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VISIONS AND REFLECTIONS

# Our impasse in developing a malaria vaccine

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**Abstract** Malaria presents a challenge to world health that to date has been beyond the abilities of researchers to conquer. This critique presents some of the strategies employed by the parasite to overcome immunity and the immunological challenges that we face to develop vaccines. A conclusion is that a vaccine must identify novel antigens or epitopes that are not normally immunogenic and which are therefore not under immune pressure and most likely to be conserved between different strains. Such antigens are most likely to be targets of cellular immunity. The case for a whole parasite blood stage vaccine is presented based on these premises.

**Keywords** Malaria · Vaccine · Immune response · Natural immunity

## Introduction

It will soon be 30 years since the cloning of malaria antigens in Melbourne [1] and New York [2] with the bold promise that a malaria vaccine would be available in the near future. The near future has now long passed, but the need has not. Malaria still kills more children than any other disease and the ability to find drugs to prevent and treat malaria is an ongoing challenge. A vaccine candidate to prevent infection with *Plasmodium falciparum*, the most deadly of the malaria parasite species, is in Phase III trials in various African countries, but there are questions over its mode of action, efficacy, and durability. A number of other vaccine candidates are far less advanced, in spite of many years of development. So, what are the challenges that are holding us back? This article will attempt to address some of these from an immunological perspective.

There are six parasite species within the genus *Plasmodium* that can infect humans: *P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P.ovale wallikeri*, and *P. knowlesi*, with the latter being a zoonosis and the two different ovale species having only recently been split [3]. Most progress has been made with *P. falciparum* vaccines, which is appropriate considering that this species is responsible for the vast majority of malaria-associated mortality. To think of vaccine development constructively, it is necessary to consider the life cycle (see Fig. 1). For all species, infection commences when an Anopheline mosquito bites and injects sporozoites into the lymph or circulation. Those that continue the life cycle are those that travel ultimately to the liver in a period of time up to about 1 h and there invade a hepatocyte after interacting with Kupffer and other cells. Sporozoites develop in a process of schizogony in the hepatocyte over a varying period of time, with *P. falciparum* sporozoites taking 5.5–7 days to each develop into approximately 30,000 merozoites per infected hepatocyte. These then rupture the liver cell and leave in merosome rafts [4] that travel to the lung where they are dissociated into individual merozoites that are free to invade a red blood cell. Depending on the species, the life cycle within a single red cell last 2 or 3 days during which time a single merozoite can divide and produce 8–24 new merozoites, each to invade another red cell. Thus exponential growth occurs resulting in a variety of pathogenic effects and host defense mechanisms.

*P. falciparum* can attain a higher blood parasite density than the other species and can exceed 300,000 per μl of blood, although typically much lower peak parasitemias are

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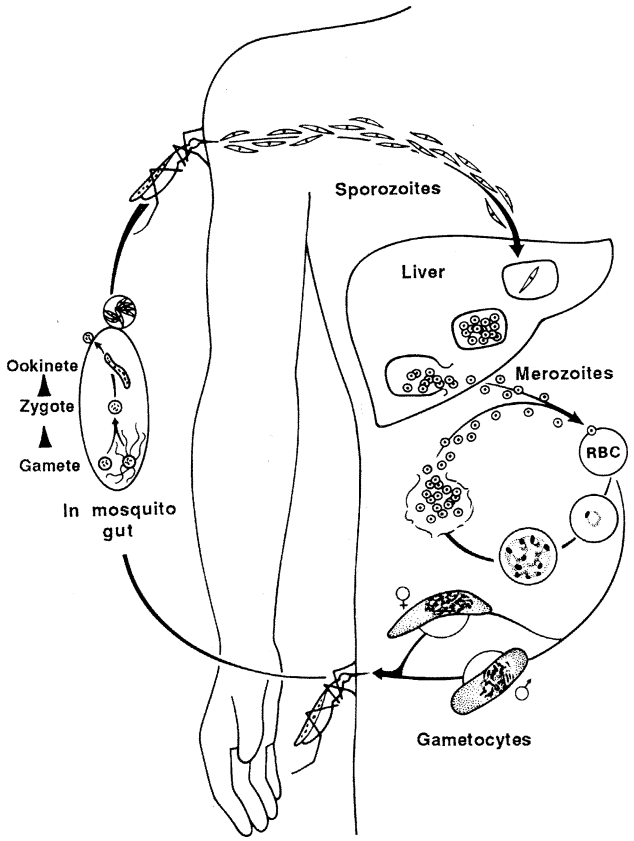


