CRISPR/CAS9 genetic modification of plasmodium falciparum and transgenic parasites in malaria vaccine research

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CHAPTER 1

Introduction

Malaria, the parasite and disease

Malaria is a vector-born disease of global health importance [1] with 216 million cases in 91 countries in 2016 resulting in around 445.000 deaths [2]. The greatest burden of malaria is in sub-Saharan Africa, where it takes the lives of more than 1,200 children each day [2]. Malaria is caused by a protozoan unicellular parasite, *Plasmodium*, which is transmitted by *Anopheles* mosquitoes. Five *Plasmodium* species are responsible for malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [3]. Most clinical cases are caused by *P. falciparum* and *P. vivax*, with *P. falciparum* being the deadliest [1, 3]. *P. falciparum* infections can cause severe anaemia, fever and organ damage, including cerebral complications; in contrast, *P. vivax* infections are usually not fatal but can be severe with recurrent clinical episodes of malaria associated with morbidity [4]. *P. malariae* and *P. ovale* infections are less well studied but the severity of illness caused by these parasites is similar to *P. vivax* malaria [4]. *P. knowlesi* is primarily a zoonotic infection encountered in Southeast Asia that can cause severe malaria [5].

The P. falciparum life cycle

Figure 1 depicts the life cycle of *P. falciparum*. A malaria infection in humans begins with the inoculation of *Plasmodium* sporozoites into the host dermis by the bite of an infected female *Anopheles* mosquito [4, 6]. The sporozoites can take 1-3 hours to exit from this site. Here they rely on gliding motility to penetrate a blood vessel, entering the blood stream and migrating to the liver [7]. Sporozoites recognise hepatocytes and infect these cells after the sporozoite become activated through a mechanism that involves interactions of host and parasite membrane proteins. Entry is gained by proteins that are released from the apical organs of the sporozoite, specifically the micronemes and rhoptries. Once hepatocyte infection is established, the parasite grows and divides in the next 2-10 days. These liver-stage (LS) or exo-erythrocytic forms (EEF) mature and release up to 40.000 merozoites per infected hepatocyte into the blood stream [3]. Once released into the circulation, the merozoites invade erythrocytes, where they grow, divide and form new merozoites, which upon release invade new red blood cells, initiating the repeated asexual replication cycles. All symptoms of malaria are associated with the blood-stage infection [8]. Within a red blood cell *P. falciparum* parasites progress over the course of 48 hours through the ring and the trophozoite stage before replicating into 8-32 merozoites at the schizont stage (schizogony) [1]. During the schizogony cycles in red blood cells, a proportion of parasites stop asexual division and undergo a developmental switch, initiating sexual development by development into male or female gametocytes (gametocytogenesis). These gametocytes mature through five defined stages over the course of 8-11 days [1, 9]. Once ingested by a mosquito the mature male and female gametocytes emerge from the red blood cell and rapidly produce gametes (gametogenesis), with the male gametocyte dividing into eight flagellated microgametes (exflagellation) and the female gametocyte developing into a single macrogamete. The female gamete is fertilised by

the male in the mosquito midgut, resulting in a diploid zygote, which elongates into an ookinete that penetrates the mosquito gut wall. The ookinete develops into an oocyst that undergoes cycles of replication to form several thousand sporozoites (sporogony), over a period of 9-12 days. These oocysts then burst to release the sporozoites that migrate to the salivary glands, resulting in mosquitoes that are infectious to humans [9, 10].

