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**Author:** Lin, Jingwen  
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CHAPTER 7

Conclusions and Discussion
The principal aim of the studies described in this thesis was to generate growth- and virulence-attenuated attenuated blood stage parasites in the rodent malaria model *P. berghei*, which may serve as immunizing agents and as tools to study correlates of disease and protection. Specifically, we aimed to create genetically attenuated blood stage parasites that induce low parasitemia, self-resolving short-term blood infections, which induce protective immunity. In order to screen a large number of potential genetically attenuated (blood stage) parasites (GAP<sub>BS</sub>), we developed methods to improve both transfection technology to generate GAP and for analysing GAP growth-characteristics during a blood stage development.

1. Progress in genetic modification technology for *Plasmodium* rodent malaria parasites

Genetic modification of the rodent malaria parasites, *P. berghei* and *P. yoelii*, is limited by the paucity of drug-selection markers that permit the selection of transformed mutants, and this in turn also hampers multiple genetic modifications in the same mutant. The novel GIMO-transfection method described in Chapter 2 permits the generation of mutants that stably express heterologous proteins and are free of drug-selectable markers in their genome, thereby facilitating further genetic modification of the transgenic parasites. In addition, it provides a fast and simple way to ‘gene complement’ gene deletion/mutation mutants (i.e. restoring the wt phenotype upon restoration of the disrupted gene). The GIMO method not only simplifies and speeds up both the generation of marker-free transgenic parasites and gene complementation experiments, the application of this method also greatly reduces the numbers of animals required to generate and complement mutants.

GIMO transfection uses negative selection to remove the positive-negative selectable marker cassette, *hdhfr::yfcu* and thereby generate transgenic mutants ready for subsequent modifications. Since GIMO-transfection is a simple, fast and efficient approach to generate mutants permissive to further genetic modification, we recommend that, where possible, transfection of *P. berghei* and *P. yoelii* parasites be performed with DNA-constructs that contain the *hdhfr::yfcu* selectable marker cassette. A recent study has reported a ‘recombineering’ system for high-throughput, genome wide and efficient generation of gene targeting constructs for *P. berghei* [1]. This development can be partnered with GIMO transfection by ensuring all the targeting constructs have a positive-negative (hdhfr::yfcu) selectable marker cassette. Consequently all resulting mutants would be receptive to GIMO-transfection thereby permitting further modification (e.g.
reporter protein expression) and complementation. In addition to the use of the GIMO-transfections described in this thesis, we have generated a wide variety of new transgenic *P. berghei* reporter parasites that express fluorescent- and luminescent-markers under the control of different *Plasmodium* promoters, all of which do not contain a drug-selectable marker (data not shown). In addition, we have generated *P. berghei* parasites expressing the (immunological) reference protein ovalbumin (OVA) under the control of different promoters (data not shown), as such parasites are excellent tools to further unravel (protective) immune responses induced by growth- and virulence-attenuated GAP$_{BS}$ as described in Chapter 6.

2. Generation of growth- and virulence-attenuated attenuated blood stage parasites (GAP$_{BS}$) by targeted gene deletion

In this study, we targeted 41 genes for targeted disruption in the virulent rodent parasite, *P. berghei* ANKA, in order to generate GAP$_{BS}$ that are both growth- and virulence-attenuated and can serve as protective immunogens. Specifically, we aimed to create virulence-attenuated GAP$_{BS}$ that induce short-term blood infections with low parasitemias and are resolved by the host and induce protective immunity. The genes we targeted for deletion were selected either based on the published roles of their encoding proteins as being important for blood stage development, or based on *P. falciparum* studies where effecting their encoded protein expression produces a growth delay phenotype.

