Vaccine 33 (2015) 7469–7475

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Whole organism blood stage vaccines against malaria☆

Danielle I. Stanisic*, Michael F. Good**

*Institute for Glycomics, Griffith University, Gold Coast Campus, Parklands Drive, Southport, Queensland 4222, Australia*

ARTICLE INFO

*Article history:*
Available online 1 October 2015

*Keywords:*
Plasmodium
Malaria
Vaccine
Whole parasite
Blood stage

ABSTRACT

Despite a century of research focused on the development and implementation of effective control strategies, infection with the malaria parasite continues to result in significant morbidity and mortality worldwide. An effective malaria vaccine is considered by many to be the definitive solution. Yet, after decades of research, we are still without a vaccine that is capable of inducing robust, long lasting protection in naturally exposed individuals. Extensive sub-unit vaccine development focused on the blood stage of the malaria parasite has thus far yielded disappointing results. There is now a renewed focus on whole parasite vaccine strategies, particularly as they may overcome some of the inherent weaknesses deemed to be associated with the sub-unit approach. This review discusses the whole parasite vaccine strategy focusing on the blood stage of the malaria parasite, with an emphasis on recent advances and challenges in the development of killed and live attenuated vaccines.



## 1. Introduction

Malaria infection is caused by parasites of the genus *Plasmodium*, with *Plasmodium falciparum* and *P. vivax* being the most prevalent of the 6 species that are able to infect humans. In 2012 there were over 3.4 billion people at risk of contracting malaria, over 200 million cases and 627,000 attributable deaths [1].

There is a long history of research into the development and implementation of different strategies which aim to both prevent and treat malaria infection. These include vector control (e.g. use of insecticide treated bed nets to reduce human-vector contact), chemoprevention (*e.g.* Intermittent Preventive Treatment (IPT) for vulnerable populations such as pregnant women and infants) and the prompt diagnosis and treatment of confirmed cases with appropriate anti-malaria drugs. While these have undoubtedly contributed to the progress in reducing case incidence and mortality rates [1], a vaccine is still seen as the definitive tool to prevent morbidity and mortality.

Despite decades of research, an effective malaria vaccine remains elusive. This is due to a number of factors including: the complex nature and life-cycle of the malaria parasite; its ability to rapidly evolve and evade the host immune system; and the lack of understanding of what precisely mediates immunity. Numerous sub-unit and whole parasite vaccine approaches targeting different parasite life-cycle stages are being examined in the pursuit of an effective vaccine. Disappointing results following the efficacy testing of sub-unit vaccines in clinical trials [2–4], including the most advanced vaccine candidate, RTS,S [5], have highlighted some of the intrinsic limitations of sub-unit vaccines that need to be addressed. Conversely, recent promising results from the clinical testing of radiation attenuated sporozoites [6] have re-ignited interest in the whole parasite approach. A radiation attenuated sporozoite vaccine must be 100% effective as even one mosquito-injected sporozoite developing through to blood stage could result in a fulminant blood stage infection. For a whole parasite blood stage vaccine, even a partially effective vaccine could result in lower parasitemias and absence of symptomatic disease in recipients. This review will focus on recent advances in strategies to develop a whole parasite blood stage vaccine. It will also discuss some of the major challenges.

## 2. Evidence for a whole parasite blood stage vaccine approach

Historically, vaccine production has involved the isolation, inactivation/attenuation and injection of the whole infectious agent. This was facilitated by the development of methodology to cultivate the organisms to enable large-scale production of the killed or live attenuated vaccines and has been successfully employed for a number of organisms such as those causing the following diseases: tuberculosis, polio, measles, mumps, rubella and varicella (reviewed in [7]).

☆ Open Access provided for this article by the PATH Malaria Vaccine Initiative and ExxonMobil Foundation.

* Corresponding author. Tel.: +61 7 555 29433.
** Corresponding author. Tel.: +61 7 555 29435.
*E-mail addresses:* d.stanisic@griffith.edu.au (D.I. Stanisic), Michael.Good@griffith.edu.au (M.F. Good).

http://dx.doi.org/10.1016/j.vaccine.2015.09.057

Whole organism blood stage malaria vaccine approaches were first investigated using animal models in the 1940s. Both ducks and monkeys were protected following immunisation with killed and adjuvanted *Plasmodium lophurae* and *Plasmodium knowlesi* infected red blood cells respectively [8,9]. Protection was also observed using the human malaria parasite, *P. falciparum*, in *Aotus* monkeys [10]. These and other non-human primate studies (reviewed in [11]) involved the use of Complete Freund's Adjuvant to enhance the immunogenicity of the parasite. Although this adjuvant is incompatible with human use, killed parasites in combination with alternative adjuvants were unable to successfully immunise monkeys [9]. The requirement for a human-compatible adjuvant to enhance the immunogenicity of the parasite and the inability at that time to generate sufficient quantities of human malaria parasites to enable clinical efficacy studies, were seen as substantial obstacles to furthering this vaccine approach. Although the discovery of how to culture *P. falciparum* [12] gave some impetus to the field, the perceived difficulties of this approach together with the ability to clone and express *P. falciparum* recombinant proteins [13,14], resulted in the sub-unit vaccine approach becoming the focus of malaria vaccine development. Recently, however, the challenges and disappointments of sub-unit vaccines has led to a renewed interest in the whole parasite approach.

