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Studies using transgenic rodent malaria parasites to improve live attenuated malaria vaccines

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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,

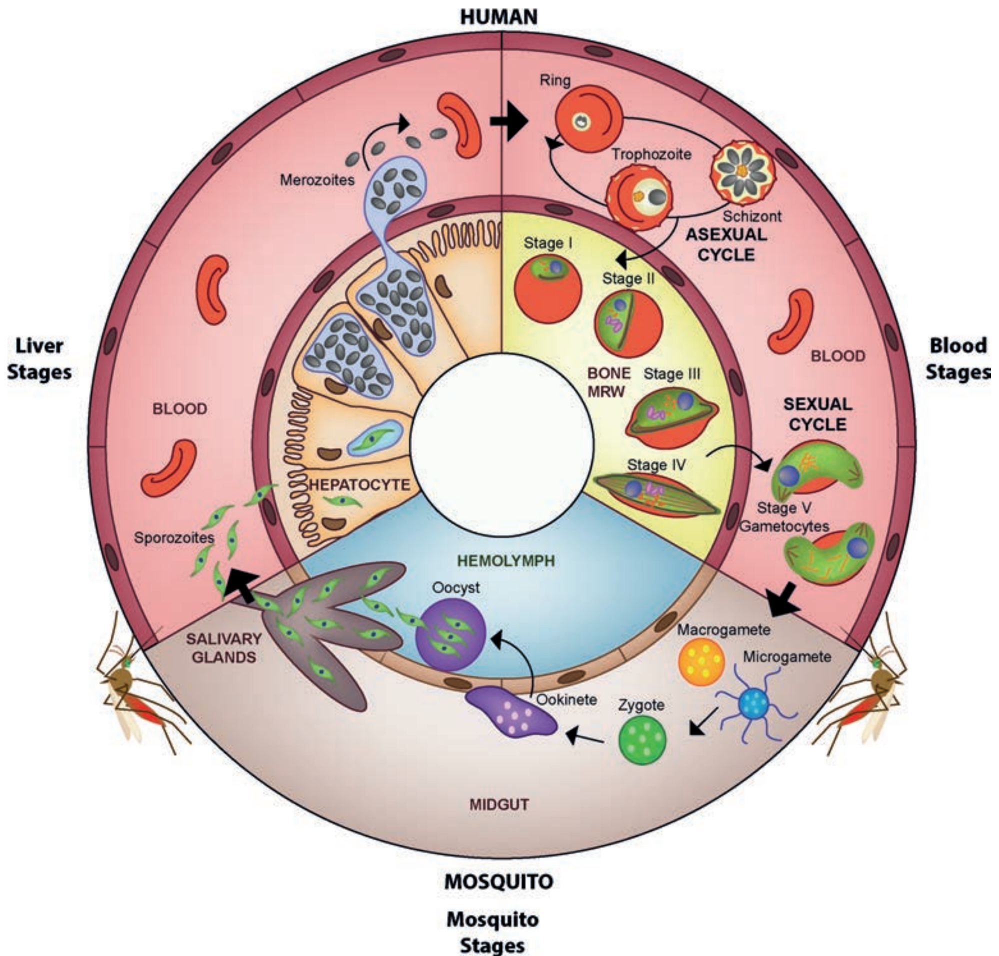

Figure 1. The life-cycle of the human malaria parasite *P. falciparum*. *P. falciparum* replication and maturation in humans (in red) and mosquitoes (in grey). This image was taken from Nilsson, S.K., et al. (2015).

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.

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