* liver stage development [65] and is essential for normal development of liver stages [57, 58]. Mutants that lack UIS4 expression have a very strong defect in liver stage development with only very few parasites developing in hepatocytes 24 hours after sporozoite infection [56]. We found that fusion of mCherry to the C-terminus of UIS4 in *Py*GAP parasites did not affect UIS4 expression; mCherry is clearly exported to the PVM/PV and these transgenic parasite develop into liver schizonts, similarly to the unmodified *Py*GAP. Parasite liver loads 44 hours after injection of three of the four adjuvant GAP sporozoites (CTB::UIS4, HspX::UIS4 and FliC::UIS4) were comparable to the parasite liver loads achieved after the administration of the same number of non-adjuvanted *Py*GAP sporozoites, indicating that fusion of these adjuvants to UIS4 did not negatively impact UIS4 expression. This *Py*GAP-like liver stage development of adjuvant GAPs was also confirmed by microscopy analyses of *in vitro* infected hepatocytes. In contrast, we were unable to detect liver stage development with the GP96 adjuvant-expressing parasite, neither *in vivo* nor *in vitro*. By analyzing additional mutants expressing Gp96, either expressed in the liver-stage parasite's cytoplasm or fused to another PVM protein, we provide evidence that fusion of this protein to proteins of the PVM interferes with parasite development. Therefore, the failure of Gp96::UIS4 sporozoites to establish a liver infection appears to be due to the incorrect or absent PVM formation after hepatocyte invasion by the parasites. The lack of PVM formation and rapid arrest during liver stage development might affect both Gp96 expression and exposure to the immune system, which may explain Gp96's inability to enhance GAP immunogenicity. However, despite the fact that Gp96::UIS4 sporozoites arrest early during liver stage development, immunization with Gp96::UIS4 sporozoites induced levels of protective immune responses similar to those of the late-arresting *PyΔfabb/f* GAP. This observation was unexpected since it has been shown that late-arresting GAP induce stronger protective immunity than early arresting parasites [8], and might suggest that protective immunity induced in the *P. yoelii*/BALB/c model employed is mainly generated against sporozoite antigens with limited contribution of antigens expressed in developing liver stages. The adjuvants were fused

to UIS4, a protein expressed only after sporozoite invasion of hepatocytes. While *uis4* is already transcribed in sporozoites, these transcripts are translationally repressed [57, 58]. Translational repression is mediated by sequences inside the open reading frame of *uis4* [57] and we found that transcripts of the *uis4* and *mCherry* fusion were also translationally repressed. This suggests that the adjuvants fused to UIS4 are not expressed in sporozoites and interact with immune responses only after the intracellular parasite aborts liver stage development.

Our failure to measure enhanced protective immune responses may be due to the inability of the selected adjuvants to induce protective immune responses that can more effectively detect and destroy developing liver stage parasites. While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, $CD8^+$ T cells and IFN-γ appear to be critical for protective immunity as they are thought to play a major role in eliminating infected hepatocytes [59, 66, 67]. Recent mechanistic investigations into immunity induced by sporozoite-based immunization have shown that protective immune responses encompass diverse and robust immune responses that include not only $CD8^+$ but also $CD4^+$ T cells and a significant contribution from antibodies against sporozoite antigens [17, 18]. The selected adjuvants are known to stimulate TLR 2 (Gp96), TLR 4 (Gp96/CTB/HSPX) and TLR 5 (FliC) on the plasma membrane of APCs [37, 39, 41, 44] and the selection was based on the hypothesis that when GAP-infected hepatocytes disintegrate and release parasite antigens they will also simultaneously release the adjuvant, with parasite antigens being taken up by APCs and the released adjuvants stimulating TLRs on the same APC. This would then result in increased inflammatory responses against parasite antigens thereby improving and increasing cellular and humoral immune responses. It is possible that the adjuvants selected do not stimulate the most appropriate adaptive response that would result in the recognition and elimination of infected liver cells.

In this study we did not directly measure the effect of the TLR-agonists on different immune cell populations in immunized mice and we only measured protective immunity by determination of the prepatent period after challenge with WT parasites. One can speculate that either the adjuvants did not activate the appropriate immune cells or that those that are involved in removal of infected liver cells are activated but this activation is not sufficient to result in a more than 10-fold increase in protective immunity (i.e. 1 day or more delay in patency).

The failure to measure enhanced protective immune responses may also be due to the immunization protocol we employed. In this study we have used the *P. yoelli*-BALB/c immunization protocol which involves immunization with a single dose of sporozoites of a late arresting *Py*GAP followed by a challenge with wild type sporozoites 14 days later [22]. The effect of the selected adjuvants on protective immunity may have been better observed after prime-boost immunization strategies where the re-call of expanded immunological memory responses may enhance protective immunity. Indeed, such strategies might also be used to examine if immunization with the adjuvant GAP

results in an increase of the duration of protective immune response compared to non-adjuvanted GAP.

In conclusion, we have tested a set of potential adjuvants and created a panel of adjuvant GAPs to assess the possibility of enhancing immunogenicity of GAP. Whilst we were not able to detect a higher that 10-fold increase in vaccine potency, we have developed an immunization-challenge protocol, as well as a *Py*GAP GIMO mother line to rapidly create adjuvant GAPs, which can be used to evaluate other immunization schedules, additional adjuvants and/or novel enhanced GAPs. Manipulation, of the host immune response to direct and increase appropriate adaptive immune responses after vaccination is of value not only to enhance GAP vaccines but also other vaccines that need to generate immune responses to target liver infections.

ACKNOWLEDGEMENTS

A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia).

REFERENCES

1. Bijker, E.M., et al., *Novel approaches to whole sporozoite vaccination against malaria.* Vaccine, 2015. **33**(52): p. 7462-7468.
2. Richie, T.L., et al., *Progress with Plasmodium falciparum sporozoite (PfSPZ)-based malaria vaccines.* Vaccine, 2015. **33**(52): p. 7452-7461.
3. Hollingdale, M.R. and M. Sedegah, *Development of whole sporozoite malaria vaccines.* Expert Review of Vaccines, 2017. **16**(1): p. 45-54.
4. Seder, R.A., et al., *Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine.* Science, 2013. **341**(6152): p. 1359-1365.
5. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites.* Journal of Infectious Diseases, 2002. **185**(8): p. 1155-1164.
6. Roestenberg, M., et al., *Protection against a Malaria Challenge by Sporozoite Inoculation.* New England Journal of Medicine, 2009. **361**(5): p. 468-477.
7. Sissoko, M.S., et al., *Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.* Lancet Infectious Diseases, 2017. **17**(5): p. 498-509.
8. Butler, N.S., et al., *Superior Antimalarial Immunity after Vaccination with Late Liver Stage-Arresting Genetically Attenuated Parasites.* Cell Host & Microbe, 2011. **9**(6): p. 451-462.
9. Kreutzfeld, O., K. Müller, and K. Matuschewski, *Engineering of Genetically Arrested Parasites (GAPs) For a Precision Malaria Vaccine.* Frontiers in Cellular and Infection Microbiology, 2017. **7**: p. 198.
10. Singer, M. and F. Frischknecht, *Time for Genome Editing: Next Generation Attenuated Malaria Parasites.* Trends in Parasitology, 2017. **33**(3): p. 202-213.
11. van Schaijk, B.C.L., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.
12. Mikolajczak, S.A., et al., *A Next-generation Genetically Attenuated Plasmodium falciparum Parasite Created by Triple Gene Deletion.* Molecular Therapy, 2014. **22**(9): p. 1707-1715.
13. Kublin, J.G., et al., *Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects.* Science Translational Medicine, 2017. **9**(371).
14. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination (vol 22, pg 614, 2016).* Nature Medicine, 2016. **22**(6): p. 692-692.
15. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(10): p. 2711-2716.
16. Van Braeckel-Budimir, N. and J.T. Harty, *CD8 T-cell-mediated protection against liver-stage malaria: lessons from a mouse model.* Frontiers in Microbiology, 2014. **5**.
17. Doll, K.L. and J.T. Harty, *Correlates of protective immunity following whole sporozoite vaccination against malaria.* Immunologic Research, 2014. **59**(1-3): p. 166-176.
18. Van Braeckel-Budimir, N., S.P. Kurup, and J.T. Harty, *Regulatory issues in immunity to liver and blood-stage malaria.* Current Opinion in Immunology, 2016. **42**: p. 91-97.
19. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Current Opinion in Biotechnology, 2012. **23**(6): p. 908-916.
20. Gonzalez-Aseguinolaza, G., et al., *Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines.* Journal of Experimental Medicine, 2002. **195**(5): p. 617-624.

21. Li, X.M., et al., *Colocalization of a CD1d-Binding Glycolipid with a Radiation-Attenuated Sporozoite Vaccine in Lymph Node-Resident Dendritic Cells for a Robust Adjuvant Effect.* Journal of Immunology, 2015. **195**(6): p. 2710-2721.

22. Othman, A.S., et al., *OX40 stimulation enhances protective immune responses induced after vaccination with attenuated malaria parasites.* Frontiers in Cellular and Infection Microbiology, 2018. **(In press)**.

23. Anderson, R.J., et al., *A self-adjuvanting vaccine induces cytotoxic T lymphocytes that suppress allergy.* Nature Chemical Biology, 2014. **10**(11): p. 943-949.

24. Brown, L.E. and D.C. Jackson, *Lipid-based self-adjuvanting vaccines.* Curr Drug Deliv, 2005. **2**(4): p. 383-93.

25. Tiptiri-Kourpeti, A., et al., *DNA vaccines to attack cancer: Strategies for improving immunogenicity and efficacy.* Pharmacology & Therapeutics, 2016. **165**: p. 32-49.

26. Moyle, P.M., *Biotechnology approaches to produce potent, self-adjuvanting antigen-adjuvant fusion protein subunit vaccines.* Biotechnology Advances, 2017. **35**(3): p. 375-389.

27. Chauhan, N., et al., *An overview of adjuvants utilized in prophylactic vaccine formulation as immunomodulators.* Expert Review of Vaccines, 2017. **16**(5): p. 491-502.

28. McDonald, D.M., S.N. Byrne, and R.J. Payne, *Synthetic self-adjuvanting glycopeptide cancer vaccines.* Frontiers in Chemistry, 2015. **3**.

29. Kalnin, K., et al., *Incorporation of RG1 epitope concatemers into a self-adjuvanting Flagellin-L2 vaccine broaden durable protection against cutaneous challenge with diverse human papillomavirus genotypes.* Vaccine, 2017. **35**(37): p. 4942-4951.

30. Chua, B.Y., et al., *A self-adjuvanting lipopeptide-based vaccine candidate for the treatment of hepatitis C virus infection.* Vaccine, 2008. **26**(37): p. 4866-4875.

31. Gomez-Samblas, M., et al., *Self-adjuvanting C18 lipid vinil sulfone-PP2A vaccine: study of the induced immunomodulation against Trichuris muris infection.* Open Biology, 2017. **7**(4).

32. Azmi, F., et al., *Self-adjuvanting vaccine against group A streptococcus: Application of fibrillized peptide and immunostimulatory lipid as adjuvant.* Bioorganic & Medicinal Chemistry, 2014. **22**(22): p. 6401-6408.

33. Moyle, P.M. and I. Toth, *Self-adjuvanting lipopeptide vaccines.* Current Medicinal Chemistry, 2008. **15**(5): p. 506-516.

34. Carapau, D., et al., *Protective Humoral Immunity Elicited by a Needle-Free Malaria Vaccine Comprised of a Chimeric Plasmodium falciparum Circumsporozoite Protein and a Toll-Like Receptor 5 Agonist, Flagellin.* Infection and Immunity, 2013. **81**(12): p. 4350-4362.

35. Cui, B.F., et al., *Flagellin as a vaccine adjuvant.* Expert Review of Vaccines, 2018. **17**(4): p. 335-349.

36. Plant, A. and N.A. Williams, *Modulation of the immune response by the cholera-like enterotoxins.* Current Topics in Medicinal Chemistry, 2004. **4**(5): p. 509-519.

37. Stratmann, T., *Cholera Toxin Subunit B as Adjuvant-An Accelerator in Protective Immunity and a Break in Autoimmunity.* Vaccines, 2015. **3**(3): p. 579-596.

38. Bolhassani, A. and S. Rafati, *Heat-shock proteins as powerful weapons in vaccine development.* Expert Review of Vaccines, 2008. **7**(8): p. 1185-1199.

39. Strbo, N., et al., *Secreted heat shock protein gp96-Ig: next-generation vaccines for cancer and infectious diseases.* Immunologic Research, 2013. **57**(1-3): p. 311-325.

40. Ding, Y., et al., *Heat-Shock Protein gp96 Enhances T Cell Responses and Protective Potential to Bacillus Calmette-Guérin Vaccine.* Scandinavian Journal of Immunology, 2016. **84**(4): p. 222-228.

41. Jung, I.D., et al., *Enhancement of Tumor-Specific T Cell–Mediated Immunity in Dendritic Cell–Based Vaccines by Mycobacterium tuberculosis Heat Shock Protein X.* The Journal of Immunology, 2014. **193**(3): p. 1233.

42. Kim, H.Y., et al., *Heat shock protein X purified from Mycobacterium tuberculosis enhances the efficacy of dendritic cells-based immunotherapy for the treatment of allergic asthma.* Bmb Reports, 2015. **48**(3): p. 178-183.

43. Andersen-Nissen, E., et al., *Evasion of Toll-like receptor 5 by flagellated bacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(26): p. 9247-9252.

44. Kang, X.L., Z.M. Pan, and X.N. Jiao, *Amino acids 89-96 of Salmonella flagellin: a key site for its adjuvant effect independent of the TLR5 signaling pathway.* Cellular & Molecular Immunology, 2017. **14**(12): p. 1023-1025.

45. Lin, J.W., et al., *The Subcellular Location of Ovalbumin in Plasmodium berghei Blood Stages Influences the Magnitude of T-Cell Responses.* Infection and Immunity, 2014. **82**(11): p. 4654-4665.

46. Montagna, G.N., et al., *Antigen Export during Liver Infection of the Malaria Parasite Augments Protective Immunity.* Mbio, 2014. **5**(4).

47. Lin, J.-w., et al., *A Novel 'Gene Insertion/Marker Out' (GIMO) Method for Transgene Expression and Gene Complementation in Rodent Malaria Parasites.* PLOS ONE, 2011. **6**(12): p. e29289.

48. Salman, A.M., et al., *Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins*, in *Malaria Vaccines: Methods and Protocols*, A. Vaughan, Editor. 2015, Springer New York: New York, NY. p. 257-286.

49. Haeberlein, S., et al., *Protective immunity differs between routes of administration of attenuated malaria parasites independent of parasite liver load.* Scientific Reports, 2017. **7**.

50. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nature Protocols, 2006. **1**(1): p. 346-356.

51. Annoura, T., et al., *Quantitative Analysis of Plasmodium berghei Liver Stages by Bioluminescence Imaging*, in *Malaria: Methods and Protocols*, R. Ménard, Editor. 2013, Humana Press: Totowa, NJ. p. 429-443.

52. Silvie, O., et al., *Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity.* Nature Medicine, 2003. **9**(1): p. 93-96.

53. Hafalla, J.C.R., I.A. Cockburn, and F. Zavala, *Protective and pathogenic roles of CD8+T cells during malaria infection.* Parasite Immunology, 2006. **28**(1-2): p. 15-24.

54. Ploemen, I.H.J., et al., *Visualisation and Quantitative Analysis of the Rodent Malaria Liver Stage by Real Time Imaging.* Plos One, 2009. **4**(11).

55. Otto, T.D., et al., *A comprehensive evaluation of rodent malaria parasite genomes and gene expression.* Bmc Biology, 2014. **12**.

56. Mueller, A.K., et al., *Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(8): p. 3022-3027.

57. Silvie, O., et al., *Post-transcriptional silencing of UIS4 in Plasmodium berghei sporozoites is important for host switch.* Molecular Microbiology, 2014. **91**(6): p. 1200-1213.

58. Silva, P.A.G.C., et al., *Translational Control of UIS4 Protein of the Host-Parasite Interface Is Mediated by the RNA Binding Protein Puf2 in Plasmodium berghei Sporozoites.* Plos One, 2016. **11**(1).

59. Tarun, A.S., et al., *Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8(+) T cells.* Journal of Infectious Diseases, 2007. **196**(4): p. 608-616.

60. Orito, Y., et al., *Liver-specific protein 2: a Plasmodium protein exported to the hepatocyte cytoplasm and required for merozoite formation.* Molecular Microbiology, 2013. **87**(1): p. 66-79.

61. Helm, S., et al., *Identification and Characterization of a Liver Stage-Specific Promoter Region of the Malaria Parasite Plasmodium.* Plos One, 2010. **5**(10).

62. De Niz, M., et al., *In Vivo and In Vitro Characterization of a Plasmodium Liver Stage-Specific Promoter.* Plos One, 2015. **10**(4).

63. Schnider, C.B., et al., *BioID Reveals Novel Proteins of the Plasmodium Parasitophorous Vacuole Membrane.* mSphere, 2018. **3**(1).

64. Silvie, O., R. Amino, and J.C. Hafalla, *Tissue-specific cellular immune responses to malaria pre-erythrocytic stages.* Current Opinion in Microbiology, 2017. **40**: p. 160-167.

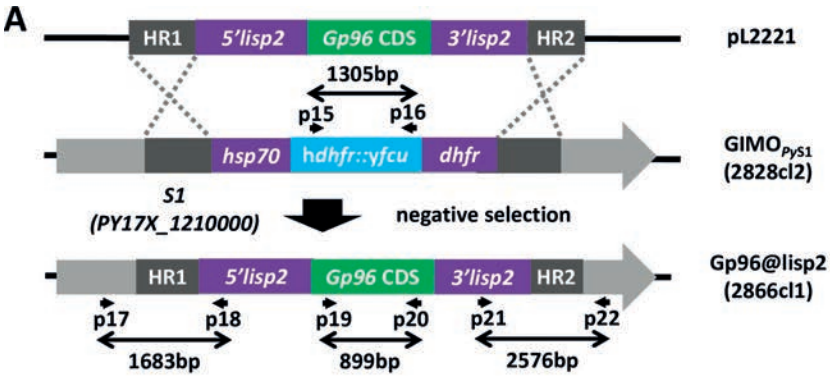
65. Matuschewski, K., et al., *Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage.* Journal of Biological Chemistry, 2002. **277**(44): p. 41948-41953.

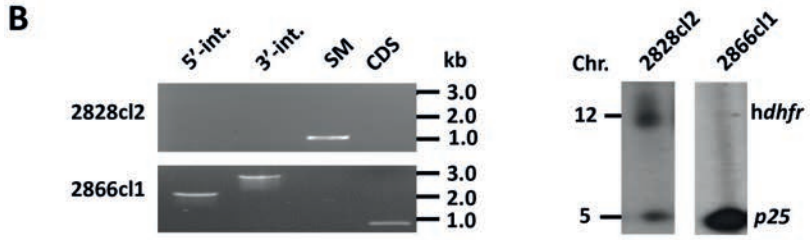
66. Jobe, O., et al., *Genetically attenuated Plasmodium berghei liver stages induce sterile protracted protection that is mediated by major histocompatibility complex class I-dependent interferon-gamma-producing CD8(+) T cells.* Journal of Infectious Diseases, 2007. **196**(4): p. 599-607.

67. Mueller, A.K., et al., *Genetically attenuated Plasmodium berghei liver stages persist and elicit sterile protection primarily via CD8 T cells.* American Journal of Pathology, 2007. **171**(1): p. 107-115.

SUPPLEMENTARY DATA

Supplementary Figure S1. Generation, genotype and phenotype analysis of a transgenic *P. yoelii* line expressing Gp96 under control of the promoter region of the liver specific *lisp2* gene. **(A)** Schematic representation of the introduction of the *Gp96*-expression cassette into the $GIMO_{PyS1}$ parasite (line 2828cl1; see **Figure S2**). Construct pL2221 contains the *Gp96* coding DNA sequence (CDS) flanked by the *lisp2* promoter region and the *3' pbdhfr UTR*. This construct is integrated into the modified *P. yoelii s1* locus of $GIMO_{PyS1}$ that contains the *hdhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination at the homology regions (HR1, HR2). Negative selection with 5-FC selects for parasites that have the SM cassette replaced by the *Gp96* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. **(B)** Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2221 in line 2866cl1 parasites. PCR shows the absence of the *hdhfr::yfcu* marker and the presence of the *Gp96* CDS. 5' integration PCR (5'-int; primers p17/p18), 3' integration PCR (3'-int; primers p21/p22), *hdhfr::yfcu* (SM, primers p15/p16), *Gp96* CDS (primers p19/p20). Primer locations and product sizes are shown in **A,** and primer sequences are presented in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker in the *s1* locus on chromosome 12 in 2866cl1 parasites. **(C)** Oocyst and sporozoite production in *A. stephensi* of the Gp96@lisp2 parasite line and the parent wild type (WT) *P. yoelli* line (line 1971cl1). Oocyst and sporozoite numbers were determined at day 8 and day 14, respectively, after mosquito infection. **(D)** Parasite liver loads in mice at 44 hour after infection with 1×10^4 sporozoites of the Gp96@lisp2 parasite line (3 mice) and the parent wild type (WT) *P. yoelli* line (line 1971cl1) (17 mice). Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU; middle panel). The left panel shows images of real time *in vivo* imaging of luciferase expressing liver stage parasites in mice at 44 h after injection of sporozoites. The right panel shows a Kaplan-Meier curve illustrating the prepatent period (day at which a parasitemia of 0.5–2% is observed).

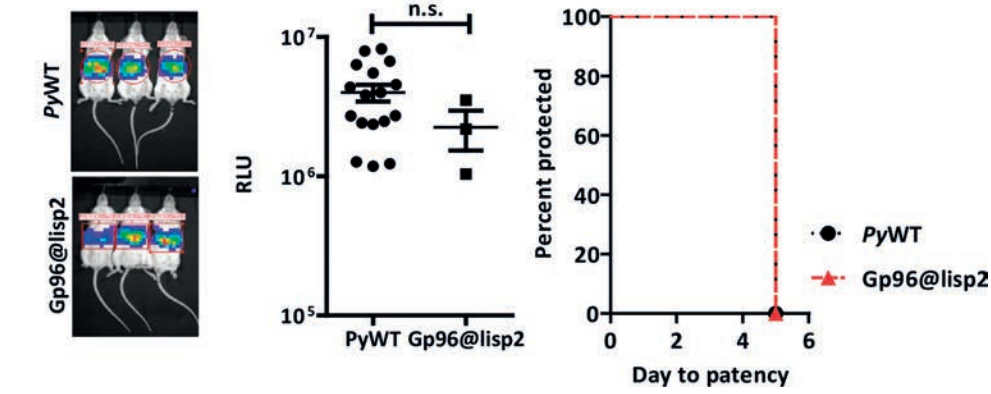


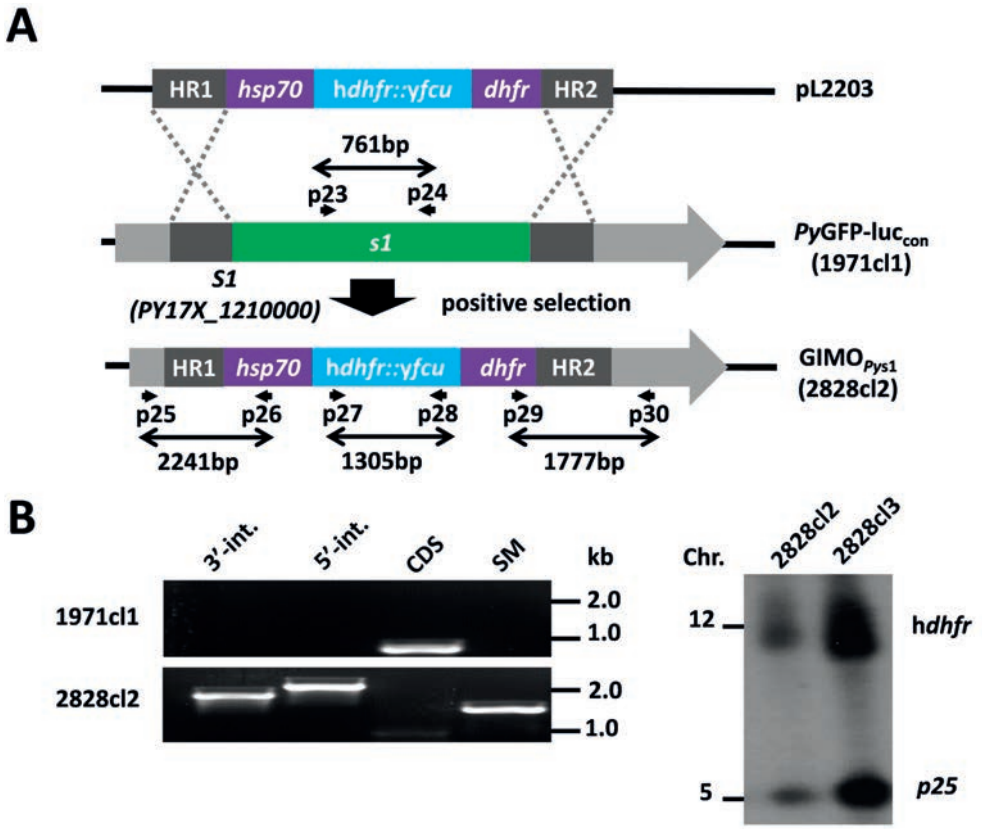


C

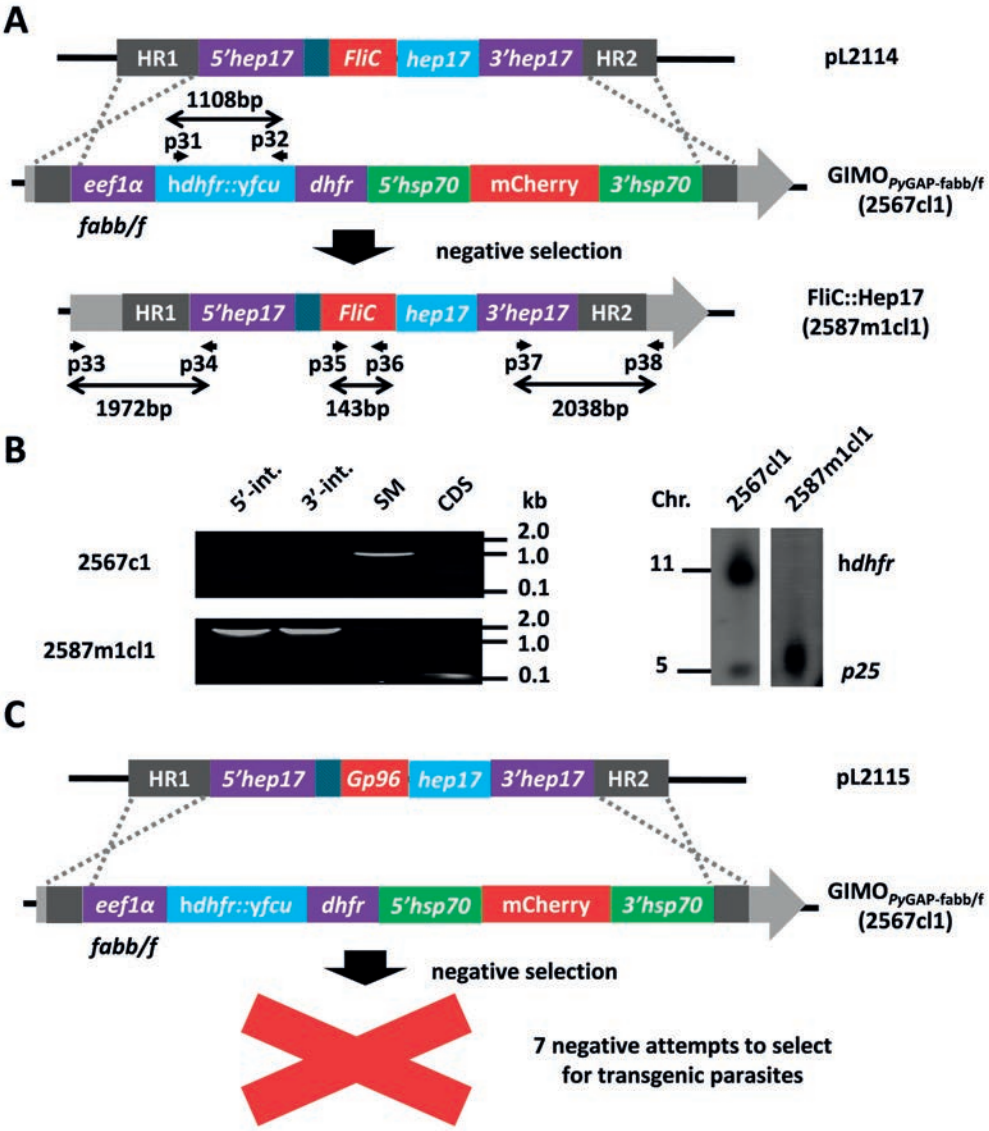
Mutant	Oocyst no.[a] Mean ± sd	Sporozoite no.[a] (x10^3)
Gp96@lisp2	282 ± 50.4	16.8 (1 exp.)
P. yoelii (1971cl1)	147 ± 103.4	19 ± 11.3

[a] (Mean) number of oocysts/sporozoites per mosquito





Supplementary Figure S2. Generation and genotype analysis of the *P. yoelii* GIMO parent line ($GIMO_{Pys1}$) for introduction of expression cassettes into the *s1* locus. (**A**) Schematic representation of the introduction of the *hdhfr-yfcu* selectable marker (SM) cassette into the *s1* gene locus of the parent *P. yoelii* parasite (line 1971cl1). Construct pL2203 contains the *hdhfr-yfcu* SM flanked by the *hsp70* promoter region and the *3' pbdhfr UTR*. This construct is integrated into the *s1* locus by double cross-over homologous recombination at the *s1* homology regions (HR1, HR2). Positive selection with pyrimethamine selects for parasites that have the *s1* coding sequence replaced by the SM cassette, thereby creating a GIMO line (2828cl2) for introduction of adjuvant expression cassettes. Location of primers used for PCR analysis and sizes of PCR products are shown. (**B**) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2203 in parasites of line 2828cl2. PCR shows the presence of the *hdhfr::yfcu* marker. 5' integration PCR (5'-int; primers p25/p26), 3' integration PCR (3'-int; primers p29/p30), *hdhfr::yfcu* (primers p27/p28), *s1* (CDS; primers p23/p24). Primer locations and product sizes are shown in **A** and primer sequences in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette marker in the *s1* locus on chromosome 12 in two clones of the 2828 line.



Supplementary Figure S3. Introduction of the Flic::*hep17* fusion gene into GIMO$_{PyGAP\text{-}fabb/f}$ and genotype analysis of the FliC::HEP17 parasite line. (A) Schematic representation of the introduction of the FliC::*hep17* expression cassette into the genome of GIMO$_{PyGAP\text{-}fabb/f}$ (line 2567cl1). Construct pL2114 contains the *flic* coding sequence (CDS) fused to the *hep17* open reading frame (ORF) which is flanked by the *hep17* promoter and *3'-UTR* regions. This construct is integrated into the modified *fabb/f* locus of GIMO$_{PyGAP\text{-}fabb/f}$ by double cross-over homologous recombination at the homology regions (HR1, HR2). This locus contains the *hdhfr::yfcu* selectable marker (SM) cassette with the SM under control of the *P. berghei eef1α* promoter and an *mCherry* expression cassette with *mCherry* under control of the *hsp70* promoter. Negative selection with 5-FC selects for parasites that have the SM cassette replaced by the FliC::*hep17* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2114 in in ▸

parasites of line 2587m1cl1. PCR shows the absence of the *hdhfr::yfcu* SM marker and the presence of the *FliC* CDS. 5' integration PCR (5' int; primers p33/p34), 3' integration PCR (3' int; primers p37/p38), *hdhfr::yfcu* (SM; primers p31/p32), *FliC* (CDS; primers p35/p36). Primer locations and product sizes are shown in **A** and primer sequences in **Table S1**). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker from the *fabb/f* locus on chromosome 11 in 2587m1cl1 parasites. (**C**) Schematic representation of (unsuccessful) attempts to introduction of the *Gp96::hep17* expression cassette into the genome of $GIMO_{PyGAP\text{-}fabb/f}$ (line 2567cl1). Construct pL2115 contains the *Gp96* coding sequence (CDS) fused to the *hep17* open reading frame (ORF) which is flanked by the *hep17* promoter and *3' UTR* regions. This construct aims at integration into the modified *fabb/f* locus of $GIMO_{PyGAP\text{-}fabb/f}$ by double cross-over homologous recombination at the homology regions (HR1, HR2). This locus contains the *hdhfr::yfcu* selectable marker (SM) cassette with the SM under control of the *P. berghei eef1*α promoter and an *mCherry* expression cassette with *mCherry* is under control of the *hsp70* promoter. Negative selection with 5-FC aims at selecting for parasites in which the SM cassette has been replaced by the *GP96::hep17* expression cassette.

