Immune Responses in Malaria

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Evidence accumulated through the years clearly indicates that antiparasite immune responses can efficiently control malaria parasite infection at all development stages, and under certain circumstances they can prevent parasite infection. Translating these findings into vaccines or immunotherapeutic interventions has been difficult in part because of the extraordinary biological complexity of this parasite, which has several developmental stages expressing unique sets of stage-specific genes and multiple antigens, most of which are antigenically diverse. Nevertheless, in the last 30 years major advances have resulted in characterization of a number of vaccine candidates, exploration of the repertoire of host immune responses to the various parasite stages, and also identification of significant hurdles that need to be overcome. Most important, these advances strengthened the concept that the induction of host immune responses that target all developmental stages of *Plasmodium* can efficiently control or abrogate *Plasmodium* infections and strongly support the notion that an effective vaccine can be developed. This vaccine would be a critical component for programs aimed at controlling or eradicating malaria.

In this review, we address immune responses to the various stages of parasite development—preerythrocytic, asexual stages in red cells, and sexual and mosquito stages. Our expanding understanding of these responses and their targets provides a foundation for the development of vaccines directed at the three major developmental stages of malaria parasites.

IMMUNE RESPONSES TO PREERYTHROCYTIC (PE) ANTIGENS

Antibody Responses to PE Antigens

Early studies in rodent malaria showed that immunization with attenuated *Plasmodium ber-*

ghei sporozoites induced antibodies that recognized the sporozoite surface and neutralized their infectivity (Nussenzweig et al. 1967). Subsequent studies in which humans were immunized with attenuated *Plasmodium falciparum* sporozoites confirmed the protective efficacy of the sporozoite-induced immune responses (Rieckmann et al. 1979). Humans naturally exposed to parasite infection in endemic areas also develop antiparasite responses, as indicated by studies in malaria-endemic areas of Africa and Asia, which reported that antiparasite antibodies are most frequently detected in individuals older than 50 years and in only a minority of children (Nardin et al. 1979; Tapchaisri

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et al. 1983; Druilhe et al. 1986). In vitro assays with sera from endemic areas showed that sporozoite-reactive sera inhibit sporozoite invasion of hepatocytes in vitro (Hollingdale et al. 1984; Hoffman et al. 1986; Mellouk et al. 1986; Hollingdale et al. 1989).

The antibodies that bind to sporozoites recognize different antigens. Among these, the circumsporozoite protein (CSP) was the first antigen identified in rodent and human malaria sporozoites (Nussenzweig and Nussenzweig 1989). A CSP-based vaccine (RTS,S) has undergone a phase III vaccine trial, and is discussed in detail in Healer et al. (2016). Anti-CSP antibodies bind the entire surface of sporozoites and induce the shedding of the CSP (Stewart et al. 1986). Most of them recognize the repeat domain of this protein, which is conserved in all strains of *P. falciparum* (Zavala et al. 1985a). Most importantly, they inhibit sporozoite infectivity in vivo and in vitro (Zavala et al. 1985b; Persson et al. 2002). Longitudinal studies, focused on antibodies specific for the CSP repeats showed an age-dependent distribution of antibodies as had been observed using an immunofluorescence antibody (IFA) assay (Zavala et al. 1985b; Del Giudice et al. 1987, 1990).

Studies in endemic areas revealed that the presence of anti-CSP antibodies correlated with transmission exposure and increased with age (Campbell et al. 1987; Esposito et al. 1988; Marsh et al. 1988). Studies on the carboxy- and amino-terminal regions flanking the repeat domain indicate that they contain important functional domains that enable sporozoite infectivity (Coppi et al. 2011). Sera from endemic areas contain antibodies against nonrepeat regions of the CSP, and the presence of *P. falciparum* amino-terminal-specific antibodies has been associated with the development of clinical immunity (Bongfen et al. 2009). Recently, it was shown that antibodies against this amino-terminal region strongly inhibit sporozoite infectivity in vivo (Espinosa et al. 2015).