**Fig. 1** The life cycle of the malaria parasite

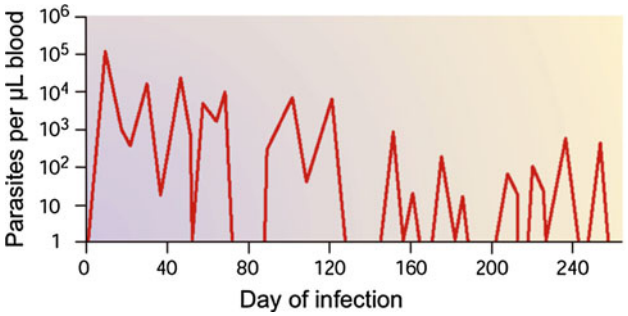


**Fig. 2** *P. falciparum* parasite density in the blood of a patient (modified from [6]). Each new peak represents the expression of a new variant antigen on the surface of infected red cells. Antibodies develop and clear the infection but expression then switches to a different variant antigen. PfEMP1 is the predominant variant antigen

reached. Parasite-encoded proteins are expressed on the red cell membrane and these, among other effects, render *P. falciparum*-infected red cells adherent to small blood vessels in deep tissue during the latter part of the red cell life cycle (trophozoites, schizonts). This has a beneficial effect for the parasite by enabling it to avoid the spleen at a time when the gross alteration of the infected red cell morphology would result in it being removed from the circulation. Thus, the mature red cell forms of *P. falciparum* are not seen in peripheral blood smears. Eventually, the sexual forms (gametocytes) develop within the red cells. These gametocytes mature but remain within the red cell membranes until the mosquito takes them up during a blood meal. In the midgut of the mosquito, the gametocytes emerge from the red cells as gametes and fertilize giving rise to ookinetes and then oocysts from which sporozoites develop and migrate via the hemocoele of the mosquito to the salivary glands awaiting an opportunity to be delivered to the next potential human host.

*Plasmodium falciparum* has an enormous impact on human well-being, accounting for the lives of up to 1 million people (mostly young children) each year, as well as causing significant morbidity. Infection of the brain (cerebral malaria) where small vessels are occluded and where cells of the immune system may also cause pathology, is a major cause of morbidity and mortality, particularly in areas of low endemicity and where non-immune adults are exposed to the parasite [5]. Severe anemia is the other major cause of morbidity and mortality, particularly amongst younger children and where transmission is more intense [6].

A goal of vaccine development must be to either prevent blood stage infection completely or limit parasite growth and subsequent parasite density within the blood compartment. For the former, most would agree that a vaccine acting to kill sporozoites before they enter the liver or kill infected hepatocytes is required (a 'pre-erythrocytic' vaccine) whereas for the latter, a vaccine acting to prevent merozoite invasion of red cells or one that causes destruction of infected red cells is required (i.e., a 'blood stage' vaccine). Rather than discussing both of these vaccine approaches separately, the immunological challenges that are common to both approaches will be discussed after first discussing briefly the immunological impediments to developing immunity as a result of infection.

