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Challenges of assessing the clinical efficacy of asexual blood-stage *Plasmodium falciparum* malaria vaccines

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In the absence of any highly effective vaccine candidate against *Plasmodium falciparum* malaria, it remains imperative for the field to pursue all avenues that may lead to the successful development of such a formulation. The development of a subunit vaccine targeting the asexual blood-stage of *Plasmodium falciparum* malaria infection has proven particularly challenging with only limited success to date in clinical trials. However, only a fraction of potential blood-stage vaccine antigens have been evaluated as targets, and a number of new promising candidate antigen formulations and delivery platforms are approaching clinical development. It is therefore essential that reliable and sensitive methods of detecting, or ruling out, even modest efficacy of blood-stage vaccines in small clinical trials be established. In this article we evaluate the challenges facing blood-stage vaccine developers, assess the appropriateness and limitations of various in vivo approaches for efficacy assessment and suggest future directions for the field.

Introduction

Plasmodium falciparum malaria remains the pre-eminent tropical parasitic disease responsible for an estimated 216 million infections and 655,000 malaria deaths worldwide in 2010,[1] a potentially conservative estimate.[2] Application of control measures for *P. falciparum* have been associated with reductions in the number of cases in some areas.[1] This epidemiological shift and the linked belief that malaria elimination may be a realistic goal,[3,4] has led many to argue for a change in the strategic direction of malaria vaccine research; away from reduction of severe disease in children[5] and toward prevention of infection, clinical disease and transmission in both adults and children.[6]

This drive toward elimination has been used as an argument in favor of pre-erythrocytic and transmission blocking vaccines [TBV, or Vaccines Interrupting Malaria Transmission (VIMT)], and against asexual blood-stage vaccines (BSV).[7] In particular, it has been suggested that while vaccines targeting the blood-stage of *P. falciparum* could reduce severe disease, they could also increase the reservoir of asymptomatically parasitemic individuals and thus actually impede efforts to reduce transmission.[7] However, in light of disappointing levels of efficacy reported in the target infant age group in the interim analysis of the Phase III trial of the leading pre-erythrocytic vaccine RTS,S,[8] and in the continued absence of any highly effective malaria vaccine targeting any stage of the parasite lifecycle, it remains vital that the merits of all promising new approaches are assessed. The focus needs to remain on achieving high levels of protective efficacy in humans with any safe and suitable formulation, and to this end there remain important reasons to pursue the development of BSVs (**Box 1**).

Foremost, avoidance of a symptomatic case would represent the best possible outcome of malaria vaccination for the individual and remains the primary goal of a BSV. However, achieving this goal (which does not necessitate sterilizing immunity), is not mutually exclusive with a second vitally important outcome; reducing malaria transmission. The relationships between blood-stage immunity to malaria, asexual parasitemia, gametocytemia, and infectivity to mosquitoes are complex and incompletely understood.[9] Nonetheless, the balance of evidence suggests that reduced asexual parasite density (as would be achieved by an efficacious BSV) is associated with reduced infectivity to mosquitoes.[9-11] Moreover, invasion of erythrocytes is a necessary step in gametocytogenesis. It therefore seems likely that an effective anti-merozoite vaccine would reduce gametocyte density, infectivity, and transmission, both directly (by reducing erythrocyte invasion by sexually-committed merozoites), and indirectly (by reducing the density of asexual progenitors of sexual-stage parasites). Investigation of these complex relationships will become a vitally important area, should any new-generation BSV formulation demonstrate significant efficacy.

Interventions that reduce exposure to *P. falciparum* also reduce the acquisition of naturally-acquired immunity (NAI) in malaria endemic populations and have already led to significant changes in malaria epidemiology.[1] Multiple sites have observed a shift in the peak age of clinical malaria to older children and an increase in the median age of hospitalization.[12-14] The development of resistance both in *Anopheles* mosquitoes to certain insecticides and of malaria parasites to chemotherapeutic agents[3,15] mean that populations with little or no immunity to blood-stage infection could be vulnerable to epidemics of severe disease in the

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Box 1. Reasons to pursue development of vaccines targeting the blood-stage of malaria infection

- Naturally-acquired immunity (NAI) largely arises against the blood-stage of infection.
- An effective blood-stage vaccine (BSV) could reduce severity of disease and mortality while allowing continued antigenic exposure and generation of NAI; although it remains possible a subunit BSV may afford protection by immune mechanisms not associated with NAI.
- An effective BSV could potentially reduce malaria transmission by reducing gametocyte densities.

