AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

MINIREVIEW

Transmission-Blocking Vaccines: Old Friends and New Prospects

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ABSTRACT In the progression of the life cycle of *Plasmodium falciparum*, a small proportion of asexual parasites differentiate into male or female sexual forms called gametocytes. Just like their asexual counterparts, gametocytes are contained within the infected host's erythrocytes (RBCs). However, unlike their asexual partners, they do not exit the RBC until they are taken up in a blood meal by a mosquito. In the mosquito midgut, they are stimulated to emerge from the RBC, undergo fertilization, and ultimately produce tens of thousands of sporozoites that are infectious to humans. This transmission cycle can be blocked by antibodies targeting proteins exposed on the parasite surface in the mosquito midgut, a process that has led to the development of candidate transmission-blocking vaccines (TBV), including some that are in clinical trials. Here we review the leading TBV antigens and highlight the ongoing search for additional gametocyte/gamete surface antigens, as well as antigens on the surfaces of gametocyte-infected erythrocytes, which can potentially become a new group of TBV candidates.

KEYWORDS gametocytes, *Plasmodium falciparum*, transmission blocking, malaria

Malaria control challenges arising from a combination of antimalarial-drug resistance and insecticide resistance (1) have reignited interest in the development of effective malaria vaccines and drugs that target multiple stages of the parasite's life cycle. Consequently, there has been increased focus on characterizing the sexual-stage parasite in order to gain an in-depth understanding of essential processes such as metabolism, sequestration, and infectiousness to the mosquito (2–5). The sexual-stage parasite begins its life cycle as sexually committed (sc) merozoites contained within an sc schizont in an infected red blood cell (RBC) (Fig. 1). When this sc schizont matures, it bursts the RBC and releases the sc merozoites, which subsequently invade surrounding RBCs. Within the RBC, sc ring-stage parasites form and then differentiate into mature transmissible gametocytes. For most *Plasmodium* parasites, the times required for gametocyte and asexual maturation are similar, and all erythrocytic stages continue to circulate in the peripheal blood. However, in the case of the most virulent human malaria parasite, *Plasmodium falciparum*, which is the primary focus of this review, an sc ring develops through five morphologically distinct developmental phases (stages I to V) over the course of 10 to 12 days to become a mature male or female gametocyte (6). Immature gametocytes (stages I to IV) are not observed in peripheral blood, and early histological studies, as well as recent molecular studies, have identified the bone marrow as a major sequestration site (7, 8). Apart from the bone marrow, immature gametocytes have been identified in other organs, such as the spleen, gut, brain, and heart (9). Following maturation to stage V, *P. falciparum* gametocytes are released and circulate in the blood for 4 to 6 days before dying. Continuous production with each asexual cycle ensures that mature stage V gametocyte-infected RBCs (giRBCs) circulate

Citation Acquah FK, Adjah J, Williamson KC, Amoah LE. 2019. Transmission-blocking vaccines: old friends and new prospects. Infect Immun 87:e00775-18. https://doi.org/10.1128/IAI.00775-18.

Editor Anthony T. Maurelli, University of Florida

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Accepted manuscript posted online 8 April 2019
Published 21 May 2019

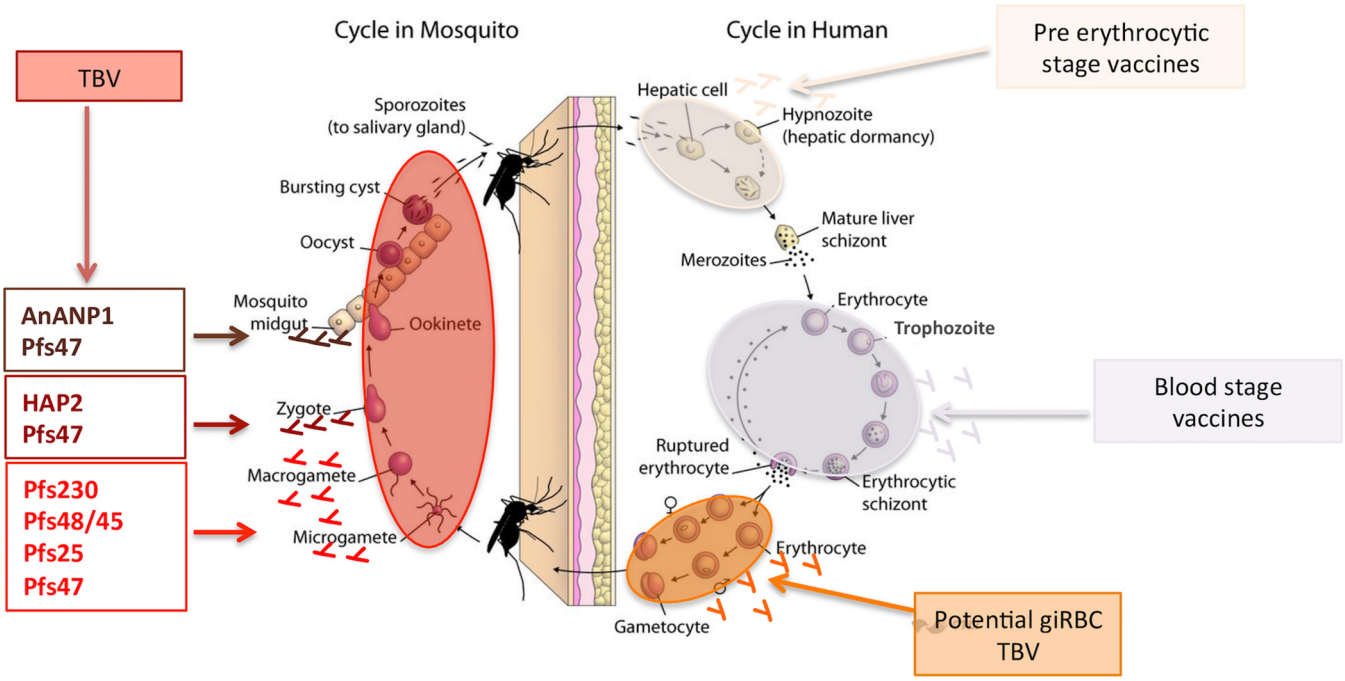

FIG 1 Life cycle of *P. falciparum*. Pre-erythrocytic-stage malaria vaccines target the sporozoites that are released from an infected mosquito into the human host and infected liver cells. Blood-stage vaccines target the merozoites released from the liver schizont as well as preventing the development of the asexual-stage-infected erythrocyte and the resulting merozoites produced from the erythrocytic schizont. Potential giRBC vaccines will target male and female gametocytes that develop within the erythrocyte. Transmission-blocking vaccines prevent the eventual development of oocysts and sporozoites within the mosquito. (Adapted from an Open Courseware image from the Johns Hopkins Bloomberg School of Public Health [188].)

continuously for several weeks (5, 10), making them accessible for uptake when a female mosquito takes a blood meal. The transcriptomes and proteomes of gametocytes have been found to contain sexual-stage-specific gene transcripts and proteins, some of which are expressed only in either immature or mature gametocytes (11–16).

