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# Murine infection models for vaccine development

## The malaria example

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**Abbreviations:** CQ, chloroquine; CSP, circumsporozoite protein; GAPs, genetically arrested parasites; MQ, mefloquine; MSP1, merozoite surface protein 1; PQ, primaquine

Vaccines are developed and eventually licensed following consecutive human clinical trials. Malaria is a potential fatal vector-borne infectious disease caused by blood infection of the single-cell eukaryote Plasmodium. Pathogen stage conversion is a hallmark of parasites in general and permits unprecedented vaccine strategies. In the case of malaria, experimental human challenge infections with *Plasmodium falciparum* sporozoites can be performed under rigorous clinical supervision. This rare opportunity in vaccinology has permitted many small-scale phase II anti-malaria vaccine studies using experimental homologous challenge infections. Demonstration of safety and lasting sterile protection are central endpoints to advance a candidate malaria vaccine approach to phase II field trials. A growing list of antigens as targets for subunit development makes pre-selection and prioritization of vaccine candidates in murine infection models increasingly important. Preclinical assessment in challenge studies with murine Plasmodium species also led to the development of whole organism vaccine approaches. They include live attenuated, metabolically active parasites that educate effector memory T cells to recognize and inactivate developing parasites inside host cells. Here, opportunities from integrating challenge experiments with murine Plasmodium parasites into malaria vaccine development will be discussed.

### Introduction

Clinical development and licensure of a safe, affordable, accessible and protective malaria vaccine remains a fundamental medical challenge. A vaccine is urgently needed because malaria continues to be the most relevant arthropod-transmitted infectious disease despite enormous investments into malaria control programs.[1-3]

The causative pathogen, an obligate intracellular protist of the genus Plasmodium, is injected into the human host upon a nightly bite by a previously infected female Anopheles mosquito. However, disease onset does not occur until the parasite propagates inside erythrocytes and is preceded by a clinically and diagnostically silent population expansion phase in the liver.[4-6] Since parasite stage conversion follows an intrinsic developmental program and is non-reversible, three consecutive phases in the human host can be distinguished and targeted by vaccines, (1) sporozoites and liver infection,[6-8] (2) asexual blood stage infection, the exclusive cause of malaria,[9-13] and (3) sexual stages that eventually colonize the insect vector.[14-17]

Experimental evidence is robust for all three stages as vaccine targets. Hence, an idealized, perfect malaria vaccine would elicit robust immune responses against all life cycle stages simultaneously. Experimental proof-of-principle studies in murine malaria models established that vaccination with liver arrested, metabolically active parasites could elicit lasting sterile protection against sporozoite challenge.[18-20] All existing anti-sporozoite and –liver stage vaccines exploit this population bottleneck of the parasite life cycle, i.e., mosquito-to-man transmission, but need to be 100% effective to prevent a subsequent blood infection.[6,8,21,22] Active immunization with purified native or recombinant blood stage antigens, exemplified by the merozoite surface protein 1 (MSP1; PF3D7_0930300; gi:929796), resulted in low blood infection upon high dose parasite challenge in murine models.[23,24] Similarly, passive and active immunization against gamete and ookinete surface antigens reduced colonization and sporozoite formation in the Anopheles vector to very low levels.[25,26]

Despite these encouraging results from pre-clinical research in animal models only very few vaccine candidates were advanced to field-testing in malaria-endemic countries. These first generation malaria vaccines all share in common that they contain small portions of one or more selected *Plasmodium falciparum* antigens. In the absence of robust correlates of protection in naturally acquired immunity to malaria,[12,27-29] they are based on the expectation that vaccine-induced responses against these distinct subunits can elicit lasting protection against clinical disease and malaria-related deaths after three immunizations. Because the precise mechanisms and targets of protective immunity in humans remain unverifiable, studies of Plasmodium infections in