Box 2. Challenges for the development of an efficacious vaccine targeting the blood-stage of *P. Falciparum* infection

- Antigen selection from a wide range of potential targets.[18,42]
- Antigenic polymorphism.[16,18,43]
- Apparent need for extremely high antibody titers for anti-merozoite antigen vaccines to achieve protection.[29,39,44]
- Lack of consensus regarding mechanisms of blood-stage immunity and strategies for vaccine design (e.g., antibody- vs. T cell-inducing), coupled with uncertainty of in vitro assay utility.[45-47]
- Difficulty identifying sufficiently immunogenic (cell-mediated and humoral) vaccine regimens in humans.
- Access to sufficiently immunogenic protein vaccine adjuvants with an acceptable reactogenicity profile.[48]

future, something that an effective BSV could protect against as much as a pre-erythrocytic vaccine. An effective BSV would also lead to prevention of disease while still allowing some low-level exposure to parasites. Importantly this could allow for vaccine-induced responses to be boosted by infection and thus immunity maintained for longer.[16]

In the absence of a highly effective product, the case remains strong to scientifically investigate all avenues that may aid in the development of a highly effective vaccine formulation against *P. falciparum*. Given that only a fraction of potential blood-stage antigens have been evaluated as vaccine targets[17] and new candidate vaccines will continue to approach clinical development, it remains essential that robust, sensitive and reliable methods of detecting even modest clinical efficacy of BSV be established.[18] For the remainder of this article, we review the challenges facing the field in these specific endeavors.

Challenges for BSV Developers—Vaccine Strategies

A wide variety of BSV approaches have been proposed, ranging from induction of antibody to infected red blood cell (iRBC) surface antigens,[19,20] through the induction of cellular immunity against blood-stage antigens,[21] to vaccination with whole blood-stage parasites.[22-24] The most widely used approach however has been the induction of anti-merozoite antibodies mostly against a small group of merozoite antigens which were originally identified because of their immuno-dominance.[16,25] Further insight into the mechanisms of blood-stage immunity and efficacy of such candidate BSVs has been gained from rodent and non-human primate models of *P. falciparum* infection.[26-28] Such studies originally validated the protective potential of vaccines targeting well-characterized and historically-identified antigens such as merozoite surface protein 1 (MSP1), MSP3 and apical membrane antigen 1 (AMA1),[29-33] which have also been repeatedly associated with protective NAI.[34] However, despite the clinical assessment of multiple subunit vaccines against these targets, results of clinical trials in humans have been disappointing with only a minority of candidates demonstrating any hint of efficacy, and none in terms of the primary efficacy endpoint of a Phase IIa/b study.[21,35-41] The experiences gained from so many studies of these candidates have identified a core set of challenges that face developers of BSV (**Box 2**).

Challenges for BSV Developers—In Vitro Assays as Predictors of Vaccine Efficacy

Anti-malarial antibody responses induced by vaccination can be assessed by a variety of laboratory assays. ELISA-based methods typically provide information on the magnitude of an antibody response measured against a recombinant antigen, while immunofluorescence assays (IFA) can confirm recognition of native parasite antigen. Other in vitro assays have also been developed in attempt to measure the functional activity of vaccine-induced antibodies against the parasite, as opposed to their titer or magnitude as often determined by ELISA. The development of any assay that could predict BSV efficacy would have extremely important applications, potentially reducing the cost and ethical risks associated with extensive human efficacy testing of new candidates and helping to prioritize progression of only the most promising vaccines to clinical studies. However, in the absence of a BSV that unambiguously protects humans, it is not possible for any preclinical assay to be completely validated as being predictive of such protection. Extensive efforts have been expended on assessing in vitro assay readouts and disease outcomes observed following natural exposure in immuno-epidemiological studies. However, interpretation of these studies with regard to vaccine development is hindered by the fact that vaccine-induced and NAI may be mediated by different mechanisms. It is also often difficult to perform such assays with parasites representing currently circulating *P. falciparum* populations, rather than historically-established culture-adapted lines, making interpretation of the results even more difficult. Given the biological and immunological complexity of the *P. falciparum* blood-stage, it seems likely that more than one immunological response is capable of contributing to protection and therefore unlikely that a single assay will predict all mechanisms of blood-stage immunity.

