Maria M. Mota · Ana Rodriguez *Editors*

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Immune Response to Infection and Vaccination

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Whole-Sporozoite Malaria Vaccines

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1 Introduction

More than two centuries have passed since Edward Jenner's breakthrough postulates on whole-organism vaccination and more than one since Pasteur's formulation of the idea of immunization through microorganism attenuation [1]. Nowadays, health-care systems around the world rely on numerous successful vaccines to prevent morbidity and mortality in a cost-effective manner [2]. However, despite significant advances in molecular biology, approximately half of all currently approved vaccines continue to rely on the use of live attenuated microorganisms to elicit immunity against the diseases caused by their non-attenuated counterparts [1].

Malaria is one of the most important infectious diseases for which an effective vaccine is still lacking [3]. The gold standard strategies for malaria immunization rely on the administration of live *Plasmodium* sporozoites, the malaria parasite form transmitted to man by mosquitoes. Sporozoite-based vaccination strategies aim at preventing the parasite's life cycle progression from hepatic stages to the symptomatic blood stages of infection while eliciting potent preerythrocytic immune responses. Such whole-sporozoite malaria vaccination strategies are unique in their potential to induce sterile protection against a new infection and have led to the development of various vaccine candidates, currently ongoing preclinical and clinical development. This chapter will explore in detail our current knowledge of the protective immunity elicited by *Plasmodium* sporozoite-based malaria vaccination.

Although it was not until the 1960s that protective immunity against human malaria was first demonstrated [4, 5], the long list of classical studies exploring the use of sporozoites for immunization against malaria spans over a century, with the first studies using heat-inactivated avian parasites dating back to 1910 [6]. However, it was the discovery of the preerythrocytic stages of *Plasmodium* [7], followed by the establishment of a mouse model of malaria [8], that enabled the laboratory production of all stages of the parasite's life cycle [9], which eventually led to the landmark demonstration that live sporozoites attenuated by X-irradiation (RAS) could be used to elicit sterile protection against a new infection [10, 11]. This discovery was soon expanded to humans with the demonstration that volunteers could be protected against homologous and heterologous strains of *P. falciparum* parasites [12–14] (Fig. 1).

The excitement generated by these successes in both animal models and humans ensured that a large number of studies would continue into the following decades, aiming at optimizing the potential of sporozoite-based immunization, as well as at characterizing the immune responses elicited by these strategies [36, 60–64]. Progress also included exploring alternative methods that did not rely on the use of radiation, such as the administration of sporozoites under the cover of antimalarial drugs [37, 38]. Such strategies are based on sporozoite administration with the concomitant administration of a drug that does not interfere with the parasite's ability to complete liver-stage development but prevents blood-stage replication, enabling the induction of a strong immune response in the liver while avoiding the onset of pathology.

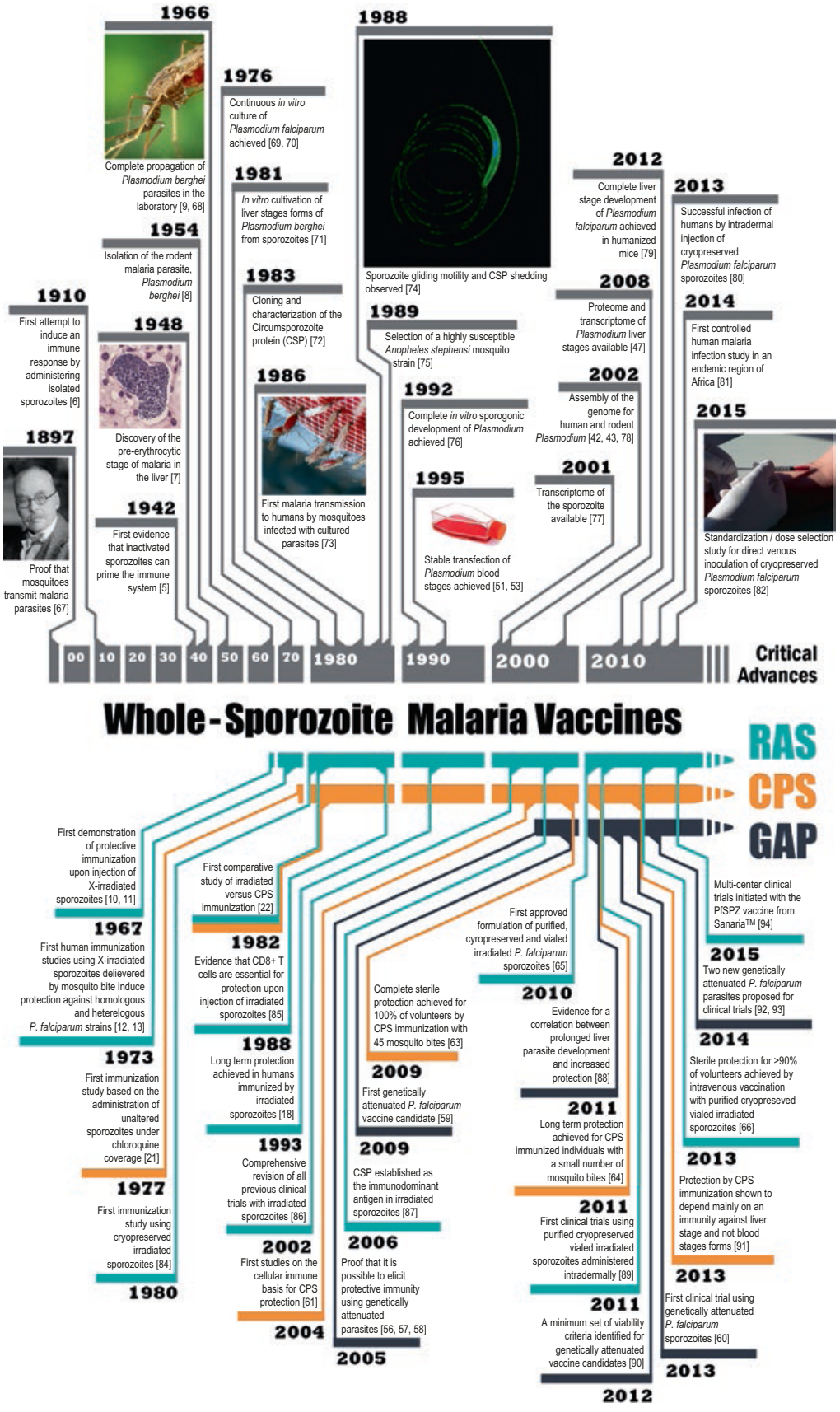

Fig. 1 Timeline of critical events in whole-sporozoite malaria vaccine development. *Top* [5–9, 15–35], *bottom* [10, 13, 36–59]

Nonetheless, some of the potential limitations of sporozoite-based vaccination strategies were already evident in the 1970s [65]. Technical procedures to manufacture and store the large amounts of sporozoites required for immunization, as well as to provide safe and efficacious administration to humans, were always a concern and justified the skepticism with which some of these advances were received [66]. These difficulties, along with extraordinary developments in molecular biology methods, led to a substantial shift from sporozoite-based vaccines to subunit vaccination approaches, based on only one or a few parasite antigens with high immunogenic potential [67]. Drawing upon studies aiming to characterize the basis of the protective responses elicited by X-radiation-attenuated sporozoites, the circumsporozoite protein (CSP) [68–70] emerged as the strongest contender for a subunit vaccine and constitutes the basis of the currently most advanced of such vaccine candidates, RTS,S [71–79]. However, the initial optimism generated by RTS,S over 20 years ago has been steadily decreasing over the years [80]. The latest field studies in endemic regions show that vaccine efficacy is substantially inferior to what are the commonly accepted standards for mass distributed vaccines [81–83].

As the reduced efficacy of subunit vaccines became increasingly evident at the beginning of the new century, a renewed call for the development of sporozoite-based immunization strategies took place. At around the same time, malaria research was also entering into the post-genomic era, with the genome sequences of various *Plasmodium* parasite species becoming available, as well as transcriptomic and proteomic data from different parasite developmental stages [15–17, 84–87]. This knowledge fed the search for genes playing essential roles in distinct points of the parasite's life cycle, which could be targeted for deletion using well-established methodologies for stable transfection of *Plasmodium* parasites [18, 19, 88–92]. This led to the design and construction of genetically attenuated parasites (GAPs), whose liver-stage development is arrested by deletion of specific gene(s). Initial studies in mice showed that GAPs were able to elicit a strong immune protection [39, 40, 93]. Shortly afterwards, the first *P. falciparum* GAPs were developed, paving the way for the clinical development of genetically attenuated sporozoites as human vaccine candidates [41, 42]. Concomitantly, strategies that entailed the induction of immunity through sporozoite administration under chemoprophylaxis (CPS) were brought back to the spotlight, with pivotal studies being carried out in mice [43, 94] and in humans [44, 45]. Also in the last decade, substantial progress was made towards overcoming some of the technical hurdles faced by RAS vaccination [46], which eventually culminated in the demonstration of the induction of sterile protection by the intravenous injection of aseptically purified, metabolically active, radiation-attenuated *P. falciparum* sporozoites [47] (Fig. 1).

Recent clinical studies played an important role in consolidating and extending the knowledge accumulated throughout the years regarding the immune responses elicited by sporozoite-based immunization approaches. We now possess a significant understanding of the protective mechanisms at play during whole-sporozoite vaccination. Undoubtedly, our ever-increasing knowledge of these mechanisms will play an instrumental role in guiding future efforts toward the development of a truly effective malaria vaccine.

2 Radiation-Attenuated Sporozoites (RAS)

Radiation-attenuated sporozoites (RAS) have been considered the "gold standard" of whole-sporozoite vaccination [95]. The concept of RAS vaccination was originally described in the late 1960s, when sterile protection from *Plasmodium* challenge was achieved in mice immunized by the mosquito bite administration of *P. berghei* sporozoites attenuated through the X-irradiation of the mosquito vector [10]. These initial studies with rodents paved the way for early studies in humans that established that immunization of volunteers through the bites of irradiated mosquitoes carrying *P. falciparum* or *P. vivax* sporozoites afforded protection against a subsequent *P. falciparum* or *P. vivax* infection [12–14, 96–98]. Between 1989 and 1999, an additional 11 volunteers were immunized by the bites of irradiated *P. falciparum*-infected mosquitoes at the Naval Medical Research Center. Of note, volunteers in these studies averaged nine immunizations with a mean of over 1000 immunizing mosquito bites per volunteer over 9–10 months before the first challenge [51]. Challenges were performed by the bites of mosquitoes infected from cultured *P. falciparum* gametocytes [26, 99], which has since become the method of choice for controlled human malaria infections (CHMIs). A total of 26 challenges were performed in these studies, and protection was observed in 24 of them, with the reported persistence of protective immunity of at least 42 weeks [51].

Despite the protective immunity elicited by these immunization protocols, for a long time, large-scale vaccination with irradiated sporozoites was considered unfeasible, due to the large numbers of parasites required and the need to deliver them alive by infected mosquitoes. As we will see, the twenty-first century brought along significant developments in this regard, to the extent that many researchers no longer consider RAS vaccination impractical. However, recent advances notwithstanding, earlier studies not only established the potential of attenuated sporozoites for malaria vaccination but also began to shed light on the immune responses that govern sterilizing immunity against *Plasmodium* infection.

2.1 Immune Correlates in RAS Immunization

2.1.1 Antibody and T-Cell Responses in Mice and Monkeys

Antibodies have been shown to be effective at limiting the number of parasites that successfully reach the liver [100]. CSP is the most abundant protein on the surface of *Plasmodium* sporozoites and plays a pivotal role in their characteristic gliding motility [101] (Fig. 1). Antibodies against the repeat region of CSP function by blocking both sporozoite motility and invasion of host cells [102]. Passive protection of mice and monkeys by monoclonal or polyclonal antibodies against the central repeat of *P. berghei*, *P. yoelii*, or *P. vivax* CSP has been amply demonstrated (reviewed in [103]). The efficacy of the protective humoral responses targeted to the

extracellular, infective sporozoite depends on the concentration and specificity of the anti-sporozoite antibody response [104, 105]. However, besides humoral responses, cell-mediated immune responses of the RAS-immunized host also play a crucial role in the generation of immunity against sporozoite challenge.

$CD8^+$ T cells have been shown to be important for eliminating parasites that successfully invade and replicate within hepatocytes, making the liver stage the primary target for the vaccine-inducible T-cell responses and the presumed target of the RAS vaccination model [100]. It has been suggested that antigens present in the skin following sporozoite injection are presented in the skin-draining lymph nodes, where they prime specific $CD8^+$ T cells which then migrate to the liver [106], where $CD8^+$ T-cell-mediated elimination of *Plasmodium* hepatic stages occurs [107]. The transporter associated with antigen processing (TAP)-associated processing of exoerythrocytic antigens was also shown to be critical for the presentation of liver-stage antigens by infected hepatocytes [108], and, very recently, it was proposed to play a crucial role in $CD8^+$ T-cell-mediated, *P. berghei* RAS-induced sterile protection [109].

The role of T cells in the protection conferred by RAS immunization was originally established by results showing that whereas immunization of T-cell-deficient mice did not result in the generation of protective immunity, B-cell-deficient mice could still be protected following immunization with irradiated sporozoites [110]. Later, passive and adoptive transfer studies in mice further demonstrated that irradiated sporozoites induce potent cell-mediated immunity in the absence of antibodies [111, 112]. Selective depletion of T-cell subsets in RAS-immunized mice by treatment with monoclonal antibodies against $CD4^+$ or $CD8^+$ T cells subsequently showed that, whereas in vivo depletion of $CD4^+$ T cells did not reduce immunity, depletion of $CD8^+$ T cells completely reversed protection [50, 113]. $CD8^+$ T cells were also shown to play an essential role in sterile immunity of *rhesus* monkeys following attenuated sporozoite vaccination [114]. The exact effector mechanisms utilized by the protective memory $CD8^+$ T cells to eliminate liver-stage parasites are yet to be fully elucidated. Studies in mice immunized with *P. berghei* RAS have suggested that IFNγ produced in response to parasite-specific stimulation of $CD8^+$ T cells is a critical effector molecule for protection [113, 115, 116]. However, whether protective mechanisms are indeed mediated by IFNγ produced by $CD8^+$ T cells or by cytolytic perforin and granzyme through direct contact in the absence of IFNγ is not yet absolutely clear (reviewed in [100, 117, 118]). $CD3^+$ γδ T cells have also been implicated in RAS-elicited protective immunity against *P. yoelii* in the absence of αβ T cells [119]. Besides, a role for interleukin (IL)-12, nitric oxide (NO), and NK cells for RAS-induced protective immunity initiated by $CD8^+$ T cells has also been proposed [120, 121].

The pivotal role of $CD8^+$ cytolytic T-cell-mediated protection following RAS vaccination of mice is now fully established, although the quantity or functionality of the protective $CD8^+$ T-cell response can differ based on the rodent model [95]. In fact, although most studies suggest that effective RAS immunization in mice appears to depend on $CD8^+$ T cells, in some strains of mice, protection mediated by $CD8^+$ T cells is not absolute, in which case $CD4^+$ T cells are also crucial [103]. Depletion of $CD8^+$ T cells in some strains of mice does not affect protective immunity against

P. yoelii [122]. Moreover, *P. yoelii* and *P. berghei* RAS-induced sterile immunity has been observed in $CD8^+$ T-cell-deficient mice, where protection appears to be mediated by antibodies and $CD4^+$ T-cell-derived IFNγ [123].

