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Abstract

A vaccine providing sterile immunity against malaria has been shown to be possible with antigens from the pre-erythrocytic stages of malaria. Therefore, it is reasonable to focus vaccine development efforts on the pre-erythrocytic stages, consisting of both sporozoites and liver stage parasites, where it is expected that sterile immunity against the parasite can be elicited to block the development of blood stage infection, clinical disease, and resulting parasite transmission. Accordingly, we will review the preclinical and clinical studies of malaria pre-erythrocytic efforts as well as highlight the advances, trends, and roadblocks encountered in these efforts.

Keywords: *Plasmodium falciparum*, malaria, vaccine, clinical trial, CSP, TRAP, CelTOS, LSA, EPI300, STARp, EXP-1, immune response, humoral, cellular, CD4+, CD8+

1. Introduction

Immunity to malaria correlates with age and develops only after years of repeated exposure to bites from infected mosquitoes [1, 2]. Most adolescents in malaria-endemic regions have developed a level of protective immunity that provides resistance to clinical disease but does not fully eliminate infection [1]. This lack of sterile immunity does not prevent further spread of the disease, as some level of parasitemia persists in these individuals. A complete understanding of how the malaria parasite avoids clearance remains unknown. However, mechanisms such as extreme allelic variation, strain diversity, as well as the unique complexity of the malaria parasite life cycle are undoubtedly involved. In this regard, identification of the conserved, critical epitopes of the parasite that are directly associated with eliciting
protection may provide the best viable target node for vaccine development. In addition, recent data from mouse models of malaria suggest that T cell exhaustion significantly impairs development of effective immune responses and contributes to chronic malaria infection [3–5]. Notwithstanding the fleeting immunity that individuals receive from frequent infection with the parasite, it suggests that there are targets for the immune system to attack.

Importantly, exposure to attenuated sporozoites that can infect hepatocytes but do not develop into the blood-phase of malaria can induce immunity that eliminates parasitic infection (i.e., sterile immunity). This has been demonstrated by conducting immunizations with irradiated sporozoites both in mouse models of malaria [6] as well as in humans with different strains of *Plasmodium falciparum* [7–10]. Similarly, induction of sterile immunity can occur in individuals undergoing chloroquine or mefloquine chemoprophylaxis. The resulting “chemically attenuated” *Plasmodium falciparum* sporozoites (CPS-CQ or CPS-MQ), which undergo full liver stage development but do not develop to clinical blood-stage infection [11, 12], can induce sterile immunity [12–15]. Of note is that sterile protection may not fully develop when sporozoites do not progress at least partially through liver stage development, potentially providing stage transcendent protection [16, 17]. These discoveries demonstrate that a vaccine providing sterile immunity against malaria is possible with antigens from the pre-erythrocytic stages of malaria. Therefore, it is reasonable to focus vaccine development efforts on the pre-erythrocytic stages, consisting of both sporozoites and liver stage parasites, where it is expected that sterile immunity against the parasite can be elicited to block the development of blood stage infection, clinical disease, and resulting parasite transmission.

1.1. Correlates of immunity

The correlates of immunity for sterile protection to malaria are not well defined [18, 19]. In both mice and humans immunized with irradiated sporozoites, sterile protective immunity is believed to be directed to both sporozoites and liver stages of malaria [1, 18]. Antibodies to sporozoites can opsonize sporozoites or neutralize invasion into hepatocytes, and potentially act synergistically with T cells to confer sterile immunity [20]. Additionally, several monoclonal antibodies against the major sporozoite surface antigen, the circumsporozoite protein (CSP), are capable of mediating protection upon passive transfer into animals [21].

Regarding cellular response, studies in mice involving depletion of CD4+ and CD8+ T cells indicate a T cell response is essential for protective immunity [22–24]. In mice immunized with irradiated sporozoites, IFN-γ produced by CD4+ and CD8+ T cells is believed to be the main antiparasitic effector produced by the immune response [18, 23, 25, 26]. IFN-γ may activate effector cells, such as natural killer cells and macrophages that can target and kill parasite-infected liver cells. CD8+ CTLs that recognize *Plasmodium* antigens presented by major histocompatibility complex (MHC) class I molecules on parasite-infected liver cells also may target those cells for destruction [25, 27–29]. This has led to identifying epitopes and vaccination approaches against malaria that elicit CD8+ T cell-mediated IFN-γ secretion and development of CTLs [25, 28, 30]. However, it is important to note that in studies of mice with incapacitated CD8+ T cell function, CD4+ T cell-dependent sterile immunity can be elicited upon immunization with irradiated sporozoites [31]. Additionally, studies evaluating peptide
vaccines in mice indicate that CD8+ T cells may not be necessary for sterile immunity but rather CD4+ T cells provide a critical role in immunity [18, 32–35]. Thus, in mice there is not a definitive marker for sterile immunity, and it is plausible that different immune responses may jointly contribute to protection.

In humans, immunization with irradiated sporozoites induces protective immunity consisting of both humoral and cell-mediated responses. Cell-mediated immunity comprises of a mixed population of CD4+ and CD8+ T cells producing IFN-γ and cytotoxic activity, both of which have been shown to possess antiliver stage activity [19, 25]. However, upregulation of CD8+ T cells does not always correlate with protective immunity in humans [10, 19, 25]. For example, immunity elicited in humans under chloroquine chemoprophylaxis with \textit{Plasmodium falciparum} sporozoites is associated with CD4+ T cell production of IFN-γ, TNF, and IL-2 [12, 14], but not with upregulation of CD8+ T cells. Although the role of CD8+ T cells in mediating sterile protection in humans is still unclear, it appears that multiple factors including antibody, CD4+ and CD8+ T cells collectively contribute to overall protective immunity in humans.

1.1.1. Immunological challenges

Two genetic restrictions impact development of vaccines to malaria. The first genetic restriction is related to the extreme heterogeneity of the \textit{Plasmodium} genome and resulting proteome, and existence of a large variety of different circulating strains (particularly for \textit{P. falciparum}) that produce antigens that vary in sequence. As a result of this extreme heterogeneity, in many cases, efforts to identify antigens that can elicit protection against a broad range of malaria strains have been limited to the antigens that are the least variable. In this case, proteins selected for vaccine development are restricted to those relatively conserved across strains, which limits the available vaccine targets. The second restriction involves the population of HLA alleles on each person’s cells that recognize these vaccine candidates and are involved in the induction of an immune response. Because of HLA polymorphism, individuals in a population will not generally recognize the same epitopes in an antigen. That is, a single individual in a given population will have HLAs that are capable of binding only a subset of the epitopes present in a vaccine. This means that a single epitope may only stimulate an effective immune response in a small percentage of any population, depending on the representation of the relevant HLA allele(s) in that population [28, 36–38]. The combined effect of these two restrictions is that an effective vaccine must provide many antigenic epitopes to ensure an effective immune response to the spectrum of circulating \textit{Plasmodium} strains is elicited in a significant percentage of a population.

The need for a multiantigen vaccine has resulted in a number of studies identifying conserved epitopes in \textit{Plasmodium} proteins that can stimulate CD4+ or CD8+ T cells from individuals either exposed to malaria or immunized with sporozoites [36–38]. The approach has been refined to identify promiscuous peptides capable of binding more than one type of HLA, resulting in antigens that can affect immune response in a larger percentage of the population. Incorporation of these epitopes into chimeric recombinant proteins for processing by immune cells to produce the peptides and stimulate T cells has been done [39, 40]. Examples of this approach are the multiepitope strings in ME-TRAP [41] and EP1300 (Section 4). Unfortunately,
Another confounding element to vaccine development, in general, is the elicitation of regulatory T cells (Tregs). It is understood that Tregs represent a “self-check” for providing immune tolerance to self and preventing the damaging effects of immune overreaction. For example, Tregs are involved in shutting down immune responses after they have successfully eliminated invading infectious agents, as well as their endogenous role of preventing autoimmunity [46]. However, some pathogens have been found to exploit development of Treg responses in order to evade elimination and persist in the human host. Tregs can also be induced by vaccination and some vaccine development efforts, such as those against tumors and chronic infection (e.g., tuberculosis), have included steps to overcome the constraints of such vaccination-induced Treg responses that result in suppression of immunity [47]. One somewhat successful approach pursued by biotechnology and pharmaceutical companies is development of adjuvant formulations, small molecules, and monoclonal antibodies to overcome the induction of and/or inhibit Treg function [48]. This area of study is also important regarding the development of malaria vaccines, as there is a growing set of data to support the conclusion that induction of Tregs during acute malaria infection limits the generation of immune memory and increases susceptibility to infection [49]. Of particular concern for malaria vaccine development efforts is that in the infant target population, functional Tregs are present in high numbers [49]. Therefore, tools to identify and remove Treg epitopes from malaria vaccines could be critical to the development of candidates that induce strong T cell responses.

1.1.2. Antigen discovery efforts

The understanding that infection with attenuated sporozoites can confer sterile immunity led to a new focus on discovery of liver-stage antigens [1]. Serological approaches to antigen discovery have been used in malaria research; however, sera from chronically infected individuals predominantly recognize the blood-phase of infection [50]. Therefore, discovery of liver-stage antigens has required methodologies that exploit recent discoveries of immunity targeting the liver-phase of infection. For example, immunological approaches using sera from individuals on antimalarial therapy (CPS-CQ or CPS-MQ) while in malaria-endemic regions were applied to screen libraries expressing Plasmodium peptides [51, 52]. Later, similar approaches were applied using blood from individuals immunized with irradiated sporozoites to evaluate expression libraries for reactivity with antibodies [53, 54], or T cell responses with peripheral blood mononuclear cells (PBMCs) [55]. These types of approaches continue to be a source of new antigens [56].

The availability of numerous genomic sequences from Plasmodium species has greatly facilitated such studies and made possible further large-scale genomic screening approaches that apply transcriptional analysis using isolated sporozoites with targeted comparisons to different phases in the Plasmodium lifecycle [57–61]. When applied to vaccine discovery, the genomic screens typically include a selection of proteins shown to react with antibodies or responsive T cells from individuals immune to Plasmodium. On a smaller scale, specific stages of the parasite’s lifecycle have been evaluated using proteomic analysis [54, 62, 63]. These
efforts have been augmented by extensive cross-comparison of results to prioritize those antigens identified by more than one approach. Many of these antigens, which show a relatively conserved primary protein structure, are being evaluated and developed as components of subunit vaccines against malaria (Section 5).