Supplementary Table S1. List of primers used in this study

Primer ID	Leiden code	Sequence	Product (bp)	Description
Primers for conformation PCR analysis of PyΔfabb/f GIMO (2668cl1)				
p1	7903	ACGAATGGGCTATGTTATTG	2271	Forward 5′ Integration
p2	8081	CGGTCCCATGGGCAATTATAATTTATTGGG		Reverse 5′ Integration
p3	4698	GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
p4	4699	GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
p5	7922	GTCTCTTCAATGATTCATAAATAGTTGG	1339	Forward 3′ Integration
p6	7904	GTTGCAATTTCCCCTACAAC		Reverse 3′ Integration
Primers for conformation PCR analysis of mCherry::UIS4 (2696cl1)				
p7	4698	GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
p8	4699	GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
p9	7903	ACGAATGGGCTATGTTATTG	1808	Forward 5′ Integration
p10	7254	CACCATAAAAGGAAAGGATCAACC		Reverse 5′ Integration
p11	5451	GCATACATATATACCTTTCAGCAC	1428	Forward mCherry CDS
p12	5514	CGTACCTGCTCGACATGTT		Reverse mCherry CDS
p13	8132	TACTTCTCGAGCAATTGACTAGTTAATTCATTATGAGGGTAATTCAG	2174	Forward 3′ Integration
p14	7904	GTTGCAATTTCCCCTACAAC		Reverse 3′ Integration
Primers for conformation PCR analysis of adjuvants mutants (2690cl1/2692cl2/2694cl1/2698cl2)				
	7903	ACGAATGGGCTATGTTATTG	1808	Forward 5′ Integration
	7254	CACCATAAAAGGAAAGGATCAACC		Reverse 5′ Integration
	8132	TACTTCTCGAGCAATTGACTAGTTAATTCATTATGAGGGTAATTCAG	2174	Forward 3′ Integration
	7904	GTTGCAATTTCCCCTACAAC		Reverse 3′ Integration
	4698	GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
	4699	GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*

Supplementary Table S1. (continued)

Primer ID	Leiden code	Sequence	Product (bp)	Description
	5451	GCATACATATATACCTTTCAGCAC	1102	Forward CTB CDS
	8190	TCTTAACTAGTATTTGCCATACTAATTGCGGCAATCG		Reverse CTB CDS
	7960	CACATCTGGGAATCAGACTC	1747	Forward gp96 CDS
	7956	GATAAGATCTTTACAATTCATCCTTCTCTGTAGATTCC		Reverse gp96 CDS
	5451	GCATACATATATACCTTTCAGCAC	1153	Forward HspX CDS
	8011	GATAAGATCTGTTGGTGGACCGGATCTG		Reverse HspX CDS
	5451	GCATACATATATACCTTTCAGCAC	811	Forward fliC CDS
	6747	CCCAGTCTGACCTCGACTCC		Reverse fliC CDS
Primers for conformation PCR analysis of Gp96@lisp2 (2866cl1)				
p15	4592	GGTAAGAAGACCTGGTTCTC	1182	Forward h*dhfr*
p16	8273	CATCGATTCACCAGCTCTGAC		Reverse h*dhfr*
p17	8532	CCATGCTTTTCCTGAGAGTG	1683	Forward 5′ Integration
p18	6549	CGATCTACATGTGAAGAAAAT		Reverse 5′ Integration
p19	8511	GAACACGGCTTGCTAAACTTC	899	Forward gp96 CDS
p20	7956	GATAAGATCTTTACAATTCATCCTTCTCTGTAGATTCC		Reverse gp96 CDS
p21	8509	GATCCCAATTGGCATGCAGAAAAACCTAAAAGAGGTAATACCC	2576	Forward 3′ Integration
p22	6512	AGAAGAAGGAGATGTTGAAGC		Reverse 3′ Integration
Primers for conformation PCR analysis of pyΔs1 GIMO (2828cl2)				
p23	6521	AACTGCAACTATGCTCATGTG	761	Forward *Pys1* CDS
p24	6522	GCTACAATTCGTACTTCCAC		Reverse *Pys1* CDS
p25	8532	CCATGCTTTTCCTGAGAGTG	2241	Forward 5′ Integration
p26	8081	CGGTCCCATGGGCAATTATAATTTATTGGG		Reverse 5′ Integration
p27	8272	ATCATGCAAGACTTTGAAAGTGAC	1305	Forward h*dhfr*
p28	6347	AACACAGTAGTATCTGTCACC		Reverse h*dhfr*
p29	5226	CATATAAACACAAATGATGTTTTTTC	1777	Forward 3′ Integration

Supplementary Table S1. (continued)

Primer ID	Leiden code	Sequence	Product (bp)	Description
p30	6512	AGAAGAAGGAGATGTTGAAGC		Reverse 3' Integration
Primers for conformation PCR analysis of fliC::Hep17 (2587m1cl1)				
p31	4698	GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
p32	4699	GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
p33	7903	ACGAATGGGCTATGTTATTG	1972	Forward 5' Integration
p34	7852	ATATAGCTGGTTATTATTTTGATG		Reverse 5' Integration
p35	7947	TACCCAATTGATGAACGAAATCAACAACAACCTG	143	Forward fliC CDS
p36	7948	GATAAGATCTTTCGTTCAGGCGCTGGGTG		Reverse fliC CDS
p37	5215	CCTTCAATTTCGGATCCACTAGTATCATAAAAAGTTTCGACTC	2038	Forward 3' Integration
p38	7904	GTTGCAATTTCCCCTACAAC		Reverse 3' Integration
Primers for generation of GIMO$_{PyGAP\text{-}fabb/f}$ construct (pL2138)				
	8080	GATTCCTGCAGGAGCTCCCATATCATTAACACTATC	1078	Forward *Pyhsp70* promoter
	8081	CGGTCCCATGGGCAATTATAATTTATTGGG		Reverse *Pyhsp70* promoter
Primers for generation of mCherry::uis4 construct (pL2154)				
	8130	TACTTCTGCAGCTTAAGGATATTCTGCGATTTTTCTTG	1764	Forward *Pyuis4* promoter + CDS
	8131	CTTAGGTACCAATGCCTCGAGCTCTATGTATGGGTCAAATGGTTTATC		Reverse *Pyuis4* promoter + CDS
	8132	TACTTCTCGAGCAATTGACTAGTTAATTCATTATGAGGGTAATTCAG	938	Forward 3'UTR *Pyuis4*
	8133	GCTTAGGTACCCCGCGGGAAAACCATGAGCTATGATAC		Reverse 3'UTR *Pyuis4*
	7739	TTATTCTCGAGATGGTGAGCAAGGGCGAGGAG	711	Forward mCherry CDS
	8148	TCTTAACTAGTCCCTTTGTACAGCTCGTCCATG		Reverse mCherry CDS

Supplementary Table S1. (continued)

Primer ID	Leiden code	Sequence	Product (bp)	Description
Primers for generation of adjuvant:uis4 constructs				
	8189	TACTTCTCGAGATGATTAAATTAAAATTTGGTGTTTTTTTTACAG	394	Forward *ctb CDS*
	8190	TCTTAACTAGTATTTGCCATACTAATTGCGGCAATCG		Reverse *ctb CDS*
	8146	TACTTGAGCTCATGAGGGTCCTGTGGGTGTTG	2428	Forward *gp96 CDS*
	8147	TCTTACAATTGCAATTCATCCTTCTCTGTAGATTCC		Reverse *gp96 CDS*
	8144	TACTTCTCGAGATGGCCACCACCCTTC	460	Forward *hspx CDS*
	8145	TCTTAACTAGTCAATTGGTTGGTGGACCGGATCTG		Reverse *hspx CDS*
	8152	TACTTCTCGAGATGAACGAAATCAACAACAACCTG	145	Forward *flic CDS*
	8153	TCTTAACTAGTTTCGTTCAGGCGCTGGGTG		Reverse *flic CDS*
Primers for generation of $GIMO_{PyS1}$ construct (pL2203)				
	8450	GATCCAAGCTTGGGCCCCACCCTTAAAAAAAAGCCTAATG	1040	Forward 5'UTR *Pys*1
	8451	TACTGCTGCAGCCCGGGGTATATGAAATTAAAATTTTTTTTTGATGAG		Reverse 5'UTR *Pys*1
	8452	TACTGGGTACCCCCGGGCTAATAGAAATTTGGAAAAAAAGAAATATAG	937	Forward 3'UTR *Pys*1
	8453	TACTTGCGGCCGCGCCGTATTTGTTTACATACATATGTG		Reverse 3'UTR *Pys*1
Primers for generation of gp96@lisp2 construct (pL2221)				
	8507	TACTTGGGCCCGGGTCTAGATCCATTCTATACTATTTATTGG	1060	Forward *Py*lisp2 promoter
	8508	TACTTGAGCTCGAGTTTTTATGTGTAAAAAAGTAAAATG		Reverse *Py*lisp2 promoter
	8509	GATCCCAATTGGCATGCAGAAAAACCTAAAAGAGGTAATACCC	883	Forward 3'UTR *Py*lisp2
	8510	TAACTTGCGGCCGCCCGGGTACCGAATTAGTCAATATAATTAGTCATC		Reverse 3'UTR *Py*lisp2
Primers for generation of FliC & Gp96 construct (pL2114 & 2115)				
	7838	TACGACTTAAGTGTCAATAATATTTATTTTGGTACAC	1481	Forward *Pyhep17* promoter + sp*
	7839	ATTATGGTACCATTCAATTGATACTTGTTTTTTCCATAAGCATC		Reverse *Pyhep17* promoter + sp*

Supplementary Table S1. (continued)

Primer ID	Leiden code	Sequence	Product (bp)	Description
	7840	GGCAATTGGGCAAGATCTGGTAAAAATGGCAAATATGG	1539	Forward *Pyhep17 CDS + 3'UTR*
	7841	GTCACGGTACCATTGTTTGTGGTCATAACATAG		Reverse *Pyhep17 CDS + 3'UTR*
	7947	TACCCAATTGATGAACGAAATCAACAACAACCTG	143	Forward *flic CDS*
	7948	GATAAGATCTTTCGTTCAGGCGCTGGGTG		Reverse *flic CDS*
	7955	GTACCCAATTGATGAGGGTCCTGTGGGTGTTG	2430	Forward *gp96 CDS*
	7956	GATAAGATCTTTACAATTCATCCTTCTCTGTAGATTCC		Reverse *gp96 CDS*

h = human, *Py* = *Plasmodium yoelii*, *sp = signal peptide

CHAPTER 5

GENERATION AND ANALYSIS OF GENETICALLY ATTENUATED RODENT MALARIA PARASITES THAT ARREST LATE DURING LIVER STAGE DEVELOPMENT

Ahmad Syibli Othman[1,2], Blandine M Franke-Fayard[1], António M. Mendes[3], Surendra K. Kolli[1], Séverine Chevalley-Maurel[1], Catherin Marin-Mogollon[1], Helena Nunes-Cabaço[3], Hans Kroeze[1], Fiona J. van Pul[1], Jai Ramesar[1], Miguel Prudêncio[3] Chris J. Janse[1] and Shahid M. Khan[1]*

[1]Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[2] Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[3] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal
* The corresponding author

Thesis Chapter

ABSTRACT

Vaccination with live, attenuated sporozoites provide strong protective immunity against malaria infection in both mice and humans. Using rodent models of malaria it has been shown that attenuated parasites that arrest late into liver-stage development provide superior immunity to those that arrest early. It is essential that attenuated-parasite vaccines cannot establish a pathogenic blood infection. We attempted to create rodent malaria mutants that show complete growth arrest during late liver-stage development by deleting different combination of two genes from the *Plasmodium berghei* parasite genome. The genes *lisp1*, *lisp2*, *mei2* and *palm,* were selected as they encode proteins that play an important role in the final maturation of the parasite's liver-stages. We showed that three genetically attenuated parasites (GAPs), Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1* and Δ*lisp1*Δ*palm,* produced blood-stage infections in mice after infection with 5×10^4 sporozoites. In contrast no blood-stage infections were observed in mice infected with 5×10^4 or 2-3 $\times 10^5$ Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* sporozoites. However, after a high dose of 5×10^5 sporozoites of either mutant, some mice developed a blood infection. The Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* mutants replicate and arrest late into liver-stage development as shown *in vitro* by the presence of large intrahepatic stages that express the merozoite proteins MSP1 and AMA1, and *in vivo* through the detection of high parasite liver-loads 44 hours after infection. We compared the immunogenicity of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* late-arresting GAPs (LA-GAPs) with that of an early-arresting GAP (Δ*b9*Δ*slarp*) in both BALB/c and C57BL/6 mice. Our results showed no significant differences exists in the induction of protective immunity between the two late-arresting GAPs and that the LA-GAPs were not significantly more protective than the early-arresting GAP. Overall, our results show that two double gene-deletion mutants were highly but not completely attenuated and that unexpectedly these LA-GAPs are not significantly more protective than parasites that arrest during liver-stage infection.

INTRODUCTION

Complete protection against a malaria infection has been demonstrated after immunization with live attenuated sporozoites both in rodent models of malaria and in humans [1-3]. Such sterile protection against a malaria infection in humans has been obtained after immunization with *Plasmodium falciparum* (*Pf*) sporozoites, which have been attenuated by radiation or administered under chemoprophylaxis [4-6]. A prerequisite for induction of protective immunity by whole sporozoite-based (wsp) vaccines is that sporozoites retain their capacity to invade liver cells after administration, as heat-killed sporozoites are unable to induce strong protective immunity [7, 8]. Vaccination with radiation-attenuated sporozoites (Irr-Spz) is currently the most advanced wsp vaccination strategy and is currently under evaluation both in the clinic and in the field [2, 9]. A number of studies have demonstrated that Irr-Spz vaccination can elicit strong protective immunity in humans [9-11]. However, in order to achieve high levels of protection, multiple immunizations with high doses of attenuated sporozoites are required [6, 9]. In the context of mass administration of wsp vaccines in malaria-endemic countries, multiple immunizations with high sporozoites doses have considerable implications for cost of goods due to the complicated production of sporozoites. A major challenge is to produce a highly immunogenic live-attenuated vaccine, that requires the fewest attenuated sporozoites per dose, and the fewest doses to induce sustained sterile protection against a malaria infection.

In rodent models of malaria it has been shown that immunization with sporozoites of genetically-attenuated parasites (GAPs) can induce similar, or even better, levels of protective immunity compared to Irr-Spz [1, 12-14]. Genetic attenuation of sporozoites is based on the deletion of one or multiple genes that play an important role during liver-stage development, resulting in complete growth arrest of the parasites in the liver and thereby preventing the development of a blood-stage infection after immunization with GAP sporozoites. An advantage of GAP sporozoites compared to Irr-Sporozoites is that homogenous sporozoites populations can be produced with defined genetic identity and an attenuation phenotype that can be designed to induce optimal protective immunity. Moreover, the use of GAP sporozoites provide manufacturing advantages compared to Irr-Spz [1, 13, 14]. Most notably because sporozoites do not need to be irradiated before they are vialed and because their production poses little risk to the individuals who produce the vaccine as GAP sporozoites are unable to establish a pathogenic blood-stage infection. GAP studies in rodent malaria models have been critical for the generation of several *P. falciparum* GAP vaccine candidates, which are currently undergoing clinical evaluation [15-19]. Moreover, rodent models of malaria have been used to explore different approaches to enhance immunogenicity of wsp vaccines [1, 13, 14]. For example, it has been demonstrated that co-administration of adjuvants during immunization with attenuated sporozoites can enhance protective immune responses [20-22]. In addition, it has been shown that immunization of mice with GAP whose growth is arrested late during liver-stage development can induce higher levels of protective immunity than immunization

with GAP that arrest early after invasion of hepatocytes. Specifically, it has been shown that late-arresting GAP (LA-GAP) induce greater numbers of a broader range of $CD8^+$ T cells, which results in increased protection against a malaria infection compared to immunization with early-arresting GAPs [12], most probably due to a greater number and repertoire of antigens expressed by LA-GAP. This may also explain the high degree of protective immunity observed when humans are immunized by fully infectious sporozoites under chemoprophylactic treatment with chloroquine [5, 23]. In this immunization approach, liver-stage development progresses normally but the merozoites that are released from the liver and infect erythrocytes are killed by chloroquine. This wsp approach induces sterile protection against parasite challenge but requires approximately 60-fold fewer cumulative sporozoites numbers than immunization with Irr-Spz which arrest early during liver-stage development [24]. A prerequisite for a GAP vaccine for humans use, is that the GAP sporozoites are unable to establish a potentially pathogenic blood-stage infection which requires that parasite growth is completely arrested during development in the liver. Consequently, multiple gene deletions in the same GAP are considered necessary, each governing independent but essential processes during liver-stage development [25]. Currently, three *P. falciparum* GAPs have been developed for clinical evaluation, all of which are early-arresting GAPs, that halt development soon after hepatocyte invasion [26-28]. In these GAPs either two or three genes, which encode proteins that play a vital role in early liver-stage development, have been deleted. Three of the selected proteins, P52, P36 and B9 are all members of the so-called 6-Cys gene family and all participate in the formation and maintenance of the parasitophorous vacuole (PV) inside the infected hepatocyte [29, 30] and the fourth protein, SLARP/SAP1, is involved in the regulation of gene expression [31, 32]. In contrast to the creation of early-arresting GAPs, the generation of safe LA-GAPs has been challenging. Although several genes have been identified that encode proteins that play an important role during late liver-stage development, deletion of those genes did not result in complete growth arrest in rodent models of malaria. Examples include multiple proteins involved in type II fatty acid synthesis pathways (FAS II, i.e. Fab proteins) [33, 34], a transcription factor with AP2 domain(s) (AP2-L) [35], biotin-protein ligase 1 (HCS1) [36] and proteins involved in formation and egress of merozoites from liver-schizonts, i.e. liver merozoite formation protein (PALM) [37], putative liver stage protein 1 (LISP1) [38], liver-specific protein 2 (LISP2; also known as sequestrin) [29, 39] and ZIP domain-containing protein (ZIPCO) [40]. Only the deletion of the genes encoding FabB/F [33] and MEI2-like RNA-binding protein (PlasMei2; hereafter referred to as *mei2*) have been reported to result in complete growth arrest in the rodent parasite *P. yoelii* [41]. However, studies in *P. falciparum* have shown that parasites lacking FabB/F expression are unable to complete mosquito stage development [42].

In an effort to identify GAPs that arrest late into liver-stage development and are unable to establish a blood infection, we investigated whether deleting combinations of genes that have been shown to have a role in late liver-stage development, could synergize to create fully arrested GAPs. Our studies show that the combination of the following deleted

genes, *mei2-palm* or *mei2-lisp2*, had the strongest attenuation phenotype in *P. berghei* and both Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* parasites were arrested in growth late during liver-stage development. Our observation of the strong attenuation of Δ*mei2*Δ*lisp2* is in line with a recent study where a similar phenotype was observed for the equivalent genetic deletion in *P. yoelii* [43]. However, we show that infection with high doses of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* sporozoites can result in 'breakthrough' blood infections. Moreover, unexpectedly, mouse immunization studies indicated that there was no improvement in protective immunity generated by LA-GAPs compared to an early-arresting GAP, which arrests soon after hepatocyte invasion. We discuss the implications of these findings both in the context of LA-GAP vaccine development and why late-arresting parasites do not provoke higher protective immunity despite the presence of much greater antigen load and diversity.

MATERIALS AND METHODS

Experimental animals and wild type and transgenic *P. berghei* lines

Female OF1, BALB/c ByJ and C57BL/6 mice (6-7 weeks; Charles River Laboratories, France) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042 and 14207) and Instituto de Medicina Molecular (IMM Lisboa). The Dutch Experiments on Animal Act and Animal Care Committee of IMM Lisboa (ACCiMM) are established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All experiments were performed in accordance with relevant guidelines and regulations. Four *P. berghei* (*Pb*) lines were used: i) the reference 'wild type' *Pb*ANKA parasite line 676m1cl1 (*Pb*ANKA-GFP-Luc_{con}; mutant RMgm-29; www.pberghei.eu) which contains the fusion gene *gfp-luc* gene under control of the constitutive *eef1*α promoter integrated into the neutral *230p* gene locus (PBANKA_0306000) and ii) the parent line *Pb*ANKA parasite line 1868cl1 (*Pb*ANKA-$mCherry_{hsp70}$+$Luc_{eef1\alpha}$; line RMgm-1320; www.pberghei.eu) which contains the fusion gene *mcherry* gene under control of the strong *hsp70* promoter and *luciferase* gene under control of the constitutive *eef1*α promoter integrated into the neutral *230p* gene locus (PBANKA_0306000). Both lines does not contain a drug-selectable marker. Also, two previously generated of early-arresting GAP mutant parasite lines (in *P. berghei* ANKA background) were used Δ*slarp* (1839cl3; mutant RMgm-1140; www.pberghei.eu) and Δ*b9*Δ*slarp* (1844cl1; mutant RMgm-1141; www.pberghei.eu).

Generation of marker-free *mei2* and *lisp1* single gene-deletion mutants

The *mei2* (PBANKA_1122300) and *lisp1* (PBANKA_1024600) genes were deleted in *P. berghei* by standard methods of transfection [44] using gene-deletion plasmids that were obtained from PlasmoGEM (Wellcome Trust Sanger Institute, UK). For deletion of *mei2,* construct *Pb*GEM-300555 (pL2206) and for deletion of *lisp1*, construct *Pb*GEM-334115

(pL2204) were used (http://plasmogem.sanger.ac.uk/) [45]. Both constructs contain the h*dhfr::yfcu* selectable marker (SM) cassette with the *P. berghei eef1*α promoter region and 3' terminal sequence of *pbdhfr*. Before transfection, the constructs were linearized by digesting with NotI. Parasites of line 1868cl1 were transfected with constructs pL2206 (exp. 2834) and pL2204 (exp. 2832) using standard transfection technologies and transformed parasites selected by positive selection with pyrimethamine [44]. Selected parasites were cloned by limiting dilution and mutants 2834cl2 and 2832cl3 were used for genotype analysis and to generate the SM-free gene deletion mutants. To remove the h*dhfr::yfcu* SM cassette from the genomes of 2834cl2 and 2832cl3, these parasites were selected (negative selection) by treatment of infected mice with 5-Fluorocytosine (5-FC) as described [46]. This treatment selects for parasites that have undergone homologous recombination between the two 3'-UTR of *pbdhfr* untranslated regions present in the integrated constructs pL2206 and pL2204, flanking the h*dhfr*::*yfcu* cassette and thereby removing the SM [47]. Selection and cloning of the parasites resulted in the SM-free single gene-deletion mutants Δ*mei2* (2834cl2m1cl1) and Δ*lisp1* (2832cl3m0cl1) that were used to generate the double gene-deletion mutants (see next section).

Generation of double gene-deletion mutants

Parasites of Δ*mei2*, which contains a disrupted *plasmei2* gene and is SM-free, was used to delete the following genes in independent transfection experiments: *lisp1* (PBANKA_1024600), *lisp2* (PBANKA_1003000) and *palm* (PBANKA_0101100). In addition, parasites of Δ*lisp1*, which contains a disrupted *lisp1* gene and is SM-free, was used to delete the following genes in independent transfection experiments: *lisp2* (PBANKA_1003000) and *palm* (PBANKA_0101100). To delete the *lisp1*, *palm* and *lisp2* genes, the gene-deletion constructs *Pb*GEM-334115 (pL2204), *Pb*GEM-266100 (pL2205) (http://plasmogem.sanger.ac.uk/) [45] and pL1462 (Annoura *et al.*, 2014; http://www.pberghei.eu/index.php?rmgm=930) were used, respectively. The PlasmoGEM constructs were obtained from Wellcome Trust Sanger Institute, UK. The PlasmoGem constructs contain the h*dhfr::yfcu* SM and pL1462 contains the *dhfr* gene of *Toxoplasma gondii* flanked by the *pbdhfr* promoter region and the 3'-UTR of *pbdhfr*. Transfection with linearized constructs, positive selection of transfected parasites with pyrimethamine and cloning of selected parasites were performed as described [44]. This resulted in the 5 following lines/clones: Δ*mei2*Δ*lisp2* (line 2900cl3), Δ*mei2*Δ*lisp1* (line 2901cl1), Δ*mei2*Δ*palm* (line 2903cl3), Δ*lisp1*Δ*lisp2* (line 2961cl1) and Δ*lisp1*Δ*palm* (line 2961cl1) that were used for genotype and phenotype analyses (see next sections).

Genotyping single- and double gene-deletion mutants

Correct integration of the constructs and deletion of the genes were verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis [44]. To show integration of the PlasmoGem constructs containing h*dhfr::yfcu* SM or

removal of the h*dhfr::yfcu* SM by negative selection, the PFG-separated chromosomes were hybridized with a mixture of two probes: a probe of the h*dhfr* gene and a ~800 bp fragment of the 5'UTR of PBANKA_0508000 located on chromosome 5 [48]. To show correct integration of the construct pL1462 containing the *tgdhfr* SM the PFG-separated chromosomes were hybridized with a probe recognizing the 3'-UTR of *pbdhfr* [44]. PCR primers used to confirm correct integration of the constructs are listed in **Table S2**.

Mosquito infection, analysis of oocysts and preparation and injection of sporozoites

Infection of *A. stephensi* mosquitoes, collection and counting of sporozoites by manual dissection of mosquito salivary glands (at day 21 days after feeding) were performed as described [22, 26]. Mosquitoes were kept at a temperature of 21°C and 80% humidity. Salivary glands were collected in RPMI medium, homogenized and filtered (40 µm Falcon, Corning, Amsterdam, NL). The free sporozoites were counted in a Bürker counting chamber using phase-contrast microscopy. For intravenous (IV) administration, sporozoites were suspended in RPMI-1640 medium and per mouse 200 µl was injected into the tail vein.

Determination of parasite liver load and prepatent period after infection, immunization or challenge

Parasite liver loads in live mice, after infection, immunization or challenge, were quantified by real time *in vivo* imaging as previously described [49]. Time and dose schedules of immunization and challenge by intravenous injection of sporozoites are indicated in the Results section and in the Figures. Parasite liver loads were visualized and quantified by measuring luciferase activity of parasites in whole bodies of mice at 44, 56 and 65 h after injection of sporozoites using the IVIS Lumina II Imaging System (Perkin Elmer Life Sciences, Waltham, USA). During measurements mice were anesthetized using the isofluorane-anesthesia system (XGI-8, Caliper Life Sciences, Hopkinton, USA). D-luciferin was dissolved in PBS (100 mg/kg; Caliper Life Sciences, USA) and 60 µl injected subcutaneously in the neck. Measurements were performed within 8 min after the injection of D-luciferin. Quantitative analysis of bioluminescence of whole bodies was performed by measuring the luminescence signal intensity (RLU; relative light units) using the ROI (region of interest) settings of the Living Image® 4.5.5 software. Mice were monitored for blood-stage infections by Giemsa-stained blood smears made at day 4 to 30 after infection or challenge. The prepatent period (measured in days after sporozoites challenge) is defined as the day when a blood-stage infection with a parasitemia of 0.5–2% is observed [48].

Analysis of liver stage development *in-vitro*

Sporozoites were isolated from salivary glands of infected *A. stephensi* mosquitoes 21 days after an infectious blood meal as described above. The human-hepatoma cell line Huh7 was used for *in vitro* cultures of the liver stages as described [33]. Isolated sporozoites

(5×10^4) were added to monolayers of Huh7 cells on coverslips in 24 well plates (with a confluency of 80–90%) in 'complete' RPMI supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2% (vol/vol) penicillin-streptomycin, 1% (vol/vol) GlutaMAX (Invitrogen), and maintained at 37°C with 5% CO_2. At 24, 48 and 72 hours after infection, nuclei were stained with Hoechst 33342 at a final concentration of 10 µM and live imaging of mCherry-expressing parasites and liver-stage size were measured using Leica LAS X software by determining the area of the parasite at its greatest circumference using the mCherry-positive area (μm^2). The following exposure times: Alexa 488/FITC: 0.7 s; mCherry: 0.7 s; Hoechst 0.136 s (1x gain) were used.

For immunofluorescence analyses of liver-stages, the infected cells at 24, 48 and 72 h post infections were fixed with 4% paraformaldehyde in PBS for 30 min and cells were permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The infected cells were incubated overnight at 4°C with the rabbit anti-*P. yoelii* MSP1 [50] and rat anti-*P. falciparum* AMA1 [51] as a primary antibodies in 10% fetal calf serum in PBS, washed 3 times with PBS at RT, followed by incubation for 1 h with secondary conjugated antibodies anti-rabbit IgG Alexa Fluor®488 (Invitrogen) or anti-rat IgG FITC (Thermofisher). Nuclei were stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10 µM (Sigma, The Netherlands) for 30 min at RT. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed onto of the cells and sealed with nail polish. Stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (40x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa 488/FITC: 0.7 s; mCherry: 0.7 s; Hoechst 0.136 s (1x gain).

Statistics

All data were analyzed using the GraphPad Prism software package 5.04 (GraphPad Software, Inc). For sporozoites number and *in-vivo* imaging (RLU) analysis, statistical analysis was performed using the unpaired Student's *t*-test. For the survival analysis, statistical analyzes to determine differences in protection after challenge were performed using a Kaplan–Meier survival plot, and survival curves were compared using the Log-rank (Mantel-Cox) test. Survival was considered as the complete absence of parasites in blood. The significance threshold were 0.05 in all analysis.

RESULTS

Generation of five double gene-deletion mutants; removing genes with a reported role in late liver-stage development

To screen for genetically attenuated parasites (GAP) that fully arrest growth late during liver-stage development we first generated two single gene-deletion mutants lacking either *plasmei2,* hereafter called *mei2*, (PBANKA_1122300) or *lisp1* (PBANKA_1024600). To delete these two genes, we used two PlasmoGem constructs *Pb*GEM-300555 KO

and *Pb*GEM-334115 KO, respectively [45]. These constructs are designed to replace the complete open reading frame (ORF) of these genes by the h*dhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination. The two gene-deletion mutants, Δ*mei2* and Δ*lisp1*, were generated in the reference reporter line 1868cl1, which expresses mCherry and luciferase under control of the constitutive *hsp70* and *eef1a* promoters, respectively [52]. Transfection followed by positive selection with pyrimethamine, cloning and genotyping of transformed parasites was performed using standard technologies [44], resulting in isolation of the following cloned lines 2834cl2 (Δ*mei2*) and 2832cl3 (Δ*lisp1*) (**Figure S1, S2**). Subsequently, negative selection was applied by treating mice with 5-FC that were infected with parasites of the two lines. This selection procedure selects for parasites with the h*dhfr::yfcu* SM cassette removed [53]. Negative selection, followed by cloning, resulted in the isolation of the following cloned lines that are drug-selectable marker free, 2834cl2m1cl1 (Δ*mei2*) and 2832cl3m0cl1 (Δ*lisp1*) (**Figure S1, S2**). These two lines have been used to create the following five double gene-deletion mutants Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*lisp1*, Δ*mei2*Δ*palm*, Δ*lisp1*Δ*lisp2*, and Δ*lisp1*Δ*palm* (**Figure S3, S4**). To delete *lisp2* (PBANKA_1003000) we used the construct reported in Annoura *et al*. [29], which replaces the complete *lisp2* ORF by a *tgdhfr* SM cassette by double cross-over homologous recombination. To delete *lisp1* and *palm* (PBANKA_0101100) we used the PlasmoGem constructs *Pb*GEM-334115 KO and *Pb*GEM-266100 KO, respectively [45]. These constructs are designed to replace the complete ORF by the h*dhfr::yfcu* SM cassette. Transfection followed by positive selection with pyrimethamine, cloning and genotyping of transformed parasites were performed using standard technologies [44], resulting in isolation of the five cloned lines Δ*mei2*Δ*lisp2* (2900cl3), Δ*mei2*Δ*lisp1* (2901cl1), Δ*mei2*Δ*palm* (2903cl3), Δ*lisp1*Δ*lisp2* (2961cl1), and Δ*lisp1*Δ*palm* (2963cl1) (**Figure S3, S4**). Parasites of all five lines showed normal, wild type (WT) blood-stage growth as determined during the cloning procedure (data not shown). In addition, they all produced salivary gland sporozoites in *A. stephensi* mosquitoes in numbers that that were in the range of WT *P. berghei* ANKA parasites (**Table S1**).

Screening double gene-deletion mutants for liver-stage growth arrest and absence of blood infections

To screen for complete liver-stage growth arrest, we infected C57BL/6 mice with 5×10^4 sporozoites of the five double gene-deletion mutants and the single gene-deletion mutant Δ*mei2*. C57BL/6 mice were selected as they are highly susceptible to infection of the liver by *P. berghei* sporozoites. In this screen the single gene-deletion mutant Δ*lisp2* and Δ*palm* were not included since *P. berghei* mutants lacking these genes have been analyzed previously for breakthrough blood infections [29, 37]. For Δ*lisp2* we previously reported that 43-100% of Swiss mice infected with 5×10^4 sporozoites developed blood infections, although with a delay in prepatent period of 2-3 days compared to mice infected with the same number of wild type (WT) parasites [29]. For Δ*palm*, Haussig *et al.*, [37] reported

that 20% of C57BL/6 mice infected with 10^5 sporozoites developed blood infections, with a delay in prepatent period of 4-8 days compared to mice infected with the same number of WT parasites.

We first determined parasite liver loads in mice infected with 5 × 10^4 Δ*mei2*, Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*palm*, Δ*mei2*Δlisp1, Δ*lisp1*Δ*lisp2 and* Δ*lisp1*Δ*palm* sporozoites by *in vivo* imaging at 44 hours (h) after injection (**Figure 1A**). As an early-arresting GAP control we also infected mice with the same number of Δ*slarp* sporozoites. We could measure parasites in the liver at 44 h in the 6 lines. At this time point the control parasites, Δ*slarp*, that arrest early after invasion of hepatocytes are not detectable by *in vivo* imaging (**Figure 1A**). At 44 h post infection mice infected with the double gene-deletion mutants Δ*mei2*Δ*palm and* Δ*mei2*Δ*lisp1* have lower parasite liver loads compared to mice infected with either WT or the single gene-deletion mutant Δ*mei2* (**Figure 1A**).

Some mice infected with 5 × 10^4 Δ*mei2*Δ*lisp1*, Δ*lisp1*Δ*lisp2* and Δ*lisp1*Δ*palm* sporozoites developed blood infections, specifically 50%, 100% and 30% of the mice, respectively (**Table 1, Figure 1A**). These mice that established a blood-stage infection had a delay to blood-stage patency, but while Δ*lisp1*Δ*lisp2*-infected mice were delayed 2-3 days longer than in WT infected mice, Δ*mei*Δ*lisp1* and Δ*lisp1*Δ*palm* were delayed by 5-9 days (**Table 1, Figure 1A**). Mice infected with 5 × 10^4 Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*palm* and Δ*mei2* sporozoites did not develop blood infections (**Table 1, Figure 1A**). The absence of blood infections in mice infected with the single gene-deletion Δ*mei2* was unexpected since 50% of the mice infected with the double gene-deletion mutant Δ*mei2*Δ*lisp1* developed blood infections. Next we infected mice with higher doses of sporozoites (2-5 × 10^5) with these three (Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*palm* and Δ*mei2*) mutants. At a dose of 2 × 10^5 sporozoites 3 out of 10 mice infected with Δ*mei2* sporozoites developed blood infections with a long prepatent period of 12-14 days, demonstrating that parasites lacking only *mei2* do not completely arrest in the liver (**Table 1**). No mice developed blood infections when infected with 2-3 × 10^5 Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* sporozoites. However, at the highest dose, 5 × 10^5 sporozoites, we observed blood infections in one out of ten mice infected with Δ*mei2*Δ*lisp2* and one out of three mice infected with Δ*mei2*Δ*palm*, with prepatent periods of 12 and 14 days (**Table 1**). These observations indicate that all five double gene-deletion mutants are not completely attenuated in the rodent parasite *P. berghei*.

