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CHAPTER 1

Introduction

1. Malaria and malaria vaccines

Humans develop malaria after being inoculated, via the bite of an infected female mosquito, with the unicellular protozoan parasite, *Plasmodium*. There were an estimated 219 million cases of malaria and 660 000 deaths in 2010 and it is one of the world's most important global health challenges ([1]; http://www.who.int/malaria/publications/world_malaria_report_2012/en/). Indeed, a recent systematic analysis on global malaria mortality showed that malaria was the underlying cause of death for 1.24 million individuals in 2010 and that malaria mortality therefore is likely to be larger than previously estimated [2]. Many prevention and elimination initiatives, such as distribution of insecticide-treated nets, indoor residual spraying with insecticides or implementation of drug treatment programmes, are underway to limit both the incidence and spread of the infection, as well as to limit the severity of the disease (http://www.who.int/malaria/publications/world_malaria_report_2012/en/). While these measures have contributed to the global decline in malaria, they are all under threat to the acquisition of drug resistance, either by *Plasmodium* parasites to antimalarial drugs or mosquitoes to insecticides [3-5]. Among the human malaria parasites, *P. falciparum* is the species responsible for most severe disease and accounts for the largest numbers of deaths, and therefore has been the target of most antimalarial drugs and vaccine development efforts.

Problems with the costs and logistics involved in mass drug administration campaigns targeting *Plasmodium* infected and at risk populations in resource and infrastructure poor settings, as well as the above mentioned acquisition of drug resistance, mean that vaccination remains the most (cost) effective method of malaria disease control, and ultimately eradication [6-8]. To date, disease elimination or eradication in humans has only been effectively achieved through the mass administration of vaccines [9]. However, a vaccine against malaria has not been licensed and indeed, only 1 vaccine candidate RTS,S has advanced to Phase III clinical testing (http://www.who.int/vaccine_research/links/Rainbow/en/index.html). Several features of a *Plasmodium* infection would appear to hamper the development of a vaccine against malaria. Specifically, for most of its development, the malaria parasite is intracellular, developing either inside hepatocytes or erythrocytes and thereby limiting its recognition and removal by the immune system. Moreover it exists as many morphologically and antigenically different forms, and parasite molecules most readily detected by the host immune response exhibit high levels of antigenic diversity (highly diverse allelic polymorphisms) and several of these critical parasite proteins are immunologically variant (where the genome encodes multiple copies of antigenically diverse but functionally related proteins). As the parasite

is itself eukaryotic, a number of parasite antigens may provoke relatively weak immune responses that do not contribute to protection against infection [6-8].

While *P. falciparum* blood stages can be effectively propagated *in vitro*, understanding the complexity of the host response to a malaria infection is best performed *in vivo.* And while very powerful, the expense and safety concerns of performing experimental studies in humans and non-human primates have limited their use. Consequently, rodent malaria parasites are recognized as valuable models to investigate the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing (http://www.lumc.nl/con/1040/ 81028091348221/810281121192556/81107074 0182556/811070744452556/).

1.1. The malaria parasite life cycle

Plasmodium parasites infect a wide variety of hosts, including birds, reptiles and mammals via an insect vector, usually a mosquito. There are five species associated with infecting humans, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [10]. Most of the severe pathologies and deaths due to malaria are associated with *P. falciparum* and it is consequently the most studied human malaria parasite (http://www.who.int/malaria/ publications/world_malaria_report_2012/en/).

1.1.1. The *P. falciparum* life cycle

One of the main difficulties in developing an effective vaccine against *P. falciparum* is the complex lifecycle of the parasite with its many different developmental stages (Figure 1). *P. falciparum* can only undergo sexual reproduction in *Anopheles* mosquitoes, its definitive host. The human being the intermediate host where parasites only reproduce asexually, both in the liver and the blood. When an infected female mosquito takes a blood meal, it injects parasites, sporozoites, into the skin and these then enter the bloodstream and migrate to the liver where they invade hepatocytes. Within the hepatocyte, the sporozoite grows and multiplies forming a hepatic schizont that contains several thousands of daughter parasites, the so-called exo-erythrocytic merozoites. These merozoites are released into the blood stream where they invade red blood cells (RBC). The development of the *P. falciparum* schizonts in the liver take 7–10 days, but the infection of the liver is not associated with any clinical symptoms. After merozoite invasion of a RBC, the parasite grows and multiplies, forming a blood-stage schizont containing 16–32 daughter parasites (merozoites) in a 44–48 hour period. When schizonts rupture, they release merozoites that can invade new RBC initiating a proliferative stage of development with new waves of parasites being released into the blood approximately every 48 hours. In other *Plasmodium* species that infect humans, replication inside RBC

