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Genetically engineered, attenuated whole-cell vaccine approaches for malaria

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Abstract

Malaria remains one of the most significant infectious diseases affecting human populations in developing countries. The quest for an efficacious malaria vaccine has been ongoing for nearly a century with limited success. The identification of malaria parasite antigens focused efforts on the development of subunit vaccines but has so far yielded only one partially efficacious vaccine candidate, RTS/S. The lack of high vaccine efficacy observed to date with subunit vaccine candidates raises doubts that the development of a single antigen or even a multi-antigen malaria subunit vaccine is possible. Fortunately, it has been demonstrated in animal studies and experimental clinical studies that immunizations with live-attenuated sporozoite stages of the malaria parasite confer long lasting, sterile protection against infection, providing a benchmark for vaccine development. These early successful vaccinations with live-attenuated malaria parasites did not however, promote a developmental path forward for such a vaccine approach. The discovery of genetically engineered parasite strains that are fully attenuated during the early asymptomatic liver infection and confer complete sterile protection in animal malaria models support the development of a live attenuated sporozoite vaccine for *Plasmodium falciparum* and its accelerated safety and efficacy testing in malaria challenge models and in malaria endemic areas.

Keywords

malaria; plasmodium; vaccine; elimination; sporozoite; liver stage; pre-erythrocytic stages; mosquito; attenuation

Introduction

Malaria remains a major global cause of disease and death and disproportionately affects developing, resource-poor regions of the world. Annually, 300–500 million clinical malaria cases result in approximately one million deaths, the majority of which are children under the age of five in sub-Saharan Africa.¹ Malaria is a mosquito-borne disease and hence it can be controlled at the level of human and mosquito. Currently, drug treatment of infected individuals, preventive drug treatment of populations at high risk of infection as well as insecticide-treated bed nets and indoor-insecticide spraying for mosquito control constitute the main weapons for malaria control.² However, malaria control is a never-ending battle and requires long-term sustainability and commitment. The complexity of the *Plasmodium* life cycle, the distinct lifestyle of each parasite life stage and their inherently distinct

antigenic makeup, necessitate the development of a portfolio of diverse strategies for malaria vaccine development approaches. The pre-erythrocytic (PE) stages of infection, encompassing the mosquito-transmitted sporozoite (SPZ) stage and the liver stages (LSs) that form after SPZ infection of the liver constitute an attractive target for vaccine development because they are low in numbers, when compared to the blood stages, completely asymptomatic and provide a relatively large window of opportunity for an effective immune response to eliminate the parasites (5–7 days for *P. falciparum*). Furthermore, to the best of our knowledge, PE stages do not exhibit substantial antigenic variation and antigenic polymorphisms during PE stages might be limited, making it less probable that a vaccine will fail against heterologous parasite strains.

The PE phase of the malaria parasite begins with the bite of an infected *Anopheles* mosquito when SPZs are injected into the skin of the mammalian host.^{3,4} SPZs migrate in the skin and when they make contact with a blood vessel, traverse the endothelium of the vessel and enter the blood stream. They are then carried to the liver by blood flow where they exit the blood vessel to infect hepatocytes and form LSs.⁵ Within a hepatocyte the LS is shielded from the host cell cytoplasm by a vacuole, known as the parasitophorous vacuole (PV). This barrier prevents free access of LS proteins to the hepatocyte proteolytic machinery, however, the parasite does target proteins to the PV membrane (PVM),^{6,7} and may even export proteins to the hepatocyte cytoplasm and nucleus.⁸ It is within the liver that a remarkable amplification of parasite numbers occurs—one LS spawns tens of thousands of red blood cell-infectious merozoites. Importantly, re-infection of hepatocytes does not occur and once in the blood, the parasite continuously infects red blood cells, a deadly cycle that causes all malaria-associated pathology.⁹ The major obstacle for a successful PE vaccine lies in the required threshold for vaccine efficacy: to protect against infection, the PE vaccine must provide *complete* protection from SPZ infection. Development of a single LS parasite results in full-blown blood stage infection and the immune response against PE stages might not give protection against the blood stage.