## Immunological challenges to developing natural immunity

The major immunological challenge inhibiting the development of immunity is best exemplified by a figure demonstrating the fluctuating course of parasite density in the blood of an infected individual (Fig. 2). Over a period of several months, the parasite attack seems to come in waves. We now understand that, in large part, each wave corresponds to the expression of one type of protein from the PfEMP1 family expressed on the surface of the infected red cell. This is a very important family of virulence proteins of which there are about 60 members in each genotype with sequential expression as a result of antigenic

variation and switching within a single clone. These same proteins are responsible for the sequestration of the parasite within the deep tissues of the host. As each parasite wave develops, so also does the antibody response to it. Ultimately, the antibodies then clear the parasite from the circulation; the parasite then switches expression to a different PfEMP1 gene, and that variant clone then grows unaffected by the antibodies that arose to the previous PfEMP1 protein. This cycle then repeats. However, Fig. 2 is only an exemplar. The rate of acquisition of natural immunity will vary between individuals and will depend on frequency of exposure, access to treatment, age of the individual when exposed, and the presence of other infections or co-morbidities. The individual represented in Fig. 2 may be well on the course to the development of natural immunity.

As can been seen in Fig. 2, the height of the variant parasite peaks does diminish over time. This could be the result of a diminution in parasite vitality over time or to the development of an immune response(s) to conserved, non-variant, parasite antigens. However, this process clearly takes an extended period of time and during this phase the infected child is at great risk. We need to better understand the mechanisms responsible for this decline in the height of the sequential peaks.

Other classes of antigens exist as allelic forms and do not undergo antigenic variation from within a single clone. These antigens are located on the surfaces of merozoites, gametes, or sporozoites. Antibodies to these antigens, if present in sufficient titer, can neutralize the parasite and block invasion of sporozoites into hepatocyte or merozoites into the red cells, or block the fertilization of gametes. As such, these have been major candidates for vaccine development. An example of antibodies causing inhibition of merozoite invasion but where antigenic polymorphism is an obstacle to the development of immunity involves AMA1 (apical membrane antigen 1). Antibodies are able to reduce invasion into human red cells of cultured *P. falciparum* merozoites of the homologous strain [7]. However, invasion inhibition of heterologous parasites was much reduced.

Antigenic diversity is similarly an impediment to the development of immunity to the pre-erythrocytic stages of the life cycle. Sporozoites can be blocked by antibody prior to entry into hepatocytes. Although the dominant epitope recognized by antibodies ([NANP]n from the circumsporozoite protein [CSP]) is totally conserved in *P. falciparum*, the T cell epitopes are known to be highly polymorphic [8] and once inside hepatocytes, T cells provide the major means of attack [9, 10]. Furthermore, the polymorphisms abrogate antigen recognition [11]. While the full extent of antigenic diversity for the T cell epitopes of the *P. falciparum* CSP is not known (with 70 unique haplotypes described so far for the CSP), the T cell response of individuals to any given haplotype is limited [8]. Together, these immunological challenges represent an enormous obstacle to an individual developing T cell-mediated immunity to the intra-hepatic pre-erythrocytic stages of malaria.

## Immunological challenges to vaccine-induced immunity

### The magnitude and duration of the antibody response

As mentioned, the repetitive [NANP]n B cell epitope of the *P. falciparum* CSP is completely conserved among all strains sequenced to date. This knowledge formed the basis for the first malaria vaccine clinical trial [12]. In that study, volunteers were vaccinated with a recombinant fusion protein consisting of part of the CSP (the repeats) adjuvanted with alum. One of six volunteers was protected from malaria following the bites of infected mosquitoes. To improve the immunogenicity of the vaccine, various manipulations of the CSP were made and different adjuvants trialed [13], culminating in the development of the most advanced sub-unit malaria vaccine candidate to date, RTS,S, consisting of part of the CSP fused to hepatitis B surface antigen and expressed with free Hep B surface antigen to enable the polypeptides to form virus-like particles [14]. These were presented with novel adjuvants (AS series) containing monophosphoryl lipid A (MPLA) and QS21 (summarized in [13]). Initially, these were trialed in naive American volunteers who were challenged with infected mosquitoes. The results were variable but in one study using AS02 as the adjuvant, six of seven volunteers were protected from infection following the bite of infectious mosquitoes. A recent review of RTS,S [13] summarized the results of all studies and demonstrated that of 214 malaria-naive adults vaccinated with RTS,S with an AS adjuvant, 85 (39.7%) were protected from malaria following deliberate mosquito challenge.