can vary from 24–72 hours. The rapid increase in parasite numbers, destruction of RBC and the ability of infected RBC (iRBC) to attach to uninfected red blood cells and host tissue (sequestration) contribute to the clinical symptoms associated with *P. falciparum* infections [11]. *P. falciparum* infections lead to severe symptoms and death if untreated; the most common clinical features of severe malaria are high fever, respiratory distress, vascular obstructions, metabolic acidosis, multi-organ derangement, severe anaemia and neurological syndrome known as cerebral malaria (CM) [11]. CM is believed to be associated with iRBC sequestration in brain microvasculature and is thought to be enhanced by the proinflammatory status of the host and virulence characteristics of the infecting parasites [12].
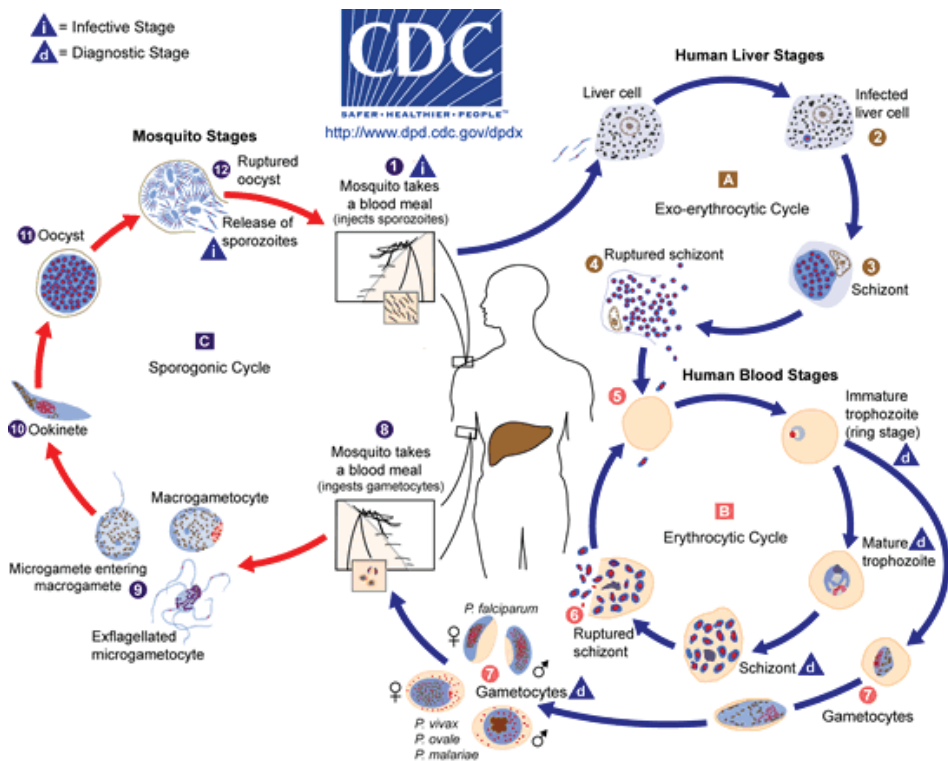
Figure 1. The life cycle of *Plasmodium falciparum*.

P. falciparum replication and maturation in humans (blue arrows) and mosquitoes (red arrows). This image was taken from Center for Disease Control and Prevention website (http://www.cdc.gov/malaria).

Some merozoites that invade RBC do not proceed with asexual multiplication but differentiate into male or female gametocytes, the sexual precursor cells of gametes. These gametocytes are responsible for transmission between host and mosquito and

once taken up by mosquitoes rapidly develop into male and female gametes in the mosquito midgut where fertilisation takes place. The resulting zygote matures into a motile ookinete, which can traverse the mosquito's midgut wall and attach onto the hemocoel side of the midgut where it differentiates into an oocyst. Through sporogony the oocyst forms thousands of daughter parasites, sporozoites. When these are released from the oocyst, they invade the mosquito salivary glands where they further mature and become infectious to humans.

1.1.2. The life cycle of *P. berghei*, the rodent malaria parasite used as a model in this study

In this study we have used the rodent malaria model, *Plasmodium berghei*. This parasite is the most genetically tractable of the four murine *Plasmodium* species (*P. berghei*, *P. vinckei*, *P. chabaudi* and *P. yoelii*). Several different *P. berghei* strains have been isolated, either from its natural host *Grammomys surdaster* (thicket rat) or from the natural vector *Anopheles dureni*. These parasites are infectious to laboratory rodents such as mice and rats, and can infect *A. stephensi* in the laboratory (http://www.lumc.nl/con/1040/810280 91348221/810281121192556/811070740182556/811070746282556). Rodent parasites are recognized as valuable model organisms to investigate human malaria, because they are similar in most essential aspects of morphology, physiology and lifecycle and the manipulation of the complete lifecycle of these parasites, including mosquito infections, is simple and safe [13].

