Review article

Immunity to asexual blood stage malaria and vaccine approaches

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Summary The development of a malaria vaccine seems to be a definite possibility despite the fact that even individuals with a life time of endemic exposure do not develop sterile immunity. An effective malaria vaccine would be invaluable in preventing malaria-associated deaths in endemic areas, especially amongst children less than 5 years of age and pregnant women. This review discusses our current understanding of immunity against the asexual blood stage of malaria – the stage that is responsible for the symptoms of the disease – and approaches to the design of an asexual blood stage vaccine.

Keywords: asexual blood stage, malaria, vaccine.

Malaria and Plasmodium life cycle

Malaria, a parasitic infection, is an important cause of mortality and morbidity in many parts of the world. Each year, an estimated 300–500 million people are affected worldwide. In reality, the true figure could be greater than three times this number.1 Malaria kills 1–2 million people each year, mostly children under the age of 5 years and a significant number of pregnant women in sub-Saharan Africa.2 It is a devastating infectious disease that not only affects the health system, but also slows the rate of long-term economic growth and development. The emergence of drug-resistant strains of the parasite has exacerbated the situation, and global climate change, disintegration of health services, human migration and population displacement have also contributed.2 In recent years, there have also been more cases of malaria in travellers to endemic countries.

Malaria is caused by unicellular protozoan parasites of the Plasmodium genus.3 There are four species of malaria parasites that infect humans: P. falciparum, P. vivax, P. ovale and P. malariae. The most severe form of malaria is caused by P. falciparum. The severity of the disease depends largely on the species and strain of the infecting parasite, and the immunological status of the person who is infected.

Cyclical fevers are the hallmark of malaria and typically occur shortly before or at the time of red blood cell (RBC) lysis as schizonts rupture to release new infectious merozoites (see below). This occurs every 48 h in P. vivax, P. ovale and P. falciparum, and every 72 h in P. malariae infection. Intense fever is accompanied by nausea, headaches and muscular pain, amongst other symptoms. In patients infected with P. vivax and P. ovale, relapse may recur months to years after initial infection. This is caused by re-activation of the silent liver-stage form of the parasites (hypnozoites). Renal failure, hypoglycaemia, hepatic dysfunction, severe anaemia, pulmonary oedema, convulsions and shock are complications in severe malaria. Cerebral malaria is a frequent presentation of severe P. falciparum infection and has been attributed in part to the unique ability of the parasites to alter the surface of infected RBC so that they bind to endothelial surfaces causing obstruction of cerebral blood flow.4 Recent observations suggest that pro-inflammatory cytokines and nitric oxide induced by parasite material also contribute to the pathogenesis of cerebral malaria.5 Malaria during pregnancy can cause miscarriages, foetal death, low birth weight and premature delivery.

The life cycle of Plasmodium comprises a sexual stage, which takes place in the stomach of the mosquito, and an asexual stage with multiplication in the vertebrate host (Fig. 1). The disease is transmitted from one infected person to another by the bite of female Anopheles mosquitoes. Soon after the female mosquito has ingested blood from an infected person, male gametocytes fertilize female gametocytes. Mobile products of this fertilization, the ookinete, burrow through the stomach wall and develop into oocysts in the lining of the gut. When the cysts rupture, they release sporozoites which enter the salivary glands. Within 60 min of inoculation into a vertebrate host by the mosquito, the sporozoites move to the liver and invade hepatocytes where they remain for 9–16 days and undergo asexual amplification. During this pre-erythrocytic stage, the host is asymptomatic.

The erythrocytic stage begins when the infected liver cell bursts, releasing merozoites into the bloodstream. Within 1–2 min of release, each merozoite attaches to specific receptors on the RBC membrane via ligands on the surface of the merozoite. Subsequently, the host RBC membrane invaginates so that the merozoite moves into the erythrocyte. Residing in the parasitophorous vacuole, the parasite undergoes development from the early ring stage trophozoite to the late trophozoite and then, after mitotic divisions, to the schizont stage, which contains 6–32 merozoites, depending on the parasite species. When the erythrocytic schizont ruptures, the merozoites spill into the blood and each one continues the life cycle by invading another RBC. During this repeated cycle, some merozoites differentiate into male and female gametocytes, which can be taken up by mosquitoes...
during a blood meal. Then the infectious cycle of *Plasmodium* can repeat itself.

It is the asexual blood stage that is responsible for the symptoms of the disease. There is therefore a significant effort to develop a vaccine against this stage of the life cycle, which could limit parasite growth and consequently prevent or minimize clinical disease. The successful development of an asexual blood stage vaccine is critically dependent upon our understanding of immunity to asexual blood stage parasites.

**Immunity to erythrocytic stage**

Naturally acquired immunity to malaria takes as long as 10–15 years of exposure to develop. Even with repeated infection, protective immunity is non-sterile, and it is species-, stage-, strain- and variant-specific. Individuals in malaria endemic areas frequently have premunition, i.e. parasitaemia and antibodies (Ab) without symptoms. Acquired protective immunity induced by malaria parasites involves both Ab-mediated and cell-mediated immunity.

