Arrested Plasmodium liver stages as experimental anti-malaria vaccines

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Eukaryotic pathogens typically follow a complex life cycle, including host switch and morphologically distinct forms. Parasite stage conversion offers exceptional opportunities for whole organism vaccine development. In the case of Plasmodium, the causative agent of malaria, disease is exclusively caused by asexual blood stages that invade and replicate within erythrocytes. Pathogenic blood stage infections are preceded by a silent parasite growth phase inside the liver. Two alternative experimental whole organisms vaccine strategies that lead to arrested Plasmodium liver stages elicit potent, lasting immunity against re-infection. Live irradiation- or genetically arrested parasites are metabolically active and correspond to classical attenuated vaccines. Specific antimalarial treatment during experimental natural sporozoite infections prevents a febrile malaria episode and, simultaneously, induces effective anti-liver stage immunity. Translation of these strategies into a safe, affordable and accessible pediatric anti-malaria vaccine requires major bioengineering and pharmaceutical improvements, respectively, but holds promise for a truly effective immunization scheme against the most prevalent and fatal vector-borne disease.
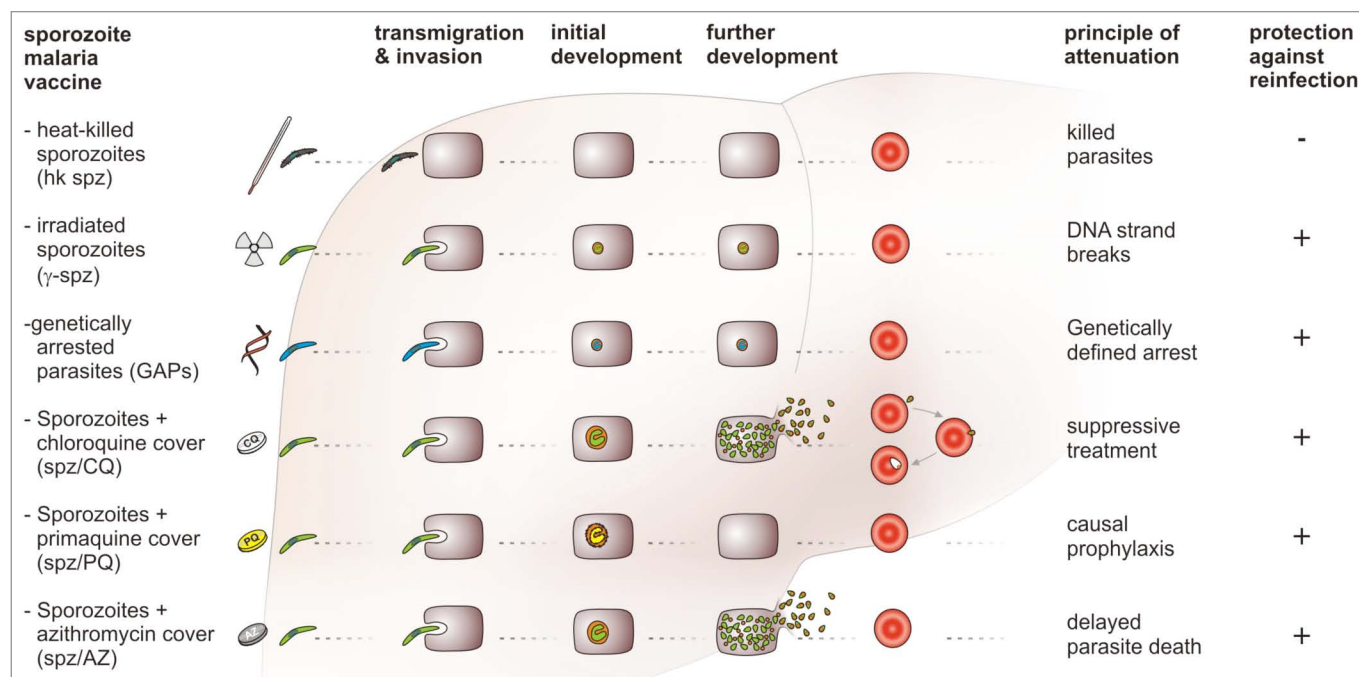
Introduction