Like the four human malaria parasites, *P. berghei* is transmitted by *Anopheles* mosquitoes and it infects the liver after being injected into the bloodstream by a bite of an infected female mosquito. After a short period (a few days) of development and multiplication, these parasites leave the liver and invade RBCs. The multiplication of the parasite in the blood causes the pathology such anemia and damage of essential organs of the host such as lungs, liver and spleen. *P. berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice. These symptoms are to a certain degree comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite [14]. The complete genome of *P. berghei* has been sequenced and it shows a high level of similarity with the genome of the human malaria parasite *P. falciparum* [15]. Despite the similarities between the life cycle stages of *P. berghei* and *P. falciparum*, some important differences exist. The *P. berghei* asexual blood cycle takes 22–24 hours to complete, unlike the 44–48 hours required by *P. falciparum*. *P. berghei* parasites have, like the human parasite *P. vivax*, a strong preference for invading and growing in immature RBCs (reticulocytes), whereas *P. falciparum* can invade both mature and immature RBCs. The *P. berghei* development in the liver does not, like *P. falciparum*,

take 7–10 days but only 48–52 hours. When studying host-parasite interactions, pathology and immune responses induced by *Plasmodium* infections in rodents, not only do the differences between the parasites but also clearly the differences between the rodent and human host have to be taken into consideration, since differences in host physiologies and immune responses will strongly influence how a host will cope with an infection and manifest malarial disease. In a recent review, the relevance of rodent *Plasmodium* infections in mice and rats as models of severe disease induced by *P. falciparum* infections in humans, have been discussed in depth [11]. For example, cerebral malaria is one of the most severe of all malaria pathologies observed in humans infected with *P. falciparum*, and C57BL/6 mice infected with *P. berghei* ANKA strain can also develop cerebral pathologies resulting in death, termed experimental cerebral malaria (ECM). Currently, the *P. berghei* ANKA-C57BL/6 system is the only available experimental model for the study of cerebral malaria *in vivo* [14], however, the similarities between the inflammatory context and the induced immuno-pathology between humans and rodent infections is under considerable debate [11,16].

1.2. Malaria vaccine intervention strategies

Most initiatives to develop a malaria vaccine target one or more of the following 3 stages of parasite development: 1) pre-erythrocytic vaccines that target the sporozoites and/or liver stages; 2) erythrocytic vaccines targeting merozoites or iRBC; and 3) transmission blocking vaccines that targets sexual and ookinete stages within the mosquito midgut [8]. Specifically:

Pre-erythocytic vaccines aim to generate antibody responses against sporozoites and thereby preventing hepatocyte invasion and/or T-cell (cellular) immune responses against intra-hepatic parasites that can kill intracellular liver stages. These vaccines prevent an infection from progressing beyond the asymptomatic liver stage and into a malaria infection in the blood [8]. However, to be successful such vaccine it has to be 100% effective in preventing blood stage infection, as any liver stage parasite that escape immune detection could generate an unregulated and potentially fatal blood stage infection.

Erythrocytic vaccines aim to generate antibody responses that target either merozoite antigens or parasite antigens expressed on the iRBC surface, therefore blocking parasite entry into a RBC or opsonising parasite/iRBC for phagocytic destruction. There is a strong rationale for the development of vaccines based on antigens of blood-stage parasites as most naturally acquired (albeit partial) protective immunity in humans is believed to target *Plasmodium* antigens expressed at the blood stage of development. Ideally, these

vaccines should reduce parasite densities to levels that do not cause disease [17]. Due to parasite antigenic variation, polymorphism and mutation, sterile immunity is likely to be very difficult for single antigen subunit blood-stage vaccines, but vaccination with such vaccines may allow the host to mount a better and more effective immune response thereby reducing the risk of the individual developing severe clinical disease [8,18].

Transmission-blocking vaccines target the stages of the parasite that establish an infection in a mosquito, specifically the sexual stages and/or the ookinetes, or even target mosquito midgut proteins. Vaccines targeting the parasite mainly rely on inducing a neutralizing antibody and/or antibody-complement based responses to eliminate parasites in the mosquito midgut, i.e. gametes and zygotes/ookinetes. These vaccines aim to reduce transmission and thereby limiting new infections within the community, and such vaccines do not provide protection against a malaria infection for the immunized individual. Consequently, they are likely to be deployed as part of multi-stage vaccine strategies, for example with vaccines that limit disease in the host and transmission to the population as a whole. Further, the importance of transmission blocking vaccines is that they may contribute to the eventual removal of reservoir populations of parasites in semi-immune individuals who carry the parasite but do not exhibit malaria symptoms [8].