**Table 1**
Characteristics of whole organism vaccine strategies.

| Type of vaccine | Characteristics |
|---|---|
| Killed vaccines | Advantages:<br>• Unable to replicate in the host.<br>• Cannot revert to virulence and result in infection<br>• Cannot be transmitted to susceptible individuals<br>Disadvantages:<br>• Most require the use of adjuvants to enhance immunogenicity<br>• May require multiple doses to induce protective immunity |
| Live attenuated vaccines | Advantages:<br>• Able to replicate in recipient so a lower number of parasites/fewer doses may be required.<br>• Generally associated with longer lasting protection.<br>Disadvantage:<br>• Failure/instability of attenuation and reversion to virulence |

## 3. Advances in whole parasite blood stage vaccine strategies

A major advantage of the whole parasite vaccine approach is the broad array of antigens that such a vaccine presents to the immune system. Many are highly conserved between different parasite strains. This helps overcome two of the issues which have impacted on the efficacy of sub-unit vaccine candidates – immunological non-responsiveness and antigenic polymorphism. However, one of the key questions for the development of a malaria vaccine, is what type of immune response do we need to induce to result in robust protection. Immunity induced by natural exposure to the whole parasite during malaria infection is sub-optimal and takes several years to develop; therefore a whole parasite vaccine must improve on naturally acquired immunity by inducing either a different type or magnitude of immune response.

In the early 1900s, malariatherapy, which involved a protracted blood stage malaria infection, was a recognised treatment for neurosyphilis. A retrospective examination of these records suggests the induction of clinical and anti-parasite immunity during a second blood stage infection, manifesting as a decrease in parasitemia and febrile episodes [15]. More recently, multiple low doses of *P. falciparum* parasitised red blood cells (pRBC) were administered to malaria naïve individuals, with each infection terminated prior to patency with anti-malaria treatment [16]. Protection in 3 out of 4 volunteers was evident and was associated with the induction of vigorous parasite-specific cellular responses in the absence of detectable parasite-specific antibody, although the possibility that residual drug contributed to this protection could not be ruled out [17]. These immune responses differed from those that follow a natural (patent) infection where antibody responses are evident. Subsequent rodent studies also showed that multiple sub-patent malaria infections terminated by anti-malaria treatment could induce robust homologous and heterologous infection with induction of a similar antibody-independent protective immune response [18]. The results from a recent clinical study suggest that this protective immunity may be reliant on prolonged exposure to the malaria parasite during multiple asexual cycles. Multiple immunisations of sporozoites via infected mosquito bite while taking chloroquine prophylaxis induced immune responses to the *P. falciparum* blood stage, but these responses were not protective [19]. It was thought that the prophylactic levels of chloroquine present in the volunteers' blood resulted in a short-lived parasitemia, and this exposure to the blood stage was not sufficient to generate protective immune responses [19]. These results collectively suggest that persistence of the blood stage parasite at low levels is associated with induction of protective immunity. Although the infection/drug treatment model is not a feasible malaria vaccine approach, it does inform vaccine development.

A number of approaches are currently being pursued to further this strategy and develop either a killed or live, attenuated blood stage malaria vaccine (Table 1). Each approach has its own advantages and disadvantages, which have implications for vaccine design.

## 4. Immunisation with killed blood stage parasites

As mentioned above, early work with rodents and monkeys demonstrated that vaccination with killed parasites/parasite lysate (including merozoites) can induce protective immunity (reviewed in [11]), when co-administered with Complete Freund's Adjuvant. A more recent study investigated the protective efficacy of low doses (100 and 1000) of frozen/thawed pRBC in the adjuvant CpG-ODN [20]. Homologous and heterologous protection was observed following immunisation, although low grade parasitemia was observed in vaccinated mice. Protection was dependent on $CD4^+$ T cells, IFN-γ and nitric oxide. This low dose parasite approach was chosen based on immunological considerations with low doses of parasites shown to induce robust T cell responses [18] without the deletion of effector cells that has been observed with higher parasite doses [21]. The induction of robust, protective T cell responses using a low dose of killed, adjuvanted parasites is very encouraging as the feasibility of producing large numbers of parasites during scale-up for human vaccine production is considered a limitation of the whole parasite approach.

Further development of this strategy requires the identification and testing of adjuvants appropriate for use in humans (reviewed in [22]). Issues encountered with the selection and utilisation of these compounds includes: limited access to novel or existing proprietary adjuvants; unacceptable safety profile of the final formulation; and a lack of understanding of the type of immune response that is required.

## 5. Immunisation with radiation-attenuated parasites

Early studies involving vaccination of rodents with radiation-attenuated parasites demonstrated that protection from lethal infection could be induced (reviewed in [11]). More recently, the effectiveness of radiation-attenuated *P. berghei* in inducing protection against both parasitemia and experimental cerebral malaria (ECM) in susceptible mice was investigated [23]. A radiation dose of 100 kilorad was used to generate a non-replicating, avirulent form of the parasite. These irradiated parasites were able to induce protection against high level parasitemia, severe disease and ECM in different susceptible rodent strains following administration of a single non-adjuvanted high parasite dose ($10^7$). Protection was not observed following administration of multiple low parasite doses ($10^3$). The influence of parasite dose on degree of protection induced by irradiated parasites is in agreement with earlier studies (reviewed in [11]). While the degree to which irradiated parasites are able to persist to stimulate the immune system without causing severe disease may influence their efficacy as a vaccine, using sub-optimal doses of irradiation in this study resulted in *P. berghei* parasites escaping attenuation and initiating fulminant infections and severe disease [23]. Enhancement of protective immunity may be facilitated by the use of an avirulent parasite, such as the *P. berghei* XAT parasite (a radiation-attenuated derivative of the P. berghei NK65 strain) which is capable of initiating a low level, self-resolving infection in immune competent mice [24]. It induces robust long-lived immunity, with recipient mice developing a very low self-limiting parasitemia following lethal *P. berghei* challenge. The use of an avirulent parasite generated by irradiation in humans would however present a number of obvious challenges with concerns of possible reversion to a virulent phenotype and its use in immunocompromised/immunodeficient individuals.

