Advances and challenges in malaria vaccine development

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Malaria caused by *Plasmodium falciparum* remains a major public health threat, especially among children and pregnant women in Africa. An effective malaria vaccine would be a valuable tool to reduce the disease burden and could contribute to elimination of malaria in some regions of the world. Current malaria vaccine candidates are directed against human and mosquito stages of the parasite life cycle, but thus far, relatively few proteins have been studied for potential vaccine development. The most advanced vaccine candidate, RTS,S, conferred partial protection against malaria in phase II clinical trials and is currently being evaluated in a phase III trial in Africa. New vaccine targets need to be identified to improve the chances of developing a highly effective malaria vaccine. A better understanding of the mechanisms of naturally acquired immunity to malaria may lead to insights for vaccine development.

Introduction

There are over 500 million cases of malaria annually among the world's poorest populations (1). Malaria claims the lives of nearly a million children each year in Africa alone (2). The parasite that causes the most deadly form of malaria, *Plasmodium falciparum*, is spread by the highly prevalent mosquitoes *Anopheles gambiae* and *An. funestus*. After decades of neglect, funding from the international community to fight malaria has increased substantially in recent years (3). Increased funding has supported the scale-up of malaria control interventions such as the procurement and distribution of artemisinin-based combination therapy (ACT), the antimalarial drug class of choice, and insecticide-treated bed nets (ITNs), as well as other mosquito vector control strategies (3). In certain areas of Africa, these interventions have been linked temporally to recent declines in the incidence of malaria of more than 50% (4); however, the incidence of malaria in other areas of Africa and other regions of the world, such as Amazonia, is static or increasing (4, 5). Unfortunately, the widespread implementation of ACTs and ITNs is hampered by the poor health care infrastructure of many malaria-endemic countries. Moreover, *P. falciparum* has proven adept at acquiring and rapidly spreading resistance to antimalarial drugs, and even now resistance may have been acquired in Asia to the artemisinin derivatives (6). Vector control is also threatened by the inevitability of the emergence of insecticide-resistant mosquitoes (7). There is no question that a key tool for the control, elimination, or even possible eradication of malaria, in addition to antimalarial drugs and vector control, is an effective vaccine.

Thus far, we have no malaria vaccine, and it is not clear that a highly effective vaccine is in the pipeline. This may be due in part to a relative scarcity of research funding; in recent years global funding for malaria vaccine development has barely reached 25% of the approximately $684 million invested in the development of a still-elusive HIV/AIDS vaccine (8). In addition, malaria vaccine development is hindered by the sheer complexity of the parasite and its life cycle (9, 10), extensive antigenic variation (11), and a poor understanding of the interaction between *P. falciparum* and the human immune system (12).

The bright spot in terms of vaccine development is that *P. falciparum* infection induces something that HIV does not: clinical immunity. In areas of intense *P. falciparum* transmission, where individuals are infected by hundreds of mosquito bites each year, immunity to severe, life-threatening disease is usually acquired early in childhood, whereas immunity to mild disease is not typically acquired until late adolescence (13, 14). Data from transmigrant studies suggest that adults may acquire immunity more rapidly than children (15, 16); however, even in adults who have had decades of *P. falciparum* exposure, sterile immunity to blood-stage infection rarely develops, and an occasional episode of fever can occur (13). Thus, the immunity ultimately acquired by adults confers protection against the disease caused by the blood stages of *P. falciparum*, and not protection from infection per se. The hope is that knowledge of the immune mechanisms and their *P. falciparum* targets that ultimately provide protection from disease in adults can be used to develop a vaccine that would induce in a child a facsimile of adult immunity. Alternatively, by understanding the clinically silent stages that precede the blood-stage infection (i.e., sporozoite and hepatocyte stages), it might be possible to evoke, by vaccination, protective immune responses that do not normally develop in natural infection, namely, responses that prevent the blood-stage infection from occurring at all. Both broad approaches to vaccine development are being taken, but given the enormous complexity of *P. falciparum* infections, the effort is relatively small, targeting less than 0.5% of the thousands of potential *P. falciparum* antigens (refs. 9, 10, and Tables 1, 2, and 3). Compounding the difficulty of the vaccine effort are the large gaps in our understanding of *P. falciparum* infection biology — how *P. falciparum* invades its target cells and causes disease. These gaps can be closed, but only with adequate research support and the recruitment of experts in all facets of *P. falciparum* immunology and biology. With increased funding, vaccinologists can broaden their scope of exploration, increasing the probability of success.

In this review we discuss the stages in the *P. falciparum* life cycle that are targeted for vaccine development (Figure 1), the progress to date

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Table 1
Sporozoite and liver-stage malaria vaccines in clinical development