1.1.3. Surrogate models of protection

One of the challenges in vaccine development is the need for an animal model that clearly correlates to immunity and efficacy in humans. Mice are frequently used to evaluate antigens for both the resulting immune response and efficacy to preventing infection. Both inbred and outbred strains have been evaluated. While inbred strains (e.g., BALB/c and C57BL/6) provide more consistent results, outbred strains (e.g., CD1 and ICR) are believed to better represent the diversity of the immune response that is encountered in humans due to the polymorphism of the MHC (HLA in human). As *Plasmodium* strains that infect humans do not infect rodents, mouse models of malaria either require (1) use of rodent malaria species (e.g., *P. berghei*, and *P. yoelii*) and immunization with the rodent malaria ortholog of the target antigen, which is generally divergent from the corresponding vaccine target from a *Plasmodium* species that infects humans (e.g., *P. falciparum* and *P. vivax*); or (2) development of chimeric parasites where the rodent malaria ortholog of the target antigen has been replaced by the corresponding protein from a human malaria species. In some cases, orthologs of the vaccine targets do not exist in the rodent malaria species, making evaluation in animal models difficult (e.g., LSA-1 and SIAP2 [61], and SIAP2 [61, 64]). While chimeric rodent malaria strains that express the *P. falciparum* antigen have still been used in this type of scenario [33], the impact upon the physiology of the parasite, the host-pathogen interaction, and resulting response to vaccination is not clear. In rare cases, orthologs are sufficiently similar between the rodent and human malaria species to permit immunization using the human malaria antigen and challenge using a wild-type rodent malaria species [65]. While mouse models of malaria can be useful, immune response and level of protection to a target antigen can vary when different rodent malaria strains/species or chimeric models are used, making analysis difficult as it is not clear which of these datasets may be most applicable to or predictive of *P. falciparum* in humans [19, 28].

The use of nonhuman primate (NHP) models provides a more phylogenetically related model of human disease for both the host and parasite. However, cost and the regulations concerning research with NHPs have limited the use of these models. Even when used to evaluate immunity and safety of vaccines, NHPs are typically not challenged with sporozoites, so vaccine efficacy cannot be determined [66–68]. Further, interpretation of results using a NHP model must be balanced with the realization that old-world monkeys do not have the ortholog to the human HLA-C locus, which is one of the three classical human MHC class I loci [69]. In humans, HLA-A and HLA-B (the other two classical human MHC class I loci) exhibit extreme diversity through extensive allelic polymorphism in each class I gene [70]. Whereas in Rhesus *macaque* (the best characterized old-world monkey), there are very limited morphotypes for class I genes, but relative to humans there are more types of class I genes and extensive diversity of the combination of the genes resulting in a potential 10-fold greater diversity of proteins in NHPs [70, 71]. Therefore, the limited overlap of NHP and human MHC, with each species
containing unique MHC genes, and the much larger repertoire of MHCs in NHP makes it difficult to extrapolate results in NHP immune response to predict the response in humans. Thus, the immune response of NHPs to peptides developed for a human vaccine must be cautiously interpreted, and extrapolation of the results to define an appropriate dosage for use in humans may be problematic. Because of these limitations, many antigens have rapidly progressed to testing in humans with very limited efficacy testing in animals.

Lastly, it is noteworthy that several research groups have recently developed humanized mouse models that mimic human immune system for the purpose of testing human malaria vaccines. These humanized mice can mount both human T cell and antibody-mediated immunity in the context of HLA-restriction [72–74]. Other efforts have been made toward creating a mouse model having human liver [75–79]. These humanized liver mice can sustain development of human malaria parasites from sporozoites to blood stages.

2. Pre-erythrocytic vaccines based on CSP

2.1. Structure and antigenicity

One of the most studied of malaria proteins is the sporozoite surface antigen CSP, which forms a dense coat surrounding the parasite. This protein is involved in sporozoite motility and invasion where contact of the N-terminal region of CSP with hepatocytes triggers cleavage of the N-terminal region from the remainder of the protein [80–82]. This cleavage event is required for sporozoite infection of hepatocytes [80, 81, 83]. Apart from the central repeat region, CSP contains two regions of high conservation: Region I in the N-terminal third of the protein that includes the proteolytic cleavage site, and Region II in the C-terminal third of the protein; the latter has some hepatocyte binding activity [82, 84] and is located in a section of CSP enriched in T cell epitopes (Figure 1).

Figure 1. Schematic of full-length CSP identifying important epitopes.

Over 30 years ago, NANP repeats in the central repeat region of the protein were identified as the target of protective antibodies [85–88]. The T1 epitope, which is located in the *P. falciparum* CS minor repeat region, comprised of alternating NANPNVDP repeats, was originally identified by human CD4+ Th1-type T cells from a volunteer immunized with irradiated *P. falciparum* sporozoites [87]. Multiple CD4+ and CD8+ T cell epitopes have been identified in
the C-terminal region of CSP, and these are recognized by murine and human cells. Several CD4+ T cell epitopes in this region, labeled as T*, UTC, or TH2 in various publications, include one or more epitopes termed “universal” as they are recognized by a broad spectrum of HLA class II molecules. The T* epitope is present in the C-terminus of CSP amino acids 326–345 and promotes both cytotoxic and helper CD4+ T cell responses. The T* epitope was identified using CD4+ T cell clones derived from volunteers immunized by multiple exposures to the bites of irradiated *P. falciparum* (NF54 strain) infected mosquitoes [89]. The T* epitope spans part of a polymorphic region as well as a portion of the conserved Region II. Different CD4+ T cell clones selected from these irradiated sporozoites volunteers recognized truncated peptides (8–18 amino acids long), all of which contained the RII sequence [89]. Importantly, the T* epitope, unlike the T1 epitope, is presented *in vitro* by multiple DR alleles reflecting a broad MHC class II binding pattern, thus suggesting a “universal CD4+ T cell epitope” for malaria [89, 90].

It has been speculated that CSP-specific T cells are required to provide protection through help for antibody production and CTL function, as well as direct cytokine-mediated antiparasite activity [22, 91]. However, the regions of CSP containing immunodominant T cell epitopes are also highly polymorphic among circulating *P. falciparum* strains [92], which is problematic regarding their use in vaccines.

### 2.1.1. RTS,S (Mosquirix)

The most advanced malaria vaccine (RTS,S) is a virus-like particle (VLP) consisting of a fragment (central repeat and C-terminal regions, amino acids 207–395) of the CSP fused to a hepatitis B virus surface antigen (Figure 1). Phase 3 clinical trials of RTS,S/AS01 (commercially known as Mosquirix), a liposomal adjuvant formulation AS01 which contains monophosphoryl lipid A (MPLA) and QS-21, have provided evidence for high levels of antiCSP antibody correlating with reduced clinical malaria episodes [93]. Interestingly, individuals immunized with irradiated sporozoites or RTS,S generate antibodies to the repeat region, which represents the immunodominant region of the protein under these circumstances; however, adults in endemic regions naturally infected with the parasite, and thus partially immune from disease, have high levels of antibodies against the C-terminus and other nonrepeat regions [94]. In malaria nonexposed individuals immunized the with RTS,S vaccine, protection correlated with CD4+ and CD8+ T cells responses to the C-terminal region of CSP [95], while in other studies, sterile immunity was correlated with antirepeat antibodies [96]. In endemic areas, protection against clinical disease in RTS,S immunized infants also correlated with antiCSP antibodies [97]. Altogether, the protective efficacy of the CSP-based RTS,S/AS01 vaccine is a clear demonstration that a recombinant subunit vaccine containing a portion of the protein delivered on a heterologous VLP carrier can provide partial protection in humans. As 30–50% protection against clinical disease was obtained in RTS,S immunized infants and children in endemic areas, it is clear that there is room for improvement upon the RTS,S vaccine.

### 2.2. Vaccines containing CSP repeats

Whereas there are several multiantigen pre-erythrocytic vaccines that include CSP repeat units along with other CSP epitopes, the vaccines described in this section contain only CSP repeat
units or CSP repeat units combined with epitopes from other malaria antigens. One of the earliest vaccine trials with epitopes from CSP was a Phase I trial with controlled human malaria infection (CHMI) where 35 volunteers were immunized with a three-unit NANP repeat peptide (NANP), conjugated to tetanus toxoid (TT) and adjuvanted with alum [98]. As the CSP repeats are a known B cell epitope, the three volunteers with the highest ELISA and immunofluorescence assay (IFA) titers were challenged with *P. falciparum* sporozoites (strain NF54); one of these volunteers was sterilely protected. A second trial with CHMI was conducted with this same vaccine where 202 volunteers were immunized and four volunteers with the highest titers were challenged; however, no sterile protection was seen in the challenged volunteers [99]. The results from these trials spurred development of vaccines containing minimal epitopes from CSP that contain CSP repeat units as well as the combination of CSP repeat units with epitopes from other malaria antigen vaccine targets.

One effort to explore combination of CSP repeat units with another malaria vaccine target includes development of a series of influenza virosome-based vaccines. However, virosome-based antigen developed contains a slight variation of the CSP repeat units, NPNA rather than NANP [100]. The resulting vaccine incorporating a constrained (NPNA)$_3$ repeat was termed PEV302. In a Phase I study, 16 volunteers received PEV302 and eight volunteers received a combination vaccine containing PEV302 as well as an influenza virosome containing a portion of domain III from AMA-1, termed PEV301 [101]. Sera collected from volunteers were used to assess ELISA and IFA titer to the constrained repeat peptide and *P. falciparum* (NF54) sporozoites, respectively. Of the 21 volunteers immunized with PEV302, 19 demonstrated titers ≥10$^2$ after three immunizations [102]; however, a challenge was not conducted as part of this study. A second trial that included CHMI was conducted with these vaccines where the PEV301 and PEV302 epitopes were combined (known as PEV3A) and administered together to 12 volunteers [103]. In this same trial, 13 volunteers were co-immunized with PEV3A and ME-TRAP. Both groups were challenged; however, all volunteers developed parasitemia. The ELISA titers to UK-39 constrained repeat peptide and IFA titers *P. falciparum* (NF54) sporozoites elicited in both studies were similar with all individuals in the second study demonstrating titers ≥10$^3$.