2.1 Genes encoding rhomboid proteases

We included all 8 genes encoding *Plasmodium* rhomboids for targeted deletion because of important roles that several of rhomboid proteases have in host cell invasion and pathogenesis in *Plasmodium* and *Toxoplasma* infections [2,3]. In addition, it has been shown that *P. berghei* and *P. yoelii* mutants lacking rhomboid 1 (ROM1) show a reduction in their blood stage growth rates [4,5]. In Chapter 4, we show successful generation of gene-deletion mutants for *rom1*, *3*, *9* and *10*, while multiple attempts to disrupt *rom4*, *6*, *7* and *8* were unsuccessful. However, blood stages of all 4 gene-deletion mutants showed normal growth and virulence characteristics, indicating that these proteins are redundant and/or that their functions can be fulfilled by other (possibly rhomboid) proteases. It had been reported that *P. berghei* and *P. yoelii* mutants lacking ROM1 exhibit a slight growth defect and are less virulent in mice than wild type parasites [4,5]. In contrast, we were unable to detect either a growth or virulence phenotype of two independent *P. berghei*
\( \Delta \text{rom1} \) lines. The cause for these discrepancies in blood stage phenotypes between our and the \( P. \text{berghei} \) mutant reported by Srinivasan \textit{et al.} [4] is unknown. Cloned lines of wild type \( P. \text{berghei} \) ANKA parasites can differ in their growth and virulence characteristics [6] and environmental factors have been shown to influence the course of infections in mice [7]. It is therefore possible that the growth and virulence phenotype of the single \( \Delta \text{rom1} \) mutant reported by Srinivasan \textit{et al.} [4] may be unrelated to the disruption of \( \text{rom1} \). The interclonal and environmental induced differences in growth and virulence characteristics of \( P. \text{berghei} \) blood stages emphasize the importance to analyse the phenotype of at least two independently-derived mutants in gene-deletion studies, and/or to perform gene complementation.

Interestingly, mutants lacking rhomboid 3 (ROM3) expression exhibit a strong and clear phenotype during mosquito-stage development. While \( P. \text{berghei} \) mutants lacking ROM3 are capable of producing normal numbers of oocysts, these oocysts show a complete absence of sporozoite formation. This is the first apicomplexan rhomboid identified to play such a vital role in sporogony. Mutant oocysts show clear signs of stalled DNA replication and fail to form individual sporozoites, and remain highly vacuolated. Further research is needed to identify the substrates of ROM3 in oocysts. We identified a number of possible substrates of ROM3, based on the published proteome data of oocysts and sporozoite proteins (www.plasmodb.org), which are predicted to contain a single transmembrane domain and encode a signal peptide. However, since we observed the expression of ROM3 in gametocytes and ookinetes, but not in developing and mature oocysts, it is very much possible that the ROM3 substrate(s) is (are) also present and cleaved in gametocytes/ookinetes.

### 2.2 Genes encoding hemoglobinases

We chose to target 12 \( P. \text{berghei} \) genes that encode proteins with predicted or possible roles in hemoglobin (Hb) digestion because of the importance that Hb digestion has in parasite blood stage development [8]. Further, it has been shown that \( P. \text{berghei} \) and \( P. \text{falciparum} \) mutants lacking expression of certain hemoglobinases, while viable, exhibit reduced growth rates, and some mutants in \( P. \text{berghei} \) are both growth and virulence attenuated [9,10]. As shown in Chapter 5, we were able to successfully generate gene deletion mutants for 9 of the 12 ‘hemoglobin digestion genes’, which indicates a high level of redundancy in the Hb degradation pathway. The viability of mutant parasites lacking hemoglobinases, indicates either that other enzymes can compensate their function(s) or that \( P. \text{berghei} \) can obtain necessary amino acids from other sources, for example, from the catabolism of proteins other than Hb or by scavenging free amino acids from
the reticulocyte cytoplasm or serum (see also below). Four of the nine mutants showed normal growth characteristics in mice, whereas 5 mutants showed a significantly reduced growth rate compared to wild type parasites. Four of these 5 mutants are reduced in their virulence, specifically, do not induce experimental cerebral malaria (ECM) in susceptible mice. In addition to these single gene-deletion mutants, we also generated a double gene-deletion mutant, Δpm4Δbp2, lacking two hemoglobinases plasmepsin-4 (PM4) and berghepain-2 (BP2). The 2 endoproteases are responsible for initial and critical cleavage of the native Hb and it was therefore highly unexpected to be able to generate this double gene-deletion mutant, since it indicates that Δpm4Δbp2 parasites may survive inside reticulocytes without Hb digestion. The initial cleavage of native Hb is mediated by aspartic and papain-like cysteine endoproteases in digestive vacuole (DV). In the P. falciparum DV, there are four aspartic proteases termed plasmepsins and two papain-like cysteine proteases termed falcipains capable of hydrolyzing native Hb [11–15]. In P. berghei PM4 is the syntenic ortholog of all four P. falciparum plasmepsins I-IV [10] and berghepain 2 (BP2) is the syntenic ortholog of the DV falcipains (falcipain 2 and 3) (www.plasmodb.org). The simultaneous absence of these two enzyme activities in P. berghei was therefore expected to result in the absence of Hb proteolysis in the DV. We show that Δpm4Δbp2 parasites can complete asexual development in reticulocytes without hemozoin (Hz) formation, a detoxification product of Hb degradation. These observations have important implications for Plasmodium drug development and drug resistance, in particular for human malaria parasites (e.g. P. vivax) that can develop inside reticulocytes in which Hb digestion may not be essential (see below). The presence of low Hz amounts in a proportion of Δpm4Δbp2 parasites indicates that Hb can still be degraded to some degree in the absence of PM4 and BP2. At present we cannot formally exclude a role of other P. berghei proteases in the initial step of Hb digestion, which would compensate (albeit poorly) for the loss of PM4 and/or BP2. Further research is needed to investigate whether the remaining low-level of Hz formation in Δpm4Δbp2 parasites is due to specific cleavage of some Hb molecules by other enzymes or results from a non-specific disassembly of the Hb tetramer.