The recent interest is not only in malaria control but in eradication, these different vaccination strategies may either be effectively brought together or different immunization methods used to best protect the populations at risk from malaria, such as young children, pregnant women, travellers, semi-immune adults etc [18,19].

2. Aim of this study

Despite major efforts over the past 50 years to develop one, there is currently no licensed malaria vaccine available. The most advanced vaccine is the sub-unit vaccine RTS,S that is based on the immunodominant sporozoite surface antigen, circumporozoite protein (CSP). It targets the sporozoite/liver stage of *P. falciparum* and has advanced to Phase III clinical trials. The preliminary results from the trial have shown a 55.8% reduction in the acquisition of clinical malaria, a 34.8% efficacy against severe malaria in young children aged between 5 to 17 month [20], and a 26% efficacy against severe malaria in infants 6–12 weeks of age [21]. The limited success achieved in inducing sterile and long-lasting protective immunity against malaria using subunit vaccines has led to renewed interest in whole-organism vaccination strategies [22,23]. Indeed, most licensed vaccines against other infectious diseases are based on killed or live attenuated whole organisms (http://

www.cdc.gov/vaccines/). It has been shown in studies with rodent and primate models of malaria and in experimental clinical studies with humans, that sterile immunity could be achieved by immunization with live radiation-attenuated sporozoites (RAS) [24-26], which has constituted the gold standard for the malaria vaccine research field. In rodent models of malaria (both *P. berghei* and *P. yoelii*), similar or even better levels of protective immunity have been achieved by immunization using genetically-attenuated sporozoites [27]. These genetically attenuated parasites (GAP) in which critical genes in liver stages are deleted (GAP_{LS}), after the invasion in hepatocytes, abort liver stage development at different time points, inducing CD8+ T-cell immunity that are capable of killing liver stage parasites, which is comparable to RAS [28], (see Section 3.1). In addition to immunization with attenuated sporozoites, it has been demonstrated that both in rodent and primate models, partial to full protective immunity can be achieved through immunization with either killed or radiation attenuated blood stages or with low doses of viable blood stage parasites followed by drug cure (reviewed in detail [23,29]). Moreover, it has been shown in humans that experimental blood stage immunity can be induced by repeated administration of ultra-low doses of infected RBC under drug cover, which induces CD4+ T-cell based protective immunity [30].

The renewed interest in whole parasite malaria vaccine strategies, due to the lack of significant progress on subunit vaccines, not only exists in developing a vaccine consisting of attenuated sporozoites, but also in exploring possibilities for inducing protective and long-lived immunity by immunization with whole blood stages (see Section 3.2). It has recently been shown in rodent malaria models that immunity against malaria can be achieved by immunization using genetically attenuated blood stage parasites (GAP_{BS}), which are growth and/or virulence attenuated through genetic modification [31-34]. Infection of mice with these GAP_{BS} resulted in self-resolving infections, and after a single infection with these parasites, all convalescent mice were protected against subsequent parasite challenge for prolonged periods. These results show that infection of mice with growth and virulence attenuated malaria parasites can induce long-lasting protective immunity [31-34], (see also Section 3.2). The major advantages of immunization with GAPs over immunization with radiation attenuated parasites are that they constitute a homogeneous population of parasites with defined genetic identity and attenuation phenotype [35]; moreover, gene insertion techniques also permit the introduction of transgenes into GAP genome that may enhance attenuation phenotype and/or improve the potency of the vaccine [35].

The aim of this study is to identify additional rodent GAPs that demonstrate growth- and virulence-attenuation of their blood stages, specifically GAP_{BS} that shows only short,

low-parasitemia blood infection and that can induce protective immune responses (see Section 3.2 and 5). In order to screen for a larger array of GAPs, we first aimed at improving methods for both the generation of genetically attenuated parasites and for analysing blood stage growth attenuation of GAP$_{BS}$ (see Section 5).

3. Whole parasite based vaccine approaches against *Plasmodium*

Most of the licensed human vaccines available today belong to one of three categories— live attenuated microbes (e.g. measles, mumps), killed/inactivated microbes (e.g. Polio, rabies) or protein subunit/conjugate (e.g. Hepatitis B, HPV) (http://www.cdc. gov/vaccines/). A large number of subunit-vaccine candidates against *Plasmodium* malaria parasites have been tested in animal models and humans, mainly as a protein (antigen) formulation or expressed by a (DNA or viral) vector system in order to generate protective immunity [36]. Most malaria antigens that have been selected as subunit- vaccine candidates have been characterized as targets of natural immunity, most often associated with strong humoral responses [37]. However, the most advanced leading subunit pre-erythrocytic vaccine candidate RTS,S showed only limited efficacy as in Phase III testing with clinical malaria episodes in children being reduced by only 30—50% [20,21]. Progress on clinical trials of blood stage subunit-vaccines has been slow: the testing of more than 10 candidate subunit vaccines targeting *Plasmodium* blood stages have not progressed to or further than Phase 2 trials, with only 3 candidates having reached Phase 2b trials [38]. The limited success with subunit-vaccine development has renewed interest in developing vaccines consisting of whole, killed or attenuated, parasites [23,35]. While sustained and sterile immunity has been achieved using live liver stage parasites attenuated by radiation or genetic modification or administered under curative doses of chemoprophylaxis [35,39,40], full protective immunity with either killed sporozoites or blood stage parasites have so far been unsuccessful [29,39].