Once in the mosquito midgut, mature male and female gametocytes of all *Plasmodium* species egress out of the RBC and differentiate into male and female gametes, respectively. Subsequently, mating (fertilization) occurs, resulting in the production of a motile ookinete within 24 h. The ookinete migrates through the midgut epithelium and forms an oocyst, where sporozoites mature over the course of 10 to 12 days before they are released and migrate to the mosquito's salivary glands. The infectous sporozoites are then released into a new host when the infected mosquito takes a blood meal.

The majority of current first-line antimalarial drugs are schizonticides that target the asexual parasite; a few of these, such as chloroquine and artemisinin, have some level of efficacy against early/young gametocytes (stages I, II, and III) (17), but not against late-stage gametocytes (stages IV and V) (18–20). The ineffectiveness of first-line antimalarials against transmissible mature gametocytes allows malaria transmission to continue despite the effective clearance of asexual parasites. Given the prolonged gametocyte maturation period in *P. falciparum*, a patient can remain infectious for more than a week after drug treatment (19, 20). It is thus imperative to develop new tools, such as transmission-blocking (TB) drugs and transmission-blocking vaccines (TBV), to effectively clear all gametocytes. A reduction in gametocyte carriage can result in reduced malaria transmission and the eventual eradication of the disease. This review focuses on *P. falciparum* malaria and covers well-known TBV candidate antigens as well as exploring a new class of gametocyte antigens, which may have the potential to elicit TB antibodies. The lack of an *in vitro* culture system for *Plasmodium vivax*, the other major cause of human malaria, has hindered *de novo* vaccine discovery. An effective alternative strategy has been to target the *P. vivax* homologs of *P. falciparum* TB candidates (21).

TABLE 1 Characteristics of some important sexual-stage antigens

Gene (protein)	TMD[a]	Localization	Reference(s)
PF3D7_0209000 (Pfs230)	0	Low-level protein expression begins in stage IIb gametocytes and peaks in stage III gametocytes. Gene expression persists from early gametocytes through macrogametes to zygotes.	2, 35, 36, 66, 159
PF3D7_1031000 (Pfs25)	1	Surfaces of emerged (extracellular) macrogametes through to ookinetes. As with Pfs28, gene expression begins in stage V gametocytes but remains in a translationally repressed state until gametocyte egress from the giRBC.	35, 100, 159, 160
PF3D7_1346700 (Pfs48/45)	1	As with Pfs230, low-level protein expression begins in stage IIb gametocytes and increases in stage III gametocytes. Gene expression persists from male and female gametocytes through sporogonic macrogametes to the zygote.	2, 35, 37, 71, 159
PF3D7_1346800 (Pfs47)	2	Protein expressed on the surfaces of extracellular female gametocytes and gametes through to ookinetes. Low levels of gene transcripts are found in early stage II/III gametocytes, and levels are increased in stage IV/V.	42, 79, 91
Pf3D7_1014200 (HAP2)	2	Surfaces of male gametocytes and microgametes.	44

[a]TMD, transmembrane domain (predicted using TMHMM [169]) obtained from PlasmoDB (142).

TRANSMISSION-BLOCKING VACCINE DEVELOPMENT

***P. falciparum* gametocyte and gamete surface antigens.** Emergence from the RBC within minutes of mosquito feeding effectively exposes all the proteins that were present on the gametocyte membrane to the contents of the blood meal, including antibodies and complement, which remain active in the midgut (22, 23). P230 and P48/45 are two of the best-characterized gametocyte membrane proteins that are exposed following gamete emergence from the RBC. The mosquito midgut environment also triggers the expression of additional gamete surface proteins from mRNA stored in the parasite's cytoplasm, such as P25 (24) and HAP2 (25). In contrast to gametocyte membrane proteins, these gamete-specific proteins are not exposed to the immune response of the human host, and thus, antibodies are not expected to be naturally acquired but can be induced by vaccination. Antibodies recognizing either type of gamete surface antigen have been shown to block/prevent the completion of the sporogonic life cycle of the parasite within the mosquito and to result in the development of transmission-reducing (TR) immunity or complete TB immunity (TBI) upon vaccination (26–31). Another set of antibodies that have been found to prevent the completion of the sporogonic life cycle of the malaria parasite target mosquito midgut proteins, including *Anopheles* alanyl aminopeptidase N (AnAPN1) (32).

Parasite proteins expressed on the RBC surface during gametocyte development in the human host could be another type of TBI target antigen. It is well established that intraerythrocytic asexual parasites export proteins to the RBC surface, but evidence for this in giRBCs has been more elusive and represents an opportunity to identify novel vaccine candidates. Potential giRBC surface proteins are discussed in detail under "Potential giRBC surface antigens" below.

Transmission-blocking antibodies. Early work using avian and murine *Plasmodium* species clearly demonstrated that anti-gamete antibodies bind to the surfaces of gametes and prevent the progression of parasite development within the mosquito midgut (33–35). The establishment of *in vitro* culture for the human malaria agent *P. falciparum* allowed the isolation of gametes that were used to produce murine monoclonal antibodies (MAbs) that recognized the gamete surface and also blocked oocyst production (35–38) in an experimental membrane-feeding assay. This assay allows mosquitoes to feed on gametocyte-infected red blood cells that have been mixed with test antibodies and is the gold standard for measuring TB activity (TBA) (39–41). Monoclonal antibodies with TBA or transmission-reducing activity (TRA) were then used to identify specific target proteins on the gamete surface: first Pfs48/45, Pfs230, and Pfs25, and later Pfs28 (Table 1). Pfs48/45, Pfs230, and Pfs25 are still being developed as TBV candidates and are discussed in detail below, while antibodies against Pfs28 were not found to be as as effective but did enhance the TBA of antibodies against Pfs25 (42).

Polyclonal antibodies targeting the male gamete protein HAP2 and a MAb targeting female-specific Pfs47, a paralog of Pfs48/45, have been shown to reduce transmission significantly (43, 44) and are included in the discussion. An *Anopheles* midgut antigen, AnAPN1, with the potential to block the transmission of malaria in a parasite strain- and species-transcending manner (45), has also been identified and is discussed below.