Vaccine	Antigen/platform	Current phase (trial location and subject age)	Challenge model	ClinicalTrials.gov ID (status)	Sponsors/ collaborators	Comments (refs.)
RTS,S	CSP coexpressed with HBsAg viral particles, AS01	Phase III (Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, Tanzania; 6–12 wk and 5–17 mo)	Natural infection	NCT00866619 (ongoing)	GSK, MVI	Approximately 30%–50% efficacy in phase II challenge studies in US and field trials in Africa (19)
FP9 CS and CS MVA	CSP expressed in FP9 (prime) and MVA (boost)	Phase I/IIa (UK, adults), phase Ib (Gambia and Kenya, adults)	Sporozoite challenge	NCT00121771 (completed)	GMP, LSHTM, MRC, Oxford Univ., MVI	No efficacy (49)
PfCS102	Recombinant CSP, montanide ISA 720	Phase I/IIa (Switzerland, adults)	Sporozoite challenge	NCT01031524 (completed)	Swiss TPH, CHUV, RUNMC	No efficacy (48)
ICC-1132	VLP consisting of HBc-expressing CSP epitopes, Seppic ISA 720	Phase I/IIa (UK, adults)	Sporozoite challenge	(Completed)	Oxford Univ., MVI	No efficacy (50)
Ad35 CS	CSP expressed in adenovirus 35	Phase I (US, adults), phase I (Burkina Faso, adults)	N/A	NCT00371189, NCT01018459 (ongoing)	DMID/NIAID, CNRFP, Crucell	–
AdCh63 ME-TRAP and MVA ME-TRAP	ME-TRAP expressed in AdCh63 (prime) and MVA (boost)	Phase I/II (UK, adults)	Sporozoite challenge	NCT00890760 (ongoing)	Oxford Univ.	–
FMP011	Recombinant LSA1, AS02A or AS01B	Phase I/IIa (US, adults)	Sporozoite challenge	NCT00312702, NCT00312663 (completed)	WRAIR, GSK, MVI	No efficacy (119)
PfLSA-3-rec	Recombinant LSA3, alum, or Montanide ISA 720	Phase I/IIa (Netherlands, adults)	Sporozoite challenge	NCT00509158 (completed)	RUNMC, Institut Pasteur	–
EP-1300	DNA polyepitope (CSP, TRAP, LSA-1, EXP-1) via electroporation	Phase I (US, adults)	N/A	NCT01169077 (pending)	DMID/NIAID	–
PfSPZ	Radiation-attenuated sporozoite	Phase I/IIa (US, adults)	Sporozoite challenge	NCT01001650 (ongoing)	Sanaria Inc., NMRC, Univ. of Maryland, WRAIR, MVI	–
Pf GAP p52$^-$/p36$^-$	Genetically attenuated parasite; KO of sporozoite-expressed *P52* and *P36*	Phase I/IIa (US, adults)	Sporozoite challenge	NCT01024686 (ongoing)	SBRI, WRAIR	–

CHUV, Centre Hospitalier Universitaire Vaudois, Switzerland; CNRFP, Centre National de Recherche et de Formation sur le Paludisme, Burkina Faso; DMID, NIAID Division of Microbiology and Infectious Diseases; FP9, Fowlpox strain 9; GAP, genetically attenuated parasite; GMP, Gates Malaria Partnership; GSK, GlaxoSmithKline; HBc, hepatitis B core antigen; LSHTM, London School of Hygiene and Tropical Medicine; ME, multiple epitope; MRC, Medical Research Council, U.K.; MVA, modified vaccinia virus Ankara; MVI, Malaria Vaccine Initiative; NMRC, Naval Medical Research Center, U.S.; RUNMC, Radboud University Nijmegen Medical Centre; SBRI, Seattle Biomedical Research Institute; Swiss TPH, Swiss Tropical and Public Health Institute; VLP, virus-like particle; WRAIR, Walter Reed Army Institute of Research.

in this effort, and last, the gaps in knowledge that, if filled, would have the greatest impact on the development of an effective vaccine.

The *P. falciparum* life cycle and vaccine targets

The *P. falciparum* life cycle in humans includes the pre-erythrocytic stage, which initiates the infection; the asexual erythrocytic stage, which causes disease; and the gametocyte stage, which infects mosquitoes that transmit the parasite (Figure 1). The pre-erythrocytic cycle begins when a female *Anopheles* mosquito inoculates a small number of *P. falciparum* sporozoites into the skin or directly into the bloodstream. Sporozoites travel to the liver and infect a small number of hepatocytes. A single sporozoite gives rise to tens of thousands of asexual parasites called merozoites (17). Merozoites are released into the bloodstream around one week after the initial liver infection, when infected hepatocytes burst, leaving no residual parasites in the liver. The pre-erythrocytic stage does not cause clinical disease (18), and there is no convincing evidence for naturally acquired protective immunity to this stage in individuals living in malaria-endemic areas (13). Thus, this stage would appear to be an unattractive vaccine target. Nonetheless, as will