Analysis of liver-stage development of two double gene-deletion mutants with the strongest attenuation phenotype

Next we analyzed in more detail the late liver-stage development of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm*, the mutants with the strongest attenuation phenotype. First we analyzed the persistence of attenuated parasites in the liver by *in vivo* imaging. In WT-infected mice luminescence signals in livers decrease between 48 h and 52 h, as merozoites are released from infected hepatocytes and enter the blood circulation and at 60 h luminescence signals emanate from the whole body of the mouse as luminescent parasites are inside red blood

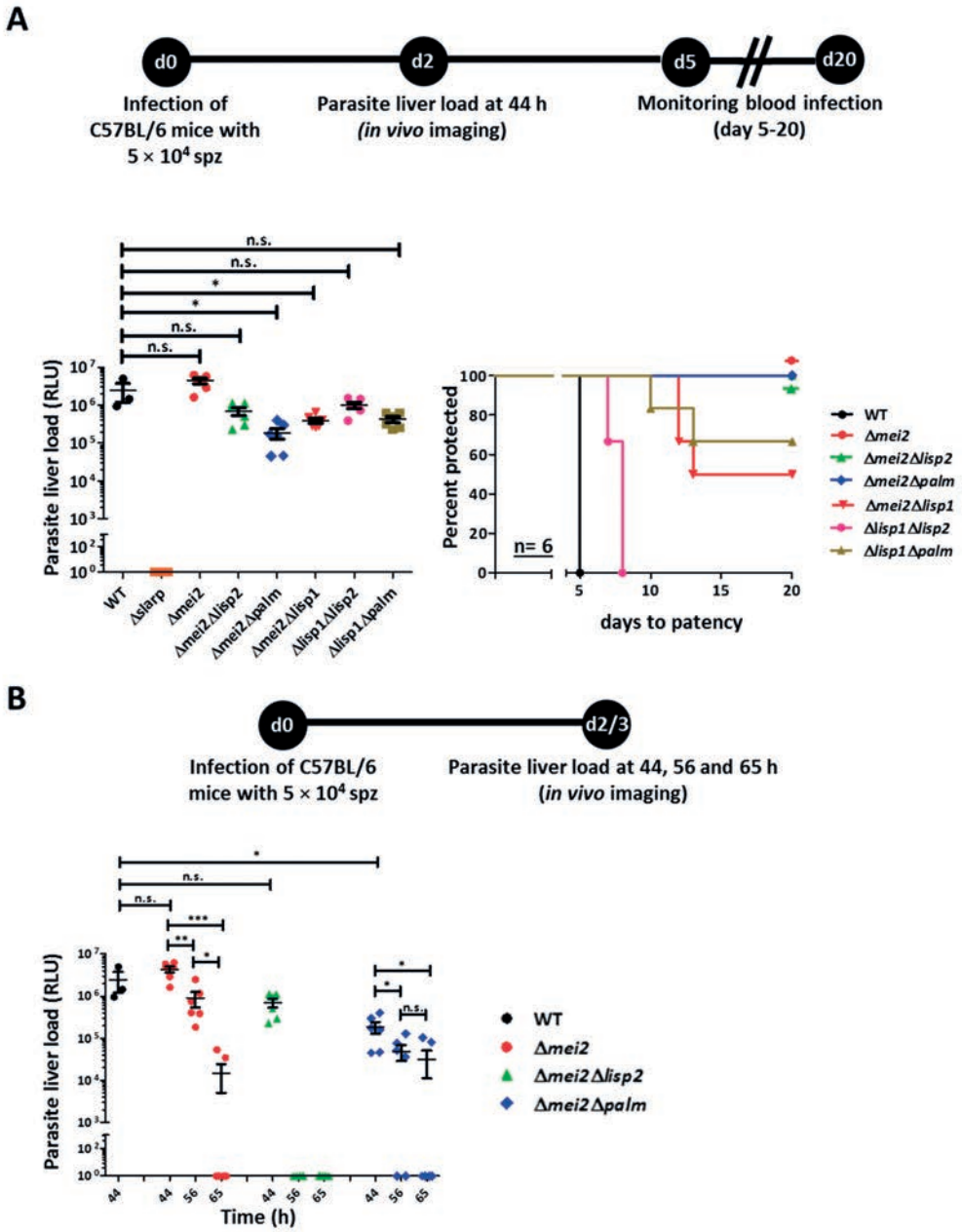


Figure 1. Parasite development in the liver and attenuation-phenotype after infection of C57BL/6 mice with sporozoites of different gene-deletion mutants. (A) The time line shows infection of C57BL/6 mice with sporozoites of gene-deletion mutants, determination of parasite liver load by *in vivo* imaging and determination of the prepatent period of blood infection. Left: Parasite liver loads in mice (n=6) at 44 h after intravenous injection of 5 × 10^4 sporozoites of WT and 7 gene deletion mutants. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). Significance values (unpaired two-tailed *t* test): WT vs Δ*mei2*Δ*palm* **p* = 0.02; WT vs ▸

Δ*mei2*Δ*lisp1* **p* = 0.04). n.s. – not significant. Right: The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of 0.5–2% is observed). Data are shown of groups of 6 mice. Significance values [Log-Rank (Mantel-Cox) test]: WT vs Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*palm*, Δ*mei2*Δ*lisp1*, Δ*lisp1*Δ*lisp2*, Δ*lisp1*Δ*palm* ***p* = 0.004. **(B)** The time line shows infection of C57BL/6 mice with sporozoites of gene-deletion mutants and determination of parasite liver load by *in vivo* imaging. The graph shows parasite liver loads in mice (n=6) at different hours (h) after intravenous injection of 5×10^4 sporozoites (44, 56 and 65 h) of 3 gene-deletion mutants. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). Significance values (unpaired two-tailed *t* test): **p* < 0.05; ***p* < 0.01; ****p* < 0.001. n.s. – not significant.

Table 1. Development of blood stage infections in C57BL/6 mice infected intravenously with a single dose of sporozoites (sporozoites) of different parasite mutants.

Parasite line or mutant	No. of sporozoites per dose (× 10^4)	No. of mice patent[a]	No. of days to patency[b]
WT	5	3/3	5
Δ*mei2*	5	0/6	n.a.
	20	3/10	9 - 10
Δ*mei2*Δ*lisp2*	5	0/6	n.a.
	20	0/10	n.a.
	30	0/3	n.a.
	50	1/10	12
Δ*mei2*Δ*lisp1*	5	3/6	12 - 14
Δ*mei2*Δ*palm*	5	0/6	n.a.
	30	0/5	n.a.
	50	1/3	9
Δ*lisp1*Δ*lisp2*	5	6/6	7 - 8
Δ*lisp1*Δ*palm*	5	2/6	10 - 13

[a] The number of mice that developed a blood stage infection
[b] The time of blood stage parasitemia between 0.5-2% WT - wild type; n.a. – not applicable

cells and distributed across all organs [54]. In contrast, in mice infected with Δ*mei2* and Δ*mei2*Δ*palm* parasites can still be detected in the liver at 56 and 65 h, without luminescent parasites in the blood circulation, although the mice had lower liver loads compared to 44 h (**Figure 1B**). Specifically, liver loads at 56 h in Δ*mei2*- and Δ*mei2*Δ*palm*-infected mice had decreased 2-5 fold compared to 44 h and in both groups of mice we could not detect liver signals in 4 out of the 6 mice at 65 h. In contrast, in Δ*mei2*Δ*lisp2*-infected mice no parasites could be detected in the liver at both 56 and 65 h (**Figure 1B**). These observation indicate differences between the two double gene-deletion mutants; while Δ*mei2*Δ*lisp2*

infected hepatocytes are lost or removed after 44 h, Δ*mei2*Δ*palm* infected hepatocytes can persist up to 65 h.

Next we analyzed Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* development in cultured Huh7 hepatoma cells. We measured the size parasites within a hepatocyte at 24, 48 and 72 h. At 24 and 48 h little or no differences were observed between the size of WT parasites and Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* parasites (**Figure 2**) However, at 72 h the liver-stages of both mutants were significantly larger than WT liver-stages, with a mean size of 1399 μm^2 for Δ*mei2*Δ*lisp2* and 1191 μm^2 for Δ*mei2*Δ*palm* compared to 507 μm^2 or 630 μm^2 for WT parasites at 48 and 72 h, respectively (**Figure 2**). These observations may suggest that, *in vitro*, liver-stages of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* can continue growing and/or dividing. However, *in vivo* we could not detect Δ*mei2*Δ*lisp2* parasites in mice livers after 56 h (**Figure 1B**). To examine the developmental progress of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* during late liver-stage development, we examined the expression of two merozoite genes, *msp1* and *ama1*, which are expressed late during liver-stage development. In *P. berghei* blood-stages the merozoite surface membrane protein MSP1 is expressed in early schizonts [55] whereas the micronemal protein AMA1 is expressed in late schizonts [51]. In rodent *Plasmodium* parasites we have previously measured MSP1 expression in infected hepatocytes at 44 h using anti-MSP1 antibodies [56]. By immunofluorescence analyses using anti-MSP1 and anti-AMA1 antibodies, we found that both Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* liver-schizonts expressed MSP1 and AMA1 at 72 h, and this staining was comparable to 72 h WT liver-schizonts (**Figure 3**). These observations indicate that the increase in size of parasites inside the hepatocytes at these later time points of development is associated with the expression of merozoite antigens, including antigens that are expressed later into schizont development (i.e. AMA1).

Analysis of immunogenicity of two double gene-deletion mutants with the strongest attenuation phenotype

We compared the immunogenicity of the late-arresting GAPs (LA-GAPs), Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm*, with protective immunity of an early-arresting GAP (Δ*b9*Δ*slarp*), using similar immunization protocols that have been used to compare immunogenicity of a mid-to-late arrester GAP with the early-arresting Δ*b9*Δ*slarp* GAP [57]. First, we immunized BALB/c mice using an immunization scheme employing single doses of 1,200 (high), 800 (medium), or 400 (low) sporozoites, followed by a challenge with 10^4 GFP-luciferase expressing WT sporozoites 3 weeks after immunization (**Figure 4A**). At 44 h after challenge, we measured parasite liver loads in the immunized mice by *in vivo* imaging. In mice immunized with Δ*mei2*Δ*lisp2* and Δ*b9*Δ*slarp*, we were only able to detect liver-stage parasites in one Δ*b9*Δ*slarp* immunized animal. In contrast, 2 out of 5 mice in each of the Δ*mei2*Δ*palm* LA-GAP immunization groups, WT liver-stage parasites could be detected in the liver at 44 h (**Figure 4B**).

All mice immunized with medium or high doses of the two LA-GAP sporozoites showed sterile protection against WT challenge and immunization with the early-arresting GAP also

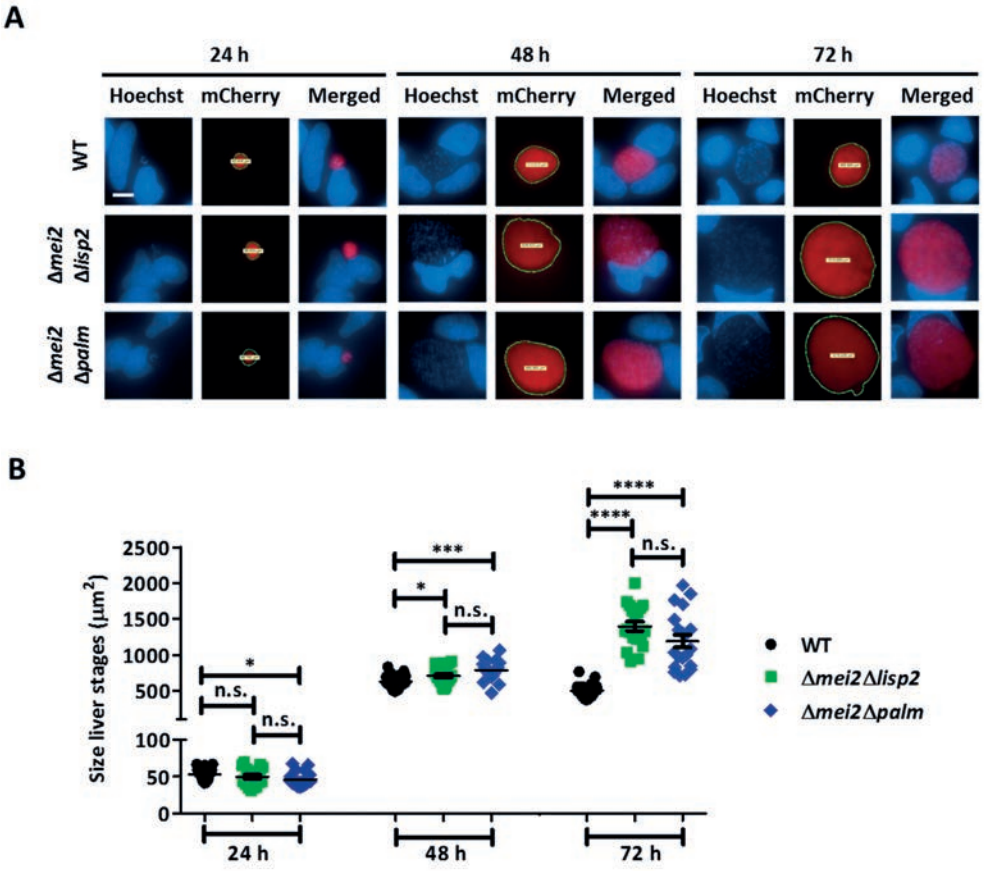


Figure 2. Size of liver-stage parasites of gene-deletion mutants at different time points after infection of hepatocytes. Upper panel: Representative images of live parasite at different time points after infection of Huh7 hepatocytes with sporozoites of two gene-deletion mutants, showing mCherry expression. Liver-stages express cytoplasmic mCherry (under control of the constitutive *hsp70* promoter). Liver-stage size was measured by determining the area of the parasite at its greatest circumference (green circles) using the mCherry-positive area (μm²). Nuclei of parasites and hepatocytes were stained with Hoechst (blue). Lower panel: Size of liver stages, determined as described above; at least 20 parasites were measured at each time point. Significant differences (unpaired two-tailed *t*-test): *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. n.s. – not significant. Scale bar: 10 μm.

induced complete protection at these higher doses (Figure 4C and Table 2). After the low dose immunization, all 5 mice immunized with LA-GAP Δ*mei2*Δ*lisp2* were protected, whereas 2 mice developed a blood infection after Δ*mei2*Δ*palm* GAP immunization and 1 mouse after immunization with the early-arresting GAP (Figure 4C). As expected, challenging naive BALB/c mice with 10,000 WT sporozoites resulted in detectable parasite liver loads and all 5 mice developed blood infections with a prepatent period of 5 days (Figure 4B,C). As summarized in Table 2, there was no significant difference in protective efficacy in BALB/c mice induced by immunization with either of the two LA-GAPs compared

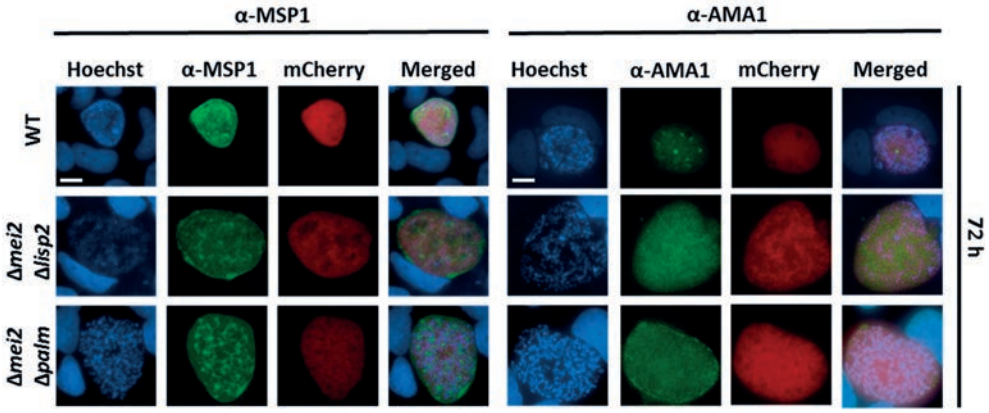


Figure 3. Expression of merozoite proteins MSP1 and AMA1 in liver stages of gene-deletion mutants at 72 hours after infection of hepatocytes. Representative images of fixed liver-stages at 72 hours after infection of Huh7 hepatocytes with sporozoites of two gene-deletion mutants, showing MSP1 and AMA1 expression. Liver-stages express cytoplasmic mCherry (under control of the constitutive *hsp70* promoter). Parasites are stained with anti-MSP1 (α-MSP1; green; Alexa-488) and anti-AMA1 antibodies (α-AMA1; green; FITC) and the nuclei of the parasite and hepatocyte are stained with Hoechst (blue). Scale bar: 10 μm.

to the early-arresting GAP. Interestingly, all high- and mid-dose Δ*mei2*Δ*palm* immunized mice did not develop a WT blood-stage infection despite the detectable presence of WT parasites in some mice at 44 h after challenge. This indicates that WT parasites were cleared late from the liver in the Δ*mei2*Δ*palm* immunized mice.

We performed a second immunization experiment in BALB/c mice, aimed to identify potential differences in maintenance and duration of protection between the two LA-GAPs and the early-arresting GAP. In this experiment BALB/c mice were immunized with a single dose of 800 sporozoites and challenged 6 weeks later with 3×10^3 parasites. As a control, we challenged immunized mice with 3×10^3 sporozoites at weeks 6. In the repeat early challenge experiment, we again detected no parasite liver loads at 44 h after WT challenge in the LA-GAP Δ*mei2*Δ*lisp2* and the early-arresting GAP immunized mice, but we detected higher WT-parasite liver loads at 44 h in the LA-GAP Δ*mei2*Δ*palm* immunized mice (**Figure 5A**). These higher parasite liver load in LA-GAP Δ*mei2*Δ*palm* immunized mice is comparable to what we observed in the first experiment (**Figure 4A**). Unexpectedly, in this experiment all immunized mice challenged after 3 weeks developed blood infections, although with a prepatent period delay of 2-4 days compared to naïve mice (**Figure 5A**; **Table 2**). This was unexpected since in the first experiment all mice that were immunized with a dose of 800 sporozoites were completely protected against WT challenge at 3 weeks. The difference between the two experiments is that the first experiment was performed in Leiden (The Netherlands) whereas the second experiment was performed in Lisbon (Portugal). In both laboratory the same BALB/c mice were used (BALB/c ByJ strain obtained from Charles River, France).

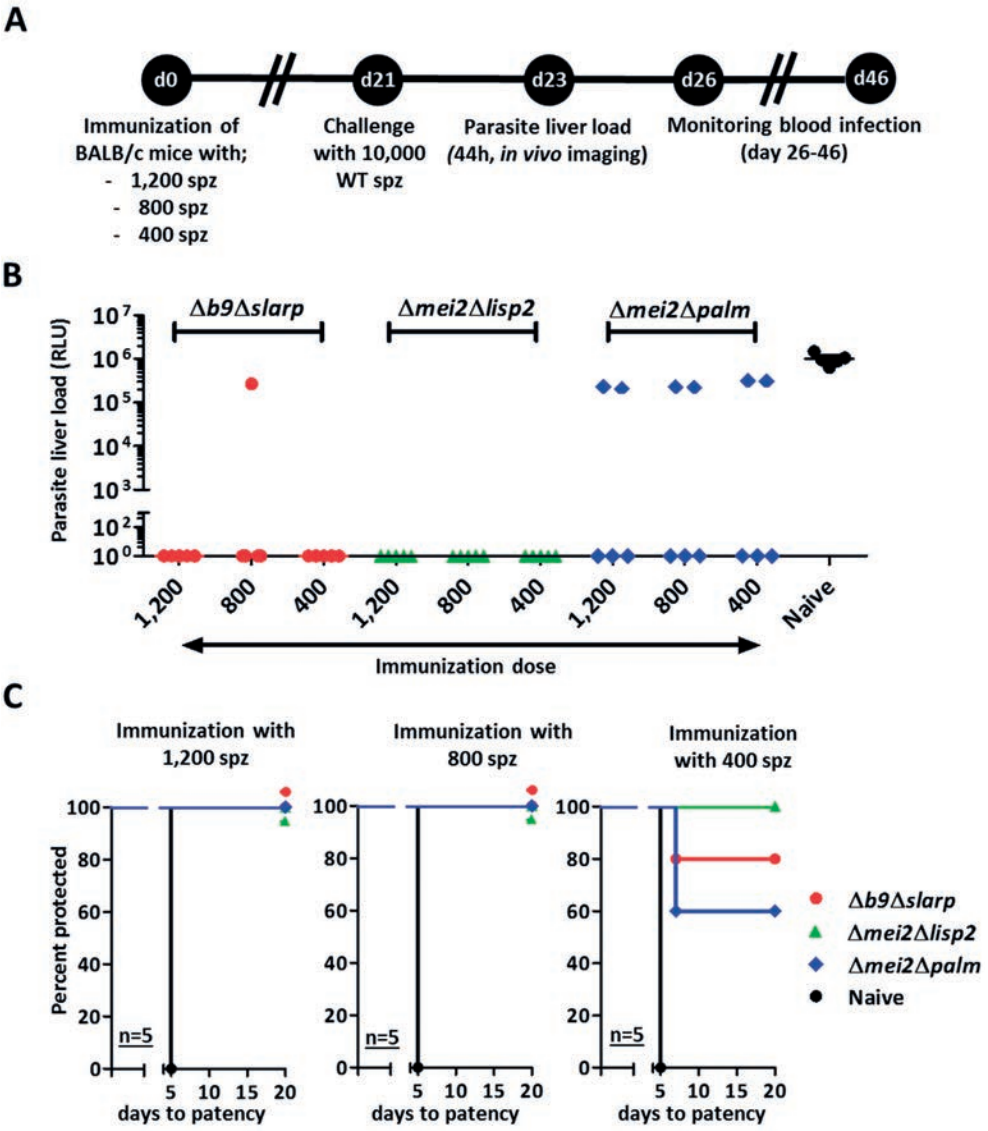


Figure 4. Analysis of protective immunity induced by immunization of BALB/c ByJ mice with sporozoites) of gene-deletion mutants (challenge of mice after 3 weeks; Leiden experiment). (A) The time line shows immunization of BALB/c mice with sporozoites of gene-deletion mutants, challenge of mice with wild type (WT) sporozoites, determination of WT parasite liver load by *in vivo* imaging and determination of the prepatent period of blood infection. (B) Parasite liver loads in mice (n=5) at 44 h after challenge of the immunized mice with 1 × 10^4 WT sporozoites. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). (C) The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of 0.5–2% is observed). Data are shown of groups of 5 mice. Significance values [Log-Rank (Mantel-Cox) test]: Naive vs Δ*b9*Δ*slarp*, Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* $^{**}p < 0.005$. No significant differences between mice immunized with early-arresting Δ*b9*Δ*slarp* and mice immunized with Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm*.

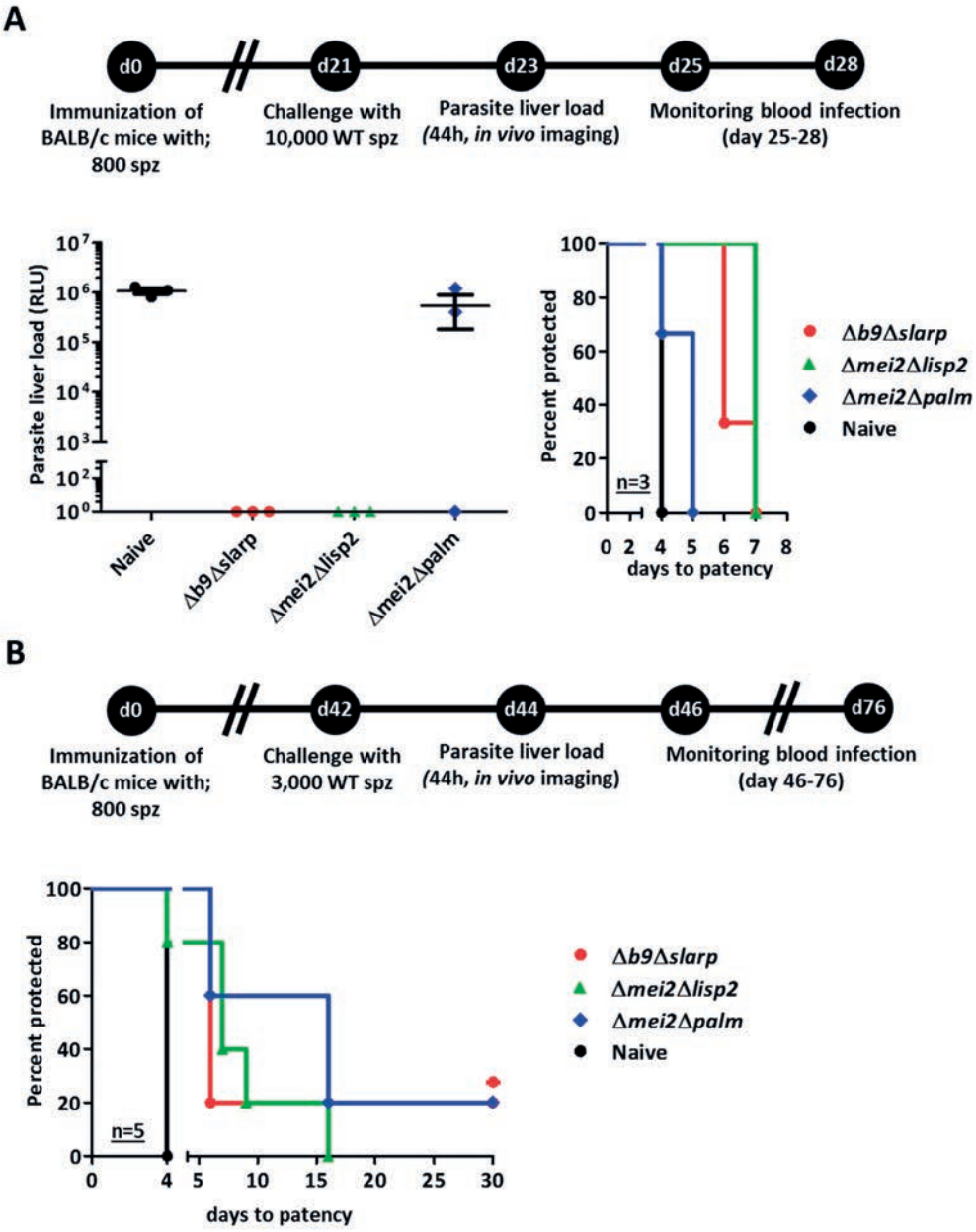


Figure 5. Analysis of protective immunity induced by immunization of BALB/c ByJ mice with sporozoites of gene-deletion mutants (challenge of mice after 3 and 6 weeks; Lisbon experiment). (A) The time line shows immunization of BALB/c mice with sporozoites of gene-deletion mutants, challenge of mice with wild type (WT) sporozoites after 3 weeks, determination of WT parasite liver load by *in vivo* imaging and determination of the prepatent period of blood infection. Left: Parasite liver loads in mice (n=3) at 44 h after challenge of the immunized mice with 1 × 10^4 WT sporozoites. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). Right: The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of

0.5–2% is observed). Data are shown of groups of 3 mice. Significance values [Log-Rank (Mantel-Cox) test]: significant longer prepatent period of Δ*b9*Δ*slarp* and Δ*mei2*Δ*lisp2* immunized mice compared to naive, non-immunized and Δ*mei2*Δ*palm* immunized mice *p = 0.02. (B) The time line shows immunization of BALB/c mice with sporozoites of gene-deletion mutants, challenge of mice with wild type (WT) sporozoites after 6 weeks, determination of WT parasite liver load by *in vivo* imaging and determination of the prepatent period of blood infection. The graph shows Kaplan-Meier curves illustrating the prepatent period (day at which a parasitemia of 0.5–2% is observed). Data are shown of groups of 5 mice. Significance values [Log-Rank (Mantel-Cox) test]: Naïve vs Δ*b9*Δ*slarp* **p = 0.008; Naïve vs Δ*mei2*Δ*lisp2* *p = 0.04; Naïve vs Δ*mei2*Δ*palm* **p = 0.008. No significant differences between mice immunized with early-arresting Δ*b9*Δ*slarp* and mice immunized with Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm*.

Table 2. Protective immunity after challenge with 10^3 wild type sporozoites of BALB/c mice immunized with early-arresting GAP (Δ*b9*Δ*slarp*) or LA-GAPs (Δ*mei2*Δ*lisp2*, Δ*mei2*Δ*palm*) sporozoites.

Mouse strain	Parasite line	No. of doses and sporozoite number (× 10^3)	No. of mice patent[a]	No. of days to patency[d]
BALB/c ByJ (experiment in Leiden)	Δ*mei2*Δ*lisp2*	1 x 0.4	0/5[b]	n.a.
		1 x 0.8	0/5[b]	n.a.
		1 x 1.2	0/5[b]	n.a.
	Δ*mei2*Δ*palm*	1 x 0.4	2/5[b]	7
		1 x 0.8	0/5[b]	n.a.
		1 x 1.2	0/5[b]	n.a.
	Δ*b9*Δ*slarp*	1 x 0.4	1/5[b]	7
		1 x 0.8	0/5[b]	n.a.
		1 x 1.2	0/5[b]	n.a.
	Naïve	–	5/5[b]	5
BALB/c ByJ (experiment in Lisbon)	Δ*mei2*Δ*lisp2*	1 x 0.8	3/3[b]	4 - 5
		1 x 0.8	5/5[c]	4 - 16
	Δ*mei2*Δ*palm*	1 x 0.8	3/3[b]	7
		1 x 0.8	4/5[c]	6 - 16
	Δ*b9*Δ*slarp*	1 x 0.8	3/3[b]	6 - 7
		1 x 0.8	4/5[c]	6
	Naïve	–	3/3[b]	4
		–	2/2[c]	4

[a] The number of mice that developed a blood stage infection
[b] Mice were challenged with 1 x 10^4 WT sporozoites 21 days after immunization
[c] Mice were challenged with 3 x 10^3 WT sporozoites 42 days after immunization
[d] The time of blood stage parasitemia between 0.5-2%
n.a. – not applicable

In the delayed challenge study, performed using 3 × 10^3 WT sporozoites at 6 weeks after immunization, we observed that most early-arresting or LA-GAP immunized mice develop a blood-stage infection (**Figure 5B; Table 1**); however, the prepatent periods are considerably delayed from 2-12 days compared to naïve mice. However, no significant differences exist between the prepatent periods of the two LA-GAP immunized mice

and the early-arresting GAP immunized mice (**Figure 5B**). Only 2 mice were protected after delayed challenge, one in the group immunized with the early-arresting GAP and one in the group of LA-GAP Δ*mei2*Δ*palm* immunized mice (**Figure 5B**). Combined these observations indicate the absence of large differences in immunogenicity between the LA-GAP and early-arresting GAP in the BALB/c model used.

Next we immunized C57BL/6 mice as these mice are known to be a more stringent model for inducing protective immunity against *P. berghei* liver-stages [58]. The C57BL/6 mice were immunized three times at weekly intervals either with 10^4 (high) or 10^3 (low) sporozoites (**Figure 6A**). Two weeks after the final immunization mice were challenged with 10^4 WT sporozoites. In the groups of mice immunized with the low dose of 10^3 sporozoites, all mice, except one (immunized with LA-GAP Δ*mei2*Δ*lisp2*), developed blood infections (**Figure 6B, Table 3**). Most immunized but unprotected mice developed a 2-4 days delayed blood-stage infection compared to naïve mice, regardless of the GAP used. There were no significant differences in the prolongation of the prepatent period between the mice immunized with 10^3 (low) sporozoites of the LA-GAPs or the early-arresting GAP.

In the groups of mice immunized with the high dose of 10^4 sporozoites, 5 out of 7 mice were protected from infection when mice were immunized with the LA-GAP Δ*mei2*Δ*lisp2* and 6 out of 8 mice were protected from infection when mice were immunized with the early-arresting GAP. However, after high dose immunization with the LA-GAP Δ*mei2*Δ*palm* only one mouse was protected and the remainder had a 2-3 days delay to blood-stage patency compared to naive mice. With the high sporozoites dose (10^4) immunization Δ*mei2*Δ*palm* immunized mice have significant shorter prepatent periods compared to Δ*mei2*Δ*lisp2* and the early-arresting GAP whereas no differences exist between mice immunized with Δ*mei2*Δ*lisp2* and the early-arresting GAP (**Figure 6B**).

DISCUSSION

In this study we generated five different double gene-deletion mutants, removing genes that have been shown to play a role in late liver-stage parasite development, in order to create fully arrested LA-GAPs. Three out of five mutants, Δ*mei2*Δ*lisp1*, Δ*lisp1*Δ*lisp2* and Δ*lisp1*Δ*palm* led to breakthrough blood infections in mice after infection with a (low) dose of 5×10^4 sporozoites. In all these mutants *lisp1* was one of the two deleted genes. LISP1 has been reported as not playing a role in liver-schizont maturation or in the formation of the merozoites, but rather as being involved in the release of liver merozoites [38]. Parasites lacking LISP1 expression display a 10-15 fold reduction in the number of merozoites released from hepatocytes and ensuing capacity to generate a blood-stage infection in mice [38]. In our study 50% of the mice infected with the double gene-deletion mutant Δ*mei2*Δ*lisp1* developed blood-stage infections after inoculation with 5×10^4 sporozoites. This was unexpected since the same number of sporozoites of the Δ*mei2* single gene-deletion mutant did not lead to a blood-stage infection. This could be due to experimental variation and it is possible that with a larger sample size Δ*mei2*-infected

Figure 6. Analysis of protective immunity induced by immunization of C57BL/6 mice with sporozoites of gene-deletion mutants. (A) The time line shows immunization of C57BL/6 mice with 3 doses of sporozoites of gene-deletion mutants, challenge of mice with wild type (WT) sporozoites, determination of WT parasite liver load by *in vivo* imaging and determination of the prepatent period of blood infection. The graph shows the parasite liver loads in mice (n =7 - 8) at 44 h after challenge of the immunized mice with 1 × 10^4 WT sporozoites. Mice were immunized with 3 doses of either 10^4 or 10^3 sporozoites. Parasite liver loads were determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). Significance values (unpaired two-tailed *t* test): Naive vs 1,000 Δ*b9*Δ*slarp* **p* = 0.017; Naive vs 1,000 Δ*mei2*Δ*lisp2* ***p* = 0.008; Naive vs 1,000 Δ*mei2*Δ*palm* ***p* = 0.001; Naive vs 10,000 Δ*mei2*Δ*palm* ****p* = 0.0002). (B) The Kaplan-Meier curves illustrate the prepatent period (day at which a parasitemia of 0.5–2% is observed). Data are shown of groups of 7 - 8 mice. Significance values [Log-Rank (Mantel-Cox) test]: Naive vs Δ*b9*Δ*slarp*, Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* ****p* < 0.001.

