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Advances and challenges in malaria vaccine development

Ruobing Wang, Joseph D. Smith and Stefan H.I. Kappe*

Malaria remains one of the most devastating infectious diseases that threaten humankind. Human malaria is caused by five different species of *Plasmodium* parasites, each transmitted by the bite of female *Anopheles* mosquitoes. Plasmodia are eukaryotic protozoans with more than 5000 genes and a complex life cycle that takes place in the mosquito vector and the human host. The life cycle can be divided into pre-erythrocytic stages, erythrocytic stages and mosquito stages. Malaria vaccine research and development faces formidable obstacles because many vaccine candidates will probably only be effective in a specific species at a specific stage. In addition, *Plasmodium* actively subverts and escapes immune responses, possibly foiling vaccine-induced immunity. Although early successful vaccinations with irradiated, live-attenuated malaria parasites suggested that a vaccine is possible, until recently, most efforts have focused on subunit vaccine approaches. Blood-stage vaccines remain a primary research focus, but real progress is evident in the development of a partially efficacious recombinant pre-erythrocytic subunit vaccine and a live-attenuated sporozoite vaccine. It is unlikely that partially effective vaccines will eliminate malaria; however, they might prove useful in combination with existing control strategies. Elimination of malaria will probably ultimately depend on the development of highly effective vaccines.

Although malaria has been eliminated from most developed countries, it remains a major global cause of disease and death, and disproportionately affects developing, resource-poor regions of the globe. Annually, 300-500 million clinical malaria cases result in approximately 1 million deaths with the primary mortality occurring in children under the age of five in sub-Saharan Africa (Ref. 1). Malaria is a mosquito-borne disease and hence it can be controlled at the level of both human and mosquito. Currently, drug treatment of infected individuals, preventive drug treatment of populations at high risk of disease, and insecticide-treated bed nets and indoor-insecticide spraying for mosquito control constitute the main weapons to control malaria (Ref. 2). However, malaria control is a

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never-ending battle and requires long-term sustainability and commitment. History shows that if control efforts are terminated before malaria is completely eliminated, it resurges with a vengeance.

Only four of the numerous malaria-causing *Plasmodium* parasite species are regularly transmitted to humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale. Plasmodium knowlesi*, a nonhuman primate malaria parasite, can also infect humans (Refs 3, 4), but is mainly a zoonotic infection. All human malaria parasites are transmitted when infected female *Anopheles* mosquitoes bite and deposit the parasite's sporozoite (SPZ) stages into the host skin during salivation. Experimental evidence using rodent malaria parasite-infected mosquitoes indicates that a single mosquito can release up to a few hundred SPZs during a blood meal (Ref. 5). SPZs invade blood vessels and are transported within the blood stream to the liver. Here, they exit the bloodstream, infect hepatocytes and form a liver stage (LS) that grows asymptomatically inside hepatocytes for up to 7 days before releasing tens of thousands of infectious merozoites into the bloodstream. SPZs and LSs together constitute the pre-erythrocytic phase of infection. The erythrocytic stage of infection commences when the merozoites released from the liver infect individual erythrocytes. Parasites then replicate as intraerythrocytic stages, and each infected cell releases up to 20-32 new merozoites to invade new red blood cells, destroying the erythrocyte in the process. During blood-stage infection, some intraerythrocytic parasites develop into sexual stages called gametocytes, which are taken up by mosquitoes during a blood feed. Male and female gametes fuse and form a zygote which then initiates infection in the mosquito, a process that progresses through complex developmental changes, taking 2–3 weeks and ultimately resulting in the accumulation of infectious SPZs in the mosquito salivary glands, which, when transmitted, initiate infection of a new host.

The pathogenesis of malaria is multifaceted, and development of severe disease depends on the parasite species that causes infection and the immune status of the infected host. The majority of deaths are caused by *P. falciparum* infection and have been attributed to the greater multiplication potential of the parasite to infect all stages of red blood cells and adherence of parasite-infected erythrocytes in the microvasculature (Ref. 6). *P. vivax* infections were thought to cause limited morbidity, but the severity of infection might have been underestimated in the past and is now increasingly well documented (Ref. 7).

Repeated natural infection with malaria results in acquired immunity that affords protection against severe disease and high parasitaemia, but does not result in sterilising immunity (Ref. 8). Many individuals that have an asymptomatic malaria infection carry gametocytes, and in consequence, directly provide a parasite reservoir for continued transmission (Ref. 9). By contrast, immunisations with whole live SPZ preparations that were conducted in experimental animal models of malaria and in malaria-naive humans, confer sterile protection against challenge with infectious parasites (Ref. 10). Both naturally acquired semi-immunity and experimentally induced sterile immunity with SPZs have served as paradigms in the quest for malaria vaccines. Here, we review the state of malaria vaccine research and highlight advances as well as challenges in the development of protective vaccines. We will not discuss transmission-blocking vaccines, but refer the reader to reviews on this topic in the Further Reading section.

Whole-cell pre-erythrocytic parasite vaccination approaches

Irradiation-attenuated sporozoites

In 1967, Ruth Nussenzweig and co-workers demonstrated that immunisation of mice with irradiation-attenuated SPZs (irrSPZs) of the rodent malaria parasite *P. berghei* completely prevented onset of blood-stage parasitaemia after infectious SPZ challenge (Ref. 11). This was a landmark finding that set the standards for immunological protection against malaria infection. Irradiation introduces random mutations and breaks in the SPZ DNA. When dosed adequately, the parasite survives and remains infectious to the hepatocyte. However, LS development terminates during early hepatocyte infection (Ref. 12). The safety and efficacy of irrSPZs is dependent on a precise irradiation dose; too little irradiation allows

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the parasite to complete LS development and cause blood-stage infection, too much irradiation completely inactivates the SPZs, and inactivated SPZs do not induce significant protection. In mice, irrSPZ-induced protection is mediated mainly by $CD8^+$ T cells, which target the infected hepatocyte, and antibodies against the major SPZ surface protein circumsporozoite protein (CSP), which block SPZ infection (Ref. 13). Humans immunised with *P. falciparum* irrSPZs (immunised by the bite of irrSPZ-infected mosquitoes) have been effectively protected from subsequent challenge with homologous and heterologous infectious *P. falciparum* SPZs (Refs 10, 14, 15). Given the high levels of protection achieved by irrSPZ immunisation in many malaria models, the development of a *P. falciparum* irrSPZ vaccine has been proposed by Stephen Hoffman (Ref. 16), whose company Sanaria (http://www.sanaria.com) has developed a manufacturing process for the aseptic production of irrSPZs in mosquitoes. The irradiation dose is tightly controlled and isolation, purification, formulation and cryopreservation of irrSPZs under good manufacturing practices (GMPs) have been established. A first-generation vaccine called the PfSPZ Vaccine has been produced. Currently, the PfSPZ Vaccine administered by intradermal or subcutaneous inoculation by needle and syringe is being tested in a Phase I clinical study with experimental challenge in malaria-naive volunteers to assess its safety, immunogenicity and protective efficacy (Stephen Hoffman [Sanaria Inc., USA], pers. commun.). Calculations based on the previous *P. falciparum* irrSPZ immunisation trials, where >1000 cumulative infectious mosquito bites confer sterile protection that lasted for at least 10 months, suggest a protective dose of >100,000 irrSPZs (Ref. 16). This is in good agreement with irrSPZ studies in rodent malaria models, where the inoculation dose can be precisely determined. However, a recently published paper demonstrated that as few as three intravenous doses of 750 *P. yoelii* irrSPZs confer complete sterile protection against challenge with infectious SPZs for at least 2 weeks in mice (Ref. 17). Thus, fewer than 100,000 irrSPZs might be necessary to induce sterile protection if given intravenously in humans. However, other routes of administration might require high numbers of irrSPZs.

