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EDITORIAL

Genetically attenuated malaria parasites as vaccines

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Despite the significant gains made with control efforts, malaria continues to be a dominant infectious disease threat in many parts of the world [1]. Malaria in humans is caused by infection with five distinct *Plasmodium* parasite species exhibiting complex biology that is controlled by genomes encoding for more than 5000 genes. Malaria parasite infections constitute extreme challenges for the human immune system. Immune responses can develop against thousands of parasite antigens, many of which are expressed in a stage-specific manner, and antigens targeted preferentially by the immune system have evolved to be highly polymorphic. Furthermore, the parasite forms that establish the asymptomatic first phase of infection within hepatocytes, called preerythrocytic stages, are distinct from blood stage parasites, which replicate in red blood cells. Natural immunity to the preerythrocytic stages is believed to be insignificant because few infectious sporozoite stages of the parasite are transmitted by the bite of an *Anopheles* mosquito. In contrast, blood stage infection, with its large parasite biomass, causes disease and elicits a pronounced immune response. Yet, blood stages employ immune evasion strategies and perturb immune cell function that render immune responses only partially effective and at best allow infected individuals to merely control parasite burden and pathogenic sequelae [2]. Complete immunity to malaria is hence not effectively engendered by natural infection. Accordingly, the quest for a highly protective subunit malaria vaccine, mainly focused on the most lethal human malaria parasite *Plasmodium falciparum*, has met with limited success.

Despite substantial efforts over decades, the subunit vaccine RTS,S is the only malaria vaccine candidate that has undergone full clinical development [3]. It contains a large portion of the *P. falciparum* circumsporozoite protein (CSP) that coats the sporozoite surface, fused to the envelope protein of the hepatitis B virus, and adjuvant to augment the immune response to vaccination. Immunization with RTS,S induces high antibody titers to CSP. The vaccine was designed to protect against sporozoite infection and initially conferred approximately 50% sterile protection in direct controlled human malaria infection (CHMI) trials conducted via mosquito bite in adult, malaria-naive subjects. However, the vaccine was never extensively tested for durability, nor for protection against heterologous parasite strains using CHMI. Thus, it did not come as a surprise that the degree of protection observed with RTS,S in phase III field trials in Africa was moderate and of limited durability [4]. Thus, a future subunit malaria vaccine needs improvement, and this might be achieved by adopting different immunization schedules, optimization of the immunogen, the application of different vaccine technology platforms, and addition of further parasite antigens.

Whereas subunit malaria vaccine efforts continue with unpredictable outcome, whole-cell attenuated malaria vaccine approaches have experienced a remarkable renaissance over the past decade. These efforts mainly focus on the preerythrocytic stages and employ a simple principle: immunization with large numbers of infectious sporozoites engenders powerful immune responses against sporozoites as well as against the intrahepatocytic replicating forms of the parasite called liver stages. Together these preerythrocytic stages contain thousands of distinct parasite proteins, many of which are potential antibody targets of sporozoites as well as T-cell targets of liver stage-infected hepatocytes. The ultimate challenges for this type of vaccine rest with safety, i.e. vaccination should not result in clinical malaria infection but should elicit optimal immunity that engenders protracted sterilizing protection against multiple strains of *P. falciparum*. Historically, the first whole sporozoite vaccination technology to be explored and currently most advanced in clinical studies is radiation-attenuated sporozoites (RAS). Irradiation causes DNA damage in sporozoites, allowing them to retain infectivity. However, upon infection of hepatocytes, DNA damage blocks parasite DNA replication and in consequence, developmental arrest of the early liver stage within hepatocytes. This causes the death of the parasite within the infected hepatocyte or death of both the parasite and the infected cell, allowing for parasite antigen presentation and priming of immune responses. Experimental immunization trials with *P. falciparum* RAS delivered by the bites of >1000 infected mosquitoes in the 1970s demonstrated that RAS conferred near-complete protection against homologous CHMI [5]. More recently, vialed, cryopreserved RAS have been administered by intravenous injection and conferred robust and durable protection against homologous CHMI [6,7] as well as to a lesser extent against heterologous CHMI [8] and, importantly, naturally acquired malaria infection in a setting of seasonal transmission [9]. These trials demonstrate the safety and efficacy of RAS vaccination. RAS vaccine efforts have been driven by the biotech company Sanaria and

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utilize a mosquito-based sporozoite production platform, thereby demonstrating the potential for manufacturing scale-up, and a reasonably practical means of administration for whole sporozoite vaccines. Although RAS have a considerable safety record, their immunogenicity appears to be suboptimal. To achieve robust and protracted protection against a homologous and a heterologous *P. falciparum* strain, immunizing sporozoite doses have to be exceedingly high (1–3 million RAS) and have to be delivered by direct venous administration.

Building on the RAS experience, the question arises as to whether it is possible to further improve the immunogenicity and efficacy of a whole sporozoite vaccine. It stands to reason that if the live parasite immunogens were to retain replication competence within hepatocytes, parasite biomass would increase, the antigen repertoire would diversify, and thus, such a whole parasite immunization could elicit broader and more robust immune responses than RAS. This principle has indeed been demonstrated by infection-treatment vaccination (ITV), in which subjects undergoing prophylactic treatment with the blood stage antimalarial chloroquine were immunized with fully infectious sporozoites. Here, liver stage development proceeds unaffected, but parasites that are released from the liver and infect red blood cells are subsequently killed by chloroquine. ITV engenders sterile protection against CHMI (tested after the chloroquine effect has waned) but strikingly requires an approximately 60-fold lower cumulative sporozoite dose when compared to RAS [10,11]. Unfortunately however, the continuous administration of an antimalarial drug during immunization is considered an impractical element of vaccination and as such it is difficult to envision ITV as a viable modality for mass vaccinations.