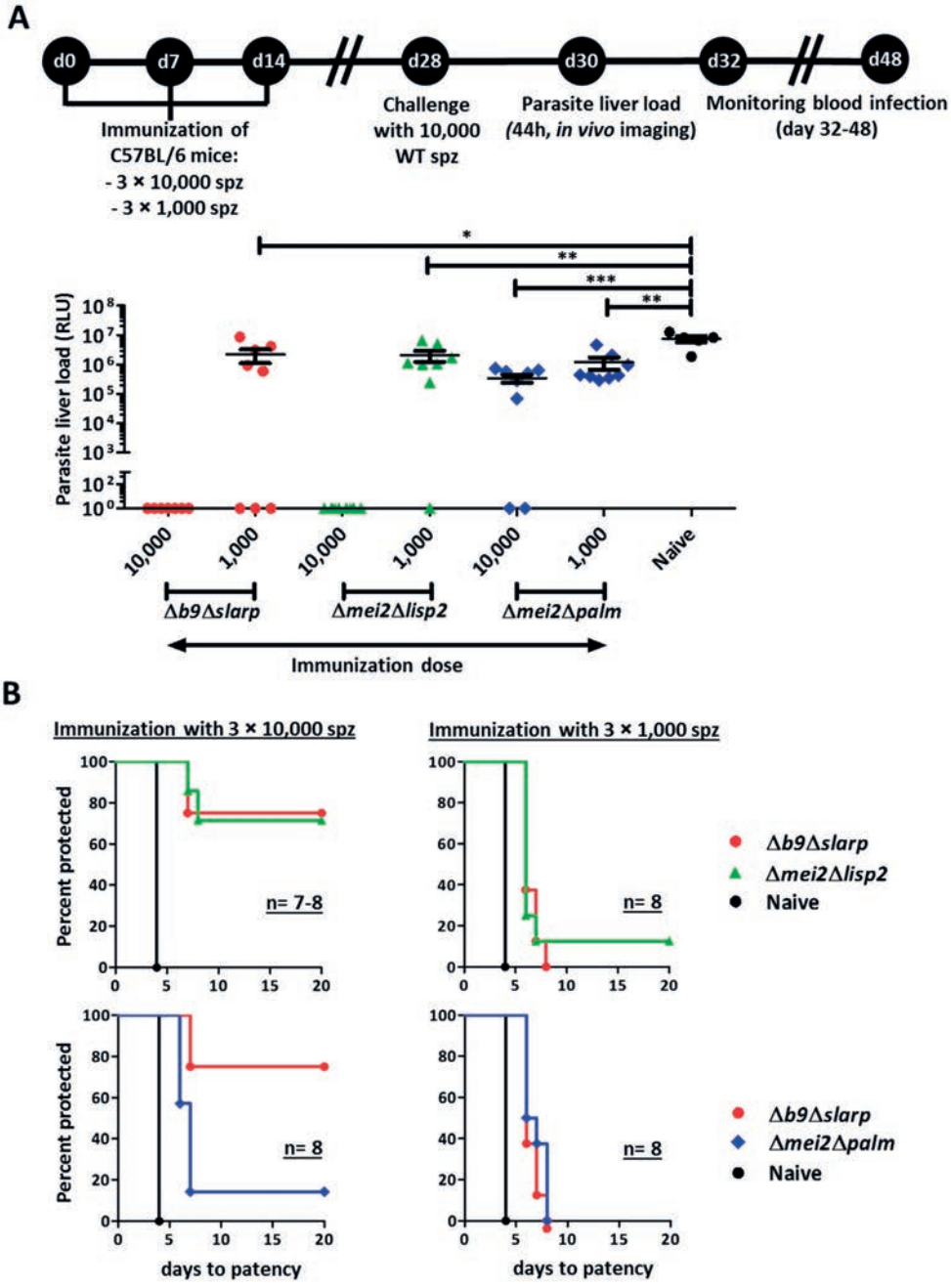
Table 3. Protective immunity after challenge with 10^3 wild type sporozoites of C57BL/6 mice immunized with early-arresting GAP (*Δb9Δslarp*) or LA-GAPs (*Δmei2Δlisp2, Δmei2Δpalm*) sporozoites.

Mouse strain	Parasite line	No. of doses and sporozoite number (× 10^3)	No. of mice patent[a]	No. of days to patency[c]
C57BL/6	Δ*mei2*Δ*lisp2*	3 x 1.0	7/8[b]	4 - 6
		3 x 10	2/7[b]	6 - 8
	Δ*mei2*Δ*palm*	3 x 1.0	8/8[b]	4 - 8
		3 x 10	7/8[b]	6
	Δ*b9*Δ*slarp*	3 x 1.0	8/8[b]	4 - 7
		3 x 10	2/8[b]	6
	Naïve	–	5/5[b]	4

[a] The number of mice that developed a blood stage infection
[b] Mice were challenged with 1 x 10^4 WT sporozoites 21 days last after immunization
[c] The time of blood stage parasitemia between 0.5-2%

mice with blood-stage infections would have been detected. However, while the single-gene deletion mutants lacking either *lisp2*, *lisp1* or *palm* are known to be only partially arrested in the liver [29, 37, 38], it has been reported that *P. yoelii* mutants lacking *mei2* have a very strong attenuation phenotype and blood infections were only observed in mice infected with very high doses of sporozoites [41, 43]. Therefore, it is possible that the deletion of *lisp1* (a gene responsible for parasite release from hepatocytes) in addition to *mei2* (which appears to affect parasite nuclear division and merozoite formation) could have resulted in parasites that are retained within the infected hepatocyte for a longer period of time, allowing for some infectious merozoites to form and give gave rise to the blood-stage infections.

The combination of deleting *mei2* with either *lisp2* or *palm* resulted in mutants that showed the strongest attenuation phenotype. The additive effect on the level of attenuation of combining *lisp2* or *palm* with *mei2* was demonstrated when mice were infected with 2 × 10^5 sporozoites. In these experiments we were unable to detect blood-


A
d0
d7
d14
d28
d30
d32
d48
Immunization of C57BL/6 mice:
- 3 × 10,000 spz
- 3 × 1,000 spz
Challenge with 10,000 WT spz
Parasite liver load (44h, in vivo imaging)
Monitoring blood infection (day 32-48)
Parasite liver load (RLU)
*
**
***
**
10,000
1,000
10,000
1,000
10,000
1,000
Naive
Δb9Δslarp
Δmei2Δlisp2
Δmei2Δpalm
Immunization dose
B
Immunization with 3 × 10,000 spz
Immunization with 3 × 1,000 spz
Percent protected
n= 7-8
n= 8
n= 8
n= 8
Δb9Δslarp
Δmei2Δlisp2
Naive
Δb9Δslarp
Δmei2Δpalm
Naive
days to patency
days to patency

stage infections after infection with either Δ*mei2*Δ*lisp2* or Δ*mei2*Δ*palm,* whereas 30% of the mice established a blood-stage infection when infected with the same number of Δ*mei2* sporozoites. The additive effect on attenuation of combining *lisp2* and *mei2* was also recently reported in *P. yoelii*, where Δ*mei2*Δ*lisp2* infected mice did not establish blood-stage infections, following injection of up to 5 × 10^5 sporozoites, whereas 3 out of 30 mice infected with 2 × 10^5 sporozoites of the *P. yoelii* Δ*mei2* mutant did produce blood-stage infections [43]. The additive effects may be explained by the different roles the three proteins have on the full maturation of parasite liver-stages. MEI2 is predicted to be a RNA binding protein and is located in distinct cytoplasmic structures reminiscent of RNA-storage 'P-bodies' [41]. *P. yoelii* liver-stage schizonts lacking this protein exhibited an abnormal DNA segregation phenotype and failed to form merozoites [41]. PALM is a *Plasmodium* apicoplast protein and its absence in rodent parasites affects merozoite formation in late liver-schizonts, with reduced merozoite segregation and merosome formation [37]. LISP2 has been shown to be specifically expressed during parasite liver-stages and is present in the parasitophorous vacuole (PV) that surround the parasites and also in the cytoplasm of infected hepatocytes. Absence of this protein also results in reduced and delayed merozoite formation, as observed for mutants lacking MEI2 and PALM [59], and although all three proteins affect correct merozoite formation, their distinct cellular localization indicates that may play diverse roles in different biological processes.

Given that *P. yoelii* mutants lacking both *mei2* and *lisp2* [43] did not result in a breakthrough blood infection even after 5 × 10^5 sporozoites, our observed a breakthrough blood infection in one out of ten mice infected with 5 × 10^5 *P. berghei* Δ*mei2*Δ*lisp2* sporozoites was unexpected. However, differences in attenuation phenotype between identical *P. yoelii* and *P. berghei* gene-deletion mutants have been reported in previous studies. For example, *P. yoelli* mutants lacking the gene encoding β-ketoacyl-ACP synthase II gene (*fabb/f*) show a much stronger attenuation phenotype [33] than *P. berghei* mutants lacking the orthologous *fabb/f* gene [34, 56]. Interestingly, while both *P. berghei and P. yoelii* lacking *fabb/f* show varying degrees of attenuation in liver-stage development, *P. falciparum* parasites lacking this gene are unable to complete development in the mosquito [60]. Further, while a complete attenuation phenotype has been reported for *P. yoelii* parasites lacking two genes, *p52* and *p36*, encoding proteins critical for the formation and maintenance of the PV [28], *P. berghei* parasites lacking these genes are not completely attenuated and these mutants can develop blood infections [56]. This discrepancy in attenuation between different *Plasmodium* species was further demonstrated by the observation that *P. falciparum* sporozoites lacking *p52* and *p36* could produce a blood infection in humans [19]. Specifically, in a phase I clinical study where volunteers were exposed to bites of 200 infected mosquitoes, one out of six volunteers became blood-stage positive.

Currently, only two genetically attenuated *P. falciparum* mutants have been generated, informed by studies performed in rodent malaria models, which have advanced into clinical studies. Both of these *P. falciparum* GAPs arrest early after hepatocyte invasion

and consist of 2 or 3 gene-deletions; in these parasites *slarp* has been deleted in combination with either *b9* [26] or with *p52* and *p36* [19]. Recently, a clinical safety study has been performed in human volunteers using the *P. falciparum* GAP lacking *slarp, p52 and p36*. Ten out of ten volunteers exposed to the bites of 150-200 triple gene-deletion GAP infected mosquitoes remained blood-stage negative [18]. The limited number of methods to rapidly and cost-effectively test the attenuation and protective efficacy of *P. falciparum* GAPs limits the direct down-selection of *P. falciparum* GAPs, and presently very few studies have been performed in humans that can confirm the predictive value of rodent models in assessing the attenuation level of GAPs that lack certain genes or gene combinations. Rodent *Plasmodium* parasites take about 2 days to complete liver-stage development while this process takes a week in *P. falciparum*. Thus, the deletion of liver-stage specific genes may result in different levels of attenuation in different *Plasmodium* species. Therefore, while the deletion of *mei2* in combination with either *palm* or *lisp2* results in very strong, but incomplete, attenuation in rodent parasites, additional studies in *P. falciparum* are required to investigate if deletion of the same combination of genes results in complete attenuation in *P. falciparum*.

We have examined the development of the two GAPs with the strongest late-arrest attenuation phenotype, Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm*, and compared the phenotypes to that of an early-arresting GAP (i.e. Δ*b9*Δ*slarp*). The *P. berghei* early-arresting GAP, Δ*b9*Δ*slarp*, has been analysed in different studies [26, 57]. This GAP lacks the expression of the SLARP/SAP1 protein which is involved in gene expression in sporozoites [26, 31] and the B9 protein that is located in the PV, [29]; this GAP aborts development soon after hepatocyte invasion. The *P. berghei* Δ*b9*Δ*slarp* GAP is able to generate strong protective immunity against WT *P. berghei* challenge in both BALB/c and C57BL/6 mice [26]. In the present study we have shown, *in vitro*, that Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* liver-stage parasites develop into large dividing schizonts that express both early and late merozoite proteins, MSP1 and AMA1. Surprisingly, mature schizonts of both mutants develop into significantly larger parasites than WT liver-schizonts. This may suggest that these parasites continue growing and/or replicating aberrantly and may therefore express more antigens than WT parasites at late stages of development. A remarkable difference between the two LA-GAPs is the difference in their persistence in the liver as detected by *in vivo* imaging of luminescence signals, which indicate that Δ*mei2* and Δ*mei2*Δ*palm* parasites may persist longer than Δ*mei2*Δ*lisp2* parasites. Additional studies are required to identify the mechanisms underlying the enlargement of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* liver-schizonts. Despite its enhanced development *in vitro*, the LA-GAP Δ*mei2*Δ*lisp2* cannot be detected *in vivo* in the liver at 56 h post infection, whereas Δ*mei2* and Δ*mei2*Δ*palm* GAPs can still be detected up to 65 h post infection. The faster disappearance of parasites from the liver may result from different factors; Δ*mei2*Δ*lisp2* liver-schizonts may more rapidly rupture *in vivo*, resulting in release of the non-infectious merozoites or the liver-schizonts may disintegrate faster and thereby losing luminescence signals. It is also possible that infected hepatocytes may undergo apoptosis or are more rapidly cleared by immune

factors. Again, additional investigation is required to unravel the mechanisms underlying differences in persistence and clearance of LA-GAPs in the liver.

In one study by Butler *et al.* [12] employing rodent malaria models, it was found that late-arresting GAPs induce stronger protective immune responses compared to early-arresting GAPs. It is presumed that this increase in immunogenicity arises from the greater amount and repertoire of antigens present in LA-GAP compared to early-arresting parasites [12]. It was shown that immunization with late-arresting GAP (LA-GAP) induced greater numbers of a broader range of $CD8^+$ T cells compared to early-arresting parasites. Butler *et al.* compared the immunogenicity of a *P. yoelii* LA-GAP lacking expression of FabB/F with an *P. yoelii* GAP lacking expression of *slarp/sap*1 and with radiation attenuated parasites, both of which arrest early after hepatocyte invasion. The higher levels of protective immunity induced by LA-GAP were observed in both BALB/c, Swiss Webster and C56BL/6 mice [12].

Based on the results of the Butler study, we anticipated that immunization of mice with two LA-GAPs generated in this study would also result in significantly enhanced protective immunity compared to the early-arresting GAP, in both BALB/c and in C57BL/6 mice. All the more so since the LA-GAPs Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* appear to continue development at late liver-stages that may result in even more antigens per parasite. Therefore, our observations that protective immunity induced after Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* immunization is not significantly different compared to Δ*b9*Δ*slarp* immunization, neither in C57BL/6 nor in BALB/c mice, was highly unexpected.

The difference between our study and the Butler study might be explained by differences between the early-arresting GAPs or the LA-GAPs used in the different studies. However, differences in immunogenicity of the early-arresting GAP seems unlikely, since in both studies the early-arresting GAP lack the *slarp/sap1* gene, which would result in both mutants having a similar arrest phenotype and, likely, antigen profile. With respect to differences in the LA-GAP used, we do show that the Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* mutants develop in very large schizonts expressing proteins that include proteins expressed late into liver-stage development. In the Butler study an LA-GAP was used that lacks the *fabb/f* gene; rodent parasites lacking this gene arrest during the maturation of liver-schizonts [33, 56] and it has not been reported whether these parasites are enlarged or persist longer in mouse livers than WT parasites. It is therefore surprising that Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* do not induce better protective immune responses than early-arresting parasites, since they are likely to express the same (if not more) antigens as those found in parasites lacking *fabb/f*.

Another possible explanation for the differences in immunogenicity between LA-GAP and early-arresting GAP observed in ours and the Butler study, is the use of GAPs that are generated in different rodent *Plasmodium* species. We used *P. berghei* to create our GAPs whereas Butler *et al.* generated the GAP lacking *fabb/f* in *P. yoelii*. Differences in T- or B-cell epitopes of antigens of these two species may influence the immunogenicity of parasites in different mouse strains. Moreover, it has been shown that these two rodent *Plasmodium* species differentially regulate key immune-effector pathways resulting in

differences in memory $CD8^+$ T cell-mediated immunity against liver-stage antigens [61]. It is also interesting to note that we observed differences in immunogenicity of our LA-GAPs in BALB/c mice in two different laboratories. Specifically, while in the Leiden laboratory were able to generate full protection in BALB/c mice after immunization with a single dose of 800 LA-GAPs sporozoites, none of the BALB/c mice were protected using the same LA-GAP dose in the Lisbon laboratory. In both laboratory the same BALB/c mice were used (BALB/c ByJ strain obtained from Charles River, France).

Combined, our observations indicate that (1) additional gene-deletion GAPs may need to be screened to identify GAPs that completely arrest during late liver-stage development and (2) the hypothesis that the greater the amount and diversity of antigens in a LA-GAPs the more likely it is to generate stronger protective immunity, requires reconsideration. Additional studies are required to define what immune mechanisms contribute to the induction, maintenance and deployment of adaptive immune response after LA-GAP immunization and the role of different rodent malaria parasites and different mouse strains in inducing protective immune responses. Using different *Plasmodium* species and employing different immunization protocols it might be possible to unveil these processes. These studies have important implications for the development of LA-GAPs for the human parasite *P. falciparum*. However, only by clinically evaluating of both the safety and the immunogenicity of LA-GAP will we be able to draw conclusions on the benefits of using LA-GAP compared to early-arresting GAP for vaccination.

ACKNOWLEDGEMENTS

A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia).

REFERENCES

1. Bijker, E.M., et al., *Novel approaches to whole sporozoite vaccination against malaria.* Vaccine, 2015. **33**(52): p. 7462-8.
2. Richie, T.L., et al., *Progress with Plasmodium falciparum sporozoite (PfSPZ)-based malaria vaccines.* Vaccine, 2015. **33**(52): p. 7452-7461.
3. Hollingdale, M.R. and M. Sedegah, *Development of whole sporozoite malaria vaccines.* Expert Review of Vaccines, 2017. **16**(1): p. 45-54.
4. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites.* Journal of Infectious Diseases, 2002. **185**(8): p. 1155-1164.
5. Roestenberg, M., et al., *Protection against a Malaria Challenge by Sporozoite Inoculation.* New England Journal of Medicine, 2009. **361**(5): p. 468-477.
6. Seder, R.A., et al., *Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine.* Science, 2013. **341**(6152): p. 1359-1365.
7. Alger, N.E. and J. Harant, *Plasmodium-Berghei - Heat-Treated Sporozoite Vaccination of Mice.* Experimental Parasitology, 1976. **40**(2): p. 261-268.
8. Hafalla, J.C.R., et al., *Priming of CD8(+) T cell responses following immunization with heat-killed Plasmodium sporozoites.* European Journal of Immunology, 2006. **36**(5): p. 1179-1186.
9. Sissoko, M.S., et al., *Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.* Lancet Infectious Diseases, 2017. **17**(5): p. 498-509.
10. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination (vol 22, pg 614, 2016).* Nature Medicine, 2016. **22**(6): p. 692-692.
11. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(10): p. 2711-2716.
12. Butler, N.S., et al., *Superior Antimalarial Immunity after Vaccination with Late Liver Stage-Arresting Genetically Attenuated Parasites.* Cell Host & Microbe, 2011. **9**(6): p. 451-462.
13. Kreutzfeld, O., K. Muller, and K. Matuschewski, *Engineering of Genetically Arrested Parasites (GAPs) For a Precision Malaria Vaccine.* Front Cell Infect Microbiol, 2017. **7**: p. 198.
14. Singer, M. and F. Frischknecht, *Time for Genome Editing: Next-Generation Attenuated Malaria Parasites.* Trends Parasitol, 2017. **33**(3): p. 202-213.
15. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Current Opinion in Biotechnology, 2012. **23**(6): p. 908-916.
16. van Schaijk, B.C.L., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.
17. Mikolajczak, S.A., et al., *A Next-generation Genetically Attenuated Plasmodium falciparum Parasite Created by Triple Gene Deletion.* Molecular Therapy, 2014. **22**(9): p. 1707-1715.
18. Kublin, J.G., et al., *Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects.* Sci Transl Med, 2017. **9**(371).
19. Spring, M., et al., *First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers.* Vaccine, 2013. **31**(43): p. 4975-83.
20. Gonzalez-Aseguinolaza, G., et al., *Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by*

malaria vaccines. Journal of Experimental Medicine, 2002. **195**(5): p. 617-624.

21. Li, X.M., et al., *Colocalization of a CD1d-Binding Glycolipid with a Radiation-Attenuated Sporozoite Vaccine in Lymph Node-Resident Dendritic Cells for a Robust Adjuvant Effect.* Journal of Immunology, 2015. **195**(6): p. 2710-2721.
22. Othman, A.S., et al., *OX40 stimulation enhances protective immune responses induced after vaccination with attenuated malaria parasites.* Frontiers in Cellular and Infection Microbiology, 2018. (**In press**).
23. Mordmuller, B., et al., *Sterile protection against human malaria by chemoattenuated PfSPZ vaccine.* Nature, 2017. **542**(7642): p. 445-449.
24. Bijker, E.M., et al., *Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(19): p. 7862-7867.
25. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Curr Opin Biotechnol, 2012. **23**(6): p. 908-16.
26. van Schaijk, B.C., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.
27. Mikolajczak, S.A., et al., *A next-generation genetically attenuated Plasmodium falciparum parasite created by triple gene deletion.* Mol Ther, 2014. **22**(9): p. 1707-15.
28. Labaied, M., 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**(8): p. 3758-68.
29. Annoura, T., et al., *Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development.* Faseb Journal, 2014. **28**(5): p. 2158-2170.
30. Arredondo, S.A. and S.H.I. Kappe, *The s48/45 six-cysteine proteins: mediators of interaction throughout the Plasmodium life cycle.* Int J Parasitol, 2017. **47**(7): p. 409-423.
31. Silvie, O., K. Goetz, and K. Matuschewski, *A sporozoite asparagine-rich protein controls initiation of Plasmodium liver stage development.* Plos Pathogens, 2008. **4**(6).
32. Aly, A.S.I., et al., *SAP1 is a critical post-transcriptional regulator of infectivity in malaria parasite sporozoite stages.* Molecular Microbiology, 2011. **79**(4): p. 929-939.
33. Vaughan, A.M., et al., *Type II fatty acid synthesis is essential only for malaria parasite late liver stage development.* Cellular Microbiology, 2009. **11**(3): p. 506-520.
34. Yu, M., et al., *The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites.* Cell Host Microbe, 2008. **4**(6): p. 567-78.
35. Iwanaga, S., et al., *Identification of an AP2-family Protein That Is Critical for Malaria Liver Stage Development.* Plos One, 2012. **7**(11).
36. Dellibovi-Ragheb, T.A., et al., *Host biotin is required for liver stage development in malaria parasites.* Proceedings of the National Academy of Sciences, 2018.
37. Haussig, J.M., K. Matuschewski, and T.W.A. Kooij, *Inactivation of a Plasmodium apicoplast protein attenuates formation of liver merozoites.* Molecular Microbiology, 2011. **81**(6): p. 1511-1525.
38. Ishino, T., et al., *LISP1 is important for the egress of Plasmodium berghei parasites from liver cells.* Cellular Microbiology, 2009. **11**(9): p. 1329-1339.
39. Kumar, H., et al., *Protective efficacy and safety of liver stage attenuated malaria parasites.* Scientific Reports, 2016. **6**.
40. Sahu, T., et al., *ZIPCO, a putative metal ion transporter, is crucial for Plasmodium liver-stage development.* Embo Molecular Medicine, 2014. **6**(11): p. 1387-1397.

41. Dankwa, D.A., et al., *A Plasmodium yoelii Mei2-Like RNA Binding Protein Is Essential for Completion of Liver Stage Schizogony.* Infection and Immunity, 2016. **84**(5): p. 1336-1345.

42. van Schaijk, B.C.L., et al., *Type II Fatty Acid Biosynthesis Is Essential for Plasmodium falciparum Sporozoite Development in the Midgut of Anopheles Mosquitoes.* Eukaryotic Cell, 2014. **13**(5): p. 550-559.

43. Vaughan, A.M., et al., *A Plasmodium Parasite with Complete Late Liver Stage Arrest Protects against Preerythrocytic and Erythrocytic Stage Infection in Mice.* Infection and Immunity, 2018. **86**(5).

44. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nature Protocols, 2006. **1**(1): p. 346-356.

45. Schwach, F., et al., *PlasmoGEM, a database supporting a community resource for large-scale experimental genetics in malaria parasites.* Nucleic Acids Research, 2015. **43**(D1): p. D1176-D1182.

46. Orr, R.Y., N. Philip, and A.P. Waters, *Improved negative selection protocol for Plasmodium berghei in the rodent malarial model.* Malar J, 2012. **11**: p. 103.

47. Burda, P.C., et al., *A Plasmodium phospholipase is involved in disruption of the liver stage parasitophorous vacuole membrane.* PLoS Pathog, 2015. **11**(3): p. e1004760.

48. Salman, A.M., et al., *Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins*, in *Malaria Vaccines: Methods and Protocols*, A. Vaughan, Editor. 2015, Springer New York: New York, NY. p. 257-286.

49. Annoura, T., et al., *Quantitative Analysis of Plasmodium berghei Liver Stages by Bioluminescence Imaging*, in *Malaria: Methods and Protocols*, R. Ménard, Editor. 2013, Humana Press: Totowa, NJ. p. 429-443.

50. Holder, A.A. and R.R. Freeman, *Biosynthesis and processing of a Plasmodium falciparum schizont antigen recognized by immune serum and a monoclonal antibody.* J Exp Med, 1982. **156**(5): p. 1528-38.

51. Kocken, C.H., et al., *Precise timing of expression of a Plasmodium falciparum-derived transgene in Plasmodium berghei is a critical determinant of subsequent subcellular localization.* J Biol Chem, 1998. **273**(24): p. 15119-24.

52. Prado, M., et al., *Long-term live imaging reveals cytosolic immune responses of host hepatocytes against Plasmodium infection and parasite escape mechanisms.* Autophagy, 2015. **11**(9): p. 1561-1579.

53. Gomes, A.R., et al., *A genome-scale vector resource enables high-throughput reverse genetic screening in a malaria parasite.* Cell Host Microbe, 2015. **17**(3): p. 404-413.

54. Ploemen, I.H., et al., *Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging.* PLoS One, 2009. **4**(11): p. e7881.

55. Kauth, C.W., et al., *The merozoite surface protein 1 complex of human malaria parasite Plasmodium falciparum: interactions and arrangements of subunits.* J Biol Chem, 2003. **278**(25): p. 22257-64.

56. Annoura, T., et al., *Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates.* Vaccine, 2012. **30**(16): p. 2662-70.

57. van der Velden, M., et al., *Protective Efficacy Induced by Genetically Attenuated Mid-to-Late Liver-Stage Arresting Plasmodium berghei Deltamrp2 Parasites.* Am J Trop Med Hyg, 2016. **95**(2): p. 378-82.

58. Jaffe, R.I., G.H. Lowell, and D.M. Gordon, *Differences in susceptibility among mouse strains to infection with Plasmodium berghei (ANKA clone) sporozoites and its relationship to protection by gamma-irradiated sporozoites.* Am J Trop Med Hyg, 1990. **42**(4): p. 309-13.

59. Orito, Y., et al., *Liver-specific protein 2: a Plasmodium protein exported*

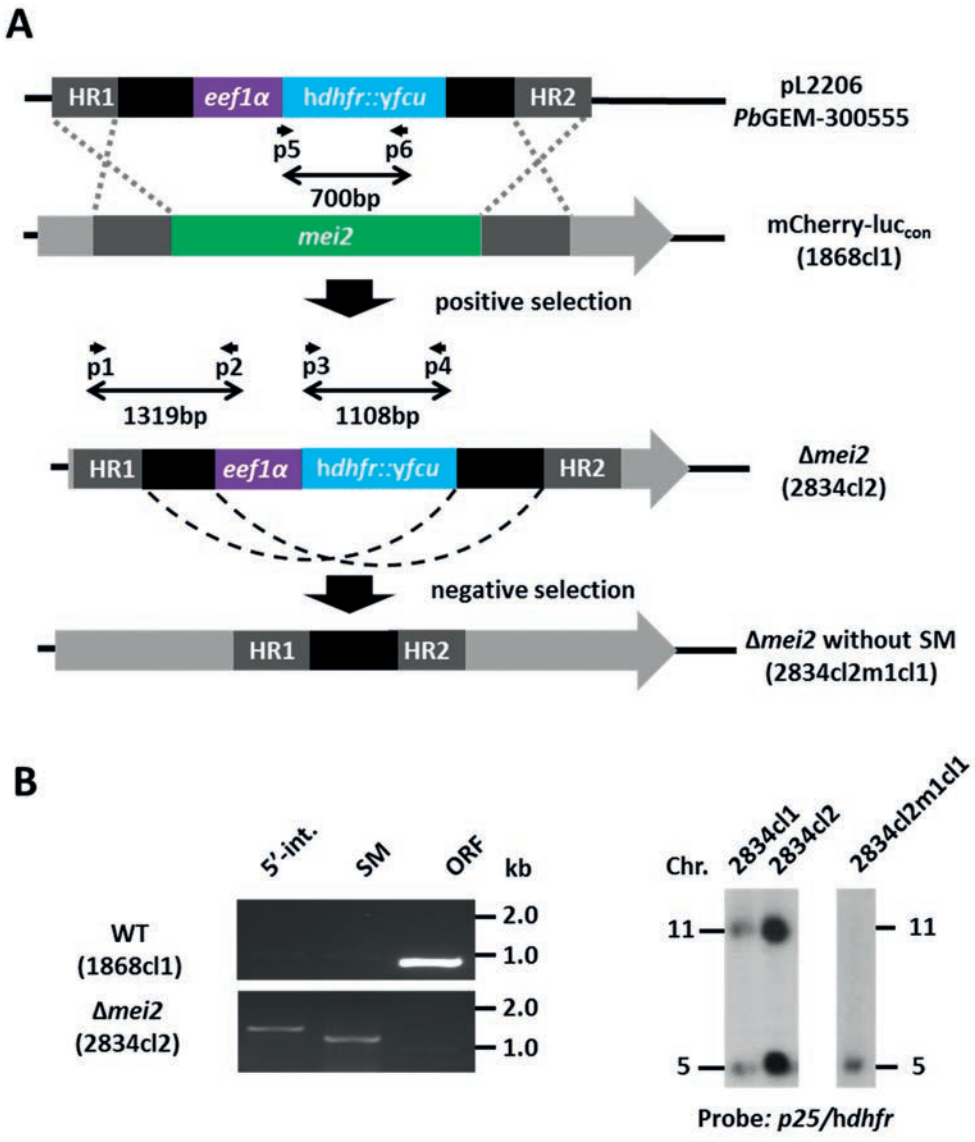
to the hepatocyte cytoplasm and required for merozoite formation. Mol Microbiol, 2013. **87**(1): p. 66-79.

60. van Schaijk, B.C., et al., *Type II fatty acid biosynthesis is essential for Plasmodium falciparum sporozoite development in the midgut of Anopheles mosquitoes.* Eukaryot Cell, 2014. **13**(5): p. 550-9.

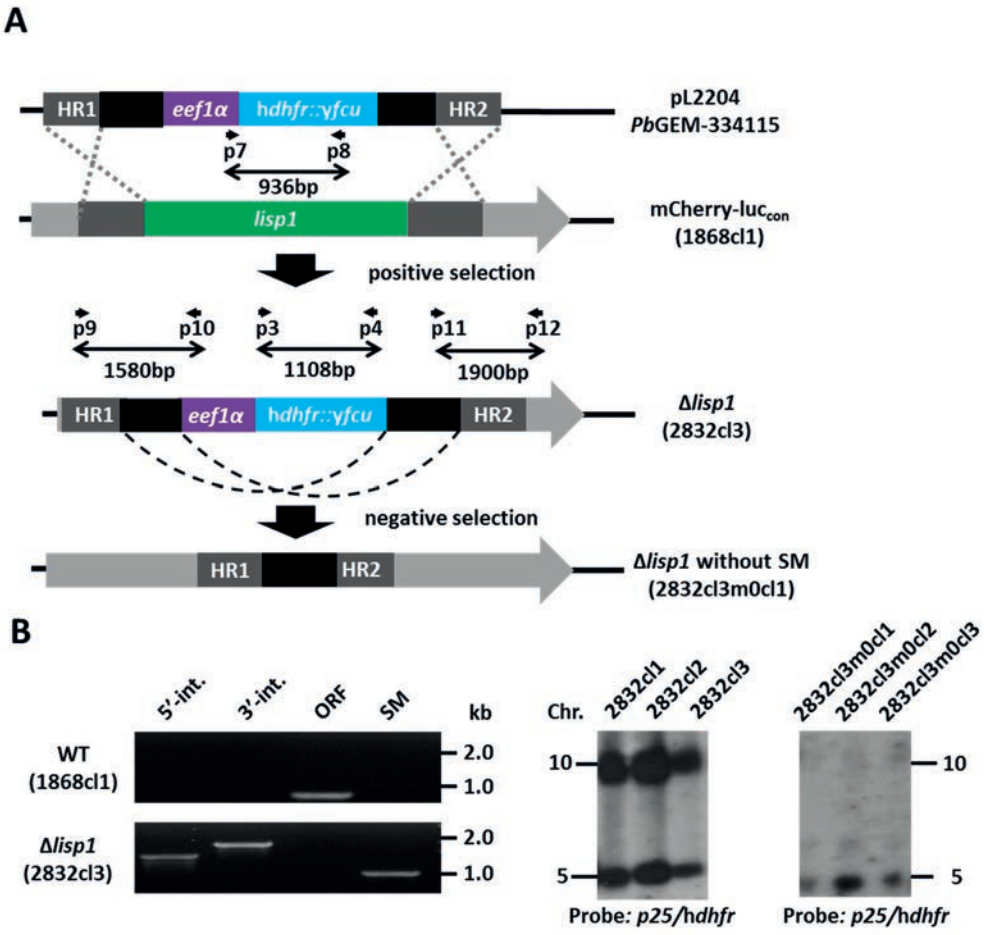
61. Butler, N.S., N.W. Schmidt, and J.T. Harty, *Differential effector pathways regulate memory CD8 T cell immunity against Plasmodium berghei versus P. yoelii sporozoites.* J Immunol, 2010. **184**(5): p. 2528-38.