A study in the 1980s examined radiation attenuation of *P. falciparum*, with surviving parasites demonstrating an attenuated growth rate, although reversion was observed following sub-inoculation into culture [25]. Although a number of studies have examined radiation attenuation of parasites, only recently has an attempt been made to characterise the cellular and molecular basis of this attenuation method [26]. Interestingly, parasites that survived attenuation in this study had a normal growth profile, with the authors suggesting that they arose from a few parasites that were either not affected by irradiation or were able to self-repair. Given the concerns related to attenuation escapees and reversion of attenuated parasites to a virulent form, this observation emphasises the need for a deeper understanding of the mechanism of radiation attenuation to further this vaccine approach.

## 6. Immunisation with chemically attenuated parasites

A novel approach that has recently been applied to the development of a whole parasite vaccine is the use of chemicals to attenuate the parasite. *Seco*-cyclopropyl pyrrolo indole analogues were originally shown to have anti-malaria activity when directly administered to parasitemic mice [27]. Additionally, mice injected with treated sporozoites exhibited sterile immunity following challenge [28]. It is thought that these compounds affect parasite replication by irreversibly alkylating parasite DNA in polyA rich regions. These compounds are genotoxic, so their use in a vaccine is informed by the "EU Guidelines on the limits of Genotoxic Impurities" which is produced by the European Medicines Agency and has been adopted by the Australian Therapeutic Goods Administration. We have demonstrated that the residual amount of free Tafuramycin-A which would be present in a vaccine dose, is well below the limits described in these guidelines (DI Stanisic and MF Good, unpublished data). As the chemical binds to the parasite DNA in an irreversible manner, reversion to a virulent phenotype is unlikely.

We recently demonstrated that mice vaccinated with a single dose of $10^6$ *P. chabaudi* pRBC treated with 2 μM of Centanamycin or Tafuramycin-A were protected from homologous and heterologous challenge (Fig. 1) and this protection was long-lived, lasting up to 6 months [29]. Both parasite-specific $CD4^+$ and $CD8^+$ T cell responses were induced, with protection mediated by $CD4^+$ T cells. Parasite-specific antibodies were not detected. Many of the antigenic targets of T cells are intracellular, and are not under immune pressure so they may be highly conserved between different *Plasmodium* strains/species. The efficacy data demonstrated that this vaccine approach was able to overcome *Plasmodium* strain/species differences, possibly by inducing protective T cell responses that targeted conserved antigens. In these mice, protection was dependent on the red cell membrane remaining intact, suggesting that this is critical for the interaction between the attenuated parasites and cells of the immune system. While patent parasitemia did not develop in the vaccinated mice, parasite DNA was detected in the blood of vaccinated mice for over 100 days. Whether this reflects live parasite or just DNA from nonviable parasites remains to be determined, but this persistence of parasite material may be crucial for the induction and maintenance of robust protective immunity. When blood was transferred from these vaccinated mice to naïve BALB/c mice, the recipient mice did not develop parasitemia, suggesting that the parasites were not viable at the time of transfer or that viable parasites were resident in the tissues.

We were also able to attenuate *P. falciparum* with these compounds and *in vitro* experiments indicated that the parasites were not able to replicate *in vitro*. We have recently undertaken a pilot study in human volunteers to determine the correct dose of chemical to attenuate *P. falciparum* so that it does not result in a patent infection *in vivo* (DI Stanisic and MF Good, unpublished observations). Safety and immunogenicity of chemically attenuated parasites was also examined. Future work will focus on optimisation of parasite dose, characterising the immune response and evaluating the protective efficacy of these chemically attenuated *P. falciparum* pRBC in humans.

An alternate strategy could exploit the dependence of the malaria parasite on the apicoplast for intraerythrocytic development in the human host. Tetracyclines such as doxycycline exert their anti-malarial effects on *P. falciparum* by inhibiting production of proteins that are encoded by the apicoplast genome, thereby leading to loss of apicoplast function [30]. Interestingly, these drugs cause a delayed death phenotype in *P. falciparum*, i.e. the effects are evident only in the progeny of the drug-treated parasites, allowing for a single cycle of growth before the apicoplast fails to replicate, ultimately resulting in parasite death. It was recently published that it was possible to rescue these apicoplast deficient parasites by providing them with isopentenyl pyrophosphate (IPP), a product of isoprenoid precursor biosynthesis, a process that normally occurs in the apicoplast [31]. Despite the loss of the apicoplast, *P. falciparum* grew *in vitro* when they were provided with exogenous IPP. This could be exploited for vaccine production. Large volumes of apicoplast knockout parasites could be generated *in vitro* in the presence of IPP. Following injection into the bloodstream of study participants, in the absence of IPP, *in vitro* results suggest that the parasites would continue to develop at most for an additional cycle which would benefit the vaccine's immunogenicity. An obvious advantage of this approach is there is very little chance of reacquisition of the apicoplast and therefore reversion to the wild type phenotype.

