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Correlates of protective immunity following whole sporozoite vaccination against malaria

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Abstract

Human infection with *Plasmodium* parasites remains a serious global health crisis, leading to more than 600,000 deaths annually. Currently, no licensed vaccine is available to alleviate this malaria disease burden and vaccination with the most advanced anti-malarial vaccine candidate, RTS,S, provides limited protection that wanes over time. To date, the only vaccination strategy capable of inducing complete, longlasting protection in human subjects is administration of attenuated whole sporozoites. Several approaches for vaccination with attenuated whole sporozoites have been clinically tested in humans and include vaccination with radiation or genetically attenuated sporozoites or with virulent sporozoites concurrent with administration of anti-malarial drug cover. Rodent studies with these three attenuated whole sporozoite vaccination approaches provide insights into the immune-correlates of vaccine-induced protection. The majority of these studies have identified a critical role for liver-stage parasite directed CD8 T cells in providing protection with possible contributions from *Plasmodium*-specific CD4 T cells or antibodies. Together, rodent and human vaccination studies with attenuated whole sporozoite vaccination may lead to an understanding of the correlates of protective immunity against malarial disease, and the development of new, highly efficacious vaccines.

Keywords

Malaria; Plasmodium infections; sporozoite; genetically attenuated parasites; infection-treatment vaccination; radiation-attenuated sporozoites

Introduction

Despite interventions such as insecticide impregnated bed nets and prophylactic/therapeutic drugs, more than 600 thousand deaths and greater than 200 million clinical cases occur annually due to malarial disease [1]. Malarial disease is primarily due to infection with *Plasmodium falciparum* or *P. vivax*, although *P. ovale, P. malariae*, and *P. knowlesi* have also been known to cause disease in humans [1]. *P. falciparum* accounts for approximately 90% of the mortality and largely affects young children in Sub-Saharan Africa [1]. Thus, it

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is of great importance to develop anti-malarial vaccines that reduce the disease burden due to *Plasmodium* infections. However, the need for a highly efficacious, long-lasting vaccine is still unmet. The most advanced anti-malarial vaccine is RTS,S, a subunit vaccine consisting of *P. falciparum* circumsporozoite protein fused with Hepatitis B surface antigen, and an adjuvant [2, 3]. RTS,S vaccine trials demonstrate approximately 30–50% efficacy in reducing malarial disease burden in young children, but the protection is not long-lasting [4, 5]. Thus, continued research into the immunological requirements for highly efficacious, long-lasting vaccine-induced protection is necessary to eventually lead to worldwide eradication of malarial disease.

Plasmodium parasites have a complex biphasic lifecycle within a human or rodent host. Infection begins when a mosquito harboring *Plasmodium* releases sporozoites into the dermal tissues of the host during a blood meal. Sporozoites will then actively invade the bloodstream and travel to the liver where they will eventually infect a hepatocyte to initiate the liver-stage of the lifecycle. This stage of the lifecycle is asymptomatic for the host and differs in duration for mice (two days) compared to humans (~7–10 days). During liver-stage infection, a single parasites will differentiate and replicate within hepatocytes, developing into liver schizonts containing ~30,000 merozoites/hepatocyte. Membrane bound merozoites are then released into the liver sinusoids and, after disruption of the membrane in the lungs [6], are released with the capacity to infect red blood cells. This begins the blood stage of the lifecycle. Similar to the liver-stage, the parasite will differentiate and replicate within red blood cells and eventually rupture the red blood cell, with release of merozoites to infect new red blood cells. It is during the blood-stage of the infection that the host experiences symptoms of infection such as fatigue, fever, nausea, and anemia [7]. In approximately 5% of *P. falciparum* infections, symptoms can become severe and can include respiratory distress, seizures, and coma [1, 7].

Successful vaccination against *Plasmodium* requires understanding the components of a protective immune response (T cells, B cells, antibodies, ect), and the antigenic targets of this protection. The *Plasmodium* lifecycle involves both liver- and blood-stages of infection, in which the parasite alters protein expression based on stage-specific requirements for infection, survival, differentiation, and replication [8]. Consequently, this will lead both stage-specific and cross-stage targets of anti-malarial immunity [9, 10]. It seems likely that vaccines to target all stages of the parasite lifecycle may provide optimal immunity. However, to date the most successful demonstration of vaccine-induced sterile protective immunity (i.e. no blood-stage infection after *Plasmodium* sporozoite challenge) in humans has resulted from liver-stage directed immunity through attenuated whole sporozoite vaccination (WSV) approaches (Table 1). This review will focus on the immunological correlates of protection following WSV.