Phase II studies were then conducted in different African countries among both adults and children. In these studies, vaccine efficacy was calculated from measuring the time to first infection, as opposed to absolute prevention of malaria. Efficacy was calculated in this way due to the nature of natural challenge where there is no control of the time to exposure nor the amount of exposure. Many studies involving children and adults have been conducted with different AS adjuvants. Protection, as determined in this manner, ranged from 11% (28/74 vs. 37/75, over a 14-week period) for adults in a malaria-intense region of Kenya [15] to 71% for adults in The Gambia (over a 9-week period) [16]. However, from inspection of the curves plotting the cumulative incidence of disease in these Phase II studies in Africa, it appears that after a period of 1–3 months following vaccination (during which time no

or few vaccinated subjects develop malaria) both vaccinated and control subjects then acquire malaria at a similar rate (Fig. 3). The vaccinated group thus appeared to behave as if they were completely protected initially but then their level of protection is similar to the non-vaccinated group (i.e., non-existent). However, at any time after vaccination, fewer people who have been vaccinated will have had malaria compared to non-vaccinated individuals. This initial period of protection correlates with the higher titer of serum antibodies present in vaccinees during this period. While the level of CSP-specific antibody following vaccination does in general correlate with protection (either following direct challenge or following natural exposure) (summarized in [13]), the rapid diminution of antibody levels following vaccination appears to be responsible for the lack of durable protection (e.g., see antibody levels in [17]). The reasons why antibody levels are not maintained are not known, but could relate to the inability of sporozoites to naturally boost vaccine-induced antibody responses which in turn could relate to exposure to only low numbers of sporozoites or to the polymorphic nature of the T cell epitopes on the CS protein [8], or both. Nevertheless, it is reasonable to expect that if the antibody response was higher to commence with and/or persisted longer then the level and duration of efficacy of the vaccine would be improved. This is a goal of ongoing research.

## The impact of allelic polymorphism on vaccine efficacy

Although the [NANP]n epitope present on the CSP is completely conserved among *P. falciparum* strains, for other antigens the target B cell epitopes are often polymorphic. Has this had an effect on vaccine efficacy in trials to date? A recent Phase IIb blood stage vaccine study in Malian children using AMA1-C (which combines two different strains of AMA1) was unsuccessful [18]. Although it is tempting to speculate that the reason for this was that AMA1 is highly polymorphic (62 known polymorphic residues) and that <3% of the circulating strains were represented in the vaccine, a follow-up analysis comparing pre- and post-immunization AMA1 sequences in the infecting parasite strains suggested that vaccination did not alter the genetic diversity of the population [19]. However, the results of their study do not argue against the barrier posed by genetic diversity; rather that failure to show any protective effect (strain-specific or otherwise) was likely due to poor immunogenicity and low resultant titer of the alum-adjuvanted vaccine in their particular study. This view is supported by a recent study of AMA1 vaccination of monkeys where strain-specific protection was observed using the more potent montanide adjuvant and where it correlated with the amount of antibody induced [20]. Thus, the impact of genetic diversity (for AMA1) has not yet been adequately tested in humans. Based on hope that the impact of genetic diversity will not be ultimately detrimental (as the pre-clinical data from the monkey experiments suggest it might be), a number of Phase I clinical studies have recently begun and have examined the safety and immunogenicity of AMA1 with more potent adjuvants (e.g., AS02 [21]; CpG 7909 [18]). We await the outcome of Phase II efficacy studies using these or other adjuvants that can induce a high antibody response, but we know that there are hundreds of unique AMA1 haplotypes [22], raising the fear that it may not be possible to collate enough allelic AMA1 strains into a vaccine to provide coverage against the majority of strains worldwide. Nevertheless, there are attempts to group different alleles into families [23, 24] or to generate diversity-covering mutated recombinant proteins [25] with the hope and with some justification that there may be sufficient cross-reactivity to enable a vaccine with a limited number of allelic strains to be developed.