The circumsporozoite protein (CSP) is an immunodominant antigen of SPZs.^{10,11} Most of the early malaria vaccine development efforts were focused on this protein and today several formulations (peptide, recombinant, DNA, viral-vector) encompassing different segment(s) of CSP alone or in combination with other antigens have been evaluated. The most advanced malaria subunit vaccine candidate RTS,S is based on CSP. RTS,S formulated with ASO-type adjuvants has demonstrated efficacy.¹² In Phase IIb trials among children living in Mozambique,¹³⁻¹⁵ RTS,S/ASO2A delayed the time to new infection by 30% and reduced episodes of severe malaria by 58% in the first six months,¹⁵ which persisted 21 months after the immunization.¹⁴ Two new adjuvant formulations enhanced RTS,S efficacy from a 30% reduction of all malarial episodes to a 56% reduction (RTS,S/ASO1E) in children 1–4 years old¹⁶ and 65% (RTS,S/ASO2D) in infants.¹⁷ It is unlikely that partially effective vaccines such as RTS,S will eliminate malaria but they might prove useful in combination with existing malaria control strategies. Elimination of malaria will ultimately depend on the development of highly effective vaccines. Do we know whether development of such vaccines is possible? The answer is yes because numerous studies have shown that immunizations with live SPZs confer complete protection against malaria.

Immunizations with live attenuated SPZs damaged by a particular irradiation dose are very effective in inducing complete protection against infectious SPZ challenge.¹⁸ Immunizations with inactivated SPZs however, do not confer protection.^{19,20} Attenuated SPZs infect hepatocytes and form LSs and thus elicit a cellular immune response against the infected hepatocyte.²¹ Long lasting sterile protection was demonstrated with irradiation-attenuated SPZ vaccines in animal models of malaria and importantly also in malaria naïve human volunteers.^{18,22,23} These findings provided a powerful paradigm for an anti-infection

malaria vaccine. Live SPZs and LSs express thousands of potential antigens and the attenuated SPZ model will be critical in identifying new potential antigens for inclusion into second-generation subunit vaccines. However, it is quite conceivable that sterile protection can only be achieved by a multi-pronged immune response that targets many antigens of the infectious parasite and this might make it difficult to design a fully protective subunit vaccine.

Advances in genetic engineering have allowed the creation of a number of live-attenuated parasite lines using gene knockouts in rodent models. These genetically attenuated parasites (GAPs) confer long lasting, sterile protection against infection in rodents. Importantly, work in the rodent malaria models has demonstrated that GAPs can be created to exhibit distinct biological and antigenic features, thus enabling the design of live-attenuated vaccine strains that are safe and elicit sterile protection with low dose immunizations. The creation of the first *P. falciparum* GAPs have recently been reported,²⁴ paving the way for the clinical development of a GAP vaccine for human use. Initiatives in production process development for live attenuated sporozoite vaccines (see Hoffman et al. in press in this issue) might overcome roadblocks that currently prevent the large-scale generation of GAPs. Here we review that state of research and development for a live-attenuated malaria vaccine.