Sporozoites and intrahepatic parasite stages contain over 1000 potential antigens [31]. This has led to alternative suggestions that the effectiveness of RAS immunization might result from broad immune responses against multiple antigen targets [124] or, instead, from a few immunodominant antigens that mediate the protection [52]. Several studies have pointed to CSP as a crucial determinant of T-cell responses following RAS immunization. The first evidence for a role for CSP in $CD8^+$ T-cell-mediated immunity elicited by irradiated sporozoites came from passive transfer and in vitro studies that identified epitopes in the carboxyl terminus of the CSP of *P. berghei* [125] and *P. yoelii* [126], which are recognized by T cells from immunized mice. An epitope of *P. falciparum* CSP was later identified in studies with mice immunized with sporozoites or with recombinant vaccinia virus expressing *P. falciparum* CSP [127]. More recently, it was shown that in the absence of T-cell-dependent immune responses to CSP, protection induced by immunization with two doses of irradiated *P. yoelii* sporozoites was greatly reduced [52]. Another study has shown that protective immunity induced by *P. yoelii* RAS in transgenic mice expressing K^d molecules under the MHC-I promoter is mediated by CSP-specific, Kd-restricted $CD8^+$ T cells [128], further supporting the notion that CSP is indeed an immunodominant protective antigen in mice. However, it has also been shown that, after RAS hyperimmunization, complete $CD8^+$ T-cell-mediated protection can occur in transgenic mice that are T-cell tolerant to CSP and cannot produce antibodies [52]. In an effort to identify these non-CSP-protective T-cell antigens, the synthetic peptides corresponding to 34 *P. yoelii* sporozoite antigens that were predicted to contain strong $CD8^+$ T-cell epitopes were screened for the presence of peptide-specific $CD8^+$ T cells secreting IFNγ in splenocytes of the same hyperimmunized transgenic mice. This study revealed that the numbers of IFNγ-secreting splenocytes specific for the non-CSP antigen-derived peptides were 20–100 times lower than those specific for the CSP-specific peptide. Besides, when mice were immunized with recombinant adenoviruses expressing selected non-CSP antigens, the animals were not protected against challenge with *P. yoelii* sporozoites, despite the large numbers of $CD8^+$-specific T cells generated [129]. While the immunodominance of CSP has been largely accepted, its role in immunity has been questioned by studies employing the expression of heterologous CSP proteins by *P. berghei* and *P. yoelii* sporozoites [130, 131]. Interestingly, adoptive transfer of $CD4^+$ CTLs from mice immunized with *P. berghei* RAS that recognized an unidentified non-CSP sporozoite and blood-stage antigen conferred protection to naïve mice [132]. Nevertheless, immunization of mice with a peptide representing a $CD4^+$ T-cell epitope in the N-terminus of *P. berghei* [133] or *P. yoelii* [134] CSP also conferred protection. It has also been argued that protective subdominant antigens may be expressed both by the sporozoite and by liver-stage parasites to allow recognition by $CD8^+$ T cells primed in the lymph nodes by sporozoites, an expression that may be critical to induce protective $CD8^+$ T cells [100]. In any event, the overwhelming evidence accumulated over the last few decades strongly suggests that CSP plays a pivotal role in the protection conferred by RAS immunization.

2.1.2 Early Studies of Antibody and T-Cell Responses in Humans

Ever since their identification, anti-CSP antibodies have been considered candidates for mediating protective immunity against malaria [67]. RAS immunization of human volunteers has been shown to elicit the production of antibodies to both the repeat and the flanking regions of CSP [61, 63], with antibody levels paralleling the serum inhibitory activity of sporozoite invasion of hepatoma cells in vitro [63]. Antibodies against the immunodominant B-cell epitope of *P. falciparum* CSP, identified as a repetitive $(NANP)_3$ sequence [69, 135, 136], inhibit sporozoite infectivity in vitro [70, 137] and in vivo [138]. However, the efficacy of a $(NANP)_3$ peptide-based vaccine was limited by the absence of *Plasmodium* T-cell epitopes [139, 140], in agreement with the notion that, in naturally exposed individuals, anti-CSP antibody levels seem to correlate with exposure but not necessarily with protection (reviewed in [141]). Antibodies against *P. falciparum* sporozoite surface protein 2 (SSP2) have also been identified in RAS-immunized volunteers protected against malaria [142].

CSP-specific cellular immune responses were described in RAS-immunized volunteers as determined by the proliferation of peripheral blood mononuclear cells (PBMCs) upon in vitro stimulation with recombinant *P. falciparum* CSP [61, 143]. An epitope mapping to the 5′ repeat region of *P. falciparum* CSP was identified in T-cell lines and clones obtained from a sporozoite-immunized human volunteer [144]. Another epitope (peptide 326–345) of the *P. falciparum* CSP was later shown to be recognized by human cytolytic class II-restricted $CD4^+$ T cells [145]. This epitope contains both a polymorphic [146] and a conserved [69] region of *P. falciparum* CSP, consistent with strain cross-reactivity of RAS-induced protection. A subsequent analysis of the genetic restriction of a series of T-cell clones derived from three RAS-immunized volunteers showed that peptide 326–345 can be presented by multiple MHC class II HLA-DR alleles [147]. Most of the clones analyzed in this study recognized a series of variant peptides representing the amino acid substitutions found in the polymorphic region of different *P. falciparum* strains, suggesting that polymorphism might not constitute a serious obstacle for recognition of this region of CSP by $CD4^+$ T cells [147].

Despite the wide array of direct evidence regarding the contribution of *Plasmodium*-specific $CD8^+$ T cells in protection in mouse models of RAS immunization, the inability to conduct $CD8^+$ T-cell depletion studies in humans makes direct evidence of $CD8^+$ T-cell involvement in immunity harder to obtain. However, as we will see below, liver-stage-specific cytokine-producing $CD8^+$ T-cell responses are induced by RAS immunization in humans and may indeed contribute to protection.

2.1.3 RAS Immunization-Based Discovery of Preerythrocytic *P. falciparum* Antigens

Following the discovery that particular HLA class I and class II alleles were associated with resistance to severe malaria in Gambian children [148], an HLA-based approach termed reverse immunogenetics has been employed to identify epitopes

from preerythrocytic-stage antigens of *P. falciparum*. This approach enabled the identification of CTL epitopes for two common HLA-B alleles in the liver-stage antigen 1 (LSA-1) protein of *P. falciparum* [149, 150] as well as epitopes for several class I antigens that are found at high frequencies in Caucasians and/or Africans, including CSP, SSP2, and sporozoite threonine- and asparagine-rich protein (STARP) [149, 151].

Antigen discovery studies in naturally infected populations have been significantly complemented and extended by analyses performed in the context of RAS immunization. Proliferative T-cell responses to *P. falciparum* SSP2 have been reported in protected RAS-immunized volunteers suggesting that this antigen is a target of protective immunity [142]. HLA-B8-restricted responses against two epitopes of SSP2, one of which is conserved and the other variant among *P. falciparum* isolates, have also been reported in two *P. falciparum* RAS-immunized individuals [152]. Later, a panel of 27 open reading frames (ORFs) previously identified by a proteomic analysis [31], representing antigens potentially expressed in the sporozoite and intrahepatic stage of *P. falciparum* life cycle, was interrogated, leading to the identification of 16 antigenic proteins recognized by *P. falciparum* RAS-immunized volunteers [153]. Peptides derived from four previously well-characterized *P. falciparum* preerythrocytic-stage antigens [CSP, SSP2, LSA-1, and exported protein 1 (EXP-1)] were analyzed in parallel. Noteworthy, the reactivity against several of the newly identified antigens, most notably antigen 2 (later designated CelTOS—cell-traversal protein for ookinetes and sporozoites [154]), exceeded those observed against the well-characterized antigens [153]. In a subsequent study, protein microarrays representing 23 % of the entire *P. falciparum* proteome were probed with plasma from individuals either showing sterile protection or not protected against infectious *P. falciparum* challenge, following experimental *P. falciparum* RAS immunization [155]. Overall, a markedly different pattern in antibody recognition was observed between the protected and non-protected groups of individuals, in agreement with previously obtained data [156]. A panel of 19 preerythrocytic-stage antigens, 16 of which were novel, was identified as strongly associated with RAS-induced protective immunity, which may form a potential signature associated with sporozoite-induced protection [155]. More recently, 27 out of 151 recombinant *P. falciparum* preerythrocytic antigens selected bioinformatically were recognized by plasma from RAS-immunized volunteers. Twenty-one of these antigens induced detectable antibody responses in mice and rabbits. Moreover, PBMCs from RAS-immunized subjects elicited positive ex vivo or cultured ELISpot responses against peptides from 20 of 22 of these antigens [157]. This study also showed that peptides spanning the full length of *P. falciparum* CelTOS recalled significantly higher positive responses from protected than non-protected subjects [157].

Collectively, these results suggested that immune responses to *Plasmodium* are complex and multispecific. These data further suggest that serodominance does not necessarily correlate with sporozoite-induced immunity and that some of the novel antigens identified by recently employed strategies may represent good candidates for vaccine development [153, 155].

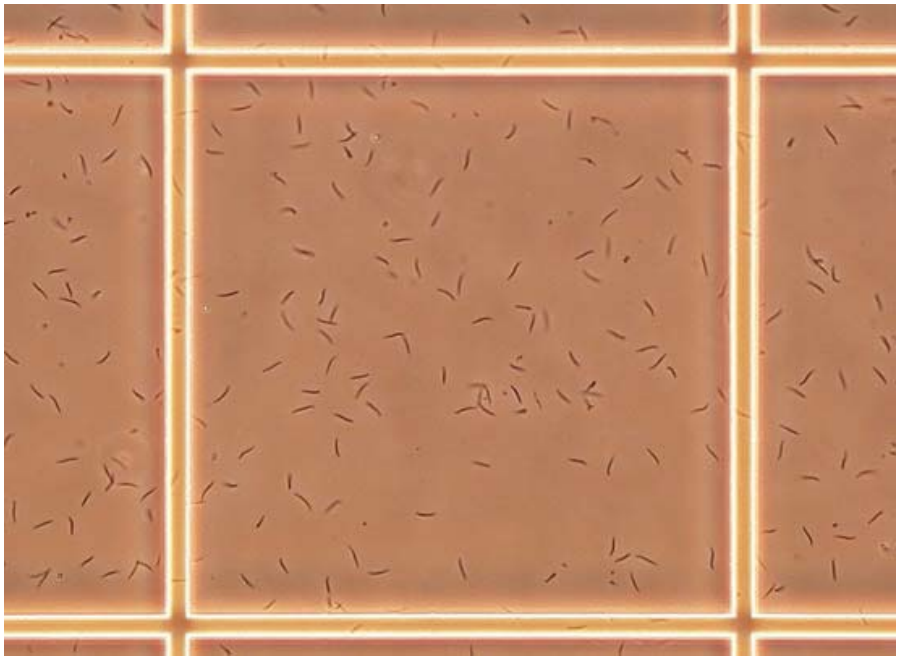

Fig. 2 Purified PfSPZ. The purification process significantly reduces the amount of salivary gland and mosquito material contaminating the PfSPZ (200× magnification). Photo kindly provided by Sanaria™ Inc.

2.2 Recent Progress in RAS Immunization

The twenty-first century witnessed a renewed interest in RAS immunization, which, to a significant extent, was due to the efforts of the company Sanaria™. This company has developed a radiation-attenuated, metabolically active, non-replicating *P. falciparum* sporozoite vaccine, the PfSPZ vaccine (Fig. 2) [46, 66]. The vialed PfSPZ vaccine is aseptic purified and cryopreserved in liquid nitrogen vapor phase (LNVP) [158], by an undisclosed method. Non-attenuated PfSPZ, cryopreserved by the same method, seems to be substantially less infective than their freshly dissected counterparts [159]. Nevertheless, they have been successfully employed in various CHMI studies where they have been administered by intradermal (id; [33, 34, 160]), intramuscular (im; [160–162]), and intravenous (iv; [35, 162]) routes, paving the way to PfSPZ vaccine immunization studies in humans.

2.2.1 The PfSPZ Vaccine Immunization and the Search for Immune Correlates of Protection

The first attempt at vaccination with the PfSPZ vaccine to humans was reported in 2011, when the vaccine was administered to 80 volunteers by id or subcutaneous (sc) injection, prior to challenge with infectious *P. falciparum* sporozoites [54]. Although the vaccine administered by these routes proved safe, it was only poorly

immunogenic and protective, with only two individuals protected out of 44 immunized volunteers challenged by the bites of five *P. falciparum*-infected mosquitoes. Immunized volunteers produced generally low levels of T-cell responses in response to a stimulus with PfSPZ, as measured by IFNγ production, which increased with vaccine dose and number of boosts for the sc but not for the id group of volunteers [54]. Furthermore, PBMCs from immunized volunteers showed a low frequency of PfSPZ-specific $CD4^+$ T cells producing any combination of the IFNγ, tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) cytokines and no $CD8^+$ T-cell responses whatsoever [54]. Also of note, responses to whole sporozoites were more pronounced than to any of the individual *P. falciparum* proteins CSP, SSP2, LSA-1, or CelTOS. The titers of antibodies against whole *P. falciparum* sporozoites and *P. falciparum* CSP were found to increase with vaccine dose and number of doses, but immune sera revealed at best a modest capacity to inhibit *P. falciparum* sporozoite invasion in vitro [54]. These results prompted additional studies in nonhuman primate (NHP) and rabbit models to assess the impact of the route of immunization on protection. The results obtained strongly indicated that immunogenicity and protective efficacy could be greatly improved by iv administration of the PfSPZ vaccine. The suboptimal immunogenicity and protection elicited by vaccine administration by other routes may be explained by the reduced liver load that results from the latter, as was observed in mouse studies employing *P. berghei* [163, 164] and *P. yoelii* [164] sporozoites.

Collectively, the data obtained in this study argue in favor of iv vaccine administration to elicit sterile protection by RAS immunization. Indeed, this is the subject of a groundbreaking study in 2013, where various doses of the PfSPZ vaccine were administered iv to 40 volunteers, prior to challenge with fully infectious *P. falciparum* sporozoites [47]. The results of this study showed that the vaccine was protective, with six out of six and three out of nine volunteers immunized with five and four doses of 135,000 PfSPZ vaccine, respectively, fully protected against malaria. Humoral responses in protected and unprotected subjects were assessed 2 weeks after the last immunization by measuring antibody titers against CSP and PfSPZ, as well as by in vitro assays of inhibition of PfSPZ invasion (ISI). A dose-dependent increase in anti-CSP and anti-PfSPZ antibody titers, as well as in ISI, was observed. However, despite a clear trend for higher antibody titers and ISI in protected than in unprotected individuals, neither of these parameters could directly predict protection. Multiparameter flow cytometry was also employed to assess the frequency of PfSPZ-specific IFNγ, IL-2, TNF-α, or perforin-producing $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+$ γδ T cells and NK cells from PBMCs 2 weeks after the final immunization. Although no absolute correlates of cell-mediated protection could be derived from this analysis, important information about the immune responses elicited by RAS immunization could be obtained: (1) a dose-dependent increase in frequency of PfSPZ-specific $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells producing any combination of IFNγ, IL-2, or TNF-α was observed; (2) there was no significant difference between protected and unprotected individuals for PfSPZ-specific $CD3^+CD4^+$ IFNγ-producing T cells, whereas protected subjects

showed a trend toward higher and more consistent PfSPZ-specific $CD3^+CD8^+$ IFNγ-producing T-cell responses; (3) no differences were observed between protected and unprotected subjects in terms of the quality of the PfSPZ-specific $CD3^+CD4^+$ T-cell responses, while a somewhat higher percentage of PfSPZ-specific $CD3^+CD8^+$ T cells that produced IFNγ only was observed in protected than in non-protected individuals; and (4) the overall frequency of $CD3^+$ γδ T cells increased in subjects who received the highest dose of vaccine. Finally, ELISpot analyses showed that IFNγ responses to PfSPZ were highest in the group that received the highest vaccine load, but IFNγ responses to CSP, SSP2, LSA-1, and CelTOS were low or undetectable in all subjects that received 135,000 PfSPZ vaccine [47].