Genetically attenuated sporozoites

The availability of genome sequences for a number of *Plasmodium* species, the generation of stage-specific gene expression data and the ability to genetically manipulate the parasite, have all enabled the search for genes that have essential roles for parasite survival at distinct points during the life cycle. The identification of such genes has allowed the generation of genetically attenuated parasites (GAPs) by precise genetic engineering techniques. Recently, pre-erythrocytic stages of the rodent malaria parasites *P. berghei* and *P. yoelii* were attenuated by deletion of genes encoding proteins called UISs (upregulated in infectious sporozoites), which are expressed at the pre-erythrocytic stage. UIS3 and UIS4 (Refs 18, 19, 20) are proteins of the LS parasitophorous vacuole membrane (PVM), the principal host–parasite interface that forms during liver infection (Refs 19, 21). Deletion of *UIS3* and *UIS4* led to complete arrest of early LS development after hepatocyte infection, but $uis4^-$ parasites showed occasional breakthrough infections when large numbers of SPZs were used for immunisation. This was not observed with $uis3^-$ parasites (Refs 19, 20, 22). Deletion of another *UIS* gene, *P52*, which encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein (Refs 23, 24) and *P36*, a gene encoding a putative secreted protein (Ref. 24), also resulted in developmental arrest at the early stage of hepatocyte infection; but $p52^-$ and $p36^-$ parasites caused breakthrough infections. However, simultaneous deletion of both genes ($p52^-$ $p36^-$) resulted in complete attenuation, with no breakthrough infections (Ref. 25). Immunisation of mice with $uis3^-$, $uis4^-$, $p52^-$ or $p52^-$ $p36^-$ SPZs induced complete long-lasting protection against infectious SPZ challenge (Refs 18, 19, 20, 23, 25), demonstrating that rodent malaria GAPs are highly efficacious vaccines. In some instances, protection could be achieved with a single dose of 10,000 GAP SPZs (Ref. 20). The GAP-induced protection was mediated mainly by $CD8^+$ T cells (Refs 20, 26, 27), but antibodies also contributed to protection (Ref. 20). More recently, an additional promising GAP strain has been created. Deletion of the SPZ

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asparagine-rich protein I (SAP1) resulted in early LS developmental arrest. No breakthrough infection occurred when *sap1*$^-$ SPZs were injected into mice, and mice immunised with *sap1*$^-$ SPZs were completely protected against infectious SPZ challenge (Ref. 28). Interestingly, the *sap1*$^-$ SPZs showed lack of expression of a number of genes, including *UIS3*, *UIS4* and *P52*, making the *sap1*$^-$ GAP a quasi-multiloci attenuated strain (Ref. 28). Together, the rodent malaria GAP data demonstrate that safe and protective attenuated malaria parasites can be created by genetic engineering. Is it possible to genetically engineer attenuated human malaria parasites? To that end, the single and simultaneous deletions of the *P52* and *P36* loci in *P. falciparum* were recently reported (Refs 29, 30). The *p52*$^-$ *p36*$^-$ parasites appeared to be normal throughout most of the life cycle, including SPZ production of the attenuated lines. However, *p52*$^-$ *p36*$^-$ parasites exhibited complete LS growth arrest in vitro and in a humanised mouse model carrying human hepatocytes (Ref. 30). Dual gene deletions might alleviate safety concerns for the use of GAPs as a vaccine in humans. To assess safety and preliminary efficacy, the *P. falciparum p52*$^-$ *p36*$^-$ GAP line has been selected for advancement into a Phase I clinical study, with experimental challenge in malaria-naive volunteers, to assess its safety, immunogenicity and protective efficacy (Ref. 30).

Together, the evidence garnered from vaccination with live-attenuated SPZs using animal models of malaria and *P. falciparum* demonstrates that this type of whole-cell immunisation elicits immune responses that completely protect against infection for extended periods of time.

Infectious sporozoites

Why does repeated natural exposure to infectious SPZs in malaria-endemic areas not induce protection against infection? One potential explanation is that normal SPZs are somehow qualitatively different from attenuated SPZs and do not induce or even subvert immune responses. However, immunisation of mice with infectious SPZs and treatment with chloroquine (a drug that kills blood stages) or primaquine (a drug that kills LSs) confers complete protection against challenge after drugs are cleared from the immunised animals (Refs 31, 32). Furthermore, vaccination of human volunteers that had been given chloroquine along with three doses of 15 bites from *P. falciparum*-infected mosquitoes conferred protection against challenge with infected mosquitoes after the drug had waned from volunteers (Ref. 33). These infection-treatment vaccination experiments indicate that the aforementioned qualitative difference between normal and attenuated SPZs cannot be substantiated. What then is the apparent reason for the lack of protection against infection in endemic areas? A second potential explanation is the suppression of immunity against pre-erythrocytic stages by blood-stage parasites that circulate in many individuals when they become exposed to new infectious mosquito bites. There is evidence from rodent malaria studies that this suppression does indeed occur (Ref. 34). However, this mechanism has recently been challenged by research showing that mice with blood-stage infections can develop robust, protective T cell responses against pre-erythrocytic stages (Ref. 35). A third potential explanation might lie in the SPZ dose that is received during a natural infectious bite. We currently do not know how many SPZs are transmitted per bite in endemic areas. The dose might be as low as one, or as high as the few hundred shown for rodent malaria parasites (Ref. 5). Thus, each SPZ inoculation by individual mosquitoes might not be enough to elicit any protective response at all, or might induce immune tolerance.

Subunit vaccine development for pre-erythrocytic stages

Subunit vaccines targeting sporozoites

CSP is an immunodominant antigen of SPZs (Refs 36, 37). Most early malaria vaccine reasearch focused on this protein, and today, several formulations (peptide, recombinant proteins, DNA, viral-vector) encompassing different segment(s) of CSP alone or in combination with other antigens have been evaluated. The most advanced malaria vaccine candidate RTS,S is based on CSP. RTS,S consists of a fusion protein between the CSP central repeats (R) and C-terminal regions (which include B cell and T cell epitopes) (T) fused to the hepatitis B surface antigen (HBsAg) (S) and prepared in a 1:4 ratio with unfused HBsAg (S) (Ref. 38). RTS,S formulated with adjuvant

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system AS-0X has demonstrated anti-infection efficacy (Ref. 38). In Phase IIb trials in children living in Mozambique (Refs 39, 40, 41), RTS,S AS02A delayed the time to new infection by 30% and reduced episodes of severe malaria by 58% in the first 6 months (Ref. 41), which persisted for 21 months after immunisation (Ref. 40). Two new adjuvant formulations enhanced RTS,S efficacy from a 30% reduction of all malaria episodes to up to 56% (RTS,S AS01E) in children 1–4 years old (Ref. 41), and to 65% (RTS,S AS02D) in infants (Ref. 42).