Table 2
Blood-stage malaria vaccines in clinical development

Vaccine	Antigen/platform	Current phase (trial location and subject age)	Challenge model	ClinicalTrials.gov ID (status)	Sponsors/ collaborators	Comments (refs.)
AMA-1-C1	Recombinant AMA1, FVO and 3D7, Alhydrogel	Phase I/II (Mali, 2–3 yr)	Natural infection	NCT00341250 (completed)	DIR/NIAID, MRTC	No efficacy (69)
FMP2.1	Recombinant AMA-1, 3D7, AS01B or AS02A	Phase I/IIa (US, adults), phase II (Mali, 1–6 yr)	Sporozoite challenge (US), natural infection (Mali)	NCT00385047, NCT00460525 (completed)	DIR/NIAID, WRAIR, MRTC	No efficacy in *P. falciparum*–naive adults (120)
PfAMA-1-FVO	Recombinant AMA1, FVO, Alhydrogel, AS02A, or Montanide ISA 720	Phase Ib (Netherlands and Mali, adults)	N/A	NCT00730782 (completed), NCT00431808 (ongoing)	AMANET, MRTC, EVI, RUNMC, GSK	Safe and immunogenic in *P. falciparum*–naive adults (121)
AMA-1-C1	Recombinant AMA1, FVO and 3D7, Montanide ISA 720	Phase I (Australia, adults)	N/A	NCT00487916 (completed)	DIR/NIAID, QIMR	–
AMA-1-C1 + CPG	Recombinant AMA1, FVO and 3D7, Alhydrogel + CPG 7909	Phase I/IIa (UK, adults)	Blood-stage challenge	NCT00984763 (ongoing)	DMID/NIAID, Oxford Univ., NIHR	–
AdCh63 AMA1 and MVA AMA1	AMA1 expressed in AdCh63 (prime) and MVA (boost)	Phase I/IIa (UK, adults)	Sporozoite challenge	NCT01095055 (ongoing)	Oxford Univ., MRC, EMVDA, NIHR	–
FMP1	Recombinant MSP1(42), 3D7, AS02A	Phase IIb (Kenya, 12–47 mo)	Natural infection	NCT00223990 (completed)	WRAIR, KEMRI, MVI, USAID, GSK	No efficacy (73)
MSP1(42)-C1	Recombinant MSP1(42), FVO + 3D7, Alhydrogel	Phase I (US, adults)	N/A	NCT00340431 (completed)	DIR/NIAID, MVI	–
MSP1(42)-C1 + CPG	Recombinant MSP1(42), FVO + 3D7, Alhydrogel + CPG 7909	Phase I (US, adults)	N/A	NCT00320658 (completed)	DIR/NIAID, JHSPH	Safe and immunogenic (79)
FMP010	Recombinant MSP1(42) FVO, AS01B	Phase Ia (US, adults)	N/A	NCT00666380 (completed)	WRAIR, GSK, USAID	–
AdCh63 MSP1 and MVA MSP1	MSP1 expressed in AdCh63 (prime) and MVA (boost)	Phase I/IIa (UK, adults)	Sporozoite challenge	NCT01003314 (ongoing)	Oxford Univ., MRC, EMVDA, NIHR	–
BSAM-2	Recombinant AMA1 + MSP1(42), Alhydrogel + CPG 7909	Phase I (US and Mali, adults)	N/A	NCT00889616 (ongoing)	DIR/NIAID, MRTC, JHSPH	–
PfCP2.9	Recombinant chimeric AMA1 + MSP1(19), Montanide ISA 720	Phase I (China, adults)	N/A	NCT00284973 (completed)	Shanghai Wanxing Bio-Pharmaceuticals, MVI	Safe and immunogenic (81)
MSP3-LSP	MSP-3 long synthetic peptide, alum	Phase IIb (Mali, 12–48 mo)	Natural infection	NCT00652275 (ongoing)	AMANET, MRTC	–
GLURP-LSP	GLURP long synthetic peptide, alum, Montanide ISA 720	Phase I (Netherlands, adults)	N/A	(Completed)	EVI, RUNMC	Safe and immunogenic (72)
GMZ 2	GLURP + MSP3, alum	Phase I (Germany, adults; Gabon, adults and 1–5 yr)	N/A	NCT00397449 (ongoing), NCT00424944 and NCT00703066 (completed)	AMANET, EVI	Safe and immunogenic in *P. falciparum*–naive adults (71)
JAIVAC-1	MSP1(19) + EBA-175, Montanide ISA 720	Phase I (India, adults)	N/A	CTRI/2010/091/000301 (ongoing)	EVI, ICGEB, Govt. of India	–
EBA-175 RII-NG	Recombinant EBA-175, aluminum phosphate	Phase I (US and Ghana, adults)	N/A	NCT00347555 (completed), NCT01026246 (ongoing)	DMID/NIAID, NMIMR	Safe and immunogenic in *P. falciparum*–naive adults (70)
SE36	Recombinant SERA5, alum	Phase Ia (Japan, adults) phase Ib (Uganda, adults)	N/A	ISRCTN78679862 (completed), ISRCTN71619711 (ongoing)	Osaka Univ.	Safe and immunogenic in *P. falciparum*–naive adults (78)
Combination B	Recombinant MSP1, MSP2, RESA, Montanide ISA 720	Phase I/IIb (Papua New Guinea, 5–9 yr)	Natural infection	(Completed)	Swiss TPH, PNG-IMR	↓ Parasite density (74); no efficacy against blood-stage challenge in earlier trial (122)

AMANET, African Malaria Network Trust; DIR, Division of Intramural Research, NIH; EMVDA, European Malaria Vaccine Development Association; EVI, European Vaccine Initiative; ICGEB, International Centre for Genetic Engineering and Biotechnology, India; JHSPH, Johns Hopkins Bloomberg School of Public Health; KEMRI, Kenya Medical Research Institute; MRTC, Malaria Research and Training Center, Mali; NIHR, National Institute of Health Research, UK; NMIMR, Noguchi Memorial Institute for Medical Research, Ghana; PNG-IMR, Papua New Guinea Institute of Medical Research; QIMR, Queensland Institute of Medical Research, Australia; RESA, ring-infected erythrocyte surface antigen; USAID, United States Agency for International Development.

Table 3
Transmission-blocking, multistage, and *P. vivax* vaccines in clinical development

Vaccine type	Vaccine	Antigen/platform	Current phase (trial location and subject age)	Challenge model	ClinicalTrials.gov ID (status)	Sponsors/ collaborators	Comments (refs.)
Transmission-blocking	PpPfs25	Recombinant Pfs25, Montanide ISA 51	Phase I (US, adults)	N/A	NCT00295581 (completed)	DIR/NIAID, JHSPH	Immunogenic, but local and systemic reactogenicity (123)
Transmission-blocking	Pfs25-Pfs25	Recombinant Pfs25 conjugated to itself	Phase I (U.S., adults)	N/A	NCT00977899 (pending)	NICHD/NIH	–
Multistage	PEV301 and PEV302	CSP and AMA1 mimetopes incorporated into influenza virosomes	Phase Ia (Switzerland, adults), phase Ib (Tanzania, 5–45 yr)	N/A	NCT00400101 NCT00513669 (completed)	Swiss TPH, BRTU, Mymetics, Pevion	Safe and immunogenic (124, 125)
Multistage	PEV3A + FFM ME-TRAP	CSP and AMA1 peptides incorporated into influenza virosomes + FP9 ME-TRAP (prime) and MVA ME-TRAP (boost)	Phase I/IIa (UK, adults)	Sporozoite challenge	NCT00408668 (completed)	Oxford Univ., MRC, Pevion, Swiss TPH	Possible ↓ blood-stage growth rate in some vaccinees (126)
Multistage	NMRC-M3V-D/ Ad-PfCA	CSP and AMA1 encoded by DNA (prime) and expressed in adenovirus 5 (boost)	Phase I/IIa (US, adults)	Sporozoite challenge	NCT00870987 (completed)	NMRC, WRAIR, GenVec, USAID	–
Multistage	NMRC-M3V-Ad-PfCA	CSP and AMA1 expressed in adenovirus 5	Phase I/IIa (US, adults)	Sporozoite challenge	NCT00392015 (ongoing)	NMRC, WRAIR, GenVec, USAID	–
Multistage	FP9 PP and MVA PP	Six fused liver- and blood-stage antigens expressed in FP9 (prime) and MVA (boost)	Phase I/II (UK, adults)	Sporozoite challenge	NCT00375128 (completed)	EMVI, Oxford Univ., Wellcome Trust, WRAIR	No efficacy[A]
Multistage	AMA1 MSP1 TRAP	AMA1 + MSP1, MSP1 + TRAP expressed in AdCh63 (prime) and MVA (boost)	Phase I/IIa (UK, adults)	Sporozoite challenge	NCT01142765 (pending)	Oxford Univ., MRC, EMVDA, NIHR	–
P. vivax vaccine	PvCS	*P. vivax* CS-derived long synthetic peptides, Montanide ISA 720 or 51	Phase Ib (Colombia, adults)	N/A	NCT01081847 (completed)	MVDDC	–
P. vivax vaccine	VMP001	*P. vivax* recombinant CSP, AS01B	Phase I/IIa (US, adults)	Sporozoite challenge	NCT01157897 (ongoing)	WRAIR, MVI, GSK	–
P. vivax vaccine	SPZ-Irrad	*P. vivax* live irradiated sporozoites	Phase I/IIa (Colombia, adults)	Sporozoite challenge	NCT01082341 (pending)	MVDDC, NHLBI/NIH	–
P. vivax vaccine	PpPvs25	Recombinant Pvs25, Montanide ISA51	Phase I (US, adults)	N/A	NCT00295581 (completed)	DIR/NIAID, JHSPH	Immunogenic, but local and systemic reactogenicity (123)