3.1. Immunization with attenuated sporozoites

In 1967, a study using *P. berghei* in rodents demonstrated that complete immune protection can be achieved by delivering live but attenuated sporozoites damaged by a specific dose of irradiation [24]. In contrast to killed sporozoites, which induce strong humoral responses, mostly to the major sporozoite surface protein—CSP, radiation attenuated sporozoites infect hepatocytes but fail to replicate and elicit antibody and cellular responses against sporozoites and infected hepatocyte [41]. Long lasting sterile

protective immunity induced by irradiated sporozoites was not only demonstrated in animal models of malaria, but also importantly in humans where malaria naïve volunteers were immunized by bites of infected mosquitoes delivering irradiated *P. falciparum* sporozoites [26,42,43]. These findings provided the paradigm for a whole organism malaria vaccination, demonstrating that complete protective immunity against a malaria infection was achievable.

In rodent models of malaria, similar or even higher levels of protective immunity has been achieved by immunization using genetically attenuated sporozoites [27,35]. These liver-stage genetically attenuated parasites (GAP$_{LS}$) were created by targeted deletions of genes that result in developmental arrest in liver stage after invasion of attenuated sporozoites and have been shown to induce high level of sterile protective immunity in mice [35,44-46]. Techniques to create multiple gene deletion could ensure the safety of GAP$_{LS}$, specifically to ensure that any parasites that are able to survive without the presence of one gene are be unable to develop without the presence of another. Moreover, genes have been identified that when removed create GAP$_{LS}$ that arrest at late into liver development and induce protective immunity that is better than that induced by irradiated sporozoites [27]. Equivalents of some of the liver stage GAP$_{LS}$ candidates have been also generated in the human parasite *P. falciparum*, and importantly, showed similar phenotypes. For example, a *P. berghei* GAP$_{LS}$ lacking P36p expression arrests in liver stage and can confer long lasting protective immunity in mice [44], and a *P. falciparum* GAP lacking expression of the same gene (termed P52 in *P. falciparum*) also arrests during liver stage development [47].

Up until now, it has only been possible to establish *P. falciparum* infections in humans via the bite of infected mosquitoes. To overcome this limitation for vaccine delivery, the company Sanaria Inc. has produced aseptic sporozoites that can be administered to humans by needle inoculation. These sporozoites are reared in sterile mosquitoes and have been purified and cryopreserved, and formulated for use in humans in compliance with all regulatory requirements [48].

3.2. Immunization with attenuated or killed blood stage parasites

Whole *Plasmodium* blood-stage formulations used for immunization have generally consisted of iRBC. These formulations have included killed parasites in adjuvant, radiation-attenuated iRBCs or infection with wild-type iRBC administered under curative doses of chemotherapy, and they have been used to immunize both rodents and primates [23,29,49,50]. The results of these immunizations, while varied in their protective efficacies for the different combinations, have demonstrated protective immunity

including complete protection against a challenge with wild-type parasites. Furthermore, in an immunization study in humans, evidence was found for the generation of complete protective immunity against *P. falciparum* that was achieved via repeated inoculations of ultra low numbers of iRBC (~30), resulting in sub-patent infections that were controlled by curative dose of chemotherapy [30]. These studies were remarkable in that not only they showed that immunization with whole blood stages can induce complete protection in humans, but also that protective immunity could be achieved using only limited amounts of parasite material and in the absence of a major antibody response [23].

Currently practical limitations exist for immunization strategies that require humans be infected with parasites inside RBCs, either killed or attenuated. For example it is unclear if regulatory authorities would approve, as part of a mass vaccination program, the intravenous administration of infected red blood cells to humans [23,37]. Nonetheless, such studies can provide important insights into how protective immune responses can be induced and maintained against *Plasmodium* blood stages [51,52]. Similar to immunization studies using genetically attenuated parasites that arrest in the liver (GAP$_{LS}$) [35], studies into blood-stage vaccination would clearly benefit from creating genetically attenuated blood stage parasites (GAP$_{BS}$) in animal models that induce limited, self-resolving infections that are virulence-attenuated and that can provoke strong and long-lasting immunity without the induction of malarial symptoms or additional pathologies. Such parasites can be instructive tools to uncover important correlates of protection and disease, and to better understand how iRBC are detected and eliminated by host immune response.