Whole Cell Pre-Erythrocytic Parasite Vaccination Approaches

Irradiation-attenuated sporozoites

Ruth Nussenzweig and co-workers demonstrated in 1967 that immunization of mice with irradiation-attenuated sporozoites (irrSPZs) of the rodent malaria parasite *P. berghei* completely prevented onset of blood stage parasitemia after infectious SPZ challenge.¹⁸ This was a landmark finding that set the standards for immunological protection against malaria infection. Irradiation of SPZs introduces random mutations and breaks into their DNA. When dosed adequately the SPZ survives and remains infectious to the hepatocyte. However, LS development terminates during early hepatocyte infection. The safety and efficacy of irrSPZs is dependent on a precise irradiation dose; too little irradiation allows the parasite to complete LS development and cause blood stage infection, too much irradiation inactivates the SPZs and inactivated SPZs do not induce significant protection.²⁵ In mice, irrSPZ-induced protection is mediated mainly by CD8⁺ T cells, which target the infected hepatocyte but also by antibodies against the major sporozoite surface protein CSP, which block sporozoite infection.^{3,26} Humans immunized with *P. falciparum* irrSPZs (immunized by the bite of irrSPZ-infected mosquitoes) have been effectively protected from subsequent challenge with infectious *P. falciparum* SPZs.^{22,23,27} Given the high levels of protection achieved by irrSPZ-immunization, the development of a *P. falciparum* irrSPZ vaccine was proposed by Stephen Hoffman.²⁸ His company Sanaria Inc., (<http://www.sanaria.com>) has developed a manufacturing process for the aseptic production of irrSPZs in mosquitoes. The irradiation dose is tightly controlled and isolation, purification, formulation and cryopreservation of irrSPZs under good manufacturing practices (GMPs) have been established. A first generation vaccine called the PfSPZ Vaccine has been produced. Currently, the PfSPZ Vaccine administered by intradermal or subcutaneous inoculation by needle and syringe is being tested in a phase 1 clinical study with experimental challenge in malaria naïve volunteers to assess the safety, immunogenicity and protective efficacy of the PfSPZ Vaccine (Stephen Hoffman, Sanaria Inc., MD, personal communication). Calculations based on the previous *P. falciparum* irrSPZ immunization trials, where >1,000 cumulative infectious mosquito bites confer sterile protection that lasted for at least ten months, approximate the protective dose at >100,000 irrSPZs.²⁸ This is in good agreement with irr-SPZ studies in rodent malaria models, where the inoculation dose can be precisely determined. However, a recently published paper showed that repeated immunizations of mice by the bite of 10–15 irrSPZ-infected mosquitoes conferred protection against infectious

SPZ challenge although, this was determined only by assessing a reduction in LS burden, not the occurrence of blood stage patency.²⁹ Furthermore, another recent paper demonstrated that as little as three doses of 750 *P. yoelii* irrSPZs confer complete sterile protection against challenge with infectious SPZs for at least two weeks in mice.³⁰ Therefore, the minimum dose of irrSPZs required for complete protection might be lower than previously thought. Additionally, immunizations with irrSPZs of one rodent parasite species, *P. yoelii* partially protected against challenge with SPZs of another species, *P. berghei* and vice versa,³¹ giving reason to hope that partial cross-species protection might also occur with human parasite species.

Genetically attenuated sporozoites

The availability of genome sequences for a number of *Plasmodium* species,³²⁻³⁴ the generation of stage-specific gene expression data and the ability to genetically manipulate the parasite, has enabled the search for genes that play essential roles for parasite survival at distinct points during the life cycle. The identification of such genes has enabled the informed genetic engineering of the parasite to produce GAPs (Fig. 1). Recently, PE stages of the rodent malaria parasite species *P. berghei* and *P. yoelii* were attenuated by deletion of PE stage-expressed genes known as *UISs* (Upregulated in Infectious Sporozoites). The expression of *UIS* genes is highly upregulated when SPZs gain infectivity for the mammalian host after transitioning from the mosquito midgut to the mosquito salivary glands.³⁵ *UIS3* and *UIS4*,^{6,36,37} are proteins of the LS PVM, the principal host-parasite interface during liver infection.^{6,38} Deletion of *UIS3* and *UIS4* led to complete arrest of LS development soon after hepatocyte infection but *uis4*⁻ parasites showed occasional breakthrough infections when high numbers of SPZs were used for immunization.⁶ However, this was not observed with *uis3*⁻ parasites.^{36,37} Deletion of a further two sporozoite-expressed genes, *P52*, which encodes a putative GPI-anchored protein^{39,40} and *P36*, a gene encoding a putative secreted protein,⁴⁰ also resulted in developmental arrest at the early stage of hepatocyte infection but *p52*⁻ and *p36*⁻ parasites caused breakthrough infections. However, simultaneous deletion of both genes (*p52*⁻/*p36*⁻) resulted in complete attenuation with no breakthrough infections.⁴¹ Immunization of mice with *uis3*⁻, *uis4*⁻, *p52*⁻ or *p52*⁻/*p36*⁻ SPZs induced complete long-lasting protection against infectious SPZ challenge (Table 1),^{6,36,37,39,41} demonstrating that rodent malaria GAPs are highly efficacious vaccines. In some instances protection could be achieved with a single dose of 50,000 GAP SPZs, as shown for immunizations with *P. yoelii uis4*⁻ SPZs³⁷ but this is dependent on the mouse strain used.⁴² The GAP-induced protection was mediated mainly by CD8⁺ T cells^{37,43,44} but antibodies also contributed to protection.³⁷ More recently, research into an additional promising GAP strain was published. Deletion of the sporozoite asparagine-rich protein 1 (*SAP1*) resulted in early LS developmental arrest. No breakthrough infections occurred when up to two million *sap1*⁻ SPZs were injected into mice. Mice immunized with three doses of 10,000 *sap1*⁻ SPZs were completely protected against infectious SPZ challenge for at least 210 days⁴⁵ and recent unpublished data from our laboratory have shown that mice immunized with as few as three doses of 1,000 *sap1*⁻ SPZs are completely protected against infectious SPZ challenge for at least 90 days (Aly AS, SBRI, USA, unpublished results). Interestingly, the *sap1*⁻ SPZs lacked expression of a number of other genes including *UIS3*, *UIS4* and *P52*, rendering it a quasi-multi-loci attenuated strain.⁴⁵