### 2.3. Vaccines containing minimal CSP epitopes

It has been suggested that malaria, along with other infectious diseases (e.g., HIV), has resisted classical vaccine strategies by utilizing a method of stimulating either strain-specific or incomplete, nonprotective immunity, which appears to have arisen from having co-evolved with humans for millions of years [104]. Further, studies with CSP in particular have suggested mechanisms other than allelic variation for parasite escape, even suggesting that the immune-dominant repeat region presents as an immunogenic “decoy” that elicits nonneutralizing antibody responses [105]. In general, the majority of vaccine developed strategies have targeted pathogens that display little antigenic variation, are highly conserved among isolates, and are readily inhibited by immune responses stimulated by native antigens, which is not the case for malaria. Furthermore, that only partially protective immune responses result after years of malaria exposure suggests that the parasites may be utilizing a “cloaking” strategy to hide
in plain sight of the host immune system such as incorporation of human-like protein sequences to induce Treg expansion [106]. Indeed, for vaccine targets, it has been shown that the degree of cross-conservation of predicted epitopes with the human genome inversely correlates with their immunogenicity [107], and in the case of malaria, Tregs (via PD1 upregulation) are responsible for a low frequency of precursor T cells [5]. Therefore, utilizing a minimal, well-designed epitope set for one or more targeted antigens may provide the most effective means to circumvent the parasites’ avoidance mechanisms.

Efforts to combine the CSP repeat unit with other CSP epitopes include development of a series of synthesized peptide constructs containing (NANP)₃ (also known as B), the T1 epitope (NANPNVDP)₂, and in later studies, the T* epitope (Figure 1). A Phase I trial of a synthetic multiple antigenic peptide (MAP) immunogen, which includes four linked TIB peptides adjuvanted in alum with or without QS21, found that better ELISA and IFA titers were achieved with QS21 formulations [108]. This study also demonstrated that recognition of the T1 epitope by T cells is highly MHC-restricted with T cell responses limited to high responder MHC class II genotypes (present in only 25–35% of the population). None of the volunteers in this study were challenged. To address limitations of the T1 epitope, the T* epitope (which is recognized in the context of many human MHC class II molecules) was included in a branched synthesized peptide immunogen containing four linked TIBT* peptides as well as a linked Pam3Cys as endogenous adjuvant [109]. In a small Phase I trial of the tetra-branched TIBT* peptide in 10 volunteers of diverse HLA types, the majority of the volunteers (8/10) seroconverted following the first dose and reached peak antirepeat antibody titers of $10^3$–$10^4$ following three immunizations [110]. The immunized volunteers developed T*-specific Th1-type CD4+ T cell responses. CD4+ T cell clones derived from PBMC up to 10 months after peptide immunization recognized the T* epitope in the context of multiple HLA DR and DQ molecules and secreted high levels of IFN-γ and variable levels of TNF-α [111]. The immunized volunteers in this study were not challenged. Note that up to 1 mg of immunogen was safely administered per dose in these clinical studies.

Additional clinical trials have investigated novel delivery platforms and adjuvants to enhance immunogenicity of CSP minimal T and B cell epitopes. A VLP based on the hepatitis B virus core antigen, ICC 1132, was engineered to express the T1, B (NANP)₃, and T* epitopes [112]. A total of four clinical studies were conducted with this antigen. Phase I trials using alum as adjuvant elicited suboptimal antibody and cellular responses [113, 114]. Use of a more potent water-in-oil emulsion-based adjuvant, Montanide ISA 720, enhanced not only immunogenicity, but also reactogenicity in a NHP model [115]. Two clinical studies were conducted with ICC 1132 formulated in Montanide ISA 720; however, due to the potential for increased reactogenicity, only a single administration was given. In the first study with ICC 1132 in ISA 720, escalating doses of antigen (from 20 to 50 μg) were administered [116]. Both ELISA and IFA titers with a single dose of ICC 1132 in ISA 720 were improved as compared to those achieved with three doses of ICC 1132 in alum. A second clinical study was conducted where a single 50μg dose of ICC 132 in ISA 720 was administered to 11 volunteers that were subsequently challenged; however, none of the volunteers were protected [117]. A summary of Phase I clinical studies, with CSP vaccine candidates, is provided in Table 1.
<table>
<thead>
<tr>
<th>Category</th>
<th>RTS,S</th>
<th>PI CS 282–383</th>
<th>NANP-TT</th>
<th>(T1B)4</th>
<th>(T1BT*)4</th>
<th>ICC-1132</th>
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<td><strong>Epitopes</strong></td>
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<td>NANP, aa 302–383 (282–383)</td>
<td>(NANP)3</td>
<td>NANNP, NYDP</td>
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<td>NYDP (T1), (T*) aa 326–345</td>
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<td>MAP</td>
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<td>HBcAg (truncated)</td>
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<td><strong>IFA: % titer &gt;10</strong></td>
<td>100% [6400]</td>
<td>100% [4755]</td>
<td>75% [654]</td>
<td>50% [ND]</td>
<td>100% [2364–2810]</td>
<td>25–67% (+QS21) [216]</td>
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<td><strong>ELISA: % Seroconv. (titer &gt;10), coat Ag</strong></td>
<td>100%, NANP</td>
<td>100%, NANP</td>
<td>100%, NANP</td>
<td>33–57%, NANNP</td>
<td>28–383</td>
<td>63–100% (T1B)4</td>
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<td><strong>Cellular proliferation</strong></td>
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<td>ND</td>
<td>ND</td>
<td>0%</td>
<td>63% (ISA)</td>
<td>0%</td>
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<tr>
<td><strong>Protection (# protected/# challenged)</strong></td>
<td>86% (6/7) 50μg x 3 29% (2/7)</td>
<td>13% (1/8)</td>
<td>0% (0/6)</td>
<td>33% (1/3)</td>
<td>ND</td>
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Percentages indicate percent responders for IFA, ELISA, and cellular proliferation; ND, not determined; GMT, geometric mean titer.

**Table 1.** Summary of clinical trials of CSP vaccine candidates (including RTS,S CHMI studies).
2.4. Long synthetic CSP peptide vaccines

In addition to vaccine studies using minimal T and B cell epitopes, clinical studies have investigated immunogenicity of long synthetic peptides containing the entire N- or C-terminus of *P. falciparum* CSP [118]. Of interest is the Pf CSP 282-383 study where a long synthetic peptide including C-terminus amino acids 282–383 from CSP (PfCS102) was synthesized and administered to volunteers as a formulation in either alum or Montanide ISA 720 [119]. Elevated ELISA and IFA titers were seen with ISA 720 formulation as compared to alum. In a second clinical trial, PfCS102 formulated in ISA 720 or AS02A elicited IFN-γ secreting CD8+ T cells specific to malaria in some individuals [120]. Tenfold higher antibody and cellular responses were obtained with the AS02 adjuvant formulation. However, no volunteers were challenged in these studies. Note that synthesized peptide doses of ≥100μg were administered in this study.

2.5. Preclinical pipeline of novel CSP-based vaccines

Additional preclinical studies of VLP have utilized a woodchuck hepatitis core antigen engineered to express CSP repeats in the loop region and multiple universal T cell epitopes at the C-terminus, termed Mal-78-3T VLP [125]. Mice immunized intraperitoneally (i.p.) with Mal-78-3T VLP, formulated in alum/QS21 or ISA 720 adjuvant, developed high levels of antirepeat antibodies. Sterile immunity was obtained in Mal-78-3T immunized mice of three different strains following challenge by transgenic sporozoites expressing *P. falciparum* CSP repeats.

The minimal T and B cell epitopes have also been tested as a recombinant protein using a TLR5 agonist, flagellin, as adjuvant in murine studies [126, 127]. Chimeric flagellin protein containing multiple T1BT* modules, or nearly full-length *P. falciparum* CSP, elicited high levels of antirepeat antibody. The chimeric protein was immunogenic without addition of exogenous adjuvant when delivered subcutaneously or as a needle-free intranasal vaccine.

In addition to the TLR 5 agonist, a TLR-7 agonist, imiquimod, was also found to be a potent adjuvant for vaccines containing minimal T and B cell epitopes. Immunization of mice with the linear T1BT* peptide subcutaneously followed by application of topical TLR-7 agonist imiquimod induced protective antibody-mediated immunity as well as splenic CD4+ T cell responses [128]. Recent Phase I/II studies have demonstrated that topical imiquimod can also enhance immunogenity and protective efficacy of a seasonal flu vaccine [129, 130], supporting the use of the murine model for identification of promising TLR agonist adjuvants for human trials.

Of interest are murine studies demonstrating that micro- and nanoparticle vaccines containing minimal T and B cell epitopes were immunogenic without the addition of exogenous adjuvants. Layer by Layer (LbL) microcapsules were constructed by sequential layering of positively charged poly-l-lysine and negatively charged poly-L-glutamic acid with derivatized peptides containing T1BT* epitopes added to the final layer [131]. Mice immunized with LbL microparticles in PBS elicited sporozoite neutralizing antibodies that blocked *in vitro* invasion of transgenic *P. berghei* sporozoites expressing *P. falciparum* CSP repeats into human hepatoma
cells and reduced parasite liver burden following challenge by exposure to the bites of mosquitoes infected with the transgenic rodent parasites. Protection was comparable to levels obtained following immunization with TIBT* peptide in Freund’s adjuvant.

Self-assembling proteins that form nanoparticles (SAPNs) have also been examined in murine studies [132, 133]. The SAPN is comprised of 60 monomers of a recombinant linear protein containing trimeric and pentameric coiled-coil domains separated by a flexible linker with T and B cell epitopes expressed at N- or C-terminus. Following expression in *Escherichia coli*, monomers self-assemble to form spherical SAPN of ~40 nm in diameter. A *P. falciparum* SAPN was constructed comprised of monomer containing four copies of the NANP repeats at the C-terminus and three previously identified *P. falciparum* CSP CD8+ T cell epitopes (KPKDELDY, MPNDPNRNV, and YLNKIQN) at the N-terminus, along with a designed pan-DR binding T cell epitope termed PADRE. All strains of mice immunized with two or three doses of SAPN developed high ELISA titers that persisted over 52 weeks and CD8+ T central memory cells secreting IL-2 and IFN-γ. Mice challenged by i.v. injection of transgenic rodent parasites expressing full length *P. falciparum* CSP protein showed 90–100% protection, with 50% of SAPN immunized mice still protected against sporozoite challenge at week 52. Protective immunity was mediated by both sporozoite neutralizing antirepeat antibodies and CD8+ T cells.