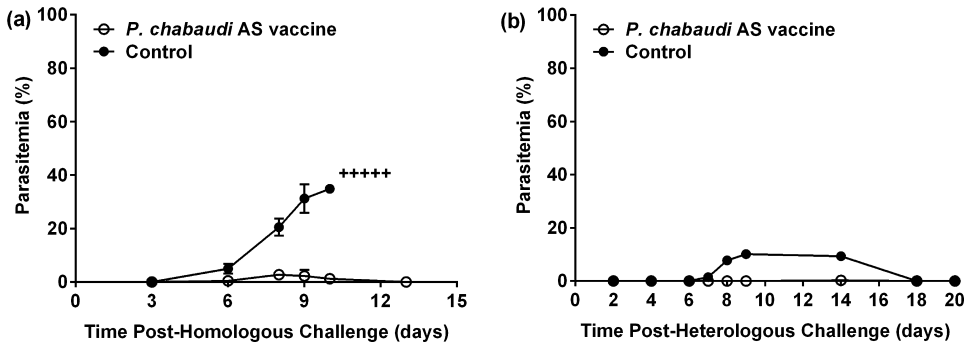


**Fig. 1.** Protection induced in mice by a single immunisation with $1 \times 10^6$ *P. chabaudi* AS parasitised red blood cells attenuated with Tafuramycin-A. Mice were challenged with (a) *P. chabaudi AS* or (b) *P. yoelii*. Crosses indicate when mice succumbed to infection.

## 7. Immunisation with genetically attenuated parasites

This strategy involves the identification and inactivation of critical parasite metabolic processes. Targeted gene knockouts are created followed by functional analyses to look for attenuated growth of the parasite. A recent study utilised a forward genetics approach examining the growth of *P. falciparum* insertion mutants [32]. By identifying growth attenuated parasites, this strategy may be a useful tool for identifying novel gene targets for a genetically attenuated vaccine.

The first study to demonstrate that genetically attenuated blood stage parasites could be used as a whole parasite vaccine approach, involved the generation of a *P. yoelii* parasite with the purine nucleoside phosphorylase (PNP) gene disrupted [33]. The enzyme product is critical for purine recycling and salvage. Injection of mice with a single dose of $2 \times 10^4$ or $2 \times 10^5$ PNP-deficient parasites demonstrated growth attenuation compared to controls; however, average parasitemias of up to 30% were observed in the recipient mice. Complete protection against blood stage challenge was observed in these mice. Although the parasitemias following receipt of the PNP deficient parasites were much higher in mice than would be allowed in human volunteers, this study provided proof-of-concept for this approach.

Targeted deletion of parasite nucleoside transporter 1 (NT1), a plasma membrane permease that mediates purine uptake, results in a parasite that can only grow when purines are supplied in supraphysiological concentrations. A *P. yoelii* XNL NT1 knockout (Pynt1$^-$) was generated, injected into mice and shown to be severely growth attenuated [34]. Although mice that received 5,000 Pynt1$^-$ pRBC developed a low-grade transient parasitemia, mice injected with 50 Pynt1$^-$ pRBC did not develop patent parasitemia following vaccination and exhibited sterile protection following lethal *P. yoelii* YM challenge. Partial protection was observed following *P. berghei* challenge. Interestingly, both αβ T cells and B cells were shown to be essential for protective immunity. In this study, parasitemia was measured by blood smear only. Given the robust protection that was observed in the recipient mice, it would be of interest to know if there was persistence of parasite material, as detected by PCR. Similar to the dependence of the apicoplast deficient parasites on IPP, NT1 deficient *P. falciparum* could be grown *in vitro* in the presence of purines, to generate large volumes of parasites for vaccine production. When injected into volunteers, the lack of sufficient purines would result in growth arrest.

Aspartic proteinases such as Plasmepsin-4 contribute to haemoglobin digestion in the parasite; thus it was predicted for *P. falciparum* that disruption of these genes would result in parasite death. Interestingly this was not observed, with disruption of multiple plasmepsin genes resulting only in a mild growth defect when compared to the wild type parasite [35]. In a recent study the gene for Plasmepsin-4 in *P. berghei* was disrupted and although it had only a mild effect on the development and growth rate of the parasite, there was a major effect on the virulence of these parasites, compared to the parental parasite both in terms of lethality and ability to induce experimental cerebral malaria (ECM) [36]. Protection was observed in mice subsequently challenged with homologous or heterologous parasites. Further work with virulence attenuated parasites involved the sequential disruption of both the Plasmepsin-4 and Merozoite Surface Protein-7 genes, resulting in a parasite whose virulence attenuation was greater than either of the individual knockout strains [37]. Inoculation with these double knockout parasites also resulted in a self-resolving infection (in some mice there was a reduction in peak parasitemia), failure to induce ECM in susceptible mice and induction of strong homologous and heterologous protection when recipient mice were challenged. While strong protection was induced against disease, it is likely that the administration of virulence attenuated parasites that resulted in patent parasitemias in humans would be associated with the development of symptoms/pathology. This data does however provide further support for the development of a genetically attenuated parasite vaccine.