**Antibody-mediated immunity**

It is well established that B cells and Ab play a crucial role in immunity to malaria. It has been demonstrated that naturally acquired immunity to malaria in individuals living in endemic areas, which takes several years to develop, is dependent largely on the acquisition of a repertoire of specific, protective Ab directed against the polymorphic target antigen, *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). Passive transfer of monoclonal Ab (mAb) against parasite antigens confers protection in naive mice. Treatment of *P. falciparum* infected Thai patients with IgG extracted from immune African adults results in reduction of parasite load and clinical symptoms. Immunoglobulin (Ig) μ-chain gene-targeted (μ-MT) mice lacking B cells are unable to clear parasites from *P. chabaudi* infection, and instead develop chronic parasitaemia. Infected μ-MT mice treated with antimalarial drug develop acute parasitaemia during secondary infection that resembles a primary infection, although with a reduced parasite density and the subsequent chronic infection is not resolved, indicating that B cells are
required for development of protective immunity. Adoptive transfer of B cells from immune donors at a later stage of infection restores the ability of deficient mice to complete parasite clearance, confirming the critical role of B cells in the clearance of blood stage parasites.14 Studies in B-cell deficient mice demonstrate that B cells also play an essential role in switching from a Th1 response to a Th2 response (see below), which is critical for the complete resolution of *P. chabaudi* infection.15,16

The degree of protective immunity in humans17–19 monkeys20 and mice21,22 has been shown to correlate with the level of Ab against asexual blood stage antigens, and is dependent on Ab isotypes. The IgG subclass responses against ring-infected erythrocyte surface antigen (RESA), merozoite surface protein (MSP) 1, MSP-2 and crude *P. falciparum* antigen in people living in exposed areas are partly determined by host genetic factors and are age-dependent.23 Cytophilic Ab of the IgG1 and IgG3 subclasses are considered to be the most important Ab for protection against *P. falciparum* malaria in humans.23 Acting in collaboration with effector cells such as monocytes and macrophages, they mediate opsonization and Ab-dependent cellular inhibition. A seroepidemiological study has shown that increased levels of *P. falciparum*-specific IgG1 and IgG3 in individuals living in endemic areas are associated with lower parasitaemia and reduced risk of malaria pathology.24,25 Protection attributable to parasite-specific IgG3 is age-associated, with greater levels of protection seen in adults. In addition to IgG1 and IgG3, IgG2 may be involved in protection. High levels of IgG2 to RESA and to MSP2 are associated with resistance to *P. falciparum* at the end of the transmission season and levels tend to be higher in older individuals who are better protected against infection and disease.26 In contrast, levels of IgG4 to parasite extract, RESA, MSP1 and MSP2 are lower in individuals who do not develop malaria than in susceptible individuals and are positively correlated with risk of infection. It has been suggested that IgG4 competes with cytophilic Ab for antigen recognition and may therefore block cytotoxicity mediated by Ab-activated effector cells.

In mice, the cytophilic isotype, IgG2a, is associated with protection against *Plasmodium* infection.27–29 IgG2a is predominant during the primary ascending parasitaemia in mice infected with *P. c. chabaudi* AS followed by an IgG1 response during the chronic stage of infection, as a consequence of Th1 to Th2 switching.30 It has been shown that IgG1 and IgG2b can confer protection against lethal challenge infection with *P. yoelii* YM in mice immunized with MSP119.31 IgG3 may also be important, as passive transfer of anti-MSP119 IgG3 into naive recipients resolves *P. yoelii* infection.30,31

Antibody responses directed against surface proteins of the merozoite may function either by blocking RBC invasion or by making the merozoite susceptible to phagocytosis. Parasite antigen-specific Ab play an important role in controlling parasitaemia via Ab-dependent cellular inhibition (ACDI), whereby binding of antibodies to phagocytes via Fc receptors leads to inhibition of parasite growth.13,26,32,33 It has been demonstrated that specific Ab initiate parasite clearance by opsonization, thus enhancing the activity of phagocytic cells34 or initiating complement-mediated damage.35,36 Merozoite surface protein 1-specific Ab not only inhibit secondary processing of the MSP1 precursor37 but also bind to MSP119, thus preventing the merozoite from binding to the RBC surface38 (see below). Serum Ab from malaria-immune donors can also simply bind to surface-accessible regions of the merozoite forming an immune complex, leading to agglutination and inhibition of merozoite dispersal.39

Antibodies to Plasmodium falciparum14 and the Duffy binding protein40 prevent cytoadherence and may thus prevent development of cerebral malaria. Blocking cytoadherence also prevents infected RBC being sequestered in the periphery, thus presumably allowing them to be removed by the spleen.41 Antibodies can disrupt spontaneous binding of uninfected RBC to *Plasmodium*-infected RBC (rosetting)42 which has also been associated with cerebral malaria.

Despite the importance of Ab responses for protection against malaria, it seems that not all Ab are protective. Polyclonal Ab specific to MSP2, but not mAb specific to the same antigen, enhance invasion of multiple merozoites into RBC.44,45 Furthermore, these MSP2-specific Ab at high-titre fail to induce complement-mediated damage. In another example, mAb against MSP119 which inhibit RBC invasion by merozoites and prevent MSP-1 secondary processing, can be blocked by other mAb to the same antigen.42 These studies illustrate the importance of identifying epitopes that induce protective Ab when designing a vaccine against malaria.