[A]Adrian V.S. Hill, University of Oxford, personal communication. BRTU, Bagamoyo Research and Training Unit, Tanzania; MVDDC, Malaria Vaccine and Drug Development Center, Colombia; NHLBI, National Heart, Lung, and Blood Institute; NICHD, Eunice Kennedy Shriver National Institute of Child Health and Human Development.

be detailed below, the most advanced vaccine in development is a protein expressed at this stage that covers the parasite surface, the circumsporozoite (CS) protein (19).

Each merozoite exiting the liver into the bloodstream can invade an erythrocyte and multiply up to 20-fold every two days in cycles of erythrocyte invasion, replication, erythrocyte rupture, and release of infectious merozoites. In a nonimmune person, this results in as many as 10^8 asexual blood-stage parasites per milliliter of blood in a matter of a week, and symptoms can occur as early as three days after the blood-stage infection begins (18, 20). The rapid increase in parasites that the host suddenly experiences suggests that clinically immune individuals control the rapidly progressive blood-stage infection by high levels of preexisting antibodies (21) or possibly effector $CD4^+$ T cells (22, 23), since there may not be time for memory B or T cells to differentiate into effector cells before the onset of symptoms (24). It appears that adults in endemic areas do maintain levels of circulating antibodies sufficient to control the blood-stage disease, as shown by the ability to rapidly resolve fevers and reduce parasite levels to below detection via the transferral of IgG from malaria-experienced adults to children with fevers and high parasitemias (25).

A small percentage of blood-stage asexual parasites convert to sexual forms, or gametocytes, by poorly understood mechanisms (26), and these forms are able to infect female *Anopheles* mosquitoes. The *P. falciparum* male and female gametes undergo fertilization in the mosquito midgut, the only time in the life cycle when the parasites are diploid. Approximately 24 hours later, a small number of parasites (ookinetes) invade the midgut epithelial cells and travel through the cells to the hemolymph, where they replicate and form an oocyst containing thousands of sporozoites. This is the only stage in the life cycle in which the parasite replicates extracellularly. After the oocyst ruptures, sporozoites invade salivary glands and migrate to the salivary duct, from which they are injected into humans by blood-feeding mosquitoes, initiating a new human infection. The mosquito stage is an attractive target for a transmission-blocking vaccine, as the parasite in the mosquito midgut is present extracellularly and in very small numbers. Thus, one vaccine strategy is to immunize humans with mosquito-stage *P. falciparum* proteins, eliciting antibodies that would be taken up with the blood meal and disrupt *P. falciparum* development in the mosquito midgut (27).

Pre-erythrocytic vaccines

As described above, complete immunity to the pre-erythrocytic stage does not appear to be acquired naturally in endemic areas, as clinically immune adults are commonly infected with blood-stage parasites (13). However, experimental data suggest that it might be possible to induce immunity to the pre-erythrocytic stage. Roestenberg et al. (28) inoculated volunteers with sporozoites by the bites of *P. falciparum*–infected mosquitoes three times at 28-day intervals. During this period volunteers received chloroquine prophylaxis, which only has activity against blood-stage parasites, resulting in transient blood-stage infections. After 28 days without chloroquine, the volunteers were inoculated again with sporozoites through exposure to infected mosquitoes. Volunteers previously exposed to infected mosquitoes did not become infected, as monitored by the appearance of parasites in the blood, whereas all volunteers in the control group initially exposed to uninfected mosquitoes developed blood-stage infections. Protection was associated with a pluripotent effector memory T cell response (28). If the observed protection was due to an immune-mediated block of the pre-erythrocytic infection, this predicts that live attenuated sporozoite-based vaccines targeting the pre-erythrocytic stage might be effective. However, the possibility that blood-stage immunity induced by the transient blood-stage infection may have contributed to protection in this study cannot be ruled out.