Whole sporozoite vaccination approaches

The first WSV approach to achieve complete, sterile protection from *Plasmodium* challenge was developed in the 1960s wherein mice were immunized with the bites from mosquitoes harboring infectious *P. berghei* parasites that were attenuated due to irradiation of the mosquito vector [11]. Importantly, this approach termed radiation attenuated sporozoite

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(RAS) vaccination, also provided sterilizing immunity when tested in human subjects [12]. Of note, sterilizing immunity in humans required the bites of >1000 irradiated infected mosquitoes. Because of the ability of RAS vaccination to protect humans, this WSV approach is commonly considered the “gold standard” of malaria vaccination. However, the future application of this vaccination approach depends on overcoming concerns with the safety of administering an irradiated whole parasite vaccine, as well as the logistics of field application. In regards to safety, RAS vaccination requires the sporozoites be sufficiently irradiated to prevent completion of liver-stage infection, but not over irradiated to lose immunogenicity and thus the capacity to induce immune-mediated protection [13]. Additionally, these sporozoites are hand-dissected from laboratory-reared mosquitoes, subjected to assays to insure sterility, then cryopreserved for long-term storage – all factors that complicate the scalability of production [14]. Further, application to the field requires knowledge of total dose required for protection, timing of booster immunizations, and preservation of the vaccine in a field lacking a cold-chain network. Extensive work has been done to address these concerns over the last several years [14]. Currently, the RAS vaccination approach is being aggressively developed and tested in safety and clinical trials in hopes that it can be eventually distributed in the field [14–17].

An approach that seeks to overcome some of the safety concerns inherent to RAS vaccination is the use of genetically attenuated parasite (GAP) vaccination. In this approach, gene specific deletions are created, that do not affect mosquito or blood stage replication, but specifically prevent the production of proteins essential for liver-stage development as a means of attenuating the sporozoite without losing immunogenicity. Because of the deletion of a gene(s) in the GAP approach, it is arguably a more controlled attenuation than the irradiation used for attenuation in RAS vaccination. Further, GAP vaccination may elicit more potent immunity than RAS due to the longer development period the parasite can undergo in the hepatocyte. Indeed, this has been demonstrated to be the case in a mouse model of RAS versus late liver-stage arresting GAP vaccination [10]. Successful GAP vaccination requires deletion of an essential gene for complete liver-stage development to attenuate the parasite and prevent progression to blood-stage infection. Should this attenuation fail, clinical disease may occur after vaccination. In the first human clinical trial of GAP vaccination, one of six patients experienced blood-stage infection (breakthrough parasitemia) following high dose vaccination with *P. falciparum p52-/p36-* parasites [18]. Importantly, immunization with GAP parasites lacking homologs of these genes in *P. yoelii* led to no breakthrough in mouse studies using a *P. yoelii p52-/p36-* model [19]. Unfortunately, this occurrence highlights the major disadvantage of GAP vaccination approaches that are primarily tested using rodent *Plasmodium* before moving into *P. falciparum* for evaluation in human subject studies. GAP vaccination overcomes safety concerns of irradiating sporozoites inherent to the RAS approach, but its success requires absolute attenuation of liver-stage development through gene deletion.

An alternative approach to RAS or GAP vaccination is infection-treatment vaccination (ITV), also referred to also as chemical prophylaxis sporozoite (CPS) vaccination [20], whereby the subject is administered virulent sporozoites via mosquito bite or needle injection while concurrently receiving an anti-malarial drugs that prevent blood stage

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infection [20–23]. To date, human subject studies have focused on the use of chloroquine drug administration. Chloroquine does not alter the liver-stage infection, but blood-stage infection is halted after a single round of infection due to the blood-stage parasite-specific action of the drug [22, 24]. ITV appears to be the most potent WSV approach based on rodent and human challenge studies [22, 23]. Although concerns about chloroquine resistance of *Plasmodium* will likely prevent the direct application of this specific approach to the field, it does provide a platform to understand the mechanistic requirements of immunity required to provide complete, sterilizing protection (i.e. no blood-stage infection) against sporozoite challenge.

Potent CD8 T cell responses following WSV

Radiation attenuated sporozoite (RAS) vaccination

The first direct evidence of the role of CD8 T cells in vaccine-induced protection against *Plasmodium* was demonstrated using mouse models of RAS vaccination. Using distinct rodent *Plasmodium* species, two independent studies showed that antibody-mediated depletion of CD8 T cells prior to challenge abrogated protection in RAS-vaccinated mice [25, 26]. Not long after these studies, adoptive transfer studies using a circumsporozoite protein (CSP)-specific CD8 T cell clone generated from RAS vaccinated mice conferred protection to naïve mice against challenge with sporozoites [27, 28]. Many additional studies using various sporozoite doses, number of immunizations, mouse strain, and parasite species combinations have further identified a critical role for CD8 T cell-mediated protection following RAS vaccination of mice [29–33].

