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Visions & Reflections (Minireview)

Hitting malaria before it hurts: attenuated *Plasmodium* liver stages

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Abstract. Continuous natural exposure to *Plasmodium* transmission by infectious *Anopheles* mosquitoes leads to a gradual acquisition of immunological competence against malaria. The partial immunity, observed in adolescents and adults living in endemic areas, reduces morbidity and mortality without preventing parasite infection. In experimental animal models, long-lasting sterilizing immunity can be achieved with genetically attenuated *Plasmodium* liver stages. Can these findings be translated to accomplish sterile protection against natural malaria transmission in the high-risk group, young infants in sub-Saharan Africa?

Keywords. Malaria, *Plasmodium*, protective immunity, liver stage, whole organism vaccine.

The silent *Plasmodium* liver stages

Management of virtually any vector-borne infectious disease is amongst the most difficult medical missions and typically consists of the classical triad, i.e. vector control programs, exposure prophylaxis and clinical management. Intriguingly, once a vaccine against the transmitted pathogen becomes available, the corresponding disease is usually well contained. Examples for successful vaccinations of arthropod-transmitted infectious diseases include the mosquito-transmitted yellow fever virus and tick-borne encephalitis. However, vaccines are not yet available against many vector-borne diseases, such as the Dengue fever virus and *Plasmodium*, the unicellular eukaryote that causes malaria. For these pathogens, we are currently witnessing a constant spread and increase in incidences, despite worldwide comprehensive control efforts [1, 2].

In principle, there are two major life cycle phases of the malarial parasite that can be attacked by immunization efforts: the clinically silent liver stage development and the pathogenic intra-erythrocytic replication (Fig. 1). Malaria transmission occurs during the mosquito bite when several dozen sporozoites are injected from the mosquito salivary glands into the skin of the host [3]. Sporozoites actively move away from the site of injection, enter a capillary and eventually reach the liver. Here, they migrate from the liver sinusoid through the space of Disse to take residence in their final target cell, a hepatocyte that is permissive for replication [4]. Inside the liver, the parasite undergoes a dramatic transformation and replication program that ultimately results in the formation and egress of up to 30 000 infectious merozoites, which specifically invade erythrocytes and commence the pathogenic blood stage cycle (Fig. 1).

It is this final phase of the *Plasmodium* liver stage development when we need to worry about malaria. Up until then we have a window of opportunity of at least 7 days for innovative intervention strategies.

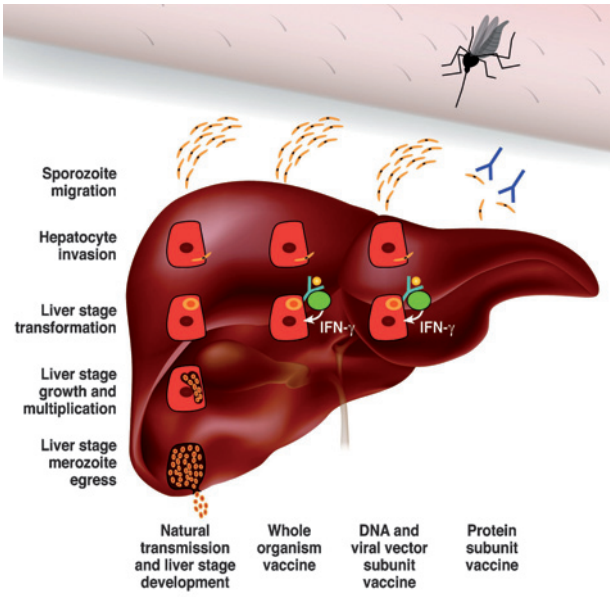

Figure 1. Liver stage development of the *Plasmodium* parasite and potential immune intervention strategies. *Plasmodium* sporozoites (amber) are injected into the skin during the brief probing phase of an infected *Anopheles* mosquito (top). Sporozoites actively penetrate a blood capillary and eventually reach the liver (bottom). Upon natural liver stage development (far left), sporozoites first invade a suitable hepatocyte and transform into early liver stages. The liver stages commence growth and undergo multiple rounds of replication to ultimately produce several thousand liver stage merozoites. Merozoites egress from the infected hepatocyte and invade host erythrocytes where they initiate the pathogenic red blood cell cycle. Upon immunization with a whole-organism vaccine (center left), an infected hepatocyte is recognized by interferon γ-secreting effector T cells (green) through MHC class I presentation (light blue) of protective liver stage antigens (yellow). Once the protective antigens have been identified, DNA vaccines (center right), which may reproduce sterile immunity, can be developed. These subunit vaccines deliver the protective antigens intracellularly and induce T cell priming. The sporozoite inoculation rate can be reduced by high anti-sporozoite antibody titers (far right). Combinations of protein and DNA subunit vaccines may lead to a substantial reduction of the initial parasite load after egress of the first-generation merozoites, which in turn may reduce complications during subsequent expansion of the blood stages.