Malaria, the health problem

Malaria incidence and transmission depends on environmental suitability for local mosquito vectors, which includes altitude, climate, vegetation, and implementation of control measures. The intensity of the host-vector-host transmission depends on factors related to the parasite, the vector, the human host and the environment [11]. Currently, half of the world's population is at risk of malaria, with some population groups at higher risk of developing severe disease than others. These include infants, children under 5 years of age, pregnant women and immunosuppressed patients [2]. Early diagnosis and treatment reduces disease, prevents death and directly contributes to a reduction in malaria parasite transmission [4, 11]. However after considerable global success in malaria control over

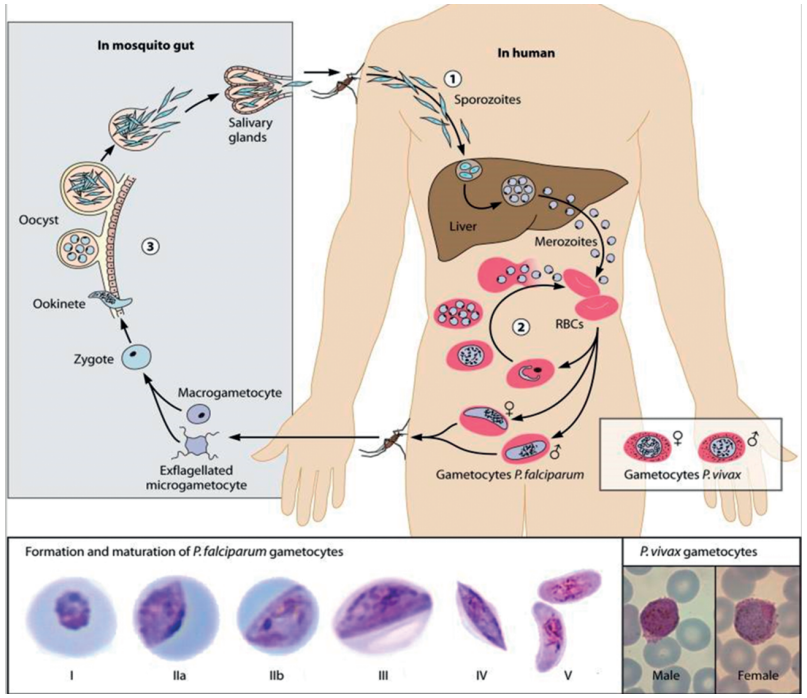

Figure 1. Schematic representation of the *P. falciparum* life cycle. Upper panel, *P. falciparum* development in mosquitoes (grey box) and in humans. Lower panel, the five developmental stages of *P. falciparum* gametocytes and mature *P. vivax* gametocytes. This image was taken from Bousema, T., et. al. (2011)[10].

the past 10-15 years, progress has now stalled according to the WHO World Malaria report 2017 [2]. A major problem is insufficient funding at both domestic and international levels, resulting in gaps in coverage of the use of insecticide-treated nets, antimalarial drugs, indoor residual spraying (IRS) with insecticides and other lifesaving tools [2]. Moreover, there is a global increase in resistance to front-line antimalarial drugs, such as chloroquine, sulfadoxine/pyrimethamine [12] and recently to artemisinin [13]. This highlights the need for novel drug and vaccine intervention programs against both the parasite and vector. In addition to novel insecticides and approaches to reduce transmission by mosquitoes, the identification of new classes of drugs as well as vaccines that target different stages of the parasites are required to protect the groups most at risk [2, 12] and to develop the most cost-effective means to prevent, eliminate and eradicate malaria [11].

Malaria, vaccine and drug development

Malaria vaccines are generally classified in different types of vaccines, defined by the different life-cycle stages of the parasite that are targeted by the vaccine. Specifically, (1) pre-erythrocytic vaccines, which induce antibodies and/or cell-mediated immune responses that block sporozoite invasion of hepatocytes or remove infected hepatocytes [11]; (2) blood-stage vaccines that are designed to block merozoite invasion of red blood cells or to eliminate infected red blood cells [12]; and (3) transmission blocking vaccines (TB vaccines) that generate antibodies that can block transmission of parasites in the mosquito by either blocking parasite fertilisation or zygote development. Pre-erythrocytic vaccines have been shown to prevent infection and can induce sterile protection against malaria. Blood-stage vaccines are likely to reduce the overall parasite burden in the blood and therefore reduce malaria symptoms and TB vaccines may be an effective means to reduce the spread of malaria within a population [12].