A number of gene-deletion mutants generated in both rodent and human parasites have been reported that exhibit moderate to severe reduction in their blood-stage multiplication rates. However, the first growth- and virulence-attenuated GAP$_{BS}$ was only recently reported for the rodent model malaria parasite *P. yoelii* YM (a lethal strain); this GAP$_{BS}$, which lacks the gene encoding purine nucleoside phosphorylase (PNP), is virulence-attenuated and produces a self-resolving infection in mice [31]. Importantly, after a single infection with this parasite, all convalescent mice were protected against subsequent wild-type parasite challenge for prolonged periods (>5 months). Since then, other rodent malaria GAP$_{BS}$ have also been reported which show growth- and virulence-attenuation and that induce self-resolving infections after which mice are protected against wild type challenge. These include a GAP$_{BS}$ in *P. yoelii* XNL (a non-lethal strain) that lacks the gene encoding nucleotide transporter 1 (NT1), which is strongly attenuated and generates a self-resolving infection in mice [33]. Other GAP$_{BS}$ characterized in the rodent model *P. berghei* include GAP$_{BS}$ lacking expression of rhomboid 1 [53], plasmepsin-4

(PM4) [32], and a GAP$_{BS}$ that lacks both PM4 and MSP7 (a merozoite-specific protein) [34]. These GAP$_{BS}$ that have been created in *P. berghei* ANKA do not cause experimental cerebral malaria (ECM) in ECM-susceptible mice [34]. These studies show that not only is it possible to generate growth- and virulence-attenuated blood stage parasites by targeting specific genes in the parasite genome, but also that strong and long-lasting protective immune responses can be induced in mice that have resolved their infections. However, despite growth- and virulence-attenuation, most of the reported GAP$_{BS}$ still produce infections with relatively high parasitemias (parasite loads). An ideal GAP$_{BS}$ should result in infections with low parasitemias that spontaneously resolve shortly after parasites are introduced into the blood. An infection with low (sub-patent) parasitemias was only achieved with low dose inoculation of Δ*nt1* mutant generated in non-lethal *P. yoelii* XNL in mice [33]. This sub-patent, self-resolving infections generated strong cellular and humoral immune responses that provided complete protective immunity in BALB/c, C57BL/6 and SWISS mice [33]. However, this mutant was not created in a virulent rodent parasite line (i.e. *P. yoelii* YM or *P. berghei* ANKA), where the kinetics and virulence phenomena of a gene-deletion mutant might be substantially different.

As the research in rodent malaria model can serve as a template to create *P. falciparum* GAPs to be used as live-vaccines in humans, the research on generation of GAP$_{BS}$ is better focused on identification of GAP$_{BS}$ in normally virulent rodent *Plasmodium* parasite strains. The double gene-deletion mutant Δ*pm4*/Δ*msp7* showed greater growth- and virulence attenuation than either of the single gene-deletion mutants, Δ*pm4* and Δ*msp7* [34]. The Δ*pm4*/Δ*msp7* infections could be resolved in BALB/c, C57BL/6 and CD1 mice, whereas only BALB/c mice could survive Δ*pm4* infections. However, creating an equivalent *pm4* and *msp7* deficient *P. falciparum* parasites is complicated, as it is not clear what the functional *msp7* ortholog in *P. falciparum* is, being a member of a multigene family [34]. Therefore, up to now, there is no ideal GAP$_{BS}$ that could be translated to *P. falciparum*.

4. Genetic modification of malaria parasites

Reverse genetic technologies have been widely applied to gain an understanding of *Plasmodium* gene function and to provide an insight into the biology of malaria parasites and their interactions with the host [54-56]. Targeted gene disruption and loss-of-function analyses have provided insight into *Plasmodium* gene function and biology, and protein-tagging methodologies have helped reveal the pattern of expression, localization and transportation of *Plasmodium* proteins. Furthermore, reverse genetics is increasingly being used to generate parasites that express transgenes encoding heterologous proteins, for example fluorescent and/or luminescent reporter proteins. Such reporter parasites

have been instrumental to visualize and analyse parasite-host interactions, in real-time, *in vitro* and *in vivo* [57-59]. Transgenic parasites expressing luciferase have also been used to develop sensitive and simple assays to measure blood and liver stage parasite drug sensitivity both *in vitro* and *in vivo* [60-62]. Genetic modification technologies have been developed for different *Plasmodium* species (*P. falciparum*, *P. knowlesi*, *P. cynomolgi* and three rodent parasites *P. berghei*, *P. yoelii* and *P. chabaudi*) [54,63]*.* The availability of efficient reverse-genetic technologies for the rodent parasites *P. berghei* and *P. yoelii* and the fact that these parasites can be followed throughout their complete life cycle in laboratory conditions, both *in vitro* and *in vivo*, have made them the most frequently used animal malaria models for so called functional genomics, helping ascribe functions to *Plasmodium* genes, and also for analysing host-parasite interactions through the use of transgenic parasites expressing heterologous proteins such as GFP or luciferase [54].