Sera from *Plasmodium*-infected individuals have also been shown to have TRA (30, 31, 41, 46–48), which has been associated with high antibody titers against gametocyte surface antigens Pfs230 and/or Pfs48/45 in some studies (46, 47, 49). A recent study of plasma from adults living in an area of malaria endemicity confirmed that naturally acquired antibodies targeting recombinant Pfs230 region CMB (amino acids [aa] 444 to 730) and Pfs48/45 region 10C (aa 159 to 428) had TRA. However, the study also demonstrated that the antibodies that remained after immunodepletion of anti-CMB and 10C-reactive antibodies had TRA and recognized the surfaces of intact gametes lacking both Pfs48/45 and Pfs230. Together, the data suggest the presence of gamete surface antigens in addition to Pfs230 and Pfs48/45 that could be new TB targets (50). To identify potential candidates, a protein microarray was used to compare the reactivity profiles of plasma from two samples, one with $\geq$90% TRA and the other with $<$10% TRA. Antibody reactivity was higher against Pfs230, Pfs48/45, and 43 other gametocyte proteins in the plasma with high TRA. This set of proteins provides candidates that need to be further evaluated for their ability to induce antibodies that block transmission in the mosquito midgut and are discussed in detail under "Current transmission-blocking vaccine candidates" below.

In contrast to antigens expressed in both gametocytes and gametes, naturally induced antibody responses to Pfs25 and Pfs28 (Table 1) have not been reported (51, 52), which is expected, since the antigen is expressed only after gametocyte egress from the erythrocyte within the mosquito. This lack of exposure during a natural malaria infection has raised concerns about the ability of a vaccine targeting an antigen expressed only in the mosquito midgut to be boosted by a natural malaria infection. To overcome this lack of natural stimulation, repeat immunizations may be necessary. Antibodies that target mosquito midgut antigens are similarly not primed in the human host during an infection and will need repeated immunizations. The attractiveness of this class of antibodies is the potential to disrupt the sporogonic life cycle of all malaria parasites (45).

An alternative TB strategy is to target parasite antigens exposed on the surfaces of giRBCs that are accessible to antibodies. In contrast to the finding of the robust immune responses against antigens on the surfaces of erythrocytes infected with asexual-stage parasites (iRBCs) (53), few studies have identified natural immune responses against antigens on the surfaces of giRBCs (54). One study involving Gambian children found that antibodies from gametocyte carriers recognized the surfaces of mature gametocytes produced from the *P. falciparum* parasite strain 3D7 (55). Although the targets of the gametocyte-recognizing antibodies were not determined, the study supported the existence of gametocyte-specific antigens on the surfaces of giRBCs. If present, parasite-produced antigens on the giRBC surface would be good targets for TBV candidates (53), since the clearance of gametocytes from the circulation would result in the interruption of malaria transmission.

Transmission-blocking immunity. The identification of MAbs that effectively block parasite transmission in a standard membrane-feeding assay (SMFA) clearly demonstrates the importance of antibodies in TBI, but the roles of other immune effector mechanisms are less well studied. There is no major histocompatibility complex class I (MHC-I) expression on the host RBCs, and therefore, gametocyte antigens cannot be presented directly to $CD8^+$ T cells, suggesting a minimal role for $CD8^+$ T-cell responses. However, in contrast to asexual parasites, unless mature intraerythrocytic stage V gametocytes are taken up by a mosquito, they die after circulating in the human host for 4 to 6 days and are cleared from the body. This provides antigen-presenting cells (APCs) the opportunity to take up gametocyte antigens and load them onto MHC-II

molecules for presentation to CD4$^+$ T cells. Once activated, CD4$^+$ T cells could enhance antibody production and memory B-cell formation. Effector CD4$^+$ T cells are also important sources of cytokines that activate macrophages and other immune effector cells. Although the role of CD4$^+$ T cells in the induction of antibodies against asexual-stage *P. falciparum* parasites has been studied extensively (56), their role in inducing gametocyte-specific antibodies is not well established (57–59). In general, antibody responses against gametocyte antigens tend to be lower than anti-asexual-antigen responses, but the intensity and breadth of the response increases over the season, suggesting boosting during reexposure (60). The reduced response to gametocytes could be due to the low number of gametocytes produced during an infection, as well as to decreased CD4$^+$ T-cell help. There is some evidence that titers of antibodies against some gametocyte antigens decline quickly (60, 61), but this decline is antigen and epitope specific, suggesting that it is not due to a general suppression of CD4$^+$ T-cell help. The marked epitope specificity indicates that the interplay between antigens, B cells, APCs, and T cells needs to be tested for each candidate vaccine in order to determine the optimal immunization strategy.

The cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) are secreted from a number of cell types (including NK cells, $\gamma\delta$ T cells, and macrophages) as well as *Plasmodium*-activated CD4$^+$ T cells and have been identified as factors that inactivate gametocytes in symptomatic malaria infections (62). However, whether cytokine levels are altered by giRBC exposure remains an open question. Complement-mediated lysis of giRBCs has also been suggested (63), as well as nonopsonic phagocytosis of young (stage I and II) giRBCs (64). Immune components of the host's blood, including antibodies, white blood cells, cytokines, reactive nitrogen species, and complement proteins, among others, are available to target all the various sporogonic parasite forms, as they remain active in the mosquito midgut hours after ingestion (65). The human complement system has been implicated in reducing transmission in the mosquito midgut, since the TR activity of antibodies against some antigens, such as Pfs230, requires complement (66, 67).

Current transmission-blocking vaccine candidates. Initially, the four *P. falciparum* sexual-stage antigens identified as targets of transmission-blocking monoclonal antibodies were advanced as transmission-blocking malaria vaccine candidates: two (Pfs230 and Pfs48/45) are antigens whose expression begins in intracellular gametocytes within the human host, and the other two (Pfs25 and Pfs28) are antigens whose expression begins in extracellular gametocytes within the mosquito. Since then, three additional candidates have been discovered: two expressed by the parasite, HAP2 (32) and Pfs47 (68), and one mosquito midgut protein, AnAPN1 (69) (Fig. 2), which is discussed below. Homologs of all these antigens are also expressed by *P. vivax*, the other major cause of human malaria, and to date, all but HAP2 have also been reported to be targets of TRA antibodies (70, 71).