Thrombospondin-related adhesive protein (TRAP) (Robson et al. 1988) is a parasite antigen also considered as a vaccine candidate. This is a transmembrane protein containing adhesive domains that enable the motility of sporozoites

in mosquitoes and vertebrate hosts, mediating their migration from skin to the liver. Early studies showed that sera from individuals immunized with *P. falciparum* sporozoites had antibodies against TRAP and these antibodies inhibited sporozoite infection of hepatocytes in vitro (Rogers et al. 1992). In Mali, the presence of antibodies against TRAP was associated with lower parasitemia, protection against infection (Scarselli et al. 1993; John et al. 2003), and protection against cerebral malaria (Dolo et al. 1999). Antibodies against TRAP are short lived in children, waning significantly during the dry season (John et al. 2003), as also observed with anti-CSP antibodies (Marsh et al. 1988).

LSA1 is a 197-Kd molecule consisting of a large number of repeated sequences, expressed exclusively in *P. falciparum* during early liver stages and no ortholog exists in rodent parasites (Guerin-Marchand et al. 1987; Zhu and Hollingdale 1991). This antigen is widely recognized by sera from individuals living in endemic areas (Fidock et al. 1994; Kurtis et al. 2001). Studies using sera from children in Gabon reported an association between anti-LSA1 titers and partial resistance to infection (Domarle et al. 1999). Another antigen, CelTOS (cell-traversal protein for ookinetes and sporozoites) (Kariu et al. 2006) is a conserved protein present in two different motile stages and appears to elicit cross-species protection (Bergmann-Leitner et al. 2010). Limited epidemiologic information on CelTOS indicates that individuals living in endemic areas develop cellular and humoral responses against this antigen (Anum et al. 2015).

Studies in Kenya that evaluated antibody responses to CSP, LSA-1, and TRAP, indicated that antibody levels against these three antigens, instead of a single antigen, displayed a stronger correlation with lower incidence and reduced risk of clinical malaria, as well as with diminished severity of disease (John et al. 2005, 2008). Recent studies using high-throughput assays to screen hundreds of *Plasmodium* antigens revealed that, compared to blood-stage reactivity, there was an infrequent reactivity to preerythrocytic antigens (Doolan et al. 2008; Crompton et al. 2010). These studies associated pro-

tection with the breadth of antigens recognized instead of responses to single antigens.

Overall, studies with sera from individuals living in endemic areas and volunteers immunized with irradiated sporozoites indicate that the development of antibodies against sporozoite antigens is clearly age and dose dependent, and their magnitude is limited. There is a consensus that antibodies induced by natural exposure may in some areas correlate with partial protection but they do not confer sterile immunity.

T-Cell Responses to Preerythrocytic (PE) Antigens

The notion that T cells can be an effective anti-parasite immune mechanism derives from studies in rodent models showing that CD8⁺ and CD4⁺ T cells inhibit the development of malaria liver stages (Tsuji and Zavala 2003). Studies in endemic areas indicate that naturally exposed individuals are able to mount specific CD8⁺ T-cell responses to CSP and TRAP, but these responses are low in magnitude and detectable only in a minority of individuals (Doolan et al. 1993; Flanagan et al. 2003). Studies in some endemic areas failed to detect CD8⁺ T-cell responses against any of the overlapping peptides representing the entire CSP sequence, including most of the variant isolates (Doolan et al. 1991). A study in Kenya suggested a degree of protection against anemia among individuals that produced interferon γ (IFN- γ) in response to TRAP peptides (Ong'echa et al. 2003). Human vaccine trials using recombinant viruses as immunogens have shown the induction of CD8⁺ T-cell responses against these antigens and an association with partial protection (Ewer et al. 2013).

CD4⁺ T cells against CSP are also found in individuals living in endemic areas. Early studies showed that several epitopes are recognized in both the CSP of *Plasmodium vivax* and *P. falciparum*, and these responses appear to be more frequent against polymorphic epitopes (Good et al. 1988; Zevering et al. 1994). The presence of CSP-specific CD4⁺ T cells in individuals living in endemic areas did not show

correlation with clinical immunity (de Groot et al. 1989; Riley et al. 1990; Esposito et al. 1992). T-cell responses against other antigens such as HEP17 (Doolan et al. 1996) and STARP (Fidock et al. 1994) have also been identified; however, these antigens are expressed in liver and asexual stages and therefore the target of their potential protective effect remains to be defined.