A slightly different scenario is unfolding with respect to the other leading blood stage subunit vaccine, merozoite surface protein 1 (MSP1). It is known that antibodies to the carboxyl terminal segment of MSP1 can inhibit invasion of homologous strain merozoites into red cells and data support an association between naturally acquired antibodies to the carboxyl terminus of the molecule (MSP1–42), of which the 19-kDa tail contains the target B cell epitope, and a decrease in the incidence of malaria attacks [26]. An early vaccine study in monkeys showed that six of seven animals immunized with MSP1–42 with a potent adjuvant were protected

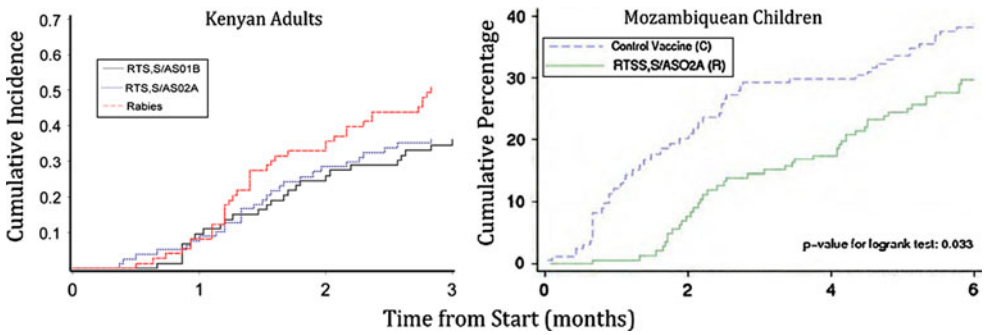


**Fig. 3** Rate of acquisition of malaria in two separate studies of RTS,S, involving Kenyan adults (*left*) and Mozambiquean children (*right*). Vaccines received different formulations of RTS,S, or control vaccines (as indicated). See Refs [15] and [60]

against high parasitemia, but five of the six nevertheless required treatment for anemia [27]. The sole animal that was completely protected had the highest pre-challenge anti-MSP1 titer (>200,000). They also showed that immune rabbit serum could discriminate between parasites of the vaccine strain and parasites of a heterologous strain in a growth inhibition assay, although there was nevertheless significant growth inhibition of heterologous parasites. This gave hope that a MSP1–42 vaccine might protect against multiple strains. In a follow-up study, monkeys were immunized with either of two allelic forms of MSP1–42, or a combination of both [28]. One of five monkeys vaccinated with the FVO MSP1 strain could control an FVO strain infection while one of seven vaccinated with 3D7 MSP1 strain could control an FVO infection and three of six vaccinated with the combination could control the FVO challenge infection. These results give some hope that allelic polymorphisms within MSP1–42 might not present the same challenges as those within AMA1 do. There are only seven known amino acid polymorphisms in this region of MSP1. However, a daunting task appears to be the need to induce an antibody response of sufficient magnitude.

In a recent Phase IIb clinical trial involving children in western Kenya, no protection was seen following vaccination with 3D7 strain MSP1–42 adjuvanted with AS02 [29]. Whether allelic polymorphism was responsible is currently being investigated. It is certainly possible that insufficient antibody was induced and lack of B cell immunological memory may be a factor. Data from our own laboratory show that in mice, *Plasmodium* infection can lead to deletion of MSP1-specific memory B cells and long-lived plasma cells, thus compromising humoral immunity [30]. It is also possible that an expanded population of atypical memory B cells, as has been observed in Malian adults and children [31], might have contributed to the low antibody response. The authors of the Malian study suggested that the atypical memory B cells may give rise to short-lived plasma cells. It was noted that in the Phase IIb clinical study in Kenya, antibody levels declined approximately sixfold over a 6-month period following vaccination, which was consistent with a loss of plasma cells and/or specific memory B cells. This, together with the documented need for a high-antibody response in order to protect monkeys (see above), as well as mice [32], suggests that in order to be effective, potent but safe adjuvants will be required for an MSP1-based vaccine.