RTS,S vaccine efficacy could be attributed partially to the AS-0X adjuvants that contain monophosphoryl lipid A (MPL) and the saponin derivative QS21. MPL is a powerful Toll-like receptor 4 (TLR4) agonist that stimulates many antigen-presenting cells (Ref. 43). All RTS,S AS-0X formulations induced high levels of CSP-specific antibody and $CD4^+$ T cell responses, but failed to induce $CD8^+$ T cells (Refs 38, 44, 45). Competition with strong anti-HBsAg T cell responses induced in parallel (Ref. 46), and the absence of the N-terminal region of *P. falciparum* CSP in RTS,S might have limited the anti-infection efficacy of the vaccine (Ref. 47). A second SPZ protein, thrombospondin-related anonymous protein (TRAP), is also considered to be a primary target of anti-infection immunity (Ref. 48), but was not protective in field trials (Refs 49, 50).

An alternative delivery system for SPZ antigens is the use of recombinant viral vectors, which induce strong and persistent immune responses with acceptable safety profiles (Refs 51, 52, 53). Both adenovirus (Ad)5-CSP and Ad35-CSP induced similar antibody titres and better interferon-γ (IFN-γ; IFNG) responses in mice when compared with the RTS,S AS01B vaccine (Ref. 54). However, in the first efficacy trial with two doses of Ad5-CSP (1×10^{10} plaque-forming units) failed to protect naive volunteers from *P. falciparum* challenge using infected mosquito bites, although significant IFN-γ responses were mounted. Unexpectedly, a second dose of Ad5-CSP did not boost the IFN-γ or antibody responses to the levels achieved by the first dose, which might have contributed to the poor efficacy (Tom Richie [US Military Malaria Vaccine Program, Naval Medical Research Center, USA], pers. commun.).

The major obstacle that the adenoviral approach faces is the possible effect of pre-existing adenovirus immunity. Two methods have been used to address this problem, including: (1) the use of adjuvants and (2) the use of nonhuman viral vectors. The Ad35-CSP vaccine formulated with aluminum phosphate adjuvant significantly increased both T and B cell responses against CSP in mice (Ref. 52). Simian adenoviral vectors have been shown to induce strong protective immunity against sporozoite challenges in mice with pre-existing anti-Ad5 antibodies (Ref. 55). These new approaches appear to be promising for malaria vaccine development, although the failure of an adenovirus HIV vaccine trial is a concern for the use of adenovirus vectors (Ref. 56). However, the difficulties associated with this trial may be HIV-specific and thus not relevant to malaria.

Subunit vaccines targeting liver-stage parasites

Although the CSP-based RTS,S vaccine is the most advanced malaria vaccine candidate, it is not 100% efficacious and will need to be combined with other antigens. Indeed, research has shown that CSP transgenic mice that are immunotolerant to CSP are still protected following irrSPZ immunisation (Ref. 57), and in addition, mice immunised with *P. berghei* irrSPZs expressing only heterologous *P. falciparum* CSP are completely protected against wild-type *P. berghei* SPZ challenge (Ref. 58). Taken together, these results demonstrate that CSP is dispensable for induction of sterile immunity. Adoptive transfer of CSP-specific T cell receptor transgenic T cells can prevent SPZ invasion of hepatocytes but cannot eliminate LS parasites in the liver (Ref. 59). These results indicate that non-CSP proteins expressed by SPZs or LSs contribute to the induction of protective immunity.

Liver-stage antigen 1 (LSA1) is the only currently known LS-specific antigen, and naturally acquired anti-LSA1 responses are associated with resistance to *P. falciparum* infection in malaria-endemic regions (Refs 60, 61, 62). An Ad35-LSA1 vaccine (rAd35.LSA1) or recombinant LSA1 protein induced comparable IFN-γ and antibody responses in mice (Ref. 63). A recombinant *P. falciparum* LSA1 protein vaccine that includes the N- and C-terminal regions and two of the 17 amino acid repeats was immunogenic and induced antibody and cytokine responses in mice. However, this

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vaccine did not protect malaria-naive individuals from *P. falciparum*-infected mosquito bites in a challenge trial (Ref. 64).

Liver-stage antigen 3 (LSA3) is expressed by SPZs, LS and blood-stage parasites. Although controversy exists regarding whether LSA3 is predominantly a LS antigen or a blood-stage antigen, recombinant *P. falciparum* LSA3 protein or long synthetic peptides protected chimpanzees and *Aotus* monkeys against *P. falciparum* SPZ challenge (Refs 65, 66, 67). *Aotus* monkeys were protected by immunisation with the N-terminus alone, or with the N-terminus in combination with the repeat region of LSA3, indicating that T cells induced by LSA3 may confer complete protection (Ref. 66). Encouragingly, a DNA vaccine encoding LSA3 protected chimpanzees from *P. falciparum* infection even though both antibody- and T-cell-specific counts were low in the protected animals (Ref. 68).

Multistage and multivalent vaccines

Combinations of vaccine candidates that target different stages of the parasite life cycle are also being developed. PEV3A, which includes peptides from *P. falciparum* CSP and AMA1 (apical membrane antigen 1), induced functional antiparasite antibodies that slowed parasite growth in vitro and were associated with a delay of onset and low parasitaemia in vivo (Ref. 69). Ad5-vectored *P. falciparum* CSP and AMA1 combination vaccines are also being developed through a partnership between the US Military Malaria Vaccine Program, GenVec and USAID (United States Agency for International Development). Concurrently, the US Military Malaria Vaccine Program and GenVec working with the Malaria Vaccine Initiative (MVI) are also developing 'next-generation' multivalent adenovirus vectors that contain optimised expression cassettes encoding transgenes, one with CSP, LSA1 and the novel pre-erythrocytic antigen Ag2 CelTOS (Ref. 70), and a second with the C-terminal 42 kDa fragment of merozoite surface protein 1 ($MSP1_{42}$) and AMA1. Further development of the pentavalent vaccine hinges on favourable results from the initial trial (Tom Richie, pers. commun.). MSP1 immunisation also induces partial protection against SPZ challenge in mice (Refs 71, 72). This suggests that antigens consecutively expressed by all parasite stages are capable of inducing effective anti-infection as well as anti-disease immunity in the host.