One notable target absent in most CSP-based constructs is the N-terminus of CSP, which contains both T and B cell epitopes as well as the highly conserved Region I (Figure 2). Further, within Region I, resides the proteolytic cleavage site and a ligand-binding domain which have been shown to be key requirements for sporozoite infection of hepatocytes [80, 81, 83]. Antibodies raised to this region have been shown to confer 90% protection in animal passive transfer studies [134]. Therefore, efforts are now being pursued to identify and incorporate this epitope into some of the strategies described in this section.

![Figure 2. Diagram of LSA-1 (adapted from [164]).](image)

### 3. Vaccines containing liver stage antigens

#### 3.1. Cell-traversal protein for ookinetes and sporozoites (CelTOS)

##### 3.1.1. Structure and antigenicity

As its name suggests, CelTOS is expressed in both the ookinetes and sporozoite stages of malaria, and functions in cell transversal [135]. This protein is highly conserved across multiple
species of *Plasmodium* including those that cause malaria in rodents (*P. berghei*) and primates (*P. falciparum* and *P. vivax*) [135]. Identification of CelTOS (182 AA—GenBank: AAN36249.1) as a potential protective antigen built upon a previous proteomic analysis identifying expressed proteins in specific phases of the *P. falciparum* lifecycle [62]. Using prototypical HLA supertypes, algorithms were developed to identify potential peptides that could bind to HLA supertypes, and peptides from 27 proteins were screened for stimulating PBMCs from volunteers vaccinated with irradiated sporozoites and subsequently challenged with infection by *P. falciparum* [55]. From these, CelTOS was identified as stimulating the highest number of effector T cells producing IFN-γ overall [55]. Subsequently, Kaiser et al. identified CelTOS using a suppression-subtractive hybridization approach in *P. yoelii* sporozoites versus merozoites [58].

In malaria-naive volunteers infected with irradiated sporozoites, peptides from CelTOS stimulate IFN-γ production from effector T cells in eight out of 12 volunteers [55]. Six out of 35 Ghanaian adults demonstrated effector T cells to CelTOS [136]. In mice immunized with irradiated sporozoites, low levels of IFN-γ producing CelTOS-specific CD8+ T cells are induced [137].

### 3.1.2. Preclinical and clinical trials of CelTOS vaccine

Vaccines comprised of CelTOS recombinant protein or expression vectors (i.e., DNA and viral vectors) have demonstrated protective efficacy against malaria in mouse models. CelTOS administered as a recombinant protein with the adjuvants Montanide ISA 720 or glucopyranosyl lipid A stable emulsion (GLA-SE) elicited CelTOS-specific antibodies, CD4+ and CD8+ T cells, and protection (10–76% protection, depending on dose and mouse strain) from infective challenge with *P. berghei* in BALB/c and CD1 mice [65, 138, 139]. This protection was demonstrated using homologous and heterologous challenge, demonstrating the unique level of conservation of CelTOS even across species of the parasite [65, 138]. Note that a challenge was not conducted with GLA-SE formulations. In mice vaccinated with recombinant CelTOS, protective immunity did not correlate with the level of antibody production, and passive immunization did not provide significant protection from infection [138]. In one study, self-adjuvanting bacterial vectors expressing CelTOS were used to vaccinate BALB/c mice and sterile immunity (40–60% protection, depending on dose) was demonstrated even though an antibody response to CelTOS was not detected [140]. In these studies, clearance of parasites infecting the liver plays a major role in protective immunity, which is likely potentiated by CD4+ and CD8+ T cells [138, 141]. Protective immunity in BALB/c mice was dependent on the mixed response of both the CD4+ and CD8+ T cells to CelTOS [138, 140, 141]. Studies using viral vectors encoding CelTOS did not demonstrate protective immunity in either BALB/c or CD1 mice, even though an antibody response and moderate levels of CelTOS-specific CD4+ and CD8+ T cells were induced, and control cohorts immunized with CSP in the same prime/boost protocol did demonstrate sterile immunity [33, 137].

Two Phase I trials with CHMI have been performed with a recombinant CelTOS protein produced in *E. coli* (FMP012) formulated with either GLA-SE or AS01B and tested for safety and efficacy (ClinicalTrials.gov: NCT01540474; ClinicalTrials.gov: NCT02174978). Malaria-
naïve adults (18–50 years of age) were vaccinated three times with FMP012 GLA-SE or four times with FMP012 AS01B, and adverse events were monitored. Antibody titers to FMP012 were monitored. Vaccinated volunteers along with nonvaccinated controls were challenged with *P. falciparum* to determine the efficacy of the immune response as determined by time to parasitemia evaluated by blood smear. Results for neither trial have been published.

### 3.2. Exported protein-1 (EXP-1)

#### 3.2.1. Structure and antigenicity

Exported protein 1 (EXP-1) (162 AA—GenBank: AAN35808.1) was discovered by screening an *E. coli* expression library of blood stage antigens using a monoclonal antibody to *P. falciparum* and was designated antigen 5.1 (Ag5.1) [142]. It was subsequently rediscovered by other screening approaches and designated circumsporozoite-related antigen (CRA), EXP-1, QF116, and *P. yoelii* hepatocyte erythrocyte protein 17 (HEP17) [143–147]. Adults in malaria-endemic regions produced antibodies to the protein and EXP-1-dependent CD4+ and CD8+ T cells [37, 38, 143]. EXP-1 was incorporated in some of the earliest subunit vaccines for malaria tested in humans [148], and peptides from this protein continue to be incorporated into vaccines for malaria, such as ex23 used in ME-TRAP [44].

EXP-1 is conserved across strains of specific *Plasmodium* species [143, 147]. The demonstration that this protein elicited an immune response that provided protection across a diverse spectrum of murine malaria strains in challenged mice [149] led to analysis identifying supertype-peptides that elicited response from a variety of HLA types [37]. In addition to being a vaccine target, the protein is a glutathione S-transferase that has been associated with artemisin metabolism, making it a potential drug target for malaria [150].

The immune responses of people in malaria endemic regions demonstrate that EXP-1 elicits antibody and IFN-γ responses from CD4+ and CD8+ T cells. Indonesian and African adults had detectable antibodies that react with EXP-1 [151–153]. In African children in malaria endemic regions, the presence of antibody responding to an EXP-1 peptide encompassing amino acids 101–162 were associated with decreased infection [154]. Low level IFN-γ and CD4+ T cells were seen in response to EXP-1 in West Africans, although CD8+ T cells were not detected [151]. A similar limited response of CD8+ T cells and low level CD4+ T cells by Kenyans was noted with only one peptide, EXP-10, stimulating CD8+ T cells [37, 38]. Adults from Gambia and Tanzania with natural immunity to malaria develop EXP-1-dependent CTL and the EXP-1 peptide EX23 has also been shown to elicit a CTL response [155]. In another study, African children naturally exposed to malaria only developed low levels of EXP-1-dependent IFN-γ expression [156]. However, the peptides used for this study (EXP2, EXP80, and EXP91) were previously demonstrated to give no CD8+ T cell response in African adults [37], and may not reflect the potential for EXP-1 as a vaccine component. This potential is demonstrated in Caucasian adults, that when immunized with irradiated sporozoites developed EXP-1-dependent CD8+ T cells as well as CD4+ T cells [37, 55].
3.2.2. Preclinical and clinical trials of EXP-1

EXP-1 provided protection from malaria in a mouse model where protection was dependent on the development of CD8+ T cells and expression of IFN-γ or nitric oxide [149]. This study resulted in an early emphasis on CD8+-dependent IFN-γ production in the development of subunit vaccines against malaria.

5.1-(NANP)19 is a recombinant protein expressed in *E. coli* that contains EXP-1 fused with 19 repeats of the tetramer NANP from CSP [157]. Thirteen adults were vaccinated subcutaneously with 5.1-(NANP)19 using two or three immunizations containing 50 or 400 μg of protein without adjuvant [148]. The vaccine was safe and only caused reactions at the site of injection. All volunteers developed antibody to the NANP peptide, and six developed 5.1-(NANP)19-specific effector T cells. Seven volunteers were subsequently challenged with *P. falciparum* by bites from infected mosquitoes. While all seven volunteers developed parasitemia, one did not develop symptoms of malaria.

In a study with 194 semiimmune children (6–12 years old) immunized with 5.1-(NANP)19, all but eight children had considerable levels of antibody to EXP-1 [158]. None of the nonvaccinated children developed malaria in this study over the 12 weeks of observation, and the protective efficacy of the vaccine could not be determined.

EXP-1 has been tested in a number of clinical trials as a component of multiantigen vaccines: EP1300, L3SEPTL, ME-TRAP, and MuStDP5. See the discussion in Section 5, Table 3.

3.3. Liver-stage antigen-1 (LSA-1)

3.3.1. Structure and antigenicity

Liver stage antigen 1 (LSA-1) is expressed after parasites have invaded hepatocytes and antigen accumulates in the parasitophorous vacuole [52, 159]. The function of this antigen is not known. LSA-1 was discovered using antisera from volunteers who remained on chloroquine while in a malaria endemic region to obtain antibodies to the liver stage of malaria. The antisera were used to screen recombinant DNA expression libraries [52]. Subsequently, a peptide ls94 from LSA-1 was found to bind to HLA-B53, which is MHC class I associated with resistance to severe malaria in West Africa. This indicated that class-I restricted CD8+ T cells are important for protective immunity against malaria in Africans [160, 161].

LSA-1 (UniProtKB/Swiss-Prot: Q25893) is a 1909 amino acid protein from the *P. falciparum* strain NF54 [162]. Even though strain 3D7 is derived from NF54 [163], the 3D7 strain produces a protein that is 1162 amino acids in length. The amino terminal and carboxyl terminal ends of the protein are nonrepetitive, whereas the middle of the protein contains multiple repeats (Figure 2). This repeat region results in significant variation of the protein between strains of *P. falciparum* [162].