More recently, another study assessed long-term protection of human volunteers following PfSPZ vaccination. In this report, 7 out of 9 and 6 out of 11 volunteers immunized with four doses of 270,000 iv-administered PfSPZ were protected following CHMI at 3 and 21 weeks after immunization, respectively. Five of the individuals protected at 21 weeks were re-challenged at 59 weeks after immunization and none developed parasitemia [165]. This study further showed that im injection of PfSPZ was substantially less efficient in inducing protection than iv administration of the vaccine, as only 3 out of 8 subjects vaccinated im with 2.2×10^6 PfSPZ were protected 3 weeks after vaccination, and all three developed parasitemia upon repeat CHMI at 25 weeks [165]. On average, anti-CSP and anti-PfSPZ antibody levels measured 2 weeks after the last immunization were significantly higher in protected than in non-protected individuals who underwent CHMI 3 and 21–25 weeks after the final immunization. Interestingly, the percentage of IFNg-, IL-2- and/or TNFα-producing *P. falciparum*-specific $CD4^+$ and $CD8^+$ T cells in the blood did not correlate with outcome at the 3-week or the 21- to 25-week CHMI. However, the absolute frequency of unstimulated $V\delta2^+$ T cells as a percentage of total lymphocytes correlated with outcome at the 21- to 25-week CHMI, while the frequency of the $V\delta2^+$ T cell subset measured prior to the first immunization also correlated with outcome of CHMI at 3 and 21–25 weeks [165]. Nevertheless, it is important to note that neither the antibody nor the cellular correlates found in this study constitute absolute predictors of protection, as no clear threshold of antibody levels or $V\delta2^+$ T cell frequency exists, above which all subjects are protected and below which none are.

Despite the encouraging results obtained with the PfSPZ vaccine, several challenges remain. These include the high numbers of parasites required for immunization, the requirement for iv delivery, the relatively high number of doses required for protection, the possibility that the vaccine might be strain specific, and the duration of protection after immunization [166, 167]. However, efforts to tackle these and other issues are currently being made by Sanaria™ [168] and others. With five stage 1 clinical trials already completed, and an additional seven stage 2 trials in the USA, Europe, and Africa planned to address a set of well-defined objectives [59], significant progress in the clinical development of the PfSPZ vaccine can safely be expected in the near future.

3 Genetically Attenuated Parasites (GAPs)

3.1 Creation of GAPs

As discussed above, human test subjects immunized with *P. falciparum* RAS can be effectively protected from a subsequent challenge of fully infectious *P. falciparum* wild-type sporozoites. In 2005, employing rodent models of malaria, it was reported that similar sporozoite attenuation can be achieved by targeted gene deletion, by removing genes essential for liver-stage development, thereby creating so-called genetically attenuated parasites (GAPs) [40, 93]. Importantly, mice immunized with rodent *Plasmodium* GAPs can induce sterile immunity that is comparable to RAS immunization [169]. An advantage of GAPs over RAS immunization is that GAPs constitute a homogeneous population of parasites with a defined attenuation phenotype. This removes issues related to the delivery of correct doses of irradiation in order to ensure sufficient attenuation without killing the parasites or leaving them competent to produce a blood infection. The conceptual basis of vaccines consisting of GAP, just like RAS, is that after inoculation sporozoites invade but only partially develop in the liver. In the years following the first description of GAPs, a number of gene deletion mutants have been described in the rodent malaria models, *P. berghei* and *P. yoelii*, which are completely or partially attenuated, and manifest an arrest phenotype at different points during liver-stage development which provokes different levels of protective immunity (Fig. 3) [171].

The generation of GAPs, which can serve as a malaria vaccine, requires the creation of genetically engineered sporozoites that can invade liver cells but are unable to complete hepatic development. Furthermore, these parasites must provoke a strong protective immunity against a subsequent human malaria infection. These two features are critical to the development of a malaria vaccine consisting of attenuated parasites: first and foremost it must be safe (i.e., complete attenuation in the liver) and next it needs to be potent (generates strong protective immunity). *Plasmodium* genes that may govern exclusively sporozoite and/or liver-stage-specific functions were first identified by characterizing mRNA transcripts that are only expressed (or highly upregulated) in salivary gland sporozoites [30, 172]. Deletion of some of these gene candidates from the genomes of rodent malaria parasites resulted in the creation of some of the first GAPs [39, 93]. Moreover, as with irradiated sporozoites, immunization with sporozoites lacking expression of these proteins generated sterile protective immunity against malaria in mice. Independent studies, also in rodent models, investigating proteins of the *Plasmodium* 6-Cys protein family revealed that one member of this family, P52, was vital for parasite development in the liver and immunization of mice with sporozoites lacking P52 could also induce protective immunity [40]. Mutants lacking the 6-Cys protein P52 either by itself or in combination with the deletion of the gene encoding the closely related protein, P36, were among the first GAP to be created in *P. falciparum* [41, 55, 173]. Recently, a newly identified member of the 6-Cys protein family, B9, has

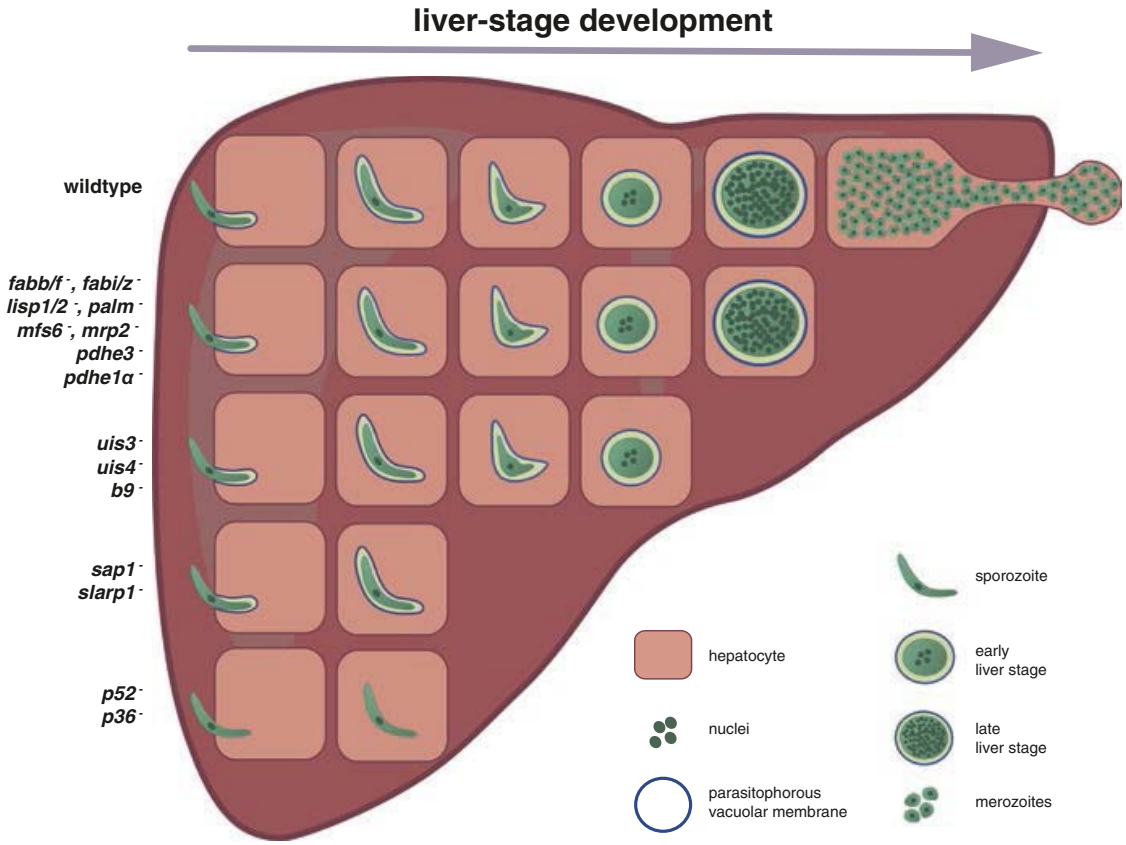

Fig. 3 Liver-stage developmental arrest of GAPs in rodent malaria models. Adapted from [170] and kindly provided by Britta Nyboer

also been shown to be critical to parasite development in the liver, and mutants lacking this protein have been shown to elicit strong protective immunity in rodent models [58, 174]. After the initial identification of these rodent GAPs, a number of other gene deletion mutants have been identified that are completely or partially attenuated and shown to induce protective immunity [171, 175] (Fig. 3). However, of all the GAP candidates in rodent models that could be advanced as a potential *P. falciparum* GAP vaccine, only mutants lacking the gene *slarp* (alternatively known as *sap1*), which encodes a protein involved in the regulation of transcripts in sporozoites, have been shown to fully arrest during liver-stage development, even at very high sporozoite doses (up to 5×10^6 sporozoites inoculated intravenously) [176–178]. Recently, GAPs generated in both *P. berghei* and *P. falciparum* lacking the gene *mrp2* that arrest mid-late during liver-stage development are only the second mutant that appears to be fully arrested in the liver cell even at very high sporozoite doses [179]. All other GAPs have been shown to be capable of leading to breakthrough blood infections when the dose of sporozoites is increased [171]. Consequently, the GAPs that are most advanced for human use and currently ready for clinical evaluation consist of mutants where *slarp*/*sap1* has been removed in addition to the deletion of one or more of the 6-Cys genes from the *P. falciparum* genome. Specifically, the *P. falciparum* GAPs "*p52(−)/p36(−)/sap1(−)*" and "Δ*b9*Δ*slarp*" have both been examined in blood-stage culture, in mosquitoes, in cultured human hepatocytes, and in mice engrafted with human liver tissue and have

been shown to only exhibit a growth defect after liver cell invasion [57, 58]. They completely abort intrahepatic development, demonstrating that they are likely to be safe for use in humans; currently these GAPs await clinical testing to evaluate their protective efficacy in humans. Importantly, these *P. falciparum* GAPs are expected to arrest early in hepatic development, soon after invasion of hepatocytes by sporozoites.

It has been reported that a late-arresting rodent GAP was a superior immunogen to irradiated sporozoites and early-arresting GAPs [53]. GAPs that progress into late liver-stage development will express both a greater quantity and a greater diversity of antigens, compared to early-arresting GAPs and RAS. The hypothesis is that parasites that arrest late into liver-stage development will not only expose the immune system to a greater magnitude of antigens (a mature liver stage contains up to 40,000 times more protein than an invading sporozoite) but also to a greater diversity of antigens, as they will express antigens expressed in irradiated sporozoites as well as antigens expressed later in the liver and in blood stages (Fig. 3).

3.2 Immunity Induced After GAP Immunization

Depending on the type and/or strength of attenuation, parasites arrest at different time points during liver-stage development or shortly thereafter. Heat-killed or over-irradiated sporozoites fail to invade hepatocytes and hence do not confer protection [180]. This emphasizes the importance of the preerythrocytic stage and the requirement for metabolically active, attenuated parasites that progress at least to early hepatic development before they arrest [181]. Sterile protective immunity in whole-sporozoite experimental vaccinations highly depends on the parasite species, mouse strains, and the immunization regimens. As such, sterile protection is more easily achieved in the *P. yoelii*/Balb/c experimental model than in the *P. berghei*/C57/Bl6 model [121, 182].

Protective immunity has been reported to rely on $CD8^+$ T cells and IFNγ as the key mediators of protection in all different GAP models [169, 183, 184]. Depletion of $CD4^+$ T cells did not abrogate protection, although adoptive transfer of $CD4^+$ T cells from, e.g., *uis3(−)*-GAP-immunized mice protected 50 % of naïve animals from wild-type infection, indicating a role also for this T-cell subset in GAP-induced immunity. Humoral responses to CSP were measured in the *P. berghei* GAP model but appeared to be dispensable since B-cell-deficient mice were capable of sterile protection [183].

Similarly to RAS, GAP immunization induces a significant increase in intrahepatic $CD8^+$ T cells displaying a T effector memory phenotype (Tem) that is clearly associated with long-term protection [185, 186]. Concerning direct effector mechanisms, Trimnell et al. hypothesized that contact-dependent cytotoxicity is mediated by $CD8^+$ T cells since sterile protection was abolished in perforin-deficient mice. Moreover, $CD8^+$ T cells induced apoptosis in infected hepatocytes in vitro [169, 186].

One dose of *P. yoelii uis4(−)* sporozoites was sufficient to induce protection against challenge infection in a Balb/c mouse background [184] that is mediated by the significant upregulation of specific effector CD8⁺ T-cell activation markers such as CD11c. It was suggested that the upregulated CD11c expression phenotype on effector CD8⁺ T cells induced by *P. yoelii* GAP vaccination is not only responsible for their potent inflammatory function but also marks their highly proliferative but short-lived, antigen-specific effector activity, which will ultimately lead to controlling both infectious sporozoite hepatocyte invasion and intrahepatic differentiation [187].

A second generation of GAP leading to a late arrest in liver-stage development was introduced more recently. Immunization with *Pyfabb/f(−)* parasites that are depleted of a gene encoding an essential enzyme of the apicoplast-localized, type II fatty acid biosynthesis (FAS II) pathway induced long-lasting protection in rodent models [53, 188], despite later evidence of breakthrough infections by *Pbfabb/f(−)* parasites [55]. The results of the underlying study further suggested that a larger number of effector memory CD8⁺ T cells with a broader antigenic specificity were elicited by immunization with *Pyfabb/f(−)* GAPs as compared to early-arresting GAP or even RAS. These characteristics of *Pyfabb/f(−)*-induced immune responses correlated with enhanced protection, and thus it was postulated that late-arresting GAP confers superior protective immunity than early-arresting attenuated parasites. Besides, it was shown that this superior protection is stage transcending, i.e., mice immunized with *Pyfabb/f(-)* are not only sterile protected against sporozoite-induced challenge infection but also enjoy protective immunity after intravenous challenge with homologous *P. yoelii* blood-stage parasites [53].