Immunological mechanisms of protection

Both live-attenuated irrSPZ (Refs 10, 11) and GAP vaccines (Refs 18, 19, 20, 23, 25, 26, 28, 73) provide complete, long-lasting protection, and immune mechanisms involved have been extensively analysed (Fig. 1). $CD8^+$ T cells have a critical role in the protection of mice immunised with irrSPZs (Refs 74, 75), whereas interleukin-4 (IL-4)-secreting $CD4^+$ T cells are essential for the development of $CD8^+$ T cell responses to LS parasites (Ref. 76). Loss of protective immunity in β_2 microglobulin$^{-/-}$ mice (which have no $CD8^+$ T cells or natural killer T cells) and in mice depleted of $CD8^+$ T cells, indicates the indispensable role of $CD8^+$ T-cell-mediated effector mechanisms (Refs 77, 78). In studies with *P. berghei* and *P. yoelii*, adoptive transfer of $CD8^+$ T cell clones specific for CSP epitopes conferred complete protection against SPZ challenge in naive mice (Refs 79, 80, 81). $CD8^+$ T cell responses can also be detected in humans protected by immunisation with *P. falciparum* irrSPZs (Ref. 82). Furthermore, CSP-specific $CD8^+$ T cells have been found to efficiently lyse infected hepatocytes in vitro (Refs 79, 83, 84). Since irrSPZs protected perforin (PRF1) and granzyme B (GZMB) double-knockout mice (which lack the ability to kill cells through cytolytic activity) from infectious SPZ challenge, it was speculated that $CD8^+$ T cell killing of infected hepatocytes is primarily through IFN-γ-induced nitric oxide (NO), rather than by direct cytolytic activity (Refs 13, 85). Indeed, it has been shown that IFN-γ responses are correlated with protective immunity against the pre-erythrocytic stage, although the importance of $CD8^+$ T-cell-derived IFN-γ in particular is yet to be fully established (Refs 86, 87). Sterile protection obtained with a *P. berghei* GAP was found to correlate with IFN-γ-producing $CD8^+$ T cells (Ref. 26). Conversely, IFN-γ-independent $CD8^+$-T-cell-mediated protective immunity has also been demonstrated (Ref. 88), and IFN-γ secretion by $CD8^+$ T cells is not essential for protecting mice against *P. yoelii* SPZ challenge (Ref. 89). Recently, Trimnell and colleagues demonstrated that contact-dependent $CD8^+$ T cell elimination

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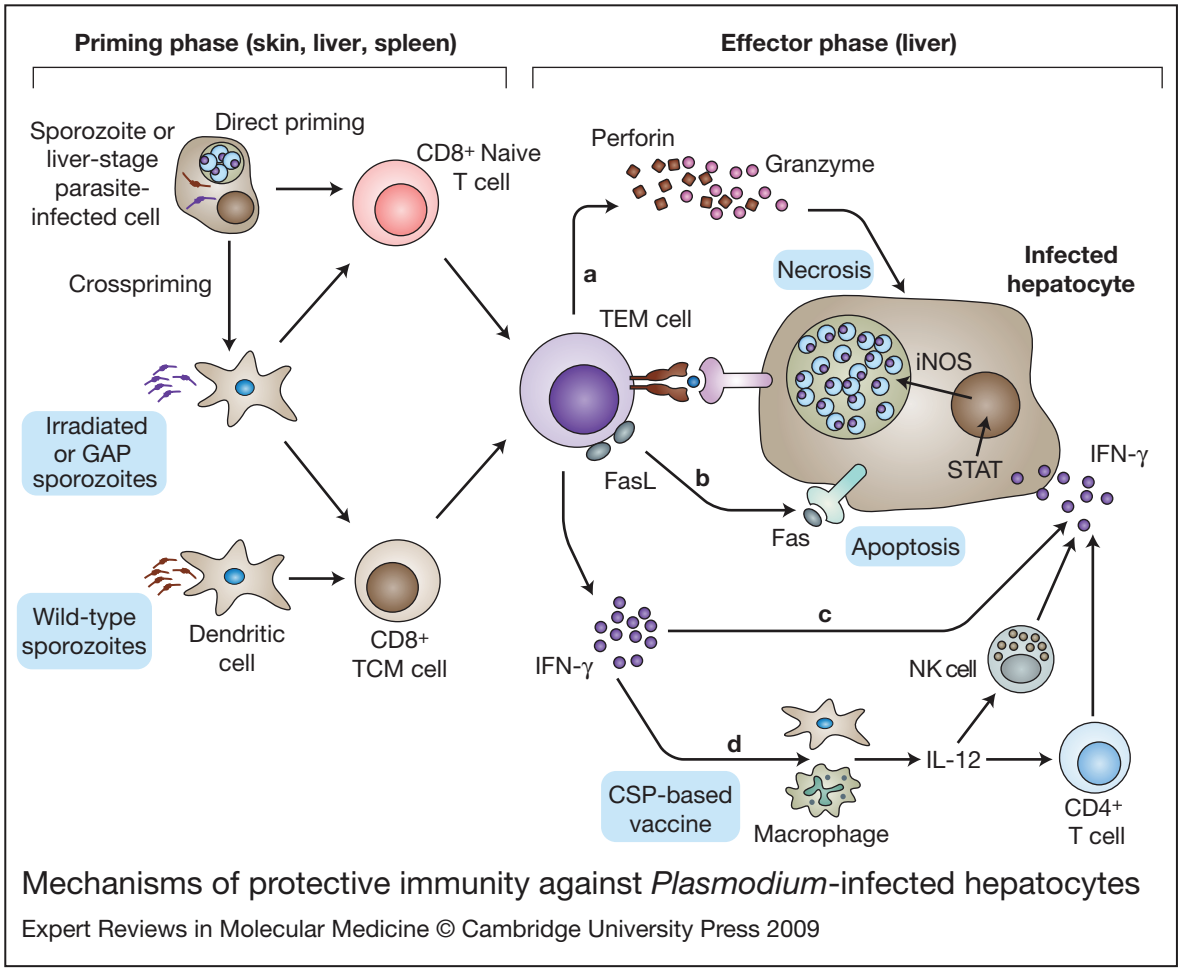


Figure 1. Mechanisms of protective immunity against *Plasmodium*-infected hepatocytes. In the protective model of live-attenuated sporozoite vaccines [irradiated sporozoites (irrSPZs) or genetically attenuated parasites (GAPs)], elimination of pre-erythrocytic-stage parasites is primarily dependent on antigen-specific and major histocompatability complex (MHC)-restricted $CD8^+$ T cells, whereas recombinant vaccines, such as the CSP-based RTS,S vaccine, are primarily dependent on $CD4^+$ T cells. Naive $CD8^+$ T cells can be primed by dendritic cells (DCs) that encounter SPZs at skin-draining lymph nodes, by parasite-traversed or -infected hepatocytes in the liver (direct priming), or by other DCs that uptake antigens provided by traversed and infected hepatocytes, or hepatocytes that die in the liver (crosspriming). Primed $CD8^+$ T cells will then proliferate and differentiate to effector (TEM) or central memory (TCM) $CD8^+$ T cells. $CD8^+$ T cells can mediate killing of liver stage (LS)-infected hepatocytes via several mechanisms: (a) release of perforin and granzyme B, which causes necrosis of the infected hepatocyte; (b) apoptosis, a programmed cell death through the Fas–FasL signalling pathway; (c) release of IFN-γ, via signal transducers associated with transcription (STAT) to activate inducible nitric oxide synthase (iNOS), which then induces the NO pathway to destroy the infected hepatocyte; (d) IFN-γ can also promote IL-12 release from DCs, macrophages or other cells to activate the natural killer (NK) and $CD4^+$ T cells that consequently increase the IFN-γ-dependent killing of LS-infected hepatocytes

of LS-infected hepatocytes is the major effector mechanism in *P. yoelii* GAP-induced sterile protection (Ref. 90). Therefore, the relative importance of different $CD8^+$ T cell effector mechanisms might differ between distinct vaccine models.