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**Table 1.** Murine Plasmodium infection models

| Plasmodium species | Strains | Mouse strain | Characteristics |
|---|---|---|---|
| P. berghei | NK65,ANKA | C57bl/6 | Robust vaccine model |
| | ANKA | | Malaria clinical syndromes |
| *P. yoelii* | 17X | Balb/c | Modest vaccine model |
| *P. chabaudi* | AS | A/J, DBA/2 | Susceptible to blood infection |
| | | C57bl/6 | Resistant to blood infection |
| *P. vinckei* | *vinckei* | Balb/c | Lethal infections |

mice can establish new concepts and prevent failures in human clinical trials.

In the following, an appraisal of current and upcoming anti-malaria strategies that originate from murine infection models will be presented. Brief overviews of murine Plasmodium parasites and the current state of clinical trials with subunit vaccine candidates will introduce the pre-clinical development opportunities and limitations of malaria vaccine discovery and development.

## Murine Infection Models

Five Plasmodium species infectious to humans share most biological, clinical and molecular features with closely related Plasmodium species that infect other mammals.[30] With the isolation and adaptation of a rodent Plasmodium species, named *Plasmodium berghei*, to laboratory rats and mice, an experimental infection model became available.[31] Additional isolates from wild rodent hosts and Anopheles vectors in Central and West Africa yielded a repertoire of subspecies and strains of four distinct Plasmodium species infectious to inbred and outbred mice (**Table 1**). Despite the evolutionary distance of their hosts, the chromosomal arrangements and overall gene repertoire between the most dangerous human parasite *P. falciparum* and rodent Plasmodium species are remarkably conserved.[32]

Infection experiments with *P. berghei* sporozoites are particularly informative since they (1) permit access to all aspects of the life cycle with the broadest selection of modified parasites,[33] (2) are performed in C57bl/6 mice, the most wide-spread mouse strain, where knockout and transgenic substrains are available,[34] (3) represent a robust vaccine model, i.e., C57bl/6 mice are difficult to protect[35,36] and (4) recapitulate central aspects of malaria complications, including experimental cerebral malaria,[37] acute lung injury[38] and malarial anemia[39] (**Table 1**). The related species, *P. yoelli*,[40] is typically examined in Balb/c mice. In this model, sterile immunity is easily achieved[36,41] (**Table 1**). While it offers opportunities for vaccine discovery[42] this model is also prone to over-interpretation of protective efficacy. *P. chabaudi* displays some striking similarities to the course of blood infection by human Plasmodium species[43] (**Table 1**). However, the value for anti-blood stage vaccine development is limited by the current lack of genetically modified *P. chabaudi* parasites. The fourth rodent parasite, *P. vinckei*, is notoriously underrated despite its exceptionally high virulence.[44] Major research investments, including generation of genome data, establishment of transfection technology and adaptation to Anopheles transmission will be required toward a potentially very important murine vaccine and infection model. Together, murine Plasmodium species offer a wide range of experimental opportunities. Limitations, such as absence of species-specific antigens, can be overcome by established transgene expression in *P. berghei*[45] or by exploring humanized mouse models[46,47] for infections with human parasites.

## Malaria Subunit Vaccines in Advanced Clinical Trials

The first malaria vaccine to enter phase II trials in malaria-endemic countries was a candidate synthetic malaria vaccine, termed SPf66. Designed as a low-complexity peptide, multi-subunit vaccine, it contains one small MSP1 peptide, two NANP repeat peptides of the major sporozoite surface protein, termed circumsporozoite protein (CSP; PF3D7_0304600/PFC0201c; gi: 160161) and peptides of still unknown origin.[48] Human challenge studies and phase II trials in Colombia were reported surprisingly successful,[49,50] prompting independent phase III trials.[51-54] The first study in 1–5 y old children in Tanzania concluded that SPf66 was safe, immunogenic and reduced the risk of clinical malaria by ~30%.[51] Partial vaccine efficacy reportedly extended well beyond the first year of follow-up.[52] In contrast, a phase III trial in infants aged 6–11 mo in The Gambia could not detect any significant differences between the SPf66 and polio groups.[53] This negative result was confirmed in a trial in Thailand,[54] triggering discontinuation of SPf66 vaccine development. In the absence of a murine infection model to provide an immunological framework for a SPf66-based strategy, no improvements to the vaccine design can be made.