An additional development in GAP research has been the attenuation of LSs by the inactivation of a LS-essential metabolic pathway involved in the synthesis of fatty acids. A bacterial-like type II fatty acid synthesis pathway (FAS II) is encoded by the *Plasmodium* genome.³⁴ Recent transcriptome and proteome data obtained from *P. yoelii* LSs demonstrated the presence of FAS II enzymes and transcript abundance of FAS II enzyme-

encoding genes was highest in liver stages when compared to other life cycle stages.⁴⁶ The fatty acid chain elongation module of FAS II consists of four key enzymes—FabB/F, FabG, FabI and FabZ. Deletion of FabI in *P. berghei*⁴⁷ and either FabB/F or FabZ in *P. yoelii*⁴⁸ had no effect on blood stage replication and gametocytogenesis providing evidence that FAS II was dispensable for this part of the life cycle. During initial LS development, no apparent defect could be observed in any of the FAS II knockout lines. However, late in LS development, the parasite failed to routinely generate exoerythrocytic merozoites. In the *P. berghei fabi*⁻ parasites this caused a severe delay in the onset of blood stage patency⁴⁷ whereas the *P. yoelii fabb/f*⁻ and *fabz*⁻ parasites failed to cause a blood stage infection altogether.⁴⁸ Thus, FAS II is necessary only for late liver stage development. The complete developmental arrest of *P. yoelii fabb/f*⁻ parasites without breakthrough infections allowed our laboratory to test whether this GAP strain induces protection. Indeed, mice immunized with three doses of 10,000 *fabb/f*⁻ SPZs were completely protected against infectious SPZ challenge for at least 210 days. Strikingly, a single dose immunization of 10,000 *fabb/f*⁻ SPZs conferred complete protection for at least a month (Vaughan AM, SBRI, USA, unpublished data). Since FAS II knockouts develop significantly and form large LS schizonts, it is possible that they expose more antigens to the immune system and thus induce a broader, more diversified immune response. However, this hypothesis remains to be investigated.

Another major question is whether the route of immunization determines the efficacy of GAP vaccines. Most published GAP immunization/protection data report intravenous immunization regimens. However, there is published evidence that GAPs protect by other routes of administration such as subcutaneous or intradermal immunizations, shown for the *P. berghei p52*⁻ GAP⁴⁹ and the *P. berghei uis3*⁻ GAP.³⁶ Data from our laboratory has demonstrated that subcutaneous immunizations with the *P. yoelii sap1*⁻ GAP confers complete protection against infectious SPZ challenge (Aly AS, SBRI, USA, unpublished results).

