Publisher: Taylor & Francis & Informa UK Limited, trading as Taylor & Francis Group

Journal: *Expert Review of Vaccines*

DOI: 10.1080/14760584.2021.1874923

***Plasmodium* infection and drug cure for malaria vaccine development**

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Funding: This manuscript has been funded by National Health and Medical Research Council (Australia) grants.

Declaration of interest: M F Good has received grants from the National Health and Medical Research Council (Australia). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer Disclosures: Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Author Contributions: All authors contributed to the writing and revision of this manuscript.

Abstract

Introduction: Despite decades of research into the development of a vaccine to combat the malaria parasite, a highly efficacious malaria vaccine is not yet available. Different whole parasite-based vaccine approaches, including deliberate *Plasmodium* infection and drug cure (IDC), have been evaluated in pre-clinical and early phase clinical trials. The advantage of whole parasite vaccines is that they induce immune responses against multiple parasite antigens, thus lowering the impact of antigenic diversity. Deliberate *Plasmodium* IDC, as a vaccine approach, involves administering infectious, live parasites in combination with an anti-malarial drug, which controls the infection and enables induction of protective immune responses.

Areas covered: We describe the evaluation of the *Plasmodium* IDC approach, using sporozoites or asexual blood-stage parasites in pre-clinical malaria models and in clinical studies in malaria-naïve individuals.

Expert Opinion: Studies in malaria-naïve volunteers have demonstrated that sporozoite IDC approaches can induce superior sterile homologous protective immunity; further optimization may augment the strain-transcending nature of this protective immune response to enable strong protection against heterologous strains. There has been less progress with asexual blood-stage IDC however, pre-clinical and clinical data demonstrate induction of protective

immunity. The IDC whole parasite approach is extremely promising for development of an effective malaria vaccine.

Keywords: Anti-malarial drugs, Controlled human infection, Immunity, Malaria, Malaria vaccine, Controlled Infection Immunization (CII), Chemoprophylaxis with sporozoites (CPS), Chemoprophylaxis vaccination (CVac).

Article Highlights

- Malaria is a major global public health threat, causing severe illness and death particularly in children <5yrs of age. A highly effective malaria vaccine is urgently needed.
- Deliberate *Plasmodium* infection and drug cure (IDC), as a malaria vaccine approach, involves administering infectious, live parasites in combination with an anti-malarial drug, which controls the infection and enables induction of protective immune responses.
- The *Plasmodium* IDC vaccine approach is being developed using sporozoites or asexual blood-stage parasites contained within red blood cells. They target the sporozoite/liver-stage and blood-stage parasites respectively.
- The sporozoite approaches (chemoprophylaxis with sporozoite immunization by mosquito bite (CPS), and chemoprophylaxis vaccination with purified sporozoites administered by injection (CVac)) induce strong sterile protection against challenge with homologous sporozoites both in rodent models of malaria and in malaria-naïve individuals. Further optimization may induce equivalent levels of protective immunity against heterologous parasites. Route of administration, sporozoite dose, choice and

timing of administration of anti-malarial drug are all critical variables for the induction of protective immunity.

- Progress with the asexual blood-stage parasite vaccine approach (Controlled infection immunization (CII)) is behind that of the sporozoite vaccine approaches, however the existing pre-clinical and clinical data demonstrate induction of strong homologous protective immunity. For blood-stage CII, parasite persistence following immunization is critical for the induction of protective immunity. Additional studies will be needed to evaluate the protective efficacy of the blood-stage CII vaccine approach against homologous and heterologous parasites in clinical studies.
- Further development and optimization of the *Plasmodium* IDC approaches are required for the development of a highly effective malaria vaccine; however, it remains a promising vaccine strategy for the induction of a broad protective immunity.

1.0 Introduction

Malaria is a major global public health threat, causing severe illness and death particularly in children <5yrs of age. In 2018, there were an estimated 228 million clinical cases and 405,000 deaths attributable to infection with the malaria parasite [1]. Nearly 85% of global malaria deaths in 2018 occurred in 19 countries of the African region and India [1].

1.1 Life-cycle

Malaria is transmitted to humans through the bite of a *Plasmodium* spp.-infected female *Anopheles* mosquito. During a bloodmeal, sporozoites in the salivary glands of the mosquito are transferred into the dermis of human skin (Figure 1) [2]. They then migrate to the liver via the bloodstream, infect hepatocytes and mature into hepatic schizonts. *P. vivax* and *P. ovale* have dormant liver-stage parasites (hypnozoites) that may reactivate, leading to relapses months or even years after the recovery of patients from the first episode of malaria. Upon rupture of an infected hepatic schizont, tens of thousands of merozoites are released into the bloodstream where they invade erythrocytes and continue to develop through the different erythrocytic stages (rings, trophozoites and schizonts), before the erythrocyte ruptures to release daughter merozoites. These merozoites also invade erythrocytes and the ongoing cycles of asexual replication enable parasite growth in the blood. During each replication

cycle, some of the parasites develop into sexual-stage gametocytes which are ingested by mosquitoes during a bloodmeal. In the mosquito, male and female gametocytes mate within the gut forming diploid zygotes, which then become ookinetes. The ookinetes migrate to the midgut of the mosquito, pass through the gut wall and form oocysts. Sporozoites are formed after meiotic division of the oocysts. Sporozoites migrate to the salivary glands of the female *Anopheles* mosquito, ready to transmit the infection to another human host and continue the cycle of transmission.

1.2 Current control methods for the malaria parasite

The current methods of malaria prevention and vector control, such as the use of insecticide-treated nets, indoor residual spraying and preventive therapies for pregnant women and infants, have been effective in significantly reducing the number of malaria cases over the last decade [1]. However, limitations such as the development of resistance against insecticides by mosquitoes [3], environmental changes and development of resistance against existing anti-malarial drugs by the parasite [4] dictate an urgent need to identify additional methods for the prevention and eradication of malaria.

An efficacious vaccine will be the most effective means of preventing infection with the malaria parasite. Two approaches currently being investigated for the development of a malaria vaccine are: sub-unit vaccines containing single or multiple recombinant malaria antigens and whole parasite vaccines containing killed or attenuated (e.g. chemical, irradiated or genetic) whole malaria parasites. Sub-unit vaccines, including the most advanced malaria vaccine MosquirixTM, demonstrated limited efficacy when evaluated in residents of malaria-endemic regions [5-9]. This is thought to be due in large part to heterogeneity in the vaccine antigens and inadequate antibody and memory responses. Whole parasite vaccines are a feasible alternative as this approach maximises the number of antigens included in the

vaccine, reducing the impact of antigenic variation and primarily inducing a cellular immune response, which targets both surface and intracellular antigens [10]. There are a number of whole parasite vaccine candidates currently in development, containing either whole sporozoites (to target the pre-erythrocytic stage) or parasitized red blood cells (pRBCs) to target the erythrocytic stage.

1.3 *Plasmodium* infection and drug cure as a vaccine approach

One approach for the development of a whole parasite vaccine involves inducing protective immunity by infecting individuals with viable sporozoites or asexual blood-stage parasites and curtailing the infection with drug treatment. For sporozoite vaccine approaches, the anti-malarial drug treatment targets liver-stage and/or blood-stage parasites, whereas for asexual blood-stage vaccines, the drug treatment targets the blood-stage parasites only. This exposes the recipients to very low-level blood-stage infections (reviewed in [11]). Multiple cycles of infection and drug treatment may be required to generate optimal protective immunity.

Historically, deliberate infection of humans with *Plasmodium* parasites has been employed for a number of reasons including as a treatment (malariotherapy) for neurosyphilis in the 1920-1960s [12,13] and to test novel anti-malarial drugs and vaccines (reviewed in [11]). Malariotherapy relied on the malaria parasites inducing fever which is thought to kill the *Treponema pallidum* organisms. Retrospective analyses of the malariotherapy studies suggest that the observed decrease in parasitemia and febrile episodes during second infections with the same or different strain/species of *Plasmodium* reflected the induction of partial clinical and/or anti-parasite immunity [14]. Together with observations regarding the development of clinical immunity following multiple infections in individuals living in malaria endemic areas (reviewed in [15]), these data support developing deliberate, controlled malaria infection as a vaccine approach.

This *Plasmodium* infection and drug cure (IDC) approach is based on the *in vivo* inactivation of live, whole *Plasmodium* spp. parasites via concurrent or subsequent administration of an anti-malarial drug, in a controlled manner over a stipulated time (reviewed in [11]). This review will discuss immunization with sporozoites and blood-stage *Plasmodium* spp. parasites, combined with the administration of anti-malarial drugs prophylactically or as a treatment, as a vaccination approach (Figure 2a) (Table 1).

2.0 *Plasmodium* infection and drug cure with sporozoites

In 1976, the protocol for *in vitro* culture of malaria parasites was published by Trager and Jensen [16]. This significantly advanced malaria research by providing an *in vitro* process for the cultivation of blood-stage parasites and for generating *Plasmodium*-infected mosquitoes using cultured gametocytes. The first controlled infection with *P. falciparum*, via the bites of sporozoite-infected mosquitoes fed on laboratory cultures, was trialled in 1986 in healthy human volunteers in the USA [17]. More recently, cryopreserved, vialled sporozoites (Sanaria® PfSPZ Challenge) have also become available, which allows infection with a precise number of sporozoites via parenteral injection [18].

Plasmodium IDC with sporozoites entails multiple immunizations by either the bites of laboratory reared *Plasmodium*-infected mosquitoes or direct injection of purified sporozoites via needle and syringe, in combination with an anti-malarial drug. Chloroquine (CQ) has been widely utilized with sporozoites as it allows complete intra-hepatic parasite development and stops the development of blood-stage parasites [19]. Other drugs, some of which act at different points in the *Plasmodium* life-cycle, such as mefloquine (MQ)[20], azithromycin (Az) [21], artesunate [22], isopentaquine [23], arteether [24], piperazine (PPQ)

[25], primaquine (PQ) [26] and pyrimethamine (PYR) [27] have also been investigated in the context of *Plasmodium* infection and drug cure with sporozoites (Figure 2b). The use of PQ in any clinical *Plasmodium* IDC regimen would entail additional safety considerations due to the risk of dose-dependent PQ-induced hemolysis in glucose-6-phosphate (G-6-PD) deficient individuals [28]. Vaccine efficacy is usually determined by whether patent blood-stage parasitemia develops following parasite challenge. Where blood-stage infection does develop, other endpoints such as reduction in liver parasite load can also be examined. In human studies, blood-stage parasitemia data generated by sensitive qPCR methods can be applied into mathematical models to estimate liver parasite burden [29,30].