As such late-arresting live attenuated parasites are promising for next-generation vaccine design, it is now imperative to gain a more detailed overview about the underlying immunological functions triggered during experimental vaccination, before such attenuated parasites can be optimized for human clinical trials. Protective immunity induced by the late-arresting experimental whole-organism model parasite *Pyfabb/f(−)* was comprehensively studied over the last few years. It was recently found that not only CD8⁺ T cells but also CD4⁺ T cells contribute to protective responses and that the co-stimulatory molecule CD40 is key in mediating robust protection. CD40 signaling was demonstrated to control intrahepatic DC licensing during *Pyfabb/f(−)* immunization and seems to be instrumental for establishing robust CD8⁺ effector and memory T-cell responses [189]. Very recently, the immunity mechanisms underlying stage-transcending protection exclusively induced by late-arresting *Pyfabb/f(−)* GAP [53] were reported to involve both the cellular and humoral arm of the immune system. Hence, it is proposed that during experimental vaccination with late-arresting GAPs, the immune system gets equipped to establish immunity against both the clinically silent, intrahepatic and pathological, blood-stage phase. As for the humoral response, it is thought that antigenic targets are shared between the hepatic and blood-stage phase and protective antibodies rely on F_c-mediated functions such as F_c receptor binding and complement fixation [190]. Indeed, a study by Keitany et al. showed for the first time that functional antibodies are induced in *Pyfabb/f(−)* GAP-immunized mice and inhibit

liver cell invasion as well as the capacity to decrease intrahepatic parasite burden [191]. It is a matter of course that such GAPs not only inform about the underlying immunological mechanisms involved in protection against parasites but will also be ideal to further isolate novel antigens and/or correlates of (transcending) sterile protection. The latter will ideally assist in the development of novel subunit-anti-infective vaccination strategies. Interestingly, a study headed by Chen et al. reported the identification of *Py*Tmp21 as a promising protective antigen candidate that shows the capacity to reduce intrahepatic parasite burden and contribute to sterile protection during *Pyfabb/f(−)* GAP experimental immunization [192, 193].

Experimental immunization of rodents with a knockout parasite line for the *Plasmodium*-specific apicoplast protein important for liver merozoite formation (PALM) elicits potent and long-lived protective immunity against sporozoite-induced malaria by only two immunization doses. Indeed *palm(−)* knockout parasites are very similar to the *Plasmodium* late-arresting *fabb/f(−)* strain, as PALM seems to be important for the successful transition from liver to blood-stage parasites, i.e., display a growth arrest at a very late time point in intrahepatic development [194]. Indeed, a thorough study of the underlying immunity mechanisms of *palm(−)*-vaccinated rodents revealed persistence of some intrahepatic antigen-specific IFNγ-secreting $CD8^+$ memory T cells in aging immunized mice [195, 196].

The involvement of innate immunity in GAP-mediated immune responses still remains to be investigated. GAP immunization of *RAG1 (−/−)* mice that lack adaptive immune cells (which are traditionally considered to mediate memory responses to vaccinations) resulted in a delayed disease outbreak after challenge with viable sporozoites, suggesting that the innate immune system in these mice is sufficient to confer partial protection after vaccination, e.g., through NK memory cells [183]. However, the cellular events and molecular pathways underlying the process of memory generation in NK cells remain largely elusive.

Regarding antigenic specificity, only CSP-specific, IFNγ-secreting $CD8^+$ T cells, as well as CSP-specific antibodies, were reported to be elicited by GAP immunizations. Indeed, Kumar et al. measured no significant differences in these CSP-specific responses in Balb/c mice that were either immunized with *Py*RAS or *Pyuis3(−)* and *Pyuis4(−)* genetically attenuated parasites. The authors therefore postulated conserved and similar mechanisms of protection irrespective of the method of attenuation [183, 197].

Recently, the results from a first clinical trial demonstrated safety of *Pf*GAP vaccination in humans using *Pfp52(−)/p36(−)* double knockout parasites. Initial immunological profiling showed CSP-specific antibody titers after 200-bite exposure of volunteers and moderate memory responses of $CD8^+$ and $CD4^+$ T cells to CSP and non-CSP antigens such as LSA-1 or AMA-1. Of importance, a breakthrough infection was observed in one of the *Pf*GAP-immunized volunteers suggesting that development of more severely attenuated *Pf*GAP, possibly with multiple gene deletions, should be pursued in future developments [42].

It has been demonstrated that immunization of mice with late liver-stage-arresting GAP elicits broader and better $CD8^+$ T-cell responses when compared to irradiated (early-arresting) sporozoites, producing superior long-lasting sterile protection

against a malaria infection [53]. These parasites are believed to present a greater diversity and amount of parasite antigen to the host immune system. However, *P. falciparum* parasites lacking FabB/F expression fail to produce sporozoites [198]. Thus, discrepancies in attenuation phenotypes and potency between GAPs of different *Plasmodium* species indicate that the importance of the same gene for liver-stage development may differ between rodent models and *P. falciparum*. Consequently, the identification of *Plasmodium* genes that are critical to mid-/late liver-stage development to create late-arresting *P. falciparum* GAPs is actively being sought, and a number of gene expression studies on rodent and human sporozoite and/or intrahepatic liver stages are currently being mined to identify these genes.

4 Chemoprophylaxis and Sporozoite (CPS) Immunization

While sterile protection has been most elaborately investigated using RAS, induction of protective preerythrocytic immunity is most potent and efficient when sporozoite and liver-stage development remain fully intact, while asexual-stage multiplication is abrogated at the earliest stages [59, 199, 200]. Attempts with targeted deletions of genes encoding late-stage liver antigens are promising, but, as outlined above, safety has thus far not been appropriately ensured in humans because of the occurrence of breakthrough infections with asexual-stage parasites [42, 175, 199]. Successes, however, have been reported with the use of antimalarial drugs in combination with wild-type parasites: chemoprophylaxis and sporozoites (CPS) immunization (or infection-treatment-vaccination (IVT)) can safely induce fully protective immune responses as was first demonstrated in rodents [37, 38, 43, 201]. The first drug used in these *P. berghei* studies to kill parasites at early blood-stage level was chloroquine, which disrupts heme detoxification and does not affect preerythrocytic stages [202, 203]. Other antimalarials have since been successfully used in rodents, including artesunate, azithromycin, clindamycin, mefloquine, piperaquine, primaquine, and pyrimethamine with variable effects on parasite liver-stage development [204–208]. Similar results have been obtained in the CHMI model where malaria-naïve adult volunteers receiving mosquito bites from a total of 36–45 *P. falciparum*-infected mosquito under chloroquine prophylaxis (Fig. 4a) show complete protection against a homologous challenge 3–5 months after the last immunization and (partial) protection up to 2.5 years later [44, 45, 56]. Chloroquine and mefloquine are equipotent as prophylactic drugs [209], and other partner drugs are under investigation in ongoing trials [59]. As in murine models [43, 210, 211], induction of sterile CPS-induced protection is dose dependent (Fig. 4b) [209]. Both partial and full protection against a heterologous *P. falciparum* strain after CPS immunization have been demonstrated in a rechallenge trial [212] and are under further investigation (clinicaltrials.gov NCT02098590).

The potency of the CPS-chloroquine regimen is unprecedented compared to radiation-attenuated sporozoite (RAS) immunization and to protection obtained

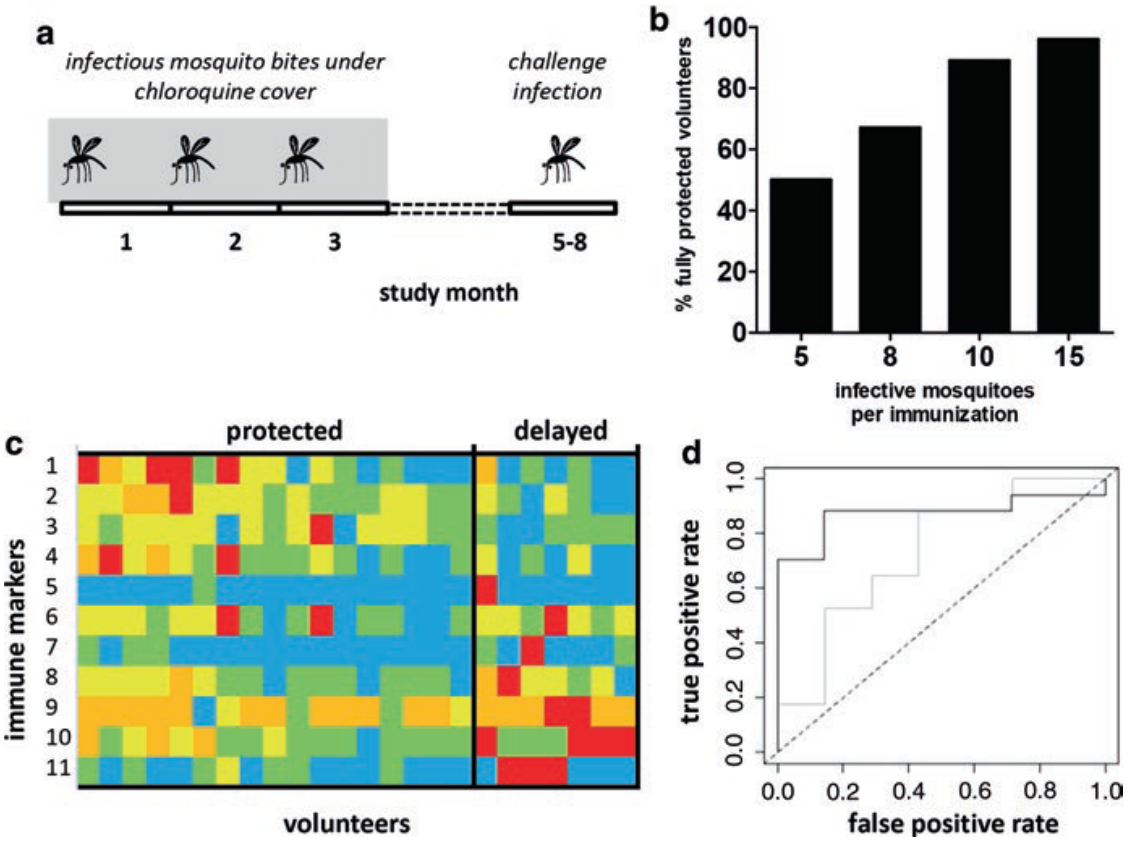

Fig. 4 CPS immunization regime, protection, and cellular immune signature in human volunteers. (**a**) Malaria-naïve adult volunteers are subjected monthly to bites of *P. falciparum*-infected mosquitoes while taking chemoprophylaxis. Protection is assessed by challenge infection via infected mosquitoes 3–5 months after the last immunization. (**b**) Sterile protection defined as the absence of parasitemia by microscopy until 21 days after challenge infection is dose dependent as assessed for $n = 58$ volunteers enrolled in four clinical trials using either chloroquine or mefloquine prophylaxis [44, 56, 209]. (**c**) *Rows* show eleven biomarkers that can distinguish $n = 17$ fully protected and $n = 7$ partially protected/delayed volunteers in a dose escalation CPS trial [209], identified by penalized logistic regression. *Colors* indicate the degree of responses, with strongest responses in *red*. (**d**) Accuracy of prediction of protection from mosquito bite challenge assessed with the area under the receiver operator curve based on leave-one-out cross validation (LOOCV), using one parameter alone (parasite-specific degranulating $CD107a^+$ $CD4^+$ T cells, *light gray line*, AUC 0.73) or in combination with ten additional markers (AUC 0.87, *dark gray line*) (unpublished data)

after natural infections [59, 213]. While the dogma that protective malaria immunity is difficult to acquire is challenging, possible explanations including the broader antigenic repertoire, higher antigen load, and potential immunomodulatory effects of chloroquine remain speculative [200]. The CPS model, however, opens avenues for a better understanding of immune mechanisms of protective immunity, identification of immune correlates of protection, and novel target antigens for inclusion in subunit vaccines. Moreover, clinical development of a vaccine based on the CPS regime is currently explored with cryopreserved sporozoites as PfSPZ-CVac (clinicaltrials.gov NCT02511054 and NCT02115516 and [59, 214]).

4.1 Parasite Life Cycle Target for CPS-Induced Protection

The mechanism of CPS-induced immunity appears to be determined by specific parasite/host combinations regarding both the immune effector mechanisms involved and the parasite life cycle stage targeted. Most evidence points to preerythrocytic stages as targets for protective immunity: (1) human CPS-immunized volunteers are protected from challenge by mosquito bite, but not after a blood-stage challenge [56], and (2) CPS immunization of C57/Bl6 mice with *P. berghei* or Balb/c mice with *P. yoelii* with 10,000 or more sporozoites intravenously [37, 43, 203, 207, 208, 215, 216] results in preerythrocytic immunity and reduction of liver parasite burden [43]. Intravenous immunization with high numbers of *P. chabaudi* sporozoites also induces preerythrocytic immunity, while presumably low numbers of sporozoites as delivered by mosquito bites primarily induce immune responses against blood stages in this model [211]. Blood-stage immunity induced by CPS immunization has been reported also in the *P. yoelii* model [208, 215], where control of parasite growth was clearly antibody dependent [208, 215]. More strikingly, there is evidence that exposure to blood stages only can also convey preerythrocytic immunity ([201, 211] and Nganou-Makamdop et al. unpublished data). The combined data suggest that antigens shared between liver and blood stages [17] create the possibility for the existence of stage-transcending protection [217].

Since clinical follow-up of prolonged parasitemia in the CHMI model is limited for obvious safety reasons, CPS studies in humans primarily highlight induction of liver-stage immunity [56]. There are nevertheless hints for the presence not only of immune recognition but also of blood-stage immunity: (1) although humoral responses directed against blood-stage antigens are minimal ([44, 218] and Behet et al. unpublished data) and show no growth inhibition activity in vitro [56], IFNγ responses to blood-stage parasites are enhanced both in vivo [56] and in vitro [44, 45, 209, 219], and (2) immunized volunteers show a somewhat reduced peak parasitemia after blood-stage challenge [56]. These data feed the possibility that at least some functional blood-stage immunity might be present which only becomes apparent after a number of asexual multiplication cycles required for boosting. This hypothesis is in line with a correlation between prepatent period, as determined by thick smear, and the interval between a positive qPCR and positive thick smear as a proxy for parasite growth rate (Bijker et al. unpublished data), in partially protected volunteers (24). Since prepatency itself was not dependent on parasite density at the time of positive qPCR, these data suggest that parasite growth becomes slower when parasitemia is longer lasting. Thus, the CPS model efficiently induces sterile protection that is, in principle, accomplished at the preerythrocytic level. In addition, a blood-stage component may be present, which may contribute in case of partial protection and might become more evident in individuals possessing some degree of anti-disease immunity that allows longer follow-up. Albeit insufficient by itself, the short period of low-grade parasitemia as obtained under chloroquine prophylaxis in humans might be an asset for induction of at least some contributing blood-stage immunity. Indeed, low parasitemia has been shown before to efficiently induce blood-stage protection in the CHMI model [220].

4.2 Humoral Immunity After CPS Immunization

Parasite-specific antibodies induced by CPS immunization may interfere at several levels with preerythrocytic parasite development where sporozoite integrity, motility (gliding), hepatocyte invasion, and/or intrahepatic development may be compromised. In the *P. berghei* murine model, the functional contribution of antibodies to CPS-induced preerythrocytic protection is unclear: in CPS-immunized C57/Bl6 mice, antibodies induced against sporozoites and liver stages inhibit hepatocyte infection in vitro [208], but are unable to confer protection against sporozoite challenge in vivo [43, 208]. In human volunteers, CPS immunization induces both a robust plasma antibody and long-lasting memory B-cell response [218]. This humoral response is largely directed against preerythrocytic parasite antigens with CSP being the major target identified thus far [44, 209, 218, 221]. Plasma IgG from CPS-immunized volunteers inhibits sporozoite traversal in vitro, as well as liver-stage infection in human liver-chimeric mice following challenge by *P. falciparum*-infected mosquito bites [222]. However, anti-CSP titers per se appear to be a correlate of exposure rather than protection in the human CPS model [218]. In fact, anti-CSP titers are the lowest in individuals that become already protected during the immunization period [218]. Since CSP is not only expressed in sporozoites but also in liver stages [32, 223, 224], one possible explanation might be that at least a proportion of the parasites is eliminated by CPS-induced immune responses early during liver-stage development. It remains to be investigated to what level human anti-CSP antibodies will be sufficient to prevent sporozoite infectivity. There is evidence that targets other than CSP might be involved to accomplish sterile protection [52, 130]. The use of protein microarrays probing the antibody compartment for proteome-wide antigen reactivity [156, 225] has already revealed novel protection-associated antigen targets in RAS-immunized individuals [155], and similar investigations are currently ongoing in CPS-immunized volunteers.