The idea of a pre-erythrocytic vaccine took shape with the landmark observation by Ruth Nussenzweig that vaccination of mice with irradiated sporozoites resulted in protection (29) and, further, that protection could be achieved by immunization with the CS protein (CSP) alone (30). Development of human pre-erythrocytic vaccines began with the cloning of the *P. falciparum* CSP (31) and the entry of SmithKline with the Walter Reed Army Institute of Research (WRAIR) into vaccine development in 1985. This research led to the development of the RTS,S vaccine, which consists of hepatitis B surface antigen (HBsAg) particles with 25% of the HBsAg fused to the central repeat and thrombospondin domain of the CSP formulated in the adjuvant AS01 (19, 32) (Figure 2). In a series of phase II clinical trials, 30%–50% of malaria-naive adults immunized with RTS,S were protected against challenge by mosquitoes infected with the homologous *P. falciparum* clone (32–37). Protection correlated with CS-specific antibody and $CD4^+$ T cell responses (37), although reanalysis of the data suggests that the contribution of T cell immunity to protection may be minimal (38). In phase II field trials in the Gambia (39) and Kenya (40), RTS,S conferred short-lived protection against malaria infection in approximately 35% of adults, although results from the Kenya trial did not reach statistical significance. Approximately 30%–50% of children and infants immunized with RTS,S in phase II trials conducted in Mozambique, Tanzania, and Kenya were protected from clinical malaria (41–45); however, protection was generally short-lived. In field trials, immunization with RTS,S induced antibodies that correlate with protection from *P. falciparum* infection (46, 47) but not clinical disease (41, 45, 46).

The mechanism by which a vaccine that targets the sporozoite and liver stages protects against blood-stage disease remains unclear. It is possible that RTS,S induces protection against clinical malaria by temporarily reducing the number of merozoites emerging from the liver. This may lead to prolonged exposure to subclinical levels of asexual blood-stage parasites, which in turn allows boosting of naturally acquired blood-stage immunity (46). The RTS,S vaccine entered a phase III clinical trial in 2009 (Table 1). Based on results from phase II trials, RTS,S is likely to provide only partial protection. However, barring any unpredictable adverse effects, the vaccine could benefit millions of children by reducing the disease burden. It is also possible that the vaccine, if widely used, could have a greater impact on disease than predicted from the phase II trials in unforeseen ways by, for example, decreasing *P. falciparum* transmission. Conversely, disrupting the normal acquisition of malaria immunity through natural infection without providing complete protection could leave older children at risk of severe disease. Efforts to improve the efficacy of CSP-based vaccines with alternative adjuvants (48) or viral vectors (49, 50) have been unsuccessful to date; however, several studies are still ongoing (Table 1). Preclinical research efforts are going toward inducing higher levels of CSP-specific antibody (51). In one study the CS repeat peptide conjugated to the mosquito stage ookinete surface protein Pfs25 induced high levels of uncommonly long-lasting antibodies to both vaccine components in mice (51). In principle, this vaccine strategy could confer protection against liver infection and block transmission by the mosquito vector.