**Variant-specific immunity**

It has been shown that natural immunity to *P. falciparum* malaria is associated with the acquisition of variant-specific Ab that agglutinate infected RBC.10,47 The sera of *P. falciparum*-infected children obtained during the convalescent stage of infection agglutinate infected RBC from the same child but not infected cells from other children within the same region.47 In contrast, sera from immune adults in the same area agglutinate infected RBC from most of the children. When adult endemic sera are added to mixed parasite populations, the agglutinates that form contain only one strain of parasite, indicating that these adults have acquired a repertoire of variant-specific antibodies, rather than antibodies against conserved determinants.48

*In vitro* studies of clones derived from a single parent line of *P. falciparum* have demonstrated that variant-specific agglutinating Ab are directed against distinct forms of the molecule, *P. falciparum* erythrocyte membrane protein-1 (PfEMP1), and that *P. falciparum* is capable of spontaneously switching the form of this molecule which is expressed, an immune evasion strategy known as antigenic variation.49

*Plasmodium falciparum* erythrocyte membrane protein-1 is a variant protein of approximately 300 kDa, expressed on the surface of RBC infected with late stage parasites and coded for by approximately 50 genes of the "var" multigene family.50,51 *Plasmodium falciparum* erythrocyte membrane protein-1 mediates cytoadherence of infected RBC to endothelial receptors such as CD36, ICAM-1,52 and chondroitin sulphate A,53 a process that is believed to be involved in the pathogenesis of cerebral malaria and placental malaria. Changes in PfEMP1 expression correlate with changes in adhesive phenotype.54,55

Episodes of clinical malaria are associated with a transient increase in the level of variant specific antibodies that
recognize the infecting strain.\textsuperscript{47,56,57} The prevalence of variant-specific antibodies increases in an age-dependent manner, and this correlates with a decline in both the prevalence and density of parasitaemia.\textsuperscript{17} Therefore, in a natural setting, clinical immunity probably develops once an individual acquires antibodies against multiple PfEMP1 variants, which may explain why natural immunity takes several years to develop.

Some parasite isolates are recognized much more widely than others when tested against a panel of plasma samples, suggesting that some variants of PfEMP1 are more frequently expressed.\textsuperscript{57,58} Interestingly, infected RBC from children with severe malaria are agglutinated more frequently than those from children with mild malaria.\textsuperscript{57,58} This agglutination frequency decreases with the increasing age of the infected child. These data suggest that parasite variants associated with severe disease may occur more frequently in young children with low immunity. It has been hypothesized that variants with optimal cytoadherence characteristics may expand rapidly and dominate the infections in these children.\textsuperscript{58} On the other hand, in older children, pre-existing immunity may select for novel variants that may be less cytoadherent and consequently less pathogenic.\textsuperscript{57}

Despite the fact that natural immunity seems to depend on the acquisition of variant-specific antibodies to PfEMP1,\textsuperscript{58} it has been shown that the molecule does in fact contain cross-reactive epitopes.\textsuperscript{59} Monoclonal Ab specific to the cysteine-rich interdomain region 1 (CIDR1) of PfEMP1, which mediates adhesion of infected RBC to CD36, cross-react with multiple strains of parasites.\textsuperscript{59} This region has been shown to be highly conserved among parasite strains, although naturally it is poorly immunogenic.\textsuperscript{60} Such epitopes, which are unlikely to be under immune pressure since they are not targets of natural immunity, could potentially be effective vaccine candidates.

**Cell-mediated immunity**

CD4 T cells are classified into two major subsets according to their pattern of cytokine production. Th1 cells produce interleukin (IL)-2, interferon (IFN) γ, and tumour necrosis factor, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10.\textsuperscript{61} In general, Th1 cells are responsible for cell-mediated immunity (CMI). They activate macrophages and other cells to produce mediators through the release of inflammatory cytokines. T helper 2 cells regulate humoral immunity by providing help to B cells for the production of Ab. T helper 2 cells promote the production of IgG subtypes that are associated with allergies and helminthic infections, such as IgG1 in mice and IgG4 in humans. T helper 1 cells enhance the production of Ab that promote opsonization and phagocytosis, mainly IgG2a and IgG3 in mice, and IgG1 and IgG3 in humans. Hence, Th1 and Th2 cells mediate distinct immune responses. They also cross-regulate the differentiation and activities of each other via the cytokines produced. Interferon γ produced by Th1 cells inhibits the development and proliferation of Th2 cells, whereas IL-4 and IL-10 produced by Th2 cells antagonize the development of Th1 cells. Both Th1 and Th2 cells are involved in protective immunity against blood stage malaria, and the balance of cytokines produced by these two subsets is crucial in determining the outcome of the disease.

Activation of malaria-specific CD4 T cells bearing αβ T cell receptors (TCR) can be initiated soon after they recognize malaria antigens in the context of major histocompatibility complex (MHC) molecules on antigen-presenting cells such as dendritic cells\textsuperscript{62} or macrophages.\textsuperscript{63} It has been shown that CD4 T cells alone are able to confer protection against malaria. Severe combined immunodeficient (SCID) mice reconstituted with T cells from immune donors suppress growth of \textit{P. chabaudi adami}, suggesting that T cells contribute to immunity.\textsuperscript{64} B cell-deficient mice are able to suppress acute infections with \textit{P. c. adami}, \textit{P. vinkei petteri}, and \textit{P. c. chabaudi} CB at the same rate as normal mice.\textsuperscript{64} When CD4 T cells are depleted using anti-CD4 mAb, the mice lose the ability to control parasite growth. These results indicate that CD4 T cells can act independently of B cells in resolution of parasitaemia.