$CD8^+$ T cell priming might occur in skin-draining lymph nodes after immunisation by the bite of irrSPZ-infected mosquitoes (Ref. 59), whereas hepatic $CD8^+$ dendritic cells (DCs) may be responsible for priming $CD8^+$ T cells by intravenous immunisation with irrSPZs

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(Ref. 91). Both wild-type SPZs and irrSPZs are processed for class I presentation by DCs. However, DCs pulsed with wild-type SPZs appear to stimulate only memory $CD8^+$ T cells, whereas DCs pulsed with irrSPZs are capable of activating both effector and memory $CD8^+$ T cells (Ref. 92).

Little is known about if and how hepatocytes process and present malaria antigens to $CD8^+$ T cells. Bongfen and co-workers investigated CSP processing in primary mouse hepatocytes exposed to wild-type SPZs or cell-traversal-deficient *P. berghei* SPZs (Ref. 83). They demonstrated that parasite-infected hepatocytes process CSP through proteasomes, whereas traversed non-infected hepatocytes utilise aspartic proteases to degrade the CSP left behind by traversing SPZs (Ref. 93). These observations suggest that the parasite may avoid host immune surveillance by diverting CSP-specific $CD8^+$ T cells to attack traversed but uninfected hepatocytes (Ref. 90). CSP also suppresses the respiratory burst in Kupffer cells, thus supporting their successful traversal (Ref. 94). Furthermore, CSP actively promotes LS parasite growth in infected hepatocytes by inhibiting the NF-κB pathway to downregulate pro-inflammatory cytokine release (Ref. 95). All these data suggest that CSP is an immunodominant antigen that is the target of protective immune responses but is also responsible for immune evasion to ensure the survival of the parasite within its host cell.

The search for correlates of protection

To date, the most commonly used marker of protection is IFN-γ in conjunction with a few other cytokines and immune cell surface markers. In general, IFN-γ detected by enzyme-linked immunosorbent spot (ELISPOT) assay in 7–14 day peripheral blood mononuclear cell (PBMC) culture (but not in fresh PBMCs) has been associated with naturally acquired or vaccine-induced T cell immunity against infection. Susceptibility to infection, however, has been associated with $CD4^+$ $CD25^+$ regulatory T cells (Ref. 96). In the livers of mice protected by irrSPZs, both effector (TEM) and central (TCM) memory $CD8^+$ T cells were identified (Ref. 97), but long-lasting protection depended on $CD8^+$ TCM cells expressing the IL-15 receptor CD122 (Ref. 98). However, a single dose of simian adenovirus-induced protection was associated with $CD8^+$ TEM cells, whereas a poxviral vector that did not protect mice from *P. berghei* infection had predominant $CD8^+$ TCM cells (Ref. 55), indicating that the readiness of TEM cells has a major role in protection against infection. Several cytokine responses (IFN-γ, TNF-α and IL-2) were also greater in mice immunised with adenovirus vector than those in mice immunised with poxvirus vector (Ref. 55). Recently, it was demonstrated that a threshold T cell frequency (≥1% of CSP-specific $CD8^+$ T cells in blood, $>10^6$ in spleen and $>2 \times 10^5$ in liver) is required for long-term protective immunity against *P. berghei* SPZ challenge. This threshold is 100- to 1000-fold higher than the number of memory $CD8^+$ T cells required for protection against a bacterial or viral pathogen (Ref. 99). *P. berghei* is known to be less infectious than *P. yoelii* in mice, indicating that much higher threshold numbers of $CD8^+$ T cells will be needed for induction of sterile protection against *P. yoelii* challenge, and potentially against *P. falciparum* in humans.

Subunit vaccine development for erythrocytic stages

During blood-stage development, malaria parasites have a largely intracellular lifestyle and are only briefly exposed when they are released as merozoites and invade new red blood cells (Ref. 6). In the case of *P. falciparum* and *P. vivax*, merozoite release occurs once every 48 hours. Erythrocyte invasion occurs very rapidly, within 30–60 seconds (Ref. 100), and therefore it is assumed that high-titre antibodies will be required for invasion-blocking vaccine approaches to be effective. Once inside the red blood cell, erythrocytes offer a relatively immunoprivileged site for parasite replication because they lack a nucleus and MHC molecules, and therefore are limited in their ability to mount effective antipathogen defences. Nevertheless, humans exposed to malaria parasites gradually acquire considerable anti-blood-stage immunity, which varies with age and transmission intensity (Ref. 101). Passive immunoglobulin-transfer studies suggest that antibodies are a crucial component of protective immunity (Refs 102, 103); however, little is known about the molecular targets and mechanisms of naturally

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acquired immunity to malaria. Current blood-stage vaccine efforts are focused on a small number of candidate antigens and three types of subunit vaccine approaches: (1) invasion-blocking vaccines, (2) antidisease vaccines for severe and pregnancy-associated malaria, and (3) antitoxin vaccines (e.g. GPI).

Invasion-blocking vaccines

Red blood cell invasion is a multi-step process that involves several proteins at the parasite and erythrocyte surface. Parasite invasion has been divided into four steps: attachment, reorientation, tight junction formation and entry (Fig. 2) (Refs 100, 104). Invasion-blocking vaccine strategies have been highly focused on three proteins, MSP1, AMA1 and the *P. vivax* Duffy binding protein (PvDBP). MSP1 is believed to function at initial attachment (Ref. 105), AMA1 at parasite reorientation and entry (Ref. 106) and DBP during tight junction formation for *P. vivax* (Refs 107, 108) (Fig. 2). Antibodies against all three proteins can inhibit parasite invasion; however, antigen polymorphism, redundancy of invasion pathways and the kinetics of erythrocyte invasion pose significant challenges for vaccine development.

MSP1

MSP1 is synthesised as a GPI-linked 195 kDa precursor protein and undergoes proteolytic processing into four protein fragments (Refs 109, 110), which then assemble into a complex with at least two other proteins, MSP6 and MSP7, at the merozoite surface (Refs 111, 112). During merozoite invasion, the C-terminal 42 kDa fragment of MSP1 ($MSP1_{42}$) undergoes secondary processing (Refs 113, 114). Antibodies that interfere with the secondary processing of $MSP1_{42}$ (Ref. 115) or target the C-terminal $MSP1_{19}$ region (Refs 116, 117, 118, 119, 120) can inhibit erythrocyte invasion or parasite growth, either directly, or possibly through Fc-dependent pathways (Ref. 121). The most advanced MSP1 vaccine candidates are based on the C-terminal $MSP1_{42}$ fragment. Anti-$MSP1_{42}$ sera can inhibit *P. falciparum* growth in vitro (Refs 122, 123, 124) and inhibit the secondary processing of $MSP1_{42}$ (Ref. 115), but only conferred strain-specific protection in a *P. falciparum-Aotus* challenge model (Ref. 125). Although safe and immunogenic in Phase I trials (Refs 126, 127, 128), an $MSP1_{42}$-based vaccine conferred no protection in a Phase IIb trial in Kenyan children (Ref. 129). The reason for vaccine failure is unknown, but could include allelic polymorphism or antibody specificity and/or function.