Malaria is caused by unicellular, obligate intracellular eukaryotes of the genus Plasmodium that can invade and replicate within erythrocytes. This vector-borne parasitic disease continues to pose a major disease burden and death toll, primarily in infants and children living in Sub-Saharan Africa.[1] As in all apicomplexan parasites, the pathogen life cycle follows a complex developmental program, in the case of Plasmodium inside the mosquito and mammalian hosts. The signature febrile episodes are caused by the synchronized rupture and re-invasion

of the host erythrocytes. Exponential expansion of the parasite population during asexual blood stage growth leads to malaria-related morbidity and clinical symptoms. A proportion of clinical malaria cases develop life-threatening complication, which can include anemia, multi-organ failure or cerebral malaria.[2] What triggers disease exacerbation remains an open question. It is believed that contributing factors include the initial sporozoite dose inoculated during the mosquito bite, parasite virulence, host genetics and co-infections with helminths or bacteria.[3-7] Reliable biomarkers that would predict disease progression are still missing.

In endemic areas anti-malaria immunity is only acquired gradually after many repeated exposures.[8,9] The first responses to develop in children protect against severe complications without affecting mild disease or parasite burden. Over time, often not before adolescence, anti-disease immunity is mounted, leading to 'clinical tolerance' as opposed to sterilizing immunity. Field studies also show that antibody responses are short-lived and necessitate continuous re-exposure to eventually persist.[10,11] A proportion of adults remains symptom-free and parasite-positive, and hence, do not receive treatments, contributing to the continuous parasite propagation via mosquito transmission. Together, naturally acquired anti-malaria immunity is slow, incomplete, short-lived and strain-specific. Whether immunization strategies can mount more potent and lasting protective immune responses and surpass nature remains to be shown.

Before the onset of a blood stage infection Plasmodium undergoes an obligate, yet diagnostically and clinically silent, population expansion phase in the liver.[12,13] Intra-hepatic development compensates for one of the two bottlenecks, i.e., sporozoite inoculation during the short probing phase of a mosquito bite, in the Plasmodium life cycle and leads to formation of thousands of infectious merozoites. Sporozoite-to-merozoite stage conversion takes several days and represents a unique window of opportunity for anti-malaria vaccine development.[14] Recent experimental whole organism vaccine approaches in rodent malaria systems have consistently demonstrated long-lasting sterilizing immunity against reinfection. Complementary vaccine approaches aim at targeting the pathogenic blood stages and transmission stages.[15-18]

sporozoite malaria vaccine	transmigration & invasion	initial development	further development	principle of attenuation	protection against reinfection
- heat-killed sporozoites (hk spz)				killed parasites	-
- irradiated sporozoites (γ-spz)				DNA strand breaks	+
-genetically arrested parasites (GAPs)				Genetically defined arrest	+
- Sporozoites + chloroquine cover (spz/CQ)				suppressive treatment	+
- Sporozoites + primaquine cover (spz/PQ)				causal prophylaxis	+
- Sporozoites + azithromycin cover (spz/AZ)				delayed parasite death	+