Nevertheless, several studies in \textit{P. c. chabaudi} AS have shown that protective immunity to blood stage malaria requires both CD4 T cells and Ab, and sequential activation of Th1 and Th2 cells is critical for protection.\textsuperscript{65,66} Mice lacking B cells can reduce a primary acute infection to low levels but develop a chronic relapsing parasitaemia which can be eliminated by adoptive transfer of B cells.\textsuperscript{67,68}

Interferon γ is the predominant cytokine produced in early acute \textit{P. chabaudi} infection but this declines as the parasitaemia decreases, and is replaced by IL-4 and IL-10 production during the latter stages of infection.\textsuperscript{14} The data imply that Th1 cells are responsible for control of primary parasitaemia, whereas Th2 cells are required for final clearance of parasites. A Th1 response followed by a Th2 response appears crucial for effective control of parasites. Activation of Th2 cells in the early acute phase of \textit{P. c. chabaudi} infection in susceptible A/J mice results in a severe and lethal course of malaria.\textsuperscript{65} Infective dose is one factor that influences Th1/Th2 activity in malaria. Increasing the inoculum size in susceptible A/J mice leads to fulminating parasitaemia associated with elevated Th2 responses.\textsuperscript{66} Collectively, it appears that CD4 T cells are crucial for resistance to malaria. Both Th1 and Th2 cells contribute to protective immunity at different times of infection, and the balance between these two subsets determines the outcome of the disease.

The role of CD4 T cells in immunity to malaria in humans is less well understood. Humans lacking previous exposure to \textit{P. falciparum}, as well as malaria-exposed individuals, have T cells that proliferate and secrete IFNγ in response to parasite antigen and inhibit parasite growth \textit{in vitro}.\textsuperscript{57} The degree of proliferation of peripheral blood mononuclear cells (PBMC) isolated from malaria-exposed individuals and stimulated \textit{in vitro} with MSP1 or circumsporozoite protein, correlates with the number of previous malaria episodes.\textsuperscript{60} In children living in highly endemic areas, protection against \textit{P. falciparum} correlates with the level of antigen-specific proliferative responses.\textsuperscript{69} It has been shown that a shift from a Th2 response to a more pronounced Th1 response is associated with the resolution of \textit{P. falciparum} infection.\textsuperscript{70} The precise role of effector CD4 T cells in protection of humans against malaria needs further investigation.

Although CD4 T cells are critical for protection against malaria, at the same time they play a role in the pathogenesis of lethal complications. Adoptive transfer of Th1-like T cells markedly suppresses parasitaemia in immunodeficient mice
following challenge infection with a non-lethal strain of *P. yoelii*, but this is associated with an increased mortality in recipient mice.\textsuperscript{71} Likewise, TH1 cells specific to *P. berghei* inhibit parasite growth but mice develop anaemia and weight loss.\textsuperscript{72} Treatment of infected mice with anti-TNFα Ab prolongs their survival although the mice develop higher parasitaemia. Convulsions due to experimental cerebral malaria in mice infected with *P. berghei* can be prevented by depletion of CD4 T cells before infection.\textsuperscript{73} Hence, CD4 T cells may protect the host by controlling parasitaemia but they can also cause detrimental immunopathology.

The role of T cells bearing γδ TCR in immunity to blood stage malaria remains unclear. Sayles et al. (1996) showed that depletion of γδ T cells does not alter parasitaemia, anaemia or survival rates of mice infected with avirulent *P. c. adami* or virulent *P. c. chabaudi* CB, suggesting that γδ T cells do not contribute to protection.\textsuperscript{74} In contrast, others have shown that mice lacking γδ T cells develop chronic parasitaemia following *P. c. chabaudi* AS infection.\textsuperscript{75,76} The number of γδ T cells in spleens of mice infected with *P. c. adami* is influenced by the level of parasitaemia during the course of infection.\textsuperscript{77} As parasitaemia increases, the number of splenic γδ T cells increase. This is followed by a reduction in the number of these cells during clearance of parasites, suggesting that, in collaboration with macrophages and αβ T cells, γδ T cells may be involved in the immune response to the blood stage of malaria. Human γδ T cells isolated from the peripheral blood of malaria non-immune individuals can inhibit growth of the late or extracellular stages of *P. falciparum* in vitro.\textsuperscript{78} The inhibitory activity correlates with the number of γδ T cells present in the culture, suggesting that γδ T cells contribute to the control of infection. Thus, a role for γδ T cells in providing protective immunity against malaria cannot be excluded and requires further investigation.

**Mediators of cell-mediated immunity to malaria**

T cells play a central role in the elimination of blood stage malaria parasites through the release of cytokines that activate other effector cells. Cytokines involved in immunity to blood stage malaria include IL-12, IFNγ and TNFα.

Interleukin-12 is a key cytokine that initiates Th1 responses by triggering IFNγ production from natural killer (NK) and CD4 T cells.\textsuperscript{79} Interleukin-12 secretion is induced by various infectious agents, including viruses, bacteria and parasites. During malaria infection, early non-specific immune responses can be augmented by the release of IL-12 from splenic macrophages.\textsuperscript{80,81} Administration of anti-IL-12 Ab to normal mice during *P. berghei* infection results in a marked reduction of IFNγ production, showing that IL-12 is a potent inducer of IFNγ during malaria infection.\textsuperscript{82} Treatment of *P. c. chabaudi* AS susceptible AJ mice with IL-12 results in increased numbers of NK cells which spontaneously secrete IFNγ and TNFα.\textsuperscript{83} Consequently, IL-12-treated AJ mice are able to eliminate parasites and survive infection, whereas untreated AJ mice develop high parasitaemia and die. The ability of mice to control parasite growth is abrogated when the mice are depleted of NK cells, indicating that the protective effects of IL-12 are mediated by NK cells.\textsuperscript{83,84} In *P. falciparum* malaria, children with mild malaria have higher levels of plasma IL-12 compared with children who suffer from severe infection, and the levels of IL-12 are inversely correlated with parasitaemia and numbers of malaria pigment-containing neutrophils.\textsuperscript{85,86} In addition to activation of NK cells, IL-12 enhances production of IFNγ by CD4 T cells, which is also critical for protection.\textsuperscript{87} Taken together, the data indicate that IL-12 plays an important role in protective immunity to blood stage malaria by inducing IFNγ production by NK and CD4 T cells.