The established benchmark of ~30% protection against clinical malaria was subsequently met by another first generation subunit vaccine developed in 1980s, termed RTS,S. This vaccine consists of an enhanced Engerix™, a pediatric hepatitis B vaccine, and includes portions of CSP as *P. falciparum* antigen and a proprietary adjuvant, AS01.[55,56] This liposome formulation with monophosphoryl lipid A, a lipopolysaccharide derivative and ligand of toll-like receptor 4 (TLR4), and QS21, a triterpene glycoside, is a strictly essential component for efficacy. RTS,S also exemplifies the current clinical development plan for candidate malaria vaccines (**Fig. 1**). Since no infection models were developed for RTS,S, experimental challenge infections by a single exposure to laboratory-reared Anopheles mosquitoes infected with a homologous *P. falciparum* strain were the starting point for efficacy trials.[57-59] 40% of malaria-naïve adults were completely protected against a single challenge infection (**Fig. 1**). Additional tests, such as heterologous challenge with another *P. falciparum* strain to mimic diverse parasite populations in the field and re-challenge at a later time point to test for duration of protection, remained unexplored.

Instead, RTS,S was tested in a field trial with semi-immune adults in the Gambia.[60] Prevalence of *Plasmodium falciparum* infections were indistinguishable between the RTS,S/AS02 and