AMA1

AMA1 is an 83 kDa protein that is targeted to micronemes (Refs 130, 131). Before invasion, it is processed to a 66 kDa form ($AMA1_{66}$), which is transferred to the merozoite surface and subsequently associates with three other proteins, RON4, RON2 and Ts4705 (Refs 132, 133). Based on studies that were initially performed in the apicomplexan *Toxoplasma gondii*, the AMA1 complex forms a moving junction that translocates over the zoite surface as the parasite enters the parasitophorous vacuole (Ref. 134). The moving junction is believed to form a barrier that excludes antibodies from entering the parasitophorous vacuole (Ref. 134). Antibodies that inhibit AMA1 complex formation (Ref. 133) or disrupt the circumferential redistribution or proteolytic shedding of AMA1 can block parasite invasion (Refs 135, 136), but AMA1 polymorphism severely compromises vaccine efficacy. AMA1 contains at least 64 variable residues, and inhibitory antibodies are highly strain-specific (Refs 137, 138, 139). Single antigen or bivalent AMA1 vaccines were immunogenic in Phase I trials (Refs 140, 141), but invasion-blocking responses were strain-specific and were not protective in human challenge models of naive malaria using a SPZ challenge (Ref. 141) or a Phase II trial in African children (Ref. 142). Thus, AMA1 polymorphism poses significant technical and economical challenges to vaccine development, and it is not yet clear whether conserved regions can form the basis for a vaccine. The safety and immunogenicity of a fusion protein between $MSP1_{19}$ and domain III of AMA1 has also been tested (Ref. 143), but this approach must also overcome obstacles faced by the individual AMA1 and MSP1 components.

Plasmodium vivax DBP

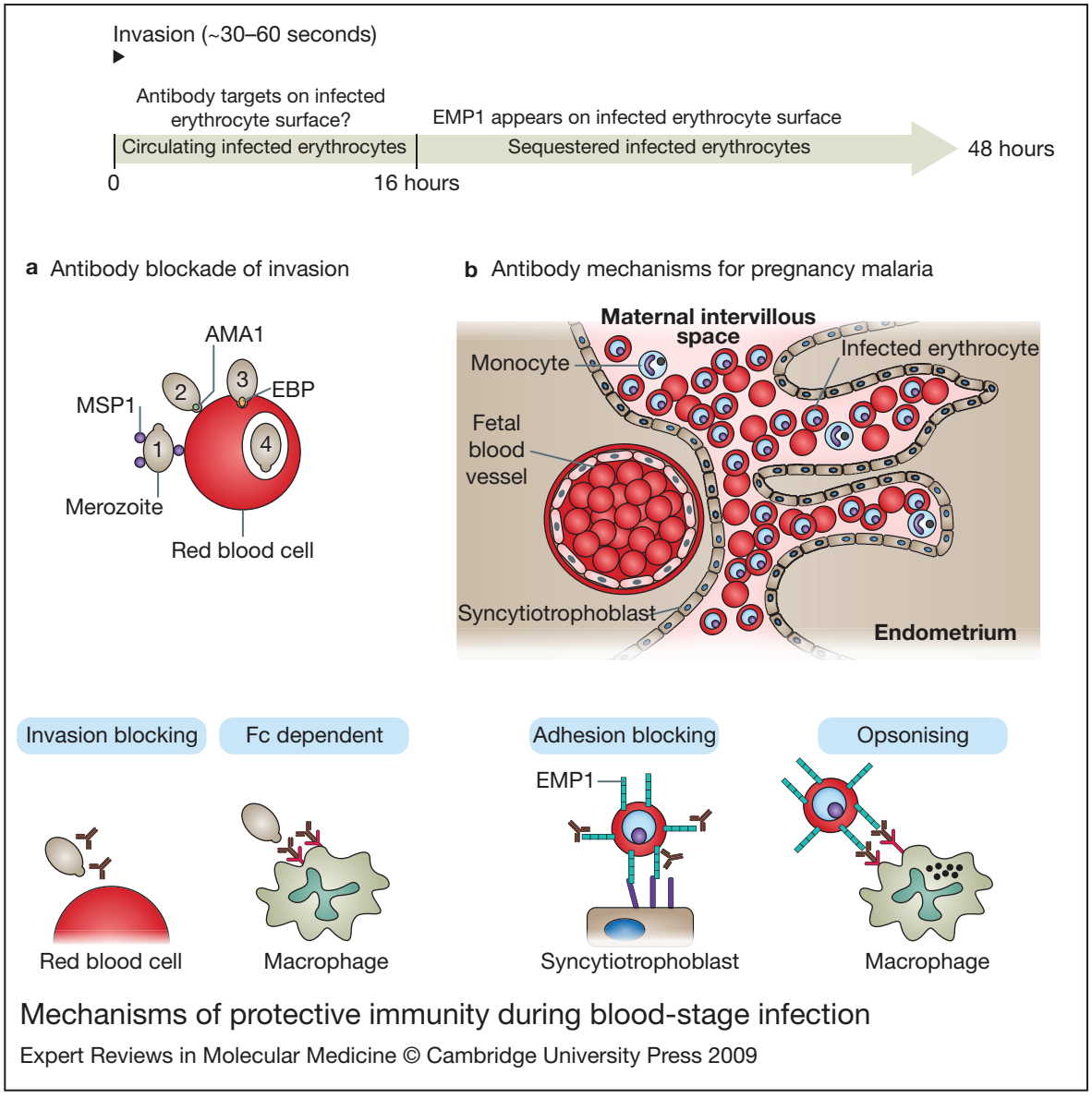
P. vivax requires the Duffy blood group antigen/receptor for chemokines (DARC) to invade reticulocytes (Ref. 144) and has nearly disappeared from West Africa, where most individuals are DARC negative. *P. vivax* binds to DARC via DBP (Ref. 145). *P. vivax* DBP is a

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member of a family of erythrocyte-binding proteins characterised by cysteine-rich binding domains, termed Duffy-binding-like (DBL) domains that confer red blood cell binding specificity (Refs 146, 147). Whereas *P. vivax* has only a single gene encoding the DBP, *P. falciparum* has four genes that encode proteins that bind different sialoglycoproteins on the erythrocyte surface (Ref. 148). Because *P. vivax* has only a single DBP gene, the parasite might be more dependent on the DARC interaction for invasion and would be unable to switch to other members to escape antibody pressure.