LSA-1 antigen stimulates IFN-γ producing T cells, composed of CD4+ and CD8+ T cells, and CTL was demonstrated from volunteers in regions with endemic malaria [36, 37, 151, 164–166]. The immune response to LSA-1 typically increased with age and exposure to the parasite
In Gabonese children, LSA-1-dependent IFN-γ producing T cells were correlated with reduced disease severity [169]. Further, naturally infected individuals often express antibodies to LSA-1, targeting primarily the central repetitive region of the protein [170], and antibody to LSA-1 is associated with a reduction in disease severity [171–173]. Immunization of malaria-naive volunteers with irradiated sporozoites produced an immune response to LSA-1 that included CD4+ and CD8+ T cells, and a weak antibody response [37, 38, 55, 174]. However, when volunteers were challenged with infection, the volunteers who responded to LSA-1 were not protected from malaria [55]. Further, in malaria-naive volunteers immunized by exposure to bites from infected mosquitoes while receiving chloroquine, 74% of the volunteers produced antibodies to LSA-1; however, the humoral response did not provide sterile protection from malaria [175].

3.3.2. Preclinical and clinical trials of LSA-1 vaccine

Rodent strains of Plasmodium do not contain orthologs of LSA-1 [64, 176], and animal models evaluating LSA-1 are limited to NHPs. Analysis of immunity to peptides from LSA-1 in combination with LSA-3, SALSA, and STARP in NHPs has been the focus of several studies wherein immunity to the peptides is altered by lipidating the peptides or incorporating the peptides in Montanide ISA-51 [66, 177, 178]. High levels of B cells and CD4+ T cells producing IFN-γ in response to LSA-1 were demonstrated. One of the peptides for LSA-1 (LSA1-J) elicited CD8+ CTLs. The NHPs were not challenged with infection by Plasmodium, so the impact of this immune response is not known.

Analysis of LSA-1 vaccination using prime/boost approaches using DNA viral vehicles is limited. A prime boost of LSA-1 in chimpanzee adenovirus 63 (ChAd63) and modified vaccine Ankara (MVA) in BALB/c and CD-1 mice demonstrated sterile protection using a chimeric strain of P. berghei expressing the P. falciparum LSA-1 protein [33]. Seven out of eight mice, for both the BALB/c and CD-1 mice, demonstrated a significant delay in time to onset of parasitemia. LSA-1-dependent IFN-γ production by CD4+ and CD8+ T cells, and antibodies were detected; however, the mediators for protection were not clear. In BALB/c mice where CD4+ or CD8+ T cells were depleted, protection was not dependent on CD8+ T cells; however, results suggested that CD4+ T cells play a significant role. Analysis of the immune response developed in rhesus monkeys using DNA priming and pox virus boosting (using the nonreplicating canary pox virus ALVAC-Pf7) was carried out using an antigen cocktail containing LSA-1, CSP, TRAP, AMA1, and MSP1 [179]. This resulted in a predominant CD4+ T cell response with secretion of IL-2. Low-level IFN-γ production was attributed to CD4+ T cells and CD8+ T cells were low and only present in animals with high CD4+ T cell responses. No antibodies to LSA-1 were detected. The animals were not challenged with infection by Plasmodium, so the impact of this immune response is not known.

Recombinant protein LSA-NRC was developed to assess efficacy of an LSA-1 vaccine in the clinic. LSA-NRC incorporates P. falciparum NF54 LSA-1 nonrepetitive N-terminal and C-terminal regions, and includes two 17-amino-acid repeats from the repetitive central region of the protein (Figure 3). This antigen was administered with AS01B (a liposomal formulation with MPLA and QS-21) in rhesus monkeys and elicited high titers of antibodies and CD4+ T
cells producing IL-2 alone or IL-2 with IFN-γ, but CD8+ T cells were not detected [180]. The NHPs were not challenged with infection by *Plasmodium*, so the impact of this immune response is not known.

![Figure 3. Diagram of LSA-NRC (adapted from [181]).](image)

Phase I trials with CHMI were carried out in malaria naïve volunteers using a recombinant LSA 1 protein (FMP011). LSA-NRC adjuvanted with either AS01B or AS02A was well tolerated, safe, and immunogenic at both low and high doses of the antigen [64]. All volunteers developed antibodies after the first dose of the vaccine. Vaccinated volunteers developed LSA-1 specific CD4+ T cells following the second dose. The low dose of LSA-NRC, with either AS01B or AS02A, resulted in LSA-1-dependent CD4+ T cells that produced both IL-2 and IFN-γ. However, the LSA-1 high-dose groups had fewer CD4+ T cells and little to no IFN-γ response relative to the low-dose cohort. None of the groups produced LSA-1-dependent CD8+ T cells. These results were similar to the immune response seen in the preclinical studies in mice (BALB/c and A/J mice) and rhesus monkeys [180, 182]. Only the high-dose groups were challenged along with nonvaccinated volunteers using the homologous 3D7 strain of *P. falciparum*. All 22 volunteers that were challenged became parasitemic without delayed onset of the erythrocytic infection.

Several clinical trials of multiantigen pre-erythrocytic vaccines that included LSA-1 as an antigen (EP1300, L3SEPTL, ME-TRAP, MuStDP5, and NYVAC-Pf7) have been conducted and are discussed in Section 4, Table 3.

### 3.4. Liver-stage antigen-3 (LSA-3)

#### 3.4.1. Structure and antigenicity of LSA-3

While LSA-3 is included with the liver-stage antigens, some report it is actually a blood stage antigen [183]. Using malaria-naïve volunteers immunized with irradiated *P. falciparum*, LSA-3 was identified by comparing antibody response of protected and nonprotected volunteers to a recombinant DNA expression library [53].
LSA-3 is composed of three nonrepeating regions (NR-A, NR-B, and NR-C) flanking two short repeat regions (R1 and R3) and one long repeat region (R2) (see Figure 4) [53]. The nonrepeat regions are well conserved across geographically diverse strains of *P. falciparum* [184]. The most significant variation is in the repeating regions, but this is due to the organization and the number of repeating subunits rather than composition of the repeating regions [184]. Vaccine development draws upon the protein from *P. falciparum* strains T9/96 and 3D7 (1586 AA GenBank: ACT22567.1).

![Figure 4. Diagram of the domains of LSA-3 (adapted from [185]).](image)

In individuals exposed to *P. falciparum*, LSA-3 elicits antibodies, and CD4+ and CD8+ T cells. West African adults demonstrated CD4+, and class I–LSA-3-dependent CTLs [151, 155]. Antibodies to LSA-3 were demonstrated in children from West Africa, and increased with age [171, 185], and malaria-naive Europeans develop antibodies to LSA-3 when immunized with irradiated sporozoites [53].

### 3.4.2. Preclinical and clinical trials of LSA-3

Rodent strains of *Plasmodium* do not contain orthologs of LSA-3 [176], and animal models evaluating LSA-3 are limited to NHPs. A murine model with the infectious challenge using a strain of *P. berghei* that was genetically modified to contain the *P. falciparum* LSA-3 gene has been reported [33]. BALB/c and CD1 mice were primed with ChAd63 and boosted with MVA encoding LSA-3, but sterile immunity was demonstrated in only 13% of the BALB/c mice. The BALB/c mice developed antibodies and moderate levels of PBMCs producing LSA-3-specific IFN-γ were developed. Both CD4+ and CD8+ T cells were produced against the antigen, and CD8+ T cells predominated.

Vaccination with LSA-3 has been shown to elicit sterile immunity in chimpanzees [53]. This study demonstrated sterile immunity using various forms of LSA-3, albeit only one chimpanzee was used for each specific antigen. LSA-3 was expressed as GST-fused recombinant peptides in three segments: DG (encompassing AA 1–60), NN (encompassing AA 369–447), and PC (encompassing AA 869–1786). The mixture of these three peptides (designated as LSA-3 GST) elicited sterile immunity when administered with the adjuvant SBAS2 (SBAS2 is an adjuvant containing MPLA and QS21) or with Montanide ISA 51. The synthetic lapidated peptides CT1, NR1, NR2, and RE were also evaluated. Vaccination with NR2 alone elicited sterile immunity. Further, NR2 administered with NR1 and Montanide ISA 51, or with NR1 and CT1 with Montanide ISA 51 also elicited sterile immunity. Studies in chimpanzees using the
same LSA-3 peptides in combination with peptides derived from LSA-1, STARP, and SALSA demonstrated that the LSA-3 peptides elicited IFN-γ production with a mixed response of CD4+ and CD8+ T cells [66].

Later studies in chimpanzees immunized with plasmid expressing the nearly full length LSA-3 also demonstrated LSA-3 provided protection, with three of the four immunized chimpanzees demonstrating sterile immunity [186]. In this study, only low levels of IFN-γ were detected from a mixed class I and class II response, and no antibodies were detected. Studies in Aotus monkeys immunized with recombinant LSA-3 without adjuvant also developed low but sustained levels of antibodies to LSA-3, and LSA-3 dependent IFN-γ production; however, neither correlated with protection [187]. Subsequent challenge with *P. falciparum* demonstrated that three of the five immunized monkeys were sterilely protected. Interestingly, immuno-fluorescence assay titers to *P. falciparum* sporozoites were highest in monkeys that were protected. These results were repeated when *Aotus* monkeys were immunized using two peptides of LSA-3, amino acids 100–222 in the amino-terminal nonrepeat region, and amino acids 501–596 in the repeat-2 region of the protein from *P. falciparum* strain T9/96 [187]. In this later study, monkeys were immunized with the recombinant peptides adjuvanted with AS02, eliciting antibody and high IFN-γ responses with sterile immunity in four out of four immunized animals.

A Phase I clinical trial with challenge evaluating the safety and efficacy of recombinant LSA-3 protein adjuvanted with aluminum hydroxide or Montanide ISA 720 was carried out, but the results of the study have not been published [188].

Clinical trials for multistage pre-erythrocytic vaccines that contain LSA-3 include L3SEPTL, ME-TRAP, and MuStDP5 and are discussed in Section 4, Table 3.

### 3.5. Sporozoite threonine-asparagine-rich protein (STARP)

#### 3.5.1. Structure and antigenicity

STARP (GenBank: CAA81224.1) was identified by screening a lambda library with antibodies developed in malaria exposed missionaries under chloroquine treatment [51]. It is a 604-amino acid protein with a central hydrophilic region (amino acids 85–489) that contains a complex repetitive structure that can vary in size between different strains of *P. falciparum*. Two peptides from this repetitive region (Rp10 and Rp45) have been evaluated in vaccines [51]. Even with variation in the repetitive structure, the protein is highly conserved across a variety of geographically separated strains [51, 189].