Interferon γ, a CMI-activating factor\textsuperscript{88} plays an important role in resistance to blood stage malaria infection. Activation of monocyte-derived human macrophages with IFNγ results in activation of phagocytic activity and killing of malaria parasites.\textsuperscript{89} Peripheral blood mononuclear cells from children with mild *P. falciparum* infection produce high levels of IFNγ when stimulated in vitro with merozoite antigens, and these children have a lower risk of re-infection. In contrast, children with severe malaria show lower levels of IFNγ production by PBMC and are more susceptible to re-infection.\textsuperscript{89} In the mouse model, infection of IFNγ-deficient mice with *P. c. chabaudi* AS results in increased morbidity and mortality,\textsuperscript{89,90} indicating a role for this cytokine in protection. Recruitment and local proliferation of macrophages are also impaired in the absence of IFNγ.\textsuperscript{91} Mice defective in IFNγ and its receptor show a predominantly Th2 response, which is associated with susceptibility to *P. chabaudi* infection.\textsuperscript{89,91} Thus, IFNγ is critical for resistance to blood stage malaria, through the stimulation of cytokine production by effector cells and enhanced activity of macrophages.

Tumour necrosis factor α production is greatly augmented during malaria infection, as shown by elevated levels of TNFα in plasma of patients with malaria\textsuperscript{85,86} and infected mice.\textsuperscript{84,92} As well as being induced by cytokines such as IFNγ,\textsuperscript{78} TNFα release by macrophages can also be directly induced by malaria parasites and their soluble antigens, such as malaria pigment (haemozoin)\textsuperscript{93} and glycosylphosphatidylinositol.\textsuperscript{94} High levels of TNFα mRNA expression in the spleens of C57BL/6 mice correlate with resistance to *P. c. chabaudi* AS infection, and administration of anti-TNFα Ab to resistant mice abrogates the immunity, indicating a protective role for TNFα.\textsuperscript{95} Mouse sera containing TNFα inhibit growth of *P. falciparum* in vitro by causing deterioration and degradation of parasites, suggesting that TNFα has a non-specific inhibitory effect on the parasites.\textsuperscript{96} In the presence of anti-*P. falciparum* Abs, addition of recombinant TNFα causes an increase in phagocytic activity of monocytes.\textsuperscript{97}

In addition to its protective capacity, TNFα is also associated with pathology of malaria. Neutralization of IFNγ with mAb results in the reduction of the levels of TNFα in serum of mice infected with *P. berghei*.\textsuperscript{98} As a consequence, the animals are protected from experimental cerebral malaria, suggesting that these cytokines contribute to pathology in *P. berghei* infection. Production of TNFα during blood stage malaria infection leads to splenomegaly\textsuperscript{99} weight loss and anaemia.\textsuperscript{100} Blocking TNFα with mAb prolongs the survival of mice.\textsuperscript{101} In humans, high levels of TNFα in children with *P. falciparum* malaria correlate with hypoglycaemia and mortality rates.\textsuperscript{102} Analysis of postmortem human tissues shows expression of TNFα in brains of patients with cerebral malaria.\textsuperscript{103} The balance between the protective and pathologic roles of TNFα is dependent on the amount, timing and
location of TNFα expression. The early presence of TNFα in the spleen confers protection against  *P. c. chabaudi* AS infection in C57BL/6 mice, whereas susceptible AIJ mice have increased levels of TNFα in sera and liver later during infection. This is consistent with a finding in  *P. falciparum* malaria, which demonstrates a correlation between high production of TNFα during acute phase infection and a rapid clinical and parasitologic cure in the patients.

The presence of IFNγ and/or TNFα promotes synthesis of nitric oxide (NO), a nitrogen organic radical. Nitric oxide plays a role in immune responses against a variety of pathogens, including fungal, helminthic, bacterial and protozoan agents. Its role in protective immunity to blood stage malaria remains controversial. Mice deficient in inducible nitric oxide synthase (iNOS), an enzyme essential for NO synthesis, are able to clear a  *P. berghei* infection at the same rate as normal mice. Blocking NO production through administration of an inhibitor of iNOS, Nω-monomethyl-L-arginine (L-NMMA), does not alter the course of  *P. yoelii* infection. These results suggest that NO does not play an important role in parasite killing. In contrast, others have demonstrated an antiparasitic effect of NO. The ability of mice transfused with a protective Th1 clone to control parasite growth is abolished after administration of L-NMMA, suggesting a protective role for NO. Therefore, it is possible that NO is involved in protective immunity by regulating the immune response rather than direct parasitic killing.

Collectively, the data demonstrate that cell-mediated immunity can be beneficial or detrimental to the malaria-infected host. To achieve desirable outcomes, the balance of mediators that are involved in the immune response to malaria must be tightly controlled. An understanding of the mechanisms by which cytokines induce protection and/or pathology in malaria will prove to be fundamental for designing vaccines and developing new therapies for malaria.