Figure 1. Experimental approaches for developmental Plasmodium liver stage arrests. Heat-killed sporozoites (hk spz) cannot enter a suitable hepatocyte and, hence, fail to elicit protection against re-infection, indicative of a minor role of antibodies against sporozoite surface proteins in lasting protective immunity. Irradiated sporozoites (γ-spz) retain their capacity to actively enter their host cells and initiate the transformation process inside a hepatocyte. They are replication-deficient because of multiple random DNA double strand breaks but persist as metabolically active parasites and elicit lasting protection. Genetically arrested parasites (GAPs) contain tailor-made, stable deletions of Plasmodium genes that exert vital functions during liver stage development. Similar to γ-spz, GAPs persist as metabolically active parasites and confer protection. Experimental sporozoite exposure (SE) under drug cover (DC) aims at preventing febrile malaria while simultaneously inducing pre-erythrocytic immune responses. SE under chloroquine cover (spz + CQ) can induce brief, but mild malaria episodes. In addition, global CQ resistance necessitates inoculation of CQ-sensitive laboratory strains under clinical surveillance. Sporozoite exposure under primaquine cover (spz + PQ) takes advantage of a causal-prophylactic drug, which kills intra-hepatic parasites. Intracellular killing by pharmacological treatment induces lasting protection. Sporozoite exposure under azithromycin cover (spz + AZ), a safe antibiotic licensed for children and during pregnancy, induces a delayed parasite death, leading to complete maturation of liver stages, yet inviable liver stage merozoites. This strategy combines the advantages of drug cover by CQ, i.e. full maturation and, hence, maximum antigen display and PQ, i.e. global parasite susceptibility to the drug and prevention of blood stage infections and malaria symptoms during the immunization phase.

Here, we present an overview of the different strategies that lead to a pre-erythrocytic life cycle arrest.

Radiation-Attenuated Sporozoites: First Generation Whole Organism Vaccines

Malaria vaccinology started with immunizations in experimental animals using killed blood stage parasites plus adjuvants[19,20] and killed or UV-inactivated sporozoites[21,22] or both parasite stages.[23] These traditional whole organism vaccine strategies offered only partial, if any, protection against re-infection. The findings established that eliciting anti-sporozoite or -blood stage immune responses, at least by the approaches available at that time, do not offer a path towards malaria vaccine development. Moreover, the experiments already indicated that vaccine strategies against a complex, slow-growing eukaryotic pathogen need to be fundamentally different from anti-viral vaccines, which often mimic naturally acquired immunity.

The first conclusive demonstration that lasting protection can be elicited by a whole organism vaccine strategy was by a series of immunization studies performed at New York University using intravenous injection of radiation-attenuated sporozoites (γ-spz or RAS) in mice[24-30] (**Fig. 1**). These findings established that sporozoites need to be viable in order to confer protection. Heat-killed sporozoites (hk spz), for instance, cannot enter a suitable hepatocyte and, hence, fail to elicit protection against re-infection[31] (**Fig. 1**). Moreover, protection following γ-spz immunization is acting exclusively against the pre-erythrocytic stages, since bypassing the life cycle by transfusion of infected blood led to a fulminant malaria infection. Subsequent work in rodent models established that protective immunity is of multifactorial nature and directed against free sporozoites through high titers of blocking antibodies and intracellular liver stages via IFNγ secreting conventional αβ T cells, as well as NK cells and γδ T cells.[13,32-34] The γ-spz immunization strategy has been swiftly translated to trials in non-human primates[35] and human volunteers.[36]

The demonstration of lasting sterile protection in a number of vaccinees that received a high number of bites from irradiated *P. falciparum*-infected Anopheles mosquitoes[36] has been confirmed in a number of subsequent small phase IIa trials and established the current gold standard for an anti-malaria vaccine.[37,38] It has been argued that the translation of γ-spz from an

experimental vaccine to a pediatric formulation is hampered by several hurdles and, hence, impractical.[39] Vaccination with γ-spz, however, most likely induces very potent, strain transcending long lasting cellular immune responses. It remains to be formally demonstrated that a subunit vaccine can achieve lasting protection against a eukaryotic pathogen. Delivery of the gold standard vaccine to the people in need is an ambitious goal that deserves sustained commitment from researchers, funding agencies and public health authorities. Towards this goal, researchers currently explore practical and efficient vaccine administration routes, sporozoite purification and freezing methods and logistics to distribute liquid nitrogen-frozen vials to rural health centers.[40,41]

An alternative vaccine candidate that emerged from the studies utilizing γ-spz was the successful cloning of the major *P. falciparum* sporozoite surface protein, termed circumsporozoite protein (CSP),[42] and the subsequent development of the RTS,S subunit vaccine, which includes portions of CSP as the malaria antigen and is the first anti-malaria vaccine candidate ever to enter testing in phase III clinical trials in Africa.[43,44]