Growth inhibition activity (GIA) assay. The most widely used and established functional assay, the assay of GIA,[49] assesses

the ability of diluted serum or purified IgG antibodies to inhibit the invasion and growth of *P. falciparum* parasites in human RBCs in vitro, and has been historically used to down-select BSV candidates for further clinical development.[50] Importantly this assay measures only cell-independent parasite neutralization by antibodies. Variations of this assay have been reported that distinguish between invasion inhibition alone vs. invasion and/or intracellular growth inhibition combined, with antibodies against different antigens reportedly acting in a different manner.[51] Miniaturized versions of the assay have also been reported that may allow for faster throughput of larger numbers of samples from field trials.[52] Other variations upon the assay have been developed, including both flow-cytometric readouts[53,54] and potentially more sensitive two-lifecycle assays (originally developed for use in immuno-epidemiological studies).[55] Although undoubtedly valuable in some contexts, these variations do not appear to be necessary in the context of vaccine development: antibody effects which could plausibly achieve protection should be readily detectable with a single-cycle assay.[38]

There have been highly conflicting results from immuno-epidemiological studies investigating the relationship between in vitro GIA and clinical outcome, compounded by considerable difference in assay methodologies and reporting between centers (reviewed in detail elsewhere).[50] Modern BSVs can induce levels of GIA that exceed those seen in NAI.[38] However, given that immune responses specifically induced by a vaccine will not be typically accompanied by the potentially multi-factorial immune responses associated with NAI, the relationship between GIA and epidemiological data are of debatable use to vaccine developers.

The available evidence from non-human primate and clinical BSV trials suggests that vaccine-induced growth inhibitory antibodies are capable of conferring in vivo protection, but neither proves this conclusively nor excludes the possibility that other antibody effects could also achieve protection. GIA has been closely associated with protective outcome of MSP1- and AMA1-based vaccine candidates in non-human primate challenge models.[30,56] However, to date in humans, only one small CHMI study has directly assessed this relationship in the context of AMA1 vaccination, and encouragingly the data indicated a possible association between in vitro GIA and in vivo parasite multiplication rates (PMR) in vaccinees.[57] Overall, however, this vaccine showed no significant efficacy in the vaccinees in comparison to controls, making interpretation of this result difficult at best, and consequently further studies are urgently required.

The establishment of an international reference laboratory at NIH-NIAID funded by the PATH-Malaria Vaccine Initiative to perform standardized assays of GIA[49] has significantly improved the ability of investigators and funders to obtain robust and comparable data.[58] The version of the assay routinely employed at this center uses purified IgG (avoiding the potential for non-specific effects due to serum constituents, and permitting testing at a wide range of supra- and sub-physiological concentrations); a single complete parasite lifecycle (permitting detection of effects of vaccines upon both invasion and intracellular growth); and a simple colorimetric readout.[59] This assay has been carefully demonstrated to give reproducible results both within and between centers.[58,60] Universal adoption of the reference center's assay method by BSV developers would greatly assist inter-center comparisons of vaccine candidates.

Antibody-dependent cellular assays. The ability of so-called "cytophilic" antibody subclasses to induce cellular immune responses mediated by Fc-dependent signaling has formed the basis of another potential in vitro assay for the assessment of blood-stage immunity. An assay measuring antibody dependent cellular inhibition (ADCI) was first described in the late 1980s and measures the antibody-dependent cellular activity of monocytes against *P. falciparum* parasites.[61] Subsequent studies demonstrated parasite clearance in passive transfer studies in humans and mice using ADCI inducing IgG,[62-64] and these data were used to design an ADCI-inducing vaccine candidate to take forward into clinical development.[35,63] However, other groups have failed to reliably reproduce the ADCI assay and as such this assay has not been widely adopted, although a recent report of methodology for a phagocytosis assay using merozoites opsonized with antibody and the THP-1 monocytic cell line may represent a new line of investigation in this area.[65]

The correlation of clearance of *P. falciparum* in Gabonese children with reactive oxygen species (ROS)[66] has led to the investigation of neutrophils as an alternative cell population for clearing blood-stage parasites. Development of an antibody-dependent respiratory burst (ADRB) assay has allowed assessment of opsonized merozoites to induce ROS from polymorphonuclear neutrophils in vitro and demonstration of correlation with protection against clinical malaria in an endemic population.[67] While promising, this result has yet to be repeated, but could provide in the future an alternative methodological approach to GIA for screening potential vaccine antigens.