SUPPLEMENTARY DATA

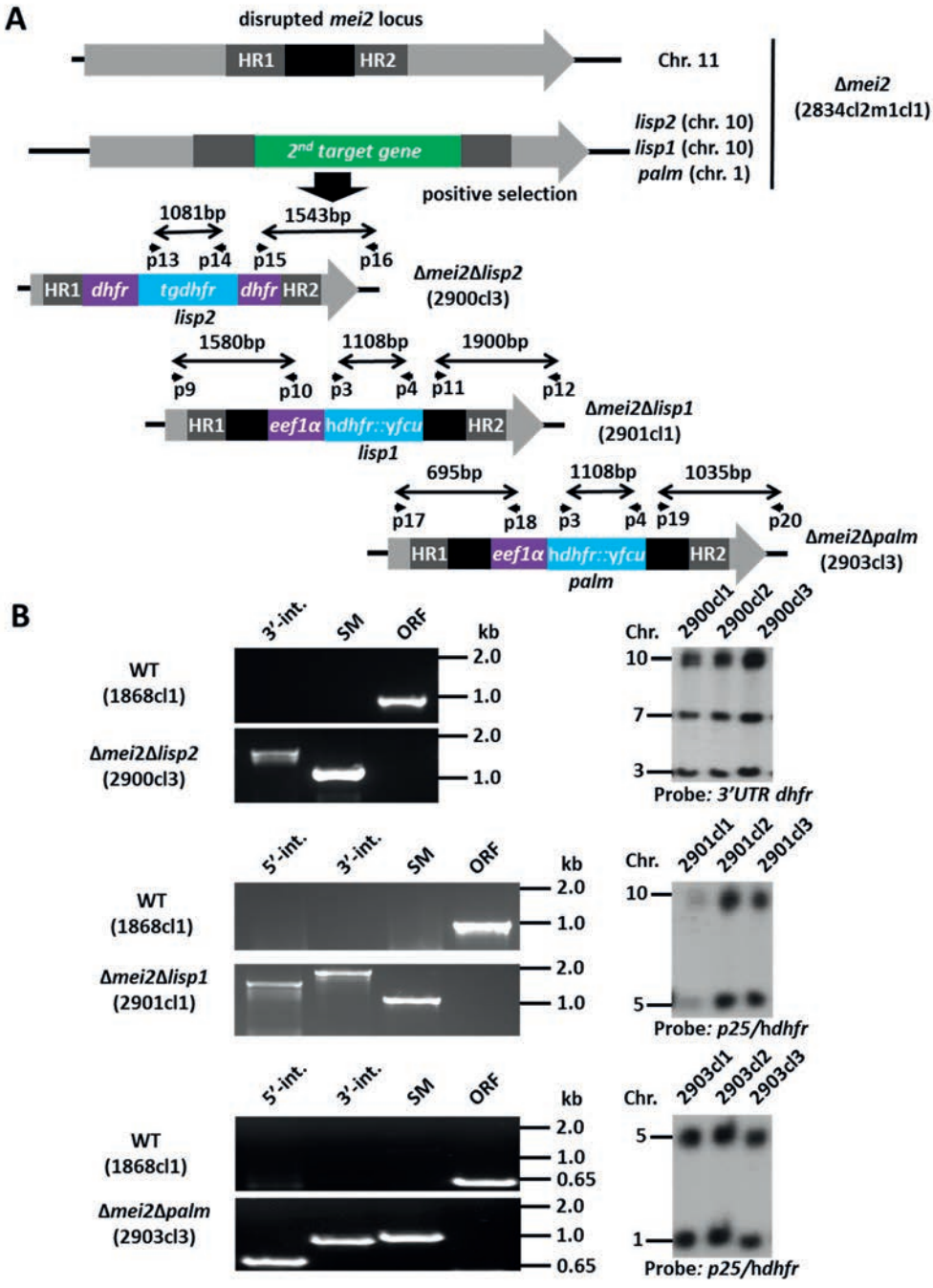
Supplementary Figure S1. Generation and genotype analysis of the selectable marker free *P. berghei* single gene-deletion mutant *Δmei2*. (**A**) Schematic representation of the introduction of the h*dhfr-yfcu* selectable marker (SM) cassette into the Δ*mei2* gene locus of the parent *P. berghei* parasite (line 1868cl1). Construct pL2206 contains the SM flanked by the *eef1*α promoter region and the 3′-UTR of *pbdhfr*. This construct is integrated into the *mei2* locus by double cross-over homologous recombination at the *mei2* homology regions (HR1, HR2). Positive selection with pyrimethamine selects for parasites that have the *mei2* coding sequence replaced by the SM cassette, resulting in parasite line 2834cl2. To remove the SM cassette from the genome of 2834cl2 parasites were selected (negative selection) by treatment of infected mice with 5-Fluorocytosine (5-FC). This treatment selects for parasites that has undergone homologous recombination between the two 3′-UTR of *pbdhfr* untranslated regions present in the integrated constructs pL2206, flanking the SM cassette and thereby removing the SM. This creates the SM free *P. berghei* Δ*mei2* single gene-deletion mutant (2834cl2m1cl1). Location of primers (p) used for PCR analysis and sizes of PCR products are shown. Details of primers are shown **Table S2**. (**B**) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2206 in parasites of line 2834cl2. PCR shows the presence of the h*dhfr::yfcu* SM (primers p3/p4); 5′ integration PCR (5′-int; primers p1/p2); *mei2* open reading frame PCR (ORF; primers p5/p6). Primer locations and product sizes are shown in **A** and primer sequences in **Table S2**. Hybridization of PFG-separated chromosomes (chr.) with a mixture of two probes (the h*dhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette marker in the *mei2* locus on chromosome 11 in two clones of the 2834 line and the absence of the SM in the *mei2* locus on chromosome 11 in SM free Δ*mei2* single gene-deletion mutant (2834cl2m1cl1).


A
HR1
eef1α
hdhfr::yfcu
HR2
pL2206
PbGEM-300555
p5
p6
700bp
mei2
mCherry-luc con
(1868cl1)
positive selection
p1
p2
p3
p4
1319bp
1108bp
HR1
eef1α
hdhfr::yfcu
HR2
Δmei2
(2834cl2)
negative selection
HR1
HR2
Δmei2 without SM
(2834cl2m1cl1)
B
5'-int.
SM
ORF
kb
WT
(1868cl1)
Δmei2
(2834cl2)
2.0
1.0
2.0
1.0
Chr.
2834cl1
2834cl2
2834cl2m1cl1
11
5
11
5
Probe: p25/hdhfr

Supplementary Figure S2. Generation and genotype analysis of the selectable marker free *P. berghei* single gene-deletion mutant *Δlisp1*. (**A**) Schematic representation of the introduction of the h*dhfr-yfcu* selectable marker (SM) cassette into the *lisp1* gene locus of the parent *P. berghei* parasite (line 1868cl1). Construct pL2204 contains the SM flanked by the *eef1*α promoter region and the 3'-UTR of *pbdhfr*. This construct is integrated into the *lisp1* locus by double cross-over homologous recombination at the *lisp1* homology regions (HR1, HR2). Positive selection with pyrimethamine selects for parasites that have the *lisp1* coding sequence replaced by the SM cassette, resulting in parasite line 2832cl3. To remove the SM cassette from the genome of 2834cl2 parasites were selected (negative selection) by treatment of infected mice with 5-Fluorocytosine (5-FC). This treatment selects for parasites that has undergone homologous recombination between the two 3'-UTR of *pbdhfr* untranslated regions present in the integrated constructs pL2204, flanking the SM cassette and thereby removing the SM. This creates the SM free *P. berghei* Δ *lisp1* single gene-deletion mutant (2832cl3m0cl1). Location of primers (p) used for PCR analysis and sizes of PCR products are shown. Details of primers are shown **Table S2**. (**B**) Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2204 in parasites of line 2832cl3. PCR shows the presence of the h*dhfr::yfcu* SM (primers p3/p4); 5' integration PCR (5'-int; primers p1/p2); *lisp1* open reading frame PCR (ORF; primers p5/p6). Primer locations and product sizes are shown in **A** and primer sequences in **Table S2**. Hybridization of PFG-separated chromosomes (chr.) with a mixture of two probes (the h*dhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette marker in the *lisp1* locus on chromosome 10 in three clones of the 2832 line and the absence of the SM in the *lisp1* locus on chromosome 10 in SM free Δ*lisp1* single gene-deletion mutants (2832cl3m0cl1).


A
HR1
eef1α
hdhfr::yfcu
HR2
pL2204
PbGEM-334115
p7
p8
936bp
lisp1
mCherry-luc con
(1868cl1)
positive selection
p9
p10
p3
p4
p11
p12
1580bp
1108bp
1900bp
Δlisp1
(2832cl3)
negative selection
Δlisp1 without SM
(2832cl3m0cl1)
B
5'-int.
3'-int.
ORF
SM
kb
WT
(1868cl1)
Δlisp1
(2832cl3)
2.0
1.0
Chr.
2832cl1
2832cl2
2832cl3
2832cl3m0cl1
2832cl3m0cl2
2832cl3m0cl3
10
5
Probe: p25/hdhfr
Probe: p25/hdhfr

Supplementary Figure S3. Generation and genotype analysis of the double gene-deletion mutants *Δmei2Δlisp1, Δmei2Δlisp2* and *Δmei2Δpalm*. **(A)** Schematic representation of the introduction of the selectable marker (SM) cassettes (h*dhfr::yfcu* or *tgdhfr*) into the *lisp2*, *lisp1* and *palm* gene loci of the single-gene deletion mutant Δ*mei2* (2834cl2m1cl1). To delete the *lisp2*, *lisp1* and *palm* genes, the gene-deletion constructs pL1462, pL2204, and pL2205 were used, respectively. pL2204 and pL2205 constructs contain the h*dhfr::yfcu* SM flanked by the *eef1α* promoter region and the 3'-UTR of *pbdhfr* and pL1462 contains the *dhfr* gene of *Toxoplasma gondii* flanked by the *pbdhfr* promoter region and the 3'-UTR of *pbdhfr*. These constructs integrate by double cross-over homologous recombination at the homology regions (HR1, HR2) of *lisp2*, *lisp1* and *palm*. Positive selection with pyrimethamine selects for parasites that have the *lisp2*, *lisp1* and *palm* coding sequences replaced by the SM cassettes of the different constructs, resulting in parasite lines 2900cl3 (*Δmei2Δlisp2)*, 2901cl1 (*Δmei2Δlisp1*) and 2903cl3 (*Δmei2Δpalm*), respectively. Location of primers (p) used for PCR analysis and sizes of PCR products are shown. Details of primers are shown **Table S2**. chr. – chromosomes. **(B)** Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of constructs pL2204, pL2205 and pL1462 in parasites of Δ*mei2* (2834cl2m1cl1). PCR shows the presence of the *tgdhfr* SM (primers p13/p14); the h*dhfr::yfcu* SM (primers p3/p4); 3' integration of *lisp2* PCR (3'-int; primers p15/p16); 5' integration of *lisp1* PCR (5'-int; primers p9/p10); 3' integration of *lisp1* PCR (3'-int; primers p11/p12); 5' integration of *palm* PCR (5'-int; primers p17/p18); 3' integration of *palm* PCR (3'-int; primers p19/p20). Primer locations and product sizes are shown in **A** and primer sequences in **Table S2**. Hybridization of PFG-separated chromosomes with a mixture of two probes (the h*dhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette in the *lisp1* and *palm* locus on chromosome 10 and 1 in three clones of the 2901 and 2903 lines, respectively. For the three clones of line 2900, hybridization with the 3'-UTR *pbdhfr* probe recognizes the integrated construct on chromosome 10, the reporter mCherry-Luc_{con} construct on chromosome 3, and the endogenous *dhfr* gene located on chromosome 7. chr. – chromosomes.

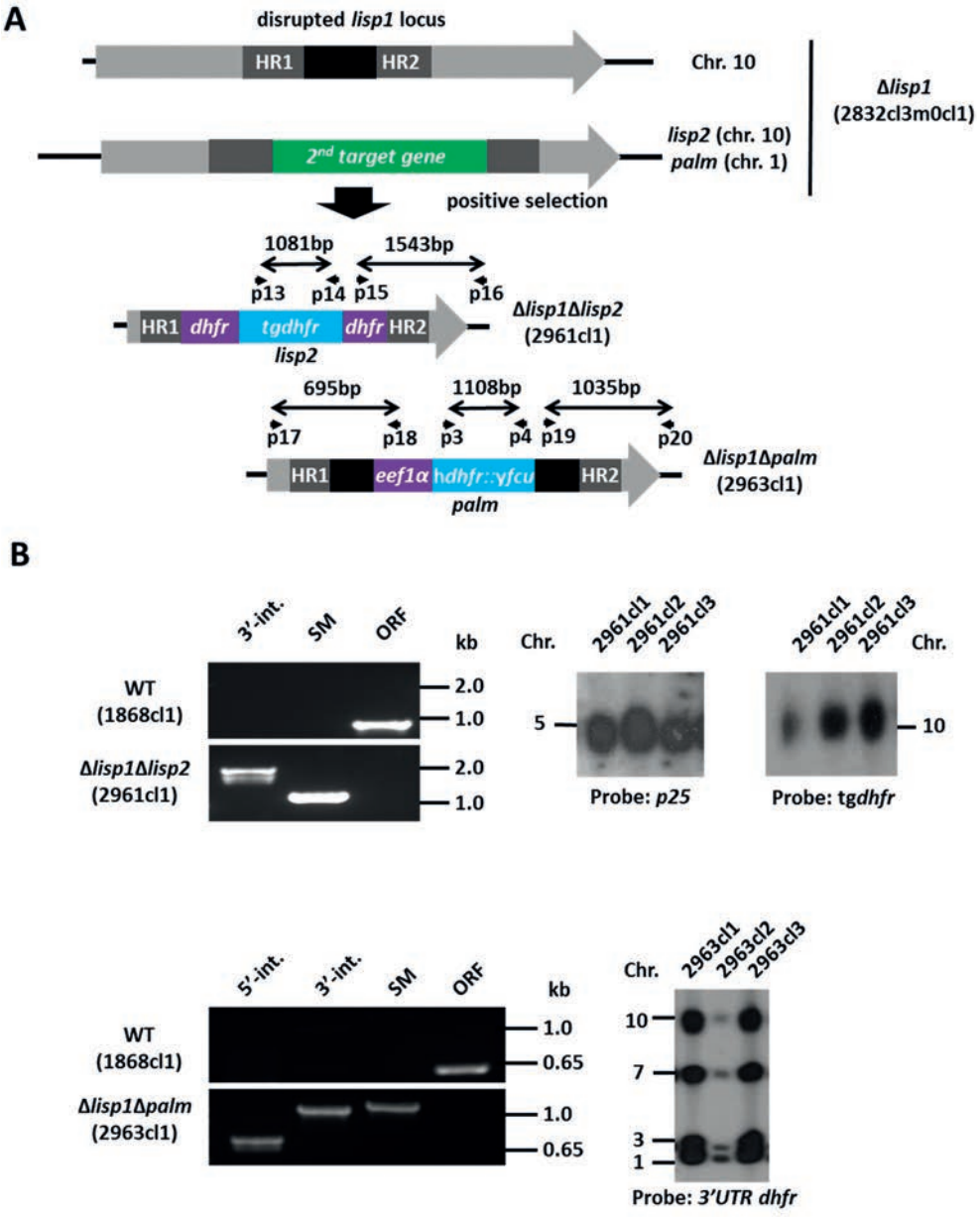

A
disrupted mei2 locus
HR1
HR2
Chr. 11
Δmei2
(2834cl2m1cl1)
2nd target gene
lisp2 (chr. 10)
lisp1 (chr. 10)
palm (chr. 1)
positive selection
1081bp
1543bp
p13
p14
p15
p16
HR1 dhfr tgdhfr dhfr HR2
lisp2
Δmei2Δlisp2
(2900cl3)
1580bp
1108bp
1900bp
p9
p10
p3
p4
p11
p12
HR1 eef1α hdhfr::yfcu HR2
lisp1
Δmei2Δlisp1
(2901cl1)
695bp
1108bp
1035bp
p17
p18
p3
p4
p19
p20
HR1 eef1α hdhfr::yfcu HR2
palm
Δmei2Δpalm
(2903cl3)
B
3'-int.
SM
ORF
kb
WT
(1868cl1)
Δmei2Δlisp2
(2900cl3)
2.0
1.0
Chr.
2900cl1
2900cl2
2900cl3
10
7
3
Probe: 3'UTR dhfr
5'-int.
3'-int.
SM
ORF
kb
WT
(1868cl1)
Δmei2Δlisp1
(2901cl1)
2.0
1.0
Chr.
2901cl1
2901cl2
2901cl3
10
5
Probe: p25/hdhfr
5'-int.
3'-int.
SM
ORF
kb
WT
(1868cl1)
Δmei2Δpalm
(2903cl3)
2.0
1.0
0.65
Chr.
2903cl1
2903cl2
2903cl3
5
1
Probe: p25/hdhfr

Supplementary Figure S4. Generation and genotype analysis of the double gene-deletion mutants *Δlisp1Δlisp2*, and *Δlisp1Δpalm*. **(A)** Schematic representation of the introduction of the selectable marker (SM) cassettes (h*dhfr::yfcu* or *tgdhfr*) into the *lisp2* and *palm* gene loci of the single-gene deletion mutant Δ*lisp1* (2832cl3m0cl1). To delete the *lisp2* and *palm* genes, the gene-deletion constructs pL1462 and pL2205 were used, respectively. Construct pL1462 contains as SM the *dhfr* gene of *Toxoplasma gondii* flanked by the *pbdhfr* promoter region and the 3'-UTR of *pbdhfr*. Construct pL2205 contains the h*dhfr::yfcu* SM flanked by the *eef1*α promoter region and the 3'-UTR of *pbdhfr*. These constructs integrate by double cross-over homologous recombination at homology regions (HR1, HR2) of *lisp2* and *palm*. Positive selection with pyrimethamine selects for parasites that have the *lisp2* and *palm* coding sequences replaced by the SM cassettes, resulting in parasite lines 2961cl1 (Δ*lisp1*Δ*lisp2*) and 2963cl1 (*Δlisp1Δpalm*), respectively. Location of primers (p) used for PCR analysis and sizes of PCR products are shown. Details of primers are shown **Table S2**. chr. – chromosomes. **(B)** Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of constructs pL1462 and pL2205 in parasites of Δ*lisp1* (2832cl3m0cl1). PCR shows the presence of the *tgdhfr* SM (primers p13/p14); h*dhfr::yfcu* SM (primers p3/p4); (3' integration of *lisp2* PCR (3'-int; primers p15/p16); 5' integration of *palm* PCR (5'-int; primers p17/p18); 3' integration of *palm* PCR (3'-int; primers p19/p20). Primer locations and product sizes are shown in **A** and primer sequences in **Table S2**. Hybridization of PFG-separated chromosomes with a mixture of two probes (the h*dhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the presence of the SM cassette marker in the *lisp2* locus on chromosome 10 in three clones of the 2961 line. For the three clones of 2963 line, hybridization with the 3'-UTR *pbdhfr* probe recognizes the integrated construct on chromosome 1, the reporter mCherry-Luc_{con} construct on chromosome 3, the endogenous *dhfr* gene located on chromosome 7 and the integrated *lisp1* gene deletion construct after SM excision on chromosome 10.

Supplementary Table S1. Sporozoite production in *A. stephensi* mosquitoes fed with different *P. berghei* gene-deletion mutants

Parasite line / mutant	Sporozoite no. (× 10^3)[a] Mean ± sd
WT	20.8 ± 4.2
Δ*mei2*	15.4 ± 3.4
Δ*mei2*Δ*lisp2*	14,9 ± 7.7
Δ*mei2*Δ*palm*	11 ± 2.9
Δ*mei2*Δ*lisp1*	14 (1 exp.)
Δ*lisp1*	n.d.
Δ*lisp1*Δ*lisp2*	17.3 (1 exp.)
Δ*lisp1*Δ*palm*	27.3(1 exp.)

[a] Mean number of sporozoites per mosquito
WT – wild type; exp. – experiment; n.d. – not done


A
disrupted lisp1 locus
HR1
HR2
Chr. 10
Δlisp1
(2832cl3m0cl1)
lisp2 (chr. 10)
palm (chr. 1)
2nd target gene
positive selection
1081bp
1543bp
p13
p14
p15
p16
HR1
dhfr
tgdhfr
dhfr
HR2
lisp2
Δlisp1Δlisp2
(2961cl1)
695bp
1108bp
1035bp
p17
p18
p3
p4
p19
p20
HR1
eef1α
hdhfr::yfcu
HR2
palm
Δlisp1Δpalm
(2963cl1)
B
3'-int.
SM
ORF
kb
WT
(1868cl1)
Δlisp1Δlisp2
(2961cl1)
2.0
1.0
2.0
1.0
Chr.
2961cl1
2961cl2
2961cl3
5
Probe: p25
Chr.
2961cl1
2961cl2
2961cl3
10
Probe: tgdhfr
5'-int.
3'-int.
SM
ORF
kb
WT
(1868cl1)
Δlisp1Δpalm
(2963cl1)
1.0
0.65
1.0
0.65
Chr.
2963cl1
2963cl2
2963cl3
10
7
3
1
Probe: 3'UTR dhfr

Supplementary Table S2. List of primers used in this study

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
Primers for conformation PCR analysis of Δmei2 (2834cl2)					
p1	8440		ACCGACCTGGTCAAAGAGGCACCAGTTTAAG	1319	Forward 5′ *mei2* integration
p2	7289		TAAAGCACAATATCTAGGATACTAC		Reverse 5′ *mei2* integration
p3	4698		GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
p4	4699		GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
p5	8768	PBANKA_1122300	TAGGCTTATATTGAGGCACAG	700	Forward *mei2* ORF
p6	8769	PBANKA_1122300	GGATGCTCTATTAAGTCCTTATG		Reverse *mei2* ORF
Primers for conformation PCR analysis of Δlisp1 (2832cl3)					
p7	4476	PBANKA_1024600	TAGTGAGTCAGCATCTCGAG	936	Forward *lisp1* ORF
p8	4477	PBANKA_1024600	TCTTCGTCGTGTATACATCCG		Reverse *lisp1* ORF
p9	4682		GCTTACAATAACAATGTATAAGTTGCC	1580	Forward 5′ *lisp1* integration
p10	4494		GGGTTGGGTGACTTTGGTGACAG		Reverse 5′ *lisp1* integration
p11	7289		TAAAGCACAATATCTAGGATACTAC	1900	Forward 3′ *lisp1* integration
p12	8770		GATAATAAACATTGAATATGGGATATTG		Reverse 3′ *lisp1* integration
Primers for conformation PCR analysis of Δmei2 and Δlisp1 double gene-deletion mutants (2900cl3/2901cl1/2903cl3/2961cl1/2963cl1)					
p13	5571		AATGAAGCGACGTATCGACC	1081	Forward *tgdhfr*
p14	5572		TGATGCGTTCCTTGTTGAGG		Reverse *tgdhfr*
p15	5226		CATATAAACACAAATGATGTTTTTTC	1543	Forward 3′ *lisp2* integration
p16	4460		GAAGAAGTATGACCATACGC		Reverse 3′ *lisp2* integration
p17	8774		CAACTCAATGTCACAGAGTTAC	695	Forward 5′ *palm* integration
p18	4494		GGGTTGGGTGACTTTGGTGACAG		Reverse 5′ *palm* integration
p19	7289		TAAAGCACAATATCTAGGATACTAC	1035	Forward 3′ *palm* integration
p20	8775		TCAATGTATATTCGATTGGTTCTC		Reverse 3′ *palm* integration

Supplementary Table S2. (continued)

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
	7041	PBANKA_1003000	CAGATTCGGCTTATCCATCTC	966	Forward *lisp2* ORF
	7040	PBANKA_1003000	CACCATTGATTTGTTCCTCAC		Reverse *lisp2* ORF
	8772	PBANKA_0101100	GCTTTAGCCATCCCGTTTC	535	Forward *palm* ORF
	8773	PBANKA_0101100	ATAACTCCTCTATATCATCGTTGG		Reverse *palm* ORF

h – human; *tg* -*Toxoplasma gondii*

CHAPTER 6

EXPRESSION OF FULL-LENGTH *PLASMODIUM FALCIPARUM* P48/45 IN *P. BERGHEI* BLOOD STAGES: A METHOD TO EXPRESS AND EVALUATE VACCINE ANTIGENS

Ahmad Syibli Othman[1,2], Jing-wen Lin[3], Blandine M Franke-Fayard[1], Hans Kroeze[1], Fiona J.A. van Pul[1], Séverine Chevalley-Maurel[1], Jai Ramesar[1], Catherin Marin-Mogollon[1], Matthijs M. Jore[4], Merribeth J. Morin[5], Carole A. Long[6], Robert Sauerwein[4], Ashley Birkett[5], Kazutoyo Miura[6], Chris J. Janse[1] and Shahid M. Khan[1]*

[1] Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[2] Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[3] Division of Pediatric Infectious Diseases, State Key Laboratory of Biotherapy, West China Second Hospital, Sichuan University and Collaboration Innovation Center, Chengdu, China
[4] Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands
[5] PATH's Malaria Vaccine Initiative, Washington DC, USA
[6] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, NIH, Rockville, Maryland, USA.
* The corresponding author

Molecular and Biochemical Parasitology, 2018; 224 : 44-49

ABSTRACT

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

INTRODUCTION

Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed [1, 2]. This hampers the screening of *Plasmodium* antigens in immunization studies for their suitability as vaccine candidate antigens. Preclinical evaluation of *Plasmodium* antigens often involves immunizing rodents with recombinant *Plasmodium* proteins followed by an examination of induced immune responses, either *in vivo* using rodent models of malaria or *in vitro* by performing functional assays with human malaria parasites incubated with immune sera [3]. Multiple factors contribute to inefficient expression of *Plasmodium* proteins, such as the high AT content of *Plasmodium* genes, large size and often unique protein structure (i.e. encoding repeated stretches of amino acids) and unique post-translational modifications [1, 4]. This is particularly evident for cysteine-rich proteins where correct folding depends on accurate formation of disulfide bridges to form domains specific for *Plasmodium* proteins [5-7]. Transgenic rodent malaria parasites (RMP) expressing human malaria parasite (HMP) proteins are increasingly used to evaluate and rank order candidate malaria vaccines before investing in scalable manufacture to support advancement to clinical testing [3]. Such transgenic RMP have been used in preclinical assays to evaluate vaccine potential of HMP proteins, both *in vivo* where mice are immunized with HMP antigens and subsequently challenged with transgenic RMP expressing the cognate HMP or in *in vitro* assays where immune sera or antibodies are evaluated for inhibition of parasite growth or invasion. Both the functional complementation of RMP genes by the HMP orthologs [3] and analysis of HMP expression using antisera, provide evidence for correct expression of functional HMP proteins in transgenic RMP [8]. Based on these studies, we reasoned that transgenic RMP can be used as expression systems to more efficiently express, screen, validate and down-select HMP antigens as potential novel malaria vaccine candidates [2, 9]. Further, the expression of conformationally-accurate *Plasmodium* proteins could be used to generate epitope-specific monoclonal antibodies, which in turn can be used to better characterize the vaccine antigen. The use of RMP would circumvent many of the above-mentioned problems associated with expression in heterologous expression systems including, but not limited to, peculiarities of post-translational modifications and *Plasmodium*-specific domains involved in protein trafficking and cellular location. As a proof of concept, we generated transgenic *P. berghei* (*Pb*) parasites that express full length Pfs48/45 from *P. falciparum* (*Pf*). The Pfs48/45 protein is expressed in *Plasmodium* gametocytes and gametes [10, 11] and contains multiple cysteine-rich domains with multiple disulfide bonds [12-14]. These constitute distinct conformational B cell epitopes that can be recognized by several monoclonal antibodies some of which have transmission-blocking (TB) activity [15]. Pfs48/45 becomes exposed on the surface of gametes once the parasite is taken up in blood meal by a mosquito and here the antigen can be targeted by antibodies and other components of the blood meal [16]. Expression of Pfs48/45 for TB

immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein [17, 18]. The limited reactivity of recombinant Pfs48/45 with monoclonal antibodies against conformational epitopes of Pfs48/45 has indicated this misfolding [19, 20].

MATERIAL AND METHODS

Experimental animals and parasites

Female OF1 and C57Bl/6 mice (6 to 8 weeks old; Charles River/Janvier) and Wistar rats (HsdCpb:WU; 175-199 gr, Harlan Netherlands BV) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042, 12043). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All experiments were performed in accordance with relevant guidelines and regulations. The following reference lines of the ANKA strain of *P. berghei* (*Pb*) were used in this study: line cl15cy1 (Janse *et al.*, 2006) and line $GIMO_{pbANKA}$ (1596cl1; RMgm-687 in www.pberghei.eu; referred to as *Pb*WT). The $GIMO_{pbANKA}$ (1596cl1) was generated in the cl15cy1 parent line and this line expresses a fusion of a drug resistance gene *hdhfr (human dihydrofolate reductase*) and a drug sensitivity gene *yfcu* (*yeast cytosine deaminase and uridyl phosphoribosyl transferase*), the so called positive-negative selectable marker (SM), constitutively expressed by the *P. berghei eef1*α promoter stably integrated into the *230p* locus [21].

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

To introduce the *Pfs48/45* gene (PF3D7_1346700) into the redundant *p230p* gene locus (PBANKA_0306000) of the *Pb* genome, we generated DNA construct pL1706. The basic gene insertion construct pL0046 was used, which contains the 5' and 3' *230p* targeting regions, the *tgdhfr/ts* selectable marker (SM) cassette and an mCherry expression cassette under the control of the *eef1*α promoter with 3' terminal sequence of *pbdhfr/ts*. The *eef1*α promoter was replaced by the *msp1* promoter (PBANKA_0831000) using *Afl*II and *Bam*HI digestion. The *msp1* promoter was amplified from genomic *Pb* ANKA DNA using primers 6145 and 6146. In addition the mCherry coding sequence (CDS) was replaced by the *Pfs48/45* CDS using *Bam*HI and *Sgr*AI digestion. The *Pfs48/45* CDS was amplified from genomic DNA of the *Pf* NF54 strain using primers 5583 and 5584. This resulted in construct pL1706. In order to introduce the expression construct in the genome of the parent GIMO *Pb*ANKA line (1596cl1) , we next removed the *tgdhfr/ts* SM by digestion of the plasmid with *Sbf*I and *Afl*II. The ends of the linearized constructs were then rendered blunt using Klenow enzyme treatment, and re-ligated. This final construct (pL1707) were analyzed via restriction digestions to confirm correct assembly. Before transfection, the construct pL1707 was linearized by digesting the plasmid with *Ksp*I.

Parasites of line 1596cl1 were transfected with this construct (exp. 1807) using standard transfection technologies and transformed parasites selected by negative selection with 5-fluorocytosine (5-FC) [21, 22]. Selected parasites were cloned by limiting dilution. Three independent clones have been obtained after the cloning and correct integration of the construct was confirmed by Southern Analysis of PFG-separated chromosomes (data not shown). Mutant 1807cl2 was used for further genotype and phenotype analysis. Correct integration of the construct into the *p230p* gene locus was performed by diagnostic PCR-analysis and Southern analysis of pulsed field gel (PFG) separated chromosomes as described previously [23]. For Southern analysis, PFG-separated chromosomes were hybridized to a mixture of two probes, one recognizing *hdhfr* and one control probe, recognizing the *p25* gene on chromosome 5 [22].

Western and IFA analyses of Pfs48/45 expression

Transgenic schizonts were obtained from short-term overnight cultures of infected blood obtained by cardiac puncture from rats or mice as previously described [24]. Leucocytes were removed from the infected blood using Plasmodipur filters before the parasites were put into short-term overnight culture. Schizonts from the short-term cultures were purified using Nycodenz gradient centrifugation, resulting in parasite populations consisting of >90% schizonts [24].

For Western analysis, purified schizont preparations were extracted in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation at 13,000 g for 5 min at room temperature (RT) and the supernatant was used for Western analysis [15]. Parasite proteins were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) for 2 h at 200 mAh. Membranes were blocked for non-specific binding in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) containing 3% skim milk (Elk, Campina, The Netherlands) overnight at 4°C. Blots were hybridized with 4 anti-*Pf*s48/45 monoclonal antibodies that recognize *Pf*s48/45 epitopes I, IIb, III and V (antibodies 85RF45.1, 85RF45.2b, 85RF45.3, 85RF45.5) [14]. One microgram of protein was loaded in each lane and for reduced reaction, the DTT was added at final concentration of 10 mM [25]. After incubation with the monoclonal antibodies the membranes were washed with PBST and incubated for 1 hour at RT with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG secondary antibody (Sigma-Aldrich) and developed in Amersham ECL Western Blotting Detection Kit according to the manufacturer's instructions (GE Healthcare). As a loading control, the membranes were also incubated with rabbit anti-*P. yoelii* MSP1 antibody [26], followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (GE Healthcare).

The amount of *Pfs48/45* protein in total schizont extract was estimated by quantitative Western blot analysis. Protein extracts of the schizont and gametocyte lysates (see above) and R0.10C recombinant protein were quantified using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Protein extracts (500 ng) were loaded on the SDS-PAGE gel and

a serial dilution series (50, 25, 12.5, 6 and 3 ng) of the recombinant *P. falciparum* P48/45 fused to GLURP R0 domain (R0.10C) was loaded on the gel. Proteins were separated by electrophoresis and transferred to nitrocellulose membrane as described above and the blot was probed with antibody 85RF45.1 (1:2000 dilution) as the primary antibody. The X-ray film was exposed to the membrane for 30 sec and developed using HQ 350XT X-ray Film Processor. The optical intensity (or Optical Densitometry (OD) values) of the signals were quantified with a BioRad GS-800™ Calibrated Densitometer using Quantity One software (Bio-Rad).

For immunofluorescence analyses (IFA), schizonts-infected red blood cells (RBC) were collected from short-term overnight cultures of infected mice blood described above [24]. The schizont-infected RBC were washed 3 times in PBS and 5 µl of packed cells resuspended in 1 ml PBS. 15-20 µl of this suspension was placed in a well of a 10-well black cell-line diagnostic microscope slide (Thermo Scientific) and allowed to air dry. The slides were fixed with 4% paraformaldehyde in PBS for 30 min and cells were permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The slides were incubated overnight at 4°C with the four different rat anti-*Pf*s48/45 antibodies and rabbit anti-MSP1 antibody (described above) in 10% fetal calf serum in PBS, washed 3 times with PBS at RT, followed by incubation for 1 hour with secondary conjugated antibodies anti-rabbit IgG Alexa Fluor®488 (Invitrogen) or anti-rat IgG Alexa Fluor®594 (Invitrogen). Nuclei were stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10 µM (Sigma, The Netherlands) for 30 min at RT. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed onto of the cells and sealed with nail polish. Stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa: 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain).