An alternate novel strategy is to generate a virulence attenuated parasite by directly targeting the virulence factor. This approach is being used to generate a genetically attenuated *P. falciparum* parasite with the target being *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) (L. Schofield and J. McCarthy, pers. comm.). PfEMP1 is a parasite adhesion ligand that binds to host tissue receptors on vascular endothelium (reviewed in [38]). It facilitates sequestration of the pRBC in the microvasculature, which prevents clearance by the spleen and contributes to the pathology of disease syndromes such as cerebral and placental malaria. It has also been associated with immunomodulation [39,40]. This protein is however, a highly variable surface antigen encoded by around 60 *var*-genes per genome, thus PfEMP1 cannot be removed by targeted gene deletion. An alternative target is the Knob Associated Histidine Rich Protein (KAHRP), which is required for the formation of the knob structures at the erythrocyte surface, where PfEMP1 is localised [41]. Without KAHRP, the pRBC cannot correctly display PfEMP1 and this prevents the adhesion of pRBC to vascular endothelium and sequestration. As it is not possible to undertake preclinical assessment of this approach in rodent models, following regulatory approval these *P. falciparum*-KAHRP-KO parasites will be directly evaluated in a first-in-man dose ranging trial to establish their safety and infectivity. This will be followed by a study to evaluate the immunogenicity and protective efficacy of these genetically attenuated *P. falciparum* parasites.

One of the major concerns discussed in relation to the safety of genetically attenuated parasites for human administration is the possible reversion to the wild type genotype/phenotype as has been seen with genetically attenuated pre-erythrocytic whole parasite vaccines approaches. Even with the use of methodology to generate

parasites with stable genetic attenuation, reversion of phenotype can occur without genetic reversion to the wild type [42]. This is discussed in more detail below. A key difference between the genetically attenuated pre-erythrocytic and blood stage vaccine approaches, is that replication of the former is completely arrested in the liver whereas for the blood stage the genetically attenuated parasites should have the capacity to replicate in the blood, albeit in a regulated way. This is a risk associated with this type of vaccine approach and would need to be monitored closely and characterised in Phase I safety trials.

## 8. Challenges for the development and clinical evaluation of whole parasite blood stage malaria vaccines

Production of a whole parasite blood stage vaccine presents a number of challenges that must be considered in the development pathway (Table 2). Some are unique to the blood stage approach due to the use of human blood products in the manufacturing process and/or final vaccine formulation. Others are inherent to vaccine design/development and have precedent in the production of other whole organism vaccine approaches, *e.g.* the irradiated sporozoite vaccine. Below, we discuss some of the challenges and possible solutions.

## 9. Regulatory and safety considerations

The use of human erythrocytes to grow the malaria parasite during the manufacturing process and/or the final formulation raises concerns regarding the possible contamination of the vaccine with infectious adventitious agents. Improved methodologies for donor and blood screening in recent years have decreased the likelihood of this occurring. A reputable source of transfusion compatible human blood products is essential, ensuring that collection and screening of these products follows current regulatory guidelines. Related to this is the source of malaria parasite that is used to initially "seed" the vaccine cultures. We have addressed this by the use of a defined malaria parasite cell bank grown at Good Manufacturing Practices (GMP) standard that has undergone rigorous screening in accordance with regulatory guidelines [43].

During the production of genetically attenuated parasites, drug resistance markers are inserted into the genome to allow for selection of the transfected gene knock-out parasites. For progression into human clinical studies, these drug resistance genes markers must be removed to address regulatory concerns relating to the use of genetically attenuated organisms. Use of the Cre/Lox or FLP/FRT recombinase technologies allows the precise, repeated and sequential removal of all drug resistance genes from gene-targeted parasites [44,45] and this is being applied to the development of *Plasmodium falciparum* genetically attenuated blood-stage parasites (L. Schofield and J. McCarthy, pers. comm.).

Possible reversion and under-attenuation need to be considered when developing live, attenuated vaccines. For genetically attenuated parasites, reversion can be addressed by utilising methodology to disrupt candidate genes to ensure that mutants cannot revert to the wild type genotype. Single crossover recombination, which only disrupts the targeted gene, can lead to reversion via the spontaneous removal of the integrated plasmid. By targeting candidate genes using double crossover recombination, the gene of interest is replaced and this results in the loss of the gene sequence thereby preventing reversion. Despite this, reversion of parasite phenotype can occur without genetic reversion to wild type. Ideally, to ensure complete attenuation, it may be necessary to perform multiple gene deletions that regulate independent biological processes, as has been undertaken with pre-erythrocytic genetically attenuated vaccine approaches. A consideration for the clinical evaluation of these vaccines is the need for a genetic marker that can distinguish the vaccine parasite from any naturally acquired infection and a defined drug sensitivity profile of the vaccine parasites, in the event that a vaccine-derived infection requires drug treatment. For other types of attenuated vaccines, *e.g.* chemically or drug-attenuated parasites, while *in vitro* work can provide preliminary data on chemical/drug doses that will adequately attenuate the parasite, ultimately this must be examined and confirmed through rigorous testing in clinical pilot studies and Phase I safety trials. An additional consideration for the eventual deployment of live attenuated, vaccines is their use in immunocompromised/immunodeficient individuals. This will need to be addressed in different populations once vaccine efficacy has been established in healthy individuals.