Challenges for BSV Developers—Assessment of Clinical Efficacy

In the absence of a validated in vitro assay of blood-stage immunity or definitive animal model, the only reliable method of assessing in vivo efficacy of promising candidate BSVs are clinical trials. The traditional gold standard in vivo assessment to date has been Phase IIb field efficacy studies. Infants or children in malaria endemic regions are vaccinated and incidence of clinical malaria infection compared with an unvaccinated or placebo control group. As well as the benefit of providing efficacy data in the target population, these studies allow collection of the most clinically relevant endpoint; protection against malaria disease in the field. However, such trials are costly, take years to perform[68] and rely on large numbers of individuals being exposed to the small but potential risks of novel vaccines in the early stages of development. Given the considerable genetic heterogeneity of *P. falciparum* parasites circulating in the field,[69] these studies may fail to observe vaccine strain-specific efficacy unless this is specifically analyzed.[39] While arguably such a vaccine may not provide overall efficacy, the detection of such positive signals would be a vital guide for continued iterative vaccine improvement.

Recently, this was exemplified by a mono-valent vaccine (called FMP2.1) targeting the 3D7 allele of the micronemal

Table 1. Relative merits of various *P. Falciparum* CHMI models

	Sporozoite—mosquito bite	Sporozoite—injection	Blood-stage
Mimicking natural route of infection	Yes	No	No
Ability to control inoculum size	+/–	++	+++
Life-cycle stages amenable to study	Sporozoite (skin and blood) Liver-stage Blood-stage	Sporozoite (blood) Liver-stage Blood-stage	Blood-stage
Availability	Limited trial centers	Potential for widespread use	Potential for widespread use
Duration of blood-stage parasite exposure	Short (2–4 d)	Variable (2–8 d)	Longer (8–9 d)
Reliably achieves 100% infection	+++	++	+++

merozoite antigen AMA1, administered in GSK's proprietary adjuvant $AS02_A$. This vaccine was reported to show significant strain-specific efficacy in a Phase IIb field trial in Malian children when compared against placebo (efficacy against clinical malaria caused by parasites with AMA1 corresponding to that of the vaccine strain was 64.3% (hazard ratio: 0.36; 95% CI: 0.08 to 0.86; p = 0.03). However no overall vaccine efficacy was observed.[39] Exemplifying the challenges of clinical BSV development, and somewhat surprisingly, this result was in contrast to a preceding Phase IIa mosquito-bite controlled human malaria infection (CHMI) study of the same vaccine in malaria-naïve US adult volunteers where no significant efficacy was reported with regard to the trial's primary endpoint (time to diagnosis by blood-film) against the vaccine homologous 3D7 clone parasite.[38] While there was no significant difference between vaccinees and controls in parasite multiplication rates (PMRs) modeled at the group rather than individual level, comparison of parasitemia measured by real-time quantitative PCR (qPCR) between vaccinees and controls from day 7–9 post challenge did show a significant reduction. Such an effect could be attributed to a reduced liver- to -blood inoculum (LBI) suggesting pre-erythrocytic efficacy, or conversely a small reduction in blood-stage parasitemia that was insufficient to translate into a significant delay in terms of patent diagnosis by thick-film microscopy. This discrepancy in efficacy results between two clinical trials, conducted in two very different settings—the field vs. CHMI, highlights the difficulties in ascertaining BSV efficacy in proof-of-concept studies.

Controlled Human Malaria Infection (CHMI) Studies

CHMI studies have been routinely performed given they provide the most rapid and relatively cost effective method for assessing vaccine efficacy in vivo without necessitating a field trial. Vaccinees are infected with either *P. falciparum* sporozoites or blood-stages under carefully controlled conditions and treated (in the majority of cases) when thick blood-film positive by microscopy.[68,70,71] qPCR performed 1–3 times daily post infection with a typical lower limit of detection of 20 parasites per mL (p/mL), allows for detailed mathematical modeling of parasite growth dynamics up until the point of diagnosis (typically 2–3 blood-stage growth cycles), and estimations of vaccine effect on the LBI and/or PMR.[68,72-75]

In the great majority of Phase IIa CHMI studies performed to date, group sizes have been in single figures reflecting both the practical and ethical limitations on the numbers of individuals that can be recruited and challenged safely. For this reason, small CHMI studies usually seek to assess proof-of-concept in the first instance, with a view to validating observed efficacy results in repeat studies or progressing to a Phase IIb field study if a highly encouraging signal is seen. Nevertheless given all vaccinees are exposed to infection, and all non-vaccinees almost without exception become infected, CHMI studies can provide sufficient statistical power both to detect and to rule out meaningful effects using small sample sizes (often 12 or fewer volunteers per group).[72] Indeed "large-scale malaria vaccine trials in the absence of evidence of efficacy in studies of controlled human infection would now be thought unethical by many"[76] **Table 1**.