The difficulties in detecting robust T-cell responses against PE antigens in individuals living in endemic areas contrasts with the relative ease at which these responses are detected in volunteers immunized with attenuated sporozoites (Seder et al. 2013) or subunit vaccines (Ewer et al. 2013). This indicates that natural exposure to parasite antigens may not be sufficient to induce robust T-cell responses. Taken together, naturally acquired antibody and T-cell responses against PE antigens are of low magnitude and infrequent, particularly in children. Although some studies indicate an association of naturally induced immune responses with clinical immunity, these responses may never confer sterile protection. This is likely because of low antigen inoculum received by individuals as mosquitoes inject only a few sporozoites (10–100) and this may not be sufficient to induce strong immune responses. Indeed, anti-PE responses induced in humans after immunization with attenuated sporozoites can confer a high degree of sterile protection, but this requires exposure to 1000 infective bites (Herrington et al. 1991) or the intravenous injection of several hundred thousand sporozoites (Seder et al. 2013). Decades of studies on the CSP protein laid the groundwork for the development and testing of the RTS,S vaccine, which is based on this protein using a platform of a hepatitis B particle (discussed in Healer et al. 2016). However, this vaccine is only partially effective and its longevity is limited. The development of a more efficient PE vaccine that could effectively contribute to eradication efforts will require a better understanding of the immune mechanisms operating in sporozoite-induced immunity, the identification of additional target antigens, and the design of a new generation of vaccines capable of inducing not only high

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antibody titers but also strong cell-mediated immune protection.

IMMUNE RESPONSES TO ASEXUAL ERYTHROCYTIC STAGES

The asexual stages of malaria parasites have been intensively investigated because they are most accessible to investigators in the blood of an infected person and because they are responsible for the pathology associated with this disease. It is known that those living in malaria-endemic areas progressively develop partial resistance to infection with *P. falciparum*, although this takes many years to attain (reviewed in Beeson et al. 2008; Langhorne et al. 2008; Crompton et al. 2014). The classic depiction of the natural history of immunity to malaria as a function of age was provided by long-term studies in Kenya (Marsh and Kinyanjui 2006). As illustrated in Marsh and Kinyanjui (2006), these studies revealed that young children are likely to have the highest parasitemias and also are more likely to develop severe malaria pathology (Milner 2016). As children age, they are still susceptible to infection accompanied by clinical symptoms but are less likely to develop severe disease. This pattern continues into adolescence and adulthood with even fewer clinical symptoms but still the periodic presence of parasites. Studies on the acquisition of immunity to *P. vivax* indicate that resistance may be acquired more rapidly than with *P. falciparum* (reviewed in Mueller 2013).

Perhaps the most important finding in our understanding of this naturally acquired immunity to *P. falciparum* malaria was published in 1961 (Cohen et al. 1961). That study established that pooled IgG antibodies from African adults living in a malaria-endemic area could dramatically drive down parasitemias when passively transferred to children with malaria. It was later confirmed and extended using IgGs from Africans transferred to Asians failing chemotherapy for malaria (Sabchareon et al. 1991). Its functionality, despite the geographic distance, provided evidence that the protective antibodies either recognized conserved determinants or a very broad range of allelic types. These experi-

ments have provided the experimental foundation for efforts to develop a malaria vaccine, historically with the main goal of protecting children against severe disease and death. Despite all this time we are not certain of the antigenic targets of those protective antibodies nor are we certain of the effector mechanisms involved in the reduction of parasite burden. Further, we do not know the contribution of T cells (beyond helper function) or other cells of the immune system to these responses. The large number of parasite-encoded proteins (approximately 5400 genes) and the allelic diversity of many of those proteins have complicated the identification of targets of protective immune responses as well as efforts to develop vaccines that elicit broadly reactive protective responses. Nevertheless, the fact that people living in endemic areas become at least partially immune to malaria provides evidence that a vaccine against this disease should be possible. Overall, progress has been slow and results of phase II clinical trials testing several blood-stage vaccine candidates have been disappointing (Healer et al. 2016). Thus, there is significant impetus for identification of new potential vaccine candidates and combinations of candidates from blood stages, expanded efforts to understand the important cells and effector mechanisms to be targeted, and exploration of immunization strategies that would elicit these responses.