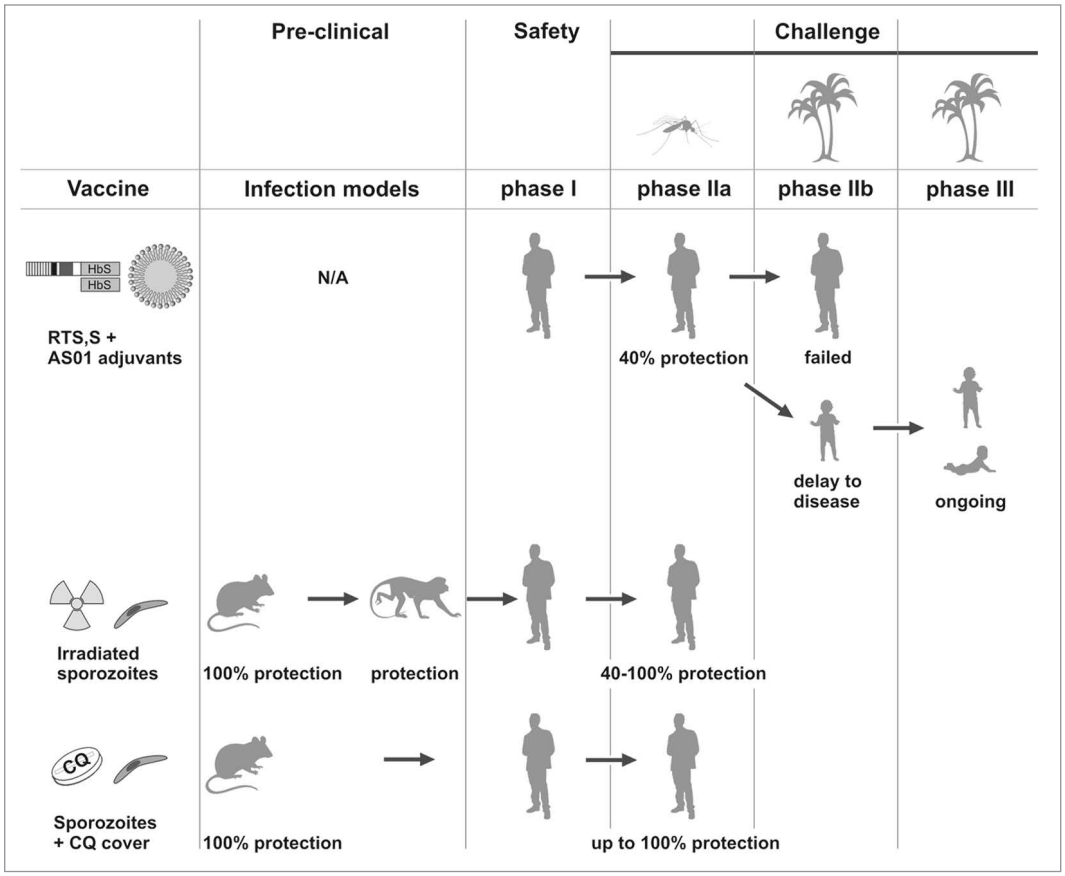


**Figure 1.** Clinical development of exemplary malaria vaccine candidates. RTS,S/AS01 (top) is currently the clinically most advanced subunit vaccine candidate. The recombinant proteins are shown as boxes (HbS, hepatitis B surface antigen) and the adjuvant AS01 as a lipid droplet. It was developed in the 80s and consistently protected 40% of adult volunteers against homologous challenge with infected laboratory-reared Anopheles mosquitoes (mosquito symbol). In a subsequent field trial (palm symbol) no protection was afforded in adults. Pictograms represent adults, 5–17 mo old children, and 6 weeks to 3 mo old infants. N/A, not applicable. Irradiated sporozoites (center) were first tested in a murine infection model in the 60s and shown to consistently protect non-human primates and adult volunteers against homologous challenge with infected laboratory-reared Anopheles mosquitoes. Sporozoite infection under chloroquine cover (bottom) was tested in a murine infection model in the 70s and recently shown to elicit robust and lasting protection in adult volunteers against homologous challenge with infected laboratory-reared Anopheles mosquitoes.

rabies control groups already 15 weeks later, and could not be improved by an additional vaccine boost[60] (**Fig. 1**). RTS,S was also tested subsequently in proof-of-concept field trials in the target group, toddlers and infants.[61-63] Numerous phase II trials have repeatedly shown a robust (~6 mo) delay to disease in the RTS,S vaccine group, which can be calculated again to ~30–50% protection against clinical disease (**Fig. 1**). Confirmation of this efficacy is currently being tested in an ongoing multi-center phase III trial. Trial completion and data analysis is scheduled toward the end of 2013. According to two interim results,[64,65] the previous outcomes were fully corroborated, yielding a significant (~6 and ~3 mo, respectively) delay to clinical disease, which can be calculated as 50% and 30% protection in the first periods of follow-up. Immunological parameters, such as anti-CSP antibody titers or persistence of antigen-specific CD4+ T cells, do not correlate with the observed delay to disease afforded by RTS,S, suggesting that a murine infection model could fill an important knowledge gap. Of note, early work in the *P. yoelii*/Balb/c infection model demonstrated that high anti-CSP repeat antibody titers did not protect against sporozoite challenge in vivo.[66]

A recent CSP-based subunit vaccine,[67] using the outer membrane protein complex of *Neisseria meningitis* as a carrier for CSP-repeat peptides, illustrates the current evidence-based pre-clinical development plan for a subunit malaria vaccine, integrating tailor-made transgenic *P. berghei* parasites for murine infection experiments[45] and immunological assessment in primates.

## Whole Cell-Based Vaccines

The first and some of the most effective human vaccines are live-attenuated pathogens. Live attenuated viruses, such as the vaccinia virus small pox vaccine, the oral polio vaccine and the yellow fever vaccine, are groundbreaking public health tools that were instrumental in successful eradication or ongoing elimination campaigns. The complex underlying immunological mechanisms and target antigens are only now being explored,[68] and it remains speculative whether effective subunit vaccines could have been engineered against these viruses.