Together, the rodent malaria GAP data demonstrate that safe and protective attenuated malaria parasites can be created by genetic engineering. Is it possible to genetically engineer attenuated human malaria parasites? To that end, the single disruption of *P. falciparum P52* was reported⁵⁰ and in another study the individual deletions and the simultaneous deletion of the *P. falciparum P52* and *P36* were reported.²⁴ The *p52/p36*⁻ dual gene deletion parasites, created by double-crossover recombination that leads to the loss of the genes, were normal throughout the initial stages of the life cycle including SPZ production of the attenuated line. However, *p52/p36*⁻ parasites exhibited complete growth arrest of LSs in vitro in a hepatocytic cell line and in a humanized mouse model carrying human hepatocytes.²⁴ Dual gene deletions might alleviate safety concerns for the use of GAPs as a vaccine in humans. To assess their safety and preliminary efficacy, the *P. falciparum p52/p36*⁻ GAP line has been selected for advance into phase I/IIa clinical trials through the administration of GAP-infected mosquito bites to human volunteers.²⁴

Together the evidence garnered from vaccination with live-attenuated SPZs using animal models of malaria and *P. falciparum* demonstrates that the whole-parasite immunization approach elicits immune responses that completely protect against infection for extended periods of time.

Infectious sporozoite vaccination

Why does repeated natural exposure to infectious SPZs in malaria-endemic areas not induce protection against infection? One proposed potential explanation is that normal SPZs are somehow qualitatively different from attenuated SPZs and do not induce or subvert immune responses. However, immunization of mice with infectious SPZs and treatment with

chloroquine (a drug that kills blood stages) or primaquine (a drug that kills LSs) confers complete protection against challenge after drugs are cleared from the immunized animals.^{51,52} Furthermore, vaccination of human volunteers that had been given chloroquine along with three doses of 12–15 bites each from *P. falciparum*-infected mosquitoes conferred protection against challenge with infected mosquitoes after the drug had waned from volunteers.⁵³ These infection-treatment vaccination experiments indicate that the aforementioned qualitative difference between normal and attenuated SPZs cannot be substantiated. What then is the apparent reason for lack of protection against infection in endemic areas? One proposed mechanism is the suppression of immunity against PEs by blood stage parasites that circulate in many individuals when they become exposed to new infectious mosquito bites. There is evidence from rodent malaria studies that this suppression does indeed occur.⁵⁴ This mechanism has however been challenged recently by research showing that mice with blood stage infections can develop robust, protective T-cell responses against PE stages.⁵⁵ An obvious potential reason for lack of protection against infection might lie in the SPZ dose that is received during natural infectious bite. We currently do not know how many SPZs are transmitted per bite in endemic areas. Laboratory transmission experiments with rodent malaria parasites indicate that the dose ranges from a few dozen to a few hundred per mosquito.⁵⁶ Thus, each individual SPZ inoculation might not be sufficient to elicit any protective response at all and might even induce immune-tolerance. Clearly, much remains unknown in this complex biological puzzle.

Immunological mechanism of protection

Complete, long-lasting protection is provided by both live-attenuated irrSPZs and GAP SPZs.^{57,58} CD8⁺ T cells play a critical role in the protection of mice immunized with irrSPZs,^{26,59} and IL-4-secreting CD4⁺ T cells are essential for the development of CD8⁺ T cell responses to LS parasites.⁶⁰ Loss of protective immunity in β_2 microglobulin^{-/-} mice (which lack CD8⁺ T cells and natural killer T cells) and in mice depleted of CD8⁺ T cells indicates the indispensable role of CD8⁺ T cell-mediated effector mechanisms.^{61,62} Protection in humans conferred by *P. falciparum* irrSPZ might also depend on CD8⁺ lymphocytes.⁶³ Since irrSPZ immunizations protected perforin^{-/-} and granzyme B^{-/-} mice (which lack the ability to kill cells through cytolytic activity) from infectious SPZ challenge, it was speculated that CD8⁺ T cell killing of infected hepatocytes is primarily through interferon- γ (IFN γ)-induced nitric oxide (NO), rather than by direct cytolytic activity.^{64,65} Indeed, it has been shown that IFN γ responses correlate with protective immunity against the PE stage⁶⁴ although the importance of CD8⁺ T cell-derived IFN γ in particular is yet to be fully established.^{66,67} Sterile protection obtained with a *P. berghei* GAP was found to correlate with IFN γ producing CD8⁺ T cells.⁴³ Conversely, IFN γ independent CD8⁺ T cell-mediated protective immunity has also been demonstrated,⁶⁸ and IFN γ secretion by CD8⁺ T cells may not be essential for protecting mice against *P. yoelii* SPZ challenge.⁶⁹ It is likely that CD8⁺ T cell IFN γ induction and cytolytic activity are both involved in the full range of immune responses to irrSPZs and GAP SPZs.