Effector Mechanisms and Assays

Selection of malaria vaccine candidates and their rapid evaluation has been hampered by numerous problems, including difficulties encountered in expressing novel malaria proteins in recombinant protein expression systems in a correct configuration and the lack of assays and markers, which predict protection in vivo. Many investigators have shown that those infected with malaria parasites produce an array of antibodies against many different asexual-stage proteins and these have usually been examined one at a time. More recent studies have extended these results using microarrays with *Escherichia coli* produced proteins (Gray et al. 2007; Doolan et al. 2008; Crompton et al. 2010).

It has been difficult to correlate responses to individual parasite proteins with protection, although it has been proposed that cytophilic IgG1 and IgG3 isotypes are more correlated with protective responses (Bouharoun-Tayoun and Druilhe 1992).

Assessing these antibodies has ranged from direct enzyme-linked immunosorbent assay (ELISA) measurements to evaluation of functional activity using assays such as in vitro parasite growth inhibition, opsonization with antibodies and leukocytes, complement fixation, measuring antibodies to proteins on the erythrocyte surface by flow cytometry or agglutination tests, and antibody-dependent cellular cytotoxicity assays. However, it has been difficult to correlate any single assay with clinical protection, so the relative contribution of these antibody effector functions in vivo is not established.

Merozoite Proteins

Merozoite proteins have been the focus of most studies of immune responses to the asexual stages of infection and work in Cowman et al. (2012) discusses the architecture of a merozoite as well as its organelles and some of the proteins involved in invasion. One of the first merozoite proteins to be investigated was the merozoite surface protein 1 (MSP1), one of a number of MSP molecules, which provide a surface coating for merozoites and likely contribute to the invasion process. Monoclonal antibodies to the carboxyl terminus of this large protein were able to protect mice passively against lethal challenge with the rodent parasite *Plasmodium yoelii* (Majarian et al. 1984; Burns et al. 1989), and subsequent studies established that both mice and Aotus monkeys could be immunized against a lethal parasite challenge using either the *P. yoelii* or *P. falciparum* MSP1 carboxyl terminus (Daly and Long 1993; Stowers et al. 2001). Later efforts to move this candidate into human clinical trials in Africa proved disappointing (Ogutu et al. 2009). Humans living in malaria-endemic areas do develop antibodies to MSP1, and in some cases these responses have been associated with protection. Apical

membrane antigen 1 (AMA1) (Deans et al. 1982; reviewed in Remarque et al. 2008) is a micronemal protein, which is translocated to the merozoite surface during invasion and, in conjunction with RON2, contributes to the moving junction on the red cell during invasion (Lamarque et al. 2011); it is also present in sporozoites. Its critical central role during erythrocyte invasion has made it a target for both vaccines and drugs, and immunization-challenge studies with PfAMA1 in Aotus monkeys showed protective responses (Stowers et al. 2002). Further, antibodies to AMA1 from a variety of animal species strongly inhibit red cell invasion in vitro, and antibodies to it are commonly found in those living in endemic areas, including young children. Nevertheless, this has presented significant problems for investigators because of the allelic diversity found in different strains of *P. falciparum* (Thomas et al. 1990), and success in human clinical trials has been limited (Sagara et al. 2009; Thera et al. 2011). However, several groups have shown in preclinical studies that immunization with a mixture of four to five alleles elicits antibodies with greater cross-strain specificity (Dutta et al. 2013; Miura et al. 2013b; Terheggen et al. 2014), and immunization with AMA1 combined with RON2 may elicit more effective responses (Srinivasan et al. 2014).

Other merozoite proteins involved in erythrocyte binding have been examined as possible targets of protective host immune responses. Most can be categorized as erythrocyte binding-like (EBL) or reticulocyte binding-like (RBL or Rh) (reviewed in Tham et al. 2012). These are located in the apical organelles and some have been shown to be targets of invasion-inhibiting antibodies. However, the existence of multiple parasite-encoded ligands has allowed these organisms to use redundant invasion pathways and potentially escape host immune responses (reviewed by Wright and Rayner 2014).