The structure of the DBL domain has been solved from the *P. knowlesi* DBP (Refs 149, 150), and used to model polymorphism and functional residues in *P. vivax* DBP. Interestingly, critical binding residues in *P. vivax* DBP mostly map to a solvent-accessible groove, but the majority of polymorphism is concentrated on the opposite surface from the predicted DARC-binding site (Refs 151, 152, 153). It is currently disputed whether polymorphism is under significant selection for escape from inhibitory antibodies, although an amino acid change at residue 171 affects



Mechanisms of protective immunity during blood-stage infection

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Figure 2. Mechanisms of protective immunity during blood-stage infection. (*See next page for legend.*)

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Figure 2. Mechanisms of protective immunity during blood-stage infection. (*Legend; see previous page for figure.*) Erythrocyte invasion occurs rapidly, within ~30–60 seconds (Ref. 100), and once inside the erythrocyte, the parasite appears to be relatively inaccessible to antibodies until approximately 14–16 h after invasion, when new parasite proteins, such as the *P. falciparum* erythrocyte, membrane protein 1 (EMP1), begins to appear on the infected erythrocyte (IE) surface. (a) Erythrocyte invasion is a complex process that has been divided into four steps: (1) attachment, (2) reorientation, (3) tight junction formation, and (4) entry. MSP1 is the most abundant protein on the merozoite surface and may function at initial attachment (Ref. 105), AMA1 at parasite reorientation and entry (Ref. 106) and *P. vivax* DBP during tight junction formation for *P. vivax* (Refs 107, 108). *P. vivax* DBP is a member of the erythrocyte-binding protein (EBP) family, which has undergone gene expansion in *P. falciparum*, leading to functional differences with *P. vivax*. Antibodies may directly block invasion by inhibiting parasite binding, preventing protein–protein interactions on the merozoite surface, or interfering with proteolytic processing or shedding of invasion ligands. There is also evidence for Fc-dependent mechanisms of protection against MSP1 (Ref. 121). (b) In pregnancy malaria, *P. falciparum*-infected erythrocytes sequester in the intervillous spaces of the maternal placenta, but are absent in fetal blood vessels. Infected erythrocytes are found directly adjacent to syncytiotrophoblast cells and also fill the intervillous space. Monocytes are recruited to infected placentas and frequently contain residual hemozoin pigment that may result from ingestion of infected erythrocytes. Potential antibody mechanisms are adhesion blocking or opsonising infected erythrocytes for phagocytosis by monocytes or activated macrophages that were recruited to the infected placenta.

invasion-blocking antibodies (Ref. 154) and many children in Papua New Guinea, aged 5–14 years, appear to have strain-specific inhibitory antibodies (Ref. 155). The most advanced *P. vivax* DBP immunogen is based on a bacterial refolded protein from the DBL-binding domain or region II (*P. vivax* DBPII) (Ref. 156). Antibodies to *P. vivax* DBPII can inhibit its interaction with DARC in vitro (Refs 157, 158, 159), as well as inhibiting parasite invasion in an in vitro invasion assay (Ref. 160). However, complete Freund's adjuvant was required to protect in a *P. vivax* and *Aotus* primate challenge model (Ref. 161).

Antidisease vaccines for severe and pregnancy-associated malaria

Once the parasite has entered the red blood cell, it might be expected that it would not display proteins at the infected erythrocyte (IE) surface, but curiously all *Plasmodium* species appear to express different clonally variant antigens at the IE surface (Ref. 162). The best characterised gene family is called *var*, or *P. falciparum* erythrocyte membrane protein 1 (EMP1) (Ref. 163) Unlike other *Plasmodium* species that infect humans, *P. falciparum* begins to sequester from the circulation approximately 14–16 h after invasion and the EMP1 extracellular domain expressed on the IE surface is responsible for this (Ref. 164). *P. falciparum* EMP1 proteins encode multiple receptor-like domains related to the binding region in the *P. vivax* DBP protein (Refs 165, 166). Each parasite genome encodes ~60 *var* genes (Ref. 167), but expresses only one at a time. Switches in *var* gene expression modify the antigenic and binding properties of IEs (Ref. 168). This trait is a major virulence determinant that allows parasites to sustain chronic infection and contributes to severe disease when *P. falciparum* IEs accumulate in brain or placenta.

The best understood paradigm for IE sequestration is placental malaria. *P. falciparum* infection during pregnancy is considered the most important nongenetic factor contributing to low birth weight in first pregnancies in Africa, and is believed to cause ~20,000 maternal deaths annually and may contribute to the deaths of 100,000–200,000 infants (Refs 169, 170). Placental malaria has been associated with the expression of a single *var* gene, termed *VAR2CSA*, which is believed to mediate IE binding to chondroitin sulphate A (CSA) in the placenta (Refs 171, 172, 173, 174). *VAR2CSA* is unusual for the *var* gene family, because a gene copy is found in all *P. falciparum* strains (Refs 173, 175), and it is thus being investigated as a syndrome-specific vaccine intervention.

VAR2CSA is a large (~350 kDa) and polymorphic protein. It is technically challenging to express the entire extracellular domain as a recombinant protein and therefore vaccine efforts have focused on expressing individual domains (Refs 176, 177, 178, 179, 180). Sequence and serological comparisons suggest that there is antigenic overlap between

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geographically diverse placental isolates (Refs 181, 182, 183), but it is not yet clear how many vaccine components will be required for an effective vaccine. Infected women frequently have five or six different placental genotypes, which could contribute to the breadth of antibody reactivity, even after a single pregnancy, and women may never acquire sterile immunity (Ref. 169). Achieving the same breadth of antibody reactivity as found in protected women by vaccination with VAR2CSA immunogens may require defining highly conserved functional epitopes, should these exist, or multivalent vaccine mixtures. Monoclonal antibodies from pregnant women appear to predominantly target polymorphic epitopes in VAR2CSA (Ref. 184), although some conserved regions in VAR2CSA might be recognised by antibodies (Ref. 185). Antibodies are thought to protect against placental malaria by inhibiting IE binding to CSA (Refs 183, 186, 187, 188) or opsonising IEs for phagocytosis (Refs 189, 190, 191, 192) (Fig. 2), but adhesion-blocking epitopes in maternal sera have not been mapped. Recent evidence suggests that antibodies to several different VAR2CSA domains can partially inhibit IE binding to CSA, but the greatest inhibitory activity was against the VAR2CSA DBL4 domain (Ref. 179). This led to the proposal that it may be possible to base a vaccine on a single VAR2CSA DBL domain. However, the breadth of inhibitory activity and the basis for this effect is not yet understood, and it has been difficult to generate adhesion-blocking responses to VAR2CSA recombinant proteins in many studies (Refs 176, 177, 178, 180).