Currently, the most extensively tested vaccine candidate for prevention of *P. falciparum* malaria is RTS,S/AS01 (RTS,S; also known as Mosquirix), a pre-erythrocytic vaccine candidate [14]. This subunit vaccine targets the sporozoite and the infected liver cell and is based on the immunodominant antigen that covers the surface of the sporozoite, circumsporozoite protein (CSP). In RTS,S the CSP fragment is fused to hepatitis B virus surface antigen and administered with the adjuvant AS01 [14]. This is the only vaccine that has shown protective efficacy against clinical malaria in a Phase III clinical trial, but protective efficacy is modest and wanes over time and may be age dependent [15]. In vaccine development against *P. vivax* the CSP protein is also seen as an important vaccine target since evidence from pre-clinical and clinical studies has indicated that immune responses against *P. vivax* CSP play a role in mediating protection against *P. vivax* infections [16].

In addition to CSP, several other antigens of sporozoites and liver-stages have been identified as target antigens for subunit vaccines, for example CelTOS (cell traversal protein for ookinetes and sporozoites) and TRAP (thrombospondin-related adhesion protein). Recombinantly produced CelTOS of *P. falciparum*, a micronemal secreted-

protein, is one of the few vaccines that has recently entered clinical testing (NCT01540474 https://clinicaltrials.gov/). A viral-vectored sub-unit vaccine directed against TRAP fused to a multi-epitope string, demonstrated some protection against malaria infection in malaria-naïve adults [17].

Most research on the development of asexual blood-stage vaccines has focused on only a few antigens, for example merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1). Antibodies against both proteins correlate with naturally-acquired immunity in multiple epidemiological studies and vaccines targeting these antigens induced protective immune responses in preclinical studies in rodents [18]. However, although some studies performed in humans have shown some efficacy, no blood-stage vaccine has reached phase III testing [19]. It is important to consider the challenges that have faced the development of blood-stage vaccines, such as high levels of antigenic polymorphism and redundant pathways of invasion of red blood cells by merozoites. Different preclinical assays are used to predict the efficacy of blood-stages vaccines [19, 20]. The growth inhibition assay (GIA) is one of the most widely used functional assays to test interventions against asexual blood-stage development. In vaccine-based studies, blood-stage parasites are co-cultured with either control or test antibodies and the percentage of reduction in parasitemia is measured after a defined culture period [19].

For TB-vaccines, the leading target antigens include the ookinete surface protein Pf25 and the gametocyte/gamete antigens Pf48/45 and Pf230. Antibodies against these antigens perform well in inhibition of transmission in comparative preclinical studies, with functionality assessed by the standard membrane feeding assay (SMFA) using mosquitoes that are fed with cultured gametocytes in the presence of antibodies, whole serum or purified IgG [21]. The most advanced TB vaccine candidates are based on the antigens P48/45 of gametes and P25 of zygotes and ookinetes and recombinant vaccines of P25 of both *P. falciparum* and *P. vivax* have progressed into Phase I trials [22-24].

Despite three decades of testing different (recombinant) sub-unit vaccines, both in the clinic and the field, only modest protection against infection has been achieved [15, 25-27], which has renewed an interest in whole parasite-based vaccine approaches [28, 29]. It was first shown in rodent models of malaria that complete protection against infection could be obtained by vaccination using live attenuated sporozoites [30, 31]. Subsequently, sterile protection against malaria was also demonstrated in humans after immunization with *Plasmodium falciparum* sporozoites, either attenuated by radiation [32, 33] or administered under chemoprophylaxis [34]. A prerequisite for induction of protective immunity using sporozoite-based vaccines is that sporozoites retain their capacity to invade liver cells after administration. 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 [35]. The most advanced live-attenuated vaccine is based on irradiation-attenuated sporozoites (Irr-Spz), which is currently being evaluated both in the clinic and in field trials [36, 37]. In rodent models, immunization

with sporozoites of genetically-attenuated parasites (GAP) can induce similar or even better levels of protective immunity compared to Irr-Spz [35, 38]. 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. Currently two *P. falciparum* GAP-based vaccines are undergoing clinical evaluation [38-42].