One of these proteins, PfRh5 (reticulocyte-binding protein homolog), has been identified as a critical merozoite component because of its binding to basigin on the red cell membrane (Crosnier et al. 2011). PfRh5 also interacts with two other parasite molecules, PfRipr (Chen et al. 2011) and CyRPA (Reddy et al. 2015).

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Although people living in a malaria-endemic area generally develop low levels of antibody to PfRh5 (Douglas et al. 2011; Tran et al. 2014), animals immunized with this antigen can produce antibodies with significant growth inhibition activity (Douglas et al. 2014) and, in the case of nonhuman primates, show protective immune responses even against heterologous parasites (Douglas et al. 2015).

Parasite Proteins on the Infected Red Cell Surface

There are several multigene families encoding important proteins exported to the infected red cell membrane. Perhaps the most important are the complex VAR proteins encoded by the large, polymorphic PfEMP1 (erythrocyte membrane protein) gene family with approximately 60 members with different *var* repertoires in different parasite strains (reviewed in Chan et al. 2014; Hviid and Jensen 2015). These large proteins have multiple variant domains, which bind ligands such as CD36 and ICAM-1 on host tissues and contribute to sequestration of *P. falciparum* parasites on the vascular endothelium (see Smith 2014 for review of adhesion domains). This has resulted in some variants being associated with specific disease pathogenesis such as cerebral malaria (Milner 2016). PfEMP1 proteins are displayed on the surface of the infected red cell for many hours and would be excellent targets for host immune responses, but their complexity, antigenic heterogeneity, and ability to switch from one PfEMP1 protein to another are daunting for vaccinology. However, it is clear that those living in malaria-endemic areas develop antibodies to the surfaces of infected red cells and these responses have been implicated in naturally acquired immunity, perhaps by sequential acquisition of antibodies caused by infection with various parasite strains (Bull et al. 1998; Mackintosh et al. 2008; Chan et al. 2012).

The most defined member of this family is VAR2CSA, a member of the PfEMP1 family, which has been implicated in pregnancy-associated malaria because of its binding to chondroitin-sulfate A (CSA) found in the placenta (Fried and Duffy 2016). Multiparous women

develop antibodies to VAR2CSA and these antibodies appear to contribute to their resistance to placental malaria after the first pregnancy (reviewed in Ataide et al. 2014). Localization of important target epitopes within this large molecule, producing correct conformation of specific domains, and some allelic variation have all complicated studies with this molecule.

Two other polymorphic gene families are the *rifins* (repetitive interspersed family) and the *stevors* (subtelomeric variant open reading frame), which encode the RIFINS and STEVOR protein families. Much less is known about these proteins and immune responses to them, although recent evidence has indicated that both the RIFINS (Goel et al. 2015) and the STEVORS (Niang et al. 2014) can promote rosetting of red blood cells; this is an aggregation of infected and uninfected red cells, which in some studies has been suggested to contribute to malaria pathogenesis (Carlson et al. 1990).

Protective Immune Responses

Given the wide array of humoral immune responses to various antigens associated with asexual stages, a meta-analysis of the literature was conducted to try to associate antibodies to specific merozoite proteins with incidence of *P. falciparum* malaria (Fowkes et al. 2010). Some limited associations were noted with proteins such as MSP3 and MSP1–19, but it is unclear whether these are cause-and-effect relationships. Overall, evidence has accumulated based on results in model systems that high levels of antibodies to merozoite antigens are likely to be required to achieve protection in humans. This is thought to be because of the short window available to attack extracellular merozoites before they enter a new red cell. Further, the major clinical trials of blood-stage vaccines have been performed with single antigens and it is likely that combinations of antigens will be required. In this context, protection from clinical malaria has been reported to be associated with both the breadth and magnitude of the antibody responses to merozoite antigens (Osier et al. 2008).