The presence of human erythrocytes in the final vaccine product has raised concerns regarding the possible induction of antibodies against red blood cell surface antigens, *e.g.* those of the ABO and Rh blood group systems. Low doses of *P. falciparum* infected red blood cells ($\leq$30,000) have been administered to 225 participants in experimental malaria infections (DI Stanisic and MF Good, unpublished data; J. McCarthy, pers. comm.) and seroconversion to donor red blood cell antigens has not been observed. These inocula contain >95% uninfected red blood cells (uRBC) so the potential antigenicity of the red blood cells is a consideration. We and others have addressed this, in part, by the use of universal donor blood group O Rh negative red blood cells. Studies conducted to-date indicate that administration of a low dose of O Rh negative red blood cells is not associated with seroconversion to donor red cell antigens. If larger numbers of pRBC are required in a vaccine dose, enrichment of these away from the uRBC for the final vaccine formulation may be necessary.

## 10. Manufacturing, storage and delivery considerations

The requirement for large volumes of culture (and the associated human blood components) as a source of malaria parasites to manufacture a whole parasite vaccine at scale, is often seen as a major limitation associated with this approach. We and others have successfully cultured *P. falciparum* at GMP standard and administered it to volunteers and this should be feasible for a low dose vaccine [43]. This may be a limiting factor for vaccines that require a large number of parasites to induce protective immunity.

As mentioned earlier, while the killed vaccine approach was superior in its ability to induce protection with as few as 100 pRBC, this was dependent on the presence of a potent Th1 adjuvant. Generally, human vaccine development has been impacted by the lack of potent, accessible human compatible adjuvants. Further development of this approach will require the identification and testing of an appropriate Th1 adjuvant.

It is likely that most live, attenuated vaccines will need to be administered intravenously. This method of delivery is being used successfully for controlled human malaria infection studies to administer sporozoites [46]. Portable vein visualisation devices, *e.g.* the "Accuvein" can be used if veins are difficult to find.

Unique challenges are associated with the deployment of a live, attenuated vaccine that requires integrity of the erythrocyte for efficacy. Thus far, our experience with the production of a chemically attenuated blood stage vaccine in pilot studies has involved the culture, attenuation and direct administration of live, fresh malaria parasites. This production strategy is not feasible for vaccine deployment. We and others are working towards the development of alternate formulation/delivery strategies, *e.g.* a cryopreserved form of the vaccine that can be rapidly thawed on site and administered. The logistics and practical considerations for liquid nitrogen transport and storage have been extensively

**Table 2**
Key challenges for the production and clinical evaluation of a whole parasite blood stage malaria vaccine.

| Challenge | Problem | Action |
|---|---|---|
| The use of human blood products (*e.g.* red blood cells) in the manufacturing process/final vaccine formulation. | Possible contamination with infectious adventitious agents. | Screening of donors and blood products according to current region-specific regulatory guidelines.<br>Use of transfusion compatible human blood products collected and screened according to current region-specific regulatory guidelines.<br>Use of a defined malaria cell bank that has been grown at Good Manufacturing Practices Standard and has undergone rigorous screening by a reputable supplier according to current region-specific regulatory guidelines |
| | Alloimmunisation (induction of antibodies against red blood cell antigens). | Use of Blood Group O Rh negative red blood cells.<br>Limit the number of total red blood cells in a vaccine dose. This could be accomplished by enriching parasitised red blood cells away from uninfected red blood cells.<br>Use of a parasite stage, *e.g.* merozoites that can be separated away from the red blood cell membrane. Note: Preliminary data has demonstrated that merozoites require the addition of an adjuvant to induce strong protective immunity.c |
| The use of live attenuated parasites in the vaccine. | Possible underattenuation/reversion of attenuated parasites to wild type. | Development of robust attenuation methodology. Preclinical *in vitro* and *in vivo* assessment of attenuation (where possible).<br>Utilising appropriate methodology to replace rather than disrupt gene of interest.<br>Perform multiple deletions of genes that regulate independent biological processes.<br>Identification of a genetic marker to distinguish the vaccine parasite from a naturally acquired infection.<br>Defined drug sensitivity profile of the vaccine parasite in the event that a vaccine-derived infection requires drug treatment. |
| | The use of drug resistance markers to select for gene knock-out parasites in the production of genetically attenuated parasites. | Removal of drug resistance markers according to appropriate methodology. |
| The use of killed parasites in the vaccine. | The requirement for a potent human compatible Th1 adjuvant to induce robust immunity. | Identification and testing of novel human compatible adjuvants. |
| Manufacturing, storage and deployment of vaccine in malaria endemic areas. | Manufacture of parasites at scale may require large volumes of culture for the required number of parasites. | Perform parasite dose ranging studies to identify the lowest dose of parasite required for protective efficacy. |
| | The requirement for intact red blood cells in the vaccine and the lack of appropriate facilities in which to culture parasites for the vaccine on site in malaria endemic areas. | Development of a cryopreserved form of the vaccine that can be rapidly thawed and administered on site. |

analysed and developed for the distribution of the attenuated sporozoite vaccine [47].