**Malaria vaccine development**

Due to the increase of malaria cases and drug resistance in many parts of the world, adequate treatment of malaria is becoming increasingly difficult, and effective controls are urgently needed. A goal of WHO is to reduce malaria-associated morbidity and mortality by 50% by the year 2010. The development of a malaria vaccine is one strategy that could prove the most cost-effective means of controlling both the transmission of infection and the impact of disease. Despite the fact that considerable effort has been invested in vaccine research for decades, no effective malaria vaccine is available. Nevertheless, increased understanding of the mechanisms of immunity to malaria and advances in biomedical technology are increasing the feasibility of producing an effective vaccine, with recent vaccine trials in mice, primates and even humans showing promise.

An ideal malaria vaccine would be safe, cheap, easy to manufacture, easy to administer and confer life-long immunity against the disease. Three main types of vaccines, against distinct stages of the life cycle, are being developed. A pre-erythrocytic/antisporozoite vaccine would prevent invasion of hepatocytes by sporozoites and/or prevent liver-stage parasites from developing to maturity. The key to developing a vaccine against this stage of the life cycle is to induce production of Ab that block invasion of the sporozoites and to induce effector CD4 and CD8 T cells that can destroy infected hepatocytes directly or via their mediators such as cytokines.

Since the asexual blood stage is responsible for clinical disease, a vaccine targeting this stage would reduce morbidity and mortality by eliminating or reducing the parasite load. In developing this vaccine, the aim would be to induce Ab that neutralize/destroy the merozoites and infected RBC, and block cytoadherence, and to activate effector CD4 T cells, which can drive a cell-mediated response.

A transmission-blocking vaccine aimed at sexual stages of the parasite life cycle would not protect individuals, but would interfere with parasite development within the mosquito. By inducing Ab that inactivate gametocytes and interfere with fertilization, such a vaccine would prevent transmission of the disease.

To develop a successful vaccine against malaria, target epitopes that have limited or no polymorphisms need to be defined. A human-compatible adjuvant and an appropriate formulation of antigen/adjuvant have to be identified for manufacture and further development. Understanding immune responses that are induced by particular vaccine candidates is fundamental to vaccine development. Not only is it essential to ensure that vaccine candidates induce protective immune responses, but also that no immunopathology results from vaccination.

**A blood stage malaria vaccine**

Since clinical symptoms of malaria manifest only during the blood stage, a vaccine against this stage of the parasite life cycle would prevent or reduce severity and complications of the disease, and perhaps eliminate malaria if sterile immunity could be achieved.

In red cells, parasites appear well located to avoid host responses. Red cells lack class I and class II MHC molecules and antigen-processing machinery. Therefore, direct T cell-mediated responses are not induced to determinants presented at the host cell surface. Only at schizont rupture is the parasite directly exposed, when, for a very brief period, daughter merozoites have to attach to and enter new red cells. Much attention has therefore been given to parasite molecules that interact with the host cells during RBC invasion as potential targets of host immune responses. A number of proteins have been identified on the merozoite surface or in the apical organelles that play a role in RBC invasion and are thought to be targets of immunity. These molecules include MSP, apical merozoite surface antigen 1 (AMA-1), the 175 kDa erythrocyte binding antigen (EBA175) and rhoptry-associated protein 1 (RAP-1), RAP-2 and RESA (Fig. 2).

**MSP1**

Merozoite surface proteins are synthesized during schizogony as single precursor polypeptides that vary in size and amino acid sequence. To date, eight  *P. falciparum* MSP have been identified and designated MSP1–8. Merozoite surface proteins are potential malaria vaccine candidates as they play important roles in the initial recognition and attachment of merozoites to the RBC surface. As proteins on the merozoite surface are exposed to the host immune system, they are
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thought to be targets of immune responses. Antibodies against MSP neutralize the parasite by agglutinating or opsonizing merozoites, preventing RBC recognition and invasion, or affecting growth of the new intracellular parasite within the RBC.

Merozoite surface protein 1 is a glycoprotein synthesized as a high molecular weight (∼185–205 kDa) precursor protein. The protein precursor is processed at least twice by protease enzymes into a number of fragments. At schizont rupture, primary processing occurs, giving rise to major fragments of approximately 83 (MSP183), 28–30 (MSP130), 38 (MSP138) and 42 (MSP142) kDa found as a non-covalently associated complex held together on the free merozoite surface by the 42 kDa fragment. At the time of merozoite invasion, secondary processing, which is a prerequisite for RBC invasion, takes place. The calcium-dependent proteolytic processing cleaves the C-terminal membrane-bound 42 kDa fragment into two products. The soluble 33 kDa fragment (MSP133) corresponding to the N-terminal region of MSP142, induces protection against P. yoelii infection and passive transfer of mAb specific to MSP133 or MSP142 suppresses parasitaemia, indicating that MSP133 and MSP142 also play an important role in protective immunity.

The whole molecule of MSP1 has been shown to induce protection against lethal P. yoelii YM and P. falciparum in mice and monkeys, respectively. Antibodies specific for MSP1 from malaria-immune donors form immune complexes and inhibit merozoite dispersal. Subsequent studies have shown that regions of MSP1 that are thought to be responsible for protection include MSP130, MSP138, MSP142 and also the N-terminal region of MSP143. A region of 115 amino acids in the middle of MSP138 (p115 MSP1) binds to glycophorin A on RBC. Antibodies induced by natural infection that are specific for p115 MSP1 inhibit invasion of RBC by P. falciparum in vitro, suggesting that this region is highly antigenic and may play a role in parasite invasion. Immunization of mice with the C-terminal proteins, MSP119 and MSP142, induces protection against P. yoelii infection and passive transfer of mAb specific to MSP119 or MSP142 suppresses parasitaemia, indicating that MSP119 and MSP142 also play an important role in protective immunity.