Other potential targets of naturally acquired immunity are the variant surface antigens

present on the membranes of infected red cells. It has been suggested that this immunity depends on acquisition of antibodies to these antigens and that the complexity and variability of protein families such as PfEMP1 requires the progressive accumulation of antibodies to different variants over time (reviewed in Chan et al. 2014; Smith 2014; Hviid and Jensen 2015). It may be that robust antibody responses to both merozoite and cell surface antigens are required to attain naturally acquired clinical immunity although there is limited data on this possibility and replicating it in vaccines remains a challenge.

In the context of elimination/eradication, the question has been raised as to the rationale for asexual-stage vaccines, especially given that they may be only partially effective. However, there are still large numbers of children susceptible to serious disease caused by malaria. Moreover, as malaria continues to decline, much larger numbers of adolescents and adults will not have the benefit of naturally acquired immunity and will be susceptible to serious illness if malaria resurges. Having a vaccine that can provide protection to these people will continue to be of relevance in malaria immunity.

Impact of Malaria Infection on the Immune System

Infection by asexual stages of plasmodia triggers an array of signals and responses in both the innate and adaptive host immune systems. Whether these responses contribute to protection or pathology or both has been difficult to evaluate, particularly in human studies. Although the evidence for a protective role of B cells and antibodies has been noted, the direct contribution of CD4⁺ T cells in protection against blood stages beyond a helper function for CD4⁺ T cells has mainly derived from rodent models of malaria. For example, it has been shown that CD4⁺ T cells can dramatically reduce infection with *Plasmodium chabaudi* in the absence of B cells (Grun and Weidanz 1981). Many other cell types have been implicated in these complex responses as well.

Innate immune cells can be activated by various pathogen-associated molecular patterns

(PAMPs); some of the PAMPs implicated include hemozoin, parasite DNA, and the glycosylphosphatidylinositol anchors of malaria proteins (reviewed in Gazzinelli et al. 2014). Receptor activation results in the release of various proinflammatory cytokines and chemokines, including TNF- α and interleukin 1 β (IL-1 β), leading to systemic pathology and many of the symptoms of malaria such as paroxysms of fever (see Milner 2016).

The long period required for acquisition of natural immunity to malaria and the lack of “sterile” immunity to this infection have led to the suggestion that dysregulation of the host immune system contributes to this incomplete immunity. In addition, reports that antibodies to some parasite antigens are short-lived has led to the suggestion that there are deficiencies in the generation and maintenance of memory B cells in malaria-infected individuals (Portugal et al. 2013; Scholzen and Sauerwein 2013). However, whether this is responsible for the slow development of naturally acquired immunity has been questioned (Hviid et al. 2015). It has also been observed that malaria-exposed individuals have a larger proportion of atypical memory B cells (Weiss et al. 2009), although their specificity is not known and this has not been directly linked with malaria infection. Additional investigations of different B-cell populations, immunologic memory, and protective immune mechanisms will be required to illuminate these issues.

IMMUNE RESPONSES TO SEXUAL AND MOSQUITO-STAGE PARASITES

Characterization of Parasite Sexual Stages

Although asexual stages are responsible for the clinical symptoms of malaria, its transmission requires differentiation to sexual stages, which can be picked up and transmitted by anopheline mosquitoes (Nilsson et al. 2015; Mitchell and Catteruccia 2016). Sexual stages II–IV are sequestered from the peripheral circulation, but eventually stage-V male and female gametocytes reemerge into the bloodstream where they can be engulfed by mosquitoes taking a blood meal. Markers of the various gametocyte

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stages, as well as proteins specific to male or female gametocytes, have been sought for some time. However, mature gametocytes no longer present *var* gene products on the red cell surface and other surface markers have not been definitively identified (Sutherland 2009). Once in the mosquito, the male and female gametocytes emerge from the red cells in the mosquito midgut as gametes where fertilization occurs. At this point, the gametes, zygotes, and ookinetes are accessible to antibodies, leukocytes, or other factors, such as complement, which may be present in the blood meal.