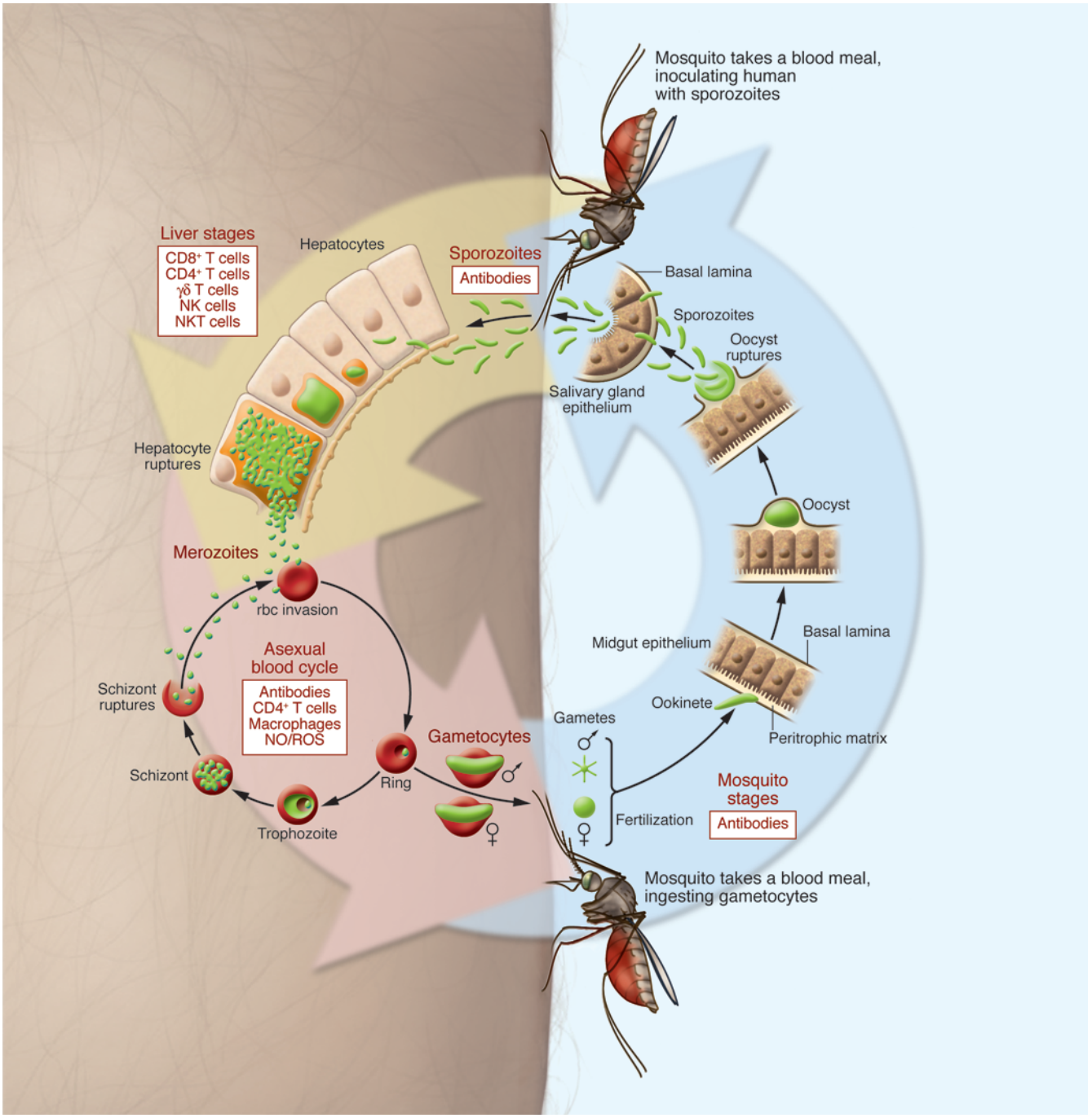

Figure 1

The *P. falciparum* life cycle. The *P. falciparum* life cycle in humans includes the pre-erythrocytic stage, which initiates the infection; the asexual blood stage, which causes disease; and the gametocyte stage, which infects mosquitoes that transmit the parasite. At each of these stages, the parasite expresses proteins that are targets of malaria vaccine candidates (Tables 1–3). The pre-erythrocytic stage begins when a female *Anopheles* mosquito inoculates sporozoites into the skin or directly into the bloodstream. Sporozoites migrate to the liver and infect a small number of hepatocytes. A single sporozoite gives rise to tens of thousands of asexual parasites called merozoites. Merozoites exit the liver into the bloodstream approximately one week later, leaving no residual parasites in the liver. The pre-erythrocytic stage does not cause disease, and complete immunity to this stage is not induced through natural *P. falciparum* infection. Merozoites entering the bloodstream begin a cycle of erythrocyte invasion, replication, erythrocyte rupture, and merozoite release that repeats approximately every 48 hours. Symptoms of malaria only occur during the blood stage of infection. Immunity that protects against disease but not infection per se can be acquired by individuals who are repeatedly infected in endemic areas. A small percentage of blood-stage asexual parasites convert to sexual forms, or gametocytes, which can infect mosquitoes. The mosquito stage is a potential target for transmission-blocking vaccines, as the parasite in the mosquito midgut is present extracellularly and in relatively small numbers. Possible immune mechanisms at each stage are indicated.

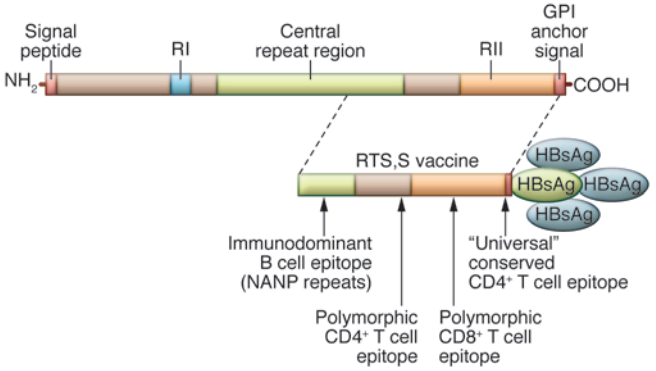

Figure 2
Schematic representation of the CSP and the RTS,S vaccine. The CSP is the predominant surface antigen on sporozoites. CSP is composed of an N-terminal region that binds heparin sulfate proteoglycans (RI), a central region containing a four-amino-acid (NANP) repeat, and a GPI-anchored C-terminal region containing a thrombospondin-like domain (RII). The region of the CSP included in the RTS,S vaccine includes the last 16 NANP repeats and the entire flanking C-terminus. HBsAg particles serve as the matrix carrier for RTS,S, 25% of which is fused to the CSP segment. The central repeat region contains the immunodominant B cell epitope, which induces antibodies that block sporozoite infection of liver cells in vitro (111, 112). RTS,S immunization induces antibodies to the central repeat region that correlate with protection from *P. falciparum* infection (46, 47) but not clinical disease (41, 45, 46). RTS,S also includes the thrombospondin domain, which binds receptors on liver cells (113). Monoclonal antibodies to the thrombospondin domain also block sporozoite invasion of liver cells, but to a lesser degree than antibodies to the repeat region (112). The CSP contains three known T cell epitopes: a highly variable $CD4^+$ T cell epitope before the thrombospondin domain (114), a highly variable $CD8^+$ T cell epitope within the thrombospondin domain (115), and a conserved "universal" $CD4^+$ T cell epitope at the C-terminus (116). RTS,S induces a moderate CS-specific $CD4^+$ T cell response that weakly correlates with protection from infection (37, 38), but RTS,S does not appear to induce a substantial CS-specific $CD8^+$ T cell response (37, 117, 118).

Efforts are also going toward developing vaccines that induce T cell immunity to the pre-erythrocytic stage through either irradiated (52) or genetically attenuated (53) sporozoites or through expression of *P. falciparum* liver-stage proteins in viral vectors (54) (Table 1). The irradiated sporozoite strategy is based on the observation that the bites of irradiated infected mosquitoes protected humans from challenge with unirradiated infected mosquitoes (55), suggesting that irradiated sporozoites in humans could be an effective vaccine, as they were first shown to be in mice (29). This approach is challenging, as protection required the bites of more than 1,000 infected, irradiated mosquitoes (56). However, this difficulty may be overcome, as it is possible to purify and cryopreserve irradiated sporozoites from aseptic mosquitoes in the quantities necessary for vaccination (52). In the first clinical trial, the irradiated, purified, cryopreserved sporozoite vaccine was safe and well tolerated, but only modestly immunogenic and protected only a few individuals. The next clinical trial will attempt to improve efficacy by optimizing the route of administration (S.L. Hoffman, personal communication). Studies are also in progress to determine whether sporozoites can be attenuated for use as vaccines by methods other than irradiation (53, 57). Recent studies in a mouse model provided evidence that infection with parasites attenuated by knockout of *Plasmodium yoelii* genes required for liver-stage development resulted in aborted hepatocyte development and induced $CD8^+$ T cells that mediated killing of infected hepatocytes through secretion of perforin and IFN-γ (57). A phase II trial to test this strategy in humans is underway (Table 1).

In mouse models of malaria, immunization with irradiated sporozoites induces $CD8^+$ T cells that kill parasite-infected hepatocytes. The known targets of $CD8^+$ T cell killing, in addition to CSP, include thrombospondin-related adhesion protein (TRAP) and liver-stage antigen (LSA). Immunization with viral vectors containing TRAP peptides led to partial protection in *P. falciparum*–naive adults from challenge by infected mosquitoes by mechanisms that involved the induction of large numbers of TRAP-specific IFN-γ–producing T cells (58). However, disappointingly, this vaccine did not induce protection in children in Africa (59). For unknown reasons, the level of TRAP-specific IFN-γ–producing T cells was considerably lower in vaccinated African children as compared with *P. falciparum*–naive adults (58, 59). Efforts to improve the T cell immunogenicity of TRAP with simian adenovirus vectors are ongoing (54) (Table 1).