Interestingly, while it has been reported that C57BL/6 mice infected with Δpm4 cannot resolve their infections and die from hyper-parasitemia [10], we found that all C57BL/6 mice infected with Δpm4Δbp2 resolve their infections. In addition, we found that BALB/c infected with Δpm4Δbp2 can resolve this infection without developing hyper-parasitemias (i.e. >50%) when i.p infected with 10^5 parasites. These results demonstrate that it is possible to generate further virulence-attenuated parasites through the deletion of multiple genes, as was also reported by Spaccapelo et al. [17] with mutants that lack expression of both PM4 and MSP7. Below we discuss in more detail the future research
on these growth- and virulence-attenuated parasites in identifying the critical host and pathogen components inducing (protective) immunity and virulence, and to better understand the differences in induced rather than acquired immune responses against a *Plasmodium* infection and this may help to create an effective and the broadest possible anti-parasite vaccine.

### 3. Future research on growth- and virulence-attenuated *P. berghei* mutants

#### 3.1 Analysis of immune responses induced by infection with growth- and virulence-attenuated parasites

Our studies show that it is possible to generate mutants with strongly reduced growth rates that do not induce ECM, and that through the deletion of multiple genes it is possible to create mutants that produce self-resolving infections in mice without producing hyper-parasitemia (Chapter 6). However, mice infected with these mutants can still develop high parasitemias ranging between 10–50%. Till now, we have not yet been able to generate mutants that produce low-level infections that resolve shortly after parasite inoculation into the blood and without developing high parasitemias (<10%). Even with parasites that have strongly reduced growth rates, both C57BL/6 and BALB/c mice are unable to rapidly mount an effective immune response that can control an acute infection. It is, however, important to note that in all our experiments the mice were infected with relatively high numbers of parasites (10⁵ – 10⁶). It is possible that starting infection with lower numbers of parasites would allow the mice to control infections before developing high parasitemias or would lead to infections with very low or even sub-patent parasitemias [18]. For *P. falciparum* it has been postulated that infection with low numbers of infected red blood cells (iRBC) under curative chemotherapy generates protective immune responses that are marked by absent or low levels of antibodies and strong cell-mediated responses, including upregulation of nitric oxide synthase, CD4+/CD8+ proliferative T-cell and INF-γ responses [19–21]. Protective immunity with *P. berghei* infections in mice have been mainly reported from immunization requiring repeated, prolonged infections cleared by drug treatment or after a self-resolving and sustained infection with an avirulent parasite line [10,22–27]. The protective immune responses in these mice are largely antibody-dependent, where the iRBC of wt challenge are opsonized and then removed in the spleen by phagocytosis [10,28,29]. These studies, and studies of experimental *P. falciparum* infections in humans where protective cellular immune responses are induced with low numbers of iRBC, would suggest that the induction of
protective immunity might require different parasite loads depending on the nature of the (cellular or humoral) immune responses required.

When studying host-cell interaction, pathology and immunity induced by Plasmodium infections in rodents, the differences between the rodent and human host have to be taken into consideration. Differences in both host physiologies and host immune responses to infections, will strongly influence both how a host copes with an infection and how a malarial disease will manifest. Clearly, further research is required to determine both parasite and host factors that can induce protective immune responses against blood stage infection and to understand the critical differences in protective immunity induced by P. falciparum infections in humans and rodent Plasmodium infection in mice. Notwithstanding these considerations, I believe that the use of attenuated blood stage parasites generated in this, and in future studies, can be very useful tools to better understand induced protective immunity against Plasmodium blood stages and may help to create an effective anti-parasite vaccine.