Sporozoite CHMI trials. To date, the majority of CHMI trials assessing BSV efficacy have been undertaken by administration of sporozoites by mosquito bite.[21,36,38] Trials using the pre-erythrocytic malaria vaccine RTS,S showed efficacy estimates to be comparable when measured by mosquito bite CHMI (number of participants sterilely protected) or field studies (number of cases of clinical malaria prevented).[77,78] However, since no BSV has yet demonstrated significant efficacy in both CHMI and field studies, no similar data exist to support mosquito bite CHMI trials as a surrogate measure of field efficacy in this context.

While mosquito bite CHMI studies have the benefit of representing the natural route of infection and an established protocol that reliably infects 100% of volunteers,[68] some have questioned its utility in the assessment of BSV efficacy.[18,72,79] Primarily, this challenge model only provides a small window of opportunity in which to observe a protective impact of vaccine-induced responses on blood-stage *P. falciparum* parasites. Typically following five infectious mosquito bites, volunteers display parasitemia detectable by qPCR in the first blood-stage parasite growth cycle (peaking on day 7–8 post-challenge). Among unvaccinated infectivity controls, a 25-fold variation between trials in the median level of parasitaemia in this first wave (range of medians 30–750) has been observed, and 75-fold variation among the controls in a single trial (range 27–2000).[80] Such levels may be dependent on a number of variables (challenge center, *P. falciparum* parasite strain (NF54 vs. 3D7), intensity of mosquito infection, mosquito feeding exposure time), but all lead to

diagnosis by thick film microscopy within a median timeframe of 2–4 days.[80] It is impossible to observe potential vaccine efficacy at levels of blood-stage parasitemia that exceed the typical peak levels observed in CHMI studies (approximately 8000–16 000 p/mL). Such a window of opportunity may thus be potentially too short for protective immune mechanisms to have a significant or measurable impact.

Second, it has been noted that CHMI trials are unlikely to fully replicate field conditions where prior exposure to malaria and other infections or immunizations may impact on vaccine efficacy.[80,81] While it has been reported in malaria endemic regions that an individual may be subjected to 35–90 mosquito bites a night (where 10% of mosquitos may be infected with *P. falciparum)*, the administration of bites from five heavily infected mosquitos almost instantaneously in the CHMI model is considered unnatural and is likely to represent a stringent test of vaccine-induced immune responses.[80]

However, five mosquito bites continue to be used as a standard infectious dose across centers in order to ensure infection of all volunteers, and this factor likely accounts for the large variability in the infectious dose mosquitoes administer.[68,80] The stringency of this human challenge model is arguably raising the bar for pre-erythrocytic and BSV candidates alike, and does not favor easy demonstration of partial vaccine efficacy, although as noted, the RTS,S vaccine candidate has succeeded in demonstrating efficacy both in CHMI studies as well as in field trials. Given that partial blood-stage immunity may need longer to exert an effect,[18] it has been argued that data from mosquito bite CHMI studies may underestimate or fail to predict field efficacy.[38,39]

A further benefit of the sporozoite CHMI model is the ability to assess a vaccine's efficacy against the pre-erythrocytic- and blood-stages of the *P. falciparum* life-cycle. This can be especially useful given that the traditional 'blood-stage' antigens such as AMA1 and MSP1 are essentially multi-stage antigens, expressed in the sporozoite[82] and late liver-stages[33] as well as on the invasive blood-stage merozoite. For example, in a recent mosquito bite sporozoite CHMI study assessing the efficacy of viral vectored vaccines encoding AMA1 and MSP1, modeling of parasite dynamics post challenge was able to demonstrate that delay in time to diagnosis seen in some vaccinees was likely attributable to a vaccine effect at the pre-erythrocytic rather than blood-stage of the parasite life-cycle.[21]