The rodent-RAS vaccination model has greatly aided in the understanding of correlates of protective immunity against *Plasmodium* as it allows comparisons between mouse strains and rodent *Plasmodium* species for the magnitude, phenotype, and functionality of the CD8 T cell response required for protection. RAS vaccination has consistently been capable of providing CD8 T cell-dependent protection in several mouse strains [29]. Further, CD8 T cell dependent protection following RAS vaccination can be achieved against different rodent *Plasmodium* species, with most studies employing *P. berhei* or *P. yoelii* [29, 30]. Taken together, these results indicate that RAS vaccination can provide CD8 T cell dependent protection in various rodent/parasite combinations. Further, the quantity or functionality of the protective CD8 T cell response can differ based on the rodent model. For instance, protection in *P. berghei* ANKA RAS vaccinated C57Bl/6 mice from challenge with homologous parasites correlated with memory CD8 T cells compromising 11% or greater of circulating CD8 T cells [30]. In contrast, a threshold of ~ 4% of the circulating CD8 T cells is required to achieve robust sterilizing immunity in BALB/c mice immunized with *P. berghei* ANKA RAS [30]. Thus, larger CD8 T cell responses are required to protect B6 mice from *P. berghei* ANKA than BALB/c mice. In the case of C57Bl/6 mice, protection following RAS vaccination was provided by CD8 T cells expressing IFNγand TNFα, whereas only IFNγexpression was detected from CD8 T cells from BALB/c mice [30]. It remains unknown if these differences in cytokine production impact the threshold of memory CD8 T cells required for protection after RAS vaccination. Further, the number of RAS vaccine administrations necessary to protect BALB/c mice from sporozoite challenge

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is lower than C57Bl/6 mice. Only two administrations of RAS vaccination are required to protect BALB/c mice from *P. yoelii* 17XNL challenge at a memory time point whereas three administrations of the same dose failed to confer a high degree of protection in C57Bl/6 mice [30]. This may be due to the differential rate of parasite replication in the liver between these two mouse strains [34], and/or the aforementioned differences in cytokine production by CD8 T cells [30]. Collectively, the spectrum of results from inbred mouse strain studies of RAS vaccination highlight the complexity of CD8 T cell protection against liver-stage malaria and the need for continued studies to understand the requirements for CD8 T cell mediated protection in humans.

RAS vaccination of human subjects has been successful in providing protection from sporozoite challenge [12, 16, 35]. Despite the plethora of direct evidence for the contribution of *Plasmodium*-specific CD8 T cells in protection from challenge in mouse models of RAS vaccination [26, 29–33], direct evidence is lacking in human studies because of the inability to conduct CD8 T cell depletion studies. However, studies have shown that RAS vaccination of human subjects elicits peripheral blood T cell responses producing IFNγ, TNFα, and IL-2 as measured following *ex vivo* sporozoite stimulation[16, 33]. Moreover, examination of the RAS vaccination-induced CD8 T cell response reveals a dose-dependent increase in the frequency of *Plasmodium*-specific $IFN\gamma^+$ CD8 T cells that correlates with protection of human subjects from challenge [16]. These studies indicate that liver-stage specific, cytokine-producing CD8 T cell responses are induced following RAS vaccination of human subjects, and may contribute to RAS vaccine-induced protection.

Genetically attenuated parasite (GAP) vaccination

Similar to RAS vaccination, CD8 T cells play a dominant role in GAP-induced protection from sporozoite challenge [10, 36–39]. Various rodent *Plasmodium* GAP with targeted deletions that affect different phases of the liver-stage lifecycle have been tested in rodent vaccination studies (see [40] for a review of rodent GAP *Plasmodium* infection and immunization studies). The main immunological appeal of the GAP vaccination strategy relative to RAS is the capacity to stop the infection late in the liver stage, thereby potentially increasing the antigenic targets for CD8 T cell recognition. Indeed, Butler et al demonstrated that vaccination with late-liver-stage arresting GAP led to a significant increase in the CD8 T cell response at both the effector phase and memory phase compared to vaccination with an early-liver-stage arresting GAP sporozoites or RAS [10, 41]. Further, vaccination with late-liver stage arresting GAP required fewer immunizations to achieve protection compared to early-liver stage arresting GAP or RAS vaccination [10]. For example, in the B6/*P. yoelii* model, two immunizations with late arresting GAP were sufficient to induce protective immunity whereas two immunizations with early arresting GAP or RAS failed to confer protection [10]. Antibody-mediated depletion of CD8 T cells in late arresting GAP immunized mice just prior to challenge abrogated protection against sporozoite infection. These results highlight the enhanced protection following late-arresting GAP vaccination relative to early-arresting GAP or RAS vaccination, which is likely due to increased diversity of antigenic targets [10].

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GAP vaccination approaches using later-liver-stage arresting parasites could be a potent approach for human vaccination, potentially reducing the dose/number of immunizations required to elicit CD8 T cell-mediated immunity in human subjects. To date, only one human clinical trial using an early arresting GAP vaccine has been published [18]. This vaccination utilized a GAP deficient in two genes, *p52* and *p36*, which arrests the parasite early in liver-stage development [19]. Human subjects received two immunizations via bites from *p52*$^{-}$/*p36*$^{-}$ *P. falciparum*-infected mosquitoes [18]. Peripheral blood *Plasmodium*-specific CD8 T cell responses producing primarily IFNγ were detected following *ex vivo* stimulation with whole sporozoites in all the human subjects at ninety days after the second immunization [18]. Whether the GAP vaccination-induced IFNγ producing CD8 T cells correlate with protection in these human subjects is unknown as protection from challenge was not assessed in this phase I study. However, it can be speculated that GAP-vaccination induced CD8 T cells may also contribute to protection following challenge. Taken together, the induction of IFNγ-producing *Plasmodium*-specific CD8 T cell responses following human vaccination with GAP suggests that GAP vaccination may elicit protective immune responses in human subjects and thus warrants further investigation.