2.1 Pre-clinical evaluation of *Plasmodium* infection and drug cure with sporozoites

2.1.1 *P. berghei* sporozoite infection and drug cure

2.1.1.1 Protection induced by *P. berghei* sporozoite infection and drug cure

Sporozoite infection under CQ cover was first studied with *P. berghei* and demonstrated the induction of stage-specific immunity against homologous sporozoite challenge [31]. Mice immunized with *P. berghei* sporozoites and CQ received an intravenous (i.v.) injection of 5×10^4 *P. berghei* sporozoites (five doses, two weeks apart) while receiving CQ during the entire immunization period. Low level blood-stage parasitemias were observed following each immunization. Following the last injection of sporozoites, a 10-day curative course of PQ was given concurrently with CQ to eradicate any remaining parasites from the liver prior to sporozoite challenge. All sporozoite + CQ immunized mice survived challenge, whereas all control mice succumbed to the infection [31]. To examine the stage-specificity of the immune response, immunized mice that survived the sporozoite challenge were re-challenged with pRBCs; however, they did not survive despite being exposed to low level blood-stage infection during the immunization period. This indicates that protection was primarily

mediated by pre-erythrocytic immunity. This was the first study to demonstrate that immunization with normal infective sporozoites in combination with suppressive doses of an anti-malarial drug (CQ) could provide protection against sporozoite challenge comparable to that observed with another leading pre-erythrocytic vaccine candidate, radiation attenuated sporozoites (RAS) [32,33]. A further comparative study established that one or two i.v. injections of 10^4 *P. berghei* sporozoites + CQ induced protection against homologous sporozoite challenge in 57% and >96% of immunized mice respectively, which was similar to what was observed in mice immunized with one or two i.v. doses of 10^4 RAS [34].

It has been suggested that the protective efficacy of the sporozoite infection + CQ regimen may not just reflect its ability to enable induction of protective immunity through exposure to a low level controlled malaria infection, it may also be due to the immunomodulatory effects of CQ [35]. *In vitro* cross-presentation of soluble viral antigens by DCs is enhanced in the presence of CQ and treatment of mice with CQ has been shown to improve CD8⁺ T-cell responses to soluble malaria parasite antigen immunization *in vivo* [36]. The immunomodulatory activity of CQ was therefore investigated in the context of the *P. berghei* sporozoite IDC regimen by directly comparing IDC with CQ or MQ (whose immunomodulatory effects do not include enhancing cross-presentation) [20]. Both drugs arrest early blood-stage parasites without an effect on pre-erythrocytic parasite stages. Mice received three i.v. doses of 2×10^4 sporozoites at weekly intervals with CQ or MQ. This study did not investigate a direct effect of CQ on cross-presentation *in vitro*; however, parasite-specific CD8⁺ T-cell responses and protection from sporozoite challenge were assessed. Replacement of CQ with MQ led to a significant reduction in the percentage of IFN- γ producing memory CD8⁺ T-cells in the liver, but similar protective efficacy against challenge was observed for both the CQ and MQ regimens. Thus, the study provided no

indication of a direct beneficial effect of CQ on the efficacy of the *P. berghei* sporozoite + CQ regimen.

Additional anti-malarial drugs have been examined in the context of the sporozoite IDC approach using the *P. berghei* model. Piperaquine is structurally related to CQ and has a prolonged elimination half-life; therefore, it was explored in the context of sporozoite IDC for single-dose chemoprophylaxis, where it is administered at the time of sporozoite injection [25]. Although a single i.v. immunization of C57BL/6 mice with 5×10^4 sporozoites and PPQ resulted in prolonged pre-patency compared to drug-only controls following challenge, all mice succumbed to blood-stage infection. In contrast, two i.v. immunizations with 10^4 *P. berghei* sporozoites and PPQ (4 weeks apart) induced complete sterile protection post-challenge, with an expansion of effector memory CD8⁺ T-cells in the liver and spleen. Induction of complete protective immunity, requiring only a single dose of anti-malarial drug at the time of sporozoite inoculation, instead of multiple doses throughout the immunization period, is a significant advance for a human sporozoite vaccine based on the IDC approach.

Sporozoite IDC with Az has also been examined. Az allows complete intrahepatic development of the parasite and through its delayed death mechanism-of-action and abrogation of apicoplast function, permits the emergence of non-infectious liver-stage merozoites [37]. Two immunizations of 10^4 *P. berghei* sporozoites + Az induced sterile protection against homologous sporozoite challenge in 80% of the mice, which was significantly higher than mice that received two immunizations with either sporozoites + CQ or RAS [21]. The time to development of parasitemia in mice immunized with sporozoites + Az and control mice following blood-stage challenge were identical, indicating that the protection induced by the sporozoite + Az regimen was attributable to pre-erythrocytic immunity.

An attenuated infection, enabled by the sporozoite IDC approach, was also examined in the context of modulating blood-stage malaria infection and severe malaria disease progression in a *P. berghei* model of experimental cerebral malaria (ECM). Sub-therapeutic doses of isopentaquine were administered prior to a single i.v. administration of 10^4 *P. berghei* sporozoites [23]. Isopentaquine has comparable liver-stage mode-of-action to PQ. Mice were monitored following this immunization; the immunized mice had a delayed patency in parasitemia of 3-4 days compared to sporozoite-only injected control mice and did not show any typical cerebral symptoms. Nevertheless, the immunized mice died 25-30 days post-infection due to hyperparasitemia-associated anaemia. Interestingly, the number of liver-stage parasites did not differ in protected animals, but the parasites were considerably less developed. The *P. berghei* sporozoite + isopentaquine regimen was associated with stronger proinflammatory responses at earlier time points compared with control mice who developed ECM. This included increased T-cell activation in the liver and spleen, increased numbers of effector T-cells, particularly $CD8^+$ T-effector cells, increased IFN- γ , TNF- α , and IL-2 production by $CD8^+$ T-cells. This was followed by subsequent downregulation of the Th1 response later in the infection, possibly mediated by IL-10. Adoptive transfer and cell depletion studies indicated that a sub-population of $CD8^+$ T-cells was responsible for protection observed in this model. This study suggested that attenuating the parasite infection at the liver-stage using drugs such as isopentaquine can modify immunopathogenesis and prevent development of cerebral malaria.

The potential immunomodulatory effect of a concurrent blood-stage infection on the induction of immunity induced by a pre-erythrocytic malaria vaccine is a consideration for eventual application in malaria-endemic regions. In a previous study using *P. yoelii*, a blood-stage infection was shown to suppress both existing $CD8^+$ T-cell responses against a liver stage-antigen and inhibit initiation of new responses [38]. This phenomenon was also

examined in the context of the *P. berghei* sporozoite + CQ regimen to determine the effect of a concurrent *Plasmodium* blood-stage infection on the induction of pre-erythrocytic immunity [39]. A modified *P. berghei* sporozoite + CQ immunization schedule was established with 10^6 pRBCs administered four days prior to each sporozoite immunization (10^4 sporozoites); the CQ administration regimen allowed 5 days of blood-stage parasite positivity following each immunization. The presence of blood-stage parasites did not negatively impact on protective immunity induced by a three dose *P. berghei* sporozoite + CQ immunization regimen, as immunized mice showed complete protection following sporozoite challenge. Although rodent studies do not necessarily predict what will occur in humans, these results are of particular interest in relation to the requirement for treatment of malaria infections in malaria endemic areas prior to administering a malaria vaccine. Drug treatment of existing blood-stage infections may be required for optimal induction of vaccine-induced responses. It would however, present additional complexities for vaccination in a malaria endemic area, both practically for the administration of anti-malarial treatment and for the subsequent timing of the vaccination due to the potential impact of the anti-malarial drugs on the live parasites administered in the vaccine.

2.1.1.2 Immune correlates of protection for *P. berghei* sporozoite infection and drug cure

Immune correlates of protection for the *P. berghei* sporozoite + CQ immunization regimen were examined in different studies [39,40]. Complete protection in *P. berghei* sporozoite + CQ immunized C57BL/6 mice was dependent on intrahepatic IFN- γ producing CD8⁺ memory T-cells [40]. Changes to memory CD4⁺ T-cells in immunized mice were minimal. These data suggest that the IFN- γ response may be useful as a predictor of the longevity of immunity. Importantly, the decreasing CD8⁺ T-cell response was boosted by re-exposure to wild-type sporozoites. Similarly, in another study, protection induced by *P. berghei* sporozoite + CQ immunization regimen was shown to be mediated by CD8⁺ T-cells, with

depletion of CD8⁺ T-cells immediately prior to sporozoite challenge resulting in all *P. berghei* sporozoite + CQ immunized C57BL/6 mice succumbing to challenge infection [39]. There was, however, a delay in the onset of patency in these CD8⁺ T-cell depleted mice compared to naïve controls, suggesting that there was residual protective immunity in the absence of CD8⁺ T-cells which may indicate a limited role for other components of the immune system. Adoptive transfer of splenocytes from *P. berghei* sporozoite + CQ immunized mice into naïve mice, also resulted in complete protection against sporozoite challenge.

It has been suggested that a combination of cellular immunity (CD8⁺ T-cells) and antibodies is responsible for protection induced by *P. berghei* sporozoite + CQ immunization [41]. In a passive transfer study, protection was observed in 90% of C57BL/6 mice transfused prior to challenge with a mixture of splenocytes and immune serum derived from *P. berghei* sporozoite + CQ immunized mice [41]. In contrast, protection against sporozoite challenge was observed in only 50% and 60% of C57BL/6 mice that received either splenocytes or immune serum respectively from *P. berghei* sporozoite + CQ immunized mice. This suggests that there is an additive protective effect of cellular and antibody-mediated immunity. To further investigate the role of cellular immunity, C57BL/6 MHC I KO (CD8⁺ T-cell deficient) or MHC II KO (CD4⁺ T-cell deficient) mice were immunized with the sporozoite + CQ regimen and compared with C57BL/6 wild-type mice. Following challenge, the immunodeficient mice were only partially protected (50-60% did not develop blood-stage parasitemia) whereas C57/BL6 wild-type mice were completely protected. These data suggest a role for both CD4⁺ and CD8⁺ T-cells. The presence of circulating CD8⁺, killer-cell lectin like receptor G1 (KLRG1)^{high}, CD27^{low}, CD44^{high}, CD62L^{low} cells was shown to be associated with protection following *P. berghei* sporozoite + CQ immunization, warranting further investigation as an immune correlate of protection [41]. KLRG1 is a co-inhibitory

receptor that is expressed on NK-cells and memory CD4⁺ and CD8⁺ T-cells and is frequently used as a marker of cellular differentiation and T-cell memory.