Protective CD8⁺ T cell priming occurs in skin-draining lymph nodes after immunization by the bite of irrSPZ-infected mosquitoes,⁷⁰ whereas hepatic CD8⁺ dendritic cells (DCs) are responsible for priming CD8⁺ T cells by intravenous immunization with irrSPZs.⁷¹ Both wildtype and irrSPZs are processed for class I presentation by DCs. However, DCs pulsed with wildtype SPZs can only stimulate memory CD8⁺ T cells, whereas DCs pulsed with irrSPZ are capable of activating both effector and memory CD8⁺ T cells.⁷²

The puzzle of persistence

It is thought that in the irrSPZ model, the maintenance of protective immunity requires persistence of growth-arrested LSs. The evidence supporting this notion comes from

experiments in which *P. berghei* irrSPZ-vaccinated rodents were treated with primaquine, a drug that kills early liver stages.⁷³ This experimental design resulted in gradual loss of protection in immunized rats, 42% loss after one month and 84% loss after three months. The primaquine had to be given within seven days after irrSPZ immunization. If primaquine was given later, it had no impact on protection. The same study detected irrLSs in the liver of immunized rats for up to six months.⁷³ The need of persistent irrLSs was confirmed in another study using *P. berghei* irrSPZ-vaccinations in mice.⁷⁴ Treatment of irrSPZ-immunized mice with primaquine had no effect on protection when mice were challenged one week after the last immunization but if challenged at five months after the last immunization, 50% of the mice lost protection.⁷⁴ For GAP SPZ immunizations, there is currently only one published study that has examined the effect of primaquine treatment on protection,⁴⁴ which used immunizations with the *P. berghei uis3⁻* GAP. The authors found that primaquine treatment of GAP-immunized mice led to complete loss of protection when mice were challenged one month after the last immunization.⁴⁴ In the *P. yoelii* GAP models however, growth arrested LSs do not persist, but nevertheless immunizations with these GAPs confer long lasting sterile protection against SPZ challenge.^{37,41} Thus, the requirement for growth-arrested LSs to persist in immunized animals in order to maintain immune protection might be exclusively associated with the *P. berghei* model but this important issue will need further investigation. Recent data from our laboratory clearly show that a triple immunization protocol with *P. yoelii uis3⁻* SPZs followed by primaquine treatment after each immunization conferred complete protection against infectious SPZ challenge given three months after the last immunization (Mikolajczak SA, SBRI, USA, unpublished data), indicating that persistent LSs are not necessary for the maintenance of immune protection in this GAP model.

Outlook and Conclusions

The limited data available for irrSPZ immunization studies in humans indicate that live-attenuated whole parasites induce sterilizing immunity against challenge with infectious *P. falciparum* SPZs. The immune protection is strain-transcending and lasts for at least ten months. Furthermore, as discussed earlier, numerous animal studies support the notion that GAP vaccinations confer sterile, long lasting protection. Within the next two years a clinical dose escalation study with a cryopreserved formulation of irrSPZs that is administered intradermally or subcutaneously and a first generation genetically attenuated SPZ vaccine that is administered by mosquito bite will likely yield clear data on the safety and protective efficacy of this class of vaccines. In the future then, the most formidable obstacle for the live-attenuated vaccine approach lies in the current need for vaccine cryopreservation in liquid nitrogen. A cryopreserved malaria vaccine requires a special cold chain and cannot be delivered in the context of the expanded program on immunization (EPI), currently the major platform through which many childhood vaccines are delivered in resource poor nations. Does that imply that live-attenuated vaccine approaches are a dead end and will remain confined within the research arena? In this context, it is of importance to consider that delivery of a malaria vaccine for disease elimination will likely require delivery platforms that are distinct from EPI, possibly mass vaccination campaigns. Furthermore, although no human vaccine is currently delivered in a cryopreserved formulation, it is of interest to note that live whole cell-parasite vaccines for veterinary applications such as a vaccine for African East Coast Fever are delivered in liquid nitrogen in resource poor countries.⁷⁵ It is conceivable that if subunit vaccine approaches continue to confer only limited protection, the delivery of an efficacious whole cell attenuated malaria vaccine will be the only means by which we can hope to eradicate malaria. If this turns out to be the case, the emphasis is likely to shift from development to delivery of a malaria vaccine in the coming decades.