People in malaria endemic regions produce antibody to STARP [190–192], with the percentage of the population related to the rate of exposure to malaria [193]. The main STARP antibody response in Africans naturally exposed to malaria or volunteers immunized with irradiated sporozoites was to the Rp10 peptide, and purified antibody prevented 90% of the sporozoites from infecting human liver cells *in vitro* [193]. In West African children, the presence of antibody to STARP was associated with protection from malaria [171]. A conserved HLA class I epitope eliciting a CTL response in adults naturally infected with *P. falciparum* has been
3.5.2. Preclinical and clinical trials of STARP

A limited number of T cell epitopes have been identified in STARP. The conserved epitope st8 was demonstrated to elicit a CD8+ T cell CTL response in an African adult exposed to P. falciparum [36]. The Rp10 epitope is both a B cell and class I T cell epitope [189]. However, in a NHP model, the Rp10 epitope elicits low levels IFN-γ response but no detectable CTL; note that the NHPs were not challenged with infection in these studies [66, 178].

Homologues of STARP have been identified in P. yoelii (PY00217 and PY05105) and P. reichenowi (PRCDC_0700500) [176, 189]. However, no studies of animals vaccinated with STARP and challenged with infection are published.

Clinical trials for multiantigen vaccines that include STARP, L3SEPTL, and ME-TRAP, are discussed in Section 4, Table 3.

3.6. Thrombospondin-related anonymous protein (TRAP)

3.6.1. Structure and antigenicity of TRAP

The TRAP protein was initially identified based on peptide motifs in common with thrombospondin and properdin [194]. Subsequently, it was demonstrated that TRAP contributed to the binding of sporozoites to hepatic cells [195], and antibodies to the protein block binding of sporozoites to hepatic cells [196]. In addition to TRAP referring to “thrombospondin-related anonymous protein,” the literature refers to TRAP as “thrombospondin-related adhesion protein” [42] and as sporozoite surface protein-2 (SSP2) [196]. TRAP antigen from P. falciparum T9/96 (559 AA-PRF: 226137) differs by 6% from the amino acid sequence of TRAP in the 3D7 strain (574 AA-CAD52497.1). The major difference between the strains is the protein from the 3D7 strain contains hepta-repeat of the PPN sequence while T9/96 only contains a single PPN.

Early development of TRAP as a component in a malaria vaccine focused on identifying peptides that were recognized by MHC I and elicited a CTL response from individuals exposed to the parasite [36–38, 41, 197, 198]. Evaluation of the immune response to TRAP in naturally infected African donors demonstrated CD4+ and CD8+ T cells. Gambian and Kenyan adults demonstrated CD8+ T cell-dependent CTL activity against TRAP [36–38, 151, 197]. In a study in Kenya, TRAP-dependent IFN-γ producing T cells correlated with reduced disease severity in children [199]. Additionally, it has been demonstrated that a CD4+ T cell response dominated over a CD8+ T cell response to TRAP in Kenyan children and adults, and the CD4+ T cells reactive with TRAP were correlated with reduced risk of clinical malaria [200].

Analysis of the immune response to TRAP in malaria-naive volunteers immunized with irradiated sporozoites (using the 3D7 clone of P. falciparum strain NF54) demonstrated CD8+ T cell-dependent CTL activity against TRAP [37, 198], and TRAP-specific CD4+ T cells [38]. In a separate study using 12 malaria-naive Caucasian male volunteers immunized with irradiated
sporozoites, TRAP was recognized by five of the volunteers and resulted in the development of TRAP responsive CD4+ T cells, CD8+ T cells, and antibodies [55].

While antibodies are recognized as important in protection from the erythrocytic phase of malaria and reducing the severity of disease, their role in providing sterile protection in the liver-stage is controversial. *In vitro*, antibodies to TRAP can block binding of sporozoites to human hepatocytes, and it has been postulated that antibodies could help to prevent infection of the liver [201]. In a study using malaria-naive volunteers infected with *P. falciparum* while receiving chloroquine to provide sterile immunity, volunteers did not produce antibodies to TRAP or detectable memory B cells [175]. A recent study, using malaria-naive volunteers vaccinated with irradiated sporozoites and subsequently challenged with the homologous strain of *P. falciparum* (3D7 clone of NF54) correlated the magnitude of the antibody response to TRAP with sterile protective immunity [54]. In this study, both sterilely protected individuals (six volunteers) and individuals who developed blood-stage parasitemia (five volunteers) developed antibodies to TRAP and other *P. falciparum* antigens, but the magnitude of the immune response was significantly higher in the protected volunteers. In a similar but separate study using 12 volunteers immunized with irradiated sporozoites, while a subset of the volunteers responded to TRAP, the immune response to TRAP was associated with volunteers who did not develop sterile immunity [55].

3.6.2. Preclinical and clinical trials of TRAP vaccine

Recent studies have tested TRAP alone and in combination with RTS,S in malaria naive adults [202]. The expressed protein was a truncated form of TRAP consisting of amino acids 26–511 with an additional hepta-histidine at the carboxyl-terminus. Volunteers immunized with TRAP in AS02 developed antibodies and modest CD4+ and CD8+ T cell responses (46 SFU/10^6 PBMCs). In an effort to determine if there was a synergistic effect of immune responses to TRAP and CSP, a clinical study with CHMI was conducted where volunteers were immunized with two 25 μg doses of TRAP or two doses of both TRAP and RTS,S (25 and 50 μg, respectively) in AS02. Following challenge, 1/11 volunteers (9%) developed sterile immunity in the cohort vaccinated with TRAP and RTS,S with no significant delayed patency in remaining volunteers. The level of protective efficacy was lower than was previously reported for immunization with RTS,S alone, suggesting potential antigenic competition.

Clinical trials of multiantigen vaccines containing TRAP, including EP1300, L3SEPTL, METRAP, MuStDP5, and NYVAC-Pi7 are discussed in Section 4, Table 3.

4. Multiantigen pre-erythrocytic vaccines

Early studies with multiantigen vaccines were based on the rational that a combination of antigens would elicit a broad immune response more comparable to that elicited by whole sporozoite vaccine. A number of multiantigen vaccines comprised of DNA, polyprotein, and recombinant viral vectors containing combinations of epitopes or entire pre-erythrocytic antigens have been tested in clinical trials (listed in alphabetical order).
4.1. EP1300

EP1300 is a multivalent DNA vaccine composed of a total of 38 CTL and 16 HTL epitopes derived from TRAP, CSP, EXP-1, and LSA-1 expressed as a single protein, and the NANP repeating epitope from CSP for antibody development (Figure 5). The epitopes are linked using spacers that facilitate proteolytic processing of the protein into individual epitopes in the body; while this worked in animals, processing may not have occurred correctly in humans.

A Phase Ia dose-escalating study in healthy malaria-naive adults (18–40 years of age) was initiated in 2010 to evaluate the safety and immunogenicity of EP1300. EP1300 was delivered using electroporation. This trial was completed in 2015 [203]. The trial demonstrated the vaccine was safe and well tolerated but did not elicit a significant immune response in the volunteers (Unpublished Results, ClinicalTrials.gov: NCT01169077).

4.2. L3SEPTL polyprotein vaccine

L3SEPTL polyprotein is comprised of six pre-erythrocytic-stage antigens, LSA-3, STARP, EXP-1, Pf516, TRAP, and LSA1-N/C, linked together to produce a 3240-aa-long polyprotein (Figure 6) [204]. In this construct, LSA-1 is modified so that only one repeat is included flanked by the conserved amino-end (amino acids 1–148) and the carboxyl-end (amino acids 1523–1909). The nucleic acid sequence expressing the recombinant fused protein is delivered as plasmid DNA using the human cytomegalovirus promoter, or in the viral vehicles MVA and Fowlpox (FP9), to provide the vaccines PP, MVA-PP, and FP9-PP, respectively.
The MVA-PP and FP9-PP vaccines were evaluated in a Phase I with challenge dose-escalation human clinical trial using malaria-naive volunteers [205]. Generally, the vaccine was well tolerated, but the immunity was lower than expected. Priming with MVA resulted in a stronger immune response than did FP9, and when FP9 was used for priming (FP9/FP9/MVA), the immune response was not significantly elicited until the MVA vehicle was used to deliver the antigens [205]. This was in contrast with the results seen in the preclinical study using FP9-PP for the priming. When delivered using MVA priming, all six proteins elicited IFN-γ-producing T cells, with stronger and more frequent responses seen for LSA-3, LSA-1, TRAP, and STARP. However, the number of IFN-γ-producing T cells was lower than had been reported in other studies [205]. Following challenge, none of the 15 vaccinated volunteers demonstrated protection or delayed onset of disease relative to the six nonvaccinated volunteers.

### 4.3. ME-TRAP

The most extensively tested multiantigen vaccine, ME (multiepitope)-TRAP vaccine, was designed to elicit T cell responses to target the hepatic stages of the malaria parasite. Nucleic acid encoding an ME string was designed to include CD8+ and CD4+ T cell epitopes from TRAP, CSP, EXP-1, LSA-1, LSA-3, and STARP (Table 2) [36, 41, 197, 198]. The ME were fused in-frame to the nucleic acid sequence encoding the entire TRAP antigen from *P. falciparum* strain T9/96, encoding a polypeptide of 789 amino acids referred to as multi-epitope thrombospondin-related adhesion protein (ME-TRAP) [44].