Infection-treatment vaccination (ITV)

Administration of virulent sporozoites concurrent with the anti-malarial drug chloroquine (termed infection-treatment vaccination, ITV or CPS [42, 43] appears to be the most potent WSV approach to date [22, 23], perhaps due to the additional target antigens provided with complete liver-stage development and aborted blood-stage infection [44]. In fact, ITV can elicit protective immune responses in C57Bl/6 and BALB/c mice with one vaccine administration, whereas RAS and GAP approaches require two or three vaccine administrations to achieve similar protection [10, 22, 23]. Therefore, ITV is able to efficiently induce potent immunity in fewer administrations. However, the prime mediators of this protective immunity differ based on the ITV rodent model. For example, Belnoue et al showed that CD4 and CD8 T cells contribute to protective immunity in a model where BALB/c mouse were administered virulent *P. yoelii* YM265 sporozoites concurrent with 10 days of chloroquine drug cover. Antibody-mediated depletion of CD4 or CD8 T cells just prior to challenge increased the liver parasite burden [22], suggesting a role for CD4 and CD8 T cells against liver-stage parasites. In another ITV model, using a different mouse strain and parasite species (C57Bl/6 mice and *P. yoelii* 17XNL parasites), surprisingly, neither CD4 nor CD8 T cells were required for protection [23]. Differences in the stringency of protection of BALB/c mice versus C57Bl/6 mice [30], ID_{50} of the parasites used to vaccinate [22], and duration and magnitude of blood-stage infection under chloroquine-cover could attribute to differences in the induction of protective immunity. However, in both these ITV models, one administration of ITV was sufficient to completely protect mice from challenge, compared to two or more administrations of RAS vaccination to achieve complete protection [29, 30]. In comparison, while one administration of ITV protected B6 mice from *P. yoelii* sporozoite challenge, at least two administrations of the same dose of late arresting GAP were required to protect C57Bl/6 mice from the same *P. yoelii* challenge [10]. Collectively, mouse models of ITV induce protection using fewer immunizations than RAS or GAP due to an increase in potential antigenic targets, but the specific rodent/parasite

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model have apparent differences in the dependence on CD4 or CD8 T cells for protection [22, 23].

Importantly, Sauerwein and colleagues showed that human subjects vaccinated through an ITV approach are protected from sporozoite challenge and this protection is associated with anti-*Plasmodium* T cell responses [21, 45]. Human subjects receiving ITV developed peripheral blood T cells that produced IFNγ, TNFα, and IL-2 cytokines following *ex vivo* stimulation with blood-stage parasites or sporozoites [21, 45]. Peripheral blood CD8 T cell responses to *ex vivo* stimulation with blood-stage parasites were very small relative to the CD4 T cell response (~0.02% vs. ~0.25% of peripheral blood, respectively), and not significantly increased relative to non-vaccinated human subject controls [21]. However, these results are not surprising as blood-stage parasites, not sporozoites, were used for the *ex vivo* stimulation and this analysis would not detect CD8 T cells specific to liver-stage antigens. Rodent malaria literature, primarily based on BALB/c models of infection, support a major role for liver-stage directed CD8 T cells in protection from challenge [10, 26, 30, 32, 38, 39]. Therefore, it would be of interest to determine the liverstage specific CD8 T cell response of human subjects receiving ITV. A rechallenge study of human subjects given ITV conducted 28 months after initial challenge revealed durable protection for four out of six subjects. In addition, this study reported durable total *Plasmodium*-specific T cells responses, which were not further subsetted into CD8 or CD4 T cells [45]. Therefore, it has yet not been reported that ITV induces potent CD8 T cell responses in humans. Unfortunately, this prevents a direct comparison of CD8 T cell responses between subjects receiving ITV or RAS vaccination. Interestingly, peripheral blood CD8 T cell responses can be used to predict protection in rodent models of WSV [30], but it is important to note the frequency of *Plasmodium*-specific CD8 T cells in the blood may not be representative of the frequency in lymphoid tissues, or more importantly, the liver. Indeed, it has been shown in a non-human primate model of RAS vaccination that the frequency of *Plasmodium*-specific CD8 T cells localized to the liver was 10-fold higher than the peripheral blood 3 to 4 months following the last immunization [16]. Similar observations have been made in rodent models [30, 43]. Whether the frequency of *Plasmodium*-specific CD8 T cells are greater in the liver than the blood of human subjects following WSV approaches is currently unknown. Collectively, human subjects receiving ITV induced T cell responses specific to sporozoite or blood-stage parasites, but the CD8 T cell portion of this response is not well-defined. Furthermore, the analysis of peripheral blood of human subjects receiving WSV may underestimate the total frequency of protective, cytokine-producing CD8 T cells throughout the host.