4.1. Generation of attenuated rodent malaria parasites by genetic modification

Genetic modification of the malaria parasite has not only been used to understand the of *Plasmodium* biology and to analyse parasite-host interactions, but also to generate GAPs (as mentioned above). These mutants permit us to explore the possibilities of whole organism vaccines against malaria. Specifically, by deleting genes from the *Plasmodium* genome critical for liver stage development, GAP_{LS} have been produced that arrest during development in the liver and induce strong protective immunity (see Section 3.1). Similarly, by deleting genes that play an important role during blood-stage growth and/or multiplication, GAP_{BS} have been generated that are both growth- or virulence-attenuated (See Section 3.2). For these purposes, the rodent models *P. berghei* and *P. yoelli*, are frequently employed to generate and analyse GAPs, both for liver and blood stages.

4.2. Generation of transgenic *Plasmodium* parasites expressing heterologous proteins

The creation of parasites that express heterologous proteins, most commonly fluorescent or luminescent reporter proteins (e.g. GFP, luciferase etc) either by themselves or fused to *Plasmodium* proteins, are now being routinely used to investigate parasite protein localization and interactions (http://www.pberghei.eu). Reference reporter parasites, which express reporter proteins either constitutively at high levels or in a stage-specific manner, are now invaluable tools in *Plasmodium* research. For example, GFP-expressing parasites have been used in conjunction with flow cytometry to provide quantitative information on the parasites development in both red blood cells [32] and hepatic cells [64]. Transgenic parasites expressing luciferase have been used to unravel and monitor

the pattern of sequestration in live mice [65,66], to visualize and quantify the *in vivo* liver stage development [62] and to screen and evaluate antimalarial activity of drugs both *in vitro* and *in vivo* [60-62]. Other transgenes have also been introduced into *Plasmodium* genome to dissect the host-pathogen interaction. Model antigen OVA (Ovalbumin) is used to examine immune responses of antigen-specific CD8+ T cells during malaria infection [67,68]. Creation of GAPs in these references lines could allow better examination and evaluation of their efficacy and potency, and this requires multiple genetic manipulation of the parasite genome. Therefore, it is has become increasingly important to develop a robust and versatile transfection system to both introduce genes and re-cycle selectable markers that permit subsequent transfections.

The application of reverse genetics in *P. berghei* and *P. yoelii* for generation of gene-deletion mutants or transgenic parasites is however restricted by the limited number (only 2) of drug resistance genes (permitting the selection of transformed parasites) that are currently available. This low number of selection markers hampers multiple genetic modifications in the genome of the same parasite line. In order to circumvent this problem, GFP has been utilized as a selection marker, permitting the selection of transformed parasites by flow cytometry [69,70]. In addition, a method has been developed for removing drug-selection markers from transformed *P. berghei* parasites by utilizing the yeast *fcu* (y*fcu*) selection marker and negative selection with the drug 5-fluorocytosine (5-FC) [71]. Both the selection of GFP-expressing mutants by flow cytometry and selection of marker-free mutants by negative selection have their limitations. They are laborious and time consuming, and also require the use of many extra animals as additional cloning steps in mice are required [72]. In Chapter 2, we present a novel transfection method in two rodent malaria parasites, *P. berghei* and *P. yoelii*, which generate transgenic mutants ready for subsequence genetic modification easily and quickly.

5. Outline and structure of this thesis

The main purpose of the studies described in this thesis was to generate and characterize genetically modified *P. berghei* ANKA parasites that are growth- and virulence-attenuated during blood stage development in mice. The availability of such GAP_{BS} parasites permits us to explore the possibilities for the use of these GAP_{BS} for inducing protective immune responses in *vivo* in mice. In addition, studies of infections with growth- and virulence-attenuated parasites may also provide insights into the development of protective immunity, the correlates of protection and disease, as well as increase our knowledge of parasite factors that underlie malarial pathology. This knowledge may help to frame studies that explore the use of whole blood stages of the human parasite *P. falciparum*

to induce strong and long-lasting protective immunity. Several GAP$_{BS}$ have already been reported in both *P. yoelii* and *P. berghei* virulent strains, however the disadvantage of these GAP$_{BS}$ is that they still result in infections with relatively high parasitemias (see Section 3.2). We therefore decided to perform a large screening study to identify additional GAP$_{BS}$ with a more severe growth- and virulence-attenuated phenotype (see below for a rationale for the choice of the different genes we have targeted for gene deletion in order to generate GAP$_{BS}$).