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Designation</th>
<th>Amino acid sequence</th>
<th>Epitope</th>
</tr>
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<tr>
<td>CSP</td>
<td>cp6</td>
<td>MNPNDPNDRNV</td>
<td>HLA class I antigens</td>
</tr>
<tr>
<td>CSP</td>
<td>cp26</td>
<td>KPKDELDY</td>
<td></td>
</tr>
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<td>EXP-1</td>
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</tr>
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<td>MINAYLKL</td>
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</tr>
<tr>
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<td>tr26</td>
<td>HLGNVYLV</td>
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</tr>
<tr>
<td>TRAP</td>
<td>tr29</td>
<td>LLMDCSGSI</td>
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<tr>
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<td>tr39</td>
<td>GIAGGLALL</td>
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</tr>
<tr>
<td>TRAP</td>
<td>tr42/43</td>
<td>ASKNKEAL</td>
<td></td>
</tr>
<tr>
<td>CSP (<em>P. berghei</em>)</td>
<td>pb9</td>
<td>SYIPSAEK</td>
<td>Mouse MHC class I antigen</td>
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<tr>
<td>CSP</td>
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<td>NANPNANPNANPNANP</td>
<td>B cell epitopes</td>
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<td>TRAP-AM</td>
<td>DEWSPCSVTCGKTR3RKE</td>
<td>Heparin binding motif</td>
</tr>
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<td>CSP</td>
<td>CSP</td>
<td>DPNANPNVPANPNV</td>
<td>Class II antigens (BCG and Tetanus toxin are not malarial antigens)</td>
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<tr>
<td>BCG</td>
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<td>QVHFQPLPAVVKLI</td>
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<td>LLMDCSGSI</td>
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<tr>
<td>BCG</td>
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<td>QVHFQPLPAVVKLI</td>
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Table 2. Peptides used in the multi-epitope domain of ME-TRAP [41].
<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Antigens (adjuvants)</th>
<th>Delivery regimen</th>
<th>Efficacy (% protected/# participants)</th>
<th>Immune response</th>
<th>Population</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3SEPTL (peptide)</td>
<td>LSA, STARP, Exp1, Pfs16, TRAP, and LSA1</td>
<td>FFM</td>
<td>0% protection (0/8)</td>
<td>85 SFU/10^6 PBMCs</td>
<td>Malaria naïve adults</td>
<td>[205]</td>
</tr>
<tr>
<td>MMF</td>
<td>TRAP and peptides from CSP, LSA-1, LSA-3, EXP-1, STARP expressed as a single protein.</td>
<td>0% protection (0/7)</td>
<td>96 SFU/10^6 PBMCs</td>
<td>Malaria naïve adults</td>
<td>[44]</td>
<td></td>
</tr>
<tr>
<td>ME TRAP</td>
<td>TRAP and peptides from CSP, LSA-1, LSA-3, EXP-1, STARP expressed as a single protein.</td>
<td>DDDDM</td>
<td>0% protection (8/8 delayed patency)</td>
<td>158-316 SFU/10^6 PBMCs</td>
<td>Malaria naïve adults</td>
<td>[42]</td>
</tr>
<tr>
<td>DDDM</td>
<td>0% protection, (6/6 delayed patency)</td>
<td>234 SFU/10^6 PBMCs</td>
<td></td>
<td></td>
<td>Gambian adults</td>
<td>[219]</td>
</tr>
<tr>
<td>DDD</td>
<td>0% protection (0/5)</td>
<td>33 SFU/10^6 PBMCs</td>
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<td></td>
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</tr>
<tr>
<td>MMM</td>
<td>0% protection (0/4)</td>
<td>44 SFU/10^6 PBMCs</td>
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</tr>
<tr>
<td>DDM</td>
<td>12.5% protection (3/8 sterile immunity, 7/8 delayed patency)</td>
<td>423 SFU/10^6 PBMCs; low titer antibodies</td>
<td>Malaria naïve adults</td>
<td>[42]</td>
<td></td>
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<tr>
<td>DDM</td>
<td>10% protective efficacy relative to control group</td>
<td>250 SFU/10^6 PBMCs</td>
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<td></td>
</tr>
<tr>
<td>FFM</td>
<td>12.5% protection (2/16 sterile immunity, 14/16 delayed patency)</td>
<td>430 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells</td>
<td>Malaria naïve adults</td>
<td></td>
<td>[45, 207]</td>
<td></td>
</tr>
<tr>
<td>MMM</td>
<td>0% protection (0/4)</td>
<td>0 SFU/10^6 PBMCs</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>FM</td>
<td>0% protection (0/5)</td>
<td>380 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells</td>
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<tr>
<td>MF</td>
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<td>100 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells</td>
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<tr>
<td>DDDMF</td>
<td>0% protection (4/4 delayed patency)</td>
<td>200 SFU/10^6 PBMCs, CD4+ T cells</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>DDFM</td>
<td>0% protection (0/3)</td>
<td>300 SFU/10^6 PBMCs, CD4+ T cells</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>FFM</td>
<td>0% protection (73% infection in vaccines, 80% infection in controls; no statistically significant decrease)</td>
<td>107 SFU/10^6 PBMCs</td>
<td>Kenyan children</td>
<td></td>
<td></td>
<td>[210]</td>
</tr>
<tr>
<td>CM</td>
<td>21% protection (3/14 sterile immunity, 5/14 delayed patency)</td>
<td>1300 SFU/10^6 PBMCs, CD4+ and CD8+ T cells producing IFN-γ, IL-2, TNF-α (protection associated with CD8+ IFN-γ producing T cells)</td>
<td>Malaria naïve adults</td>
<td></td>
<td></td>
<td>[212]</td>
</tr>
<tr>
<td>CM</td>
<td>13% protection (2/15 sterile immunity, 5/15 delayed patency)</td>
<td>2000 SFU/10^6 PBMCs, and antibodies detected</td>
<td>Malaria naïve adults</td>
<td></td>
<td></td>
<td>[213]</td>
</tr>
<tr>
<td>Vaccine</td>
<td>Antigens (adjuvants)</td>
<td>Delivery regimen</td>
<td>Efficacy (# protected/# participants)</td>
<td>Immune response</td>
<td>Population</td>
<td>References</td>
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</tr>
<tr>
<td>CM</td>
<td></td>
<td></td>
<td>67% protection (18% infection in vaccines, 47% infection in controls)</td>
<td>1450 SFU/10^6 PBMCs, CD4+ and CD8+ T cells producing IFN-γ, IL-2, TNF-α</td>
<td>African adults</td>
<td>[105, 214]</td>
</tr>
<tr>
<td>MuStDO5</td>
<td>TRAP, CSP, EXP1, LSA1, and LSA3</td>
<td>DDD</td>
<td>0% protection (0/8)</td>
<td>For both regimens: class I—41 SFU/10^6 PBMCs; class II—59 SFU/10^6 PBMCs, no antibodies detected</td>
<td>Malaria naïve adults</td>
<td>[215, 216]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDD &amp; plasmid with hGM-CSF</td>
<td>0% protection (0/23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NYVAC-Pf7</td>
<td>CSP, TRAP, LSA-1, MSP1, SERA, AMA1 and Pf625</td>
<td>NNN</td>
<td>3% protection (1/35 sterile immunity, 34/35 delayed patency)</td>
<td>9/38 volunteers developed CTL to TRAP; 2/38 volunteers developed CTL to LSA1; 30% of volunteers developed antibody to TRAP and LSA1</td>
<td>Malaria naïve</td>
<td>[218]</td>
</tr>
</tbody>
</table>

Regimens are the sequence of immunization using the vehicles D (plasmid DNA), F (attenuated fowlpox virus FP9), M (modified vaccinia virus Ankara), C (chimpanzee adenovirus 63), and N (attenuated vaccinia virus NYVAC) to provide gene sequences encoding the described antigens.

*T cells isolated by FAC, antigen-dependent T cell count not provided. SFU refers to antigen-specific IFN-γ producing T cells.

Table 3. Summary of the efficacy of multi-antigen pre-erythrocytic vaccines tested in clinical trials.

Expressing the ME-TRAP antigen *in situ* using DNA vaccines (plasmids) or viral vehicles has been employed to provide low-cost vaccination. While results in animal models using plasmids to deliver antigens were promising, the immunity elicited in humans was typically low. Heterologous prime/boost combinations, wherein the antigen remained constant but was delivered using different vehicles, reduced the development of immunity to the vehicle while provided a boost to the immune system with the target antigens [206]. In humans, the order of vehicle used for immunization was demonstrated to affect overall immune response [45, 207]. Thus, studies have focused on identifying the appropriate combination and order of vehicles that can be used to produce an elevated and expanded immune response. Plasmids encoding ME-TRAP only elicited a low-level immune response [44], and subsequent efforts have focused on viral vehicles for both priming and boosting (Table 3).

The MVA strain has proven to be a strong vehicle for boosting, and clinical trials using ME-TRAP subsequently focused on identifying an optimal priming vehicle and dosing regimen (see Table 3). Using an attenuated fowlpox virus vehicle (FP9) with MVA elicited strong immunity in malaria-naïve volunteers and limited protection [207], but provided only low-level immunity in people in malaria-endemic regions without providing significant protection [208–210] (see Table 3). The recent addition of a chimpanzee adenovirus 63 (ChAd63) for priming has provided encouraging results (see Table 3). AdCh63 priming elicits both CD4+ and CD8+ T cells at much higher levels than DNA or FP9 priming [211], and also results in a
much higher proportion of IFN-γ-secreting monofunctional CD8+ T cells that were correlated to sterile protection [212]. This combination has provided protection from infection in malaria-naive volunteers comparable to the immunity provided by CSP using the same delivery platform [212, 213]. Further, ME-TRAP using the ChAd63/MVA regimen elicited a strong immune response and provided significant protection in Africans who had prior exposure to malaria [43, 214]. Using a Cox regression analysis, vaccine efficacy was 67% during the 56 days of monitoring.

4.4. Multistage DNA vaccine operation five antigens (MuStDO5)

The MuStDO5 cocktail is composed of sequences encoding the antigens TRAP, CSP, EXP-1, LSA-1, and LSA-3 using sequences based on *P. falciparum* strain 3D7 [215, 216]. LSA-1 only encodes the nonrepetitive amino and carboxyl ends of the protein (462 amino acids), with the repetitive 727 amino acid middle domain removed. Each antigen is encoded on a separate plasmid using the same vector VCL-25 (Vical Inc., San Diego, CA, USA), with expression controlled by the promoter/enhancer of the human cytomegalovirus. The cocktail of plasmids is used for vaccination to provide each of the five antigens. In this study, the impact of including a plasmid expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) as an immune enhancer was evaluated. This vaccine is safe and well tolerated. However, protective immunity was not demonstrated, and the addition of hGM-CSF decreased the response to class I epitopes.

4.5. NYVAC-Pf7 attenuated vaccinia virus

NYVAC-Pf7 is composed of an attenuated vaccinia virus NYVAC engineered to express seven antigens (Pf7) to target pre-erythrocytic, blood stages, and transmission stages of the parasite [217]. The seven antigens include three pre-erythrocytic antigens (CSP, TRAP, and LSA-1), three blood stage antigens (MSP1, SERA, and AMA1), and the Pf625 antigen found on the sexual stage ookinete. CSP, TRAP, LSA-1, AMA1, and Pf625 are derived from *P. falciparum* 3D7 strain. The lsa-1 gene was modified to produce a protein linking amino acids 1–458 with 1630–1909, removing the sequence encoding the repeat region of the protein. Each gene is under a separate poxvirus promoter and expressed as a separate protein as follows: CSP under H6, TRAP under 42K, LSA-1 under C10LW, MSP1 under H6, SERA under 42K, AMA1 under I3L, and Pf625 under I3L (Figure 7). Each gene was inserted into defined sites in the genome of the attenuated vaccinia strain NYVAC by *in vivo* recombination to produce NYVAC-Pf7.