## Strategies to identify conserved vaccine targets

It is self-evident that a vaccine that induces an immune response similar to that induced by malaria parasite infection will induce a similar degree of protection. Given that to be effective a vaccine must induce a protective immune response in a relatively short period of time, we must aim to induce novel immune responses (i.e., different to those induced by natural infection) or immune responses to novel determinants. In other words, a vaccine must induce a quantitatively and/or qualitatively different immune response to that induced by natural infection. With respect to blood stage infection, an area that has been largely ignored is cell-mediated immunity (CMI), but evidence suggests that it may often represent a novel type of immune response.

The pioneering work of William Weidanz and colleagues [33] demonstrated that mice lacking B cells and antibody could be immunized by infection and drug cure. His group was also the first to show that CD4 + Th1 cells could adoptively transfer protection [34], something that has been now shown in various laboratories (e.g., [35, 36]). We now know that apart from CD4 + T cells, $\gamma\delta$ T cells [37] play a role and possibly CD8 + T cells as well [38, 39]). Data suggest that T cells activate other cells, such as macrophages, and that parasites are killed in the spleen [40]. Gamma interferon seems to be a critical mediator in both animal models of immunity [39, 41, 42] and in humans [38, 43].

However, in spite of the ability of CMI to control parasite growth, it is not readily induced by malaria infection. For example, only one-third of semi-immune older children in Papua New Guinea had a significant in vitro interferon-$\gamma$ response to blood stage parasites. However, those that did exhibit a strong response subsequently developed infections with a lower parasite density than those that did not [43]. Similarly, in Cameroon, it has been shown that the in vitro interferon-$\gamma$ response to crude parasite antigens as well as to defined peptides from blood-stage proteins develops only in the third year of life [44]. This is consistent with data showing that peripheral blood mononuclear cells from patients acutely infected with *Plasmodium* undergo apoptosis in vitro [45, 46] and that parasite-specific interferon-$\gamma$-secreting CD4 + T cells transferred into naive mice undergo apoptosis in the face of infection [47]. The lack of an active CMI response during the early time periods of malaria exposure, either as a result of active deletion of specific cells or for other reasons, would suggest that parasite proteins cannot then be under immune pressure from T cells. Given that T cells can theoretically respond to all parasite proteins, including critical housework proteins (that are likely to be highly conserved), the down-regulation of parasite-specific CD4 T cell responses in the face of infection may represent a parasite survival strategy. However, this information could be used to the advantage of vaccine development. If conserved target antigens for T cells can be defined, then it may be possible to induce a protective immune response

that would recognize multiple strains. One such antigen has been defined. The purine salvage enzyme, HGXPRT, which is located within merozoites and in the infected red cell cytoplasm, has been shown to be the target of protective CD4 + T cells in a rodent model of malaria [48]. The sequence of HGXPRT from *P. falciparum* is totally conserved in all strains sequenced to date.