Genetically Arrested Parasites (GAPs): Tailor-Made Vaccine Lines

Near-complete Plasmodium genome sequence data,[45] expression profiling,[46] and reliable transfection technology[47] permit molecular genetics approaches to generate Plasmodium vaccine lines with precise gene deletions.[14,48] Important requirements are (1) non-vital roles during asexual growth, where transfection is performed; (2) normal transmission, sporozoite formation inside the mosquito vector, and invasion of host hepatocytes; and (3) complete life cycle block between sporozoite entry and release of infectious merozoites from the liver. In a proof-of-concept study in the *P. berghei*/C57BL/6 model, targeted deletion of a signature liver stage protein, termed upregulated in infectious sporozoites gene 3 (*UIS3*), resulted in an early liver stage arrest and absence of blood stage infections, even when very high sporozoite doses were injected intravenously[49] (**Fig. 1**). Immunization with three doses of *uis3(-)* sporozoites mounted long-lasting sterile protection against sporozoite re-infection, but not blood stage transfusion, supporting the notion that immunity is liver stage-specific. The concept to test genetically arrested parasites (GAPs) as experimental malaria vaccines was corroborated by additional targeted deletions of other liver stage-specific genes both in the *P. berghei*/C57BL/6 and the *P. yoelii*/Balb/c models.[50-54] Protection offered following GAP-vaccination has been shown to be primarily dependent on CD8 T cells.[53,55,56]

Following the promising pre-clinical findings in rodent models, one vaccine line has been translated to *P. falciparum* by generation of *p36/p36p(-)* parasites, despite the early indications of substantial break-through infections in the rodent model.[57,58] It remains uncertain how this parasite line can be tested in human trials and highlights the need for rigorous pre-clinical testing.

In an effort to develop safe GAPs that display unconditional liver stage arrest a master regulator of gene expression in *P. berghei* sporozoites, termed *SLARP* (sporozoite and liver stage asparagine-rich protein), was targeted.[59] Loss of *SLARP* function results in complete early liver stage arrest. When tested as an experimental malaria vaccine, *slarp(-)* sporozoites induced only modest and short-lived protection against reinfection. Expression profiling demonstrated that expression of a number of signature liver stage antigens, including UIS3 and UIS4, are controlled by SLARP, offering a molecular explanation for low vaccine efficacy of *slarp(-)* immunizations and opening a rationale for discovery of protective liver stage antigens by differential profiling.[59] Experiments with the *P. yoelii* homologue, called sporozoite asparagine-rich protein 1 (SAP1), led to complete protection in the Balb/c malaria mouse model.[60] This apparent discrepancy may be attributed to the presence of an immunodominant CSP H2K^d-restricted CD8$^+$ T cell epitope,[61] corroborating the notion that the *P. berghei*/C57BL/6 model is the most difficult to protect against, and hence, serves as the most stringent preclinical model for anti-malaria vaccine development.

Sporozoite Exposure during Drug Cover (SE/DC): A 'Needle'-Free Immunization Approach?

The potential of treatment with registered antimalarial drugs during sporozoite exposure to induce potent immune responses was first tested in rodents. In these studies oral chloroquine (CQ) was given over a period of repeated sporozoite injections.[62,63] Protection was also found to be mediated primarily by CD8$^+$ T cells.[63] Roestenberg et al. could recently demonstrate the potency of this approach in human volunteers.[64] In the vaccinees multifunctional (IFNγ- and IL2-secreting) T cells of the effector memory phenotype (CD62L$^-$, CD45RO$^+$) were consistently detected, but the precise molecular and immunological mechanisms of protection remain largely undefined. Individuals, who adhere to a strict CQ cover and effectively follow a SE/CQ scheme, can occasionally be found in malaria-endemic countries and were the basis for a successful antigen discovery program, which returned a number of liver stage antigens, e.g., LSA1.[65] High anti-LSA1 antibody titers consistently correlate with a lower risk of clinical malaria.[10,66] Although circumstantial, these findings support the premise that SE/CQ might have elicited liver stage immunity in the past. Because of widespread resistance of *P. falciparum* against CQ[67] replacement drugs with CQ-like anti-blood stage activity or, alternatively, with unconventional modes of action against the liver stage are essential for the translation of this concept into a clinical intervention.