To date, the need for an insectary and skilled entomology staff have precluded the conduct of mosquito bite CHMI trials outside Europe and the USA.[68] However, the recent production of aseptic cryopreserved *P. falciparum* (PfSPZ Challenge) to Good Manufacturing Practice (GMP) by the biotechnology company Sanaria Inc. offers a practical alternative for the conduct of sporozoite CHMI trials, particularly in malaria endemic countries.[83] PfSPZ Challenge, which is stored and transferred in vapor phase liquid nitrogen, allows the administration of a known concentration of sporozoites simply by needle and syringe. Multiple trials are currently underway at numerous sites internationally to identify the optimal route and dose of administration of PfSPZ Challenge that reliably infects 100% of volunteers, however initial data have demonstrated remarkably reproducible results, even when using different batches of sporozoites.[84,85]

As well as reducing variability in infectious dose between trials, PfSPZ Challenge could be a key product for assessing the in vivo efficacy of BSVs in particular. If PfSPZ Challenge[84] can be administered in a regimen that leads to a lower burden of liver infection than that seen in mosquito bite CHMI studies, the resulting longer interval between challenge and microscope-patent parasitemia could allow for more subtle signals of partial vaccine-induced blood-stage immunity to be detected.[18] Indeed in a recent trial in Oxford, all volunteers were infected and the median time to microscopic patency was two days longer than that observed with mosquito-bite challenge.[85]

Blood-stage CHMI trials. An alternative method of performing CHMI trials is to intravenously administer infected erythrocytes from a cryopreserved inoculum originally obtained from an infected parasitemic donor.[23,70,71,86] Since the liver-stage of infection is circumvented, this method can only be used to assess vaccines targeting the blood-stage of the lifecycle and is particularly suited to this application. The thawed inoculum is prepared and administered, and a subsequent limiting dilution parasite viability assay is performed to ascertain the infectious dose. Consequently, a very low dose of parasites (30–6000 parasites in studies to date)[70] can be uniformly administered to all volunteers, allowing more accurate modeling of PMRs and thus increased sensitivity to detect subtle BSV efficacy.[18,68,70,87] Importantly, the time-frame of blood-stage parasite exposure prior to thick film microscopy diagnosis is increased to 8–9 days (unlike 2–4 days following mosquito bite challenge), and infection is initiated by much lower parasite densities – providing much improved conditions to assess partial vaccine efficacy. For vaccines demonstrating efficacy in a CHMI trial initiated by sporozoites, the blood-stage CHMI model can also provide a useful method of confirming the life-cycle stage specificity of the protective response.[88] However, one potential confounder of the model is that there is no possibility of detecting a protective antibody response against the merosomes released from the liver,[18,89] although no data exist to argue whether such a factor would be an important contributor to anti-merozoite vaccine efficacy. To date only approximately 100 volunteers have been taken part in blood-stage CHMI trials[71] (unlike > 1300 for mosquito bite challenge),[68] and only two vaccine candidates have undergone efficacy testing in this manner.[57,79]

Several factors have limited the widespread use of blood-stage CHMI studies. First, the sole source of the inoculum comes from a study performed at the Queensland Institute for Medical Research (QIMR) in 1994.[23,86] Two malaria-naïve individuals were infected with *P. falciparum* (3D7 clone) by the bite of laboratory-reared *Anopheles stephensi* mosquitoes. Thirteen to fourteen days later, when pyrexial and blood-film positive, 500 mL of blood was collected from each volunteer and infected erythrocytes cryopreserved at known parasite concentrations. Both individuals were seropositive for Epstein-Barr virus (EBV) and cytomegalovirus (CMV).[86] As a result, participants in some blood-stage CHMI studies have been restricted to those that are seropositive for EBV and CMV, considerably limiting recruitment and

leading to small and sub-optimal group sizes.[57,87] This, in combination with volunteer and regulatory body concerns regarding the potential risks of administering a blood product to healthy volunteers has considerably restricted application of the model, meaning that blood-stage CHMI trials have not been performed in the United States.[70]

However, to date, two centers (QIMR and Radboud University Nijmegen Medical Centre) have performed blood-stage CHMI studies in healthy volunteers sero-negative for EBV and CMV, with no evidence of seroconversion post CHMI (personal communication R Sauerwein).[71,88] Of note, the risk of CMV and EBV infection among recipients of standard blood donation who are sero-negative for these viruses but receive sero-positive blood has been deemed to be minimal if the donor blood has been subject to leuko-depletion, as is the case for the inoculum.[71,90] If, as is hoped, other national regulatory bodies remove EBV/CMV seropositivity as eligibility criteria, the capacity to perform blood-stage CHMI trials would increase significantly.