2.1.1.3 Factors affecting induction of protective immunity by *P. berghei* sporozoite infection and drug cure: route of administration

Previous studies using the RAS vaccine demonstrated that route of immunization can significantly affect immunogenicity and efficacy both in rodents [42] and humans [43]. Thus, examining this in the context of sporozoite IDC is important.

Mice immunized via an intradermal (i.d.) route with *P. berghei* sporozoites + CQ had reduced protection against sporozoite challenge [44]. This reduction was associated with a 30-fold lower parasite liver load in the mice who received their vaccination i.d. compared with the mice who were vaccinated via the i.v. route. Intradermal immunization with sporozoites, unlike i.v. immunization, failed to result in the expansion of effector CD8⁺ memory T-cells and led to decreased sporozoite-specific CD8⁺ IFN- γ responses in the liver and spleen. These data highlight the importance of optimization of the vaccination regimen to achieve maximal protection. Increasing the number of sporozoites administered i.d. may significantly improve protective efficacy, as was observed with the *P. yoelii* RAS vaccination model.

2.1.2 *P. yoelii* sporozoite infection and drug cure

2.1.2.1 Protection induced by *P. yoelii* sporozoite infection and drug cure

P. yoelii sporozoite IDC was examined with different anti-malarial drugs; initially this was undertaken with CQ. Two i.v. doses of 2×10^4 *P. yoelii* sporozoites + CQ induced sterile protection against homologous sporozoite challenge [45]. When a single dose of PQ was administered on the day of immunization, protection was not observed, indicating that the presence of liver-stage parasites is essential for inducing protective immunity. PQ treatment

eliminated >90% of liver-stage parasites. In this model, both CD4⁺ and CD8⁺ T-cells contributed to the protection through induction of IFN- γ and nitric oxide [45]. Protective immunity was directed at liver-stage parasites with limited protection against blood-stage parasite challenge [45].

The *P. yoelii* sporozoite IDC approach was also investigated with PQ alone [26]. BALB/c mice were injected with three doses of *P. yoelii* sporozoites. One dose of PQ was administered at the time of each sporozoite injection or at various time points up to 36 hours post-injection, and a second PQ dose was administered 48 hours after each sporozoite injection. After PQ treatment, 90% of the parasites were eliminated within 42 hours of infection. In contrast to the *P. yoelii* sporozoite + CQ immunization study, where PQ administration on the day of immunization prevented successful vaccination [45], PQ in this instance did not preclude induction of immunity as immunized mice were protected against homologous sporozoite challenge [26]; protection was maintained for at least 3 months. Immunity was stage-specific, with all immunized mice succumbing to a blood-stage challenge.

The potential direct contribution of CQ to the efficacy of the sporozoite + CQ regimen was examined for *P. yoelii* [46]. To investigate the possibility that CQ may be prolonging liver-stage development which could result in enhancement of protective immune responses, liver parasite load was measured in CQ-treated and untreated mice infected with luciferase-expressing *P. yoelii* sporozoites. This did not significantly differ between the two groups at any of the time points from 42-64 hours post immunization. Furthermore, parasites were detected in the blood of mice from both groups at the same time. While this study showed that CQ does not eliminate or delay the development of liver-stage parasites, it does not exclude CQ impacting on other aspects of liver-stage biology e.g. the repertoire of expressed antigens [46].

Sporozoite IDC with MQ was also evaluated with *P. yoelii* [47]. Two i.v. doses of 10^4 *P. yoelii* sporozoites (14 days apart) with concurrent MQ treatment (5-day regimen) induced antibodies against homologous sporozoites and CD8⁺ T-cell-mediated sterile immunity following homologous sporozoite challenge. Following heterologous challenge with *P. vinckei* sporozoites, partial protection was observed with a 2 day delay in patency and a 92% reduction in peak parasitemia [47]. A significant reduction in liver parasite burden following both homologous and heterologous challenge was observed, although to a lesser degree with *P. vinckei*.

To investigate *P. yoelii* sporozoite IDC with other anti-malarial drugs, 10^4 *P. yoelii* sporozoites were administered to Swiss mice under curative doses of arteether (a semi-synthetic derivative of artemisinin), MQ, Az or PQ [24]. Arteether and MQ target the parasite's blood-stage, Az targets both the liver-stage and blood-stage and PQ targets the liver-stage. Protection was not observed in mice even after five immunizations with *P. yoelii* sporozoites + PQ. Under arteether and MQ cover, delayed patency in blood-stage parasitemia following 10^5 homologous sporozoite challenge was observed after the 4th immunization and sterile protection against sporozoite challenge was achieved in mice that received five immunizations. Sterile protection against 10^5 sporozoite challenge in mice immunized under Az cover was observed after four IDC. The protection induced in mice immunized with CPS under arteether, MQ or Az was stage-specific as the immunized mice developed blood-stage infection following homologous challenge with 10^6 pRBCs.

The potential modulation of vaccine-induced pre-erythrocytic immunity by a subsequent blood-stage infection was also examined for *P. yoelii* sporozoite + MQ immunization [47]. To evaluate the effect of blood-stage malaria infection on pre-erythrocytic immunity, two weeks after the final immunization, mice were infected with homologous or heterologous (*P. vinckei*) blood-stage parasites. This subsequent parasite exposure to a homologous or

heterologous blood-stage malaria infection did not abrogate protection to subsequent homologous sporozoite challenge in this rodent model.

2.1.2.2 Immune correlates of protection and cross-stage immunity induced by *P. yoelii* sporozoite infection and drug cure

Using antibody-mediated cell depletion, immunity induced by two i.v. doses of *P. yoelii* sporozoites + CQ in BALB/c mice was shown to be dependent on CD4⁺ and CD8⁺ T-cells; IFN- γ and NO were also critical effectors [45]. In a two dose *P. yoelii* sporozoite + MQ regimen, CD8⁺ T-cells were also associated with protective immunity in BALB/c mice. In a further study, a single immunization with *P. yoelii* sporozoites + CQ (10 days of CQ) did not require CD8⁺ T-cells for protection against homologous sporozoite challenge [48]. Following immunization, very low levels of blood-stage parasites persisted in the blood and this transient parasitemia after CQ treatment was associated with protection for this one-dose regimen; increasing CQ dosing to 25 days reduced the efficacy of the vaccine. Persistence of parasites also enabled the generation of antibody responses against blood-stage parasites. While antibody-mediated depletion of CD4⁺ T-cells commencing at the time of immunization resulted in a fulminant blood-stage infection, depleting CD4⁺ or CD8⁺ T-cells in immunized mice prior to challenge with pRBC did not impact protective cross-stage immunity. Thus, in this one immunization dose regimen, protection appeared to be independent of T-cells and relied instead on antibodies against the blood-stage parasite.

The induction of cross-stage immunity is an ideal goal [49]. Antigenic targets that are shared between liver- and blood-stage parasites are central to a malaria vaccine that provides cross-stage protection. It is also possible that due to the nature of the IDC approach, exposure to low numbers of blood-stage parasites may induce cross-stage immunity. Although not

observed in the *P. berghei* studies (Section 2.1.1.1), it was examined in the context of *P. yoelii* sporozoite IDC.

BALB/c mice were immunized with three i.v. doses of 10^4 *P. yoelii* sporozoites and drug cure with either CQ or artesunate [22]. One month after vaccination, mice were challenged with homologous sporozoites or pRBCs. As expected, *P. yoelii* sporozoite + CQ immunization induced sterile protection against sporozoites. Following blood-stage parasite challenge, 62% of mice were sterilely protected whereas the remainder had a 1-4 day delay in the onset of parasitemia. *P. yoelii* sporozoite infection with artesunate induced total sterile protection against sporozoite challenge and delayed the onset of blood-stage infection in 73% of mice. Both groups had pre-erythrocytic stage-specific CD8⁺ effector memory T-cell responses and hepatic CD8⁺ T-cells that demonstrated cytotoxicity against liver stage parasites *in vitro*. Both groups also had antibodies that recognized pre-erythrocytic antigens, but only *P. yoelii* sporozoite + CQ immunization induced high levels of antibodies that recognized merozoite surface protein 1 (MSP1) and schizont lysate. The greater degree of cross-stage protection observed with the *P. yoelii* sporozoite + CQ immunization regimen may be explained by the differential effects of the anti-malarial drugs impacting on parasite persistence. Artesunate is a fast-acting drug and is active against all asexual blood-stages of the parasite, including early ring stages, whereas CQ acts mainly on trophozoite-stage parasites which could prolong exposure to low levels of blood-stage parasites. This is supported by the differential recognition of blood-stage antigens with the two sporozoite IDC regimens. The contribution of immune responses against shared antigens to this protection is unknown. Cross-stage immunity induced by the *P. yoelii* sporozoite + MQ regimen has also been observed, with significant protection observed against homologous and to a lesser degree, heterologous (*P. vinckei*) blood-stage challenge [47].

2.1.2.3 Factors affecting induction of protective immunity: route of administration

Unlike the *P. berghei* sporozoite IDC regimen (section 2.1.1.3), in the *P. yoelii* model, vaccine efficacy against liver-stage parasites was not dependent on the route of administration [50]. The liver parasite burdens following i.d. or i.v. challenge in mice immunized with two i.d. doses of 2×10^3 *P. yoelii* sporozoites + MQ were not significantly different from mice immunized with two i.v. immunizations of *P. yoelii* sporozoites + MQ with the same regimen.

2.1.3 *P. chabaudi* sporozoite infection and drug cure

The sporozoite IDC approach has been investigated with the *P. chabaudi* rodent model using MQ and CQ. *P. chabaudi* is an excellent model to study strain-specificity of vaccine-induced immune responses, using the different clones of *P.c. chabaudi*. Two i.v. doses of *P.c. chabaudi* CB sporozoites + MQ were shown to suppress blood-stage parasite growth in immunized mice following sporozoite or blood-stage challenge with homologous *P.c. chabaudi* CB or heterologous *P.c. chabaudi* AJ five weeks later [51]. The protective immunity was less effective in controlling heterologous parasite growth, particularly following blood-stage challenge. Conversely, *P. c. chabaudi* AJ immunization only induced partial homologous protection following sporozoite and blood-stage challenge. Understanding why some parasite strains (e.g. *P. c. chabaudi* CB) may induce a broader immunity, and the mechanisms responsible for this, are critical for the rational development of whole parasite vaccines.