While the search for an effective vaccine against malaria remains a very active area of research, the most effective means to treat and prevent malaria remains the use of drugs [43]. However, resistance to available antimalarials continues to spread, including resistance to the widely used artemisinin-based combination therapies [44]. As multi-drug resistance spreads, there is an urgent need for new antimalarial agents to control malaria infections [43]. At the forefront of antimalarial development is the Medicines for Malaria Venture (MMV), a not-for-profit, public–private partnership (www.mmv.org). The current MMV portfolio contains many promising compounds at various stages of development [43]. New classes of antimalarial compounds have been identified in high-throughput screens of large compound libraries [45]. Most of such screens involve the exposure of different life cycle stages of *P. falciparum* parasites to the compounds and the measurement of inhibition of development. For example in short-term cultures of the blood-stages and determination of inhibition of blood-stage growth and multiplication. Such screening of large compound libraries requires highly reproducible and cost-effective assays that are amenable to automation and can be performed in a small culture volumes [43, 45].

Malaria elimination is likely to require a combination of interventions, including the generation, testing and implementation of new drugs and vaccines as well as new vector control strategies.

Genetically modified malaria parasites and their use in malaria research: the aim of the studies described in this thesis

In the mid-nineties, genetic modification to create permanent modifications in malaria parasite genomes was first described in the rodent malaria parasite *Plasmodium berghei* [46]. 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 [47, 48]). In addition to gene disruption and gene mutation, methodologies have been developed for creating malaria parasites that express 'foreign' genes from other organisms, so-called transgenic parasites. Among the first transgenic mutants were rodent malaria parasites (RMP) that were modified to express fluorescent and luminescent reporter proteins. These parasites have been used to visualize and analyse parasite growth and development *in vitro* and *in vivo*, and have been valuable tools to

analyse cellular and molecular aspects of malaria parasite biology (reviewed in [49-52]), and to study host-parasite interactions and pathology [53-58]. In addition, transgenic rodent parasites have been used to develop and evaluate vaccines (reviewed in [59]). For example, chimeric RMP expressing *P. falciparum* or *P. vivax* antigens have been used to directly evaluate human malaria vaccines before their advancement to clinical testing.

Transgenic parasites expressing fluorescent or luminescent reporter proteins have also been created in the human parasite *P. falciparum* and the primate parasite *P. cynomolgi*. These transgenic parasites have been exploited in screening assays to measure (inhibition of) parasite growth at different points of the parasite life cycle. GFP- and luciferase-expressing *P. falciparum* parasites have been used *in vitro* to examine the effect of drugs and other inhibitors on blood-stage growth and on gametocytes [51, 60-63] and fluorescent *P. cynomolgi* parasites have been generated to screen for compounds that target the hypnozoite stage in the liver [64].

For RMP, the availability of transgenic parasite lines expressing different reporter proteins under the control of stage-specific or constitutive promoters has been of great benefit to research of parasite gene function and on research focused on evaluation of novel drugs and vaccines. The availability of similar *P. falciparum* transgenic reporter lines would open up possibilities to perform these studies directly with the human malaria parasite. For example, strongly fluorescent liver-stage *P. falciparum* parasites could create possibilities for enriching infected hepatocytes by flow-sorting methods, which would aid identification of novel vaccine targets, or mCherry-expressing *P. falciparum* parasites could be used to analyse parasite interactions with host cells (e.g. sporozoites with cells of the immune system or hepatocytes). Increasingly, cell-cell interactions in culture are examined using transgenic host cells expressing, for example, green fluorescent protein; therefore, the availability of transgenic *P. falciparum* parasites expressing different fluorophores can boost such studies. The creation of transgenic RMP expressing more than one transgene has permitted more elaborate and intricate studies on parasite biology and immunity. For example, fluorescent parasites that also express the immunological reporter antigen ovalbumin have been used to better understand how parasite antigens induce protection by examining interactions of infected hepatocytes with anti-OVA OT-1/2 T-cells [55]. The creation of transgenic parasites stably expressing multiple transgenes is dependent on the presence of multiple suitable target loci in the parasite genome, which can be modified without altering parasite growth and development. In *P. falciparum* the *p47* gene locus has most frequently been used to introduce transgenes [65, 66]. Therefore, the identification of other suitable 'neutral' genomic loci would greatly aid in the generation of mutants expressing multiple transgenes.