Antitoxin vaccine strategies

Another antidisease intervention is to target the malaria toxin. Despite intensive research efforts to characterise parasite factors responsible for proinflammatory and febrile responses associated with schizont rupture, the actual 'malaria toxin' is still a matter of debate. It has been proposed that the *P. falciparum* GPI is an important parasite factor that activates the host innate immune system, mainly through TLR2 and to a lesser extent TLR4 (Refs 193, 194), whereas haemozoin carrying parasite DNA was found to be a TLR9 ligand (Ref. 195). Although the primary function of the innate immune response and fever is to fight off the pathogen, excessive proinflammatory cytokines, such as tumor necrosis factor (TNF), might be involved in the pathogenesis of fatal malaria (Refs 196, 197) and could exacerbate inflammation at adherence sites, such as brain, by upregulating cell-adhesion molecules (Ref. 198). Based on this concept, a synthetic malaria GPI glycan is being explored as an antitoxic therapeutic target in experimental models of malaria in mice (Ref. 199). Immunisation of mice with synthetic GPI glycan prevented pulmonary oedema and cerebral malaria complications, but did not have an antiparasite effect, with the result that mice died of massive parasitaemia with normal kinetics (Ref. 199). The major remaining questions for antitoxin vaccines are whether GPI is the only malaria toxin, and whether this approach could prevent the inflammatory complications of infection without losing the potential beneficial effects of fever or innate responses. If proved to be safe, then antitoxin vaccines would presumably need to be combined with antiparasite vaccine components to be effective.

Clinical implications and outstanding research questions

In summary, all malaria vaccine development approaches discussed in this review face formidable obstacles, but also provide tantalising opportunities. Subunit vaccines do not yet give the desired levels of protection, but would be easiest to manufacture and deliver. Live-attenuated parasites are protective, but we do not know how to best manufacture and deliver them.

Pre-erythrocytic subunit vaccine development faces considerable challenges. Identifying which of the thousands of genes expressed at the SPZ and LSs contribute to protective immunity is one challenge, optimisation of vaccine delivery systems that elicit high-titre functional antibodies to prevent infection and/or induce cellular immunity to infected hepatocytes is another challenge. Recent advances in LS research support for the first time an efficient, pragmatic and systematic approach for the identification and prioritisation of pre-erythrocytic antigens for subunit vaccine development. These include the purification of in vivo LS parasites (Ref. 200), the description of the LS transcriptome and partial proteome of *P. yoelii* (Ref. 201), the availability of protective

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attenuated whole-cell vaccines (Ref. 18) and the application of high-throughput immune assays to identify novel pre-erythrocytic antigens targeted by antibodies and T cells in humans (Refs 70, 202, 203, 204). These are all potent tools for future investigations which should include: (1) characterisation of the immune mechanisms of protection induced by attenuated parasites; (2) identification of peripheral biomarkers that are associated with acquisition, varying degrees and duration of protective immune responses induced by whole-parasite vaccination using system biology approaches; (3) exploration of the entire *Plasmodium* SPZ and LS proteome to identify novel pre-erythrocytic antigens that contribute to sterile protection induced by attenuated parasites; (4) high-throughput in vitro assays for determination of antibody- and cell-mediated elimination of LS-infected hepatocytes that correlate with protection; (5) rational selection of vaccine delivery system(s) and adjuvant(s) that induce immune responses of the magnitude necessary to prevent infection.

The lack of immune correlates of protection is a barrier to invasion-blocking vaccine development for blood stages. It is not known whether MSP1 or AMA1 vaccines would be more effective if vaccine antibodies could be focused on functionally critical sites (e.g. complex interaction or proteolytic sites). Whereas most invasion-blocking vaccine effort has focused on *P. falciparum*, *P. vivax* might provide a more tractable system to develop 'proof of concept' for invasion-blocking approaches because *P. vivax* has more restricted blood cell tropism (Ref. 6). The selective preference for reticulocytes is an important factor limiting *P. vivax* growth and disease severity, and may provide unique opportunities for vaccine intervention. A challenge for *P. vivax* DBP vaccine development is that *P. vivax* cannot be easily grown in vitro; therefore, in vitro growth inhibition assays have used blood collected from naturally infected humans (Ref. 160). This in vitro invasion assay has relatively low efficiency, and vaccine development has relied on in vitro binding assays with *P. vivax* DBPII recombinant proteins (Refs 157, 158, 159). Major priorities include implementing standardised in vitro binding assays for antibody assessment, developing more robust in vitro invasion assays, optimisation of *P. vivax* and nonhuman primate challenge models or human challenge models, and a better understanding of how antibodies inhibit *P. vivax* invasion.

Antidisease vaccines would not prevent infection, but would target specific parasite variants associated with disease. Because severe malaria is a relatively rare complication of malaria infection, estimated to occur in ~2% of infections (Ref. 205), antidisease vaccine interventions will require large and expensive trials. As many first-time pregnant women acquire severe placental infections, trial design could be smaller and allow the antidisease vaccine concept to be tested. It is thought that a pregnancy malaria vaccine would need to be administered to women in their teenage years, similarly to human papilloma virus vaccines (Ref. 206). VAR2CSA polymorphism poses challenges, and it is not known whether vaccines can elicit antibodies against conserved regions of the protein that would not vary under vaccine pressure. Current understanding of how antibodies protect against placental malaria is limited and the lack of animal models for *P. falciparum* sequestration in the placenta are barriers to vaccine development. Whereas vaccine efforts have been highly focused on adhesion-blocking-antibody responses, more consideration may be needed for synergistic vaccines that aim for the broadest achievable adhesion-blocking and opsonising responses.

The current data available for irrSPZ immunisation studies in humans indicate that live-attenuated whole parasites induce sterilising immunity against challenge with homologous *P. falciparum* via the bite of infected mosquitoes. The protection lasts for up to 10 months and a small number of volunteers, when challenged with heterologous strains, were also protected. Furthermore, as discussed earlier, numerous animal studies support the notion that live-attenuated SPZ vaccinations confer sterile, long-lasting protection. Within the next 2 years, a clinical dose-escalation study with a cryopreserved formulation of irrSPZs that is administered intradermally or subcutaneously and a first-generation genetically attenuated SPZ vaccine that is administered by mosquito bite will probably yield unambiguous data on the safety and protective efficacy of this class of vaccines. In the future, the most formidable obstacle for the

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live-attenuated vaccine approach lies in the current need for cryopreservation in liquid nitrogen. A cryopreserved malaria vaccine will face challenges when delivered in the context of the 'Expanded Program on Immunization' (EPI), which is currently the major platform through which many childhood vaccines are delivered in resource-poor nations. Does this imply that live-attenuated vaccine approaches are a 'dead end' that will remain confined to the experimental stage? In this context, it is of interest to consider that delivery of a malaria vaccine for disease elimination will probably require delivery platforms that are distinct from EPI. Furthermore, it should be noted that live-parasite vaccines for veterinary applications are currently delivered in liquid nitrogen in resource-poor countries (Ref. 207). Thus, it is conceivable that if the subunit vaccine approaches described in this review continue to fail or confer only limited protection, the delivery of an efficacious whole-cell, live-attenuated malaria vaccine is likely to be a critical tool by which we can hope to eradicate malaria.

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Features associated with this article

Figures

Figure 1. Mechanisms of protective immunity against *Plasmodium*-infected hepatocytes.
Figure 2. Mechanisms of protective immunity during blood-stage infection.

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