Cross-stage and strain-specificity of protection were examined in a further study. Mice were immunized three times with the bites of *P.c. chabaudi* AS infected mosquitoes under CQ cover followed by a challenge 100 days after the final immunization [52]. In this model, immunization by mosquito bites did not generate pre-erythrocytic immunity. Induction of pre-erythrocytic immunity required immunization with a large number of sporozoites

(10,000), presumably to enable the development of a sufficient number of liver-stage parasites which may enhance the immune repertoire with sufficient exposure to a broad range of immunodominant and sub-dominant antigens [52]. *P. chabaudi* sporozoites administered by mosquito bites + CQ did however induce protection against homologous and heterologous (*P. chabaudi* CB) blood-stage challenge, manifesting as reduced peak parasitemia and enhanced parasite clearance rates. Homologous immunity was again more effective than heterologous immunity. This study highlights a number of important variables that can impact on the specificity of the immune response and the efficacy of the vaccine including route of infection and antigen dose.

2.1.4 *P. knowlesi* sporozoite infection and drug cure

P. knowlesi is a zoonotic malaria parasite [53]. Investigating vaccine-induced immunity to a human malaria parasite in a non-human primate model enables interrogation of the immune response beyond the peripheral blood. *Rhesus* monkeys received three *P. knowlesi* sporozoite immunizations via mosquito bites + CQ. Half of the immunized group (2/4) were completely protected against sporozoite challenge, whereas a delayed onset of parasitemia was observed in the remaining animals [54]. IgG specific for *P. knowlesi* whole sporozoites, circumsporozoite protein (CSP) and apical membrane antigen (AMA)-1 were detected in immunized monkeys, with higher sporozoite and CSP-specific antibodies observed in completely protected animals. Completely protected animals also had high frequencies of sporozoite-specific memory T-cells in blood, liver, spleen and bone marrow. Sporozoite-specific liver memory T-cells expressed the chemokine receptors CCR5 and CXCR6, whose ligands are involved in tissue homing, as well as the phenotypic marker of tissue-resident memory cells, CD69. The study design could not exclude that blood-stage immunity may have also contributed to the observed protection as the animals were exposed to transient blood-stage parasites during the immunization.

2.2 Clinical evaluation of *P. falciparum* sporozoite infection and drug cure

The nomenclature associated with the sporozoite IDC approaches was established following the first clinical study evaluating the protective efficacy of immunization with *P. falciparum* sporozoites administered by mosquito bites under CQ cover [55]. Sporozoite IDC studies using chemoprophylaxis with *P. falciparum* sporozoites administered by mosquito bite (CPS) or chemoprophylaxis vaccination with purified *P. falciparum* sporozoites administered by injection (CVac) have been widely conducted in malaria-naïve humans in a controlled clinical setting to evaluate safety, immunogenicity and efficacy [55-64]. Intravenous, i.d. and mosquito bites have all been used as routes of sporozoite administration. Intravenous and i.d. routes enable a precise dose as well as being practical and feasible for a large-scale vaccination program.

2.2.1 Homologous protection induced by *P. falciparum* sporozoite infection and drug cure

Malaria-naïve human volunteers were immunized three times with *P. falciparum* NF54 via 12-15 mosquito bites combined with CQ. Control subjects received bites from uninfected mosquitoes. Similar to the rodent models, a sub-microscopic parasitemia was detected in all ten vaccinees following the first immunization; blood-stage parasite burden decreased with each immunization. All CPS-CQ immunized subjects demonstrated complete sterile protection against homologous sporozoite challenge eight weeks after the final immunization [55]. No serious adverse events were recorded. Protection was shown to be long-lasting [57,59]. Four of six *P. falciparum* NF54 CPS-CQ immune volunteers were aparasitemic following re-challenge with homologous sporozoites 28 months after immunization, whereas the remaining two volunteers had delayed patency of parasitemia [59]. Potential immune correlates of protection are discussed in Section 2.2.4.

Similar to the rodent studies discussed previously, a further study was conducted to determine the stage-specificity of the protective immune response induced by *P. falciparum* CPS-CQ

immunization [60]. Malaria-naïve individuals were immunized three times by mosquito bites with both *P. falciparum* NF54 and 3D7 (a clone of NF54) while on CQ prophylaxis; control subjects received CQ only. Volunteers were challenged i.v. with *P. falciparum* 3D7 pRBCs or by mosquito bites with *P. falciparum* 3D7 sporozoites. As the i.v. blood-stage challenge completely bypasses the liver stage, this allowed separate assessment of protective immune responses against each life-cycle stage. Complete sterile protection was observed in vaccinees who received the sporozoite challenge whereas those who received the blood-stage challenge developed parasitemia and had similar prepatent periods and blood-stage multiplication rates as the CQ-treated controls. Despite exposure to low levels of blood-stage parasite during the immunization phase immediately prior to challenge, antibodies against blood-stage antigens were detected in only one vaccinee and IgG from vaccinees did not display *P. falciparum* growth inhibitory activity *in vitro*. Following blood-stage challenge, IFN- γ and the chemokine, Monokine induced by IFN- γ (MIG), were detected earlier in the plasma of vaccinees compared with controls.

A further study also examined whether exposure to blood-stage parasites during CPS-CQ is required for vaccine efficacy against homologous challenge. In malaria-naïve individuals, three immunizations of CPS-CQ with 12-15 *P. falciparum*-infected mosquito bites at monthly intervals in malaria-naïve individuals was directly compared with CPS-PQ/CQ, where a single dose of PQ was administered a day after sporozoite administration to kill the liver-stages and completely prevent the development of blood-stage parasites [63]. Controls received non-infectious mosquito bites and PQ/CQ. During the immunization phase, only one individual in the CPS-PQ/CQ remained qPCR negative, indicating that the dosing regimen of PQ was sub-optimal in this study as it did not prevent development of blood-stage parasitemia. Following sporozoite challenge, all CPS-CQ vaccinees had delayed parasite patency, but did not develop sterile immunity, as has been reported in other studies [55,60].

They did however develop greater blood-stage immunity than the CPS-PQ/CQ group. Interestingly, thirty percent (3/11) of the CPS-PQ/CQ vaccinees demonstrated sterile protection. The lack of sterile protection in the CPS-CQ group was attributed to a higher sporozoite inocula per mosquito at challenge than was expected; this increased number of sporozoites may have overwhelmed protective immune responses.

The protective efficacy of *P. falciparum* CPS has also been examined with MQ [61], which like CQ does not affect liver-stage development. Malaria-naïve volunteers were immunized with three doses of *P. falciparum* NF54 sporozoites by mosquito bites (four weeks apart) while receiving MQ and this was compared with CPS-CQ in the same study. The control group received MQ only. Seventy percent of volunteers immunized with *P. falciparum* CPS-MQ were protected, which was not significantly different to the 60% protection observed in individuals who received CPS-CQ. Antibody and cellular immune responses were also comparable between groups.

2.2.2 Heterologous protection induced by *P. falciparum* sporozoite infection and drug cure

The ability of *P. falciparum* CPS to induce heterologous protective immunity in humans has also been examined. Sixteen volunteers previously immunized with three rounds of 5, 10 or 15 *P. falciparum* NF54-infected mosquito bites with CQ prophylaxis and challenged with *P. falciparum* NF54 (West-Africa) sporozoites were re-challenged with the geographically and genetically distinct *P. falciparum* clone NF135.C10 (Cambodia) sporozoites, 14 months after the last immunization [65]. Three volunteers previously not protected against *P. falciparum* NF54 sporozoite challenge were also not protected against challenge with *P. falciparum* NF135.C10. Amongst thirteen volunteers who were completely protected against the original *P. falciparum* NF54 challenge, sterile protection against heterologous strain NF135.C10 was

observed in 2 volunteers (one each from the 10 and 15 mosquito bites immunized groups). Delayed patency was seen in the other 11 volunteers, indicating partial protection. There are several possible explanations for these results. Protective immune responses may have decreased as the heterologous challenge was performed 14 months rather than the usual 2-5 months after the last immunization. Additionally, the different sporozoite immunization doses (varied between volunteers from 5 to 15 mosquito bites) could have resulted in differential heterologous protection as higher doses might be needed for long-lasting protection, particularly against heterologous strains. Similar dose-dependent protection was observed in a dose-ranging study where complete homologous protection was obtained in 4 out of 5, 8 out of 9 and 5 out of 10 volunteers after CPS-CQ immunization with 45, 30 and 15 *P. falciparum*-infected mosquito bites respectively [62].

A further study examined heterologous protection in malaria-naïve individuals in a double-blind, placebo-controlled trial of *P. falciparum* NF54 CPS-CQ immunization followed by challenge with homologous or heterologous (*P. falciparum* NF135.C10 or NF166.C8 (Guinea)) sporozoites, eighteen weeks later [66]. While 5/5 individuals demonstrated sterile protection following homologous challenge, this was only observed in 2/10 (NF135.C10) and 1/9 (NF166.C8) individuals who received heterologous challenge. Six out of ten challenged with NF135.C10 had a prolonged prepatent period compared to controls. The inhibitory activity of plasma for blocking intra-hepatic development of heterologous strains *in vitro* was significantly lower than the homologous strain but this did not correlate directly with protection. There was significant diversity demonstrated in the target antigens for CPS-CQ-induced antibodies in the heterologous strains compared with *P. falciparum* NF54. Parasite-specific T-cell responses (IFN- γ and granzyme B) were detected *in vitro* in all groups.