4.3 Cellular Immunity After CPS Immunization

As in other whole-sporozoite vaccination approaches [95], cellular responses induced after CPS immunization are considered to be responsible for the key effector mechanisms driving elimination of infected hepatocytes. In the C57/Bl6 *P. berghei* CPS model, there is a sustained $CD8^+$ T-cell IFNγ response [205, 216] correlating with protective efficacy [216], and immunity can be transferred by total splenocytes [203] and $CD4^+$ and $CD8^+$ T cells [43]. Despite a strong $CD8^+$ T-cell response, protection of *P. yoelii* CPS-immunized C57/Bl6 mice, in contrast, was independent of $CD8^+$ T cells, suggesting a role for $CD4^+$ T cells or antibodies instead. CPS immunization in humans induces long-lasting cellular immune responses [44, 45, 209, 219], which are still detectable even 2.5 years after the initial immunization [45]. In line with a role for both Th1 and cytotoxic responses, CPS-immunized volunteers show increased expression of granzyme B and the Th1

transcription factor T-bet in activated and proliferating T cells during immunization [214]. However, a clear immune correlate of protection has not yet been identified, as neither polyfunctional effector memory T cells that coproduce IFNγ and interleukin-2 [44, 45] nor total IFNγ-producing $CD4^+$, $CD8^+$, or γδ T cells that can differentiate between fully and partially protected volunteers [209]. Parasite-specific degranulation by $CD4^+$ T cells and granzyme B production by $CD8^+$ T cells, in contrast, are significantly higher in fully protected individuals [209]. Nevertheless, these cytotoxic responses also do not allow for a distinct differentiation between the two groups. Given the fact that individuals differ in their "immunological fingerprint" and protection likely relies on the interaction of multiple immune effector pathways, a combination of multiple immune markers in the form of an immune signature may be of more predictive of protection than individual responses (Bijker and Teirlinck et al. unpublished data, Fig. 4c, d). Next to adaptive responses, this may also include innate lymphocytes such as NK cells but particularly γδ T cells, which are expanded following CHMI, CPS, and high-dose RAS [47, 219, 226] and show memory-like responses following CPS immunization [209, 219].

Based on observations from natural immunity in the field, the current dogma is that acquisition of protective immunity against malaria per se is a lengthy and difficult process involving many exposures and is rapidly lost in the absence of exposure. The efficient induction and persistence of immunity following CPS immunization, however, clearly refutes this statement and indicates that this may only apply to protection from disease, but not infection. A plausible explanation can be found in the composition of immune responses targeting the different parts of the life cycle involving distinct parasite immune regulation and evasion strategies [200]. CPS immunization induces protection from sporozoite challenge, in line with predominantly preerythrocytic targeted antibodies that reduce sporozoite infectivity and long-lasting cellular cytokine and cytotoxic responses. None of these responses, however, are a clear-cut correlate of protection, and further research is necessary to identify which (combination) of immune responses predicts protection. Importantly, while this immunization regimen has been primarily tested against homologous parasites, heterologous challenge studies are underway and indicate at least some protection. Future studies will need to focus on the identification of target antigens of the induced immune responses and their functionality also against heterologous parasites. Another focus should be to elucidate the potential contribution of blood-stage immunity in this model, particularly in preexposed individuals. Thereby, CPS immunization may not only aid future subunit vaccine development but—once satisfactory—also be considered for clinical development as a vaccine to be marketed for special groups including travelers or the military.

5 Other Models of Sporozoite-Based Vaccination

RAS, GAP, and CPS are, by far, the most thoroughly investigated approaches for whole-sporozoite vaccination against malaria. However, alternative strategies for eliciting protective immune responses against human-infective *Plasmodium* parasites have also been proposed and will be briefly addressed here.

5.1 Chemically Attenuated Sporozoites

Chemical attenuation of sporozoites (CAS) has been investigated as an alternative strategy for the production of a liver-stage vaccine against malaria. Similarly to other whole-sporozoite immunization approaches, the concept of CAS relies on the immune responses elicited during hepatic infection by chemically treated sporozoites, which are unable to progress into a patent blood-stage infection and cause disease. The strategy has been evaluated by the use of centamycin [227], a DNA-binding agent that is effective against blood-stage *Plasmodium* parasites and blocks sexual differentiation of the parasites in mosquitoes [228]. Centamycin-treated *P. berghei* sporozoites were shown to display a significantly reduced hepatocyte infection rate and impaired preerythrocytic development and to be unable to generate blood-stage infections in mice [229]. Immunization of mice with *P. berghei* [229] and *P. yoelii* [229] CAS was shown to produce sterile immunity against challenge with fully infectious sporozoites. Vaccination with *P. yoelii* or *P. berghei* CAS induced sporozoite-specific antibodies and CSP-specific IFNγ-producing $CD8^+$ T-cell responses at levels similar to those generated by RAS immunization [229]. More recently, it was shown that chemical attenuation of *P. berghei* parasites with isopentaquine during their liver-stage development alters disease outcome and protects mice from experimental cerebral malaria [230]. It has been argued that the CAS vaccine presents certain advantages over the RAS approach, most notably the fact that the chemical attenuation process can be strictly controlled, leading to a vaccine that is reproducibly attenuated and does not present the risks potentially associated with improper parasite irradiation [229]. However, further investigations are clearly necessary before the true potential of CAS vaccination can be fully ascertained.

5.2 Rodent Plasmodium-Based Immunization Against Human Malaria

A yet unpublished novel approach for whole-sporozoite vaccination against malaria has recently been proposed, based on the use of genetically modified rodent *Plasmodium* parasites that were genetically engineered to express and present antigens of their human-infective counterparts. The concept relies on the notion that such genetically modified rodent parasites may elicit cross-species immune responses arising from similarities between the antigenic compositions of the two species of parasites (interestingly, immunization with *P. falciparum* CelTOS has previously been shown to confer protection against *P. berghei* [231]), along with antigen-targeted responses resulting from the introduction of specific human *Plasmodium* immunogens on the rodent parasite platform. The proof of concept of this idea has been established through the use of *P. berghei*, a rodent parasite species that is widely recognized as nonpathogenic to humans. *P. berghei* is readily

amenable to genetic modification [232], and strategies to generate transgenic parasites expressing human-infective *Plasmodium* proteins have been developed [233]. A method for transgene expression and gene complementation [234] was employed to generate selectable marker-free *P. berghei* parasites that express *P. falciparum* CSP under the control of the *P. berghei* upregulated in sporozoites 4 (UIS4) promoter [*Pb*(*Pf*CS@UIS4)]. *P. berghei* was shown to effectively infect human hepatocytes of human liver-chimeric mice, a requisite for any whole-sporozoite vaccine against malaria. Immunization of mice with *Pb*(*Pf*CS@UIS4) was shown to elicit the production of antibodies against whole *P. falciparum* sporozoites as well as against *P. falciparum* CSP. Passive transfer of the immune serum of these mice into liver-humanized mice followed by challenge by *P. falciparum*-infected mosquito bites led to a significant blockage of hepatic infection by this human-infective parasite. Following completion of its preclinical validation, the *Pb*(*Pf*CS@UIS4) vaccine candidate is expected to enter Phase I/IIa clinical trials. If successful, the use of "naturally attenuated" rodent parasites for immunization against human malaria could gain further momentum, and next-generation vaccine candidates can be envisaged to protect not only against multiple species of human malaria but also against various stages of the parasite's life cycle.

6 Future Directions and Concluding Remarks

The application of a whole-sporozoite vaccination approach has raised various types of concerns, ranging from safety to deployment in the field. Safety-wise, whole-sporozoite immunization requires ensuring that sporozoites cannot complete their development process in the liver or that merozoites are eliminated as soon as they reach the blood while retaining immunogenicity [60, 235]. On the other hand, the *P. falciparum* sporozoites required for immunization must be obtained from infected mosquitoes, which must be reared under appropriate containment conditions to prevent accidental release. Sporozoites are then obtained from the dissection of mosquito salivary glands and must subsequently be purified and adequately cryopreserved for long-term storage [46]. Finally, application to the field requires knowledge of total dose required for protection and timing of booster immunizations [95], as well as a feasible vaccine administration method that ensures the development of protective immune responses.

Many important aspects to be considered for the design of a next-generation preerythrocytic vaccine may equally account for the development of a blood-stage vaccine. The ultimate question to be answered is what kind of immune response must be induced to confer high-level and long-lived protection and how to accomplish this. This task is both supported and exacerbated by the complex and not always unequivocal data from basic research, preclinical, and clinical studies. In general, it will be vital to improve the knowledge about protective immune

responses, i.e., to properly understand the difference between vaccine-induced protection and naturally acquired immunity, to exploit the data on *P. falciparum* RAS-mediated protection in humans or the large past clinical trials of RTS,S in order to determine which responses are the true mediators of protection.

A substantial amount of data advocates the need to induce antibody response against the sporozoite to prevent liver infection or at least to reduce the number of sporozoites infecting hepatocytes. The latter mechanism is assumed to contribute to a delay in prepatency in RTS,S-vaccinated individuals which consequently limits severe disease. Likewise, it seems unrealistic that complete protection can be mediated solely by humoral responses that were even shown to be dispensable in animal models. In fact, numerous studies have pointed out the essential role of T-cell responses against liver-stage parasites and have explicitly demonstrated the critical function of intrahepatic $CD8^+$ T cells in protective immunity. This has important implications for the quality and/or quantity of the $CD8^+$ T-cell response but also for the liver as the site of T-cell priming and effector function.

It seems clear that research into the immune responses induced by whole-sporozoite immunization approaches has given first insights into the immune mechanisms involved, but an actual correlate of sterile protective immunity has yet to be revealed. Such a correlate is unlikely to be a single common readout, but instead a signature of multiple immune effector pathways acting in synergy. This will likely include both phenotypic and functional readouts of humoral and cellular immune responses as well as a combination of various antigen targets. Complementing ongoing efforts using the protein microarray for genome-wide screening of antimalarial antibody responses, T cells which target antigen can also be identified by large-scale screen approaches, such as the Atlas™ assay using protein plasmid and expression libraries [225]. Application of this screening technology to samples from RAS- as well as CPS-immunized individuals is currently under way [225] and should be extended to GAP trials in the future. Such comparative analysis will be highly informative to dissect the differences and overlaps in immune signatures between the three main whole-sporozoite immunization approaches to (1) clarify why some approaches are more efficient in inducing protection than others and (2) home in on a minimal signature of antigen targets associated with protection. Target antigen identification will be useful not only for subunit vaccine design but also for generation of next-generation whole-sporozoite immunization regimens using parasites transgenic for variants of key antigens to induce efficient heterologous protection.

It can safely be said that further investigations of the immunological mechanism(s) whereby whole-sporozoite inoculation elicits protective immunity will greatly facilitate whole-sporozoite vaccine development [167]. Such knowledge, along with the expanded use of controlled human malaria infection studies [236, 237] and the envisioned progress in tackling the technical and logistical hurdles that still persist [59], can be expected to lead toward the ultimate goal of obtaining an effective vaccine with a pivotal role in the eventual global eradication of malaria.

References

1. Plotkin S (2014) History of vaccination. Proc Natl Acad Sci U S A 111(34):12283–12287
2. Ozawa S, Clark S, Portnoy A, Grewal S, Brenzel L, Walker DG (2016) Return on investment from childhood immunization in low- and middle-income countries, 2011–20. Health Aff 35(2):199–207
3. World Health Organization (2015) World malaria report 2015. World Health Organization, Geneva
4. McGregor IA (1964) The passive transfer of human malarial immunity. Am J Trop Med Hyg 13(Suppl):237–239
5. Russell PF, Mohan BN (1942) The immunization of fowls against mosquito-borne *Plasmodium gallinaceum* by injections of serum and of inactivated homologous sporozoites. J Exp Med 76(5):477–495
6. Sergent E, Sergent E (1910) Sur l'immunite dans le paludisme des oiseaux. Conservation in vitro des sporozoites de *Plasmodium relictum*. Immunite relative obtenue par inoculation de ces sporozoites. C R Acad Sci 151:407–409
7. Shortt HE, Garnham PC et al (1948) The pre-erythrocytic stage of human malaria, *Plasmodium vivax*. Br Med J 1(4550):547
8. Vincke IH (1954) Natural history of *Plasmodium berghei*. Indian J Malariol 8(4):245–256
9. Yoeli M, Upmanis RS, Vanderberg J, Most H (1966) Life cycle and patterns of development of *Plasmodium berghei* in normal and experimental hosts. Mil Med 131(9 Suppl):900–914
10. Nussenzweig RS, Vanderberg J, Most H, Orton C (1967) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. Nature 216(5111):160–162
11. Nussenzweig R, Vanderberg J, Most H (1969) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. Mil Med 134(10):1176–1182
12. Clyde DF, McCarthy VC, Miller RM, Hornick RB (1973) Specificity of protection of man immunized against sporozoite-induced falciparum malaria. Am J Med Sci 266(6):398–403
13. Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW (1974) Letter: sporozoite induced immunity in man against an Ethiopian strain of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 68(3):258–259
14. Rieckmann KH, Beaudoin RL, Cassells JS, Sell KW (1979) Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. Bull World Health Organ 57(Suppl 1):261–265
15. Carlton JM, Angiuoli SV, Suh BB, Kooij TW, Pertea M, Silva JC, Ermolaeva MD, Allen JE, Selengut JD, Koo HL et al (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. Nature 419(6906):512–519
16. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S et al (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419(6906):498–511
17. Tarun AS, Peng X, Dumpit RF, Ogata Y, Silva-Rivera H, Camargo N, Daly TM, Bergman LW, Kappe SH (2008) A combined transcriptome and proteome survey of malaria parasite liver stages. Proc Natl Acad Sci U S A 105(1):305–310
18. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE (1995) Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci U S A 92(4):973–977
19. van Dijk MR, Waters AP, Janse CJ (1995) Stable transfection of malaria parasite blood stages. Science 268(5215):1358–1362
20. Ross R (1897) On some peculiar pigmented cells found in two mosquitos fed on malarial blood. Br Med J 2(1929):1786–1788
21. Vanderberg JP, Yoeli M (1966) Effects of temperature on sporogonic development of *Plasmodium berghei*. J Parasitol 52(3):559–564
22. Haynes JD, Diggs CL, Hines FA, Desjardins RE (1976) Culture of human malaria parasites *Plasmodium falciparum*. Nature 263(5580):767–769

23. Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. Science 193(4254):673–675
24. Hollingdale MR, Leef JL, McCullough M, Beaudoin RL (1981) In vitro cultivation of the exoerythrocytic stage of *Plasmodium berghei* from sporozoites. Science 213(4511):1021–1022
25. Ozaki LS, Svec P, Nussenzweig RS, Nussenzweig V, Godson GN (1983) Structure of the *plasmodium knowlesi* gene coding for the circumsporozoite protein. Cell 34(3):815–822
26. Chulay JD, Schneider I, Cosgriff TM, Hoffman SL, Ballou WR, Quakyi IA, Carter R, Trosper JH, Hockmeyer WT (1986) Malaria transmitted to humans by mosquitoes infected from cultured *Plasmodium falciparum*. Am J Trop Med Hyg 35(1):66–68
27. Stewart MJ, Vanderberg JP (1988) Malaria sporozoites leave behind trails of circumsporozoite protein during gliding motility. J Protozool 35(3):389–393
28. Feldmann AM, Ponnudurai T (1989) Selection of *Anopheles stephensi* for refractoriness and susceptibility to *Plasmodium falciparum*. Med Vet Entomol 3(1):41–52
29. Warburg A, Miller LH (1992) Sporogonic development of a malaria parasite in vitro. Science 255(5043):448–450
30. Kappe SH, Gardner MJ, Brown SM, Ross J, Matuschewski K, Ribeiro JM, Adams JH, Quackenbush J, Cho J, Carucci DJ et al (2001) Exploring the transcriptome of the malaria sporozoite stage. Proc Natl Acad Sci U S A 98(17):9895–9900
31. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, Moch JK, Muster N, Sacci JB, Tabb DL et al (2002) A proteomic view of the *Plasmodium falciparum* life cycle. Nature 419(6906):520–526
32. Vaughan AM, Mikolajczak SA, Wilson EM, Grompe M, Kaushansky A, Camargo N, Bial J, Ploss A, Kappe SH (2012) Complete *Plasmodium falciparum* liver-stage development in liver-chimeric mice. J Clin Invest 122(10):3618–3628
33. Roestenberg M, Bijker EM, Sim BK, Billingsley PF, James ER, Bastiaens GJ, Teirlinck AC, Scholzen A, Teelen K, Arens T et al (2013) Controlled human malaria infections by intradermal injection of cryopreserved *Plasmodium falciparum* sporozoites. Am J Trop Med Hyg 88(1):5–13
34. Shekalaghe S, Rutaihwa M, Billingsley PF, Chemba M, Daubenberger CA, James ER, Mpina M, Ali Juma O, Schindler T, Huber E et al (2014) Controlled human malaria infection of Tanzanians by intradermal injection of aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites. Am J Trop Med Hyg 91(3):471–480
35. Mordmuller B, Supan C, Sim KL, Gomez-Perez GP, Ospina Salazar CL, Held J, Bolte S, Esen M, Tschan S, Joanny F et al (2015) Direct venous inoculation of *Plasmodium falciparum* sporozoites for controlled human malaria infection: a dose-finding trial in two centres. Malar J 14:117
36. Edelman R, Hoffman SL, Davis JR, Beier M, Sztein MB, Losonsky G, Herrington DA, Eddy HA, Hollingdale MR, Gordon DM et al (1993) Long-term persistence of sterile immunity in a volunteer immunized with X-irradiated *Plasmodium falciparum* sporozoites. J Infect Dis 168(4):1066–1070
37. Beaudoin RL, Strome CP, Mitchell F, Tubergen TA (1977) *Plasmodium berghei*: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. Exp Parasitol 42(1):1–5
38. Orjih AU, Cochrane AH, Nussenzweig RS (1982) Comparative studies on the immunogenicity of infective and attenuated sporozoites of *Plasmodium berghei*. Trans R Soc Trop Med Hyg 76(1):57–61
39. Mueller AK, Labaied M, Kappe SH, Matuschewski K (2005) Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. Nature 433(7022):164–167
40. van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, van Dooren MW, van Schaijk B, van Gemert GJ, Sauerwein RW, Mota MM, Waters AP et al (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. Proc Natl Acad Sci U S A 102(34):12194–12199

41. VanBuskirk KM, O'Neill MT, De La Vega P, Maier AG, Krzych U, Williams J, Dowler MG, Sacci JB Jr, Kangwanrangsan N, Tsuboi T et al (2009) Preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design. Proc Natl Acad Sci U S A 106(31):13004–13009
42. Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, Williams J, de la Vega P, Ware L, Komisar J et al (2013) First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. Vaccine 31(43):4975–4983
43. Belnoue E, Costa FT, Frankenberg T, Vigario AM, Voza T, Leroy N, Rodrigues MM, Landau I, Snounou G, Renia L (2004) Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J Immunol 172(4):2487–2495
44. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, van de Vegte-Bolmer M, van Schaijk B, Teelen K, Arens T et al (2009) Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 361(5):468–477
45. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, Arens T, Beckers P, van Gemert G, van de Vegte-Bolmer M et al (2011) Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet 377(9779):1770–1776
46. Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, Chakravarty S, Gunasekera A, Chattopadhyay R, Li M et al (2010) Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. Hum Vaccin 6(1):97–106
47. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A et al (2013) Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341(6152):1359–1365
48. Clyde DF, Most H, McCarthy VC, Vanderberg JP (1973) Immunization of man against sporozite-induced falciparum malaria. Am J Med Sci 266(3):169–177
49. Orjih AU, Nussenzweig RS (1980) Immunization against rodent malaria with cryopreserved irradiated sporozoites of *Plasmodium berghei*. Am J Trop Med Hyg 29(3):343–347
50. Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF (1988) $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. Proc Natl Acad Sci U S A 85(2):573–576
51. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M, Paul C et al (2002) Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J Infect Dis 185(8):1155–1164
52. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, Nussenzweig V (2006) The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. Nature 444(7121):937–940
53. Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SH, Harty JT (2011) Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. Cell Host Microbe 9(6):451–462
54. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, Gunasekera A, Chakravarty S, James ER, Sedegah M et al (2011) Live attenuated malaria vaccine designed to protect through hepatic $CD8^+$ T cell immunity. Science 334(6055):475–480
55. Annoura T, Ploemen IH, van Schaijk BC, Sajid M, Vos MW, van Gemert GJ, Chevalley-Maurel S, Franke-Fayard BM, Hermsen CC, Gego A et al (2012) Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. Vaccine 30(16):2662–2670
56. Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, Siebelink-Stoter R, Arens T, Teelen K, Nahrendorf W et al (2013) Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc Natl Acad Sci U S A 110(19):7862–7867

57. Mikolajczak SA, Lakshmanan V, Fishbaugher M, Camargo N, Harupa A, Kaushansky A, Douglass AN, Baldwin M, Healer J, O'Neill M et al (2014) A next-generation genetically attenuated *Plasmodium falciparum* parasite created by triple gene deletion. Mol Ther 22(9):1707–1715
58. van Schaijk BC, Ploemen IH, Annoura T, Vos MW, Foquet L, van Gemert GJ, Chevalley-Maurel S, van de Vegte-Bolmer M, Sajid M, Franetich JF et al (2014) A genetically attenuated malaria vaccine candidate based on *P. falciparum* b9/slarp gene-deficient sporozoites. Elife 3, e03582
59. Richie TL, Billingsley PF, Sim BK, James ER, Chakravarty S, Epstein JE, Lyke KE, Mordmuller B, Alonso P, Duffy PE et al (2015) Progress with *Plasmodium falciparum* sporozoite (PfSPZ)-based malaria vaccines. Vaccine 33(52):7452–7461
60. Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Druilhe P (1990) Protection against malaria induced by irradiated sporozoites. Lancet 335(8691):721
61. Herrington D, Davis J, Nardin E, Beier M, Cortese J, Eddy H, Losonsky G, Hollingdale M, Sztein M, Levine M et al (1991) Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. Am J Trop Med Hyg 45(5):539–547
62. Malik A, Egan JE, Houghten RA, Sadoff JC, Hoffman SL (1991) Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. Proc Natl Acad Sci U S A 88(8):3300–3304
63. Egan JE, Hoffman SL, Haynes JD, Sadoff JC, Schneider I, Grau GE, Hollingdale MR, Ballou WR, Gordon DM (1993) Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. Am J Trop Med Hyg 49(2):166–173
64. Silvie O, Semblat JP, Franetich JF, Hannoun L, Eling W, Mazier D (2002) Effects of irradiation on *Plasmodium falciparum* sporozoite hepatic development: implications for the design of pre-erythrocytic malaria vaccines. Parasite Immunol 24(4):221–223
65. Vanderberg J, Nussenzweig R, Most H (1970) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. J Parasitol 56(SectionII):350–351
66. Luke TC, Hoffman SL (2003) Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. J Exp Biol 206(Pt 21):3803–3808
67. Nussenzweig V, Nussenzweig RS (1989) Rationale for the development of an engineered sporozoite malaria vaccine. Adv Immunol 45:283–334
68. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M (1980) Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. Science 207(4426):71–73
69. Dame JB, Williams JL, McCutchan TF, Weber JL, Wirtz RA, Hockmeyer WT, Maloy WL, Haynes JD, Schneider I, Roberts D et al (1984) Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science 225(4662):593–599
70. Enea V, Ellis J, Zavala F, Arnot DE, Asavanich A, Masuda A, Quakyi I, Nussenzweig RS (1984) DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino acid sequence of repetitive epitope. Science 225(4662):628–630
71. Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, Heppner DG, Yuan G, Hollingdale M, Slaoui M et al (1995) Safety, immunogenicity, and efficacy of a recombinantly produced *Plasmodium falciparum* circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J Infect Dis 171(6):1576–1585
72. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, Wellde BT, Garcon N, Krzych U, Marchand M (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med 336(2):86–91
73. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, Ballou WR, Conway DJ, Reece WH, Gothard P et al (2001) Efficacy of RTS,S/AS02 malaria vaccine

against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 358(9297):1927–1934
74. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, Mandomando I, Spiessens B, Guinovart C, Espasa M et al (2004) Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 364(9443):1411–1420
75. Bojang KA, Olodude F, Pinder M, Ofori-Anyinam O, Vigneron L, Fitzpatrick S, Njie F, Kassanga A, Leach A, Milman J et al (2005) Safety and immunogenicity of RTS,S/AS02A candidate malaria vaccine in Gambian children. Vaccine 23(32):4148–4157
76. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, Manaca MN, Lafuente S, Barbosa A, Leach A et al (2007) Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 370(9598):1543–1551
77. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, Methogo BG, Doucka Y, Flamen A, Mordmuller B et al (2011) First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med 365(20):1863–1875
78. Rts SCTP, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, Adegnika AA, Mordmuller B, Issifou S et al (2012) A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med 367(24):2284–2295
79. Cohen J, Nussenzweig V, Nussenzweig R, Vekemans J, Leach A (2010) From the circumsporozoite protein to the RTS,S/AS candidate vaccine. Hum Vaccin 6(1):90–96
80. Greenwood B, Doumbo OK (2016) Implementation of the malaria candidate vaccine RTS,S/AS01. Lancet 387(10016):318–319
81. Minsoko PA, Lell B, Fernandes JF, Abossolo BP, Kabwende AL, Adegnika AA, Mordmuller B, Issifou S, Kremsner PG, Loembe MM et al (2014) Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med 11(7), e1001685
82. Tinto H, D'Alessandro U, Sorgho H, Valea I, Tahita MC, Kabore W, Kiemde F, Lompo P, Ouedraogo S, Derra K et al (2015) Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet 386(9988):31–45
83. Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, Lievens M, Leboulleux D, Njuguna P, Peshu N et al (2013) Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. New Engl J Med 368(12):1111–1120
84. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, De La Vega P, Holder AA, Batalov S, Carucci DJ et al (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 301(5639):1503–1508
85. Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, Florens L, Janssen CS, Pain A, Christophides GK et al (2005) A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. Science 307(5706):82–86
86. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P et al (2008) Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature 455(7214):757–763
87. Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, Waters AP (2005) Proteome analysis of separated male and female gametocytes reveals novel sex-specific *Plasmodium* biology. Cell 121(5):675–687
88. Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffy P, Carter R, Mendis K, Wirth D (1993) Transfection of the malaria parasite and expression of firefly luciferase. Proc Natl Acad Sci U S A 90(11):5234–5236
89. Wu Y, Kirkman LA, Wellems TE (1996) Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci U S A 93(3):1130–1134
90. van Dijk MR, Janse CJ, Waters AP (1996) Expression of a *Plasmodium* gene introduced into subtelomeric regions of *Plasmodium berghei* chromosomes. Science 271(5249):662–665

91. van der Wel AM, Tomas AM, Kocken CH, Malhotra P, Janse CJ, Waters AP, Thomas AW (1997) Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. J Exp Med 185(8):1499–1503
92. Mota MM, Thathy V, Nussenzweig RS, Nussenzweig V (2001) Gene targeting in the rodent malaria parasite *Plasmodium yoelii*. Mol Biochem Parasitol 113(2):271–278
93. Mueller AK, Camargo N, Kaiser K, Andorfer C, Frevert U, Matuschewski K, Kappe SH (2005) *Plasmodium* liver stage developmental arrest by depletion of a protein at the parasite-host interface. Proc Natl Acad Sci U S A 102(8):3022–3027
94. Belnoue E, Voza T, Costa FT, Gruner AC, Mauduit M, Rosa DS, Depinay N, Kayibanda M, Vigario AM, Mazier D et al (2008) Vaccination with live *Plasmodium yoelii* blood stage parasites under chloroquine cover induces cross-stage immunity against malaria liver stage. J Immunol 181(12):8552–8558
95. Doll KL, Harty JT (2014) Correlates of protective immunity following whole sporozoite vaccination against malaria. Immunol Res 59(1-3):166–176
96. Clyde DF (1975) Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg 24(3):397–401
97. Clyde DF (1990) Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. Bull World Health Organ 68(Suppl):9–12
98. Rieckmann KH (1990) Human immunization with attenuated sporozoites. Bull World Health Organ 68(Suppl):13–16
99. Hoffman SL (1997) Experimental challenge of volunteers with malaria. Ann Intern Med 127(3):233–235
100. Overstreet MG, Cockburn IA, Chen YC, Zavala F (2008) Protective CD8 T cells against *Plasmodium* liver stages: immunobiology of an 'unnatural' immune response. Immunol Rev 225:272–283
101. Stewart MJ, Vanderberg JP (1991) Malaria sporozoites release circumsporozoite protein from their apical end and translocate it along their surface. J Protozool 38(4):411–421
102. Stewart MJ, Nawrot RJ, Schulman S, Vanderberg JP (1986) *Plasmodium berghei* sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies. Infect Immun 51(3):859–864
103. Doolan DL, Hoffman SL (1997) Pre-erythrocytic-stage immune effector mechanisms in *Plasmodium* spp. infections. Philos Trans R Soc Lond B Biol Sci 352(1359):1361–1367
104. Charoenvit Y, Collins WE, Jones TR, Millet P, Yuan L, Campbell GH, Beaudoin RL, Broderson JR, Hoffman SL (1991) Inability of malaria vaccine to induce antibodies to a protective epitope within its sequence. Science 251(4994):668–671
105. Chai SK, Clavijo P, Tam JP, Zavala F (1992) Immunogenic properties of multiple antigen peptide systems containing defined T and B epitopes. J Immunol 149(7):2385–2390
106. Chakravarty S, Cockburn IA, Kuk S, Overstreet MG, Sacci JB, Zavala F (2007) $CD8^+$ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. Nat Med 13(9):1035–1041
107. Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, Mac-Daniel L, Ganusov VV, Zavala F, Menard R (2013) In vivo imaging of $CD8^+$ T cell-mediated elimination of malaria liver stages. Proc Natl Acad Sci U S A 110(22):9090–9095
108. Cockburn IA, Tse SW, Radtke AJ, Srinivasan P, Chen YC, Sinnis P, Zavala F (2011) Dendritic cells and hepatocytes use distinct pathways to process protective antigen from *Plasmodium* in vivo. PLoS Pathog 7(3), e1001318
109. Pichugin A, Steers N, De la Vega P, Zarling S, Chalom I, Krzych U (2016) TAP-mediated processing of exoerythrocytic antigens is essential for protection induced with radiation-attenuated *Plasmodium sporozoites*. Eur J Immunol 46(4):885–896
110. Chen DH, Tigelaar RE, Weinbaum FI (1977) Immunity to sporozoite-induced malaria infection in mice. I. The effect of immunization of T and B cell-deficient mice. J Immunol 118(4):1322–1327