## 11. Conclusion

Modern whole organism vaccine strategies provide hope that a highly effective malaria vaccine may yet be feasible. Thus far, sub-unit vaccine candidates have performed below expectations in malaria endemic areas, which may in part be due to variation in the antigens that are included in the vaccine compared with those present in circulating strains. One of the major advantages of the whole parasite vaccine approach is the broad array of antigens that are presented-many of which would be conserved between different parasite strains. Pre-clinical data provide proof-of-concept that robust immunity may be induced to both homologous and heterologous strains and species of *Plasmodium* using the whole parasite blood stage strategy. Whole parasite blood stage vaccine approaches can be broadly grouped into whole, killed and live, attenuated vaccines. Whole, killed vaccines require the identification and use of a suitable human-compatible adjuvant. Reversion to virulence and incomplete attenuation need to be carefully considered and addressed when developing live, attenuated vaccines. Despite these are technical and safety challenges, transition of these approaches into early phase clinical trials should occur quickly. With the ability to undertake challenge studies with defined strains using sporozoites or blood stage parasites, the efficacy of these vaccines will soon be known.

## Acknowledgements

We would like to thank Dr Louis Schofield (Walter and Eliza Hall Institute of Medical Research) and Dr James McCarthy (QIMR Berghofer Medical Research Institute) for providing information describing their vaccine approach. MFG is funded by an NHMRC Australia Fellowship and an NHMRC Program Grant.

*Authors' contributions*: All authors contributed to the writing of the manuscript. All authors edited and approved the final version of the manuscript.

*Conflict of interest*: MFG received grant support from Griffith University.

## References

[1] World Health Organisation. World Malaria Report 2013. World Health Organisation; 2013.
[2] Sagara I, Dicko A, Ellis RD, Fay MP, Diawara SI, Assadou MH, et al. A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali. Vaccine 2009;27:3090–8.
[3] Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, et al. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. PLoS ONE 2009;4:e4708.
[4] Sirima SB, Cousens S, Druilhe P. Protection against malaria by MSP3 candidate vaccine. N Engl J Med 2011;365:1062–4.
[5] Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, et al. Four-year efficacy of RTS S/AS01E and its interaction with malaria exposure. N Engl J Med 2013;368:1111–20.
[6] Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 2013;341:1359–65.