The current inoculum used in blood-stage CHMI trials has the benefit of more than 15 years clinical follow-up of the donors, providing reassuring data on the safety of the inoculum.[71] Given that the inoculum is finite and access limited to a small number of research institutes internationally, the current supply is unlikely to be adequate for future international research needs, especially if blood-stage CHMI studies become more widely used to assess candidate BSV efficacy. It is therefore important the research community consider supporting the creation of a new infected erythrocyte inoculum or in vitro culture expansion of the existing inoculum, so that, if applicable, safety data may begin to be amassed and provision for the assessment of future BSV candidates by blood-stage CHMI trials be maintained.[71] A similar inoculum would be especially useful to allow clinical assessment of BSVs against *P. vivax*, helping to circumvent problems culturing *P. vivax* in vitro and to avoid the potential risk of relapse following reactivation of hypnozoites in healthy volunteers post mosquito bite CHMI.

Outcome Measures in CHMI Trials

Post CHMI, volunteers are diagnosed and treated when thick blood-film positive at most trial centers, meaning microscopy is a key outcome in Phase IIa trials. While most microscopists have considerable experience reading blood-films at the high parasite densities typically seen in symptomatic patients in the field, most lack experience reliably identifying the extremely low level parasitemias seen in CHMI studies. This is likely to contribute to the considerable variability in parasitemia at diagnosis (geometric mean 8000–16 000 p/mL),[80] and is an understandable consequence given the significant role of chance in finding parasites at such low parasitemias. Conversely, since a number of trial centers process qPCR samples retrospectively, a false positive blood-film can lead to the incorrect and early treatment of a volunteer. The "Consensus SOP for Malaria Microscopy in the Context of Clinical Challenge Trials"[91] has been helpful to aid standardization across trial centers, however the lack of a definition of an 'unambiguous' parasite still allows for subjective interpretation of blood-films and variability between microscopists. While the need for two parasites to be identified on a blood-film or 'real-time' contemporaneous processing of qPCR samples reduces the likelihood of false positive blood-film results,[91] standardized training specifically in the identification of low patent parasitemia would greatly assist the field.

Although work has begun to standardize the conduct of CHMI trials and microscopy,[91] there remains much variability between trial centers in blood filtering and qPCR methodologies, as well as preferred modeling methods[18] making direct comparison of results difficult. The assay currently used in Oxford involves filtration of fresh blood to remove leukocytes using a multi-well plate (Whatman VFE), followed by DNA extraction using a commercial kit (QiaAmp, Qiagen), and a Taqman-probe based qPCR reaction, and is designed to be performed contemporaneously during an ongoing trial.[92] The group at QIMR also perform qPCR in real-time during a trial, while Radboud University Nijmegen Medical Center perform phenol-chloroform DNA extraction from whole blood, followed by the same Taqman-based qPCR assay, but do not perform the assay contemporaneously.[93] More recently a RNA-based (quantitative reverse transcriptase PCR) assay has been developed which is fully automated, rigorously quality-controlled, and can be performed using 50 μL samples of frozen whole blood.[94] This assay appears to have a number of advantages over previous techniques and holds promise for inter-center PCR method standardization.

A recent study undertaken comparing simple linear,[73] sine-wave,[74] and normal-cumulative-density-function[75] models for analysis of a large historical data set of qPCR monitoring post CHMI from our center reassuringly found that PMRs estimated by these different models were closely correlated.[73] The same was the case in a recent vaccine efficacy study where the results of both linear and sine wave modeling were reported and gave comparable results.[21] If the outputs derived from the simple and transparent linear model (essentially, linear regression) were reported for all future studies, this would help improve comparability between the outcomes of CHMI trials. However, the persisting and considerable differences in qPCR methodologies between centers, in particular with regard to blood sample processing and design of the assay itself, mean that international consensus on the standardization of laboratory protocols remains a priority for the field,[18] especially since the quality of data fed into the modeling process is likely to be more important than the choice of analytical model.[73]

Determining Efficacy of a Blood-Stage Vaccine Using CHMI Models

An efficacious BSV is anticipated to lead to a reduction in PMRs post CHMI, resulting either in a delay in time-to-diagnosis post CHMI or failure of individuals to be diagnosed post CHMI.[18] It remains to be experimentally confirmed what degree of reduction in PMR would lead to a significant delay in time-to-diagnosis by thick-film microscopy, however it is has been predicted that a vaccine reducing parasite growth by only 30% would be expected to produce approximately a 3.0 day delay to patency under blood-stage challenge (vs. a 1.5-day delay under sporozoite challenge).[87]

Box 3. Research priorities

- Standardization of methodology for in vitro assays aside from GIA.
- Standardization of qPCR methodology and modeling of data across trial centers.
- Improved sensitivity of qPCR assays to < 20 p/mL for use in controlled human malarial infection (CHMI) studies.
- Establishment of alternative inocula for blood-stage CHMI-expansion of existing 3D7 stocks; alternative strains of *P. falciparum*, as well as *P. vivax*.
- Increasing capacity for CHMI studies in malaria endemic populations.
- Demonstration of proof-of-concept in a Phase IIa CHMI study that an effective BSV can significantly impact PMR in vaccinated volunteers.