GAMETOCYTE/GAMETE ANTIGENS Pfs230, Pfs48/45, Pfs47, AND HAP2

Pfs230, Pfs48/45, and Pfs47 belong to the 6-Cys s48/45 family of proteins (72). Some essential features of this family of proteins include their surface localization on exposed, extracelluar gametes (Table 1) and the presence of at least one s48/45 domain comprising 6 positionally conserved cysteine residues (73, 74). It was initially thought that the family comprised 10 members (72); however, 5 additional members have been identified (74). Although the functions of only a few family members are known (74), five members—Pfs230 and Pfs48/45, their respective paralogs Pf230p and Pfs47, and the putative secreted ookinete protein 12 (PSOP12)—are expressed on gametocytes, and their expression persists through the gamete to the zygote stage of the sexual life cycle of *P. falciparum* (36, 73, 74). Until recently, only Pfs230 and Pfs48/45 had been shown to be TB targets and had been very well characterized (37, 75–82). The monoclonal antibodies initially available against Pfs47 did not exhibit TB activity (83), and therefore, Pfs47 had not been the focus of much previous analysis. However, the transmission-blocking potential of Pfs47 has been demonstrated recently (43).

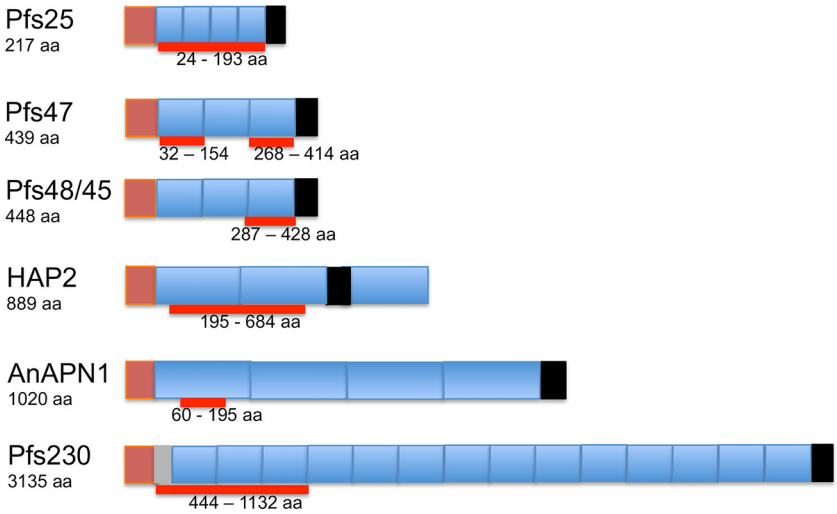

FIG 2 Schematic of TBV gene structures. Brown segments represent the signal peptide motif; the gray segment represents the prodomain; blue segments represent the main structural domains; black segments represent the GPI motif or, for HAP2, the transmembrane domain. Thick red lines represent the regions containing the active epitopes.

Pfs230. Pfs230 is a 3,135-amino-acid protein encoded by a 9.4-kb gene and is predicted to be 363 kDa long (77). It is, however, truncated to 300 kDa as the gametes emerge from the red blood cells (80). Gene disruption studies showed that Pfs230 is retained on the surface of the parasite plasma membrane through interactions with Pfs48/45 and is critical for the formation of exflagellation centers in the male gametocyte (82). Pfs230 contains 14 s48/45 6-Cys domains (67), which is the maximum number that has been found among members of the 6-Cys s48/45 family (74). The numerous cysteine motif domains of the protein have made expression of full-length, correctly folded, soluble protein difficult, but fragment expression has been achieved (85–87). Through the expression of various maltose binding protein (MBP)-tagged fragments of Pfs230 in *Escherichia coli*, antibodies generated against a region designated "C" (aa 443 to 1132) were found to reduce transmissibility to mosquitoes by as much as 80% (88). Studies on various fragments of this region, expressed in a wheat germ cell-free expression system, also indicated that the N-terminal subdomain (aa 443 to 588) was sufficient to induce transmission-blocking activity (87). Higher concentrations of antibodies against this subdomain were found to correlate positively with the age of the host in a preliminary study and were not affected by deletion of one of the two "YGE" tripeptides (85). Optimization using the *Pichia pastoris* expression system identified recombinant Pfs230 aa 443 to 730 (Pfs230D1M) as a strong transmission-blocking vaccine candidate, and two formulations (Pfs230D1M conjugated to exoprotein A [Pfs230D1M-EPA]), one with aluminum hydroxide (Alhydrogel) (ClinicalTrials registration no. NCT02334462) and one with AS01 (ClinicalTrials registration no. NCT02942277), are currently in phase I clinical trials (67).

Pfs48/45. Pfs48/45 is a 448-aa protein that is encoded by a 1.3-kb gene and is predicted to be 51 kDa long. It is a glycosylphosphatidylinositol (GPI)-anchored protein that interacts with Pfs230 and anchors it to the plasma membrane of the parasite (76). Pfs48/45 has been identified as a vital antigen required for male fertility (75). It contains three s48/45 domains and has three main epitopes. Antibodies against epitope I have the highest level of TB activity and are independently able to reduce gametocyte infectivity to mosquitoes in a complement-independent manner (89). Antibodies against epitopes II and III exhibit negligible TB activity independently (89). Epitope I has few polymorphisms, and antibody titers against epitope I have been found to be higher in adults than in younger people (85, 90), making it a suitable region for the production of a transmission-blocking vaccine candidate. A major challenge to the production of

correctly folded, functional fragments of Pfs48/45 has been the generation of a construct with precise pairing of cysteine residues during disulfide bond formation, due to the presence of numerous cysteine residues. Initial success was achieved by using *E. coli* to produce the full-length protein without the secretory signal sequence (aa 27 to 427) (81), as well as constructs comprising aa 28 to 428 (16C) or aa 159 to 428 (10C) as chimeras, both individually fused to MBP and referred to as M-Pfs16C and M-Pfs10C, respectively (91). However, neither of these constructs could be scaled up using good manufacturing practices (GMP), due to incorrect protein folding (92). Subsequently, the 10C fragment (aa 159 to 428), which contains epitopes I and II, was fused to the nonrepititive region (R0) of *P. falciparum* glutamate-rich protein (GLURP; aa 26 to 500) and expressed in *Lactococcus lactis* with much better success at GMP than that for M-Pfs10C, although the yield of correctly folded protein was low, and extensive *in vitro* refolding and purification was required to increase it (92). In order to overcome the yield-associated challenges, the 10C fragment was further reduced to a 6C fragment (aa 287 to 428) that contained only epitope I, still fused to R0 (93). In order to obtain a purified 6C fragment, without the R0 fusion partner, a tobacco etch virus (TEV) protease site was inserted between R0 and the 6C fragment to obtain pure, correctly folded Pfs48/45 6C protein (85). Correctly folded full-length Pfs48/45 has recently been produced with high yields in insect cells (*Drosophila* S2 cells) and has been found to be the target for highly potent transmission-blocking antibodies (94). No construct has reached phase I clinical trials yet, although a number are in preclinical development (68).