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

For generation of the schizont-extracts for immunization, 10 Wistar rats were infected with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 parasites and at a parasitemia of 1-3% infected blood was collected by cardiac puncture. Leucocyte removal, short-term overnight culture of infected RBC and Nycodenz gradient purification of schizonts was performed as described above. Purified schizont-infected RBC were divided into samples containing 1.1×10^9 schizont-infected RBC cells. The cells were pelleted by centrifugation (450 *g*, 8 minutes) and stored at −80°C after removal of the supernatant.

Groups of 10 C57BL/6 mice were immunized with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 schizonts by intravenous injection of 1×10^8 schizont-infected red blood cells (in 200 µl RPMI). Mice were immunized a total of four times, at 2 week intervals. Before injection, schizonts were subjected to 3 freeze (dry ice) - thaw (RT) cycles, to ensure parasites were killed. Fourteen days after the last immunization blood was collected from all mice by cardiac puncture and serum collected after centrifugation (1500×g for 10

min). Serum was stored at −20°C until further analysis. IgG was purified from the pooled serum from 10 mice, by protein G affinity chromatography (Pierce, Rockford, IL) according to the manufacturer's instructions and adjusted to a final concentration of 4 mg/ml in phosphate-buffered saline (PBS).

Standard Membrane Feeding Assay (SMFA)

IgG purified from sera obtained from immunized mice was assessed for transmission reducing (TR) activity in SMFA as previously described [27, 28] using *P. falciparum* (*Pf*) gametocytes. Briefly, *Pf* gametocyte cultures (16 to 18 days old) of *P. falciparum* NF54 (originally provided by Steve Hoffman, Sanaria, Rockville, MD) were adjusted to 0.15 to 0.2% stage V gametocytemia at 50% hematocrit. Sixty microliters of a test sample (with a defined concentration of purified mouse IgG) in 1x PBS was mixed with 100 µl of the gametocyte mixture, and the final mixture was immediately fed to 50 female *Anopheles stephensi* (Nijmegen strain, 3 to 6 days old) mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected (20 per sample) to count the number of oocysts. As assay controls both malaria-naïve human sera and an anti-Pf25 monoclonal antibody (4B7; [29]) were used to establish background and complete inhibition of oocyst formation, respectively. Significance of inhibition (% inhibition in oocyst intensity) was determined by the zero-inflated negative binomial model described previously [28].

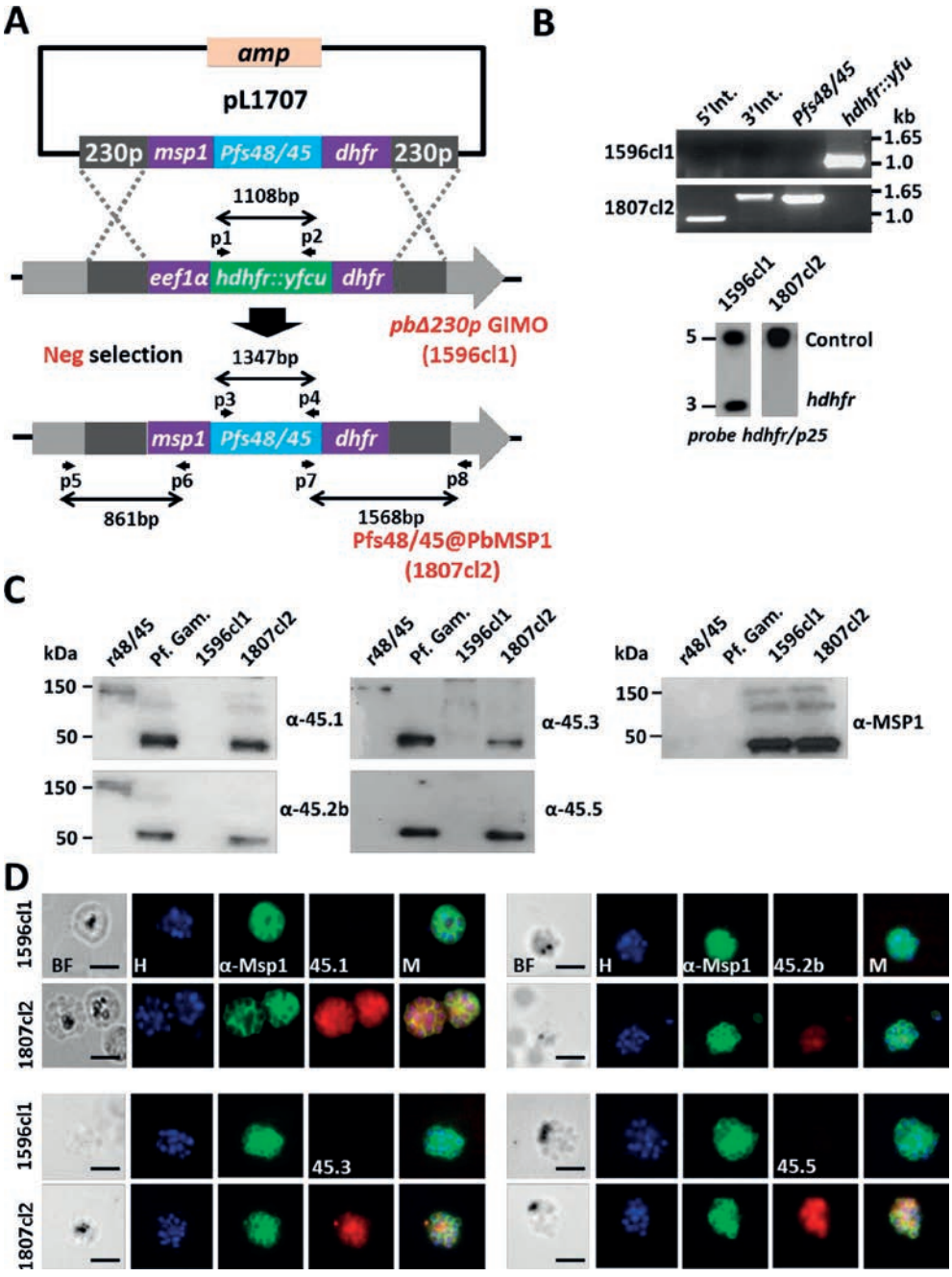
RESULTS AND DISCUSSION

In this study the coding sequence of the gene encoding *Pfs48/45* (PF3D7_1346700) was introduced into the redundant *P. berghei p230p* gene locus (PBANKA_0306000) [3, 21, 30]. The *Pfs48/45* gene was placed under control of 1.3 kb of the promoter region of the schizont-specific *Pb msp1* gene (PBANKA_0831000). This promoter was chosen since *msp1* is one of the highest transcribed genes in developing *Pb* schizonts [31] and the *Pb* schizont stage can be easily produced and purified in large quantities [24]. The transgenic parasite (Pfs48/45@PbMSP1) was generated by the method of GIMO transfection and selection [21]. Using this method transgenes can be rapidly introduced into the *p230p* gene locus in a $GIMO_{pbANKA}$ parent line by replacing the positive-negative selectable marker expression cassette by the transgene expression cassette (**Supplementary M&M** and **Figure 1A**). Correct replacement of the selectable marker cassette and insertion of the Pfs48/45 expression cassette in a cloned line of Pfs48/45@PbMSP1 (1807cl2) was confirmed by diagnostic PCR and Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (**Figure 1B**). Analysis of the growth rate of transgenic Pfs48/45@PbMSP1 parasites during the cloning period demonstrated normal growth of blood stages, comparable to wild type (WT) *Pb*ANKA parasites (i.e. all mice (n=3) achieved a 0.5-2% parasitemia on day 8, after inoculation with a single infected red blood cell). To obtain transgenic schizonts, parasites were cultured overnight using standard methods to produce and purify *Pb* schizonts (**Supplementary M&M**).

Figure 1. Generation, genotype and phenotype analyses of Pfs48/45@PbMSP1, a transgenic *P. berghei* parasite expressing *P. falciparum* P48/45 in schizonts. (A) Schematic representation of the introduction of the *Pfs48/45*-expression cassette into the $GIMO_{pbANKA}$ parasite (line 1596cl1). Construct pL1707 contains the *Pfs48/45* gene flanked by the *msp1* promoter region and the *3' pbdhfr UTR*. This construct is integrated into the modified *P. berghei 230p* locus of $GIMO_{pbANKA}$ that contains the *hdhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination at the homology regions (*230p*; grey boxes). Negative selection with 5-FC selects for parasites that have the *SM* cassette replaced by the *Pfs48/45* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (upper panel) and Southern analysis of PFG-separated chromosomes (lower panel) confirm correct integration of construct pL1707 in line 1807cl2 parasites. PCR shows the absence of the *hdhfr::yfcu* marker and the presence of the *Pfs48/45*. 5' integration PCR (5' int; primers p5/p6), 3' integration PCR (3' int; primers p7/p8), *hdhfr::yfcu* (primers p1/p2), *Pfs48/45* (primers p3/p4). Primer locations and product sizes are shown in A and primer sequences in Table S1). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker in the *230p* locus on chromosome 3 in 1807cl2 parasites. (C) Western analysis of Pfs48/45 expression in protein extracts of purified gametocytes of *P. falciparum* (Pf Gam), purified schizonts of wild type *P. berghei* (1596cl1) and purified schizonts of Pfs48/45@PbMSP1 (1807cl2). As a positive control, recombinant *P. falciparum* P48/45 fragment fused to GLURP R0 domain (R0.10C) was included (expected molecular size is 150 kDa). Blots were stained with 4 different anti-*Pf*s48/45 antibodies (45.1-3, 45.5) that recognize different epitopes. Anti-*Py*MSP1 antibody staining was used as a loading control. (D) Immuno-fluorescence analyses of Pfs48/45 expression in purified schizonts of Pfs48/45@PbMSP1 (1807cl2), and the reference parent *P. berghei* GIMO line (i.e. WT; 1596cl1). Fixed parasites were stained with four different rat anti-*Pf*s48/45 mAbs (45.1-3, 45.5) and rabbit anti-*Py*MSP1 antibody followed by secondary conjugated antibodies anti-rabbit IgG Alexa Fluor ® 488 (green) or anti-rat IgG Alexa Fluor ® 594 (red). Nuclei stained with the DNA-specific dye Hoechst 33342 (H). All pictures were recorded with the same exposure/gain times; anti-rabbit IgG Alexa Fluor ® 488 (green) 0.7 s; anti-rat IgG Alexa Fluor ® 594 (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). BF: bright field; M: merged. Scale bar: 2 µm.

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

In Western analysis all the antibodies recognize a protein of the expected size (48 kDa) in protein extracts from Pfs48/45@PbMSP1 schizonts and WT *Pf* gametocytes but not in extracts from a *Pb* line that does not express Pfs48/45 (i.e. $GIMO_{pbANKA}$ line 1596cl1). As a positive control, recombinant protein that contains a fragment of *P. falciparum* Pfs48/45 fused to the GLURP R0 domain (R0.10C) was included and as expected a 150 kDa band was present after probing with monoclonal antibodies 45.1, 45.2b and 45.3 but was not present after probing with 45.5 (**Figure 1C**) [32]. We next examined the presence of Pfs48/45 epitopes using the anti-*Pf*s48/45 antibodies by immuno-fluorescence assay (IFA) (**Figure 1D**). All antibodies recognized Pfs48/45 produced in the Pfs48/45@PbMSP1 schizonts and did not react with proteins of WT *Pb* schizonts (**Figure 1C and 1D**). These results demonstrate that transgenic *Pb* schizonts can effectively express full length



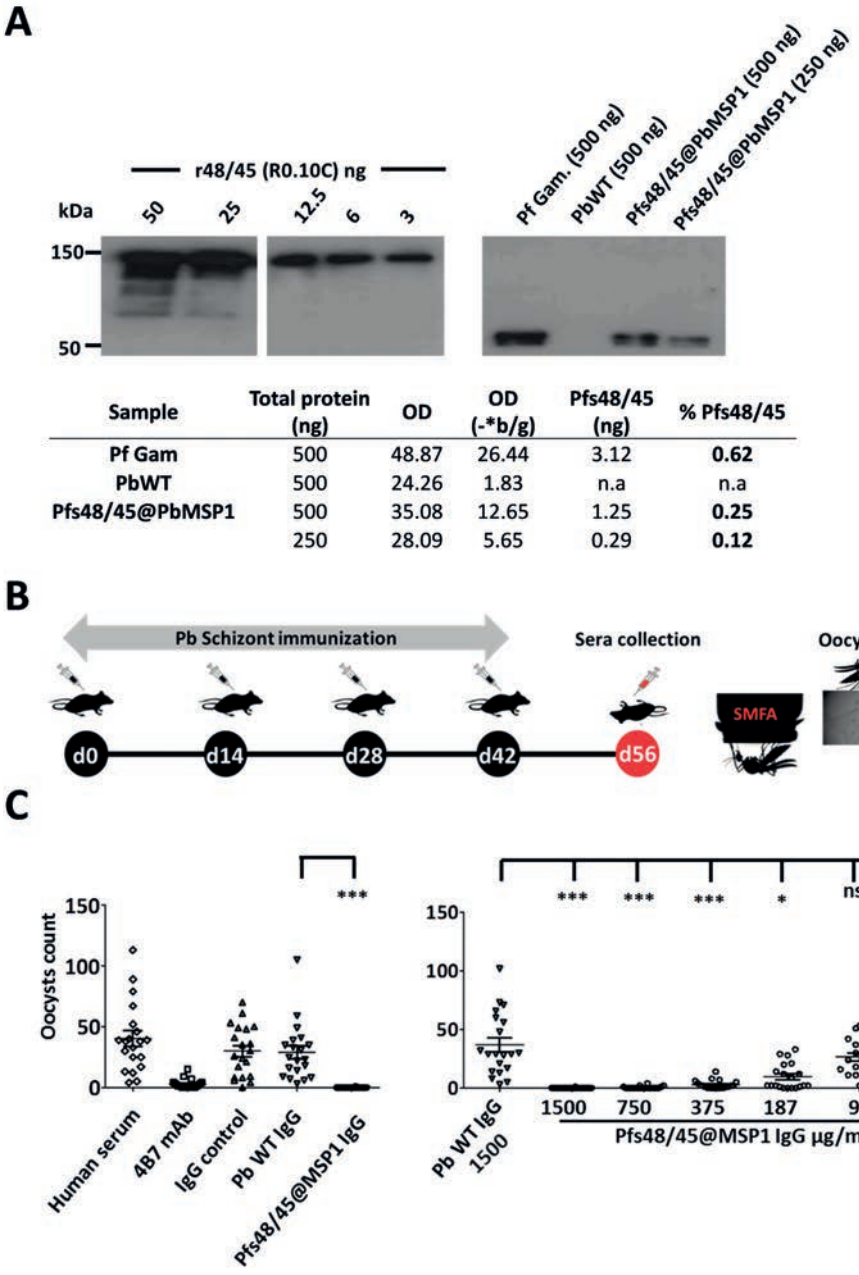
Pfs48/45, which retains a number of conformational epitopes. Pfs48/45, like MSP1, contains a GPI anchor and is present at the plasma membrane of *Pf* gametocytes/gametes [33]. The immuno-fluorescence analyses indicate that Pfs48/45 was located in the cytoplasm of the transgenic merozoites as the fluorescence signals did not completely overlap with

fluorescence signals obtained with anti-MSP1 antibodies, which stain MSP1 at the merozoite plasma membrane in mature schizonts (**Figure 1D**). Possible reasons for this observation is that GPI attachment may be different between rodent and human *Plasmodium* parasites, or that the attachment of Pfs48/45 onto *P. berghei* merozoites would require the presence of other *Plasmodium* proteins normally present in gametocytes/gametes.

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

Next, we examined if Pfs48/45@PbMSP1 transgenic schizont lysate could be used to raise sera that could block *Pf* transmission in mosquitoes, presumably by Pfs48/45 specific antibodies. Two groups of 10 C57BL/6 mice were immunized 4 times (2 week interval) with lysates of $1x10^8$ schizonts of either Pfs48/45@PbMSP1 or WT (c15cy1) parasites (**Figure 2B**). Purified schizonts were inactivated by three rounds of freezing on dry ice followed by thawing at room temperature before immunization and schizont lysates were injected intravenously. Two weeks after the final immunization, serum was collected from all animals and a serum pool made for each group. Total IgG was isolated from the pooled sera and tested for transmission-reducing activity (TR activity) in standard membrane feeding assays (SMFA) using *Pf* gametocytes (**Supplementary M&M**). *Pf* gametocytes were fed to *A. stephensi* mosquitoes in the presence of IgG obtained from mice immunized with

Figure 2. Quantification of Pfs48/45 protein in Pfs48/45@PbMSP1 schizont lysate and transmission reducing (TR) activity of IgG isolated from mice immunized with Pfs48/45@PbMSP1 schizont lysates. **A.** Pfs48/45@PbMSP1 schizont lysates (500 & 250 ng), *P. falciparum* gametocytes (Pf Gam.; 500 ng) and *P. berghei* WT schizont lysate (*Pb*WT; 500 ng) were analyzed in Western blot analysis using anti-*Pf*s48/45 monoclonal 85RF45.1 (1:2000). Densitometry analysis was performed on signals after probing Pfs48/45@PbMSP1 schizont lysate (500 ng) and a dilution series (50, 25, 12.5, 6 and 3 ng) of recombinant Pfs48/45 (r48/45; R0.10C) with antibody 45.1. The Table shows the calculated Pfs48/45 protein content (ng) and the percentage of Pfs48/45 protein in parasite samples; see **Supplementary Figure S1** for determination of Pfs48/45 in samples. *quantification performed after subtraction of background (b/g) Optical Densitometry (OD) values and **quantitation based on regression curve calculations (see **Supplementary Figure S1**). **B.** Timeline showing the immunization of mice with extracts of Pfs48/45@PbMSP1 and *Pb*WT schizont lysates and collection of sera for isolation of IgG that is tested for TR activity in standard membrane feeding assays (SMFA) of *P. falciparum* gametocytes to *Anopheles stephensi* mosquitoes (see **C**). **C.** Left panel: First SMFA with IgGs from mice immunized with purified schizonts of Pfs48/45@PbMSP1 and *Pb*WT. TR activity was determined by the mean number of oocysts 8 days after feeding, and significance of inhibition was determined by the zero-inflated negative binomial model described previously [28]. Right panel: Second SMFA with serially diluted IgGs. IgG from mice immunized with purified schizonts of Pfs48/45@PbMSP1 was titrated resulting in the concentrations shown in the Figure. Significant TR activity was detected until a concentration of 187 µg/ml (*p=0.014). Significant; *$p < 0.05$, ***$p < 0.001$.



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

schizonts of either Pfs48/45@PbMSP1 or WT. In the first experiment, IgG (1500 µg/ml) from Pfs48/45@PbMSP1-immunized mice showed 99.8% inhibition in oocyst density (p=0.001) compared to the IgG obtained from WT immunized (**Figure 2C**). Next, TR activity was determined in SMFA using a dilution series of the IgG obtained from Pfs48/45@PbMSP1-immunized mice. Significant TR activity with IgG from Pfs48/45@PbMSP1-immunized mice

was still observed at a concentration of 187 µg/ml (p=0.014) compared to the control IgG (Figure 2C). The quantitative Western blot analysis (Figure 2A) indicated that is between 0.25-0.12% of the total Pfs48/45@PbMSP1 schizont lysate was Pfs48/45 and therefore it is likely that the majority of the IgG from the immunized mice is not directed against Pfs48/45. The failure to induce TR activity of IgG of mice immunized with WT schizont lysate indicates that the small proportion of anti-*Pf*s48/45 antibodies are mediating the TR activity after Pfs48/45@PbMSP1 schizont lysate immunization. The strong TR activity mediated by the total IgG isolated from Pfs48/45@PbMSP1 immunized mice (Figure 2C), indicates that Pfs48/45 expressed in *P. berghei* can induce antibodies with potent TR activity.

Combined, our proof-of concept studies demonstrate that transgenic *Pb* schizonts can be used as a system to produce a difficult to express HMP protein that is correctly folded and retains conformational epitopes of the native protein. This opens possibilities to use this expression system to evaluate the immunogenicity of other difficult to express antigens or specific domains of these parasites. Studies using sera obtained from mice immunized with *Pf* proteins expressed by transgenic *Pb* parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using blood stage growth inhibition assays (GIA) or sporozoite-antigens using inhibition of sporozoite invasion (ISI) assays [3]. Moreover, the expression in transgenic schizonts of HMP proteins with affinity tags will allow for the purification of these HMP proteins from whole parasite lysate preparations and immunization with purified protein will mean that all of the raised immune response is due to the target antigen and will permit a more detailed analyses of antigen immunogenicity, for example to examine and clone potent inhibitory and cross-reactive B-cells/antibodies after rodent immunization [34]. The creation of transgenic parasites that express antigens from multiple life-cycles that can induce potent immune responses is also of interest to the development of whole organism vaccines [35]. For example, genetically attenuated sporozoite vaccines could be further modified to induce immune responses against multiple life cycle stages by expression in sporozoites and liver stages antigens of blood- or transmission–stages to produce a multi stage-vaccine.

ACKNOWLEDGEMENTS

We would like to thank Dr. Michael Theisen and Dr. Susheel K. Singh, Statens Seum Institute, Copenhagen Denmark, for kindly providing us with the recombinant *P. falciparum* P48/45 protein fragment (R0.10C). A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). The SMFA works were supported in part by the intramural program of the National Institute of Allergy and Infectious Diseases/NIH. This work was funded by PATH's Malaria Vaccine Initiative under Grant No. OPP48782 from the Bill & Melinda Gates Foundation.

REFERENCES

1. Flick, K., et al., *Optimized expression of Plasmodium falciparum erythrocyte membrane protein I domains in Escherichia coli.* Malaria Journal, 2004. **3**.
2. Tuju, J., et al., *Vaccine candidate discovery for the next generation of malaria vaccines.* Immunology, 2017. **152**(2): p. 195-206.
3. Othman, A.S., et al., *The use of transgenic parasites in malaria vaccine research.* Expert Review of Vaccines, 2017. **16**(7): p. 685-697.
4. Ntege, E.H., et al., *Blood-stage malaria vaccines: post-genome strategies for the identification of novel vaccine candidates.* Expert Review of Vaccines, 2017. **16**(8): p. 769-779.
5. Klein, M.M., et al., *The cysteine-rich interdomain region from the highly variable Plasmodium falciparum erythrocyte membrane protein-1 exhibits a conserved structure.* Plos Pathogens, 2008. **4**(9).
6. Thompson, J., et al., *Plasmodium cysteine repeat modular proteins 1-4: complex proteins with roles throughout the malaria parasite life cycle.* Cellular Microbiology, 2007. **9**(6): p. 1466-1480.
7. MacDonald, N.J., et al., *Structural and Immunological Characterization of Recombinant 6-Cysteine Domains of the Plasmodium falciparum Sexual Stage Protein Pfs230.* Journal of Biological Chemistry, 2016. **291**(38): p. 19913-19922.
8. Longley, R.J., et al., *Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates.* Scientific Reports, 2015. **5**.
9. Miguel-Blanco, C., et al., *Hundreds of dual-stage antimalarial molecules discovered by a functional gametocyte screen.* Nature Communications, 2017. **8**.
10. van Dijk, M.R., et al., *A central role for P48/45 in malaria parasite male gamete fertility.* Cell, 2001. **104**(1): p. 153-164.
11. van Dijk, M.R., et al., *Three Members of the 6-cys Protein Family of Plasmodium Play a Role in Gamete Fertility.* Plos Pathogens, 2010. **6**(4).
12. Annoura, T., et al., *Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development.* Faseb Journal, 2014. **28**(5): p. 2158-2170.
13. Mistarz, U.H., et al., *Expression, Purification and Characterization of GMZ2'.10C, a Complex Disulphide-Bonded Fusion Protein Vaccine Candidate against the Asexual and Sexual Life-Stages of the Malaria-Causing Plasmodium falciparum Parasite.* Pharmaceutical Research, 2017. **34**(9): p. 1970-1983.
14. Theisen, M., M.M. Jore, and R. Sauerwein, *Towards clinical development of a Pfs48/45-based transmission blocking malaria vaccine.* Expert Review of Vaccines, 2017. **16**(4): p. 329-336.
15. Outchkourov, N., et al., *Epitope analysis of the malaria surface antigen Pfs48/45 identifies a subdomain that elicits transmission blocking antibodies.* Journal of Biological Chemistry, 2007. **282**(23): p. 17148-17156.
16. Vermeulen, A.N., et al., *Sequential Expression of Antigens on Sexual Stages of Plasmodium-Falciparum Accessible to Transmission-Blocking Antibodies in the Mosquito.* Journal of Experimental Medicine, 1985. **162**(5): p. 1460-1476.
17. Milek, R.L.B., et al., *Plasmodium falciparum: Heterologous synthesis of the transmission-blocking vaccine candidate Pfs48/45 in recombinant vaccinia virus-infected cells.* Experimental Parasitology, 1998. **90**(2): p. 165-174.
18. Milek, R.L.B., H.G. Stunnenberg, and R.N.H. Konings, *Assembly and expression of a synthetic gene encoding the antigen Pfs48/45 of the human malaria parasite Plasmodium falciparum in yeast.* Vaccine, 2000. **18**(14): p. 1402-1411.
19. Milek, R.L.B., et al., *Immunological properties of recombinant proteins of the transmission blocking vaccine candidate, Pfs48/45, of the human malaria parasite Plasmodium*

falciparum produced in Escherichia coli. Parasite Immunology, 1998. **20**(8): p. 377-385.

20. Outchkourov, N.S., et al., *Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(11): p. 4301-4305.

21. Lin, J.W., et al., *A Novel 'Gene Insertion/Marker Out' (GIMO) Method for Transgene Expression and Gene Complementation in Rodent Malaria Parasites.* Plos One, 2011. **6**(12).

22. Salman, A.M., et al., *Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins*, in *Malaria Vaccines: Methods and Protocols*, A. Vaughan, Editor. 2015, Springer New York: New York, NY. p. 257-286.

23. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nature Protocols, 2006. **1**(1): p. 346-356.

24. Janse, C.J. and A.P. Waters, *Plasmodium-Berghei - the Application of Cultivation and Purification Techniques to Molecular Studies of Malaria Parasites.* Parasitology Today, 1995. **11**(4): p. 138-143.

25. Singh, S.K., et al., *A Plasmodium falciparum 48/45 single epitope R0.6C subunit protein elicits high levels of transmission blocking antibodies.* Vaccine, 2015. **33**(16): p. 1981-1986.

26. Holder, A.A. and R.R. Freeman, *Biosynthesis and Processing of a Plasmodium-Falciparum Schizont Antigen Recognized by Immune Serum and a Monoclonal-Antibody.* Journal of Experimental Medicine, 1982. **156**(5): p. 1528-1538.

27. Miura, K., et al., *Functional Comparison of Plasmodium falciparum Transmission-Blocking Vaccine Candidates by the Standard Membrane-Feeding Assay.* Infection and Immunity, 2013. **81**(12): p. 4377-4382.

28. Miura, K., et al., *Transmission-blocking activity is determined by transmission-reducing activity and number of control oocysts in Plasmodium falciparum standard membrane-feeding assay.* Vaccine, 2016. **34**(35): p. 4145-4151.

29. Barr, P.J., et al., *Recombinant Pfs25 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in experimental animals.* J Exp Med, 1991. **174**(5): p. 1203-8.

30. van Dijk, M.R., et al., *Three members of the 6-cys protein family of Plasmodium play a role in gamete fertility.* PLoS Pathog, 2010. **6**(4): p. e1000853.

31. Otto, T.D., et al., *A comprehensive evaluation of rodent malaria parasite genomes and gene expression.* Bmc Biology, 2014. **12**.

32. Theisen, M., et al., *A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages.* Vaccine, 2014. **32**(22): p. 2623-2630.

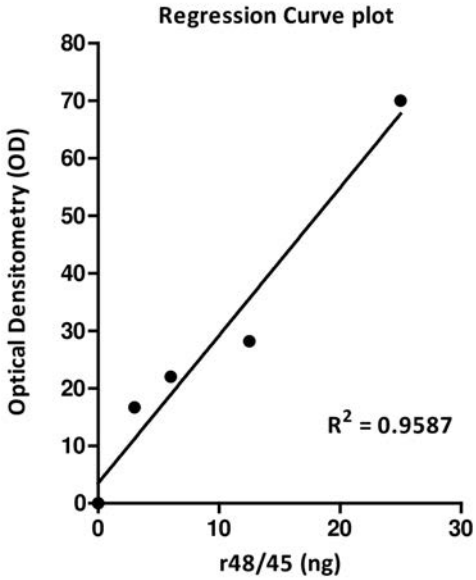
33. Eksi, S., et al., *Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production.* Molecular Microbiology, 2006. **61**(4): p. 991-998.

34. Triller, G., et al., *Natural Parasite Exposure Induces Protective Human Anti-Malarial Antibodies.* Immunity, 2017. **47**(6): p. 1197-+.

35. Bijker, E.M., et al., *Novel approaches to whole sporozoite vaccination against malaria.* Vaccine, 2015. **33**(52): p. 7462-7468.

SUPPLEMENTARY DATA

A



B

Sample	Total protein (ng)	OD	OD (-*b/g)	**Pfs48/45 (ng)	% **Pfs48/45
r48/45 (R0.10C)	50	102.99	80.56	n.a	35
	25	92.45	70.02	n.a	35
	12.5	50.63	28.20	n.a	35
	6	44.43	22.00	n.a	35
	3	39.12	16.68	n.a	35
	0	22.43	0	n.a.	n.a
Pf Gam	500	48.87	26.44	3.12	**0.62**
PbWT	500	24.26	1.83	n.a	n.a
Pfs48/45@PbMSP1	500	35.08	12.65	1.25	**0.25**
	250	28.09	5.65	0.29	**0.12**
***Background**	0	22.43	0.00	n.a	n.a

Supplementary Figure S1. Quantification of Pfs48/45 protein in Pfs48/45@PbMSP1 schizont lysate by Western blot analyses as shown in Figure 2A. Densitometry and regression curve analysis was performed on signals after probing Pfs48/45@PbMSP1 schizont lysate (500 ng) and a dilution series (50, 25, 12.5, 6 and 3 ng) of recombinant Pfs48/45 fusion protein (r48/45; R0.10C) with antibody 45.1. Regression analysis based on the dilution series of recombinant Pfs48/45 fusion (R0.10C). Optical densitometry (OD) values of signals before and after *background (b/g) subtraction and the calculated protein content and the percentage of Pfs48/45 protein of the total amount of protein in the *P. falciparum* gametocyte (*Pf* Gam), P. *berghei* WT schizont (*Pb*WT) and Pfs48/45@*Pb*MSP1 schizont lysates. ** To calculate the amount and percentage of Pfs48/45 in the protein samples we adjusted for the proportion of Pfs48/45 (approximately 35%) in the recombinant protein R0.10C (a GLURP::Pfs48/45 fusion protein).

Supplementary Table S1. List of primers used in this study

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
Primers for confirmation PCR analysis					
P1	4698		GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
P2	4699		GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
P3	6324	PF3D7_1346700	CATGCCATGGATGATGTTATATATTTCTGCGAAAAAGGC	1347	Forward *Pfs48/45*
P4	6325	PF3D7_1346700	ATAGTTTAGCGGCCGCCTAAATATATAATAATTGCTACAATTAGG		Reverse *Pfs48/45*
P5	5510		GCAAAGTGAAGTTCAAATATGTG	861	Forward 5′ Integration
P6	6192		TTAATTTGCACTTCAACATCAC		Reverse 5′ Integration
P7	6196		TTTTGGCTAAAACATTTATATTCC	1568	Forward 3′ Integration
P8	5511		AGTGACTTTCAGTGAAATCGC		Reverse 3′ Integration

Pf = *P. falciparum*, h = human

CHAPTER 7

CONCLUSIONS AND DISCUSSION

In this thesis we describe a set of studies, performed using rodent models of malaria, aimed to identify methods to improve vaccines consisting of live attenuated sporozoites, in particular genetically attenuated parasites (GAP) vaccines. Studies in rodents and humans have shown that immunization with live-attenuated sporozoites can generate protective immunity, however induction of sterile protection in humans has required immunization with multiple vaccine doses and each dose consisting of relatively high numbers of sporozoites [1, 2]. Increasing the immunogenicity of whole sporozoite (wsp) vaccines can both reduce the number of sporozoites per dose and the number of vaccine doses. In the studies described in this thesis we attempted to increase GAP immunogenicity by: (i) adding adjuvants during GAP immunization; (ii) introducing genes encoding putative immunomodulatory proteins in the GAP genome to create 'self-adjuvanting' parasites; (iii) generating GAPs that arrest late into liver-stage development (LA-GAP) to increase antigen load and diversity during immunization; and (iv) exploring possibilities to genetically modify parasite to express vaccine antigens from different life cycle stages, in order to test the ability of parasites to induce immune responses against multiple life cycle stages and to inform the creation of a 'multi-stage' GAP vaccine.

IMPROVING GAP IMMUNIZATION BY THE ADDITION OF IMMUNOSTIMULATORY MOLECULES (CHAPTER 3)

While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, T cells appear to be critical for protection and in particular $CD8^+$ T cells are thought to play a major role in eliminating infected hepatocytes. Early rodent studies using sporozoites attenuated by irradiation (Irr-Spz) have demonstrated a vital role for $CD8^+$ T cells [3, 4]. Recent mechanistic investigations into protective immune responses induced by immunization with attenuated sporozoites have demonstrated diverse and robust immune responses that encompasses both $CD8^+$ and $CD4^+$ T cells, as well as a significant contribution from antibodies [5, 6]. Nonetheless, $CD8^+$ T cells are considered to be the main effector cells in eliciting protection after sporozoites immunization [7].

Cancer immunotherapies have employed antibodies that target proteins on the surface of T cells, as treatment with these antibodies have been shown to restore, expand and enhance the function of tumor-reactive T cells. The antagonistic antibodies targeting CTLA-4 and PD-1 have been used to block inhibitory signals to T cells [8, 9], while agonistic antibodies targeting CD27, OX40 and 4-1BB on $CD4^+$ and $CD8^+$ T cells have been used to increase costimulatory signals [10-12]. These immunostimulatory antibodies have been shown to improve the control of tumors and this was associated with an increase in tumor-specific T cell function [13].