A Phase I/II clinical trial was carried out with malaria-naive adults [218]. Volunteers were immunized with either high dose (1 × 10⁸ pfu) or low dose (1 × 10⁷ pfu) of NYVAC-Pf7 at 0, 4, and 26 weeks. Vaccination with NYVAC-Pf7 resulted in delayed onset of parasitemia following challenge in all volunteers who received the vaccine, as compared to nonvaccinated volunteers. The CTL response or antibody titer did not demonstrate a relationship to the time of delay to onset of parasitemia. One vaccinated volunteer from the low-dosage group demonstrated sterile immunity. About 50% of the volunteers in each of the dosage groups developed antibodies to TRAP and LSA-1, regardless of the dosage. Low levels of CTL were developed to TRAP, with no statistical significance due to the dosage. A CTL response to LSA-1 was only...
seen in the low-dosage group. However, the CTL response did not correlate with the time to onset of parasitemia, and the one volunteer exhibiting sterile immunity did not develop a detected CTL response.

Figure 7. Genomic Organization of the NYVAC-Pf7 (adapted from [217]). The poxvirus promoters are represented with solid boxes.

5. New proteins for pre-erythrocytic malaria vaccines

New malarial proteins have been discovered through screens for pre-erythrocytic phase sporozoites, including identifying mRNA or proteins expressed in specific stages of the lifecycle of the parasite (e.g., sporozoites for the mosquito salivary glands), and typically included monitoring the immune response to the specific proteins using antibodies or T cells from volunteers vaccinated with sporozoites that were either irradiated or suppressed with chemotherapy, as discussed in the Introduction. Section 5 summarizes the preclinical studies carried out focusing on antigens analyzed beyond the initial identifying screen.

5.1. DNA vaccine encoding SAP1

Sporozoite asparagine-rich protein 1 (SAPI) was identified using suppressive-subtractive hybridization to identify *P. berghei* genes that are up-regulated in the sporozoite as it transitions from the mosquito midgut into the salivary gland [60]. In targeted deletion studies, *P. yoelii* SAP1 knockout sporozoites can invade hepatocytes but arrest in early liver stage development [220]. The *P. yoelii* genome sequence encoding the SAP1 protein domain corresponding to amino acids 3063–3227, and flanked by two CpG motifs to enhance the immune response, was cloned into pcDNA3.1(+) [221]. SAP1 encoded in plasmid DNA was evaluated for protective immunity against challenge with a homologous strain of *P. yoelii*. BALB/c mice were immunized intramuscularly with 100 μg plasmid three times with 3 weeks between each vaccination. Controls included mice vaccinated with phosphate buffered saline. For the protection study, each group was composed of ten mice. BALB/c mice were challenged by bites from mosquitoes infected with *P. yoelii* 2 weeks after the final immunization. Onset of parasitemia was evaluated using blood smears. Mice without parasitemia 14 days after challenge were considered completely protected. The mice immunized with DNA encoding SAP1 produced SAPI-
specific antibodies and moderate levels of T cells secreting IFN-γ. All 10 of the control mice vaccinated with phosphate buffered saline developed parasitemia. Two of 10 (20%) of the mice vaccinated with pcDNA3.1(+) SAP1 did not develop parasitemia.

5.2. Adenovirus vectored pre-erythrocytic antigens

This was an exploratory study to evaluate the protective immunity of newly identified pre-erythrocytic-stage antigens, selected from sporozoite and liver stage libraries based on the predicted signal sequences [137]. The P. yoelii orthologues of P36p (PY01340; Py52), Ag5 (PY00410; hypothetical protein), Sporozoite Invasion-Associated Protein-1 (PY00455; SIAP1), Kruepple-like protein (PY00839; KLP), and TRAP-like protein (PY01499; TLP), CelTOS or CSP were delivered in 10^10 virus particles of adenovirus encoding each individual antigen [137].

Protection was evaluated as the parasite burden in the liver following infection. Immunization of female BALB/c (H2-K^d) mice with adenovirus vector (Ad) expressing P36p, CelTOS, or Ag5 elicited a high IFN-γ T cell response that was comparable to the response to CSP. Following challenge, only mice immunized with Ad-CSP reduced the parasite burden. None of the other antigens reduced the burden of parasites as compared to nonvaccinated mice.

5.3. Heterologous prime boost with DNA and vaccinia viral vector

The protective immunity of the individual antigens CSP, Falstatin, PY03661, and UIS3, as well as combinations of the antigens, were evaluated using the P. yoelii model [222]. CD1 mice (14 per group) were vaccinated intramuscularly with a priming dose of the DNA vaccine, and boosted with the vaccinia vector vaccine. Parasitemia was evaluated by blood smears following challenge with 300 P. yoelii sporozoites. The combination of Falstatin and UIS3 and PY03661 provided protective immunity (57%) against challenge. Heterologous prime boost with CSP protected 36% of the mice. The addition of plasmid expressing murine GM-CSF to immunization scheme did not enhance protective immunity.

5.4. Heterologous prime boost with viral vectors ChAd63 and MVA

Humoral responses in mice were evaluated using recombinant ChAd63 and MVA viral vectors expressing eight individual P. falciparum 3D7 antigens [33]. Responses elicited following immunization with five newly identified antigens, upregulated in sporozoite 3 (UIS3), Falstatin, liver-stage associated protein 1 (LSAP1), liver-stage associated protein 2 (LSAP2), and the early transcribed membrane protein 5 (ETRAMF5) were compared to three previously characterized antigens, LSA-1, LSA-3, and CelTOS.

Protective efficacy was evaluated in both BALB/c (inbred) and CD1 (outbred) mice, with each antigen evaluated in a separate cohort of mice using heterologous prime boosts with ChAd63/ MVA. Mice were challenged with P. berghei transgenic parasites that were modified to express the cognate P. falciparum antigen. The immunity and protective efficacy against infection was evaluated for each antigen using nonvaccinated controls for comparison.

The BALB/c mice developed antibodies to all the antigens except LSAP1. High levels of IFN-γ (>1000 SFU/10^6 PBMCs) developed in mice immunized with UIS3 and LSA1. Moderate levels
of IFN-γ (>250 SFU/10⁶ PBMCs) were developed against ETRAMP5, CelTOS, LSAP2, Falstatin, and LSA-3. LSAP1 elicited very low levels of IFN-γ (<100 SFU/10⁶ PBMCs). CD8+ T cells predominated for UIS3, LSA-1, and LSA-3 cellular responses, while CD4+ T cells predominated for Falstatin. Highest levels of protection were found in BALB/c or CD1 mice immunized with LSAP2 (85 and 70%, respectively) or LSA-1 (87.5% for both murine strains). Mice immunized with CSP gave 37.5 and 33.3% sterile immunity in BALB/c and CD1 mice, respectively. Strain variation noted, with 30% of CD1, but none of the BALB/c, mice were protected following immunization with TRAP or LSAP1. Minimal or no protection was obtained in mice immunized with ETRAMP5, CelTOS, UIS3, Falstatin, or LSA-3.

6. Conclusions

Trends, advances, and roadblocks for malaria pre-erythrocytic vaccine development identified by the preclinical and clinical studies summarized in this chapter include:

• The hallmarks for protective immunity are not clear and the elements associated with sterile immunity vary in humans. Antibodies, IFN-γ, CD4+ T cells, CD8+ T cells, and cytolytic lymphocytes are believed to play essential roles in immunity, but are not always collectively demonstrated in immunity that protects from infection by Plasmodium.

• To achieve effective immunity that induces T cells across a wide population of people will require multiple and diverse T cell epitopes. Many of the studies that identified T cell epitopes in humans demonstrated that, within the limited number of volunteers in a Phase I clinical trial, only a limited subset of individuals respond to any single protein.

• Vaccines that string T cell epitopes from different proteins together in a composite recombinant protein (e.g., multiepitope strings) do not elicit significant T cell responses in humans. This is found even when other proteins in the vaccine do elicit strong immune response.

• For protective immunity in humans, the means and route of administration effect efficacy of the antigens. For example, irradiated sporozoites are more effective by intravenous verses intradermal administration, and TRAP delivered using heterologous vehicles of chimpanzee adenovirus followed by a modified vaccinia virus (heterologous prime boost regimen) provides more effective immunity than immunization using only one of these vehicles, or using other combinations of vehicles.

• Adjuvant formulations remain a critical factor for pre-erythrocytic vaccines, as highly purified subunit vaccine lack the pathogen-associated molecular patterns that trigger the innate immune responses required for initiation of adaptive immunity. Alum has been found to be a poor adjuvant for malaria subunit vaccines in Phase I/II trials. Oil-based adjuvants, e.g., ISA 50 or ISA 720, while enhancing immune responses, have been limited by increased reactogenicity. Precisely targeted adjuvants, such as TLR agonists, have been shown to enhance immunogenicity and protective efficacy of pre-erythrocytic vaccines, and hold promise as future adjuvant formulations.
While cost competitiveness is a goal for malaria vaccines, it is unlikely that this has been a primary consideration in the early development plan for many candidates as much of the focus is on efficacy. Multiple platforms and approaches have been described in this chapter including DNA-based platforms, viral vehicles, recombinant proteins, and chimeric proteins composed of peptides. In consideration of vaccine design and development, production may be an initial focus to contain costs per dose. This includes consideration for adjuvants and processes to stabilize the vaccine for delivery to the clinic. However, it is not always obvious in early development which approaches may be the most cost effective for commercial vaccine production as efficiencies may be realized through optimizing processes and scaling of production so as to reduce costs significantly relative to the costs to produce research material. Further contributors to the direct cost for vaccination are transportation and storage requirements for the vaccine, the dosage, and number of boosters required to achieve protective immunity. This cost analysis may also need to consider compliance if complex or protracted boosting schedules are required. While development may strive to contain these direct costs, the vaccine still must be effective at preventing the morbidity and mortality caused by malaria.

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