Human studies, as well as mouse models, have supported the role of liver-stage directed CD8 T cell responses in protection using WSV approaches. Evidence for the protective capacity of CD8 T cells in mouse studies has largely been determined through the use of antibody-mediated depletion of CD8 T cells just prior to challenge, through the use of various knockout mouse models, as well as cellular transfer experiments. Although each of these approaches has caveats, the relatively consistent result of CD8 T cell dependence for protection in mouse models of WSV, particularly after RAS and GAP vaccination, supports a critical role for CD8 T cells in protection from sporozoite challenge. However, WSV of

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mice has also lead to complete protection from sporozoite challenge independently of CD8 T cells, suggesting a possible role for other components of the immune response in protection [23]. Human studies of WSV approaches have measured liver-stage and blood-stage specific CD8 T cell responses following vaccination, which correlated with protection from challenge [16, 33]. Taken together, mouse and human studies of WSV have demonstrated the induction of *Plasmodium*-specific CD8 T cell responses, which directly contribute to protection in several rodent studies, and are correlated with protection in human studies.

Differential requirement of WSV-induced CD4 T cell responses for protection

While a substantial data support the role of WSV-induced CD8 T cells in protection against sporozoite challenge, the protective role of CD4 T cells is less clear. Studies using the mouse model of malaria suggest that a role of CD4 T cells in protection against sporoozite challenge may depend on the mouse strain used for evaluation. In one such study, Doolan et al. performed RAS vaccination in seven inbred mouse strains and evaluated the contribution of CD4 T cells in protection by performing CD4 T cellspecific antibody-mediated depletion. In contrast to CD8 T cells, which were required for protection in all tested mouse strains, CD4 T cells were required for protection in only three out of seven mouse strains (C57Bl/6; B6,129; B10.D2) [29]. Interestingly, two prominently used mouse strains for *Plasmodium* studies, the BALB/c and C57Bl/6, substantially differed in their requirement for CD4 T cells in protection. Namely, protection in RAS vaccinated BALB/c mice was CD4 T cell independent, while CD4 T cells played an important role in protection of RAS vaccinated C57Bl/6 mice against sporozoite challenge [29].

Although these results strongly suggest that contribution of CD4 T cells to RAS-induced protection differs between mouse strains, it should also be noted that such CD4 T cell-dependence might change over time. For instance, we observed that protection of C57Bl/6 mice against early sporozoite challenge infection, 2 weeks after *P. yoelii* 17XNL RAS-vaccination, was CD4 T cell-dependent (unpublished data). Conversely, later challenge of *P. yoelli* 17XNL RAS-vaccinated C57Bl/6 mice at a stable CD8 T cell memory time point led to CD4 T cell-independent protection. Thus, it is possible that dependence on CD4 T cells for protection following RAS-vaccination could change as stable memory CD8 T cell populations fully develop. We speculate that CD4 T cell-independent protection can be observed in all RAS-vaccinated mice if sporozoite challenge is performed later than two weeks post-immunization, thus allowing sufficient time for functionally and phenotypically stable memory CD8 T cell response to develop [46]. Whether CD4 T cell dependence for protection differs over time in other WSV approaches remains an open question. If the correlates of protective immunity differ over time after immunization, this could have significant implications for how WSV elicits protection in human subjects. Thus, further investigation into the requirement for CD4 T cells in protection following WSV approaches in early challenge or late challenge is warranted.

In addition to the potential difference in CD4 T cell dependence for RAS vaccine-induced protection based on mouse strain, it is possible the WSV approach can affect the

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requirement for CD4 T cells in protection from challenge. For instance, in a rodent GAP vaccination model using C57Bl/6 mice immunized with *uis*3$^{-/-}$ *P. berghei* NK65 parasites, CD4 T cells minimally contributed to protection [36]. Further, in an ITV model using C57Bl/6 mice immunized with virulent *P. yoelii* 17XNL, CD4 T cells were not required for protection from sporozoite or blood-stage challenge [23]. These results indicate that CD4 T cells are not required for protection in C57Bl/6 mice vaccinated with the GAP or ITV approach. However, these WSV studies all utilized different parasite species, as well as different immunization dose size and timing between immunizations. It is possible these differences could dramatically alter the immune components required for protection. Direct comparisons between mouse strain, parasite species, dose sizing and timing between immunizations, as well as consistent challenge time points in these three WSV approaches are much needed to better understand the role of *Plasmodium*-specific CD4 T cell responses in protection from sporozoite challenge. These results highlight how differences in the mouse strain and WSV approach could differentially alter the requirement of WSV-induced CD4 T cells in protection.

Vaccination of human subjects with WSV induces detectable *Plasmodium*-specific CD4 T cells responses in the peripheral blood. It has been shown that these CD4 T cells are capable of producing cytokines upon *ex vivo* stimulation with blood-stage parasites or sporozoites [16, 18, 21, 33]. Furthermore, the magnitude of the cytokine producing RAS-induced CD4 T cell response directly correlated with the administered vaccine dose [16]. Although testing the direct involvement of CD4 T cells in protection against sporozoite infection in humans is not possible, it is clear that CD4 T cells responses are elicited in human subjects by WSV vaccination and their magnitude depends on vaccine dose and correlate with observed protection.