We show that treatment of mice with an agonistic antibody against the T cell costimulatory molecule OX40 enhances protective immunity after immunization with GAP sporozoites. The increase in protection was correlated with an expansion effector $CD4^+$

and CD8$^+$ T cell subsets, in both the liver and the spleen. In addition α-OX40 treatment induced the production of effector cytokine-producing T cells in the liver and spleen. Previously it has been shown that targeting OX40 increases the magnitude of T cell responses and improves T cell functionality [14, 15]. OX40 is transiently expressed on T cells following cognate interactions between T cell receptors (TCRs) and antigen-major histocompatibility (MHC) complexes on antigen presenting cells (APCs) [15]. While OX40 is expressed on both activated CD4$^+$ and CD8$^+$ T cells, OX40 expression on CD4$^+$ T cells is greater than CD8$^+$ T cells and consequently α-OX40 treatment is expected to exert its greatest effect on CD4$^+$ T cells [15-17]. Our analyses of T cell responses in mice immunized with a single dose of GAP parasites, showed an increase in total WBC numbers in the livers and an increase in CD4$^+$ effector (CD44^{hi}CD11a^{hi}) T cells in both liver and spleen of α-OX40 treated mice compared to untreated mice. While protective immunity after sporozoite immunization is thought to largely dependent on the killing infected hepatocytes by CD8$^+$ T cells and IFN-γ [18-20], adoptive transfer of CD4$^+$ T cells from GAP-immunized C57BL/6 mice was able to provide sterile protection to 50% of naïve animals against a WT infection, indicating an important role for CD4$^+$ T cells in GAP induced immunity [19]. In addition, protective immunity induced by sporozoites of a *P. yoelii* GAP, similar to the one used in our study, was dependent not only on CD8$^+$ T cells but also CD4$^+$ T cells [21]. Since enhancement of CD4$^+$ T cell responses by OX40 stimulation may lead to an increase in humoral immunity we examined total IgG responses generated in mice after prime-boost GAP immunization, either with or without α-OX40 treatment. These studies revealed that anti-sporozoite antibodies were generated after GAP immunization and significantly more IgG was generated in mice immunized with GAP plus α-OX40 treatment compared to mice immunized with only GAP. This observation indicates that the increase in CD4$^+$ T cells after α-OX40 treatment may directly contribute to B cell maturation/activation. It is known that CD4$^+$ T cell help is necessary for an effective CD8$^+$ T cell memory response against non-inflammatory antigens, such as tumor cells and certain pathogens that may not carry sufficient danger signals [22]. Indeed, we explored the possibility of adding immune-modulatory (danger) signals into a late arresting GAP in order to increase T cells responses (**Chapter 4**). Mice depleted of CD4$^+$ T cells during immunization with sporozoites failed to exhibit a robust CD8$^+$ T cell expansion and were not protected against challenge [23, 24]. Murray *et al*. found that CD4$^+$ T cell help was also necessary to induce protection after immunization with GAP sporozoites [21]. Together, our results indicate that improving CD4$^+$ T cell activation enhances protective immunity against malaria.

Future studies

Additional studies are required to determine if CD4$^+$ T cell stimulation and expansion after anti-OX40 treatment acts only to improve humoral responses targeting sporozoites or also enhances CD8$^+$ T cell responses against infected liver cells. Also how the expansion of T cells subsets contribute to formation of immunological memory and on duration of

protection requires further investigation. If this method of adjuvant treatment can be applied to human GAP vaccines could be determined by performing small controlled human malaria infection (CHMI) studies. However, the use of adjuvants with human malaria vaccines may be difficult to deploy in the field due to costs, applicability or side-effects and therefore these pre-clinical studies in rodent models not only provide useful information of the largely unknown mechanisms underlying protective immunity but are of importance to select the most appropriate adjuvant to advance further. Although α-OX40 treatment is currently in clinical trials for cancer immunotherapy, the use of antibody-based α-OX40 treatment for vaccines for the developing world may be unrealistic as they are likely to be too expensive. Other (protein based) agents that can stimulate costimulatory responses, including agonists of OX40 are being developed as potential adjuvants in vaccine development. For example, combination therapy using the protein ligand of OX40, OX40L, fused to a cancer vaccine have been shown to reduce breast cancer metastasis, by enhancing antigen specific $CD4^+$ and $CD8^+$ T cell responses and inhibiting immunosuppressive Treg responses [25]. The co-administration of proteins like OX40L which are likely to be cheaper and easier to produce, may therefore be more practical and feasible approaches to pursue. In conclusion, this study demonstrates how specific immune response to vaccination coupled with activation of costimulatory molecules on the surface of T cells, can enhance protective immunity after sporozoite immunization and merits further investigation to see if such approaches not only increase the magnitude but also the breadth of an immune responses after vaccination. Moreover, knowing that both formation of parasite-antigen specific CD4 and CD8 T cell responses play a role in the reduction of sporozoites and elimination of infected hepatocytes, we created and analysed GAPs that encoded immunostimulatory proteins. These so called 'adjuvant GAPs' studies are described below (Chapter 4).

IMPROVING GAP IMMUNOGENICITY BY CREATING 'SELF-ADJUVANTING' PARASITES THAT ALSO EXPRESS PUTATIVE IMMUNOMODULATORY MOLECULES (CHAPTER 4)

GAP immunization in combination with exogenous adjuvants provides useful information about mechanisms underlying protective immunity. Induction of protective immune responses by GAP immunization is dependent on sporozoites migrating to the liver and invading hepatocytes. However, the administration of adjuvants at the site of GAP injection will result in systemic distribution of the adjuvant which will therefore be considerably diluted at the sites where parasite antigens are taken up by antigen presenting cells (APCs), i.e. the liver, spleen or proximal lymph nodes [26]. In order to maximize the adjuvant effect, i.e. the increase of the number of APCs that have both taken up parasite antigen and have received adjuvant induced stimulatory signals to enhance their function, it is important to maximize the adjuvant effect at the point of antigen uptake and processing [26, 27].

We therefore, explored the possibility of creating GAPs that were engineered to express immunomodulatory proteins in sporozoites and liver stages, so called 'adjuvant GAPs'.

Four different proteins were selected with known adjuvant activity: nontoxic cholera toxin B (CTB) sub-unit, mouse heat shock protein Gp96, *Mycobacterium* heat shock protein X (HspX) and *Salmonella* flagellin (FliC), shown to act as adjuvants in other vaccine studies [28-36]. The selected adjuvant molecules are thought to stimulate different Toll-like receptors (TLRs), which can not only improve antibody and $CD4^+$ T cell responses but also promote the cross-presentation of vaccine antigens directing the immune response towards the formation of cytotoxic ($CD8^+$) T cells. Parasite antigen-specific $CD8^+$ T cells are considered of particular importance in detection and clearance of *Plasmodium*-infected hepatocytes [19]. However, immunization with none of the four adjuvant GAP developed in the present study resulted in a significant increase in protective efficacy (more than 10-fold) compared to the unmodified *Py*GAP in the *P. yoelli*-BALB/c model employed in this study to measure enhanced protective immunity.

This inability to achieve significantly higher protective immunity with the adjuvant GAPs could be due to a number of factors, but is unlikely to be due to a poor expression of the adjuvant proteins. These proteins were fused to UIS4, a protein associated with the PVM, which surrounds the parasites inside a hepatocyte [37]. UIS4 is strongly expressed during *Plasmodium* liver stage development [38]. Our failure to measure enhanced protective immune responses may be due to the inability of the selected adjuvants to induce protective immune responses that can more effectively detect and destroy developing liver stage parasites.

The selected adjuvants are known to stimulate TLR 2 (Gp96), TLR 4 (Gp96/CTB/HSPX) and TLR 5 (FliC) on the plasma membrane of APCs [30, 32, 34, 39] and the selection was based on the hypothesis that when GAP-infected hepatocytes disintegrate and release parasite antigens they will also simultaneously release the adjuvant, with parasite antigens being taken up by APCs and the released adjuvants stimulating TLRs on the same APC. This would then result in increased inflammatory responses against parasite antigens thereby improving and increasing cellular and humoral immune responses. It is possible that the adjuvants selected do not stimulate the most appropriate adaptive response that would result in the recognition and elimination of infected liver cells. In this study we did not directly measure the effect of the TLR-agonists on different immune cell populations in immunized mice and we only measured protective immunity by determination of the prepatent period after challenge with WT parasites. One can speculate that either the adjuvants did not activate the appropriate immune cells or that those that are involved in removal of infected liver cells are activated but this activation is not sufficient to result in a more than 10-fold increase in protective immunity (i.e. 1 day or more delay in patency).

Future studies

The failure of the adjuvant GAPS to greatly enhance protective immune responses may be due to the immunization protocol we employed. In this study, we have used the *P.*

yoelli-BALB/c immunization protocol which involves immunization with a single dose of sporozoites of a late arresting *Py*GAP followed by a challenge with wild type sporozoites 14 days later [40]. In future studies, the effect of the selected adjuvants on protective immunity may have been better observed after prime-boost immunization strategies where the re-call of expanded immunological memory responses may enhance protective immunity in different strains of mice. Indeed, such strategies might also be used to examine if immunization with the adjuvant GAP results in an increase of the duration of protective immune response compared to non-adjuvanted GAP. Whilst we were not able to detect a higher that 10-fold increase in vaccine potency, we have developed an immunization-challenge protocol, as well as a *Py*GAP GIMO mother line to rapidly create adjuvant GAPs, which can be used to evaluate other immunization schedules, additional adjuvants and/or novel enhanced GAPs. Novel adjuvant GAPs could be tested that encode other immunomodulatory molecules that have been characterized to enhance both anti-microbial and tumor vaccines. In our study we focused on adjuvants that interact with TLRs on the APC cell surface; in case of take-up of GAP-infected hepatocytes by APC this may be an issue since both the adjuvant and parasites are intracellular within the phagosome of the APC. In this case, adjuvant signaling would be better if it were to trigger cytoplasmic pattern recognition receptors (PPRs) either inside the APC or, indeed, the infected cell. For example, the C-terminus of flagellin has a NAIP5 ligand that would potentially activate intracellular sensing pathways, which could activate hepatocyte death and/or inflammatory cytokine production and perhaps increase the adjuvant potency [41, 42]. In Chapter 5 we only used the FliC portion of Salmonella flagellin that has been demonstrated to interact with TLR5 on the surface of APCs. In future studies we could therefore create and analyze a new adjuvant GAP that encodes full length flagellin to expand the adjuvant potential of this molecule. Manipulation, of the host immune response to direct and increase appropriate adaptive immune responses after vaccination is of value not only to enhance GAP vaccines but also other vaccines that need to generate immune responses to target liver infections.

THE GENERATION AND CHARACTERIZATION OF NOVEL LATE ARRESTING GAP (LA-GAP) (CHAPTER 5)

In contrast to the creation of early arresting-GAP, the generation of safe LA-GAPs have been challenging. Several genes have been identified that encode proteins that play an important role during late liver-stage development, deletion of those genes did not result in complete growth arrest in rodent models of malaria. A prerequisite for a GAP vaccine for humans use, is that the GAP sporozoites are unable to establish a potentially pathogenic blood-stage infection which requires that parasite growth is completely arrested during development in the liver.

We tested whether dual deletion of a variety of genes, with a role in late liver stage development, could synergize to create fully arrested GAPs. Specifically, we created

genetically attenuated rodent malaria parasites (GAPs) by deleting combinations of two genes from the parasite genome and screened for complete growth arrest during late liver development. The genes we selected for further investigation were: *lisp1*, *lisp2*, *mei2* and *palm* as they have been shown to encode proteins that have been shown to play an important role for final-stages of liver stage maturation/development [43-47].

We found that three GAPs, Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1* and Δ*lisp1*Δ*palm*, could produce blood infections in mice after infection with 5 × 10^4 sporozoites. In contrast no blood infections were observed in mice infected with Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* 5 × 10^4 or 2-3 × 10^5 sporozoites. However, after a high dose of 5 × 10^5 sporozoites of either mutant, some mice developed a blood infection. Given that *P. yoelii* mutants lacking both *mei2* and *lisp2* [48] did not result in a breakthrough blood infection even after 5 × 10^5 sporozoites, it was unexpected that we observed a breakthrough blood infection in one out of ten mice infected with 5 × 10^5 *P. berghei* Δ*mei2*Δ*lisp2* sporozoites. However, differences in attenuation phenotype between identical *P. yoelii* and *P. berghei* gene-deletion mutants have been reported in previous studies. For example, *P. yoelli* mutants lacking the gene encoding β-ketoacyl-ACP synthase II gene (*fabb/f*) show a much stronger attenuation phenotype [49] than *P. berghei* mutants lacking the orthologous *fabb/f* gene [50, 51].

We have examined the development the two GAPs with the strongest late-arrest attenuation phenotype, Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* and compared the phenotypes to that of an early-arresting GAP (i.e. Δ*b9*Δ*slarp*). The additive effect on the level of attenuation of combining *lisp2* or *palm* with *mei2* is demonstrated when we infected mice with 2 × 10^5 sporozoites. In these experiments we were unable to detect blood-stage infections after infection with either Δ*mei2*Δ*lisp2* or Δ*mei2*Δ*palm*, whereas 30% of the mice established a blood-stage infection when infected with the same number of Δ*mei2* sporozoites.

The Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* mutants replicate and arrest late into liver-stage development as shown *in vitro* by the presence of large intrahepatic stages that express the merozoite proteins MSP1 and AMA1, and *in vivo* through the detection of high parasite liver-loads 44 hours after infection. Surprisingly, mature schizonts of both mutants develop into significantly larger parasites than WT liver-schizonts. This may suggest that these parasites continue growing and/or replicating aberrantly and may therefore express more antigens than WT parasites at late stages of development.

We compared the immunogenicity of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* late-arresting GAPs (LA-GAPs) with immunogenicity of an early-arresting GAP (Δ*b9*Δ*slarp*). Immunization studies in both BALB/c and C57BL/6 mice showed that there were no significant differences in the induction of protective immunity between the two late-arresting GAPs. Moreover, the LA-GAPs were not significantly more protective than the early-arresting GAP. In one study in rodent models reported by Butler *et al.* [52] it was found that late-arresting GAPs induce stronger protective immune responses compared to early-arresting GAPs. It is presumed that this increase in immunogenicity arises from the greater amount and repertoire of antigens present in LA-GAP compared to early-arresting parasites [52]. It was shown that immunization with late-arresting GAP (LA-GAP) induced greater numbers

of a broader range of $CD8^+$ T cells compared to early-arresting parasites. The difference between our study and the Butler study might be explained by differences between the early-arresting GAPs or the LA-GAPs used in the different studies. However, differences in immunogenicity of the early-arresting GAP seems unlikely, since in both studies the early-arresting GAP lack the *slarp/sap1* gene, which would result in both mutants having a similar arrest phenotype and, likely, antigen profile. With respect to differences in the LA-GAP used, we do show that the Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* mutants develop in very large schizonts expressing proteins that include proteins expressed late into liver-stage development. In the Butler study an LA-GAP was used that lacks the *fabb/f* gene; rodent parasites lacking this gene arrest during the maturation of liver-schizonts [49, 51] and it has not been reported whether these parasites are enlarged or persist longer in mouse livers than WT parasites and it has not been reported whether these parasites are enlarged or persist longer in mouse livers than WT parasites. It is therefore surprising that Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* do not induce better protective immune responses than early-arresting parasites, since they are likely to express the same (if not more) antigens as those found in parasites lacking *fabb/f*.

Another possible explanation for the differences in immunogenicity between LA-GAP and early-arresting GAP observed in ours and the Butler study, is the use of GAPs that are generated in different rodent *Plasmodium* species. We used *P. berghei* to create our GAPs whereas Butler *et al*. generated the GAP lacking *fabb/f* in *P. yoelii*. Differences in T- or B-cell epitopes of antigens of these two species may influence the immunogenicity of parasites in different mouse strains. Moreover, it has been shown that these two rodent *Plasmodium* species differentially regulate key immune-effector pathways resulting in differences in memory $CD8^+$ T cell-mediated immunity against liver-stage antigens [53].

Future studies

Currently, only two genetically attenuated *P. falciparum* mutants have been generated, informed by studies performed in rodent malaria models, which have advanced into clinical studies. Both of these *P. falciparum* GAPs arrest early after hepatocyte invasion and consist of 2 or 3 gene-deletions; in these parasites *slarp* has been deleted in combination with either *b9* [54] or with *p52* and *p36* [55]. Recently, a clinical safety study has been performed in human volunteers using the *P. falciparum* GAP lacking *slarp, p52 and p36*. Ten out of ten volunteers exposed to the bites of 150-200 triple gene-deletion GAP-infected mosquitoes remained blood-stage negative [56]. The limited number of methods to rapidly and cost-effectively test the attenuation and protective efficacy of *P. falciparum* GAPs limits the direct down-selection of *P. falciparum* GAPs, and presently very few studies have been performed in humans that can confirm the predictive value of rodent models in assessing the attenuation level of GAPs that lack certain genes or gene combinations. Rodent *Plasmodium* parasites take about 2 days to complete liver-stage development while this process takes a week in *P. falciparum*. Thus, the deletion of liver-stage specific genes may result in different levels of attenuation in different *Plasmodium* species. Therefore,

while the deletion of *mei2* in combination with either *palm* or *lisp2* results in very strong, but incomplete, attenuation in rodent parasites, additional studies in *P. falciparum* are required to investigate if deletion of the same combination of genes results in complete attenuation in *P. falciparum*.

Based on the results of the Butler study, we anticipated that immunization of mice with two LA-GAPs generated in this study would also result in significantly enhanced protective immunity compared to the early-arresting GAP, in both BALB/c and in C57BL/6 mice. All the more so since the LA-GAPs Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* appear to continue development at late liver-stages that may result in even more antigens per parasite. Therefore, our observations that protective immunity induced after Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* immunization is not significantly different compared to Δ*b9*Δ*slarp* immunization, neither in C57BL/6 nor in BALB/c mice, was highly unexpected.

Combined, our observations indicate that (1) additional gene-deletion GAPs may need to be screened to identify GAPs that completely arrest during late liver-stage development and (2) the hypothesis that the greater the amount and diversity of antigens in a LA-GAPs the more likely it is to generate stronger protective immunity, requires reconsideration. Additional studies are required to define what immune mechanisms contribute to the induction, maintenance and deployment of adaptive immune response after LA-GAP immunization and the role of different rodent malaria parasites and different mouse strains in inducing protective immune responses. Using different *Plasmodium* species and employing different immunization protocols it might be possible to unveil these processes. These studies have important implications for the development of LA-GAPs for the human parasite *P. falciparum*. However, only by clinically evaluating of both the safety and the immunogenicity of LA-GAP will we be able to draw conclusions on the benefits of using LA-GAP compared to early-arresting GAP for vaccination.

GENERATION OF TRANSGENIC PARASITES EXPRESSING ANTIGENS FROM OTHER LIFE CYCLE STAGES (CHAPTER 6)

In order to establish if transgenic parasites can express additional proteins and if these antigens are able to provoke immune responses we examined if *P. berghei* blood-stage schizonts could be used express the *P. falciparum* transmission blocking vaccine candidate antigen, Pfs48/45. The transmission-blocking vaccine candidate Pfs48/45 from the human malaria parasite *Plasmodium falciparum* is known to be difficult to express in heterologous systems, either as full-length protein or as correctly folded protein fragments that retain conformational epitopes [57, 58]. The Pfs48/45 protein is expressed in *Plasmodium* gametocytes and gametes [59, 60] and contains multiple cysteine-rich domains with multiple disulfide bonds [44, 61, 62]. These constitute distinct conformational B cell epitopes that can be recognized by several monoclonal antibodies some of which have transmission-blocking (TB) activity [63]. The distinct and complex structure of Pfs48/45 is

thought to contribute to difficulties of expression of this protein in heterologous expression systems [64].

We expressed Pfs48/45 as a transgene under control of the strong *P. berghei* schizont-specific *msp1* gene promoter (Pfs48/45@PbMSP1). We show that these Pfs48/45@PbMSP1 schizont-infected red blood cells not only produced full-length Pfs48/45 but also that Pfs48/45 retains it structural integrity, as confirmed using a panel of conformation-specific monoclonal antibodies. We confirmed that this *P. berghei* expressed Pfs48/45 could evoke antibody responses, in mice, that reduce *P. falciparum* development in the mosquito. Specifically, we showed that purified IgG isolated from mice immunized with transgenic Pfs48/45@PbMSP1 schizont lysate exhibited strong transmission-reducing activity in mosquitoes infected with *P. falciparum*, using standard membrane feeding. Quantitative Western blot analysis indicated that only 0.25-0.12% of the total Pfs48/45@PbMSP1 schizont lysate was Pfs48/45 and therefore it is likely that the majority of the IgG from the immunized mice is not directed against Pfs48/45. Further, the failure to IgG from mice immunized with WT schizont lysate to block parasite development in the mosquito, indicates that the small proportion of anti-*Pf*s48/45 antibodies are mediating the transmission reducing activity after Pfs48/45@PbMSP1 schizont lysate immunization. These results demonstrate that transgenic rodent malaria parasites expressing human malaria antigens can be used as means to evaluate immunogenicity and functionality of difficult to express malaria vaccine candidate antigens.

Future studies:

The proof-of concept studies in Chapter 6 demonstrate that transgenic *P. berghei* parasites can be used as a system to produce a difficult to express human *Plasmodium* proteins, which are correctly folded and retain the conformational epitopes of the native protein. Therefore transgenic rodent malaria parasites can be used as expression systems to more efficiently express, screen, validate and down-select human *Plasmodium* antigens as potential novel malaria vaccine candidates.

This *P. berghei* expression system may therefore be used to evaluate the immunogenicity of other difficult to express antigens or specific domains of these parasite antigens. Studies using sera obtained from mice immunized with *P. falciparum* proteins expressed by transgenic *P. berghei* parasites could be used to rank-order novel vaccine candidate antigens, not only in transmission-reduction studies but also for blood-stage antigens using blood stage growth inhibition assays (GIA) or sporozoite-antigens using inhibition of sporozoite invasion (ISI) assays [65]. Moreover, the expression in transgenic schizonts of human *Plasmodium* proteins with affinity tags would allow for the purification of these proteins from whole parasite lysate preparations. All of the raised immune response Immunization with purified protein would be due to the target antigen, and therefore permit a more detailed analyses of antigen immunogenicity, for example to examine and clone potent inhibitory and cross-reactive B-cells/antibodies after rodent immunization [66].

Tis study demonstrates that it is possible to express vaccine antigens, from different life cycle stages in a genetically modified parasite and thus it opens up possibilities to create GAPs that express antigens from multiple life-cycles, so called multi-stage GAP vaccine, which could not only protective immune responses against sporozoite and liver stages but also to blood- and mosquito-stages. Moreover, this study suggests that it might be possible to express vaccine antigens from other *Plasmodium* strains or species, i.e. *P. vivax*, to create stage- and strain-transcending GAP vaccines, respectively.

REFERENCES

1. Hoffman, S.L., et al., *The march toward malaria vaccines.* Vaccine, 2015. **33**: p. D13-D23.
2. Hollingdale, M.R. and M. Sedegah, *Development of whole sporozoite malaria vaccines.* Expert Review of Vaccines, 2017. **16**(1): p. 45-54.
3. Schofield, L., et al., *Gamma-Interferon, Cd8+ T-Cells and Antibodies Required for Immunity to Malaria Sporozoites.* Nature, 1987. **330**(6149): p. 664-666.
4. Weiss, W.R., et al., *Cd8+ T-Cells (Cytotoxic/Suppressors) Are Required for Protection in Mice Immunized with Malaria Sporozoites.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(2): p. 573-576.
5. Doll, K.L. and J.T. Harty, *Correlates of protective immunity following whole sporozoite vaccination against malaria.* Immunologic Research, 2014. **59**(1-3): p. 166-176.
6. Van Braeckel-Budimir, N., S.P. Kurup, and J.T. Harty, *Regulatory issues in immunity to liver and blood-stage malaria.* Current Opinion in Immunology, 2016. **42**: p. 91-97.
7. Silvie, O., R. Amino, and J.C. Hafalla, *Tissue-specific cellular immune responses to malaria pre-erythrocytic stages.* Current opinion in microbiology, 2017. **40**: p. 160-167.
8. Curran, M.A., et al., *PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(9): p. 4275-4280.
9. Wolchok, J.D., et al., *Nivolumab plus Ipilimumab in Advanced Melanoma.* New England Journal of Medicine, 2013. **369**(2): p. 122-133.
10. Croft, M., *Costimulation of T cells by OX40, 4-1BB, and CD27.* Cytokine & Growth Factor Reviews, 2003. **14**(3-4): p. 265-273.
11. Dawicki, W., et al., *4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses.* Journal of Immunology, 2004. **173**(10): p. 5944-5951.
12. Melero, I., et al., *Immunostimulatory monoclonal antibodies for cancer therapy.* Nature Reviews Cancer, 2007. **7**(2): p. 95-106.
13. Schaer, D.A., D. Hirschhorn-Cymerman, and J.D. Wolchok, *Targeting tumor-necrosis factor receptor pathways for tumor immunotherapy.* Journal for ImmunoTherapy of Cancer, 2014. **2**(1): p. 7.
14. Sugamura, K., N. Ishii, and A.D. Weinberg, *Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40.* Nature Reviews Immunology, 2004. **4**(6): p. 420-431.
15. Croft, M., *Control of Immunity by the TNFR-Related Molecule OX40 (CD134).* Annual Review of Immunology, Vol 28, 2010. **28**: p. 57-78.
16. Baum, P.R., et al., *Molecular Characterization of Murine and Human Ox40/Ox40 Ligand Systems - Identification of a Human Ox40 Ligand as the Htlv-1-Regulated Protein Gp34.* Embo Journal, 1994. **13**(17): p. 3992-4001.
17. AlShamkhani, A., et al., *OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand.* European Journal of Immunology, 1996. **26**(8): p. 1695-1699.
18. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination.* Current Opinion in Biotechnology, 2012. **23**(6): p. 908-916.
19. Tarun, A.S., et al., *Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8(+) T cells.* Journal of Infectious Diseases, 2007. **196**(4): p. 608-616.
20. Douradinha, B. and D.L. Doolan, *Harnessing immune responses against Plasmodium for rational vaccine design.* Trends in Parasitology, 2011. **27**(6): p. 273-282.

21. Murray, S.A., et al., *CD40 Is Required for Protective Immunity against Liver Stage Plasmodium Infection.* Journal of Immunology, 2015. **194**(5): p. 2268-2279.

22. Sun, J.C., M.A. Williams, and M.J. Bevan, *CD4(+) T cells are required for the maintenance, not programming, of memory CD8(+) T cells after acute infection.* Nature immunology, 2004. **5**(9): p. 927-933.

23. Weiss, W.R., et al., *The Role of Cd4(+) T-Cells in Immunity to Malaria Sporozoites.* Journal of Immunology, 1993. **151**(5): p. 2690-2698.

24. Overstreet, M.G., et al., *CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites.* PLoS One, 2011. **6**(1): p. e15948.

25. Malamas, A.S., et al., *Combination therapy with an OX40L fusion protein and a vaccine targeting the transcription factor twist inhibits metastasis in a murine model of breast cancer.* Oncotarget, 2017. **8**(53): p. 90825-90841.

26. Anderson, R.J., et al., *A self-adjuvanting vaccine induces cytotoxic T lymphocytes that suppress allergy.* Nature Chemical Biology, 2014. **10**(11): p. 943-949.

27. Brown, L.E. and D.C. Jackson, *Lipid-based self-adjuvanting vaccines.* Curr Drug Deliv, 2005. **2**(4): p. 383-93.

28. Cui, B.F., et al., *Flagellin as a vaccine adjuvant.* Expert Review of Vaccines, 2018. **17**(4): p. 335-349.

29. Plant, A. and N.A. Williams, *Modulation of the immune response by the cholera-like enterotoxins.* Current Topics in Medicinal Chemistry, 2004. **4**(5): p. 509-519.

30. Stratmann, T., *Cholera Toxin Subunit B as Adjuvant-An Accelerator in Protective Immunity and a Break in Autoimmunity.* Vaccines, 2015. **3**(3): p. 579-596.

31. Bolhassani, A. and S. Rafati, *Heat-shock proteins as powerful weapons in vaccine development.* Expert Review of Vaccines, 2008. **7**(8): p. 1185-1199.

32. Strbo, N., et al., *Secreted heat shock protein gp96-Ig: next-generation vaccines for cancer and infectious diseases.* Immunologic Research, 2013. **57**(1-3): p. 311-325.

33. Ding, Y., et al., *Heat-Shock Protein gp96 Enhances T Cell Responses and Protective Potential to Bacillus Calmette-Guérin Vaccine.* Scandinavian Journal of Immunology, 2016. **84**(4): p. 222-228.

34. Jung, I.D., et al., *Enhancement of Tumor-Specific T Cell–Mediated Immunity in Dendritic Cell–Based Vaccines by Mycobacterium tuberculosis Heat Shock Protein X.* The Journal of Immunology, 2014. **193**(3): p. 1233.

35. Kim, H.Y., et al., *Heat shock protein X purified from Mycobacterium tuberculosis enhances the efficacy of dendritic cells-based immunotherapy for the treatment of allergic asthma.* Bmb Reports, 2015. **48**(3): p. 178-183.

36. Andersen-Nissen, E., et al., *Evasion of Toll-like receptor 5 by flagellated bacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(26): p. 9247-9252.

37. Silvie, O., et al., *Post-transcriptional silencing of UIS4 in Plasmodium berghei sporozoites is important for host switch.* Molecular Microbiology, 2014. **91**(6): p. 1200-1213.

38. Matuschewski, K., et al., *Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage.* Journal of Biological Chemistry, 2002. **277**(44): p. 41948-41953.

39. Kang, X.L., Z.M. Pan, and X.N. Jiao, *Amino acids 89-96 of Salmonella flagellin: a key site for its adjuvant effect independent of the TLR5 signaling pathway.* Cellular & Molecular Immunology, 2017. **14**(12): p. 1023-1025.

40. Othman, A.S., et al., *OX40 stimulation enhances protective immune responses induced after vaccination with attenuated malaria parasites.* Frontiers in Cellular and Infection Microbiology, 2018. **(In press)**.

41. Tenthorey, J.L., et al., *The structural basis of flagellin detection by NAIP5: A strategy to limit pathogen immune evasion.* Science, 2017. **358**(6365): p. 888-893.

42. Reyes Ruiz, V.M., et al., *Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome.* Proc Natl Acad Sci U S A, 2017. **114**(50): p. 13242-13247.

43. Ishino, T., et al., *LISP1 is important for the egress of Plasmodium berghei parasites from liver cells.* Cell Microbiol, 2009. **11**(9): p. 1329-39.

44. Annoura, T., et al., *Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development.* FASEB J, 2014. **28**(5): p. 2158-70.

45. Haussig, J.M., K. Matuschewski, and T.W. Kooij, *Inactivation of a Plasmodium apicoplast protein attenuates formation of liver merozoites.* Mol Microbiol, 2011. **81**(6): p. 1511-25.

46. Dankwa, D.A., et al., *A Plasmodium yoelii Mei2-Like RNA Binding Protein Is Essential for Completion of Liver Stage Schizogony.* Infect Immun, 2016. **84**(5): p. 1336-1345.

47. Kreutzfeld, O., K. Muller, and K. Matuschewski, *Engineering of Genetically Arrested Parasites (GAPs) For a Precision Malaria Vaccine.* Front Cell Infect Microbiol, 2017. **7**: p. 198.

48. Vaughan, A.M., et al., *A Plasmodium Parasite with Complete Late Liver Stage Arrest Protects against Preerythrocytic and Erythrocytic Stage Infection in Mice.* Infect Immun, 2018. **86**(5).

49. Vaughan, A.M., et al., *Type II fatty acid synthesis is essential only for malaria parasite late liver stage development.* Cell Microbiol, 2009. **11**(3): p. 506-20.

50. Yu, M., et al., *The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites.* Cell Host Microbe, 2008. **4**(6): p. 567-78.

51. Annoura, T., et al., *Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates.* Vaccine, 2012. **30**(16): p. 2662-70.

52. Butler, N.S., et al., *Superior Antimalarial Immunity after Vaccination with Late Liver Stage-Arresting Genetically Attenuated Parasites.* Cell Host & Microbe, 2011. **9**(6): p. 451-462.

53. Butler, N.S., N.W. Schmidt, and J.T. Harty, *Differential effector pathways regulate memory CD8 T cell immunity against Plasmodium berghei versus P. yoelii sporozoites.* J Immunol, 2010. **184**(5): p. 2528-38.

54. van Schaijk, B.C., et al., *A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.* Elife, 2014. **3**.

55. Spring, M., et al., *First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers.* Vaccine, 2013. **31**(43): p. 4975-83.

56. Kublin, J.G., et al., *Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects.* Sci Transl Med, 2017. **9**(371).

57. Flick, K., et al., *Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli.* Malar J, 2004. **3**: p. 50.

58. Tuju, J., et al., *Vaccine candidate discovery for the next generation of malaria vaccines.* Immunology, 2017. **152**(2): p. 195-206.

59. van Dijk, M.R., et al., *A central role for P48/45 in malaria parasite male gamete fertility.* Cell, 2001. **104**(1): p. 153-64.

60. van Dijk, M.R., et al., *Three members of the 6-cys protein family of Plasmodium play a role in gamete fertility.* PLoS Pathog, 2010. **6**(4): p. e1000853.

61. Mistarz, U.H., et al., *Expression, Purification and Characterization of GMZ2'.10C, a Complex Disulphide-Bonded Fusion Protein Vaccine Candidate against the Asexual and Sexual Life-Stages of the Malaria-Causing*

Plasmodium falciparum Parasite. Pharm Res, 2017. **34**(9): p. 1970-1983.

62. Theisen, M., M.M. Jore, and R. Sauerwein, *Towards clinical development of a Pfs48/45-based transmission blocking malaria vaccine.* Expert Rev Vaccines, 2017. **16**(4): p. 329-336.

63. Outchkourov, N., et al., *Epitope analysis of the malaria surface antigen pfs48/45 identifies a subdomain that elicits transmission blocking antibodies.* J Biol Chem, 2007. **282**(23): p. 17148-56.

64. Milek, R.L., H.G. Stunnenberg, and R.N. Konings, *Assembly and expression of a synthetic gene encoding the antigen Pfs48/45 of the human malaria parasite Plasmodium falciparum in yeast.* Vaccine, 2000. **18**(14): p. 1402-11.

65. Othman, A.S., et al., *The use of transgenic parasites in malaria vaccine research.* Expert Review of Vaccines, 2017. **16**(7): p. 685-697.