In order to improve and speed up the generation of gene-insertion or gene-deletion mutants necessary for large screening assays, we sought to improve the existing transfection methods. In **Chapter 2**, we report on the development of a novel 'gene insertion/marker out' (GIMO) method in two rodent malaria parasites, *P. yoelii* and *P. berghei*, which uses negative selection to rapidly generate transgenic mutants ready for subsequent modifications. This method greatly simplifies and speeds up the generation of mutants expressing heterologous proteins, free of drug-resistance genes, and requires far fewer laboratory animals. It can be used to rapidly and more easily generate reporter parasites useful for phenotype characterization, and it also facilitates the generation of reporter parasites expressing multiple transgenes and GAP$_{BS}$ in which multiple genes have been deleted. In addition to improving methods for generation of GAP$_{BS}$, we improved the assays for analysing *in vitro* and *in vivo* growth kinetics of GAP$_{BS}$. The improved protocols of these assays, which were based on published methods to analyse growth and drug-sensitivity of blood stage and liver stage parasites [62,73], are described in **Chapter 3**. These optimised assays for analysing drug-sensitivity of *Plasmodium* blood stages to newly-developed drugs are deployed by other groups and their use has also been published [74,75].

In **Chapter 4**, we present systematic gene deletion analyses of all eight *Plasmodium* rhomboid-like proteins as a means to screen for mutants with a growth- and virulence-attenuated phenotype. Rhomboid proteases cleave membrane-anchored proteins within their transmembrane domains, and in apicomplexan parasites, these substrates include molecules involved in parasite motility and host cell invasion [76-79]. Understanding the biological functions of apicomplexan rhomboids is an active area of research and the critical roles have been identified for several of these proteases in host cell invasion and pathogenesis [76-78]. Rhomboid 1 and 4 of *Plasmodium* have been reported to play critical roles in host-cell invasion [53,80,81], through cleavage of various parasite adhesins [79]. Both *P. berghei* and *P. yoelii* mutants lacking rhomboid 1 were reported to have reduced growth rate in asexual blood stages and are virulence attenuated [82,83]. To confirm this phenotype of *rhomboid 1* mutant and to identify additional growth-attenuated mutants,

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we targeted all 8 rhomboid genes for deletion and characterized the phenotypes of the gene deletion mutants throughout the whole parasite life cycle.

In **Chapter 5** we present systematic gene deletion analyses for 12 enzymes with a possible role in hemoglobin digestion and hemozoin formation, including all 8 predicted hemoglobinases in *P. berghei*. All clinical symptoms of a malaria infection are associated with growth of *Plasmodium* parasites inside RBCs, where the parasite ingests and catabolises more than half of the host hemoglobin (Hb) [84,85]. Because *Plasmodium* has a limited capacity to synthesize amino acids *de novo*, Hb digestion is believed to be essential in supplying the parasite with amino acids [86]. During Hb degradation, free heme is released, which is cytotoxic and is rapidly detoxified by the parasite through polymerization into inert crystals known as hemozoin (Hz). Hz is released into the circulation during schizont rupture and is rapidly removed by phagocytosis by cells in the liver and spleen. Upon host-cell phagocytosis, Hz cannot be further degraded and persists for some time in host tissues, and it has long been considered as a virulence factor. Indeed, the number of pigment containing leukocytes in the peripheral blood correlates with disease severity in *P. falciparum*-infected patients [87,88], and several inflammatory and immunomodulatory effects of Hz have been reported (reviewed in [89,90]). Interestingly, evidence has been presented that both *P. falciparum* and *P. berghei* mutants lacking *plasmepsin 4* have reduced Hz production and are reduced in their growth rates [32,91]. Furthermore, *P. berghei* mutants lacking *pm4* in mice are also virulence attenuated [32]. In the studies described in chapter 5 and 6, we generated additional gene deletion mutants lacking different hemoglobinases (some in combination) to screen for mutants with a more severe reduction in growth and reduction of Hz production.

In **Chapter 6** we present experiments where we targeted a total of 41 *P. berghei* genes in this study, in order to generate genetically attenuated blood stage parasites that are growth- and virulence- attenuated (i.e. GAP_{BS}), and that may serve as immunogens and as tools to study protective immunity. Using GAP_{BS} generated in this study and studies described in Chapter 4 and 5, we examined their infection and virulence characteristics by assessing experimental cerebral malaria (ECM) in C57BL/6 mice and the development of hyper-parasitemia in BALB/c mice.

In **Chapter 7** the studies of the Chapters 2–6 are summarized and discussed, including a critical evaluation of the use of rodent malaria models for generation of GAP_{BS} and analysing their growth- and virulence-attenuated phenotypes.

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