In the *P. falciparum* CPS approach, modest protection against heterologous challenge has been observed [65,66]. Recent work has examined the biological heterogeneity of the three

clinical isolates used in these studies to better understand this strain-specific vaccine-induced protection and the implications for vaccine efficacy in malaria endemic regions. Differences in invasion and replication within human hepatocytes *in vitro* were identified [67]. This differential infectivity was directly correlated with the magnitude of the first wave of blood-stage parasites *in vivo* and correlated inversely with the prepatent period. This *in vitro* and *in vivo* data support that a greater number of blood-stage parasites were released from the livers of individuals infected with NF135.C10 and NF166.C8 compared with NF54, which will impact on the prepatent period and must be considered when interpreting any data from studies using these isolates. Their genetic diversity was also examined, with tens of thousands of variants (including SNPs, indels and small structural variants) detected between the three isolates and this was reflected in variability in immunologically important regions of the genome which has the potential to impact on vaccine efficacy [68]. At a whole genome level, these isolates were shown to be a reliable proxy for the strains circulating in the geographic region from which they originated [68]. While it may be possible to further optimize the vaccine dose and schedule to improve vaccine efficacy against heterologous challenge [56], the development of a geographically-targeted multi-strain vaccine may also be required for high levels of protective efficacy in the field.

2.2.3 Factors affecting induction of protective immunity following *P. falciparum* sporozoite infection and drug cure: route of administration, immunization dose and frequency

As rodent studies demonstrated that the route of administration of sporozoites can impact on the generation of protective immunity (sections 2.1.1.3 and 2.1.2.3), this was also investigated for *P. falciparum*. The i.d. administration of cryopreserved, infectious *P. falciparum* sporozoites (Sanaria® PfSPZ Challenge) was tested for its safety and immunogenicity in the context of CVac with CQ [64]. Volunteers were immunized i.d. with three or four doses of 7.5×10^4 sporozoites while taking CQ; controls received saline. Prior

to challenge, vaccinees had weak antibody responses against CSP, liver stage antigen 1 (LSA1), MSP1 and no detectable cellular immune responses against *P. falciparum* pRBC or sporozoites. Following challenge by *P. falciparum* NF54-infected mosquito bites, 2/8 vaccinees did not develop microscopically patent parasitemias however, they both developed transient parasitemias detectable by qPCR. The lack of protection was attributed to sub-optimal parasite exposure during the i.d. immunizations with insufficient numbers of sporozoites migrating to and developing in the liver. A further CVac study evaluated i.v. administration of sporozoites (Sanaria[®] PfSPZ challenge) to malaria-naïve volunteers while taking CQ [56]. It revealed that the protective efficacy was not only dependent on route of administration but also on the immunization dose and dosing frequency. A CVac regimen of three i.v. doses of 3.2×10^3 , 1.28×10^4 or 5.12×10^4 *P. falciparum* sporozoites + CQ at 28-day intervals induced sterile protection against i.v. challenge with homologous sporozoites in 33%, 67% and 100% of volunteers, respectively. When the completely protective regimen was administered at 5- and 14-day intervals instead of 28-day intervals, protection against 2×10^3 PfSPZ i.v. challenge was achieved in only 63% and 67% of volunteers. Increasing the number of sporozoites in the vaccine may improve protection in these accelerated vaccination schedules. Therefore, route of administration, antigen dose as well as timing of immunizations are major determinants of the protective efficacy of *P. falciparum* sporozoite IDC approaches.

2.2.4 Immune correlates of protection induced by *P. falciparum* sporozoite infection and drug cure

2.2.4.1 Cellular correlates of immunity

Numerous studies have characterized the immune response induced by *P. falciparum* sporozoite IDC approaches; this may enable identification of immune correlates of

protection. Protective immunity against homologous sporozoite challenge is associated with the induction of pluripotent effector memory T-cells [55]. In protected vaccinees, a significant increase in the proportion of multiple cytokine (IFN- γ , TFN- α , IL-2)-producing pluripotent effector memory T-cells in response to pRBCs was observed at the pre-challenge timepoint. In a sub-set of individuals who were challenged 28 months later, long-term persistence of parasite-specific pluripotent effector memory T-cell responses was detected prior to re-challenge [59], although the parasite-specific cytokine responses in the 2/6 individuals with delayed onset of parasitemia following challenge were not significantly different from the 4/6 who were completely protected. In a further study, protection was also associated with increased expression of markers of cellular cytotoxicity [62]. Higher proportions of CD4⁺ T-cells expressing the degranulation marker CD107a and granzyme B-producing CD8⁺ T-cells were observed at the pre-challenge time point following *in vitro* re-stimulation with *P. falciparum* pRBC.

The comparative *P. falciparum* CPS-CQ and CPS-MQ immunization study, which induced complete protection following homologous challenge in 60% and 70% of vaccinees respectively [61], also suggested a potential role for $\gamma\delta$ T-cells, Th1 and cytotoxic responses in vaccine-induced protection [69]. A possible suppressive role of regulatory T-cells was also identified, as proliferation of this cell population was significantly higher after the second immunization in subjects not protected against sporozoite challenge.

Most studies have identified immune responses associated with protection by comparing pre-challenge samples from fully protected vaccinees with the control group or comparing immune responses in samples from vaccinees collected at baseline and pre-challenge. This approach primarily identifies immune responses induced by vaccination; they may or may not be mediating protection. In a recent study, cellular immune responses were compared between protected and unprotected individuals vaccinated with *P. falciparum* CVac with CQ

[56]. Pre-challenge, CD4⁺ T-cells that simultaneously produced IFN- γ , IL-2 and TNF- α were more prevalent in subjects who did not develop parasitemia, whereas CD4⁺ T-cells that produced TNF- α only were more frequent in those who became parasitemic. Sterile protection was strongly associated with a higher frequency of pRBC-specific polyfunctional IFN- γ , IL-2 and TNF- α producing CD4⁺ T-cells. Protection was weakly associated with pRBC-specific IFN- γ producing CD8⁺ T-cells.

Whole blood transcriptome profiling by RNA-seq provides a broad, unbiased approach for evaluating the dynamics and composition of immune responses [70]. In individuals completely protected against homologous sporozoite challenge, differential expression of genes related to T-cell, NK-cell, protein synthesis and mitochondrial processes were detected four weeks after the first CPS-CQ immunization, suggesting induction of cytotoxic cellular responses. These signatures were not detected in protected subjects four weeks after the second immunization or 132 days after the third immunization. Similar signatures were however detected in these individuals three weeks following sporozoite challenge, suggesting an ongoing T-cell memory response after sporozoite exposure and in the absence of detectable parasitemia. Interestingly, evidence of immune interactions between different components of the immune system were observed in protected individuals in the weeks following the second and third immunizations. In contrast, in non-protected subjects, transcriptomic changes were detected straight after the third immunization and on their day of drug treatment (in the presence of patent parasitemia) with upregulation of interferon and innate inflammatory genes and downregulation of B-cell signatures. These data provide *P. falciparum* CPS-CQ-induced molecular signatures associated with protection that can be interrogated in greater detail in future studies.

2.2.4.2 Humoral correlates of immunity

The induced antibody response, including breadth, magnitude and functionality, has been examined in different studies to determine the role of antibodies in *P. falciparum* sporozoite IDC approaches and to identify potential antibody correlates of protective immunity.

Antibodies may play a role in protection, but their mechanism of action in pre-erythrocytic immunity may extend beyond direct neutralisation of sporozoites or inhibiting invasion of sporozoites. An additional mechanism of action is activation of complement. The complement system plays a role in phagocyte recruitment, pathogen opsonization and pathogen lysis via downstream C3 complement protein deposition. Antibody-mediated complement activation has been identified as an important mechanism for inhibiting invasion of *P. falciparum* merozoites, which facilitates a reduction in *P. falciparum* blood-stage replication and prevention of clinical disease [71]. To investigate this, antibodies in sera from *P. falciparum* NF54 CPS-CQ immunized volunteers, who were completely protected from homologous sporozoite challenge, were evaluated for their capacity to activate complement [72]. CPS-CQ immunization induced sporozoite-specific complement-fixing IgM and IgG1 antibodies that were capable of activating the classical complement pathway resulting in functional lysis of sporozoites and a reduction in homologous (NF54) and heterologous (NF135.C10) sporozoite infectivity of human hepatocytes *in vitro*. These data highlight the role of complement as an immune effector mechanism in pre-erythrocytic immunity induced by *P. falciparum* CPS-CQ.

Using a proteome microarray containing 809 antigens, the reactivity of pre-challenge plasma from malaria-naïve individuals who were completely protected following *P. falciparum* CPS-CQ immunization was compared with semi-immune individuals from a malaria endemic area [73]. There was substantial variation in the number of antigens recognized in the *P. falciparum* CPS-CQ-immunized individuals, however recognition of the pre-erythrocytic antigens CSP and LSA1, were common in all protected volunteers. Strikingly, while there

were 60 antigens that overlapped between the two groups, the serological recognition profile of CPS-CQ immunized volunteers was skewed towards pre-erythrocytic antigens while plasma from naturally exposed semi-immune Kenyan individuals was biased towards recognition of asexual blood-stage antigens. These results shed light on the antibody profile induced in immunized volunteers and the identification of antigens for further investigation as potential targets of protective immunity.

A further study also screened pre-challenge sera from malaria-naïve individuals who developed long-lasting sterile immunity following three immunizations of *P. falciparum* CPS-CQ for antibody functionality and against a broad repertoire of *P. falciparum* antigens that were expressed during the pre-erythrocytic stage [74]. Pooled sera from protected volunteers inhibited liver-stage development *in vitro* whereas there was limited invasion inhibitory activity specific for blood-stage parasites (2/9 vaccinees). Overall, there was a broad and varied *Plasmodium* antigen-specific antibody response detected in protected individuals. Three antigens: Pf merozoite apical erythrocyte binding ligand (MAEBL) (sporozoite, liver, asexual blood-stage expression), Pf thrombospondin-related adhesive protein (TRAP) (sporozoite, liver, asexual blood-stage expression) and Pf Schizont Egress Antigen-1 (SEA1) (sporozoite, liver-stage expression), were recognized by antibodies present in the sera of 6/9, 8/9, 9/9 protected individuals. These antibodies were not present or were variably present at low levels in mock-immunized individuals. Antibodies raised against PfMAEBL demonstrated invasion inhibitory activity against sporozoites *in vitro*. These data suggest a potential role for these antibodies in protection.

