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**Title:** Generation of genetically attenuated blood-stage malaria parasites: characterizing growth and virulence in a rodent model of malaria  
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Summary
Despite intense efforts over the past 50 years to develop a vaccine, there is currently no licensed malaria vaccine available. Optimism that a first-generation malaria (subunit) vaccine based on the *Plasmodium* circumsporozoite protein, RTS,S, would soon be licensed has been dampened by the interim results of the ongoing Phase 3 trials in Africa, which indicate that this vaccine confers only around 30% protection against severe malaria in young children and infants, and that the protection generated is only short-lived (<6 months). The limited success achieved in inducing long-lasting effective protective immunity against malaria using subunit vaccines has led to renewed interest in whole-parasite vaccination strategies, which while hard to formulate and administer, have been shown to confer long-lasting sterile immunity in humans. The aim of the work described in this thesis was to genetically engineer and characterize growth- and virulence-attenuated blood stage parasites (GAPBS) in the rodent malaria model, *P. berghei*. Specifically, GAPBS that produce only short-lived low-parasitemia self-resolving blood infections and provoke strong protective immune responses. In order to screen a large number of potential GAPBS, we first improved both transfection methods to generate these GAPBS and methods to analyse their blood stage growth-characteristics.

In Chapter 2, I describe the development of a novel genetic modification method in two rodent malaria parasites, *P. yoelii* and *P. berghei*, which helps overcome the problem of limited number of selectable markers that can be effectively used to select for *Plasmodium* mutants. This ‘gene insertion/marker out’ (GIMO) method uses negative selection to rapidly generate transgenic mutants that are free of drug selectable markers and therefore ready for subsequent modifications. This method can also be used to rapidly and more easily generate ‘reporter parasites’, which are useful for phenotype characterization of mutants and facilitate the generation of parasites expressing multiple transgenes and/or lacking multiple genes. 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, but the application of this method also greatly reduces the numbers of animals required to generate and complement mutants.

We also improved the existing methods to analyse both *in vitro* and *in vivo* the growth kinetics of GAPBS that have been created in luciferase-expressing background parasite lines and also described how luciferase-expressing reporter parasites can be used in drug screening assays both *in vitro* and *in vivo*. These improved protocols are described in Chapter 3.
In order to generate a GAP\textsubscript{bgv}, I targeted 41 genes in the virulent \textit{P. berghei} ANKA line, to specifically identify mutants that are both growth- and virulence-attenuated and that can serve as protective immunogens. The selection of these genes was mainly based on their predicted roles in parasite blood stage development or based on \textit{P. falciparum piggyBac} random mutagenesis studies, where a growth defect was observed in mutants with a piggyBac gene-insertion. Specifically, we targeted the 8 genes encoding \textit{Plasmodium} rhomboid proteases, because critical roles were published for several rhomboid proteases in host cell invasion and pathogenesis. However, we found a high degree of redundancy in this family; 4 of them (ROM1, 3, 9 and 10) were dispensable for parasite blood stage development, with no alteration in growth or virulence. We also examined the phenotype of these gene-deletion mutants throughout the complete lifecycle including development in the mosquito and in the liver (Chapter 4). We found that \textit{P. berghei} mutants lacking ROM3, although producing normal numbers of oocysts in the mosquito, show a complete absence of sporozoite formation within the oocysts. This is the first apicomplexan rhomboid identified to play a vital role in sporogony.

In addition to the rhomboid proteases, we selected 12 genes with predicted or possible roles in the hemoglobin degradation pathway for targeted disruption (Chapter 5), as hemoglobin catabolism is believed to be essential for parasite blood-stage development, and a mutant lacking expression of one of the hemoglobinases, plasmepsin-4, was shown to be growth- and virulence-attenuated. We were able to successfully generate gene-deletion mutants for 9 out of the 12 selected genes, indicating a high level of redundancy also amongst \textit{P. berghei} hemoglobinases. Four of the 9 mutants showed normal growth characteristics in mice, whereas 5 mutants showed a significantly reduced growth rate compared to wild type parasites. Unexpectedly, we were able to generate a double gene-deletion mutant lacking expression of both plasmepsin-4 and berghepain-2, the only 2 enzymes reported to initiate hemoglobin digestion. This double gene-deletion mutant was restricted to growth in young red blood cells, reticulocytes, where parasites were able to develop without any detectable hemozoin formation. Hemozoin is the detoxified byproduct of hemoglobin degradation and its absence indicates that this mutant is able to develop inside red blood cells with little or no hemoglobin digestion, and is supported by the observation that these parasites are more resistant to chloroquine compared to wild-type parasites. Chloroquine directly interacts with heme that is liberated upon hemoglobin degradation, creating a complex highly toxic to the parasite; and therefore the increase in chloroquine resistance of the double gene-deletion parasites is consistent with our observations of reduced/absent hemozoin production. These observations have important implications for \textit{Plasmodium} drug development and drug resistance, in particular for malaria parasites (e.g. \textit{P. vivax}) that can develop inside reticulocytes where
hemoglobin digestion may not be essential.

From the 41 genes targeted, we generated 22 single gene-deletion mutants and 2 double gene-deletion mutants, one already described above lacks expression of both plasmeisin-4 and berghepain-2 (Δp4Δbp2), and another lacks expression of both plasmeisin-4 and SMAC (schizont membrane-associated cytoadherence protein, Δp4Δsmac). The growth and virulence characterization of these 24 mutants is presented in Chapter 6. Nine of these mutants showed significant reduction in in vivo multiplication rates, and 7 of the 9 growth-arrested mutants did not induce experimental cerebral malaria (ECM) in ECM-sensitive (C57BL/6) mice. All 4 mutants that produce significantly reduced amounts of hemozoin fail to induce ECM. The mutants Δlap, Δapp and Δp4Δbp2 were able to produce self-resolving infections in C57BL/6 and ECM-resistant BALB/c mice.

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. However, 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 at low parasitemias. It is, however, important to note that in all our experiments the mice were infected with relatively high numbers of parasites (10^5 – 10^6). 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. These studies and those of experimental P. falciparum infections in humans, where protective cellular immune responses are induced with low numbers of infected red blood cells, would suggest that the induction of protective immunity might require different parasite loads depending on the nature of the immune (cellular or humoral) responses required. Clearly, further research is required to determine both the parasite and host factors that can induce protective immune responses against blood stages. The use of attenuated blood stage parasites can be an extremely useful tool to better understand induced rather than acquired immunity against Plasmodium and may help to create the most effective and broadest anti-malaria vaccine.