In recognition of the much more complex Plasmodium biology and very slow acquisition of naturally acquired partial

**Table 2.** Update on current clinical trials testing live, attenuated parasite strategies

| Vaccine* | Trial | Status† | ClinicalTrials.gov identifier |
|---|---|---|---|
| *Pf*γspz | Phase I/IIa | ongoing | NCT01441167 |
| *Pv*γspz | Phase I/IIa | planned | NCT01082341 |
| *Pf*spz + CQ | re-challange‡ | ongoing | NCT01660854 |
| *Pf*spz + CQ | Phase IIa | completed | NCT01218893 |
| $Pfspz_{cryo}$ | Phase I | recruiting | NCT01728701 |
| *Pf*spz + CQ or MQ | Phase IIa | ongoing | NCT01422954 |
| *Pf*spz + PQ | Phase I/IIa | ongoing | NCT01500980 |
| *Pf*GAP: *p36(-)/p36p(-)* | Phase I/IIa | suspended | NCT01024686 |

*Abbreviations: CQ, chloroquine; γspz, irradiated sporozoites; GAP, genetically arrested parasite; MQ, mefloquine; p36, 6-cysteine protein 36kDa; p36p, p36 paralog; Pf, Plasmodium falciparum; Pv, Plasmodium vivax; PQ, primaquine; spz, sporozoites; spzcryo, aseptic purified cryopreserved sporozoites. †Status as of January 2013. ‡re-challenge: Protected volunteers from previous study are being re-exposed to laboratory-reared Anopheles mosquitoes infected with a different Pf strain, termed heterologous re-challenge.

anti-malaria immunity, scientist at New York University established the proof-of-concept for a live-attenuated malaria vaccine.[69,70] Immunization of mice with irradiated sporozoites induced lasting[69] and stage-specific[70] immunity against sporozoite challenge (**Fig. 1**). These pre-clinical studies suggested that a malaria vaccine is possible and provided a rationale for advancing the irradiated sporozoite vaccine to a limited primate trial[71] and small phase I/IIa trials in humans[72-74] (**Fig. 1**). The momentum gained by the first successful human malaria vaccination[72] cannot be over-estimated. Confirmation of sterile protection was consistently achieved in follow-up trials, at least in a proportion of volunteers[73,74] (**Fig. 1**). However, apart from anecdotal evidence of lifetime protection against malaria in volunteers of the early experimental vaccine studies, no clinical data from heterologous challenge infection experiments or natural exposure in malaria-endemic countries are available.

Recent research and bioengineering investments into this benchmark malaria vaccine have resulted in the production of aseptic, purified, cryopreserved and irradiated *P. falciparum* for syringe delivery.[75] However, intradermal or subcutaneous immunization with this sporozoite vaccine failed to elicit protection.[76] In the absence of a positive control it remains unclear which of the several alteration(s) to the original protocol, i.e., exposure to irradiated infectious Anopheles mosquitoes, need attention. Ongoing clinical trials with irradiated *P. falciparum* and *P. vivax* sporozoites will further contribute to the assessment of safety and efficacy of this candidate malaria vaccine approach (**Table 2**).