Asexual blood-stage vaccines

The asexual blood stage begins with the release of merozoites into the bloodstream from ruptured infected hepatocytes. The blood stage is the only stage in the parasite life cycle that causes disease (18). Since immunity to disease develops with repeated *P. falciparum* infections, it may be possible to mimic and accelerate the acquisition of naturally acquired immunity by a vaccine. What do we know about the mechanism of this immunity? One key component of blood-stage immunity is antibodies, as demonstrated by experiments in which the transfer of IgG from immune adult Africans to partially immune African (25) or Thai (60) children rapidly reduced parasitemia and fever. Thus, it is theoretically possible to develop a vaccine that would elicit in children the antibodies that protect against disease in adults. At present, the specificity of antibodies that confer protection against malaria is not fully characterized, and as is the case for many infectious diseases, the precise mechanisms of antibody-mediated protection are unknown. The transferred IgG from malaria-immune adults did not block merozoite invasion of erythrocytes or growth of the parasites within erythrocytes in vitro (61), although this may not reflect events in vivo. However, the IgGs were shown to kill in vitro by antibody-dependent cell-mediated cytotoxicity (61), suggesting that inducing antibody responses of IgG1 and IgG3 isotypes that interact with activating Fc receptors may be desirable. Antibodies may also confer protection against blood-stage infection by blocking the binding of infected erythrocytes to endothelial cells (62); promoting the opsonization and destruction of merozoites and infected erythrocytes by phagocytic cells (63–65); and neutralizing *P. falciparum*–derived proinflammatory molecules such as glycosylphosphatidylinositol (GPI) (66, 67).

Antibody-independent mechanisms may also play a role in blood-stage immunity, although there are far less data from human studies to support this possibility. Volunteers repeatedly inoculated with *P. falciparum*–infected erythrocytes and then cured early in infection with antimalarial drugs were protected from reinfection (23). Although antibodies to *P. falciparum* were not observed in the protected volunteers, there was a Th1-biased $CD4^+$ and $CD8^+$ T cell response after exposure to malarial antigens ex vivo. However, the interpretation of this result is clouded by the possibility that the antimalarial drugs persisted at the time of challenge (68).

Thus far, relatively few blood-stage antigens are in clinical development as vaccines (Table 2). These include apical membrane antigen 1 (AMA1) (69), erythrocyte-binding antigen–175 (EBA-175) (70), glutamate-rich protein (GLURP) (71, 72), merozoite surface protein 1 (MSP1) (73), MSP2 (74), MSP3 (71, 75–77), and serine-repeat antigen 5 (SERA5) (78), all of which are highly expressed on the surface of the merozoite. Unfortunately, recent phase II trials of the most advanced blood-stage candidates, AMA1 and MSP1, did not demonstrate efficacy in African children (69, 73). Efforts to enhance the vaccine efficacy of AMA1 and MSP1 with novel adjuvants (79, 80), with viral vector prime-boost strategies (54), or by combining AMA1 and MSP1 (81) are ongoing (Table 2). However, extensive parasite genetic diversity due to the selective pressure exerted by the human immune response presents a major hurdle for blood-stage vaccine development (82, 83). For example, AMA1 is highly polymorphic, with hundreds of haplotypes that affect the ability of antibodies specific for one haplotype to block invasion by other haplotypes (84). Unless strategies are developed to overcome such genetic diversity, highly polymorphic *P. falciparum* antigens such as AMA1 are unlikely to be useful (82, 84). Another major challenge, considering that *P. falciparum* encodes approximately 5,300 genes (9), is the identification of new potential blood-stage vaccine candidates. One approach that takes advantage of the completion of the *P. falciparum* genome (9) is the use of high-throughput protein expression systems to construct microarrays of large numbers of *P. falciparum* proteins (21, 85, 86). In a recent study, an array containing 1,204 *P. falciparum* proteins was probed with plasma from *P. falciparum*–exposed children in Mali to identify antibody profiles associated with naturally acquired malaria immunity (21). An inherent drawback to this high-throughput approach is that not all proteins on the array will be properly folded and display all possible antigenic epitopes. Thus, this approach may serve as a starting point to "rule in" but not necessarily "rule out" *P. falciparum* proteins or combinations of proteins that induce protective antibodies. Another approach being taken to circumvent concerns related to protein folding and complex conformational epitopes is to screen for protective antibodies directed against predicted α-helical coiled-coil peptides derived from putative *P. falciparum* blood-stage antigens (87). Regardless of the approach, the analogous proteins determined by homology and synteny in rodent malaria could be tested for vaccine efficacy in preclinical vaccine trials.

Another starting point to search for new asexual blood-stage vaccine candidates is to focus on the parasite proteins that are required for erythrocyte invasion. However, there are a number of hurdles to this approach, as *P. falciparum* uses highly redundant, receptor-mediated pathways to invade erythrocytes, presenting an ever-moving target to the host immune response (88, 89). To initiate invasion, the merozoite first attaches to erythrocytes in a random orientation and then reorients to attach apically. The parasite ligands for initial attachment have yet to be identified and may be good targets for invasion-blocking antibodies. Two families of *P. falciparum* proteins have been identified that create the tight junction between the apical end of the parasite and the erythrocyte: the Duffy binding–like (DBL) and the reticulocytes homology (Rh) ligands. *P. falciparum* has multiple, functionally redundant members of each family (88, 89). As a consequence, it is likely that a vaccine that successfully blocks erythrocyte invasion would need to target multiple parasite ligands. By selecting conditions whereby the *P. falciparum* ligand under study is the only one available for invasion, it may be possible to determine whether an antibody will block invasion against multiple *P. falciparum* clones.