The main aim of the studies described in this thesis was to develop novel CRISPR/Cas9 methodologies to improve *P. falciparum* transgenesis. This was done in order to create novel transgenic reporter parasites that can be used to analyse host-pathogen interactions and for anti-malarial drug and vaccine research. We first focused on improving CRISPR/Cas9 gene editing technology and on introducing transgenes into the *P. falciparum* genome using a new potential 'neutral' locus. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that either express fluorescent-luminescent reporters or express a major vaccine candidate from the other major human malaria parasite, *P. vivax*. The outline of the different studies is explained in more detail below, as well as the rationale for the different approaches taken to generate these transgenic parasites.

Outline of this study

In **Chapter 2** we provide a review on the use of transgenic malaria parasites in the development of malaria vaccines targeting different stages of the parasite life-cycle. While transgenic *P. falciparum* parasites have been used in studies to evaluate both antimalarial drugs and vaccines, the majority of the studies use transgenic RMP, for which a greater number of techniques is available to genetically modify and examine the parasite throughout the complete life cycle.

Improved CRISPR/Cas9 genetic modification of P. falciparum (Chapter 3)

For rodent malaria parasites (RMP) efficient technologies have been developed for stably introducing transgenes into the parasite genome and efficient and rapid methods are available for the generation of transgenic reporter parasites that do not contain drug-selectable markers [67, 68]. Such 'marker-free' parasites make it considerably easier to further genetically modify transgenic parasites; moreover, they can be used for drug-sensitivity testing, as possible interference from an introduced drug-selection marker is absent. For RMP, a variety of transgenic reporter parasite lines have been generated in multiple strains of three different *Plasmodium* species [69]. In comparison to RMP, the technologies to genetically modify the human malaria parasite *P. falciparum* are much less efficient [48]. Traditional approaches to engineer the *P. falciparum* genome have been hampered by the limited methods available and transfection inefficiencies for introducing exogenous DNA into the parasite genome. Also, the limited number of drug-selectable markers restricts genetic engineering of *P. falciparum*; for example, performing sequential genetic manipulations in the same parasite line. Several technologies have been developed for the removal (re-cycling) of drug-selectable markers from the modified parasite genome, specifically using FLP and Cre recombinases [70, 71]. However, the application of these techniques is time consuming, as it can take 4-5 months to generate cloned 'marker-free' genetically modified parasites. The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has transformed genome editing in a wide variety of organisms [72]. This powerful genome editing technique has also been applied to *P. falciparum* and provides efficient methods to manipulate the parasite's genome, such as site-directed mutagenesis, gene disruption and the introduction of transgenes [73, 74]. Generation of *P. falciparum* transgenic reporter parasites would benefit from the availability of standard CRISPR/Cas9 plasmids that

permit the rapid introduction of different transgenes into the parasite genome without permanently integrating a drug-selectable marker cassette. Currently, no cloned reporter lines have been published that are drug-selectable marker free.

In **Chapter 3** we describe studies aimed at improving CRISPR/Cas9 genetic modification for introduction of transgenes into the genome of *P. falciparum* without the inclusion of a drug-selectable marker cassette. We describe the generation of transgenic parasites expressing GFP under control of different *P. falciparum* promoter regions that were selected based on their high (and constitutive) expression in different life cycle stages. The GFP-expression cassettes were introduced into the genome in the *p230p* gene locus, which we predicted to be a 'neutral' locus. We examined and compared the GFP-reporter expression of the three novel transgenic lines at different points during blood-stage development. However, disruption of the *P230p* locus unexpectedly resulted in parasites that could not infect and develop in mosquitoes. The phenotype of the 'gene-deletion' mutants in mosquitoes and the potential role of the P230p protein in mosquito development is described in more detail in **Chapter 4**.