111. Verhave JP, Meuwissen JH, Golenser J (1978) Cell-mediated reactions and protection after immunization with sporozoites. Isr J Med Sci 14(5):611–616
112. Egan JE, Weber JL, Ballou WR, Hollingdale MR, Majarian WR, Gordon DM, Maloy WL, Hoffman SL, Wirtz RA, Schneider I et al (1987) Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. Science 236(4800):453–456
113. Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V (1987) Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature 330(6149):664–666
114. Weiss WR, Jiang CG (2012) Protective $CD8^+$ T lymphocytes in primates immunized with malaria sporozoites. PLoS One 7(2), e31247
115. Ferreira A, Schofield L, Enea V, Schellekens H, van der Meide P, Collins WE, Nussenzweig RS, Nussenzweig V (1986) Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. Science 232(4752):881–884
116. Radtke AJ, Tse SW, Zavala F (2015) From the draining lymph node to the liver: the induction and effector mechanisms of malaria-specific $CD8^+$ T cells. Semin Immunopathol 37(3):211–220
117. Duffy PE, Sahu T, Akue A, Milman N, Anderson C (2012) Pre-erythrocytic malaria vaccines: identifying the targets. Expert Rev Vaccines 11(10):1261–1280
118. Nardin EH, Nussenzweig RS (1993) T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. Annu Rev Immunol 11:687–727
119. Tsuji M, Mombaerts P, Lefrancois L, Nussenzweig RS, Zavala F, Tonegawa S (1994) Gamma delta T cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. Proc Natl Acad Sci U S A 91(1):345–349
120. Doolan DL, Hoffman SL (1999) IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by $CD8^+$ T cells in the *Plasmodium yoelii* model. J Immunol 163(2):884–892
121. Doolan DL, Hoffman SL (2000) The complexity of protective immunity against liver-stage malaria. J Immunol 165(3):1453–1462
122. Weiss WR, Good MF, Hollingdale MR, Miller LH, Berzofsky JA (1989) Genetic control of immunity to *Plasmodium yoelii* sporozoites. J Immunol 143(12):4263–4266
123. Oliveira GA, Kumar KA, Calvo-Calle JM, Othoro C, Altszuler D, Nussenzweig V, Nardin EH (2008) Class II-restricted protective immunity induced by malaria sporozoites. Infect Immun 76(3):1200–1206
124. Hoffman SL, Doolan DL (2000) Malaria vaccines-targeting infected hepatocytes. Nat Med 6(11):1218–1219
125. Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F (1989) Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature 341(6240):323–326
126. Weiss WR, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good MF, Berzofsky JA, Miller LH, Hoffman SL (1990) Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. J Exp Med 171(3):763–773
127. Kumar S, Miller LH, Quakyi IA, Keister DB, Houghten RA, Maloy WL, Moss B, Berzofsky JA, Good MF (1988) Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium falciparum*. Nature 334(6179):258–260
128. Huang J, Tsao T, Zhang M, Tsuji M (2014) Circumsporozoite protein-specific K^d-restricted $CD8^+$ T cells mediate protective antimalaria immunity in sporozoite-immunized MHC-I-K^d transgenic mice. Mediators Inflamm 2014:728939
129. Mishra S, Rai U, Shiratsuchi T, Li X, Vanloubbeeck Y, Cohen J, Nussenzweig RS, Winzeler EA, Tsuji M, Nussenzweig V (2011) Identification of non-CSP antigens bearing CD8 epitopes in mice immunized with irradiated sporozoites. Vaccine 29(43):7335–7342
130. Gruner AC, Mauduit M, Tewari R, Romero JF, Depinay N, Kayibanda M, Lallemand E, Chavatte JM, Crisanti A, Sinnis P et al (2007) Sterile protection against malaria is independent of immune responses to the circumsporozoite protein. PLoS One 2(12), e1371

131. Mauduit M, Gruner AC, Tewari R, Depinay N, Kayibanda M, Chavatte JM, Franetich JF, Crisanti A, Mazier D, Snounou G et al (2009) A role for immune responses against non-CS components in the cross-species protection induced by immunization with irradiated malaria sporozoites. PLoS One 4(11), e7717
132. Tsuji M, Romero P, Nussenzweig RS, Zavala F (1990) $CD4^+$ cytolytic T cell clone confers protection against murine malaria. J Exp Med 172(5):1353–1357
133. Migliorini P, Betschart B, Corradin G (1993) Malaria vaccine: immunization of mice with a synthetic T cell helper epitope alone leads to protective immunity. Eur J Immunol 23(2):582–585
134. Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert PH, Mazier D, Del Giudice G (1993) Effector functions of circumsporozoite peptide-primed $CD4^+$ T cell clones against *Plasmodium yoelii* liver stages. J Immunol 150(4):1471–1478
135. Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V (1983) Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J Exp Med 157(6):1947–1957
136. Zavala F, Tam JP, Hollingdale MR, Cochrane AH, Quakyi I, Nussenzweig RS, Nussenzweig V (1985) Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. Science 228(4706):1436–1440
137. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta KT, Tapchaisri P, Chomcharn Y (1982) Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. J Exp Med 156(1):20–30
138. Herrington DA, Clyde DF, Losonsky G, Cortesia M, Murphy JR, Davis J, Baqar S, Felix AM, Heimer EP, Gillessen D et al (1987) Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Nature 328(6127):257–259
139. Good MF, Berzofsky JA, Maloy WL, Hayashi Y, Fujii N, Hockmeyer WT, Miller LH (1986) Genetic control of the immune response in mice to a *Plasmodium falciparum* sporozoite vaccine. Widespread nonresponsiveness to single malaria T epitope in highly repetitive vaccine. J Exp Med 164(2):655–660
140. Calvo-Calle JM, Oliveira GA, Watta CO, Soverow J, Parra-Lopez C, Nardin EH (2006) A linear peptide containing minimal T- and B-cell epitopes of *Plasmodium falciparum* circumsporozoite protein elicits protection against transgenic sporozoite challenge. Infect Immun 74(12):6929–6939
141. Offeddu V, Thathy V, Marsh K, Matuschewski K (2012) Naturally acquired immune responses against *Plasmodium falciparum* sporozoites and liver infection. Int J Parasitol 42(6):535–548
142. Rogers WO, Malik A, Mellouk S, Nakamura K, Rogers MD, Szarfman A, Gordon DM, Nussler AK, Aikawa M, Hoffman SL (1992) Characterization of *Plasmodium falciparum* sporozoite surface protein 2. Proc Natl Acad Sci U S A 89(19):9176–9180
143. Nardin EH, Nussenzweig RS, Altszuler R, Herrington D, Levine M, Murphy J, Davis J, Bathurst I, Barr P, Romero P et al (1990) Cellular and humoral immune responses to a recombinant *P. falciparum* CS protein in sporozoite-immunized rodents and human volunteers. Bull World Health Organ 68(Suppl):85–87
144. Nardin EH (1990) T cell responses in a sporozoite-immunized human volunteer and a chimpanzee. Immunol Lett 25(1-3):43–47
145. Moreno A, Clavijo P, Edelman R, Davis J, Sztein M, Herrington D, Nardin E (1991) Cytotoxic $CD4^+$ T cells from a sporozoite-immunized volunteer recognize the *Plasmodium falciparum* CS protein. Int Immunol 3(10):997–1003
146. de la Cruz VF, Lal AA, McCutchan TF (1987) Sequence variation in putative functional domains of the circumsporozoite protein of *Plasmodium falciparum*. Implications for vaccine development. J Biol Chem 262(25):11935–11939
147. Moreno A, Clavijo P, Edelman R, Davis J, Sztein M, Sinigaglia F, Nardin E (1993) $CD4^+$ T cell clones obtained from *Plasmodium falciparum* sporozoite-immunized volunteers recognize polymorphic sequences of the circumsporozoite protein. J Immunol 151(1):489–499

148. Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, Greenwood BM (1991) Common West African HLA antigens are associated with protection from severe malaria. Nature 352(6336):595–600
149. Allsopp CE, Harding RM, Taylor C, Bunce M, Kwiatkowski D, Anstey N, Brewster D, McMichael AJ, Greenwood BM, Hill AV (1992) Interethnic genetic differentiation in Africa: HLA class I antigens in The Gambia. Am J Hum Genet 50(2):411–421
150. Doolan DL, Houghten RA, Good MF (1991) Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. Int Immunol 3(6):511–516
151. Aidoo M, Lalvani A, Allsopp CE, Plebanski M, Meisner SJ, Krausa P, Browning M, Morris-Jones S, Gotch F, Fidock DA et al (1995) Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. Lancet 345(8956):1003–1007
152. Wizel B, Houghten RA, Parker KC, Coligan JE, Church P, Gordon DM, Ballou WR, Hoffman SL (1995) Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. J Exp Med 182(5):1435–1445
153. Doolan DL, Southwood S, Freilich DA, Sidney J, Graber NL, Shatney L, Bebris L, Florens L, Dobano C, Witney AA et al (2003) Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. Proc Natl Acad Sci U S A 100(17):9952–9957
154. Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M (2006) CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. Mol Microbiol 59(5):1369–1379
155. Trieu A, Kayala MA, Burk C, Molina DM, Freilich DA, Richie TL, Baldi P, Felgner PL, Doolan DL (2011) Sterile protective immunity to malaria is associated with a panel of novel *P. falciparum* antigens. Mol Cell Proteomics 10(9), M111.007948
156. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, Valdez C, Randall A, Molina D, Liang X, Freilich DA et al (2008) Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. Proteomics 8(22):4680–4694
157. Aguiar JC, Bolton J, Wanga J, Sacci JB, Iriko H, Mazeika JK, Han ET, Limbach K, Patterson NB, Sedegah M et al (2015) Discovery of novel *Plasmodium falciparum* pre-erythrocytic antigens for vaccine development. PLoS One 10(8), e0136109
158. Garcia CR, Manzi F, Tediosi F, Hoffman SL, James ER (2013) Comparative cost models of a liquid nitrogen vapor phase (LNVP) cold chain-distributed cryopreserved malaria vaccine vs. a conventional vaccine. Vaccine 31(2):380–386
159. March S, Ng S, Velmurugan S, Galstian A, Shan J, Logan DJ, Carpenter AE, Thomas D, Sim BK, Mota MM et al (2013) A microscale human liver platform that supports the hepatic stages of *Plasmodium falciparum* and *vivax*. Cell Host Microbe 14(1):104–115
160. Sheehy SH, Spencer AJ, Douglas AD, Sim BK, Longley RJ, Edwards NJ, Poulton ID, Kimani D, Williams AR, Anagnostou NA et al (2013) Optimising controlled human malaria infection studies using cryopreserved parasites administered by needle and syringe. PLoS One 8(6), e65960
161. Hodgson SH, Juma E, Salim A, Magiri C, Kimani D, Njenga D, Muia A, Cole AO, Ogwang C, Awuondo K et al (2014) Evaluating controlled human malaria infection in Kenyan adults with varying degrees of prior exposure to *Plasmodium falciparum* using sporozoites administered by intramuscular injection. Front Microbiol 5:686
162. Gomez-Perez GP, Legarda A, Munoz J, Sim BK, Ballester MR, Dobano C, Moncunill G, Campo JJ, Cistero P, Jimenez A et al (2015) Controlled human malaria infection by intramuscular and direct venous inoculation of cryopreserved *Plasmodium falciparum* sporozoites in malaria-naive volunteers: effect of injection volume and dose on infectivity rates. Malar J 14:306
163. Nganou-Makamdop K, Ploemen I, Behet M, Van Gemert GJ, Hermsen C, Roestenberg M, Sauerwein RW (2012) Reduced *Plasmodium berghei* sporozoite liver load associates with low protective efficacy after intradermal immunization. Parasite Immunol 34(12):562–569

164. Ploemen IH, Chakravarty S, van Gemert GJ, Annoura T, Khan SM, Janse CJ, Hermsen CC, Hoffman SL, Sauerwein RW (2013) *Plasmodium* liver load following parenteral sporozoite administration in rodents. Vaccine 31(34):3410–3416
165. Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, Enama ME, Gordon IJ, Chang LJ, Sarwar UN et al (2016) Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat Med 22:614–623
166. Good MF (2013) Immunology. Pasteur approach to a malaria vaccine may take the lead. Science 341(6152):1352–1353
167. Mo AX, Pesce J, Hall BF (2015) Exploring immunological mechanisms of the whole sporozoite vaccination against *P. falciparum* malaria. Vaccine 33(25):2851–2857
168. Hoffman SL, Vekemans J, Richie TL, Duffy PE (2015) The march toward malaria vaccines. Vaccine 33(Suppl 4):D13–D23
169. Jobe O, Lumsden J, Mueller AK, Williams J, Silva-Rivera H, Kappe SH, Schwenk RJ, Matuschewski K, Krzych U (2007) Genetically attenuated *Plasmodium berghei* liver stages induce sterile protracted protection that is mediated by major histocompatibility complex Class I-dependent interferon-gamma-producing $CD8^+$ T cells. J Infect Dis 196(4):599–607
170. Vaughan AM, Wang R, Kappe SH (2010) Genetically engineered, attenuated whole-cell vaccine approaches for malaria. Hum Vaccin 6(1):107–113
171. Khan SM, Janse CJ, Kappe SH, Mikolajczak SA (2012) Genetic engineering of attenuated malaria parasites for vaccination. Curr Opin Biotechnol 23(6):908–916
172. Matuschewski K, Ross J, Brown SM, Kaiser K, Nussenzweig V, Kappe SH (2002) Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. J Biol Chem 277(44):41948–41953
173. van Schaijk BC, Janse CJ, van Gemert GJ, van Dijk MR, Gego A, Franetich JF, van de Vegte-Bolmer M, Yalaoui S, Silvie O, Hoffman SL et al (2008) Gene disruption of *Plasmodium falciparum* p52 results in attenuation of malaria liver stage development in cultured primary human hepatocytes. PLoS One 3(10), e3549
174. Annoura T, van Schaijk BC, Ploemen IH, Sajid M, Lin JW, Vos MW, Dinmohamed AG, Inaoka DK, Rijpma SR, van Gemert GJ et al (2014) Two *Plasmodium* 6-Cys family-related proteins have distinct and critical roles in liver-stage development. FASEB J 28(5):2158–2170
175. Bijker EM, Borrmann S, Kappe SH, Mordmuller B, Sack BK, Khan SM (2015) Novel approaches to whole sporozoite vaccination against malaria. Vaccine 33(52):7462–7468
176. Aly AS, Lindner SE, MacKellar DC, Peng X, Kappe SH (2011) SAP1 is a critical post-transcriptional regulator of infectivity in malaria parasite sporozoite stages. Mol Microbiol 79(4):929–939
177. Aly AS, Mikolajczak SA, Rivera HS, Camargo N, Jacobs-Lorena V, Labaied M, Coppens I, Kappe SH (2008) Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection. Mol Microbiol 69(1):152–163
178. Silvie O, Goetz K, Matuschewski K (2008) A sporozoite asparagine-rich protein controls initiation of *Plasmodium* liver stage development. PLoS Pathog 4(6), e1000086
179. Rijpma SR, van der Velden M, Gonzalez-Pons M, Annoura T, van Schaijk BC, van Gemert GJ, van den Heuvel JJ, Ramesar J, Chevalley-Maurel S, Ploemen IH et al (2016) Multidrug ATP-binding cassette transporters are essential for hepatic development of *Plasmodium sporozoites*. Cell Microbiol 18(3):369–383
180. Hafalla JC, Rai U, Morrot A, Bernal-Rubio D, Zavala F, Rodriguez A (2006) Priming of $CD8^+$ T cell responses following immunization with heat-killed *Plasmodium sporozoites*. Eur J Immunol 36(5):1179–1186
181. Matuschewski K, Hafalla JC, Borrmann S, Friesen J (2011) Arrested *Plasmodium* liver stages as experimental anti-malaria vaccines. Hum Vaccin 7(Suppl):16–21
182. Ngonseu E, Chatterjee S, Wery M (1998) Blocked hepatic-stage parasites and decreased susceptibility to *Plasmodium berghei* infections in BALB/c mice. Parasitology 117(Pt 5):419–423