Another potential target for blood-stage vaccines is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, which is encoded by 60 or more *var* genes present in each parasite clone, with polymorphism between clones (11). The PfEMP1s are expressed on the surface of infected erythrocytes and are essential for the sequestration of the parasite in vascular endothelium to avoid destruction in the spleen (90). As one parasite clone expressing one PfEMP1 is detected by the immune system, another parasite clone expressing another PfEMP1 takes over (91, 92). PfEMP1-mediated sequestration of parasitized erythrocytes in vital organs is also thought to be responsible for severe disease such as cerebral and placental malaria. Despite the diversity of the PfEMP1, there is evidence for conservation in function and in sequences that might provide vaccine targets among this protein family. For example, only one PfEMP1, VAR2CSA, is thought to mediate parasite sequestration in the placenta (93), through binding of placental chondroitin sulfate A (94), causing pregnancy-associated malaria that can result in the mother's death and low birth weight or death of the fetus or newborn. Efforts to identify antibodies to domains of VAR2CSA that are broadly cross-reactive and block sequestration are ongoing (95). It is possible that other severe malaria syndromes such as cerebral malaria may result from a single, relatively conserved PfEMP1 that mediates sequestration of parasitized erythrocytes in the brain, a hallmark of cerebral malaria (96). To identify such PfEMP1s, convalescent sera from patients who survive severe disease can be probed against a protein array of PfEMP1 domains to identify antibody reactivity against domains that are associated with the various syndromes of severe malaria. In addition, some members of the PfEMP1 family, encoded by the so-called Type 3 Ups A *var* genes, are more structurally conserved than other PfEMP1s (97, 98), although their function remains unknown. Understanding the function of PfEMP1s encoded by group A *var* genes could indicate their potential as vaccine candidates.

Combining pre-erythrocytic and blood-stage vaccines

According to the WHO's guidelines, the efficacy of malaria vaccines in field trials is assessed as the time to first clinical malaria episode (99). By this criterion, the RTS,S vaccine is showing 30%–50% efficacy, as described above. However, an important unanswered question remains: How does partial pre-erythrocytic immunity influence the time to onset of clinical malaria, which occurs during the erythrocytic stage? As commented on above, one possibility is that a partially effective pre-erythrocytic vaccine reduces the number of infected hepatocytes, thus decreasing the number of merozoites released into the bloodstream, and allowing more time for blood-stage immunity to develop before the fever threshold is reached. If so, combining *P. falciparum* antigens that target the pre-erythrocytic and blood stages may further decrease the probability of reaching the disease threshold. This possibility provides the rationale for several multistage vaccine candidates that are currently under evaluation in clinical trials (Table 3).

Transmission-blocking vaccines

Transmission-blocking vaccines would target antigens on gametes, zygotes or ookinites, and the antibodies ingested as part of the blood meal would prevent parasite development in the mosquito midgut (27). These vaccines could be important tools for malaria elimination and could protect against epidemics if *P. falciparum* parasites

are reintroduced after a period of elimination. The feasibility of this approach is supported by the observation of transmission-blocking antibodies in individuals living in endemic areas (100, 101). However, the vaccine would need to be used in the entire population to block transmission. The vaccine would confer no protection to the vaccinated individual unless combined with an effective pre-erythrocytic (51) or erythrocytic vaccine. Transmission-blocking vaccines are not predicted to be effective in areas of intense *P. falciparum* transmission unless other measures to reduce transmission such as ITNs and insecticide spraying are employed (27).

P. falciparum proteins expressed only in the mosquito, such as Pfs25, are not polymorphic, as they are under no adaptive immune pressure in the human host (102). Gamete proteins such as Pfs48/45 and Pfs230 that are expressed in the human host are more polymorphic, but still have conserved domains present in all parasite clones studied to date. These two proteins have a six-cysteine structure unique to *Plasmodium* that presented a problem for recombinant protein production, which has now been solved (103). Pfs230 has the additional advantage of being the target of antibody-dependent complement lysis (104). In a mouse model, antibodies to HAP2, a *Plasmodium* protein thought to be involved in the fusion of male and female gametes in the mosquito midgut (105), also have transmission-blocking activity in vivo and in vitro (106).

Although the approach seems reasonable, transmission-blocking vaccines may be difficult to develop and implement. First, current evidence suggests that the levels of antibody in blood that would be required to significantly affect mosquito development may need to be extremely high (107). Conjugation of Pfs25 to a carrier such as outer membrane protein complex (OMPC) of *Neisseria meningitides* serogroup B may overcome this problem, as the conjugate induces high-titer antibody in rhesus monkeys that persists for at least two years (108). Second, it may be difficult to widely implement a vaccine that has no direct benefit to the individual receiving the vaccine but only to the vaccinated community. The combination of a pre-erythrocytic vaccine to prevent infection, such as the repeat region of the CSP, with a transmission-blocking vaccine, such as Pfs25 (51), may be an ideal vaccine strategy because, if effective, it would reduce transmission and provide some protection to vaccinated individuals. Despite the theoretical problems cited above, preclinical and clinical development of transmission-blocking vaccines is underway (Table 3) because of its promise for malaria elimination.

Although less virulent than *P. falciparum*, the capacity of *Plasmodium vivax* to cause severe, life-threatening disease is increasingly recognized, and its global burden has been historically underestimated (109). While not the focus of this review, it is important to note that efforts to develop a *P. vivax* vaccine are underway but still in the early stages (Table 3).

Summary

Malaria is a complex parasitic disease that imposes an enormous disease burden for which we currently have no vaccine. Optimism that a vaccine can be developed comes from the observations that malaria immunity can be acquired through natural and experimental infection. However, many *P. falciparum* proteins are highly polymorphic and their functions are redundant, which presents significant challenges for vaccine design. Nevertheless, we remain optimistic that with adequate research support and the recruitment of experts in all aspects of *P. falciparum* infection biology and immunity to work on this problem, a highly effective vaccine is possible.

Note added in proof. Dhingra et al. have now reported that the burden of malaria in India has been underestimated (110).

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