In a recent study, it was possible to compare antibody responses in protected and unprotected individuals vaccinated with *P. falciparum* CVac with CQ [56]. Antibody responses were evaluated using a *P. falciparum* proteome array expressing 7,455 malaria peptides, standard protein ELISAs, Immunofluorescence Assays on whole sporozoites and assessment of

invasion inhibitory activity for sporozoites. There was no significant association of any antibody responses with protection. The authors hypothesized that antibodies may play a role in protection but are not the primary effector. This same proteome array was also used to screen a larger number of sera from protected and unprotected vaccinees enrolled in multiple studies [75]. Antibody responses against six antigens were associated with susceptibility and responses against six entirely novel antigens were associated with protection. Together, this multi-antigen combination predicted protection in the CPS-immunized individuals with 83% sensitivity and 88% specificity. High reactivity against the immunodominant antigens CSP, LSA1, MAEBL, TRAP, SEA1 was not associated with protection individually or in combination; suggesting that they may be markers of exposure rather than protection for the *P. falciparum* CPS-CQ regimen [75]. This is particularly interesting for CSP, which is the major parasite antigen contained within the most advanced malaria vaccine, Mosquirix™ (RTS,S/AS01) [76].

3.0 *Plasmodium* infection and drug cure with asexual blood-stage parasites

Infection and Drug Cure with asexual blood-stage parasites (referred to as controlled infection immunization (CII) in this review) has been explored to a lesser extent compared with sporozoite IDC. Asexual blood-stage parasite CII is administered via i.v. injection of pRBCs and is typically followed by anti-malarial drug administration. Protection against blood-stage challenge is determined by the development of blood-stage parasitemia, blood-stage parasite levels, parasite multiplication rates and/or clearance rate of parasites.

3.1 Pre-clinical evaluation of *P. chabaudi*, *P. yoelii* and *P. vinckei* asexual blood-stage parasite infection and drug cure

3.1.1 *Homologous and heterologous protection*

Blood-stage CII has been investigated using different rodent malaria parasites in combination with various anti-malarial drugs such as MQ, atovaquone-proguanil (Malarone), CQ, doxycycline and Az.

To examine strain-specific protective immunity induced by blood-stage CII, *P. chabaudi* CII was examined using a combination of 10^5 *P. c. chabaudi* AS CII with Malarone initiated 48 hours after parasite inoculation, which prevented the development of a microscopically detectable blood-stage infection. Three i.v. immunizations of the *P. c. chabaudi* AS CII protected mice against both homologous and heterologous (*P. c. chabaudi* CB) blood-stage parasite challenge [77]. In all vaccinated mice following challenge, low level parasitemias were detected that were significantly lower than in the naïve control mice. Peak parasitemias in mice challenged with heterologous parasites were significantly higher than in mice challenged with homologous parasites. Serum from CII mice contained IgG specific for AS and CB pRBCs and conserved merozoite surface antigens, as measured by ELISA and immunofluorescence assays, but lacked IgG specific for variant surface antigens (VSA) of AS pRBCs. This is in direct contrast to serum from mice who experienced patent, self-resolving blood-stage malaria infections which also contained significant levels of IgG specific for the VSA. These data suggest that the VSA are less immunogenic or are expressed at relatively lower levels than the other malaria antigens. It may be that by reducing malaria parasite antigen concentrations in the blood-stage CII mice compared to those who experienced a patent blood-stage infection, this negatively impacts on the generation of adequate T help for generation of effective VSA-specific antibody responses [77]. Mice immunized by this blood-stage CII were able to control the parasitemia following challenge, suggesting that variant-specific antibodies were not critical for protection. Prominent Th1 cell-mediated immune responses (IFN- γ^+ CD4 $^+$ lymphocytes) were detected in immunized mice. Splenocyte proliferative responses to *P. c. chabaudi* AS and CB pRBC were similar in

immunized mice, suggesting that the blood-stage CII-induced cell-mediated immunity was targeting conserved antigens.

Strain-transcending immunity was also demonstrated with *P.c. chabaudi* clones used in combination with MQ, with treatment initiated 5 days after the injection of blood-stage parasites [78]. Mice received one or two doses of blood-stage CII with *P.c. chabaudi* AS or CB and following the last immunization they received a mixed strain blood-stage parasite challenge with equal numbers of the homologous and heterologous parasite strains. In mice that received a single dose of CII, there was a partial reduction in parasitemia compared with naïve mice, with the blood-stage parasitemias of each parasite strain reduced to equal degrees. In mice that received two doses of CII, the reduction in parasitemias was greater and homologous strain parasitemia reduced faster than the heterologous strain for all combinations of *P. c. chabaudi* clones tested (AS, CB, AJ, AQ). This suggests that the strain-specific component of immunity induced by blood-stage CII may depend on immune memory.

Cross-species immunity has been demonstrated in the context of *P. yoelii* blood-stage CII with MQ. Mice immunized with one dose of 10^6 *P. yoelii* blood-stage CII with MQ (starting on day 5 post-inoculation) were completely protected against homologous blood-stage infection; and the peak parasitemias in mice challenged with a heterologous species (*P. vinckei lentum*) were significantly reduced compared to parasitemias in MQ-treated control mice [47]. Similar results were obtained when mice were immunized with 10^6 *P. vinckei* blood-stage CII with MQ and challenged with homologous or heterologous parasites. Immunization induced significant levels of antibodies specific for homologous species crude parasite antigen and much lower levels against heterologous species crude parasite antigen.

A further study determined that the timepoint for initiation of anti-malarial drug treatment impacted on the development of cross-species protective immunity in the context of blood-stage CII with PYR [79]. Mice inoculated with 5×10^4 pRBCs of the non-lethal strain *P. chabaudi* AS and drug-treated on day-5 failed to develop species-transcending protection against the more virulent *P. yoelii* 17XL, with all mice succumbing to infection. In contrast, when drug treatment was administered on day-7 post-parasite inoculation, mice survived *P. yoelii* challenge with lower parasitemia levels. A predominant Th2-type response with higher IL-4 and IL-10 levels and lower IFN- γ expression was associated with protection against lethal infection.

For blood-stage CII to be a viable vaccine approach it is critical however, that anti-malarial drug treatment is initiated at the same time that the parasite is inoculated. A delay in treatment initiation and relying on individuals to return for drug treatment is a safety concern. For this reason, blood-stage CII was examined with the delayed death anti-malarial drugs doxycycline and Az [80]. Delayed death drugs target the apicoplast of the parasite, inhibiting the growth of progeny of the parasites thereby allowing the parasites to persist for an extra replication cycle [81]. Initially, BALB/c and C57BL/6 mice received three immunizations with 10^6 or 10^7 *P. chabaudi* AS or *P. yoelii* YM blood-stage parasites, with doxycycline treatment initiated on the same day (50 mg/kg for 7 days) [80]. Strong protective immunity was observed against homologous challenge. Heterologous protection was also observed in *P. chabaudi* AS-immunized BALB/c and C57BL/6 mice and *P. yoelii* YM-immunized C57BL/6 mice, although this was not as strong as homologous protection. Depletion studies demonstrated that CD4⁺ T-cells played a critical role in homologous protection in the *P. chabaudi* AS model with a broad Th1 cytokine response (IFN- γ , TNF- α , IL-2) correlated with the protection. While in the *P. yoelii* model, antibodies significantly contributed to protection. The persistence of parasites, enabled by the use of doxycycline, was crucial for

the induction of protective immunity. Mice receiving three *P. chabaudi* or *P. yoelii* CII with doxycycline had detectable parasite DNA for at least one week following immunization with complete clearance by day 14. In comparison, mice receiving three *P. chabaudi* CII with Malarone or *P. yoelii* CII with PYR, exhibited an immediate decline in parasite levels and parasites were undetectable by day 2 as measured by qPCR. Following homologous challenge, mice that were immunized with CII and doxycycline demonstrated superior protection to mice that received CII with the faster-acting drugs.

As a vaccine approach, to ensure patient safety, it would be desirable to administer the anti-malarial drug not only at the time of parasite inoculation, but also in a single dose. For this purpose, *P. yoelii* CII was next examined with the delayed death drug Az which was administered in a single dose at the same time as parasite inoculation [80]. Mice receiving a single *P. yoelii* CII with Az (500mg/kg) were strongly protected from a homologous blood-stage challenge one month later. The need for repeated infection and treatment cycles brings a new challenge for the implementation of CII in a clinical setting as well as in malaria-endemic areas. Single CII with a single dose of the drug, as reported in this study, is ideal for a blood-stage CII vaccine.

3.1.2 Cross-stage immunity

Induction of cross-stage immunity by blood-stage CII has been examined in a limited number of studies. BALB/c mice immunized with a single dose of 1×10^6 *P. yoelii* or *P. vinckei lentum* blood-stage parasites in combination with MQ (commenced on Day 4 or 5 following parasite inoculation) were challenged i.v. with *P. yoelii* sporozoites [47]. The liver-stage parasite burdens in immunized mice at 42 hours post-challenge were not significantly different from naïve, challenged mice indicating a lack of cross-stage immunity.

In contrast, immunization with one dose of 10^6 or two doses of 10^5 *P. yoelii* blood-stage CII with daily CQ treatment for 10 days starting on the day of pRBC administration was shown to induce protection against sporozoite challenge; a single dose of 10^6 also completely protected against blood-stage challenge [82]. Protection against sporozoite challenge was mediated by T-cells that were inhibitory to liver-stage parasites and antibodies targeting any blood-stage parasites that emerged from the liver. The effect of $CD4^+$ and $CD8^+$ T-cells was partly mediated by IFN- γ and was completely dependent on nitric oxide.

3.2 Clinical studies with *P. falciparum* blood-stage parasite infection and drug cure

There has been limited investigation of *P. falciparum* blood-stage CII. Malaria-naïve individuals received four cycles of 30 viable *P. falciparum* 3D7 pRBC at five-weekly intervals, with three infections treated with Malarone prior to patency (starting on day 8 post-parasite inoculation) and the last infection treated with CQ on day 14 post-parasite inoculation [83]. This *P. falciparum* blood-stage CII induced protection as characterized by the absence of parasites or parasite DNA in the blood by qPCR in three of the four volunteers following administration of the final infection and diminished parasite growth in the fourth volunteer. It is possible that residual low levels of atovaquone detected in the volunteer's plasma may have contributed towards the protection [84]. The observed protection was associated with proliferative $CD4^+$ and $CD8^+$ T-cells responses against pRBC *in vitro*, along with the IFN- γ production and higher induction of nitric oxide synthase in peripheral blood mononuclear cells. Immunity was independent of humoral responses as parasite-specific antibodies to either whole parasite or recombinant *P. falciparum* proteins (MSP1-19, MSP2, AMA1) were not detected.