The role of *Plasmodium*-specific CD4 T cell responses following WSV is not fully understood. While it is clear that that both mice and human subjects produce cytokine-producing *Plasmodium*-specific CD4 T cell response after WSV, it is not clear whether these CD4 T cells are required for protection. WSV-induced CD4 T cells appear essential for protection in some rodent models, but this may be dependent on the time sporozoite challenge is administered following immunization. Due to possible differential requirement of CD4 T cells in protection of inbred mouse models of WSV, it is possible humans will similarly have diverse requirements for WSV-induced CD4 T cells in protection from malarial disease. Thus, it remains important to study the role of *Plasmodium*-specific CD4 T cells in protection against sporozoite, or blood-stage challenge in rodent models to best understand how these CD4 T cell responses could contribute to protection in human subjects receiving WSV.

Induction of antibody responses against liver-stage and blood-stage parasite antigens following WSV

Anti-*Plasmodium* antibodies are capable of inhibiting parasite infection and contributing to parasite clearance of blood-stage infections [47, 48], but their role in protection following WSV remains largely undefined. Before the initial studies of RAS vaccination demonstrating a prominent role for liverstage directed CD8 T cells in protection against

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challenge, mouse studies of RAS vaccination revealed the induction of antibodies against CSP, a prominent liver-stage parasite protein, suggesting an important role of humoral immunity in protection [49, 50]. However, RAS vaccination of mice can elicit protection independently of B cells with as few as two immunizations [31], which indicates that antibodies are not necessary for RAS-induced protection. Taken together, WSV approaches can elicit anti-sporozoite antibody responses, but protection from challenge may be achieved independent of B cells/antibodies through CD8 T cells [31, 49, 50].

In contrast to the majority of mouse studies of WSV, recent work from Doll et al using a stringent rodent model of ITV showed that immunity from liver-stage sporozoite or blood-stage parasite did not require CD8 T or CD4 T cells, but required blood-stage targeted antibodies [23]. A single administration of 104 liver-stage *Plasmodium yoelii* 17XNL sporozoites administered with 10 consecutive days of chloroquine drug cover provided complete, sterilizing immunity from homologous parasite challenge with 103 sporozoites or 106 parasitized red blood cells. Protection correlated with the induction of IgG antibody responses against blood-stage parasite as a result of a short duration, low magnitude blood-stage infection (3–5 days, >5% parasitemia) shortly following chloroquine cessation [23]. Similar to these studies, human ITV studies have also shown presence of blood-stage parasites following vaccine administration, suggesting that blood-stage specific immune responses could contribute to protection in human models of ITV. To date, no other rodent ITV studies have demonstrated a dominant role for antibodies in protection from challenge [22, 51], which may be due to differences in whether breakthrough parasitemia occurs in a particular rodent/parasite ITV model, and likely also the length and duration of blood-stage infection. These results highlight the continued need to consider the anti-parasitic humoral immune response, which could contribute to complete, sterilizing immunity in addition to or independently of anti-*Plasmodium* CD8 T cell responses.

Despite the prominent role of liver-stage directed CD8 T cells in protection following WSV, and lack of robust evidence for T-cell independent antibody mediated protection, further studies of antibody responses may help in the development of the most potent strategy for human vaccination. To date, anti-circumsporozoite protein (CSP) antibodies are arguably the most studied antibody response following human WSV [16, 18, 21, 52]. CSP is a surface protein of the liver-stage parasite, making it an attractive target for an antibody response. Anti-CSP antibody responses have been shown to inhibit sporozoite infection of hepatocytes *in vitro* and *in vivo* [53, 54], and monoclonal CSP-specific antibodies can confer sterilizing protection against sporozoite challenge [50, 55]. Antibodies directed against CSP have been detected in many human studies of WSV [16, 18, 20, 33]. Following RAS vaccination of human subjects, anti-CSP titers correlated with the immunization dose, with protected individuals having higher antibody titers compared to non-protected individuals [16]. In human GAP and ITV vaccination studies, anti-CSP antibody responses were detected in the majority of volunteers [18, 20, 21, 52]. However, only one of six patients who received ITV had detectable CSP antibodies in a follow-up study (28 months later) [45], suggesting that ITV-induced anti-CSP antibody responses are short-lived. The contribution of anti-CSP antibodies to protection of human subjects following WSV may not be clear, but in RAS, GAP, and ITV approaches it is clear that human subjects can make anti-CSP responses.