Most studies have focused on MSP119 as a leading malaria vaccine candidate against blood stage malaria. Successful immunization with MSP119 has been reported in non-human primates and rodents. Animals that are vaccinated with the protein develop high titres of anti-MSP119 Ab, and the level of Ab correlates with the level of protection. Immune sera or purified IgG specific to MSP119 can passively transfer immunity to naive mice. B cell knockout (BKO) mice immunized against MSP119 are incapable of resolving P. yoelii YM infection. These data indicate that protective immunity induced by MSP119 is predominantly mediated by specific Ab. Monoclonal Ab specific to MSP119 prevent the secondary processing of MSP142 and inhibit merozoite invasion in vitro. Passive transfer of mAb specific to MSP119 inhibits growth of P. yoelii, and protect mice from infection. In humans, it has been shown that Ab against MSP119 comprise a large component of the total invasion-inhibitory response in P. falciparum immune individuals. Merozoite surface protein 1 is a restricted set of Ab in P. falciparum-exposed donors, with elevated IgG1/IgG3 and little, if any, IgG2 and IgG4. The level of cytophilic IgG1 is inversely correlated with parasite density, suggesting
that the IgG1 antibody response may play a role in protection in humans.

In rodent malaria, the antibody subclass responses in MSP119-immunized BALB/c mice are predominantly IgG1 and IgG2b, indicative of a Th2 type response. However, mAb of the IgG2a and IgG3 subclasses confer protective immunity to naive mice. Merozoite surface protein 1 (MSP1) specific IgG3 mAb are able to transfer protection to mice deficient in Fc receptor (Fc-γRII). suggesting that Ab-dependent cell-mediated cytotoxicity and Fc-mediated phagocytosis are not necessary for parasite clearance by this Ab. Whether the relative levels of different subclasses of MSP119 specific Ab affect the degree of protection requires further investigation.

In addition to specific Ab that are crucial for immunity induced by MSP119, complete protection against a blood stage malaria infection also requires CD4 T cells and an active immune response post challenge. Depletion of CD4 T cells from MSP119-immunized mice results in some mice succumbing to infection or rechallenge infection with P. yoelii YM. Furthermore, passive transfer of MSP119 immune serum into BKO, SCID, nude and CD4-depleted mice fails to protect the mice from lethal P. yoelii YM infection even though wild type mice are fully protected. These results imply that the transferred Ab alone cannot eliminate parasites, and that, an active immune response involving B cells and Th cells is required. Although T cells from BKO mice are capable of responding to MSP119, BKO mice that are vaccinated with MSP119 cannot control parasite growth, suggesting that MSP119-specific effector CD4 T cells may play only a minor role, if any, in immunity.

Structural determinants of MSP119 formed by the two EGF-like modules together seem to be critical for the immunogenicity of the protein. Mice are only protected when immunized with recombinant protein containing both modules but not when immunized with the individual modules, either alone or as a mixture. Reduction and alkylation of the protein abolishes the protection obtained, suggesting the existence of conformational epitopes, which are maintained by disulphide bonds.

In mice, immune responses to MSP119 are regulated by genes located in the H-2 locus. BALB/c (H-2d) and C57BL/6 (H-2b) recognize different epitopes on MSP119 as indicated by their proliferative responses to overlapping peptides spanning the length of MSP119. C57BL/10 (H-2b) mice are protected by immunization with MSP119 whereas B10.BR (H-2k) mice are not. Antibody isotypes produced in response to immunization with MSP119 differ between these two strains of mice, with C57BL/10 mice producing higher levels of IgG1. T cells from mice of these two different genetic backgrounds also differ in their production of IFNγ and TNF, but further investigation is required.

Despite the fact that MSP119 inhibitory Ab play a critical role in protection by inhibiting the secondary processing of MSP119, and preventing parasite invasion, their function may be blocked by other anti-MSP119 Ab, which are termed blocking Ab. Blocking Ab do not directly inhibit the proteolytic step or RBC invasion but instead, they compete with inhibitory Ab for binding to the merozoite surface. It has been shown that Ab against other regions on MSP1 also have blocking activity. Most importantly, naturally acquired immune individuals possess blocking Ab. This may be a means by which the parasite evades the host immune response. Nevertheless, substitution of amino acids in MSP119 abrogates the binding of blocking Ab without altering the ability of inhibitory Ab to prevent parasite invasion. This has implications for the development of a MSP119-based malaria vaccine.

**AMA1**

Apical merozoite surface antigen 1 (AMA1) is an integral membrane protein located in the apical secretory organelles or rhoptries, of developing and free merozoites. Protective immune responses induced by AMA1 have been shown in mice and monkeys. Protection of mice correlates with Ab titre, and passive immunization of mice with anti-AMA1 mAb confers protection against lethal P. yoelii YM. Rabbit anti-AMA1 Ab and human Ab from individuals living in malaria endemic areas inhibit invasion of RBC by P. falciparum. These data suggest that protective immunity induced by AMA1 is mediated by Ab. Nevertheless, the ability of AMA1-immunized mice to control parasite growth during infection with P. c. adami is partially abrogated when CD4 T cells are depleted. Since the level of anti-AMA1 Ab in CD4-depleted mice is not altered, the results suggest an important role of Ab-independent T cell-mediated immunity. This is confirmed by the ability of CD4 T cells specific for a cryptic epitope on AMA1 to adoptively transfer protection to T cell deficient (nude) mice. Thus, AMA1 induces protection against blood stage malaria, and the immunity is mediated by inhibitory Ab and CMI. A phase I AMA1 vaccine trial in humans has commenced, and the vaccine is considered safe and sufficiently immunogenic to be used in field trials.