The latter hypothesis was recently tested in rodent models using the liver-specific drug primaquine (PQ) as cover during sporozoite exposure[68] (**Fig. 1**). SE/PQ leads to a high degree of sterile protection after three rounds of immunization, including SE by bites of infected mosquitoes during the immunization phase, which most closely mimics natural transmission at night. Unfortunately, severe side effects of PQ and other 8-aminoquinolines limit the use of this intervention in malaria-endemic countries.[69] However, it will be interesting to test in a limited proof-of-concept clinical trail in humans whether SE/PQ would also induce consistent protection against reinfection.

If confirmed, the search for novel compounds that effectively kill intra-hepatic stages will gain further momentum.

More recently, studies in the *P. berghei*/C57BL/6 model established that concomitant use of antibiotics (Abx), such as azithromycin (AZ) and clindamycin, with infectious sporozoites does not lead to development of pathogenic blood stage infection despite full maturation of exoerythrocytic forms and release of hepatic merozoites.[70] This so-called "delayed death" of parasites occurs after administration of antibiotics, which target the algae-originating apicoplast, an organelle encoding the prokaryotic-type translation machinery.[71,72] When administered in a vaccination scheme, sporozoite exposure under Abx cover induces robust CD8 T-cell mediated protection against re-infection with mosquito-derived salivary gland sporozoites (**Fig. 1**). This strategy is apparently at least as powerful as the irradiated sporozoite gold-standard vaccine and might in fact be superior, since developmental arrest during late liver stage development may lead to improved live-attenuated vaccines due to the presence of a wider antigenic repertoire. Yet, correlates of protection, most likely protective CD8^{+} T-cell epitopes, remain to be identified. Systematic identification and characterization of these, still elusive signatures of protection will also inform the development of second-generation subunit vaccines.

Long-acting antibiotic drugs like AZ are being tested for mass drug administration, for instance in trachoma control programs. A recent documentation of an unanticipated substantial reduction in overall mortality after a campaign in Ethiopia[73] encourages optimism that the protective effect observed in the experimental immunizations may be translated to malaria-endemic regions. One testable hypothesis is that intermittent delivery of AZ to the most vulnerable target populations, young children and pregnant women, may have a dual role in protection against malaria. In addition to an immediate therapeutic effect against an ongoing blood stage infection, sporozoites that are being delivered after bites of *P. falciparum*-infected mosquitoes will be arrested leading to gradual acquisition of anti-preerythrocytic immunity. Because of a potential vaccine-like secondary effect, oral Abx administration together with natural sporozoite exposure could effectively complement needle-vaccinations. Abx cover will also add an additional safety level or may even boost immunity in prospected experimental human trials with GAPs.

Due to its favorable drug safety record, AZ can be used in all ages and risk groups.[74] The results of ongoing intermittent preventive treatment in pregnancy (IPTp) studies with a fixed drug combination azithromycin and chloroquine against uncomplicated malaria will inform future wider use and, perhaps, inclusion into other IPT programs.

Outlook

Development of a safe, affordable and long-lasting pediatric malaria vaccine and identification of immune correlates of protection among the abundant non-protective host responses remain research priorities in infection biology. Understanding the molecular and immunological mechanisms of the crosstalk between the parasite and the host is a prerequisite for rational anti-malaria vaccine discovery and development. Metabolically active, arrested liver stage parasites by live irradiation or molecular genetics and therapeutic cover during experimental sporozoite infections are alternative experimental whole organism vaccine strategies. Systematic immunological profiling of arrested parasites has the potential to inform translation of a whole organism anti-malaria vaccine to the human pathogen and can lead to the identification of protective antigens that have been elusive thus far. Together, precise genetic and pharmaceutical arrests of Plasmodium liver stage development are important approaches towards vaccine discovery.

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