Characterization of P. falciparum mutant (reporter) lines lacking P230p expression (Chapter 4)

In *P. falciparum* the *p47* gene locus has been most frequently used to introduce transgenes into the *P. falciparum* genome [65, 66]. We initially had selected the *p230p* gene locus to introduce transgenes as an alternative to the *p47* gene, since the P47 protein has been shown to be important for limiting the host-defence responses against the parasite in mosquitoes [75, 76] . Consequently, *P. falciparum* parasites lacking P47 expression are less efficiently transmitted by some strains of *Anopheles* mosquitoes, as they have a decreased capacity to escape the mosquito immune response. In two rodent *Plasmodium* species the male-specific P230p protein appears to be dispensable throughout the parasite's complete life cycle [77-79]. *P. berghei* and *P. yoelii* mutants lacking expression of P230p can develop in the vertebrate host and in the mosquito vector without a discernible phenotype and *p230p* knock-out parasites manifest a wild type parasite phenotype. Consequently, as P230p is non-essential, the *p230p* gene is the locus most frequently used to introduce additional transgenes into rodent malaria parasite genomes [78].

P230p and P47 belong to the s48/45 domain 6-cysteine (6-cys) family of *Plasmodium* proteins, a small family with 14 members that show stage-specific expression throughout the parasite life cycle and most members localize at the parasite surface [80]. Most members have critical roles in parasite development, either in the vertebrate host or in the mosquito vector, and several members are leading targets for malaria vaccines. These include vaccine antigens that target parasites in the mosquito, the so called transmission blocking vaccines, i.e. P48/45 and P230 which are paralogs of P47 and P230p, respectively [81-83]. In both *P. berghei* and *P. falciparum* P47 is specifically expressed in female gametocytes/gametes and is located on the surface of female gametes, zygotes and ookinetes [84]. P47 is important in protecting ookinetes from the mosquito's complement-like immune

response in both rodent and human malaria species [76, 85, 86]. In addition, *P. berghei* P47 plays an essential role in the attachment and recognition of the female gamete by the male gamete [77, 85]. In contrast, *P. falciparum* P47 does not play such a crucial role in gamete fertilization [84].

In **Chapter 4** we characterise in more detail some of the transgenic reporter lines we have described in **Chapter 3**, where the reporter cassette had been introduced into the *P230p* locus. Specifically, we examine the phenotype of these parasites during sexual blood-stage development and early mosquito stages.

Generation of a transgenic P. falciparum parasite line expressing fluorescent and luminescent protein in different life cycle stages (Chapter 5)

For RMP the availability of transgenic reporter parasites expressing different fluorescent and luminescent proteins under the control of stage-specific or constitutive has been of great benefit to research of parasite gene function and research focused on evaluation of novel drugs and vaccines. Such transgenic reporter lines for *P. falciparum* would benefit research where *P. falciparum* parasites are used (see the sections above).

In **Chapter 3** we described studies to test different *P. falciparum* promoter-GFP expression cassettes. These studies were performed in order to generate parasite lines that express fluorescent proteins at high levels throughout the complete life cycle. However, given that the insertion of transgenes into the *P. falciparum p230p* locus resulted in parasites that could no longer infect mosquitoes (**Chapter 3 and 4**), we reverted to using the standard 'neutral' *p47* gene locus for introduction of a novel reporter expression cassette. In **Chapter 5**, we describe the creation and evaluation of a reporter line that expresses a fusion of mCherry and luciferase driven by the promoter of the *etramp10.3* gene and examine these transgenic parasites in blood- and liver-stage cultures, as well as in mosquitoes. We selected this promoter because *etramp10.3* has structural similarity to the *uis4* gene of RMP and both genes have the same syntenic genomic location. In transgenic RMP lines the promoter of the *uis4* gene has been used to drive expression of multiple transgenes specifically in sporozoites and liver-stages, such as genes encoding mCherry, ovalbumin or human malaria proteins [87-93]. The *uis4* gene is highly transcribed in sporozoites and liver-stages and encodes a parasitophorous vacuole membrane (PVM) protein that surrounds the parasite in the infected hepatocyte [87]. Evidence has been presented for expression of *etramp10.3* in *P. falciparum* sporozoites and in blood- and liver-stages where the protein is located at the PVM, similar to the PVM location of UIS4 in liver-stages of RMP [94]. We chose to generate an mCherry-expressing *P. falciparum* line, as it could be used to visualise interactions of *Plasmodium* sporozoites with host-cells (e.g. immune cells or hepatocytes) which are often labelled with green fluorescent proteins. Moreover, we fused the mCherry gene to the gene encoding firefly luciferase as luciferase expression can be used to quantify parasite numbers (e.g. sporozoites and liver-stages) using simple and sensitive luminescence assays [62, 95, 96].