The concept of pre-erythrocytic vaccines

Sporozoite transmission to the human host is one of two population bottlenecks of the *Plasmodium* life cycle [3] and this limitation is compensated for by the dramatic intra-hepatic parasite replication. Any intervention prior to liver stage merozoite egress is therefore likely to be far more effective than during erythrocytic schizogony where parasite numbers quickly reach millions of infected erythrocytes per milliliter blood. *Plasmodium*, being an obligate intracellular parasite, chose two ideal host cells with respect to avoiding cellular immune responses. As a terminally differentiated cell, erythrocytes are unlikely to serve as an antigen-presenting cell, and during the hepatic phase, the parasite develops in an immunologically privileged organ that triggers tolerance rather than priming of immune responses. The host cell choice during the population expansion phase together with low transmission burden probably accounts for the very limited naturally acquired anti-pre-erythrocytic immune responses. Therefore, eliciting strong liver stage immunity by a vaccination strategy may result in protective responses that are not normally seen in endemic areas and will probably be superior to naturally acquired immunity (Fig. 1).

Currently, there are numerous clinical trials ongoing that all rely on recombinant candidate pre-erythrocytic and, to a lesser extent, blood stage antigens in various regimens, including polypeptides combined with adjuvants, DNA and viral vectors and combinations thereof [5]. There is little doubt that a recombinant vaccine will likely be the most cost-effective vaccine that can be produced at sufficient quantities to cover the endemic areas. Of equal importance, such a vaccine can be produced under good manufacturing procedures (GMP), ensuring continuous high-quality vaccine batches. However, the development of recombinant malaria vaccines has proven to be far more complex than initially anticipated. Since the success of recombinant S-antigen to elicit a protective humoral response against the hepatitis B virus in the mid 1980s, no additional recombinant vaccine has been licensed for human use. The criteria for turning *Plasmodium* genes into vaccine candidates remain particularly challenging since only very few partially protective antigens have been identified and all subunit vaccine candidates were chosen empirically. So far, the approach to accelerate vaccine development prior to vaccine discovery, which is typically seen as prerequisite for a vaccine to be effective, has not met the expectations for a protective malaria vaccine. The most advanced subunit vaccine strategy, RTS,S/AS02A, elicits high antibody titers against the major sporozoite surface antigen and effectively reduces the transmission inoculum (Fig. 1), yet fails to protect the vaccinees from acquiring pathogenic blood stages [6].

Genetically attenuated parasites as malaria vaccines

In general, live attenuated vaccines are probably the most effective. Live vaccines are so potent because they function from inside out, i.e. by infecting a cell without causing pathology. Infected host cells display antigen fragments of the pathogen on their cell surface in the context of the class I major histocompatibility complexes (MHC I). This MHC I-dependent antigen display triggers the cell-mediated arm of the immune system, which eventually activates cytotoxic T lym-

Table 1. Induction of sterilizing immune responses against natural malaria transmission.

Strategy	Model system[1]	Reference
Irradiated sporozoites	*P. berghei*/mice	7
	P. knowlesi/Rhesus monkey	8
	P. falciparum/humans	9, 10
Life sporozoites and drug treatment	*P. berghei*/mice	11, 12
Genetically attenuated parasites	*P. berghei*/mice	14, 18, 19

[1] Malaria parasite/host combinations that were used to demonstrate protection.

phocytes that recognize and kill infected cells (Fig. 1). Pioneer studies in the late 1960s and early 1970s with irradiation-attenuated *Plasmodium* sporozoites demonstrated the induction of long-lasting protective immune responses against natural malaria transmission in model animals and humans [7–10] (Table 1). However, since irradiated sporozoites consist of a very heterogenous parasite population with unknown random mutations, this approach cannot be translated to a licensed vaccine. Similarly, pharmacological inhibition of blood stage infection induces sterilizing immune responses [11, 12] (Table 1), whereas heat-killed or over-radiated sporozoites do not [13], corroborating the notion that target cell infection is vital to protection and that anti-sporozoite antibodies are not essential.