A further study examined parasite persistence and immunogenicity in blood-stage CII with doxycycline. A single immunization with 3×10^6 *P. falciparum*-infected RBCs followed by

doxycycline treatment (100 mg/day for 21 days) commencing 1 hr after parasite administration increased parasite-specific cellular responses ($CD3^+$, $CD4^+$, and $\gamma\delta$ T-cells, along with IFN- γ and TNF) [80]. Three of four individuals also produced parasite-specific IgM antibodies. This CII approach was well tolerated. Three of the four volunteers developed parasitemia detectable by qPCR; parasites were only detectable for 2 growth cycles in one of these. Persistent low levels of parasites, which were controlled by doxycycline treatment, were observed in the remaining two volunteers. However, after completion of doxycycline, the parasitemia in these volunteers increased and they required rescue treatment on day 28 with artemether-lumefantrine as per the requirements of the study protocol. Further studies are required to determine if this CII protocol induces protective immunity in humans.

4.0 Concluding remarks

The development of a highly effective malaria vaccine is essential for the control and eventual eradication of the malaria parasite. While the low and variable protection observed for sub-unit vaccine candidates in the field has been disappointing, IDC-based whole parasite vaccines are a feasible alternative strategy for the development of a malaria vaccine. Much progress has been made with the sporozoite IDC approaches, with strong sterile protection demonstrated against challenge with homologous parasites in malaria-naïve individuals. Route of administration, sporozoite dose, choice of anti-malarial drug and timing of drug administration were all critical variables for the induction of protective immunity (Table 1). There was limited evidence of cross-stage immunity. Further optimization, including the assessment of higher parasite doses, will be required to enhance strain-transcending immunity to ensure it is able to offer a broad protection. While less progress has been made with the asexual blood-stage CII vaccine approach and there has been limited clinical evaluation, the existing pre-clinical and clinical data supports the induction of strong homologous protective immunity (Table 1). Persistence of the blood-stage parasite following blood-stage CII is

critical for the induction of strong protective immunity. Furthermore, recent data support an optimized blood-stage CII regimen with concurrent administration of the parasite and a single-dose anti-malarial drug, which addresses a major safety concern with this vaccine strategy [80]. Additional clinical studies will be needed to evaluate the protective efficacy of the blood-stage CII vaccine against homologous and heterologous parasites. While further development is required for an IDC vaccine, it remains a promising approach for the development of a broadly protective malaria vaccine.

5.0 Expert Opinion

Development of a highly effective malaria vaccine that is capable of inducing a broad protective immunity against the many different parasite strains circulating in malaria endemic areas would play a key role in the eradication of the malaria parasite. *Plasmodium* IDC is one of a number of whole parasite malaria vaccine approaches that are currently being developed and at least in terms of the whole sporozoite approaches, it offers superior protection. In theory, inclusion of the whole parasite into the vaccine should limit the impact of antigenic diversity and immunological non-responsiveness; these factors contribute to the low efficacy that has been observed when sub-unit vaccine candidates have been tested in the field. To date, sporozoite IDC studies in rodents and malaria-naïve humans have demonstrated that while this approach can induce strong protection against homologous challenge, equivalent levels of heterologous immunity are not induced (Table 1). This may be addressed by further vaccine optimization. If heterologous immunity is limited by the dose of conserved antigenic target/s of protective immunity, then increasing the parasite dose may increase the strain-transcending immune response. Alternatively, if the parasite's genetic diversity is also an impediment with this vaccine approach, then including multiple representative strains in the vaccine may be required.

The data presented and discussed in this review reflect that clinical evaluation of IDC approaches for the development of a malaria vaccine have to-date focused on *P. falciparum*. The necessary reagents to develop both pre-erythrocytic [85] and blood-stage *P. vivax* [86] whole parasite vaccines are available, although there are additional considerations for *P. vivax*. These include: the challenges of long-term culture of *P. vivax* to generate sufficient material for a whole parasite blood-stage vaccine and the possibility of hypnozoite formation and infection relapse following immunization with sporozoites. While PQ or tafenoquine can be administered to clear these latent liver stages, there is a risk of PQ-induced hemolysis in G-6-PD deficient individuals. Multiple relapses in two participants in a *P. vivax* mosquito challenge study also highlighted human cytochrome P-450 isoenzyme 2D6 (CYP2D6) polymorphisms, which impact the efficacy of PQ by affecting the conversion of PQ to its active metabolite [87]. These are issues that will need to be addressed to enable the development of *P. vivax*-specific IDC-based vaccine approaches.

Substantial immunological interrogation aiming to identify mechanisms and correlates of immunity has been undertaken in a number of studies; results were not always consistent between studies (Table 1). Identifying an immune response/s that is reproducibly correlated with protection and understanding the mechanisms of immunity will be a major advantage in evaluating vaccine efficacy and informing any further vaccine optimization.

The IDC regimen employed for vaccination must also be appropriate for large-scale administration in malaria endemic areas. While sporozoite immunization by mosquito bites has been used for many studies so far, the use of purified sporozoites administered via an i.v. route will enable transitioning a sporozoite IDC-based vaccine approach into the field. The need for multi-dose administration of anti-malarial drugs is also a potential safety issue for this vaccine approach as it relies on self-administration or individuals returning to a health clinic for these drugs. Ideally, an anti-malarial drug that can be administered concurrently

with the parasite in a single dose is required. Proof-of-concept studies in rodents, demonstrated that IDC with a single dose of drug (PPQ for sporozoite CII and Az for blood-stage CII)[25,80] induced strong protective immunity. Alternatively, novel drug formulations that can reduce drug dosing frequency by modifying the rate of drug absorption may be developed to enable single dose administration. The lack of adherence to a multi-dose vaccination regimen is always a concern however, further optimization of the vaccination regimens may enable the number of vaccine doses to be reduced, particularly if natural boosting of vaccine-induced immune responses is observed.

Ultimately an IDC-based vaccine approach needs to be evaluated in malaria endemic areas in individuals who have been exposed to the malaria parasite. Few studies have examined the duration of protective immunity induced by an IDC-based approach in humans; for sporozoite CPS-CQ, protection against homologous challenge lasted for at least two years in malaria-naïve adults [59]. It will be essential to define this in malaria endemic areas in different age groups following exposure to diverse parasite strains and to determine whether this exposure is able to boost and maintain protective immune responses. This will be of particular interest as there is no evidence that the CSP-specific antibodies induced by the leading malaria vaccine, RTS,S/AS01, are boosted following natural exposure [88]. It is also unknown how the vaccine will perform in the presence of pre-existing *Plasmodium*-specific immune responses. Recent data from a Tanzanian study evaluating another whole parasite vaccine, the RAS vaccine (PfSPZ), in malaria-exposed adults, showed that despite substantial person-to-person variation in seroreactivity to an array of *P. falciparum* proteins at baseline, this reactivity profile remained largely unchanged following repeated PfSPZ vaccination [89]. This suggests that at least for this particular vaccine, only pre-existing antibody responses were boosted, and this was attributed to natural imprinting of the individual immune responses. It is unknown whether this will be relevant for cellular immune

responses. The impact of this on the vaccine's protective efficacy is unclear, however, a Malian study observed that this PfSPZ vaccine had a protective efficacy of 48% by time to first positive blood smear and 29% by proportion of participants with at least one positive blood smear during a full transmission season [90]. This demonstrates that a whole sporozoite vaccine can induce protective immunity against naturally transmitted malaria infection in the presence of pre-existing *Plasmodium*-specific immune responses. The impact of pre-exposure will be a consideration for both the evaluation of whole parasite vaccines in malaria-exposed populations and its eventual application, as it may result in greater heterogeneity in the immune response and protective efficacy than observed in malaria-naïve individuals.

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Table 1: Protective efficacy of different *Plasmodium* infection and drug cure regimens

<i>P. berghei</i>	<i>P. yoelii</i>	<i>P. vinckei</i>	<i>P. chabaudi</i>	<i>P. knowlesi</i>	<i>P. falciparum</i>	<i>Main findings</i>
Induction of homologous protection against spz challenge						<ul style="list-style-type: none">• Complete protection against homologous spz challenge was demonstrated using different <i>Plasmodium</i> infection and drug cure regimens.• In murine models, complete sterile protection was observed with one to five doses of $1-5 \times 10^4$ spz.• In humans, three doses of CVac or CPS results in sterile protection.
Outcome: Complete protection ^a						
5×10^4 spz + CQ, five i.v. doses [31]	2×10^4 spz + CQ, two i.v. doses [45]	-	-	-	CPS-CQ, strain NF54, three doses via 12-15 infected mosquito bites [55,66]	
5×10^4 spz + CQ, one i.v. dose [44]	10^4 spz + MQ, two i.v. doses [47]				5.12×10^4 Sanaria PfSPZ-CVac-CQ, strain NF54, three i.v. doses, 28 days interval [56]	
10^4 spz + CQ, three i.v. doses [44]	10^4 spz + CQ and spz + artesunate, three i.v. doses [22]				CPS-CQ, three doses via 8 NF54 and 7 3D7 infected mosquito bites [60] ^c	
Prime 5×10^4 and two boosts with 2×10^4 or three 10^4 spz + CQ, i.v. doses [40]	10^4 spz + Az four i.v. doses, 10^4 spz + arteether and 10^4 spz + MQ five i.v. doses [24]					
2×10^4 spz + CQ, three i.v. doses [41]						
10^4 spz + PPQ, two i.v. doses [25]	One 10^6 or two 10^5 blood-stage CII with CQ [82]					