66. Triller, G., et al., *Natural Parasite Exposure Induces Protective Human Anti-Malarial Antibodies.* Immunity, 2017. **47**(6): p. 1197-+.

APPENDIX &

SUMMARY

NEDERLANDSE SAMENTVATTING

CURRICULUM VITAE

LIST OF PUBLICATIONS

ACKNOWLEDGEMENTS

SUMMARY

A number of studies have shown that immunization with live, attenuated sporozoites can generate strong protective immunity against malaria infection in humans. The major challenge for sporozoite-based vaccines is to produce a highly immunogenic live-attenuated vaccine, which requires the fewest attenuated sporozoites per dose and the fewest doses in order to induce sustained sterile protection against malaria in the field. In this thesis, we describe a set of studies performed in rodent models of malaria to improve malaria vaccines consisting of sporozoites of genetically attenuated parasites (GAPs). We attempted to increase GAP immunogenicity by: (i) adding adjuvants during GAP immunization; (ii) introducing genes encoding putative immunomodulatory proteins in the GAP genome to create 'self-adjuvanting' GAP; (iii) generating GAP that arrest late into liver-stage development (known as late-arresting GAPs, or "LA-GAPs") to increase antigen load and diversity during immunization; and (iv) exploring possibilities to genetically modify parasite to express vaccine antigens from different life-cycle stages, in order to test the ability of GAPs to induce immune responses against multiple life-cycle stages and to inform the creation of a 'multi-stage' GAP vaccine.

We have used well-established rodent malaria models in combination with standard and adapted immunization protocols in order to evaluate protective immune responses induced by different GAPs and immunization approaches. In addition, we also used a variety of well-established genetic modification technologies to create a variety of (transgenic) rodent malaria parasite mutants and made use of transgenic parasites that express luminescent and fluorescent reporter proteins to analyze parasite development. In **Chapter 2** we provide a review on the use of transgenic malaria parasites in vaccine research, both for testing novel vaccines and for generation of GAP-based vaccines.

In **Chapter 3** we describe studies in which we examine if agonistic OX40 monoclonal antibody (OX40 mAb) treatment could be used to improve protective immunity induced by immunization with an LA-GAP. In cancer immunotherapies, agonistic antibodies that target T cell surface proteins such as CD27, OX40 (CD134) and 4-1BB (CD137) have been used to enhance T cell function by increasing co-stimulation. In these studies, we analyze the effect of agonistic OX40 monoclonal antibody treatment on protective immunity induced in mice immunized with GAP. We show that OX40 stimulation enhanced protective immunity after vaccination as shown by an increase in the number of protected mice and delay to blood-stage infection after challenge with wild-type sporozoites. Consistent with the enhanced protective immunity enforced by OX40 stimulation resulted in increased expansion of antigen-experienced effector ($CD11a^{hi}CD44^{hi}$) $CD8^+$ and $CD4^+$ T cells in the liver and spleen, and also increased IFN-γ and TNF producing $CD4^+$ T cells in the liver and spleen. In addition, GAP immunization plus α-OX40 treatment significantly increased sporozoite-specific IgG responses. Thus, we demonstrate that targeting T cell costimulatory receptors can improve sporozoite-based vaccine efficacy.

GAP immunization in combination with exogenous adjuvants provides useful information about mechanisms underlying protective immunity. However, the use of such adjuvants in populations where malaria is endemic may be difficult due to cost-of-goods, applicability or side-effects. Due to the limitations of co-injecting adjuvants with attenuated sporozoites, we describe in **Chapter 4** studies where we explored the possibility of creating GAPs that express immunomodulatory proteins in sporozoites and liver stages, so-called 'self-adjuvanting GAPs'. We selected four TLR agonists that can increase and direct adaptive immune responses and have the ability to improve cross-presentation of antigens as has been demonstrated in other animal and/or human studies. The selected adjuvant proteins are: (i) nontoxic cholera toxin B subunit from *Vibrio cholerae*; (ii) heat shock protein Gp96 of mice; (iii) heat shock protein X from *Mycobacterium tuberculosis*; and (iv) the TLR5 binding region of *Salmonella typhimurium* flagellin. The genes encoding the 'adjuvant' proteins were fused to a *Plasmodium* gene expressed in liver stages, *uis4*. UIS4 is located at the parasitophorous vacuole membrane (PVM) in infected hepatocytes. We fused the adjuvant proteins to a PVM protein, as it has been shown that ovalbumin (OVA) fused to proteins located in the PV/PVM induce stronger T cell responses than ovalbumin expressed in the cytoplasm of transgenic parasites. To facilitate the generation of multiple 'self-adjuvanting' lines in *P. yoelii* LA-GAP, we generated a GIMO locus in the *P. yoelii fabb/f* gene locus, thereby creating a novel *P. yoelii* GIMO GAP mother line. This line was used for the rapid introduction of the adjuvant fusion-transgenes into the *P. yoelii* genome without retention of a drug-selectable marker (SM). The four adjuvant GAPs were analysed for protective immunity using the *P. yoelli*-BALB/c screening model for assessing protective immunity after GAP immunization. When compared to immunization performed with non-adjuvanted *P. yoelii fabb/f* GAP, we were unable to observe a significant (more than 10-fold) enhancement in protection against wild-type *P. yoelii* sporozoite challenge after immunization with the four adjuvant GAPs. Several possible explanations for the inability to achieve significantly higher protective immunity with the adjuvant GAPs are discussed in **Chapter 4**. Whilst we were not able to detect a higher than 10-fold increase in vaccine potency, we have developed an immunization-challenge protocol, as well as a novel *P. yoelii* GIMO GAP mother line to rapidly create adjuvant GAPs, which can be used to evaluate other immunization schedules, additional adjuvants and novel enhanced GAPs.

In **Chapter 5** we explore the creation of novel LA-GAPs. It has been shown that immunization of mice with GAP that arrest late during liver stage development can induce higher levels of protective immunity compared to immunization with GAP that arrest early after invasion of hepatocytes. Specifically, it has been shown that LA-GAPs induce greater numbers of a broader range of $CD8^+$ T cells, which results in increased protection against a malaria infection compared to immunization with early-arresting GAP, most probably due to a greater number and repertoire of antigens expressed by LA-GAPs. Currently, three *P. falciparum* GAPs have been developed for clinical evaluation and all are early-arresting GAPs, which arrest development soon after hepatocyte invasion. In contrast to the creation

of early arresting-GAPs, the generation of safe LA-GAPs have been challenging. Several genes have been identified that encode proteins that play an important role during late liver stage development but deletion of those genes did not result in complete growth arrest in rodent models of malaria. In order to create an LA-GAP that completely arrests late into liver stage development and cannot establish a blood infection, we describe studies where we create double gene-deletion mutants using combinations of different genes that have a role in late liver stage development and could synergize to create fully arrested GAPs. Four genes, *lisp1*, *lisp2*, *mei2* and *palm* were selected that encode proteins that have been shown to play an important role for final maturation of liver stages. We created the following double gene-deletion mutants: Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1,* Δ*lisp1*Δ*palm,* Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm.* We found that three GAPs, Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1* and Δ*lisp1*Δ*palm,* could produce blood infections in mice after infection with 5×10^4 spz. In contrast, no blood infections were observed in mice infected with 5×10^4 or 2-3 $\times 10^5$ Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* spz. However, after a high dose of 5×10^5 spz of either mutant, some mice developed a blood infection. The Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* mutants replicated and arrested late into liver-stage development as shown in *in vitro* cultures by large intrahepatic stages that express the merozoite proteins MSP1 and AMA1, and *in vivo* where high parasite liver-loads are detected 44 hours after infection. We compared the immunogenicity of Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm* LA-GAPs with the immunogenicity of an early-arresting GAP (Δ*b9*Δ*slarp*). Immunization studies in both BALB/c and C57BL/6 mice showed that there were no significant differences in the induction of protective immunity between the two LA-GAPs. Moreover, the LA-GAPs were not significantly more protective than the early-arresting GAP. These studies indicate that the hypothesis that the greater the amount and diversity of antigens in an LA-GAP, the more likely it is to generate stronger protective immunity, requires reconsideration.

The creation of GAPs expressing vaccine antigens from different parasite life-cycle stages could improve GAP vaccine potency by providing stage-transcending immunity. In Chapter 6 we created transgenic parasites that express additional antigens, and examined if the introduced antigen provoked specific immune responses. Specifically, we generated *P. berghei* parasite that express the *P. falciparum* transmission-blocking vaccine candidate antigen, Pfs48/45. We expressed Pfs48/45 in *P. berghei* blood stages, as blood stages are easier to produce than sporozoites, and next we examined if these blood stage parasites could be used to provoke antibody responses against Pfs48/45. In addition to providing a template for creating 'enhanced' GAPs that express vaccine antigens from multiple parasite life-cycle stages, these studies also demonstrate the utility of *P. berghei* parasites as a *P. falciparum* protein expression system. Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed. This hampers the screening of antigens of human malaria parasites in immunization studies for their suitability as vaccine candidate antigens. We reasoned that the use of transgenic rodent malaria parasites expressing human malaria

proteins for the production of such proteins would circumvent problems associated with expression in heterologous expression systems, including peculiarities of post-translational modifications and *Plasmodium*-specific domains involved in protein trafficking and cellular location. Hence, in **Chapter 6** we also describe studies where we express full-length Pfs48/45 in the rodent parasite *P. berghei*. Expression of Pfs48/45 for TB immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein. We expressed Pfs48/45 as a transgene under control of the strong *P. berghei* schizont-specific *msp1* gene promoter (Pfs48/45@PbMSP1). Pfs48/45@PbMSP1 schizont-infected red blood cells produced full-length Pfs48/45 and the structural integrity of Pfs48/45 was confirmed using a panel of conformation-specific monoclonal antibodies that bind to different Pfs48/45 epitopes. Sera from mice immunized with transgenic Pfs48/45@PbMSP1 schizonts showed strong transmission-reducing activity in mosquitoes infected with *P. falciparum* using standard membrane feeding. These results demonstrate that transgenic rodent malaria parasites expressing human malaria antigens can be used as a means to evaluate immunogenicity and functionality of difficult-to-express malaria vaccine candidate antigens.

NEDERLANDSE SAMENTVATTING

Verschillende onderzoeken hebben laten zien dat immunisatie met levende, verzwakte sporozoieten een krachtige, beschermende afweer tegen malaria infecties kan induceren. De grootste uitdaging in het toepassen van vaccins gebaseerd op verzwakte sporozoieten is het maken van een vaccin dat een duurzame, volledige bescherming tegen een malaria infectie kan induceren met zo weinig mogelijk sporozoieten per dosis en zo weinig mogelijk doses per vaccinatieregime.

Dit proefschrift behandeld een reeks van studies uitgevoerd in knaagdiermodellen van malaria met als doel om malaria vaccins, die bestaan uit verzwakte sporozoieten van genetisch geattenueerde parasieten (GAP), te verbeteren. Wij hebben getracht de immunogeniciteit van de vaccins te verhogen door: (i) gebruik te maken van immunologische adjuvantia tijdens GAP immunisaties; (ii) introductie van genen coderend voor potentiele, immuun-modulerende eiwitten in het genoom van de GAP om zo 'zelf-adjuverende' GAPs te ontwikkelen; (iii) het genereren van GAPs waarvan de ontwikkeling in het leverstadium laat tot stilstand wordt gebracht (LA-GAP; Late Arrester GAP) en daardoor blootstelling aan meer antigenen te bewerkstelligen gedurende de immunisatie; en (iv) het verder genetisch modificeren van GAPs door genen te introduceren coderend voor antigenen van diverse ontwikkelingsstadia van de parasiet om te onderzoeken of het mogelijk is om een zogenaamd 'multi-stage' vaccin te ontwikkelen dat een afweerreactie kan opwekken tegen antigenen van diverse ontwikkelingsstadia.

Wij hebben gebruik gemaakt van veelvuldig toegepaste knaagdiermalaria-modellen in combinatie met zowel standaard als aangepaste immunisatieprotocollen voor de evaluatie van beschermende afweerreacties opgewekt door vaccinaties met de verschillende GAPs en immunisatie-strategieën. Daarnaast hebben wij in deze studies een verscheidenheid aan moleculaire technieken toegepast om diverse genetisch gemodificeerde malaria parasieten te maken en is er gebruik gemaakt van transgene malaria parasieten die luminescerende en fluorescerende indicatoreiwitten tot expressie brengen om parasiet-infecties te analyseren. In **Hoofdstuk 2** wordt een overzicht gegeven van het gebruik genetisch gemodificeerde malaria parasieten in vaccinonderzoek, zowel voor het testen van nieuwe vaccins als voor het maken van GAP vaccins.

In **Hoofdstuk 3** beschrijven wij studies waarin wij onderzoeken of behandeling met een agonistisch OX40 monoclonaal antilichaam (OX40 mAb) toegepast kan worden om de afweer na immunisatie met een LA-GAP te versterken. Agonistische antilichamen die reageren met oppervlakte-eiwitten van T cellen zoals CD27, OX40 (D134) en 4-1BB (CD137) worden toegepast in immunotherapieën tegen kanker waarin deze antilichamen door co-stimulatie de activiteit van T cellen verhogen. In deze studies hebben wij het effect van behandeling met OX40 mAb op de afweerreactie geanalyseerd in GAP-geïmmuniseerde muizen. Wij tonen aan dat stimulatie met OX40 een verhoogde afweer induceert. Dit uit zich in een groter aantal beschermde muizen en een uitgestelde infectie in het bloed na blootstelling van de geïmmuniseerde muizen aan een malaria infectie.

Overeenkomstig met de verhoogde afweer, laat OX40 stimulatie een verhoogde toename zien van effector ($CD11a^{hi}CD44^{hi}$) $CD8^+$ en $CD4^+$ T cellen in de lever en milt, alsmede een toename van het aantal IFN-γ en TNF producerende $CD4^+$ T cellen in deze organen. GAP immunisatie in combinatie met OX40 behandeling laat bovendien een significante toename zien van sporozoiet-specifieke IgG reacties. Deze studies laten zien dat stimulatie van co-stimulatoire T cel receptoren de effectiviteit van sporozoiet-vaccins kan verbeteren.

GAP immunisatie gecombineerd met toegediende adjuvantia geeft bruikbare inzichten in de onderliggende mechanismen die een rol spelen in de totstandkoming van een beschermende afweer. Echter, het gebruik van dergelijke adjuvantia in malaria-endemische gebieden is niet eenvoudig vanwege de kosten, de minder gemakkelijke toediening en eventuele bijwerkingen die de adjuvantia teweeg kunnen brengen. Vanwege deze beperkingen, beschrijven wij in **Hoofdstuk 4** studies waarin wij de mogelijkheden hebben onderzocht om zogenaamde zelf-adjuverende GAPs te ontwikkelen die immuun-modulerende eiwitten tot expressie brengen. Vier TLR agonists werden geselecteerd op hun vermogen adaptieve immuunreacties te sturen en te verhogen en welke in staat zijn om kruispresentatie van antigenen te verbeteren zoals eerder is aangetoond in studies in proefdieren of mensen. De geselecteerde adjuvant-eiwitten zijn: (i) 'nontoxic cholera toxin B subunit' van *Vibrio cholerae* (CTB); (ii) 'heat shock protein Gp96' van muizen (Gp96); (iii) 'heat shock protein X' van *Mycobacterium tuberculosis* (HspX); en (iv) 'TLR5 binding region' van *Salmonella typhimurium* flagellin. De genen coderend voor deze adjuvant-eiwtten werden gefuseerd met *uis4*, een gen dat tot expressie komt in het leverstadium. UIS4 is gelokaliseerd op het 'parasitophorous vacuole membrane' (PVM) in geïnfecteerde hepatocyten. De adjuvant-eiwitten zijn gefuseerd met een PVM-membraaneiwit omdat eerder is aangetoond dat ovalbumine (OVA), gefuseerd met membraan-eiwitten van de PVM, sterkere T cel reacties induceert dan wanneer OVA tot expressie wordt gebracht in het cytoplasma van transgene parasieten. Voor het maken van meerdere, zelf-adjuverende LA-GAP, werd een GIMO locus gegenereerd in het genoom van een LA-GAP van de knaagdier malaria parasiet *P. yoelii* voor het introduceren van transgenen, waardoor een nieuwe *P. yoelii* GIMO LA-GAP moederlijn werd gecreëerd. Deze lijn is gebruikt voor het eenvoudig en snel introduceren van de gefuseerde adjuvant-genen zonder dat selectiemarkers in het genoom aanwezig blijven. De vier resulterende zelf-adjuverende GAP lijnen werden getest op hun vermogen een beschermende afweer te induceren in het *P. yoelli*-BALB/c screening model voor analyse van afweer tegen een malaria infectie na GAP immunisatie. Vergeleken met immunisaties uitgevoerd met een niet zelf-adjuverende *P. yoelii* LA-GAP, hebben we geen significante (meer dan 10-voudig) verhoging van beschermende afweer tegen een malaria infectie kunnen aantonen na immunisatie met de vier zelf-adjuverende GAPs. Verschillende mogelijke verklaringen hiervoor worden in **Hoofdstuk 4** besproken. Alhoewel wij geen significante verbetering van de immunogeniciteit van GAP hebben kunnen bewerkstelligen, hebben wij wel een immunisatie-infectie protocol ontwikkeld alsmede een GAP GIMO moederlijn gecreëerd waarmee snel en efficiënt nieuwe zelf-adjuverende GAP lijnen gemaakt en getest kunnen

worden voor de evaluatie van nieuwe immunisatie-strategieën, additionele adjuvantia en nieuwe, verbeterde GAPs.

In Hoofdstuk 5 onderzoeken wij het maken van nieuwe' late-arrester' GAPs (LA-GAPs). Er is aangetoond in muizen dat immunisatie met GAP, waarvan de ontwikkeling laat in het leverstadium tot stilstand komt ('late arresters'), een hogere mate van beschermende immuniteit opwekt in vergelijking met immunisatie met GAP waarvan de ontwikkeling vroeg tot stilstand komt in levercellen. Aangenomen wordt dat LA-GAPs meer antigenen tot expressie brengen waardoor zij meer en een grotere verscheidenheid aan CD8 T cellen activeren tijdens de immunisatie. Dit resulteert in een verhoogde afweer tegen een infectie met malaria in vergelijking met de afweer na immunisatie met GAPs die vroeg in hun ontwikkeling in de lever worden geremd. Op dit moment zijn er drie *P. falciparum* GAPs ontwikkeld voor klinische evaluatie van de immunogeniciteit. Alle drie GAPs zijn echter GAPs die vroeg in hun ontwikkeling in het leverstadium, kort na invasie van de levercel, worden geremd. In tegenstelling tot de ontwikkeling van deze GAPs, is de ontwikkeling van LA-GAPs een grote uitdaging gebleken. Verschillende genen, coderend voor eiwitten die een belangrijke rol spelen in het late leverstadium, zijn geïdentificeerd maar uitschakeling van deze genen in knaagdiermodellen van malaria heeft niet geresulteerd in een volledige remming van de ontwikkeling van de parasiet in de lever. Om een veilige LA-GAP te ontwikkelen die laat in zijn ontwikkeling in de levercel volledig tot stilstand komt en geen doorbraak kent naar de bloedstadia, beschrijven wij studies waarin wij genetisch gemodificeerde parasieten ontwikkelen met dubbele gen-deleties waarbij verschillende combinaties van genen worden uitgeschakeld. Vier genen, *lisp1*, *lisp2*, *mei2* en *palm* werden geselecteerd waarvan bekend is dat ze een rol spelen in de ontwikkeling van de parasiet in het late leverstadium. Wij hebben de volgende dubbele gen-deletie mutanten (GAPs) gemaakt: Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1,* Δ*lisp1*Δ*palm,* Δ*mei2*Δ*lisp2* and Δ*mei2*Δ*palm.* Bij drie GAPs, Δ*lisp1*Δ*lisp2*, Δ*mei2*Δ*lisp1* en Δ*lisp1*Δ*palm*, hebben we geconstateerd dat infectie van muizen met 5×10^4 sporozoieten toch resulteerde in een bloed-infectie in een aantal muizen. Twee andere GAPs daarentegen, Δ*mei2*Δ*lisp2* en Δ*mei2*Δ*palm*, lieten geen infecties van het bloed zien na infectie van muizen met 5×10^4 or 2-3 $\times 10^5$ sporozoieten. Echter, na infectie met een hogere dosis van 5×10^5 sporozoieten bleken beide mutanten bloedinfecties te veroorzaken in sommige muizen. Wij vonden dat de ontwikkeling van de Δ*mei2*Δ*lisp2* en Δ*mei2*Δ*palm* mutanten pas laat geremd werd in leverstadium. Dit werd aangetoond door de aanwezigheid van grote, delende leverstadia in levercel kweken, die de merozoiet-specifieke eiwitten MSP1 en AMA1 tot expressie brengen en tevens door het aantonen van grote aantallen parasieten, *in vivo* in muizen, in de lever 44 uur na infectie. De immunogeniciteit van de Δ*mei2*Δ*lisp2* en Δ*mei2*Δ*palm* LA-GAPs werd door ons vergeleken met de immunogeniciteit van de GAP Δ*b9*Δ*slarp* die vroeg geremd wordt in ontwikkeling na invasie van lever cellen. Immunisatie-studies in zowel BALB/c en C57BL/6 muizen hebben aangetoond dat er geen significante verschillen zijn tussen beide LA-GAPs met betrekking tot hun vermogen een beschermende immuniteit te induceren. De LA-GAPs lieten bovendien niet meer bescherming zien dan de GAP Δ*b9*Δ*slarp* die

vroeg geremd wordt in de lever. Deze studies laten derhalve zien dat de hypothese, waarin wordt verondersteld dat de grotere hoeveelheid/diversiteit van antigenen van een LA-GAP verantwoordelijk is voor een sterkere afweerreactie in vergelijking met een GAP waarvan de ontwikkeling vroeg tot stilstand komt, opnieuw bekeken moet worden.

Het creëren van GAPs die vaccin-kandidaat antigenen van diverse stadia van de levenscyclus van de parasiet tot expressie brengen zou de effectiviteit van het GAP vaccin ten goede kunnen komen door hun vermogen een immuniteit op te wekken tegen meerdere ontwikkelingsstadia van de parasiet. In **Hoofdstuk 6** hebben we transgene parasieten ontwikkeld die additionele antigenen tot expressie brengen en hebben wij onderzocht of het geïntroduceerde antigeen specifieke immuunreacties opwekte. Meer specifiek, wij hebben een transgene lijn gemaakt van de knaagdier malaria parasiet *P. berghei,* die het zogenaamde 'transmission blocking vaccine antigen' Pfs48/45 van de humane parasiet *P. falciparum* tot expressie brengt in de bloedstadia. Dit gametocyt-specifieke antigen werd tot expressie gebracht in de *P. berghei* bloedstadia omdat bloedstadia, in tegenstelling tot sporozoieten, makkelijker te produceren zijn. Vervolgens hebben we onderzocht of de transgene bloedstadia gebruikt kunnen worden voor het induceren van specifieke immuunreacties tegen Pfs48/45. Deze studies zijn niet alleen een voorbeeld voor het maken van geoptimaliseerde GAPs die vaccin antigenen van diverse stadia tot expressie brengen, maar laten ook zien dat *P. beghei* toegepast kan worden als expressiesysteem voor *P. faciparum* antigenen. Efficiënte en conformatie-accurate expressie van *P. falciparum* eiwitten in heterologe systemen, zoals gist of bacteriën, is vaak problematisch, resulterend in verkeerd gevouwen of niet goed gemodificeerde eiwitten. Dit hindert de screening van eiwitten van humane parasieten als geschikte vaccin-kandidaten in immunisatie studies. We hebben verondersteld dat het gebruik van transgene knaagdier malariaparasieten als expressiesysteem voor eiwitten van humane malariaparasieten de problemen geassocieerd met het gebruik van heterologe expressiesystemen zou kunnen omzeilen, inclusief de problemen met betrekking tot post-translationele modificaties en expressie van *Plasmodium* specifieke domeinen betrokken bij eiwitmigratie en cellulaire lokalisatie. In **Hoofdstuk 6** beschrijven we studies waarin we het gehele Pfs48/45 eiwit tot expressie brengen in blodstadia van de knaagdier malariaparasiet *P. berghei*. Expressie van Pfs48/45 voor immunisatiestudies in gangbare expressiesystemen is moeilijk gebleken, voornamelijk door incorrecte of niet voldoende vouwing van het eiwit, wat afhankelijk is van de correcte formatie van zwavelbruggen in dit cysteïne-rijke eiwit. We hebben Pfs48/45 als transgen tot expressie gebracht onder controle van de sterke, *P. berghei* schizont-specifieke *msp-1* promoter (Pfs48/45@PbMSP1). Rode bloedcellen geïnfecteerd met Pfs48/45@PbMSP1 schizonten lieten expressie zien van het Pfs48/45 eiwit en de structurele integriteit van Pfs48/45 werd aangetoond met een reeks aan conformatie-specifieke monoclonale antilichamen gericht tegen verschillende Pfs48/45 epitopen. Sera van muizen geïmmuniseerd met transgene Pfs48/45@PbMSP1 schizonten lieten, middels standaard membraanvoedingen, een sterk gereduceerde transmissie activiteit zien in muggen geïnfecteerd met *P. faciparum*. Deze resultaten tonen aan dat

transgene knaagdier malariaparasieten, welke antigenen van humane malariaparasieten tot expressie brengen, gebruikt kunnen worden om de immunogeniciteit en functionaliteit te evalueren van malaria vaccin kandidaat antigenen, die in heterologe systemen moeilijk tot expressie kunnen worden gebracht.

CURRICULUM VITAE

Ahmad Syibli bin Othman was born on 13th of November, 1986 in Kuala Terengganu, Malaysia. In 2010, he completed his bachelor of Medical Laboratory Technology (Honours) at the Universiti Teknologi Mara in Malaysia under a Jabatan Perkhidmatan Awam (JPA) Scholarship. He continued his Master of Science degree in Human Genetics at the Universiti Sains Malaysia from 2010 to 2013 with a scholarship from the Ministry of Higher Education in Malaysia (Skim Latihan Akademik IPTA – SLAI). His master thesis, performed under the guidance of Dr. Sarina Sulong was entitled 'Application of two techniques: fluorescence *in situ* hybridization (FISH) and quantitative real time PCR (qPCR) in detection of TERT gene amplification using cancer cell lines'. In 2014, he was enrolled as a PhD student in the Leiden Malaria Research Group in the Department of Parasitology, Leiden University Medical Center (LUMC) The Netherlands with support from the Ministry of Higher Education in Malaysia (Skim Latihan Akademik IPTA – SLAI fellowship). Here, he performed studies aiming at improving live attenuated malaria vaccines and vaccination strategies. His studies c involved the generation and characterization of genetically modified rodent malaria parasites in different rodent species. He carried out his PhD under the supervision of Dr. Shahid Khan and Dr. Chris Janse. The results of this research have been presented in this thesis. After finishing his PhD, Ahmad Syibli will bring the knowledge he has gained back to Malaysia and will work as a lecturer at Universiti Sultan Zainal Abidin, Malaysia.

LIST OF PUBLICATIONS

Othman AS*, C Marin-Mogollon*, AM Salman, BM Franke-Fayard, CJ Janse and SM Khan. *The use of transgenic parasites in malaria vaccine research*. Expert Rev Vaccines 16(7): 1-13 (2017)
*Authors contributed equally to this study.

Othman AS, BM Franke-Fayard, T Imai, ETI van der Gracht, A Redeker, AM Salman, C Marin-Mogollon, J Ramesar, S Chevalley-Maurel, CJ Janse, R Arens and SM Khan. *OX40 Stimulation Enhances Protective Immune Responses Induced After Vaccination With Attenuated Malaria Parasites*. Front Cell Infect Microbiol 8: 247 (2018)

Othman AS, JW Lin, BM Franke-Fayard, H Kroeze, FJA van Pul, S Chevalley-Maurel, J Ramesar, C Marin-Mogollon, MM Jore, MJ Morin, CA Long, R Sauerwein, A Birkett, K Miura, CJ Janse and SM Khan. *Expression of full-length Plasmodium falciparum P48/45 in P berghei blood stages: A method to express and evaluate vaccine antigens*. Mol Biochem Parasitol 224: 44-49 (2018)

Othman AS, BM Franke-Fayard, S Chevalley-Maurel, C Marin-Mogollon, AM Mendes, H Nunes-Cabaço, H Kroeze, J Ramesar, M Prudêncio, CJ Janse and SM Khan. *Generation and protective efficacy testing of self-adjuvanting genetically attenuated rodent malaria parasites*. (Submitted)

Othman AS, BM Franke-Fayard, AM Mendes, SK Kolli, S Chevalley-Maurel, C Marin-Mogollon, H Nunes-Cabaço, H Kroeze, FJA van Pul, J Ramesar, M Prudêncio, CJ Janse and SM Khan. *Generation and analysis of genetically attenuated rodent malaria parasites that arrest late during liver stage development*. (Thesis chapter)

C Marin-Mogollon, FJA van Pul, S Miyazaki, T Imai, J Ramesar, AM Salman, BMF Winkel, **Othman AS**, H Kroeze, S Chevalley-Maurel, A Reyes-Sandoval, M Roestenberg, BM Franke-Fayard, CJ Janse and SM Khan. *Chimeric Plasmodium falciparum parasites expressing Plasmodium vivax circumsporozoite protein fail to produce salivary gland sporozoites*. Malar J. 17:288 (2018)

C Marin-Mogollon, M van de Vegte-Bolmer, GJ van Gemert, FJA van Pul, J Ramesar, **Othman AS**, H Kroeze, J Miao, L Cui, KC Wiliamson, R Sauerwein, CJ Janse and SM Khan. *The Plasmodium falciparum male gametocyte protein P230p, a paralog of P230, is vital for zygote formation and mosquito transmission*. (Submitted)

C Marin-Mogollon, AM Salman, KMJ Koolen, JM Bolscher, FJA van Pul, S Miyazaki, T Imai, **Othman AS**, J Ramesar, GJ van Gemert, H Kroeze, BM Franke-Fayard, S Chevalley-

Maurel, R Sauerwein, KJ Dechering, CJ Janse and SM Khan. *A P. falciparum NF54 reporter line expressing mCherry-luciferase in gametocytes, sporozoites and liver stages.* (Thesis chapter)

ACKNOWLEDGEMENTS

I would like to express my appreciation to a number of wonderful individuals for being part of this journey and making this thesis possible.

I would like to record my greatest gratitude and sincere appreciation to my beloved supervisors, Dr. Shahid Khan and Dr. Chris Janse, who gave me the opportunity to work in the Leiden Malaria Research Group. Thank you for the support, advice, patience and motivation from the very early stage of this research as well as giving me valuable experiences throughout the work. I really grateful to work under your supervision and I are indebted to them more than they know. My sincerest gratitude also goes to my Promoter Prof. Dr. Maria Yazdanbakhsh, for her continuous support throughout my studies.

I would like to thank Dr. Blandine Frank-Fayard and Séverine Chevalley-Maurel for being helpful colleagues, especially with the immunization experiments involving mosquito infections and laboratory animals. My thanks also go to Jai Ramesar, Hans Kroeze, Dr. Takashi Imai, Dr. Jingwen Lin, Fiona van Pul, Dr. Surendra Kolli, Dr. Ahmad Salman, Dr. Shinya Miyazaki and Dr. Yukiko, Brian Kruisinga, Gizem Özel and Edwin Scholl for being so supportive for the last four years. I also would like to thank all the collaborators who have contributed to my project; Dr. Ramon Arens, Esmé T. I. van der Gracht, Dr. Anke Redeker, Dr. Miguel Prudêncio, Dr. António M. Mendes, Dr. Helena Nunes-Cabaço, Dr. Matthijs M. Jore, Prof. Dr. Robert Sauerwein, Dr. Kazutoyo Miura, Prof. Dr. Carole A. Long, Dr. Merribeth J. Morine and Dr. Ashley Birkett. All of the results described in this thesis would not been obtained without their involvement.

I would like to thank the members of my thesis committee, Prof. Dr. Annemieke Geluk, Prof. Dr. Ferry A. Ossendorp, Dr. Clemens Kocken and Dr. Miguel Prudêncio for their excellent and detailed review of this thesis.

My special thanks to my dearest, best friend, sister and mentor, Catherin Marin-Mogollon. This work was not possible without her constant support and scientific help. Your friendly advice, your soothing words and your big heart helped me face all the obstacles and continue with my work. I will never forget your kindness, as well your boys, Juan Alarcon and Juan Daniel.

I also would like to thank all my colleagues of Department of Parasitology, and in particular Abena, Alice, Alwin, Angela, Arifa, Astrid, Bart, Beatrice, Bruno, Dian, Dicky, Eric, Erliyani, Eunice, Firdaus, Frank, Hermelijn, Katja, Leonard, Maria Kaisar, Marijke, Mathilde, Meta, Michelle, Mikhael, Patrick, Ron, Suzanne, Yoanne and Yvonne. Thank you for your help in the lab and the good times.

To the Leideners; Naqi, Sofea, Zack, Ija, Lubna, Abg Hafeez, Kak Nomie, Yuven, Kak Lela, Zuwairi, Farah, Angga, Suci and to all other Malaysians, thanks for sharing so many wonderful moments with me over the past few years in Leiden.

I am very grateful to the Universiti Sultan Zainal Abidin and Ministry of Higher Education in Malaysia for awarding me the 'Skim Latihan Akademik Bumiputra' and giving me the opportunity and supporting my studies in Leiden.

My deep and sincere gratitude and appreciation to my parents; my late father, Othman Bin Zit, and my mother, Zarina Binti Abd Lateh, for their continuous and unparalleled love and support. I am indebted to my parents for giving me the opportunities and experience that have made who I am. My sincere gratitude also to my parents in law, Azizan Bin Osman and Hasnah Binti Raman, for their support. I would also like to thank my brothers and sister, Balia, Safwan, Aina, Baihaqi, Asyraf, Nor Hazilah and Nu'aim as well as my brothers and sister in law for their constant support.

I would like to say to my two lovely children, Aisyah and Amiru, who are the pride and joy of my life, I appreciate all your patience and support during Ayah's PhD studies. Thank you for every one of my smiles and even more, for every one of your smiles.

At last I do not know how to begin with saying thank you to my soul mate, my dearest wife and my best friend, Nurul Alia. Thank you for being here with me and being so supportive through the toughest moments of my life. You are my inspiration and motivation for continuing to improve my knowledge and move my career forward. I'm blessing to have you during this journey.