Moreover, as discussed above, the quality and quantity of qPCR data input to the chosen model are also likely to be of critical importance in determining the accuracy of PMR estimation.

The degree of reduction in PMR following CHMI that will predict clinical efficacy in the field remains for now debated. Comparison of PMRs between malaria-naïve control volunteers in CHMI trials and semi-immune Gambian adults showed a 5.6-fold difference in PMRs, suggesting that naturally occurring blood-stage immunity impacts on PMRs and that a vaccine capable of emulating the disease-reducing effect of NAI could achieve a detectable effect in the CHMI pre-patent period.[95] However, since no individuals with clearly defined NAI have undergone CHMI to date, it remains impossible to definitively predict the effect of naturally occurring blood-stage immunity on PMR post CHMI or validate the model as a method of assessing blood-stage immunity. Nonetheless, any significant change in PMR between vaccinees and controls observed in a Phase IIa CHMI trial would provide evidence of an in vivo effect of the vaccine candidate upon parasitemia. This would be an unprecedented finding for the BSV field and would provide a compelling argument for continuation to Phase Ib/IIb field trials, especially in light of the fact that the ultimate outcome of post-patent infection in vaccinees would remain unknown and could not be explored in the context of a Phase IIa trial where volunteers are treated at first microscopic detection of parasites. Moreover, as discussed above, the power to detect such reductions in PMRs is maximized in the context of the blood-stage CHMI model, and thus this approach remains for now the most suited for testing the potential protective impact of BSV candidates.

CHMI Trials in Malaria Endemic Populations

To date, few CHMI trials have taken place in malaria endemic populations (Abdulla et al., manuscript in preparation).[18] The increased ability to perform CHMI studies in malaria endemic countries would expand international capacity for efficacy testing of vaccine candidates and allow earlier assessment of vaccine efficacy in target populations. It would also allow malaria endemic countries, which have the most to gain from malaria research, to influence the research agenda, operate independently and exercise ownership over key research pertinent to their health priorities. Performing a CHMI study in individuals with prior exposure to malaria where the exact burden and timing of infection is known will also provide a unique opportunity to gain an insight into the immunological responses associated with NAI. Documentation of the kinetics of control of blood-stage parasitemia following CHMI with varying degrees of clinical immunity could also provide key insight regarding the characteristics required of a future BSV.[18] Such a trial is planned to take place in Kenya in 2013 as part of a recently awarded European and Developing Countries Clinical Trials Partnership grant supporting the development of CHMI studies in Africa (**Box 3**).

Closing Remarks

There remain strong rationale to assess promising new BSV candidates, and it is thus essential that robust methods of detecting even modest efficacy of such vaccines in early-phase clinical trials be established. Most recently the first AMA1-based vaccine has been reported that demonstrated strain-specific efficacy in a Phase IIb field study.[39] Such a vaccine presents a potentially important opportunity to revisit and validate measures of efficacy. An informative step would be to assess the efficacy of FMP2.1/$AS02_A$ following homologous blood-stage CHMI and compare these results to efficacy data both from the field and sporozoite CHMI studies.[38,39] For new BSV candidates, two models appear potentially best suited to explore the impact of such vaccines on PMRs in Phase IIa CHMI studies—the blood-stage challenge model, as well as PfSPZ Challenge. The initiation of controlled low-level blood-stage inocula, with accurate PMR quantification by qPCR should allow for the greatest power to observe the potential impact of vaccine-induced responses. A further productive step will be to use the growing capacity to perform CHMI trials in malaria endemic populations as a possible model to better understand the mechanisms of NAI and to enable vaccine assessment in target populations. These factors in combination with a growing number of promising antigens still to be tested in clinical trials[58,96,97] support cautious optimism for the future development of a clinically effective BSV against *P. falciparum* malaria.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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