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Antibodies directed against other liver-stage targets besides CSP (i.e. LSA1 and SSP2) have been detected in human subjects following WSV [52, 56]. The protective capacity of these non-CSP-specific antibodies is currently unknown. However, total WSV-induced antibodies in human subjects may contribute to protection through inhibiting sporozoite infection of hepatocytes. For example, plasma collected three months following two-dose immunization of human subjects with GAP vaccination inhibited *P. falciparum* invasion of hepatocytes *in vitro* [57]. This inhibition of invasion was similar to plasma collected from human subjects who received 4–5 doses of RAS sporozoites [16]. Thus, both RAS and GAP vaccination of human subjects induce antibodies, which can inhibit hepatocyte invasion. It should be noted that it is likely that anti-CSP antibodies within the total plasma from GAP vaccination subjects heavily contributed to inhibition of hepatocyte infection in the GAP study as the percent of inhibition positively correlated with the anti-CSP antibody titer [57]. Collectively, human subjects receiving WSV can make antibody responses directed against liver-stage parasite antigens, which may aid in protection through inhibition of sporozoite infection.

Blood-stage specific antibodies can be detected in human subjects receiving WSV, but only if the subject was exposed to blood-stage parasites. In human RAS studies, where the subject is never exposed to blood-stage infection, antibodies directed against crude blood-stage parasite lysate or against known blood-stage antigens were not detected [16, 33]. Likewise, GAP vaccination of human subjects did not elicit anti-merozoite surface protein-1 (MSP-1) antibodies, a known blood-stage antigen, except in the one patient that had detectable blood-stage infection from vaccination breakthrough [18]. In partial contrast, antibodies directed against crude blood-stage parasite lysate were detected in humans receiving ITV, but antibodies against defined blood-stage antigens such as MSP-1, AMA-1, or GLURP were not detected [21, 45, 52]. Collectively, these results are not surprising since RAS and GAP vaccination does not lead to any blood-stage parasite exposure during vaccination whereas ITV does allow exposure to blood-stage infection at levels below detection by blood smear (subpatent), but detectable by qPCR [20, 21]. Despite the induction of blood-stage directed antibodies against crude blood-stage parasite lysate in human subjects receiving ITV, these antibodies were insufficient to sterilely protect from blood-stage parasite challenge [20]. This result clearly indicates that blood-stage specific immunity following ITV is not sufficient to sterilely protect from blood-stage parasite challenge. However, it is unknown if ITV-induced blood-stage specific immunity is capable of controlling infection and contributing to reduction of disease, since the humans undergoing blood stage challenge were treated with antimalarial drugs immediately upon detection of blood-stage parasite. Thus, exposure of human subjects to blood-stage parasites during ITV elicits blood-stage-specific antibody responses, but it is unknown if these antibodies contribute to the total protective immune response. Collectively, antibodies directed against blood-stage parasite antigens are induced during ITV, but not RAS or GAP vaccination, but these antibodies are not sufficient to protect human subjects from blood-stage parasite challenge.

Anti-*Plasmodium* antibody responses have been detected in human subjects as well as mice following WSV, but the contribution of those antibody responses in either liver-stage or blood-stage protection appears minimal and may be limited to partial inhibition of

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hepatocyte infection by anti-CSP antibodies. Overall, it is likely that protection is heavily dependent on T cell responses with antibody responses partially contributing. Recently, Felgner et al compared antibody profiles from human subjects receiving ITV to naturally exposed individuals from Kenya. Their results indicate that ITV primarily elicits an antibody profile directed against liver-stage antigens, whereas naturally exposed individuals primarily have antibody profiles directed against blood-stage antigens [52]. Based on these data, if antibodies contribute to protection following ITV, it is likely they are primarily specific for liver-stage antigens. This is in contrast to the results of a rodent model of ITV whereby blood-stage directed antibodies were induced, and were associated with complete protection from a blood-stage parasite challenge [23]. Both human and rodent models of ITV have shown a degree of blood-stage infection following CQ cessation [20, 21, 23], but the duration and magnitude of blood-stage infection in human subjects may have not been sufficient to generate robust blood-stage directed antibodies. On the other hand, natural immunity to *Plasmodium* infection requires repetitive exposure to blood-stage infections and is not sterilizing [58]. Whether there is an optimal duration and magnitude of blood-stage infection that generates potent, protective blood-stage directed antibody responses in human subjects – providing enough antigen to elicit a response, but not too much to induce negative immunomodulatory effects, is an area under current investigation. At this point, the literature supports a small role for liver-stage directed antibodies, particularly anti-CSP antibodies, in protection following WSV. Collectively, liver-stage directed antibodies appear to contribute partial, but incomplete protection from liver-stage challenge following WSV and further research defining the role of blood-stage antibodies following WSV is an area of much needed investigation.

Future directions for the field

Mouse models of WSV have demonstrated a dominant role for liver-stage directed CD8 T cell responses in protection from challenge [10, 26–33], however a substantial proportion of these studies did not directly test the role of memory CD8 T cell responses in protection. It has been well established that phenotype and functionality of CD8 T cells is dynamic and alters from an effector response (first couple weeks after initial activation by antigen), to a memory response (>2 months after antigen exposure in mouse models) [46, 59]. Nonetheless, it is clear that memory *Plasmodium*-specific CD8 T cell responses can protect against sporozoite challenge as this has been shown in some rodent GAP studies, as well as with studies using prime-boost methods to generate CSP-specific memory CD8 T cells [10, 30, 34, 60, 61]. Future studies should focus on memory anti-*Plasmodium* CD8 T cell responses in protective immunity as this best models the goal for long-term, stable protective immunity following WSV in humans.