Initial evidence that vertebrate immune responses can interfere with sexual-stage development and mosquito infection led to the concept of transmission-blocking vaccines (reviewed in Stowers and Carter 2001; Sinden 2010). The first studies were conducted in avian models of malaria when birds were immunized with killed blood containing gametocytes, making them less infectious to mosquitoes than controls (Huff et al. 1958). Almost two decades later, it was shown that chickens immunized with inactivated *Plasmodium gallinaceum* gametocytes produced a transmission-blocking activity found in the serum (Carter and Chen 1976; Gwadz 1976). Interestingly, these serum effectors did not affect the gametocytes directly but rather acted on the parasites in the mosquito. Transmission-blocking immunity was also reported with rodent and nonhuman primate malaria parasites (Gwadz and Green 1978; Mendis and Targett 1979). Identification of monoclonal antibodies with transmission-blocking activity in mosquito-feeding experiments facilitated identification of parasite antigens that were the targets of transmission-blocking activity and also established the critical role of antibodies in blocking transmission (Kaushal et al. 1983; Renner et al. 1983). These studies also resulted in a method to evaluate antibodies to sexual stages, viz., the standard mosquito membrane feeding assay (SMFA). In this assay, cultured gametocytes are incubated with antibodies and the mixture fed to anopheline mosquitoes; 1 week later, the oocysts on the mosquito midgut are enumerated. Recent efforts to qualify the SMFA and to analyze data derived

from this assay have increased its applicability to transmission-blocking studies (Churcher et al. 2012; Miura et al. 2013a).

Such antibodies with transmission-blocking activity were then used to identify the parasite antigens they targeted, primarily revealing molecules of 25, 48/45, and 230 kDa (Renner et al. 1983). More recently, efforts have been made to exploit the sequencing of plasmodial genomes as well as transcriptional and proteomic analysis to identify sexual-stage proteins or mRNAs for such proteins found in these stages and the mosquito (Hall et al. 2005; Khan et al. 2005; Silvestrini et al. 2005; Young et al. 2005). These would be possible targets for antibodies or drugs targeting gametocytes.

Identification and Investigations of Pre- and Postfertilization Proteins

For convenience, immune responses to parasite antigens have been divided into prefertilization and postfertilization proteins (reviewed in Pradel 2007; Nikolaeva et al. 2015; Wu et al. 2015).

Prefertilization Antigens

Most studies have focused on a limited set of parasite proteins and considerable difficulties have been encountered in expressing many of them in recombinant platforms, particularly antigens such as Pfs230 and Pfs48/45, which are members of the 6-cysteine protein family. These proteins are characterized by repeating motifs of six conserved cysteine residues, which are coexpressed in gametocytes and have been implicated in the process of fertilization of male and female gametes. Interestingly, activity of antibodies to Pfs230 in the SMFA is enhanced by serum complement, which apparently retains some activity in the blood meal (Read et al. 1994). Although presenting difficulties in expression, inclusion of such proteins in a transmission-blocking vaccine has the potential for boosting by natural infection, which is not the case for proteins expressed only in the mosquito vector. In addition, although some polymorphism has been reported for these molecules, it is less than that of many asexual-stage antigens (Niederwieser et al. 2001). Similarly, evidence

has been presented that the *P. vivax* protein (Pvs230) can also elicit transmission-blocking activity (Tachibana et al. 2012).

Postfertilization Antigens

Because proteins such as Pfs25 and Pvs25 have limited or no expression in the vertebrate host, they are not subject to selection by the vertebrate immune system and consequently are generally quite conserved (Tsuboi et al. 1998). However, as noted, this limits the possibility of boosting of vaccine-induced immune responses by natural infection. The best studied of the postfertilization proteins is Pfs25 and to a lesser extent its homolog in *P. vivax* (Pvs25). Each is found on the surface of the ookinete and includes four epidermal growth factor (EGF)-like domains. Structural studies have revealed Pvs25 to be a flat triangular molecule that could tile the surface of ookinetes; residues forming the triangle are conserved in P25 molecules from all plasmodial species (Saxena et al. 2006). The report of anti-Pfs25 monoclonal antibodies and polyclonal antibodies, which have transmission-blocking activity in SMFA experiments have supported its candidacy as a potential transmission-blocking vaccine (Kaslow et al. 1988). Whether effector antibodies prevent ookinete crossing of the midgut or have other roles in reducing oocyst numbers is not yet clear. Further, estimates of specific antibody concentration required to attain 50% inhibition of oocyst density in the SMFA indicate that relatively high antibody concentrations, approximately 100 µg/ml, will be required (Cheru et al. 2010), and eliciting high, long-lasting antibody titers in humans remains a challenge.