Pfs47. Pfs47 is a 439-amino-acid protein encoded by a 1.32-kb gene and predicted to be 50.8 kDa long. It is a GPI-anchored protein that is expressed only by female gametocytes and is retained on the surfaces of female gametes (83) through fertilization and development into ookinetes (95). Earlier studies of this antigen suggested a minimal role and functional redundancy, since gene disruption and monoclonal antibodies against this antigen did not lead to reduced mosquito infection, undermining its potential as a vaccine candidate (83). Subsequent genetic linkage and functional genomic studies, however, identified this antigen as the crucial antigen that protects the parasite from the mosquito's hemolymph complement-like immune system (96). The antigen downregulates JNK signaling in mosquito midgut cells, which targets the invading ookinete for subsequent complement-like removal (97, 98). The gene encoding this antigen has been found to be highly polymorphic (with 42 haplotypes) (84), especially in the region encoding the second of the three 6-Cys domains, which shows high geographic diversity. It has been reported that Pfs47 mediates immune evasion in different mosquito species in a haplotype-dependent manner (99). A recent study demonstrated that the full-length protein expressed in *E. coli* as a chimera with thioredoxin protein was immunogenic but did not induce TB immunity. Monoclonal antibodies generated against the full-length protein did not have transmission-blocking activity and were found to bind s48/45 domain 1 or 3. Domain 2 was not tested initially, because it could not be generated in *E. coli* due to toxicity. This problem was overcome by expressing a section of domain 2 (aa 178 to 267) in which the two cysteines were replaced with alanines (mD2). The original MAbs generated against full-length protein did not recognize the recombinant, so mD2 was used to generate additional MAbs, some of which were found to have significant TRA, inhibiting fertilization and subsequent ookinete formation (43). Further analysis of domain 2 identified a smaller, relatively conserved 52-aa region (aa 178 to 229) that elicited TRA antibodies in mice, and this is currently a prospective transmission-blocking vaccine candidate. This antigen is undergoing preclinical development (100).

HAP2. *P. falciparum* HAP2 (PfHAP2) is an 889-amino-acid protein encoded by a 1-intron, 2,816-bp gene. PfHAP2 belongs to the HAP2 family of proteins, which are class II viral fusion proteins with a cysteine-rich extracellular region (101). PfHAP2 is expressed only in male gametocytes and activated male gametes and therefore is referred to as the male gamete protein (44). The HAP2 gene is an essential gene, which enables

the male microgamete to fuse with and fertilize the female macrogamete (25). The conserved "fusion/cd loop" region (aa 174 to 205 in *Plasmodium berghei* and aa 178 to 207 in *P. falciparum*) has been found to be essential for HAP2 function (102). Subsequently, a study showed that antibodies against aa 355 to 609 of *P. berghei* HAP2 inhibited oocyst formation in the mosquito *in vivo*, although this inhibition was reversed at low antibody dilutions (44). Another study produced a fragment of PfHAP2 (aa 195 to 684) representing the ortholog of *P. berghei* aa 355 to 604 in the wheat germ cell-free expression system (101). Purified IgG from sera obtained from mice immunized with PfHAP2 strongly inhibited (by 97%) oocyst formation in *Anopheles* mosquitoes in the presence of complement (32). Additional studies have shown that antibodies targeting the "cd loop" region (aa 178 to 195 in *P. falciparum* and aa 174 to 191 in *P. berghei*) possess transmission-blocking activity (101). No construct has reached phase I clinical trials yet.

Pfs25. Pfs25 is a 217-aa protein encoded by a 0.65-kb gene and predicted to be a 24-kDa, GPI-anchored protein that belongs to a 13-member P25 family of proteins (103). This family of proteins is similar to the s48/45 family by virtue of having a relatively complex tertiary structure containing a large number of disulfide bridges (103). The protein is not expressed on the giRBC surface but rather is expressed after egress of the sexual-stage parasite from the giRBC (104). Vaccina virus was the first recombinant expression system found to generate correctly folded recombinant Pfs25 that bound to specific TB monoclonal antibodies (105, 106). Since then, expression in a variety of recombinant systems, including yeast (105, 107), plants (108), and algae (109), have been successful (Table 2). Monoclonal antibodies raised against correctly folded recombinant Pfs25 antigens, such as the highly effective 4B7, have been found to achieve potent TBA at high concentrations (105, 110), and MAb 4B7 is used as a reference for mosquito-feeding assays (40, 111, 112). Pfs25 is among the most advanced antigens in terms of TBV development (100); however, the lack of natural boosting within the human host continues to be a concern. A number of yeast- and plant-expressed Pfs25 products are in phase I clinical development; these include Pfs25-EPA formulated in Alhydrogel (ClinicalTrials registration no. NCT01434381, NCT01867463, and NCT02334462) or AS01 (ClinicalTrials registration no. NCT02942277) and chimeric Pfs25 fused in frame to the alfalfa mosaic virus coat protein and produced as a virus-like particle (VLP) formulated in Alhydrogel (ClinicalTrials registration no. NCT02013687). The initial trials using Alhydrogel formulations of Pfs-EPA or Pfs25-VLP showed that they were safe but did not stimulate robust TBA titers, and these results have led to the testing of alternative adjuvants, including AS01 (113–115).

AnAPN1. The most advanced mosquito-based malaria transmission-blocking vacine candidate is the *Anopheles* alanyl aminopeptidase N (AnAPN1) (116), a member of the M1 family of metallopeptidases (69). AnAP1 is a 1,020-aa residue GPI-anchored protein predicted to be 113.5 kDa long. The gene is composed of four domains, designated domains I to IV, the N-terminal domain I (aa 57 to 270), domain II (aa 271 to 523), domain III (aa 524 to 613), and the C-terminal domain IV (aa 614 to 942). AnAPN1 is found on the apical surfaces of both sugar-fed and blood-fed *Anopheles gambiae* midguts and is a ligand for both murine and human *Plasmodium* ookinetes (69). Antibodies against a 135-aa fragment of the N-terminal domain I (aa 60 to 195) are capable of inhibiting the completion of the sporogonic life cycles of both *P. falciparum* and *P. berghei* in the mosquito (69, 117). Recent epitope mapping of AnAPN1 has identified aa 98 to 123 and aa 173 to 194 as targets of antibodies that block the binding of AnAPNI to the ookinete micronemal pore-forming protein (116). AnAPN1 has been expressed in a variety of expression systems, including *E. coli*, viral vectors (recombinant chimpanzee adenovirus 63 [ChAd63] and modified vaccina virus Ankara [MVA]) (118), and HEK293 human embryonic kidney cells, as either full-length protein or fragments including all or part of the N-terminal domain I. Antibodies against full-length AnAPN1 expressed in viral vectors did not exhibit TBA (118), while those expressed in bacteria exhibited 66% to 68% inhibition of *P. falciparum* oocyte development (69). Antibodies