Fortuitously, with the advent of genetic manipulation techniques, it is now possible to embark on the rational and targeted design of attenuated malaria parasite vaccine strains. This approach has numerous principal advantages: (1) Attenuation is intrinsic and targeted by employing the precise removal of genes; (2) Attenuation is based on biological information and can be focused on achieving safety with concomitant optimal immunogenicity; (3) Sequential genetic manipulation enables a combinatorial platform to create multigene deletion attenuated strains; (4) Addition of immunostimulatory transgenes as well as antigens from different human *Plasmodium* parasite species (e.g. *Plasmodium vivax*) might further augment the immune responses as well as broadening the target profile; and (5) Attenuation, immunogenicity, and efficacy can be iteratively improved based on preclinical and clinical data.

The genetic attenuation of malaria parasites initiated with studies of rodent malaria genetically attenuated parasites (GAPs) and focused on genes that were upregulated in infective sporozoites [12]. The deletion of many of these upregulated genes from parasite genomes did not affect parasite viability during blood stage replication, the viability of sexual stages, mosquito infection, and sporozoite production but instead only caused early developmental arrest of the liver stage after hepatocyte infection. These early GAPs were robust immunogens, protecting immunized mice against sporozoite challenge [13,14]. Based on research of rodent malaria GAPs, a *P. falciparum* early liver stage-arresting triple knockout GAP (PfGAP3KO) was created ($p36^{-}/p52^{-}/sap1^{-}$), which in preclinical studies showed complete attenuation early after infection of hepatocytes with no evidence of breakthrough blood stage infection in a humanized mouse model [15]. A recent clinical study showed a favorable safety profile with no breakthrough to blood stage infection when PfGAP3KO was administered to human subjects by the bites of approximately 200 PfGAP3KO-infected mosquitoes [16]. A further *P. falciparum* GAP was recently created that arrests early during hepatocyte infection [17] and will enter clinical trials in due course. Accordingly, GAP vaccine efforts have reached important goals: full viability throughout the parasite life cycle including sporozoite viability and infectivity, specific arrest during liver stage infection, and complete attenuation. What vaccine efficacy profile can we expect from PfGAP3KO? Based on rodent malaria GAP3KO studies, this type of early liver stage-arresting GAP confers complete protection against sporozoite challenge in mice [18]. A preliminary analysis of human humoral immune responses to PfGAP3KO showed induction of potent sporozoite infection-blocking antibodies [18], but T-cell responses have yet to be analyzed. It can be speculated that vaccine efficacy might turn out to be superior when compared to RAS as PfGAP3KO is uniformly attenuated. Further clinical studies are underway to test the preliminary efficacy of PfGAP3KO against homologous CHMI, and future trials will test efficacy against heterologous CHMI as well as the safety and efficacy of an injectable PfGAP3KO formulation.

What improvements can be made to increase potency of a GAP vaccine? As mentioned, it is of great benefit to allow the whole parasite immunogen to undergo significant replication within infected hepatocytes, and this is strongly supported by ITV trials that demonstrated superior protection [10] vis-à-vis RAS immunization [6]. Furthermore, proof of concept has been generated in rodent malaria models that late liver stage-arresting GAP can be generated and engender better T-cell responses and antibody responses compared to early liver stage-arresting GAP and RAS [18,19]. These GAPs confer superior protection against sporozoite challenge with homologous and heterologous parasites when compared to early liver stage-arresting GAP and RAS and retain complete efficacy when administered intradermally. Furthermore, late liver stage-arresting GAP also confers protection against the blood stages of the parasite [18,19], which is not observed with RAS. Thus, a safe late liver stage-arresting *P. falciparum* GAP strain should be the ultimate goal of whole parasite vaccine efforts, but this has yet to be realized [20].

In conclusion, evidence in animal models of malaria and in humans demonstrates that a whole parasite vaccine expressing the full repertoire of sporozoite and liver stage antigens constitutes the most potent, broadly protective, and durable immunogen against malaria infection. Genetic engineering of the parasite provides a powerful and versatile platform to pursue the informed design of this type of live-attenuated vaccine. As such, the creation and clinical development of an optimal live-attenuated malaria vaccine should receive the highest priority from the malaria vaccine development community. The

generation of robust *P. falciparum* liver stage 'omics data sets, conducting CRISPR/Cas9 gene-editing screens to identify loci whose deletion attenuates the liver stage and the further refinement and increase in throughput of preclinical assays for testing genetically engineered *P. falciparum* vaccine strains, are a critical element in this pursuit. Furthermore, the safety and efficacy assessment of attenuated strains in human CHMI trials must be accelerated. Together such efforts will all require substantial investment by funders. Formidable other hurdles remain that need to be further addressed, which include the scalable production of attenuated sporozoites, their preservation, storage, distribution, as well as the route of administration for global immunization efforts. Only then will we have a realistic shot at a highly efficacious *P. falciparum* malaria vaccine within the next decade.

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