Recently, a defined, metabolically active, attenuated parasite line was generated by targeted gene disruption of the *Plasmodium berghei UIS3* (upregulated in infectious sporozoites gene 3) locus [14] (Table 1). Mutant parasites are phenotypically indistinguishable from wild type sporozoites prior to intra-hepatic development and elicit strong antibody responses [15]. Once inside the liver, parasites arrest in their development and, consequently, do not cause blood stage infection [14] (Fig. 1). Importantly, attenuation is independent of the host immune status, suggesting that the mutant parasites do not pose any risk such as mild or full-blown malaria [15]. These genetically attenuated parasites (GAPs) confer long-lasting sterilizing immunity that is MHC I dependent and largely mediated by interferon (IFN)-γ producing CD8+ T cells [14–16] (Fig. 1). GAPs offer an unprecedented opportunity to induce complete, sterilizing and long-lasting protection against malaria transmission with a genetically defined and homogenous vaccine strain.

A major challenge is to translate these encouraging findings to the human malaria parasite (Table 2). The generation of a safe *P. falciparum* GAP that arrests during liver stage development is highly feasible, based on the available genome data [17] and the generation of additional *P. berghei* GAPs [18, 19]. The latter findings suggest that disruption of the orthologous *P. falciparum* genes or perhaps of any liver stage-specific vital gene will result in developmental arrest. Important issues that have been experimentally addressed but need further substantial improvements include sporozoite *in vitro* propagation systems [20] to eventually avoid mosquito infections, viability of sporozoite freeze-downs [21] and the evaluation of clinically practical delivery routes for sporozoites [22] (Table 2). Fundamental limitations towards clinical approval may include (i) the presence of a heterologous selection marker in the mutant parasite line, (ii) the use of human blood and (iii) the use of non-sterile mosquitoes for GAP generation. However, with the revived interest in translating the gold-standard of an experimental malaria vaccine into a viable vaccine strategy, these road blocks are likely to be sequentially removed. A safety concern relates to parasite persistence, which is crucial for sustained sterile protection [15], yet may also elicit undesirable inflammatory responses in the liver (Table 2). However, so far, no malaria-related liver pathology has been reported from high *P. vivax* transmission areas, indicating that dormant liver stages do not cause adverse effects. Since immunization trials with irradiated sporozoites have never been advanced to transmission areas, a potential failure of GAPs to confer long-lasting sterile protection in humans upon continuous exposure cannot be excluded (Table 2).

If successful, *P. falciparum* GAPs will serve several complementary purposes: (i) the defined developmental arrest will permit expression profiling of candidate protective antigens and may, for the first time, allow a rational path for vaccine discovery, (ii) short-term visitors may receive sterilizing immunizations against malaria and (iii) the duration of vaccine-induced immunity may be substantially extended upon continuous infections important for boosting protection (Table 2). The latter perspective is the most desirable and would benefit the population in need, i.e. infants in malaria-endemic countries. Although an inclusion of a live attenuated malaria vaccine into the Program for Expanded Immunization is a distant vision, the follow-up of GAPs as the most potent vaccines against malaria may ultimately result in an effective public health tool.

Table 2. Translation of a genetically attenuated whole organism vaccine to *P. falciparum.*

Major road blocks	Status[1]	Reference
1. Proof-of-principle for *P. falciparum*		
P. falciparum orthologs of GAP genes	√	17
Generation of *P. falciparum* GAPs	ongoing	–
Attenuation of mutant parasites in the liver	crucial	–
2. *P. falciparum* GAP production		
Stable *P. falciparum* gametocytogenesis	ongoing	–
P. falciparum sporozoite *in vitro* culture	ongoing	20
3. Vaccine delivery		
Frozen sporozoites remain viable	√	21
Clinically practical delivery route	ongoing	22
4. Safety of GAPs		
No breakthrough infections	crucial	–
No chronic liver inflammation	crucial	–
5. Sustained protection		
Protection against diverse parasite strains	crucial	–
Natural boost by exposure	crucial	–

√, road block removed; ongoing, currently under investigation; crucial: not firmly established yet and major research investment required. GAP, genetically attenuated parasites.
[1] Current status towards the development of a genetically attenuated *P. falciparum* vaccine line.

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