TABLE 2 Heterologous expression of some important sexual-stage antigens

Antigen	Fragment/domain	Expression system(s)[a]	TBA (%)[b]	Hu. comp.[c]	Reference(s)
Pfs48/45	Full length (aa 1–448)	Sf9 insect cells	ND	NA	170
	aa 27–427	*Drosophila melanogaster*	Yes (>96)	Yes	94
	M-Pfs10C (aa 159–428)	*E. coli*	Yes (>90)	Yes	91
	aa 118–218	*E. coli*	ND	NA	170
	Full length (M-Pfs16C)	*E. coli*	ND	NA	91
	CH-rPfs48/45 (full length)	*E. coli*	Yes (>97)	Yes	171
	Epitope I (aa 287–428)	*L. lactis*	ND	NA	85
	Epitope I (aa 291–428)	*L. lactis*	Yes (>90)	No	93
	Epitope I+II (aa 159–428)	*L. lactis*	Yes (>90)	Yes	92
	C-terminal (domain III)	*Chlamydomonas reinhardtii*	ND	NA	172
	16C (full length)	DNA based	Yes (>90)	Yes	173
	Pfs48F1 (aa 28–401)	*Nicotiana benthamiana*	ND	NA	174
	Full length	*N. benthamiana*	ND	NA	175
	Pfs48/45+NGln (aa 28–427)	MVA, ChAd63	Yes (45.5)	Yes	118
	Pfs48/45-NGln (aa 28–427)	MVA, ChAd63	No	Yes	118
Pfs230	Prodomain (aa 443–588)	*L. lactis*	ND	NA	85
	Prodomain, C0 (aa 443–588)	WGCF system	Yes (≤82)[d]	Yes	87
	Region C (aa 443–1132)	WGCF system	Yes (≤99)[d]	Yes	87
	Pfs230C1 (aa 443–715)	WGCF system	Yes (≤91)[d]	Yes	87
	Pfs230C2 (aa 443–915)	WGCF system	Yes (≤79)[d]	Yes	87
	Region C (aa 443–1132)	*E. coli*	Yes (≤76)	Yes	67
	Pfs230D1–2 (aa 443–915)	*E. coli*	Yes (≤65)	Yes	176
	Region C (aa 443–1132)	DNA based	Yes (94.4)	Yes	177
	D1M (aa 542–736)	*P. pastoris*	Yes (≤100)	Yes	176
	Pfs230D1H (aa 443–736)	*P. pastoris*	Yes (≤100)	Yes	176
	230CMB (aa 443–730)	Plant	Yes (≤100)	Yes	86
	Region C (aa 443–1132)	HEK293 cells	Yes (100)	Yes	118
	Pfs230C1 (aa 443–731)	Baculovirus	Yes (99.5)	Yes	178
Pfs25	Pfs25B (aa 22–190)	Yeast	Yes (≤100)	No	105
	aa 22–193	*P. pastoris*	Yes (≤100)	No	179
	TBV25H (aa 22–193)	*Saccharomyces cerevisiae*	Yes (≤100)	No	180
	aa 18–202	DNA based	Yes (≤99)	Yes	181
	Full length	DNA based	Yes (≤99)	Yes	173
	aa 18–202	WGCF system	Yes (≤100)	No	182
	aa 1–217	Cell-free system	Yes (≤100)	No	183
	aa 24–193	Cell-free system	Yes (≤32)		183
	Pfs25-FhCMB (aa 23–193)	Plant	Yes (≤99)	No	49
	a-Pfs25 (aa 22–193)	Algae	Yes (≤100)	No	184
	CHrPfs25 (full length)	*E. coli*	Yes (≤100)	No	185
	Full length	*E. coli*	Yes (≤62)[e]	No	186
	Full length (aa 1–217)	Adenovirus	Yes (≤82.5)	No	187
	4B7 and 1D2 (aa 122–134)	Adenovirus	Yes (≤78.1)	No	187
	aa 22–192	MVA, ChAd63	Yes (≤99)	Yes	118
	aa 22–193	Baculovirus	Yes (≥98)	No	179
Pfs47	Full length (aa 28–414)	Insect cells	ND	No	43
	Full length (aa 28–414)	*E. coli*	No	No	43
	Pfs47-mD2-Del1 (aa 178–267)	*E. coli*	Yes (87)[f]	Yes/no	43
	Pfs47-mD2-Del2 (aa 178–229)	*E. coli*	Yes (97)[f]	Yes/no	43
	Pfs47-mD2-Del3 (aa 155–181)	*E. coli*	No	Yes/no	43
HAP2	aa 195–684	WGCF system	Yes (97)	NS	32, 112
AgAPN1	aa 60–195	*E. coli*	Yes (≤100)	Yes	45
	aa 60–195	*E. coli*	Yes (≤100)	Yes	117
	aa 60–195	Bacteria	Yes (≤87)	NS	69
	rAnAPN160-942	*D. melanogaster*	ND	NA	45
	AgAPN1 Nterm (aa 61–195)	MVA, ChAd63	Yes (48)[f]	Yes/no	118
	AgAPN1 DomI (aa 20–303)	MVA, ChAd63	No	Yes	118
	AgAPN1 (aa 20–998)	MVA, ChAd63	No	Yes	118

[a]WGCF, wheat germ cell free; MVA, modified vaccinia virus Ankara; ChAd63, chimpanzee adenovirus 63.
[b]ND, not determined.
[c]Hu. comp., human complement; NA, not applicable; NS, not stated.
[d]Reduced TBA in the absence of complement.
[e]At low IgG concentrations.
[f]Not affected by the presence or absence of complement.

against the entire N-terminal domain I expressed in viral vectors similarly lacked TBA; however, antibodies against the 135-aa fragment of domain I (aa 61 to 196) expressed in viral vectors exhibited 45% TBA only at low antibody concentrations (118). Other studies, however, have demonstrated TBA as high as 100% for antibodies raised against the same 135-aa fragment of N-terminal domain I expressed in *E. coli* (45, 117). This antigen has not yet advanced to phase I clinical development.