3.2 Defining the parasite factors that induce ECM

We found that all mutants that have reduced Hz production do not induce ECM (Chapter 6). Hz is released into the circulation at schizont rupture and it is rapidly removed by phagocytosis mainly in the spleen and liver. Upon phagocytosis, Hz cannot be further degraded and persists for prolonged periods in host tissues and has long been considered as a virulence factor. It has been shown that the number of Hz-containing leukocytes in the peripheral blood correlates with disease severity in P. falciparum-infected patients [30,31]; and several inflammatory and immune-modulatory effects of Hz have been reported (reviewed in [32,33]). Therefore, the amount of Hz that is released by the parasite in the host may play a critical role in both inducing inflammatory responses and severe pathology in the host. Since induction of ECM is correlated with pro-inflammatory status of the host [34], Hz may be a critical factor involved in inducing ECM. However, the amount of Hz, like growth rates of the parasites, may not be the only factor responsible for inducing ECM, since blood stages of several growth-attenuated mutants (Δnt1, Δlap and Δbp1) have normal Hz production but do not induce ECM (Chapter 6; unpublished results). However, the absence of ECM-inducing capacity of these mutants could still be related to reduction in amounts of released Hz in the circulation in the early phase of an infection. Mice infected with slow-growing parasites can be expected to release less Hz compared to wt parasites and therefore the Hz levels may be below the threshold that is required to induce inflammatory responses causing ECM. So far we have been unable to select mutants that have both a normal growth rate and Hz production, but do not
induce ECM. The successful selection of such mutants would indicate that other factors contribute to ECM in addition to growth rate and Hz levels.

Further research is required to unravel the relative contributions and relationship between the critical parasite and host factors that cause severe disease and elicit protective immunity. I believe that the mutants produced in this study, with their different phenotypes and properties, may help shed light on parasite factors involved in ECM induction. Such knowledge may be critical in evaluating the value of the *P. berghei* ANKA infections in C57BL/6 mouse as an experiment model of cerebral malaria for understanding human cerebral malaria, since the similarities of induced immuno-pathology between humans and rodent infections is under considerable debate [35,36].

### 3.3 Drug development and drug resistance of drugs that target hemoglobin digestion

The ability of *Plasmodium* parasites to invade RBC and produce infectious merozoites without Hz formation may have important implications for development of drugs that target Hb digestion and/or Hz formation and in understanding the development of resistance against such drugs. We found that Δ*pm4Δbp2* parasites that lack expression of PM4 and BP2 can grow with little or no Hz production, and importantly, are less sensitive to chloroquine (CQ) treatment *in vivo*. CQ directly interacts with free heme creating a heme-chloroquine complex that is highly toxic for the parasite [37], and therefore the increased CQ resistance of Δ*pm4Δbp2* is consistent with our observations of severely reduced/absent Hz formation. Interestingly, it has been previously reported that *P. berghei* lines that have been selected for CQ-resistance have a stronger preference for reticulocytes and produce less Hz [38,39]. It has been proposed that CQ-resistance in parasites with reduced Hz is due to detoxification of hemin by elevated levels of glutathione in parasites that grow inside reticulocytes, thus precluding heme-polymerization and preventing the CQ activity [39,40]. However, our observations may provide a more direct explanation for CQ-resistance and reduced Hz production in these parasites, namely that these parasites hydrolyse less Hb in reticulocytes like the Δ*pm4Δbp2*-parasites. While Δ*pm4Δbp2* parasites have an increased resistance to CQ, they retain the same sensitivity to artesunate (AS). Although the precise and critical mode of action of artemisinin and related-derivatives remains contentious, most studies concur that their activity results from activation by reduced heme iron in the DV [41,42]. Our results showing that Δ*pm4Δbp2*-parasites are more resistant to CQ but not AS, would suggest that additional, non-heme based, modes of AS action are equally, or even more effective at targeting *P. berghei* parasites *in vivo*. Therefore, the Δ*pm4Δbp2*-parasites that grow with little or no Hz formation may
be useful tools to further analyse the mode of drug action, for example, how they target and interact with molecules critical to or result from Hb digestion.

Our results on the acquisition of CQ-resistance when parasites develop in reticulocytes with limited or no Hz production may have relevance for *P. vivax*, which is restricted for growth in reticulocytes. Interestingly, mechanisms of CQ-resistance in *P. vivax* appears to be different from those in *P. falciparum* [43]. Based on our observations, we hypothesize that some *P. vivax* parasites may acquire resistance to CQ (and other drugs targeting Hb digestion) by preferentially ‘switching’ to a development mode where they are less dependent on Hb digestion for growth. Such ‘switching’ may only be possible for those *Plasmodium* species that can infect and develop in reticulocytes. It would therefore be of great interest to analyse whether in ‘hotspots’ of *P. vivax* CQ-resistance parasites develop inside the RBC with reduced Hz formation. I believe that mutants with reduced Hz production are not only useful tools to analyse the influence of Hz in inducing pathology (as explained above), but also useful tools to analyse drug activities where Hb digestion/Hz formation is believed to be critical for their mode of actions.
References