[7] Zepp F. Principles of vaccine design – lessons from nature. Vaccine 2010;28(Suppl. 3):C14–24.
[8] Thomson KJ, Freund J, Sommer HE, Walter AW. Immunization of ducks against malaria by means of killed parasites with or without adjuvants. Am J Trop Med Hyg 1947;27:79–105.
[9] Freund J, Thomson KJ, Sommer HE, Walter AW, Pisani TM. Immunization of monkeys against malaria by means of killed parasites with adjuvants. Am J Trop Med Hyg 1948;28:1–22.
[10] Siddiqui WA. An effective immunization of experimental monkeys against a human malaria parasite, *Plasmodium falciparum*. Science 1977;197:388–9.
[11] McCarthy JS, Good MF. Whole parasite blood stage malaria vaccines: a convergence of evidence. Hum Vaccin 2010;6:114–23.
[12] Trager W, Jensen J. Human malaria parasites in continuous culture. Science 1976:193.
[13] Kemp DJ, Coppel RL, Cowman AF, Saint RB, Brown GV, Anders RF. Expression of *Plasmodium falciparum* blood-stage antigens in Escherichia coli: detection with antibodies from immune humans. Proc Natl Acad Sci U S A 1983;80:3787–91.
[14] Ellis J, Ozaki LS, Gwadz RW, Cochrane AH, Nussenzweig V, Nussenzweig RS, et al. Cloning and expression in *E. coli* of the malarial sporozoite surface antigen gene from *Plasmodium knowlesi*. Nature 1983;302:536–8.
[15] Collins WE, Jeffery GM. A retrospective examination of secondary sporozoite- and trophozoite-induced infections with *Plasmodium falciparum*: development of parasitologic and clinical immunity following secondary infection. Am J Trop Med Hyg 1999;61:20–35.
[16] Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. Lancet 2002;360:610–7.
[17] Edstein MD, Kotecka BM, Anderson KL, Pombo DJ, Kyle DE, Rieckmann KH, et al. Lengthy antimalarial activity of atovaquone in human plasma following atovaquone-proguanil administration. Antimicrob Agents Chemother 2005;49:4421–2.
[18] Elliott SR, Kuns RD, Good MF. Heterologous immunity in the absence of variant-specific antibodies after exposure to subpatent infection with blood-stage malaria. Infect Immun 2005;73:2478–85.
[19] Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, et al. Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc Natl Acad Sci U S A 2013;110:7862–7.
[20] Pinzon-Charry A, McPhun V, Kienzle V, Hirunpetcharat C, Engwerda C, McCarthy J, et al. Low doses of killed parasite in CpG elicit vigorous CD4+ T cell responses against blood-stage malaria in mice. J Clin Invest 2010;120:2967–78.
[21] Xu H, Wipasa J, Yan H, Zeng M, Makobongo MO, Finkelman FD, et al. The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. J Exp Med 2002;195:881–92.
[22] Mata E, Salvador A, Igartua M, Hernandez RM, Pedraz JL. Malaria vaccine adjuvants: latest update and challenges in preclinical and clinical research. Biomed Res Int 2013;2013:282913.
[23] Gerald NJ, Majam V, Mahajan B, Kozakai Y, Kumar S. Protection from experimental cerebral malaria with a single dose of radiation-attenuated, blood-stage *Plasmodium berghei* parasites. PLoS ONE 2011;6:e24398.
[24] Miyagami T, Igarshi I, Suzuki M. *Plasmodium berghei*: long lasting immunity induced by a permanent attenuated mutant. Zentralbl Bakteriol Mikrobiol Hyg A 1987;264:502–12.
[25] Waki S, Yonome I, Suzuki M. *Plasmodium falciparum*: attenuation by irradiation. Exp Parasitol 1983;56:339–45.
[26] Oakley MS, Gerald N, Anantharaman V, Gao Y, Majam V, Mahajan B, et al. Radiation-induced cellular and molecular alterations in asexual intraerythrocytic *Plasmodium falciparum*. J Infect Dis 2013;207:164–74.
[27] Yanow SK, Purcell LA, Pradel G, Sato A, Rodriguez A, Lee M, et al. Potent antimalarial and transmission-blocking activities of centanamycin, a novel DNA-binding agent. J Infect Dis 2008;197:527–34.
[28] Purcell LA, Yanow SK, Lee M, Spithill TW, Rodriguez A. Chemical attenuation of *Plasmodium berghei* sporozoites induces sterile immunity in mice. Infect Immun 2008;76:1193–9.
[29] Good MF, Reiman JM, Rodriguez IB, Ito K, Yanow SK, El-Deeb IM, et al. Cross-species malaria immunity induced by chemically attenuated parasites. J Clin Invest 2013;123:3353–62.
[30] Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. Antimicrob Agents Chemother 2006;50:3124–30.
[31] Yeh E, DeRisi JL. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. PLoS Biol 2011;9:e1001138.
[32] Balu B, Singh N, Maher SP, Adams JH. A genetic screen for attenuated growth identifies genes crucial for intraerythrocytic development of *Plasmodium falciparum*. PLoS ONE 2010;5:e13282.
[33] Ting LM, Gissot M, Coppi A, Sinnis P, Kim K. Attenuated *Plasmodium yoelii* lacking purine nucleoside phosphorylase confer protective immunity. Nat Med 2008;14:954–8.
[34] Aly AS, Downie MJ, Mamoun CB, Kappe SH. Subpatent infection with nucleoside transporter 1-deficient Plasmodium blood stage parasites confers sterile protection against lethal malaria in mice. Cell Microbiol 2010;12:930–8.
[35] Bonilla JA, Moura PA, Bonilla TD, Yowell CA, Fidock DA, Dame JB. Effects on growth, hemoglobin metabolism and paralogous gene expression resulting from disruption of genes encoding the digestive vacuole plasmepsins of *Plasmodium falciparum*. Int J Parasitol 2007;37:317–27.
[36] Spaccapelo R, Janse CJ, Caterbi S, Franke-Fayard B, Bonilla JA, Syphard LM, et al. Plasmepsin 4-deficient *Plasmodium berghei* are virulence attenuated and induce protective immunity against experimental malaria. Am J Pathol 2010;176:205–17.
[37] Spaccapelo R, Aime E, Caterbi S, Arcidiacono P, Capuccini B, Di Cristina M, et al. Disruption of plasmepsin-4 and merozoites surface protein-7 genes in *Plasmodium berghei* induces combined virulence-attenuated phenotype. Sci Rep 2011;1:39.
[38] Chan JA, Fowkes FJ, Beeson JG. Surface antigens of *Plasmodium falciparum*-infected erythrocytes as immune targets and malaria vaccine candidates. Cell Mol Life Sci 2014;71:3633–57.
[39] D'Ombrain M, Voss T, Maier A, Hansen D, Cowman A, Schofield L. *Plasmodium falciparum* erythrocyte membrane protein-1 specifically suppresses early production of host interferon gamma. Cell Host Microbe 2007;2:130–8.
[40] Elliott SR, Spurck TP, Dodin JM, Maier AG, Voss TS, Yosaatmadja F, et al. Inhibition of dendritic cell maturation by malaria is dose dependent and does not require *Plasmodium falciparum* erythrocyte membrane protein 1. Infect Immun 2007;75:3621–32.
[41] Rug M, Prescott SW, Fernandez KM, Cooke BM, Cowman AF. The role of KAHRP domains in knob formation and cytoadherence of *P. falciparum*-infected human erythrocytes. Blood 2006;108:370–8.
[42] Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, et al. First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. Vaccine 2013;31:4975–83.
[43] Stanisic DI, Liu XQ, De SL, Batzloff MR, Forbes T, Davis CB, et al. Development of cultured *Plasmodium falciparum* blood-stage malaria cell banks for early phase in vivo clinical trial assessment of anti-malaria drugs and vaccines. Malar J 2015;14:143.
[44] O'Neill MT, Phuong T, Healer J, Richard D, Cowman AF. Gene deletion from *Plasmodium falciparum* using FLP and Cre recombinases: implications for applied site-specific recombination. Int J Parasitol 2011;41:117–23.
[45] van Schaijk BC, Vos MW, Janse CJ, Sauerwein RW, Khan SM. Removal of heterologous sequences from *Plasmodium falciparum* mutants using FLPe-recombinase. PLoS ONE 2010;5:e15121.
[46] Mordmuller B, Supan C, Sim KL, Gomez-Perez GP, Ospina Salazar CL, Held J, et al. Direct venous inoculation of *Plasmodium falciparum* sporozoites for controlled human malaria infection: a dose-finding trial in two centres. Malar J 2015;14:117.
[47] Garcia CR, Manzi F, Tediosi F, Hoffman SL, James ER. Comparative cost models of a liquid nitrogen vapor phase (LNVP) cold chain-distributed cryopreserved malaria vaccine vs. a conventional vaccine. Vaccine 2013;31:380–6.