Outcome: Partial protection						
96% and 57% ^b , 10 ⁴ spz + CQ (two and one i.v. doses respectively) [34] 80% ^b , 10 ⁴ spz + Az, two i.v. doses [21] 50% ^b , 5 × 10 ⁴ spz + CQ, one i.d. dose [44] Delay in patency (3-4 days) compared to spz-only control, one i.v. dose 10 ⁴ spz with isopentaquine, (administered prior to spz immunisation on days -2, -1 and 0) [23] 13% ^b , 10 ⁴ spz + CQ, three i.d. doses [44]	94.7% ^b , prime 5 × 10 ⁴ and two boosts with 2.5 × 10 ⁴ spz + PQ, i.v. doses [26] 90% ^b , 10 ⁴ spz + CQ, one i.v. dose [48] An equal degree of protective immunity as assessed by liver parasite burdens, 2 × 10 ³ spz + MQ two i.d. or i.v. doses [50]		Suppression of blood-stage parasite growth compared to mock-immunized control, prime 4 × 10 ² and one boost 2 × 10 ³ spz + MQ, strains CB and AJ, two i.v. doses [51]	50% ^b , spz + CQ, three doses via 15-20 infected mosquito bites [54]	80%, 88% and 50% ^b , CPS-CQ with 45, 30 and 15 NF54-infected mosquito bites, respectively [62] 70% ^b (CPS-MQ) and 60% ^b (CPS-CQ), strain NF54, three doses via 8 infected mosquito bites [61] 33% (3.2 × 10 ³) and 67% ^b (1.28 × 10 ⁴) Sanaria PfSPZ-CVac-CQ, strain NF54, three i.v. doses [56] 27% ^b , CPS-CQ/PQ, three doses via 12-15 NF54-infected mosquito bites [63]	
Induction of homologous protection against blood-stage challenge						
Outcome: Complete protection^a						• In murine models, complete protection against
	10 ⁶ blood-stage CII with MQ, one dose [47]	10 ⁶ blood-stage CII with MQ, one dose [47]				

	10 ⁶ blood-stage CII with CQ, one dose [82]					homologous blood-stage challenge was obtained with <i>P. yoelii</i> and <i>P. chabaudi</i> .
Outcome: Partial protection						
-	<p>62%^b, 10⁴ spz + CQ, three i.v. doses [22]</p> <p>Delay in the onset of blood-stage infection in 73% immunised mice, 10⁴ spz + artesunate, three i.v. doses [22]</p> <p>Reduced peak parasitemia, 10⁴ spz + MQ, two i.v. doses [47]</p> <p>Complete survival and reduced peak parasitemia, three 10⁶ or 10⁷ blood-stage CII, strain YM with 7 days Dox treatment [80]</p> <p>Low level</p>		<p>Suppression of blood-stage parasite growth compared to mock-immunized control, prime 4 × 10² and one boost 2 × 10³ spz + MQ, strains CB and AJ, two i.v. doses [51]</p> <p>Reduced peak parasitemia, improved parasite clearance rates, spz + CQ, via strain AS-infected mosquito bites [52]</p> <p>Reduced parasitemia, 10⁶ blood-stage CII with strains AS or CB with MQ, one or two i.p. doses [78]</p>	-	<p>75%^b, 3D7 blood-stage CII, four doses of 30 pRBCs, three infections treated with Malarone and the last infection treated with CQ [83]^d</p> <p>.</p>	<ul style="list-style-type: none"> • Complete sterile protection was only observed following immunization with blood-stage CII. It was not observed following immunization with spz + drug cure. • In humans, complete protection was observed in 75% of individuals immunized with 4 doses of blood-stage CII^d.

	parasitemia, 10^7 blood-stage CII, strain YM with Az [80]		Complete survival, reduced peak parasitemia, three 10^6 or 10^7 blood-stage CII, strain AS with 7 days Dox treatment [80] Low-level parasitemia, 10^5 blood-stage CII with Malarone, strain AS, three i.v. doses [77]			
Induction of cross-strain/species protection against spz challenge						
Outcome: Partial protection						
-	Two-day delay in patency and 92% reduction in peak parasitemia, 10^4 spz + MQ, two i.v. doses, <i>P. vinckei</i> challenge [47]	-	Suppression of blood-stage parasite growth, prime 4×10^2 and one boost 2×10^3 spz + MQ, strain CB, two i.v. doses, strain AJ challenge [51]	-	20% and 11% ^b , CPS-CQ, strain NF54, three doses via 15 infected mosquito bites, (NF135.C10 and NF166.C8 challenge respectively) [66] 12.5% ^b , CPS-CQ, 5, 10 or 15 NF54-infected mosquito bites, NF135.C10 challenge [65]	<ul style="list-style-type: none"> • Spz + drug cure regimens have so far failed to induce equal levels of protective immunity against heterologous spz challenge compared with homologous spz challenge. • In clinical studies, 12-20% cross-strain protection was observed following immunization with

						CPS.
Induction of cross-strain/species protection against blood-stage challenge						
Outcome: Partial protection						• Heterologous protection against blood-stage challenge has been examined for both spz and blood-stage infection and drug cure regimens; only partial protection has been observed.
-	<p>Reduced peak parasitemia on day 3 p.c, 10^4 spz + MQ, two i.v. doses, <i>P. vinckei</i> challenge [47]</p> <p>Reduced parasitemia compared to drug-treated only control, 10^6 blood-stage CII with MQ, one dose, <i>P. yoelii</i> challenge [47]</p> <p>Complete survival and reduced peak parasitemia, three 10^7 blood-stage CII, strain YM with 7 days Dox treatment, <i>P.</i></p>	<p>Reduced parasitemia compared to drug-treated only control, 10^6 blood-stage CII with MQ, one dose, <i>P. yoelii</i> challenge [47]</p>	<p>Suppression of blood-stage parasite growth, prime 4×10^2 and one boost 2×10^3 spz + MQ, strain CB, two i.v. doses, strain AJ challenge [51]</p> <p>Reduced peak parasitemia and improved parasite clearance rates, spz + CQ via strain AS infected mosquito bites, strain CB challenge [52]</p> <p>Reduced parasitemia, 10^6 blood-stage CII with strains AS or CB with MQ, one or two i.p. doses,</p>	-	-	

	<i>chabaudi</i> AS challenge [80]		<p>mixed strains AS, CB, AJ and AQ challenge [78]</p> <p>Complete survival and lower peak parasitemia, three 10^7 blood-stage CII, strain AS with 7 days Dox treatment, <i>P. yoelii</i> challenge [80]</p> <p>Low-level parasitemia, 10^5 blood-stage CII with Malarone, strain AS, three i.v. doses, strain CB challenge [77]</p> <p>Survival and lower parasitemia, 5×10^4 blood-stage CII PYR treatment on day 7, strain AS, <i>P. yoelii</i> 17XL challenge [79]</p>			
Immune responses associated with protection in pre-clinical and clinical studies						
Spz i.v.: IFN- γ producing effector memory	Spz i.v.: CD4 ⁺ and CD8 ⁺ cells, IFN- γ , NO	Spz i.v. or via mosquito bite: -	Spz i.v. or via mosquito bite: -	Spz via mosquito bite:	CPS or CVac: IFN- γ , TFN- α , IL-2 producing	Immune responses associated with protection were

<p>CD8⁺ T cells [40]</p> <p>Antibodies, CD8⁺ and CD4⁺ cells [41]</p> <p>Expansion of effector memory CD8⁺ T-cells [25]</p> <p>KLRG1 CD27^{low}, CD44^{high}, CD62L^{low} cells [41]</p> <p>CD8⁺ T effectors cells, increased IFN-γ, TNF-α, and IL-2 production [23]</p> <p>Blood-stage CII: -</p>	<p>[45]</p> <p>CD8⁺ T cells [22,47]</p> <p>Antibodies [22,47,48]</p> <p>Blood-stage CII: Antibodies [47,80, 82]</p> <p>CD4⁺ and CD8⁺ cells, IFN-γ, NO [82]</p>	<p>Blood-stage CII: Antibodies [47]</p>	<p>Blood-stage CII: Antibodies to conserved antigens, Th1 response (IFN-γ⁺ CD4⁺ lymphocytes) [77]</p> <p>Th2 response with higher IL-4 and IL-10 levels and lower IFN-γ expression [79]</p> <p>Th1 cytokine response (IFN-γ, TNF-α, IL-2) [80]</p>	<p>Antibodies, memory T-cells expressing CCR5 and CXCR6 and CD69 [54]</p> <p>Blood-stage CII: -</p>	<p>pluripotent effector memory T-cell [55,59]</p> <p>Polyfunctional IFN-γ, IL-2 and TNF-α producing CD4⁺ T-cells [56]</p> <p>IFN-γ and granzyme B T-cell responses [66]</p> <p>T-cell memory response [70]</p> <p>Cytotoxic markers (CD4⁺ T-cells expressing CD107a and granzyme B-producing CD8⁺ T-cells) [62]</p> <p>$\gamma\delta$ T-cells, Th1 and cytotoxic responses [61,69]</p> <p>Complement-fixing IgM and IgG1 antibodies [72]</p> <p>Antibodies [61, 66,</p>	<p>variable between studies.</p>
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					73,74] Blood-stage CII: Proliferative CD4 ⁺ and CD8 ⁺ T-cells, IFN- γ , NO [83]	
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Abbreviations: Az: azithromycin, CII: controlled infection immunization, CPS: chemoprophylaxis and sporozoite immunization, CQ: chloroquine, CVac: chemoprophylaxis vaccination, Dox: doxycycline, IFN- γ : interferon-gamma, IL: interleukin, KLRG1: killer-cell

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lectin-like receptor G1, MQ: mefloquine, NO: nitric oxide, PfSPZ-CVac: *Plasmodium falciparum* sporozoites chemoprophylaxis vaccination, p.c.: post-challenge, PPQ: piperazine, PQ: primaquine, PYR: pyrimethamine, spz: sporozoites, TNF- α : Tumor necrosis factor-alpha. ^a Complete protection in immunized group represents total absence of blood-stage parasitemia following challenge. For partial protection: ^b % protection in immunized group represents number protected against development of blood-stage parasitemia following challenge / number challenged; ^c Low level blood-stage parasitemia was detected by qPCR retrospectively in one of the five participants on the day of presumptive drug treatment (Day 21 post challenge)[60]; ^d residual anti-malarial drug may have contributed to this protection [84].

Figure Legends

Figure 1: The life-cycle of *Plasmodium* spp. parasites.

Figure 2: *Plasmodium* infection and drug cure as a vaccine approach to induce protective immunity. A) Schematic of *Plasmodium* infection and drug cure vaccine regimens; B) Stage-specificity of anti-malarial drugs used in the sporozoite infection and drug cure regimens for immunization, CPS and CVac. Drugs targeting liver-stage parasites arrest parasites in the liver, impacting on the release of parasites into the bloodstream while drugs targeting blood-stage parasites prevent the establishment of a blood-stage infection by preventing the development of merozoites and their subsequent release into the bloodstream. Abbreviations: Az, azithromycin; CPS, chemoprophylaxis and sporozoite immunization; CQ, chloroquine; CVac, chemoprophylaxis vaccination; MQ, mefloquine; PPQ: piperaquine; PQ, primaquine, PYR, pyrimethamine; i.d., intradermal; i.v., intravenous.