While the contribution of CD8 T cells to protection following WSV in rodent models are clear, the correlates of protection in human subjects receiving WSV are less clear (Table 2). Although it is not possible to directly test the contribution of a specific cell population in protection of human subjects from sporozoite challenge, studying the association of these cell populations with protection provides invaluable information. Thus, in order to draw parallels between mechanistic studies from rodent malaria models to human vaccination studies it is essential that both CD4 and CD8 T cell populations be assessed in human

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subjects. Further, testing the liver- or blood-stage specificity of these T cell responses will be important to further understand the targets of protective immunity. Currently, it is still unknown if a WSV approach can ever be applied on a large scale to the field, but the superior protective capacity of WSV approaches compared to the most advanced subunit approach (RTS,S) support the continued need to understand the protective correlates of these vaccination approaches whether mediated by liver-stage or blood-stage CD8 T cells, CD4 T cells, and/or antibody responses.

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Table I

Overview of whole sporozoite vaccination approaches.

Whole sporozoite vaccination approach	Liver-stage infection	Blood-stage infection	Advantages	Disadvantages
Radiation-attenuated sporozoites (RAS)	Incomplete, arrests early	None	Potent mediator of protection in humans, non-human primates, and rodents. Considered the "gold standard."	Need to for sufficient irradiation to attenuate, but not over-irradiattion to destroy immunogenicity.
Genetically-attenuated parasites (GAP)	Incomplete, arrests at different stages depending on gene-specific deletions	None	Potentially larger antigenic repertoire to be targeted for protective immune responses compared to RAS vaccination.	Gene-specific deletion must ensure attenuation during the liver-stage to prevent blood-stage infection and clinical disease.
Infection-treatment vaccination (with chloroquine drug administration)	Complete, non-attenuated	Attenuated, brief blood-stage infection. Parasite can be detected by qPCR in blood of humans [20, 21], and by Giemsa blood smear in a rodent model [23].	Exposure to liver-stage and blood-stage antigens. Provides broader antigenic repertoire for protective immune responses compared to RAS or GAP vaccination.	Requires anti-malarial drug administration to attenuate blood-stage but chloroquine-resistance of *Plasmodium* in the field is a potential issue that may arise.

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Table II

Correlates of protection following whole sporozoite vaccination approaches.

Whole sporozoite vaccination approach	**Vaccination-induced responses**		
Radiation-attenuated sporozoites (RAS)	*CD8 T cells*	*CD4 T cells*	*Antibodies*
Mouse	Required for protection [29–33].	Generally not required for protection, dependent on mouse strain [29].	Anti-sporozoite antibodies did not correlate with protection [30].
Non-human primate	IFNγ^{+}, TNFα^{+} CD8 T cells detected in liver following *ex vivo* sporozoite stimulation [33].	IFNγ^{+}, TNFα^{+}, or IL-2^{+} CD4 T cells detected in liver following *ex vivo* sporozoite stimulation [33].	N.D.*
Human	Peripheral blood IFNγ^{+}, TNFα^{+}, IL-2^{+} CD8 T cells detected following *ex vivo* sporozoite stimulation [16].	Peripheral blood IFNγ^{+}, TNFα^{+}, IL-2^{+} CD4 T cells detected following *ex vivo* sporozoite stimulation [16,33].	Anti-CSP antibodies induced [16,33].
Genetically-attenuated parasites (GAP)			
Mouse	Required for protection [10, 36–39].	Not required for protection [30,36,38].	N.D.*
Human	Peripheral blood IFNγ^{+} CD8 T cells detected following *ex vivo* sporozoite stimulation [18].	Peripheral blood IFNγ^{+}, TNFα^{+}, IL-2^{+} CD4 T cells detected following *ex vivo* sporozoite stimulation [18].	Antibodies capable of inhibiting *in vitro* hepatocyte invasion detected [18].
Infection-treatment vaccination Mouse	Requirement for protection differs by rodent/ parasite immunization model. Not required for protection in C57Bl/6-*P. yoelii* 17XNL [23]. Partially reduces liver parasite burden in BALB/c-*P. yoelii* YM265 model [9].	Not required for protection – can delete population just prior to challenge [23]. Partially reduces liver parasite burden in BALB/c-*P. yoelii* YM265 model [9].	Anti-blood-stage parasite antibodies correlated with protection in C57Bl/6-*P. yoelii* 17XNL model [23].
Human	Detectable peripheral blood IFNγ^{+}, IL-2^{+} CD8 T cell response following *ex vivo* blood-stage parasite stimulation [21].	Peripheral blood IFNγ^{+}, IL-2^{+} CD4 T cell response following *ex vivo* blood-stage parasite stimulation [21].	Anti-CSP antibodies induced [20,21].

*Indicates not discussed in review.