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Abbreviations

GAP	genetically attenuated parasite
irr	irradiated
LS	liver stage
PE	pre-erythrocytic
SPZ	sporozoite
UIS	upregulated in infectious sporozoites
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane

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Figure 1.

Liver stage (LS) developmental arrest of genetically attenuated parasites (GAPs) in rodent malaria models. Wildtype sporozoites invade hepatocytes with the formation of a parasitophorous vacuole membrane (PVM), transform into LS trophozoites and mature as LS schizonts. Schizonts eventually form infectious exo-erythrocytic merozoites (wildtype). GAP sporozoites successfully invade hepatocytes but do not develop fully as LSs. The *p52*⁻/*p36*⁻ GAP arrests without the formation of a PV. The *sap1*⁻ GAP forms a PVM and then arrests. The *uis3*⁻ and *uis4*⁻ GAPs form a PV begin to develop but arrest soon after the transition to early schizogony. The *fabb/f*⁻ GAP develops furthest and enters into late schizogony but is unable to form infectious merozoites.

Table 1

Biological features and protective efficacies of rodent malaria GAP vaccines

Knock out (KO) gene*	Localization/ Function	Phenotype of KO parasite	Mouse strain	Immunization dose $\times 10^3$ (interval in days)	Challenge dose $\times 10^3$ (day after immunization)	% Protection	Potential mediators of protection	Ref #
<i>P. berghei</i>								
UIS4	PVM*/Critical for LS development	Development arrested at early schizont Occasional breakthrough	C57BL/6	10/10 (14)/10 (28)	50 (38)	100%	Not determined	6
UIS3	PVM/Critical for LS development	Development arrested at early schizont No breakthrough	C57BL/6	10/10 (34)/10 (45) 10/10 (14)	10 (30) 10 (7)	100% 70%	IFN γ , CD8 T cells in IFN γ -/- -, or by Ab depletion, adoptive transfer	36 36
UIS3 & UIS4 (double KO)	PVM/Critical for LS development	Development arrested at early schizont No breakthrough	C57BL/6	10/10 (14)/10 (28)	10 (7, 118)	100%	IFN γ -producing CD8 T cells in $\beta 2m$ -/-, or by intracellular cytokine staining	43
P52	Micronemal protein PVM formation	Development arrested at trophozoite Occasional breakthrough	BALB/c C57BL/6 C57BL/6	50 50/20 (7) 50/20 (7)/20 (14)	10 (120) 10 (10) 10 (30)	100% 25% 100%	Not determined	39 39 39
<i>P. yoelii</i>								
UIS3	PVM/Critical for LS development	Development arrested at early schizont No breakthrough	BALB/cJ BALB/cJ BALB/cJ	10/10 (14)/10 (28) 10/10 (14)/10 (28) 50	10 (60) 10 (180) 10 (7)	100% 75% 0%	CD8 T cells by Ab depletion	37 37 37
UIS4	PVM/Critical for LS development	Development arrested at early schizont No breakthrough	BALB/cJ BALB/cJ BALB/cJ BALB/cAn	10/10 (14)/10 (28) 10/10 (14)/10 (28) 50 50	10 (60) 10 (180) 10 (30) 10 (30)	100% 100% 100% 0%	CD8 T cells by Ab depletion	37 37 37 42
SAP1	Cytoplasm regulates expression of UIS genes	Development arrested at trophozoite No breakthrough	BALB/cJ	10/10 (14)/10 (28)	10 (30, 110)	100%	Not determined	45
P52/P36 (double KO)	P52 in micronemes P36 unknown PVM formation	Development arrested at trophozoite No breakthrough	BALB/cJ	10/10 (7)/10 (14)	10 (30)	100%	Not determined	41

* PVM, Parasitophorous vacuole membrane; LS, liver stage; KO, knockout.