NOVEL GAMETOCYTE AND GAMETE SURFACE ANTIGENS

A recent study aimed to identify novel TBV candidates by probing a protein microarray containing 315 *P. falciparum* gametocyte-related proteins with human plasma from an area of malaria endemicity (50). The antibody responses of plasma samples with high (≥90%) and low (<10%) TRA were compared, and 43 proteins, in addition to Pfs230 and Pfs48/45, showed were significantly higher levels in plasma samples with high TRA. Thirteen of these 43 proteins were highlighted as possible TBV candidates based on enriched expression in gametocytes and the presence of at least one predicted transmembrane domain (TMD) or a secretory signal sequence. Two of the 13 antigens, PF3D7_1021100 and PF3D7_1324600, were specifically predicted by go_cell_comp annotation to be surface exposed, and another two, Pf11-1 (PF3D7_1038400) and gamete surface and sporozoite traversal protein/GEST (PF3D7_1449000), have previously been functionally associated with gamete emergence in the mosquito midgut (50). Except for Pf11-1, the TBA of antibodies specific for any of these antigens has not been reported. Antibodies did recognize Pf11-1 on the gamete surface during emergence but did not exhibit potent TB responses (119). Given the large size of Pf11-1 and the recent demonstration of the marked specificity of the TB epitope in Pfs47, it may be worthwhile to reassess specific protein domains in Pf11-1 as TB antibody targets. Additional work is needed to carefully characterize these 13 antigens in order to validate their potential as TBV candidates.

PARASITE-SPECIFIC INFECTED-ERYTHROCYTE SURFACE ANTIGENS

After successful RBC invasion, *P. falciparum* develops and resides in the parasitophorous vacuole (PV), which separates it from the RBC cytoplasm. As the parasite grows, it actively enlarges the PV membrane (PVM) and exports proteins across the PVM to modify both the RBC cytoplasm and the plasma membrane (13, 120–122). A number of these exported, parasite-produced proteins are exposed on the RBC surface during asexual intracellular development. Many of these surface-exposed parasite proteins belong to large multigene families, notably the surface-associated interspersed protein (SURFIN) (123), the subtelomeric variable open reading frame (STEVOR) (124), Maurer's cleft two-transmembrane domain (PfMC-2TM) (124), type A and B repetitive interspersed family (RIFIN) (125, 126), and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is encoded by *var* genes (127, 128). These multigene families are known to be highly polymorphic and thus, as a group, are simply called variant suface antigens (VSA). Erythrocyte surface exposure allows these genes to mediate the interaction of iRBCs with other cells, including uninfected RBCs and endothelial cells, and to serve as targets for immune responses. Antibodies against specific PfEMP1 family members are thought to be associated with allele-specific protection against malaria. However, the expression of only 1 of the 60 distinct PfEMP1 proteins encoded in the genome at a time, coupled with the extreme polymorphism in the population, allows immune evasion through the continual emergnce of parasites expressing distinct PfEMP1 proteins (129, 130). PfEMP1 also mediates endothelial cell adhesion, allowing iRBCs to be sequestered and thus avoid circulation (53) and clearance in the spleen. The roles of the other iRBC surface protein families are less well defined, especially for the SURFIN family, and can differ between family members. RIFIN and STEVOR family members have been identified that bind glycoproteins on the uninfected RBC surface and play roles in merozoite invasion and adhesion and iRBC sequestration (124, 131–134). Some members of the SURFIN family of proteins have also been suggested

TABLE 3 Selected potential RBC surface proteins with transcripts in gametocytes

Antigen	PlasmoDB ID	Expression profile[a]		TMD
		Gametocyte	Asexual	
SURFIN				
SURFIN 1.1	Pf3D7_0113100	II/V	All (low)	Yes
SURFIN 1.3	Pf3D7_0113600	II/V	All (low)	No
SURFIN 8.2	Pf3D7_0830800	V	All (low)	Yes
SURFIN 13.1	Pf3D7_1301800	V	All (low)	Yes
STEVOR				
STEVOR	Pf3D7_0617600	II	R, LT	Yes
STEVOR	Pf3D7_1040200	II	R, ET, LT, Sc	Yes
RIFIN				
Type B	PF3D7_0425700	II	R	Yes
Type A	PF3D7_0114700	II (low)	R	Yes
Type B	PF3D7_0115200	II (low)	R	Yes
Type B	Pf3D7_0222700	V/II	R	Yes
Type ND[b]	PF3D7_0632300	II/V	R	Yes
Type B	PF3D7_0900300	II (low)/V (low)	R/Sc	Yes
Type B	PF3D7_0900500	V	All (low)	Yes
Type A	PF3D7_0901000	II (low)/V (low)	R/ET	Yes
Type B	PF3D7_1300600	V/II	LT (low)	Yes
Type A	PF3D7_0115600	II/V	R	Yes
Type A	PF3D7_0324800	II/V	R, ET, LT	Yes
Other				
Conserved protein	PF3D7_1021100	V	All	Yes
Export protein (EP)	PF3D7_1149100	II/V	ET	No
EP (PHISTc)	PF3D7_0936800	II/V	R	Yes
FIKK4.1	PF3D7_0424500	II/V	R	Yes
MESA	PF3D7_0500800	II	ET/LT	No

[a]Roman numerals refer to stages: II, early gametocytes; V, mature gametocytes. All, all asexual-parasite stages; R, ring-stage asexual parasites; ET, early trophozoites; LT, late trophozoites; Sc, schizonts; low, low expression levels. The expression data are from Lopez-Barragan et al. (14) as published in PlasmoDB (142).
[b]ND, classification of *rif* gene not specified (14, 142).

to be involved in merozoite invasion (123); however, more studies are needed to confirm this.

None of these protein families have been well characterized in giRBCs, but if exposed on the surface, they could be targets for transmission-blocking immunity, as seen with anti-PfEMP antibodies against asexual parasites. Initally, the sequestration of immature gametocytes until they mature to stage V was thought to be due to the presence of adhesins on the surfaces of immature giRBCs that are lost/degraded upon maturity (135). The inability to identify PfEMP1 family members on immature gametocytes (13), as well as the negligible binding of giRBCs to a variety of bone marrow-derived endothelial cells (135–138) and C32 melanoma cells (139), has given rise to an alternative theory, that sequestration is not dependent on adhesins. This alternative theory suggests that the rigidity of immature gametocytes keeps them embedded within the bone marrow until they mature to stage V, regain elasticity, and return to the circulation (135, 140). However, transcripts for members of the SURFIN, STEVOR, and RIFIN families have been found in gametocytes, and both indirect and live immunofluorescence assays (IFA) indicate that the proteins are expressed on the gametocyte surface (141) (Table 3). Recent proteomic data have identified a number of proteins encoded by conserved single-copy-number genes exposed on the asexual iRBC surface that may function as blood-stage malaria vaccines (127). To identify additional gametocyte-specific giRBC proteins, a similar, careful protemic analysis of giRBCs is needed.