Generation of chimeric P. falciparum parasites that express vaccine candidate antigens from the human malaria parasite, P. vivax (Chapter 6)

Testing the next generation of *P. falciparum* vaccines and vaccine formulations is greatly aided by being able to perform immunization studies in people followed by malaria-parasite challenge in controlled human malaria infections (CHMI) [97-100]. CHMI studies have increased the speed of vaccine evaluation by using well-controlled early-phase proof-of-concept clinical studies. Such studies facilitate the down-selection of vaccine candidates and the identification of those candidates most suitable for further evaluation in more expensive and complex phase II/III trials in areas where malaria is endemic.

Although recently CHMI has also been developed for *P. vivax* [101] and has been applied to assess pre-erythrocytic vaccine candidates [102, 103], the use of *P. vivax* CHMI to rapidly screen different *P. vivax* vaccines is limited because of the lack of methods to continuously propagate *P. vivax* blood-stages in culture and to produce gametocytes *in vitro* that can be used to infect mosquitoes to produce sporozoites for challenge infections [101]. Therefore, *P. vivax* CHMI is dependent on sporozoites that have been obtained from mosquitoes fed on infected patients [101]. Moreover, *P. vivax* sporozoites can produce hypnozoites, dormant forms that can persist in the liver for prolonged periods, which requires safe and effective means to clear these forms from the liver in CHMI studies [101, 104].

In preclinical evaluation of vaccines, chimeric rodent malaria parasites (chimeric RMP) expressing *P. falciparum* and *P. vivax* pre-erythrocytic antigens have been used to analyse protective immune responses induced by *P. vivax* or *P. falciparum* vaccines *in vivo* in mice. These chimeric RMP have been used to assess the protective immune responses induced by vaccination that influence sporozoite invasion of hepatocytes both *in vitro* and *in vivo,* and the removal of infected hepatocytes *in vivo* [59]. For example, chimeric RMP have been generated where the endogenous *csp* gene has been replaced either with *P. falciparum csp* or different *P. vivax csp* alleles. These chimeric parasites produce sporozoites that are infectious to rodent hepatocytes *in vivo* and human hepatocytes in culture [59].

Based on studies with chimeric rodent parasites, we reasoned that the availability of chimeric *P. falciparum* parasites that express *P. vivax* antigens would open up possibilities to analyse protective immune responses induced by vaccination using *P. vivax* antigen-based vaccines in CHMI, bypassing the need for *P. vivax* parasite production and measures to ensure that *P. vivax* hypnozoites are removed. As a proof of concept we explored in **Chapter 6** the possibility to create, using CRISPR/Cas9 gene editing methodologies, two chimeric *P. falciparum* parasites where the gene encoding circumsporozoite protein (CSP), was replaced by *csp* genes of *P. vivax*. CSP is the major protein of the sporozoite surface [14, 109] and plays a critical role both in sporozoite formation and in sporozoite invasion of mosquito salivary glands and liver cells of the host [105-108]. CSP is the target antigen of the most advanced *P. falciparum* malaria vaccine (RTS,S) and is also an important vaccine target for *P. vivax* [110, 111].

In **Chapter 7** the results of the studies described in **Chapters 3-6** are summarized and discussed, including a discussion on the use of transgenic *P. falciparum* parasites in research aimed at developing novel drugs and vaccines

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