**RAP1/RAP2**

Rhoptry-associated protein 1 and RAP2 are located in the rhoptries of merozoites. Naturally acquired immunity against P. falciparum in Aotus monkeys correlates with the level of Ab to RAP-1, and anti-RAP1 mAb are able to inhibit merozoite invasion in vitro. Additionally, successful immunization with parasite-derived or recombinant RAP1 and RAP2 of P. falciparum in monkeys has been demonstrated. The level of Ab in immunized monkeys correlates with the degree of protection, suggesting that immunity is Ab-mediated. A clinical trial of RAP2 in humans is under development.

**RESA**

Ring-infected erythrocyte surface antigen is present in dense granules in the apical region of merozoites. It is released into the parasitophorous vacuole during merozoite invasion and translocated to the inner surface of the RBC membrane. Ring-infected erythrocyte surface antigen elicits both humoral and cellular immune responses in P. falciparum-primed donors. Naturally induced Ab from exposed individuals inhibit P. falciparum growth in vitro and the levels of Ab increase in an age-dependent manner.
PIEMP1
In addition to antigens on the surface of merozoites, parasite proteins expressed on the surface of infected RBC are regarded as potential vaccine candidates. *Plasmodium falciparum* erythrocyte membrane protein-1 is a family of variant proteins expressed on the surface of infected RBC. These proteins are coded for by the ‘var’ multigene family which contains at least 50 genes. Var genes spontaneously switch during infection, resulting in sequential expression of different PIEMP1 variants on the RBC surface. *Plasmodium falciparum* erythrocyte membrane protein-1 is involved in cytoadherence of infected RBC to the vascular endothelium and is thought to be responsible for the pathogenesis of cerebral malaria. In order to develop PIEMP1 as a potential vaccine, conserved epitopes involved in endothelial binding need to be identified.

GPI
Glycosylphosphatidylinositols (GPI) are free lipids attached to proteins found ubiquitously in eukaryotic cells. Glycosylphosphatidylinositols expressed by malaria parasites may contribute to disease pathology, since parasite GPI can induce production of TNFα by macrophages. Glycosylphosphatidylinositols could be a target for a new class of malaria vaccine, an anti-clinical disease or antitoxic vaccine. People living in malaria endemic areas express naturally elicited anti-GPI Ab. Sera from adults who are resistant to clinical malaria contain high levels of persistent anti-GPI Ab, whereas susceptible children lack or have low levels of short-lived Ab. Anti-GPI Ab responses are correlated with protection against malaria-related febrile illness and haemoglobin loss. An effective GPI vaccine may prevent or reduce the severity of disease. During the complex life cycle of *Plasmodium*, the parasites express many different antigens. Therefore, a successful vaccine may need to include a broad variety of target antigens to elicit an effective immune response. Two phase I safety and immunogenicity trials in humans with a vaccine containing three recombinant blood stage malaria antigens, *P. falciparum* MSP1, MSP2 and RESA, have been performed. These initial studies have shown that the vaccine is safe, and all three antigens induce both Ab production and T cell responses. The clinical efficacy of the vaccine still needs to be assessed in field trials. Successful immunization with a multistage multiantigen vaccine has been demonstrated in monkeys. The composition of a vaccine able to induce consistent, lasting protective immunity in humans remains to be established.

Adjuvants and malaria vaccine development
Adjuvants are substances that enhance immune responses to vaccines, promoting the induction of long-lasting humoral and cellular immunity. They modulate the immune system by up-regulating certain types of cytokines, preserving the conformational integrity of an antigen, delivering an immunogen to immune effector cells and generating a depot of antigen. Substances that are used as adjuvants include small solid particles, aluminium salts, water-in-oil emulsions, oil-in-water emulsions, immune stimulating complexes (ISCOM), liposomes, saponins, bacterial toxins and cytokines. The identification of effective adjuvants is very important for the development of a successful malaria vaccine.

The ability of adjuvants to influence immune responses to blood stage malaria vaccines has been demonstrated. Some adjuvants such as saponin and pertussis enhance protection in mice that are immunized with whole *P. yoelii* vaccines by inducing the production of IgG2a Ab. Mice immunized with the same vaccines combined with adjuvants which do not augment IgG2a production succumb to infection. Successful immunization of mice with MSP1 has been shown to be dependent on both the adjuvant used and the genotype of the responding host. Mice that are vaccinated with MSP1 in various formulations show different degrees of protection against *P. yoelii*, which correlates with the levels and isotypes of Ab produced. The formulations that are effective in BALB/c mice cannot induce protection in Swiss/Webster mice, suggesting that the fine specificity of the protective response is influence by both the host MHC haplotype and by the adjuvant.

As adjuvants can alter the quality of immune responses, selection of an appropriate formulation of antigens/adjuvant is a crucial step in malaria vaccine design. Further investigation is required to identify an effective adjuvant for use in humans, which should strongly enhance the protective immune response and have an acceptably low level of side-effects.

Conclusion
There is currently no malaria vaccine available for universal use. The process of developing a successful malaria vaccine may take several more years. However, with advances in malaria research and our understanding of malaria immunity, together with significant on-going governmental, industrial and philanthropic support, an effective vaccine will be developed.

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