The paradigm of irradiated sporozoites illustrates the power of murine infection models in informing human vaccine design and was critical for the development of alternative live attenuated malaria vaccine approaches.[22] They all follow the basic principle to infect with very high sporozoite doses and prevent onset of malaria. Because liver infection is diagnostically and clinically silent, high vaccine doses can mount lasting protection. One strategy, live unaltered sporozoite administration under continuous cover with the anti-malaria drug chloroquine (CQ), which kills blood stage parasites, has already advanced from proof-of-concept studies in mice[77-79] to a small human phase I/IIa clinical study[80,81] (**Fig. 1**). The trial results were impressive and demonstrated unprecedented lasting, sterile protection against a single homologous challenge infection by only three consecutive immunizations through exposure to 15 *P. falciparum*-infected Anopheles mosquitoes under continuous CQ cover.[80,81] These results clearly warrant major research investments and confirmation (**Table 2**). Ongoing clinical studies address whether (1) sporozoite exposure under CQ cover also elicits protection against challenge with a heterologous *P. falciparum* strain, (2) the immunization dose can be lowered, i.e., exposure to fewer Anopheles mosquitoes still induces sterile protection or (3) sporozoite exposure under cover of an alternative blood stage suppressive drug, mefloquine (MQ), elicits similar (non-inferior) or better (superior) protection. The use of antimalarial drugs that affect blood stages only, however, implies that most volunteers experience mild malaria in their first immunization round.[80] This might affect compliance and requires rigorous clinical supervision during the immunization regimes.

Proof-of-concept for two alternative strategies that might overcome these limitations has recently been demonstrated in murine infection models.[20,82] Sporozoite administration under cover with primaquine, a drug that acts on Plasmodium liver stages, was shown to be safe and protective.[82] Based on these promising results an ongoing clinical trial in humans was designed (**Table 2**). However, systematic side-by-side comparison in sporozoite challenge infections showed inferior immunity as compared with irradiated sporozoites.[83] It will be interesting to see if this outcome reproduces in the human trial, since it will exemplify the prognostic value of mouse infection models. It will also clarify to which extent more emphasis should be placed on preclinical evaluations before moving to proof-of-concept phase I/IIa trials in humans. Based on the comparison of vaccine efficacy in murine models,[83] sporozoite infection under antibiotic treatment, including azithromycin and clindamycin, both of which are licensed for pediatric applications, would seem to be the most promising way forward toward a whole organism sporozoite vaccine.[6,20,84]

Tailor-made arrests at various stages in liver stage development, maturation and merozoite release can also be generated by reverse genetics. Proof-of-principle studies with these so-called genetically arrested parasites (GAPs) were first demonstrated in the *P. berghei*/C57bl/6 model.[19] Translation of this approach to proof-of-concept studies in humans critically depends on careful preclinical studies in murine infection models in order to select multiple independent target genes that permit a complete life cycle arrest and yet induce the most potent immune responses. Two examples in the *P. berghei*/C57bl/6 model show that both requirements appear mutually exclusive.[85,86] A tight, early and pleiotropic arrest due to deletion of a master regulator of liver stage development, termed sporozoite and liver-stage asparagine-rich protein (SLARP), resulted in incomplete and short-lived protection.[85] In contrast, a very late arrest just prior to liver merozoite release elicited superior and long-lasting protection, but resulted in considerable break-through infections.[86] Accordingly,

selection of *P. falciparum* target genes that is likely to generate a safe *Pf* knockout line should be the first research priority. A first clinical trial with a mutant *Pf*GAP line[87] was discontinued (**Table 2**). Preclinical assessment in murine models demonstrated incomplete arrest in the selected target genes, providing a molecular rationale for breakthrough infections.[88] Of note, this was detectable only in the robust vaccine model, *P. berghei*/C57bl/6 mice, lending further support for a strict requirement to assess vaccine safety and efficacy in this murine infection model. In conclusion, the decision of which whole parasite strategy to move forward to clinical trials should be evidence-based and supported by robust preclinical data.

## Outlook

Strategies that led to an arrest of liver stage development or early during blood stage infection were already successfully tested in phase II clinical trials. Successful and rapid translation of vaccine strategies from murine infection models to human proof-of-concept trials demonstrates the important role of preclinical research in assessment of vaccine safety, efficacy and longevity. The proven track record of these models for malaria vaccine development warrants inclusion of similar approaches in future vaccine trials, including low-complexity subunit vaccines.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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