One difficulty with using proteins such as HGXPRT in a vaccine, however, is that there is significant homology to the human enzyme, HGPRT, and as such there is a limited amount of the protein that can be considered suitable for vaccine development, suggesting that few individuals would be responsive. We have thus looked to broaden the repertoire of suitable parasite proteins and are looking at ways to use the entire organism to induce CMI. Having observed that a patent parasite infection (i.e., high parasite load) can cause apoptosis of parasite-specific T cells (but not T cells of other specificities), we then showed that a sub-patent infection (one undetectable by microscopic examination of blood from an infected individual—i.e., very low parasite load) followed by drug cure induced a robust T cell response in human volunteers [38] and in mice [49]. The human response was characterized by an in vitro proliferative response to malaria parasites from CD4 + and CD8 + T cells, in vitro production of interferon-$\gamma$ in response to parasite stimulation, and production of nitric oxide synthase from peripheral blood mononuclear cells in vitro. Parasites challenge of volunteers immunized by such a sub-patent infection followed by drug cure resulted in apparent protection, as measured by parasite growth, although we could not exclude the possibility that residual drug may have contributed to the apparent protection. In a recent study, however, Roestenberg et al. [50] showed that volunteers immunized by the bite of 15 infected mosquitoes under cover of chloroquine protection developed a CMI response to blood-stage antigens and were protected against subsequent parasite challenge. Control subjects who received chloroquine but were not bitten by infected mosquitoes did not develop protection. One possibility is that chloroquine, by suppressing the growth of blood-stage parasites, resulted in the volunteers being exposed to a very low-dose (sub-patent) infection, with resultant induction of a potent blood-stage cellular immune response. An alternative explanation is that the volunteers developed immunity to liver-stage parasites. This would seem unlikely as the number of infected mosquitoes used to bite the volunteers was much less than the number required to induce protection, at least for when using irradiated infected mosquitoes to induce protection [51]. However, this possibility cannot be discounted. To formally discriminate between these two possibilities, it would be necessary to challenge immunized volunteers with parasitized red blood cells in place of infected mosquitoes.

While controlled infections can induce protection in certain situations (see above), how can this be translated into vaccine development? The most advanced whole-parasite vaccine approach is to use irradiated sporozoites. The ability of irradiated infected mosquitoes to induce pre-erythrocytic immunity following mosquito bite is well described for both mice and humans [52, 53]. Hoffman and colleagues [54] have discussed a strategy and approaches being considered for using this fundamental knowledge to develop a whole parasite vaccine. A related approach is to use genetically attenuated sporozoites [55] or sporozoites attenuated by chemical treatment [56]. For all of these approaches, the logistical challenges of preparing and administering purified attenuated sporozoites are not inconsiderable. Furthermore, the route of administration (via syringe) may open up other scientific difficulties as this mode of administration may differ substantially from mosquito bite. A recent paper showing that hair follicles can support parasite development following mosquito bite [57] brings this issue into sharp focus. It has also been hypothesized that vaccine strategies using the skin may induce immunosuppression to malaria vaccines [58].

A less well-developed strategy is to use whole-blood-stage parasites. Based on the observations that humans and mice could be immunized by exposure to a sub-patent live infection, we have attempted to induce immunity following exposure to low doses of killed or attenuated parasites. Low doses of irradiated rodent parasites can induce immunity (unpublished observations) and as few as 100 killed parasites adjuvanted with CpG and alum can induce protection to both homologous and heterologous species parasites via a mechanism dependent on CD4 + T cells, interferon-$\gamma$, and nitric oxide [59]. This vaccine schedule induced effector and central memory T cells. Immunity was shown to be independent of antibody. It is now hoped that this or a similar approach will be successful in humans. However, this approach is not without its own challenges. For example, it will be important to minimize any risk of auto-immune antibodies to red cells or any pathologies induced by T cells. The use of purified merozoites may be considered. Logistical problems in manufacture may be minimal, however, as it appears likely that very few whole parasites will be required.

## Conclusions

In this perspective review, an attempt has been made to outline some of the immunological challenges facing sub-unit malaria vaccine development. An underlying theme has been that the parasite has evolved ways to circumvent the processes and effects of immunity and that to induce protection via vaccination a qualitatively or quantitatively

different strategy of immune engagement must be followed. It must be appreciated that malaria presents challenges that no other organism for which a vaccine has been developed presents, viz that every malaria infection is unique in terms of the antigenic repertoire that is presented to the host. A vaccine must therefore identify novel antigens that themselves are not normally immunogenic in the whole organism (and which are therefore likely to be highly conserved in sequence) but which can nevertheless be antigenic and recognized by vaccine-induced immune responses. Emerging data suggest that the targets of T cells may represent such antigens and as such the induction of cell-mediated immunity is worthy of serious consideration as a strategy for vaccine development. By using extremely low doses of whole attenuated or killed organisms, it is possible to induce robust durable immune responses against multiple strains or species of *Plasmodium* and as such this represents a different strategy for vaccine development worthy of serious consideration.

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