POTENTIAL giRBC SURFACE ANTIGENS

***stevor*/STEVOR.** There are 226 members of the subtelomeric variable open reading frame (STEVOR) protein family (142). They range from about 27-kDa to 35-kDa proteins

and have been strongly predicted as possible gametocyte surface antigens (141, 143). STEVOR proteins have been identified on the surfaces of late-trophozoite- and schizont-infected erythrocytes (53), and some studies have also identified STEVOR proteins on the membranes of stage III to V giRBCs (141); however, limitations such as the availability of variant- and domain-specific reagents to ascertain the actual localization of the proteins weaken the report. *Plasmodium falciparum* is able to coexpress multiple *stevor* genes in a single parasite (53). Although the surface exposure of STEVOR proteins on giRBCs might suggest that STEVOR proteins play a role in gametocyte sequestration, the absence of STEVOR proteins on sequestrable early-stage (stage I and II) gametocytes (141) suggests that gametocyte sequestration is unlikely STEVOR dependent.

***rif*/RIFIN.** The repetitive interspersed (RIFIN) proteins belong to a family of 222 variable genes (142) that code for proteins ranging between 30 and 45 kDa (125, 144). These proteins, like the STEVOR proteins, have been identified on the surfaces of parasite-infected erythrocytes, merozoites, sporozoites, and gametocyes (15, 126, 145). RIFIN proteins have been suggested to be trafficked through the Maurer's cleft (MC) (126, 146) to the surface of the parasitized RBC (pRBC) (125, 144). All RIFIN proteins were initially thought to possess two putative TMDs (147). However, it has recently been confirmed that although some variants possess two TMDs, others have one (148). The RIFIN proteins can be classified, based on sequence diversity, architecture, and cellular localization, into two subgroups, RIFIN-A (RIF_A) and RIFIN-B (RIF_B) (126, 148), which are most often coexpressed in a single parasite (149).

***surf*/SURFIN.** The surface-associated interspersed gene (*surf*) family encodes 13 high-molecular-weight proteins (about 280 to 300 kDa) known as SURFIN proteins (PlasmoDB). SURFIN proteins have structural similarities with other exported and surface-exposed *Plasmodium* proteins, such as an N-terminal signal sequence similar to that of *P. vivax* VIR (PvVIR) and tryptophan-rich domains (WRD) similar to those of PfEMP1, both of which are surface-exposed proteins (123). Indirect IFA on iRBCs were used to identify the localization of some surfins. SURFIN 4.2 was found to colocalize with PfEMP1 on the iRBC membrane (123), and SURFIN 4.1 was found within the PV (150) and the iRBC cytosol (151). In contrast to the observations for *rifin* and *stevor* genes, coexpression of multiple *surf* variants in a single isolate has not yet been reported, although several copy number variants have been identified (150). Just as with *stevor* and *rifin* genes, *surf* genes are generally differentially transcribed in different stages of the intraerythrocytic parasite (14). A few family members, such as *surf* 1.3, *surf* 4.2, and *surf* 8.3, are detected in all the various asexual intraerythrocytic stages of the parasite (150), as opposed to *surf* 8.2, which is preferentially expressed in mature stage V gametocytes, and *surf* 4.1, which is preferentially expressed in schizonts (14). As opposed to *rifin* and *stevor* genes, where all family members have *Plasmodium* export element (PEXEL) motifs, some surfins, such as *surf* 8.2, are PEXEL-negative exported proteins (PNEPs). Although *surf* 4.2 has two PEXEL motifs, translocation to the iRBC surface was found to be independent of either motifs (152). The high levels of *surf* 8.2 transcripts in gametocytes (14) and the presence of a TMD and a PNEP sequence suggest that SURFIN 8.2 is exposed on the surfaces of gametocyte-infected RBCs; however, this assumption has yet to be validated.

Insights/perspectives. Additional effective transmission-blocking interventions are needed to complement current global malaria control and elimination efforts. A key process in the life cycle of *P. falciparum* parasites that enhances both disease and transmission is sequestration. Sequestration, which is thought to decrease recognition and clearance of the parasite by the spleen, has been extensively studied in the asexual parasite (127, 128, 153–157). Sequestration in the sexual-stage parasite has not gained as much attention (136), although it is assumed to be responsible for gametocyte development to maturity within the host without splenic clearance, which increases the likelihood of malaria transmission. A humanized mouse model with a human bone marrow transplant has recently been made available for the study of gametocyte sequestration (158) and should enable us to better understand its mechanisms. Al-

though there is some indirect evidence of gametocyte-specific giRBC surface antigens (55, 159, 160), no specific antigens have been identified. Enhancing immune recognition of giRBCs by using vaccine-induced antibodies to target a gametocyte-specific giRBC surface antigen could reduce the prevalence of mature and infective gametocytes, leading to a reduction in malaria transmission. A few studies have identified antibody responses that are specific to gametocyte carriage (60, 161); however, the localization of these antigens on the giRBC or gamete is not currently known. Parasite diversity is a major challenge to malaria vaccine design (162). It has been observed that the licensed pre-erythrocytic-stage circumsporozoite protein (CSP) vaccine RTS,S is more effective against an infection with parasites whose genetic backbones are similar to that of the 3D7 vaccine strain than against genetically diverse parasites (163). This finding suggests that TBVs based on a single genetic backbone will likely be more effective against vaccine-like parasites. However, diversity in sexual-stage parasites is limited relative to that of asexual- and pre-erythocytic-stage antigens (164, 165), so TBVs based on sexual-stage parasites likely will be effective against more strains.

More efforts are needed to identify and characterize giRBCs as well as to continue to explore the gamete surface and mosquito midgut in order to aid in the development of effective TB interventions. It is also important to extend the analysis of candidates identified in *Plasmodium falciparum* to *P. vivax*, the most prevalent form of the malaria parasite circulating in Asia (166–168).

Conclusion. Antigens on the surfaces of gametocytes and antigens exported by gametocytes to the surfaces of giRBCs have the potential to reduce and technically prevent malaria transmission. Apart from the well-described antigens Pfs25, Pfs230, and Pfs48/45, and the more recent demonstrations that HAP2, AnAPN1, and a region of Pfs47 may elicit TB antibodies, the presence of other gametocyte antigens that elicit TBA has yet to be discovered and/or validated. Identification of additional essential TB antigens could be important for the design and construction of effective TB vaccines.

ACKNOWLEDGMENTS

F.K.A. and J.A. are recipients of WACCBIP postgraduate fellowships, and K.C.W. and L.E.A. are supported by NIAID grant AI069314.

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