183. Mueller AK, Deckert M, Heiss K, Goetz K, Matuschewski K, Schluter D (2007) Genetically attenuated *Plasmodium berghei* liver stages persist and elicit sterile protection primarily via CD8 T cells. Am J Pathol 171(1):107–115
184. Tarun AS, Dumpit RF, Camargo N, Labaied M, Liu P, Takagi A, Wang R, Kappe SH (2007) Protracted sterile protection with *Plasmodium yoelii* pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by $CD8^+$ T cells. J Infect Dis 196(4):608–616
185. Douradinha B, Doolan DL (2011) Harnessing immune responses against *Plasmodium* for rational vaccine design. Trends Parasitol 27(6):274–283
186. Trimnell A, Takagi A, Gupta M, Richie TL, Kappe SH, Wang R (2009) Genetically attenuated parasite vaccines induce contact-dependent $CD8^+$ T cell killing of *Plasmodium yoelii* liver stage-infected hepatocytes. J Immunol 183(9):5870–5878
187. Cooney LA, Gupta M, Thomas S, Mikolajczak S, Choi KY, Gibson C, Jang IK, Danziger S, Aitchison J, Gardner MJ et al (2013) Short-lived effector CD8 T cells induced by genetically attenuated malaria parasite vaccination express CD11c. Infect Immun 81(11):4171–4181
188. Vaughan AM, O'Neill MT, Tarun AS, Camargo N, Phuong TM, Aly AS, Cowman AF, Kappe SH (2009) Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. Cell Microbiol 11(3):506–520
189. Murray SA, Mohar I, Miller JL, Brempelis KJ, Vaughan AM, Kappe SH, Crispe IN (2015) CD40 is required for protective immunity against liver stage *Plasmodium* infection. J Immunol 194(5):2268–2279
190. Sack BK, Keitany GJ, Vaughan AM, Miller JL, Wang R, Kappe SH (2015) Mechanisms of stage-transcending protection following immunization of mice with late liver stage-arresting genetically attenuated malaria parasites. PLoS Pathog 11(5), e1004855
191. Keitany GJ, Sack B, Smithers H, Chen L, Jang IK, Sebastian L, Gupta M, Sather DN, Vignali M, Vaughan AM et al (2014) Immunization of mice with live-attenuated late liver stage-arresting *Plasmodium yoelii* parasites generates protective antibody responses to preerythrocytic stages of malaria. Infect Immun 82(12):5143–5153
192. Wang R, Arevalo-Herrera M, Gardner MJ, Bonelo A, Carlton JM, Gomez A, Vera O, Soto L, Vergara J, Bidwell SL et al (2005) Immune responses to *Plasmodium vivax* pre-erythrocytic stage antigens in naturally exposed Duffy-negative humans: a potential model for identification of liver-stage antigens. Eur J Immunol 35(6):1859–1868
193. Chen L, Keitany GJ, Peng X, Gibson C, Mohar I, Vignali M, Crispe IN, Huang F, Wang R (2014) Identification of pre-erythrocytic malaria antigens that target hepatocytes for killing in vivo and contribute to protection elicited by whole-parasite vaccination. PLoS One 9(7), e102225
194. Haussig JM, Matuschewski K, Kooij TW (2011) Inactivation of a *Plasmodium* apicoplast protein attenuates formation of liver merozoites. Mol Microbiol 81(6):1511–1525
195. Hafalla JC, Bauza K, Friesen J, Gonzalez-Aseguinolaza G, Hill AV, Matuschewski K (2013) Identification of targets of $CD8^+$ T cell responses to malaria liver stages by genome-wide epitope profiling. PLoS Pathog 9(5), e1003303
196. Haussig JM, Burgold J, Hafalla JC, Matuschewski K, Kooij TW (2014) Signatures of malaria vaccine efficacy in ageing murine immune memory. Parasite Immunol 36(5):199–206
197. Kumar KA, Baxter P, Tarun AS, Kappe SH, Nussenzweig V (2009) Conserved protective mechanisms in radiation and genetically attenuated *uis3(−)* and *uis4(−) Plasmodium sporozoites*. PLoS One 4(2), e4480
198. van Schaijk BC, Kumar TR, Vos MW, Richman A, van Gemert GJ, Li T, Eappen AG, Williamson KC, Morahan BJ, Fishbaugher M et al (2014) Type II fatty acid biosynthesis is essential for *Plasmodium falciparum* sporozoite development in the midgut of Anopheles mosquitoes. Eukaryot Cell 13(5):550–559
199. Nganou-Makamdop K, Sauerwein RW (2013) Liver or blood-stage arrest during malaria sporozoite immunization: the later the better? Trends Parasitol 29(6):304–310
200. Sauerwein RW, Bijker EM, Richie TL (2010) Empowering malaria vaccination by drug administration. Curr Opin Immunol 22(3):367–373

201. Golenser J, Heeren J, Verhave JP, Kaay HJ, Meuwissen JH (1977) Crossreactivity with sporozoites, exoerythrocytic forms and blood schizonts of *Plasmodium berghei* in indirect fluorescent antibody tests with sera of rats immunized with sporozoites or infected blood. Clin Exp Immunol 29(1):43–51
202. Slater AF (1993) Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*. Pharmacol Ther 57(2-3):203–235
203. Lewis MD, Pfeil J, Heiss K, Mueller AK (2014) $CD8^+$ T cells mediate robust stage-specific immunity to *P. berghei* under chemoprophylaxis and this protective environment is not down-regulated by the presence of blood-stage infection. PLoS One 9(2), e88117
204. Bijker EM, Nganou-Makamdop K, van Gemert GJ, Zavala F, Cockburn I, Sauerwein RW (2015) Studying the effect of chloroquine on sporozoite-induced protection and immune responses in *Plasmodium berghei* malaria. Malar J 14:130
205. Pfeil J, Sepp KJ, Heiss K, Meister M, Mueller AK, Borrmann S (2014) Protection against malaria by immunization with non-attenuated sporozoites under single-dose piperaquine-tetraphosphate chemoprophylaxis. Vaccine 32(45):6005–6011
206. Friesen J, Borrmann S, Matuschewski K (2011) Induction of antimalaria immunity by pyrimethamine prophylaxis during exposure to sporozoites is curtailed by parasite resistance. Antimicrob Agents Chemother 55(6):2760–2767
207. Friesen J, Matuschewski K (2011) Comparative efficacy of pre-erythrocytic whole organism vaccine strategies against the malaria parasite. Vaccine 29(40):7002–7008
208. Peng X, Keitany GJ, Vignali M, Chen L, Gibson C, Choi K, Huang F, Wang R (2014) Artesunate versus chloroquine infection-treatment-vaccination defines stage-specific immune responses associated with prolonged sterile protection against both pre-erythrocytic and erythrocytic *Plasmodium yoelii* infection. J Immunol 193(3):1268–1277
209. Bijker EM, Teirlinck AC, Schats R, van Gemert GJ, van de Vegte-Bolmer M, van Lieshout L, IntHout J, Hermsen CC, Scholzen A, Visser LG et al (2014) Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites. J Infect Dis 210(10):1605–1615
210. Inoue M, Tang J, Miyakoda M, Kaneko O, Yui K, Culleton R (2012) The species specificity of immunity generated by live whole organism immunisation with erythrocytic and pre-erythrocytic stages of rodent malaria parasites and implications for vaccine development. Int J Parasitol 42(9):859–870
211. Nahrendorf W, Spence PJ, Tumwine I, Levy P, Jarra W, Sauerwein RW, Langhorne J (2015) Blood-stage immunity to *Plasmodium chabaudi* malaria following chemoprophylaxis and sporozoite immunization. Elife 4, e05165
212. Schats R, Bijker EM, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, van Lieshout L, Haks MC, Hermsen CC, Scholzen A, Visser LG et al (2015) Heterologous protection against malaria after immunization with *Plasmodium falciparum* sporozoites. PLoS One 10(5), e0124243
213. Bijker EM, Sauerwein RW (2012) Enhancement of naturally acquired immunity against malaria by drug use. J Med Microbiol 61(Pt 7):904–910
214. Bastiaens GJ, van Meer MP, Scholzen A, Obiero JM, Vatanshenassan M, van Grinsven T, Sim BK, Billingsley PF, James ER, Gunasekera A et al (2015) Safety, immunogenicity, and protective efficacy of intradermal immunization with aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites in volunteers under chloroquine prophylaxis: a randomized controlled trial. Am J Trop Med Hyg 94(3):663–673
215. Doll KL, Butler NS, Harty JT (2014) CD8 T cell independent immunity after single dose infection-treatment-vaccination (ITV) against *Plasmodium yoelii*. Vaccine 32(4):483–491
216. Nganou-Makamdop K, van Gemert GJ, Arens T, Hermsen CC, Sauerwein RW (2012) Long term protection after immunization with *P. berghei* sporozoites correlates with sustained IFNγ responses of hepatic $CD8^+$ memory T cells. PLoS One 7(5), e36508
217. Nahrendorf W, Scholzen A, Sauerwein RW, Langhorne J (2015) Cross-stage immunity for malaria vaccine development. Vaccine 33(52):7513–7517

218. Nahrendorf W, Scholzen A, Bijker EM, Teirlinck AC, Bastiaens GJ, Schats R, Hermsen CC, Visser LG, Langhorne J, Sauerwein RW (2014) Memory B-cell and antibody responses induced by *Plasmodium falciparum* sporozoite immunization. J Infect Dis 210(12):1981–1990
219. Teirlinck AC, McCall MB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, van der Ven AJ, Hermsen CC, Luty AJ, Sauerwein RW (2011) Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. PLoS Pathog 7(12), e1002389
220. Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, Anderson K, Mahakunkijcharoen Y, Martin LB, Wilson D et al (2002) Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. Lancet 360(9333):610–617
221. Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J, Nakajima-Sasaki R, Molina D, Teelen K, Hermsen CC et al (2013) Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. Sci Rep 3:3549
222. Behet MC, Foquet L, van Gemert GJ, Bijker EM, Meuleman P, Leroux-Roels G, Hermsen CC, Scholzen A, Sauerwein RW (2014) Sporozoite immunization of human volunteers under chemoprophylaxis induces functional antibodies against pre-erythrocytic stages of *Plasmodium falciparum*. Malar J 13:136
223. Singh AP, Buscaglia CA, Wang Q, Levay A, Nussenzweig DR, Walker JR, Winzeler EA, Fujii H, Fontoura BM, Nussenzweig V (2007) *Plasmodium* circumsporozoite protein promotes the development of the liver stages of the parasite. Cell 131(3):492–504
224. Hollingdale MR, Leland P, Leef JL, Leef MF, Beaudoin RL (1983) Serological reactivity of in vitro cultured exoerythrocytic stages of *Plasmodium berghei* in indirect immunofluorescent or immunoperoxidase antibody tests. Am J Trop Med Hyg 32(1):24–30
225. Davies DH, Duffy P, Bodmer JL, Felgner PL, Doolan DL (2015) Large screen approaches to identify novel malaria vaccine candidates. Vaccine 33(52):7496–7505
226. Teirlinck AC, Roestenberg M, van de Vegte-Bolmer M, Scholzen A, Heinrichs MJ, Siebelink-Stoter R, Graumans W, van Gemert GJ, Teelen K, Vos MW et al (2013) NF135.C10: a new *Plasmodium falciparum* clone for controlled human malaria infections. J Infect Dis 207(4):656–660
227. Sato A, McNulty L, Cox K, Kim S, Scott A, Daniell K, Summerville K, Price C, Hudson S, Kiakos K et al (2005) A novel class of in vivo active anticancer agents: achiral seco-amino- and seco-hydroxycyclopropylbenz[e]indolone (seco-CBI) analogues of the duocarmycins and CC-1065. J Med Chem 48(11):3903–3918
228. Purcell LA, Yanow SK, Lee M, Spithill TW, Rodriguez A (2008) Chemical attenuation of *Plasmodium berghei* sporozoites induces sterile immunity in mice. Infect Immun 76(3):1193–1199
229. Purcell LA, Wong KA, Yanow SK, Lee M, Spithill TW, Rodriguez A (2008) Chemically attenuated *Plasmodium sporozoites* induce specific immune responses, sterile immunity and cross-protection against heterologous challenge. Vaccine 26(38):4880–4884
230. Lewis MD, Behrends J, Sa ECC, Mendes AM, Lasitschka F, Sattler JM, Heiss K, Kooij TW, Prudencio M, Bringmann G et al (2015) Chemical attenuation of *Plasmodium* in the liver modulates severe malaria disease progression. J Immunol 194(10):4860–4870
231. Bergmann-Leitner ES, Mease RM, De La Vega P, Savranskaya T, Polhemus M, Ockenhouse C, Angov E (2010) Immunization with pre-erythrocytic antigen CelTOS from *Plasmodium falciparum* elicits cross-species protection against heterologous challenge with *Plasmodium berghei*. PLoS One 5(8), e12294
232. Janse CJ, Ramesar J, Waters AP (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. Nat Protoc 1(1):346–356
233. Salman AM, Mogollon CM, Lin JW, van Pul FJ, Janse CJ, Khan SM (2015) Generation of transgenic rodent malaria parasites expressing human malaria parasite proteins. Methods Mol Biol 1325:257–286

234. Lin JW, Annoura T, Sajid M, Chevalley-Maurel S, Ramesar J, Klop O, Franke-Fayard BM, Janse CJ, Khan SM (2011) A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS One 6(12), e29289
235. Chattopadhyay R, Conteh S, Li M, James ER, Epstein JE, Hoffman SL (2009) The effects of radiation on the safety and protective efficacy of an attenuated *Plasmodium yoelii* sporozoite malaria vaccine. Vaccine 27(27):3675–3680
236. Sauerwein RW, Roestenberg M, Moorthy VS (2011) Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev Immunol 11(1):57–64
237. Scholzen A, Sauerwein RW (2016) Immune activation and induction of memory: lessons learned from controlled human malaria infection with *Plasmodium falciparum*. Parasitology 143(2):224–235