Two recent studies have sought to compare immune responses to different transmission-blocking antigens using the SMFA (Miura et al. 2013c; Kapulu et al. 2015). In one study, proteins were produced in the wheat germ cell-free expression system and antibodies generated in mice. Detailed analysis of SMFA results showed that Pfs25 elicited IgGs with higher inhibition than anti-Pfs230 or anti-HAP2. This was the first demonstration that *P. falciparum* HAP2, a protein also involved in fertilization,

could elicit antibodies with transmission-blocking activity. In the second study (Kapulu et al. 2015), viral vectors encoding parasite proteins were used to immunize mice and the resultant antibodies compared; both anti-Pfs230-C and anti-Pfs25 gave very high levels of blockade in the SMFA.

Other postfertilization proteins, some of which have been investigated as potential transmission-blocking vaccine candidates, include micronemal proteins of the ookinete, proteins found in the cytoplasmic crystalloid organelle, as well as the enzymes such as enolase and chitinase produced by the parasite (reviewed by Pradel 2007; Nikolaeva et al. 2015; Wu et al. 2015). Many of these proteins are likely to have important roles in exodus from the blood meal, attachment to the midgut wall, invasion of the midgut, protection of the parasite, and oocyst development but have not been well studied in terms of immune responses.

Host Immune Responses to Sexual-Stage Parasites

Host immune responses to gametocyte surface membranes have been examined in some individuals from malaria-endemic areas to explore the hypothesis that such natural immune responses could either reduce gametocyte sequestration, gametocyte density in vivo, or infectivity to mosquitoes (Bousema and Drakeley 2011). However, it has proven difficult to show recognition of stage-V-specific proteins on the surface of infected red cells. Some data have been presented that sera from African children recognize these cells by flow cytometry (Saeed et al. 2008); another study reported seroreactivity to gametocytes by IFA, which correlated with reduction of gametocyte density (Baird et al. 1991). Detailed investigation of the gametocyte surface at various stages of differentiation will be important future activities.

With regard to specific antigens present in gametes or zygotes, several studies have reported antibodies in those living in malaria-endemic areas to Pfs230 (Graves et al. 1988; Healer et al. 1999; Bousema et al. 2010; Miura et al. 2013c; Jones et al. 2015) and to Pfs48/45 (Roef-

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fen et al. 1996; Bousema et al. 2010; Jones et al. 2015). Some of these responses have been correlated with transmission-reducing activity as measured by SMFA in the same sera. Evidence for the presence of antibodies to Pfs25 in human populations is inconsistent, perhaps depending on the methodology used (Riley et al. 1994; Miura et al. 2013c).

In addition to the immune responses of the vertebrate host, it is clear that the mosquito vector has an innate immune system, which can also affect the outcome of infection by plasmodia. Those responses are beyond the scope of this review but are discussed in Crompton et al. (2014).

FUTURE PERSPECTIVES

Overall, studies on antiparasite immune responses of the vertebrate host have identified a repertoire of potential immune effector mechanisms and a number of antigens as candidates for vaccine development. In the process these studies have also identified some of the many challenges that lie ahead.

The positive results obtained in the phase III trial of the RTS,S vaccine have established that a malaria vaccine is potentially feasible. Given these results, the next 5 years should see major efforts to develop a more advanced formulation, an effort that will require a more nuanced understanding of the interactions between the host and the infecting sporozoite. Characterizing the specificities and affinities of anti-CSP protective antibodies, identifying optimal immunization strategies, and addressing issues of allelic polymorphism in the CSP protein should contribute to improvements in the vaccine. In addition, expanded efforts should be made to identify other important PE antigens and to develop methodologies that can elicit antigen-specific CD8⁺ T cells in humans.

As discussed previously, the adoptive transfer of antibodies from individuals living in endemic areas drastically reduced parasitemia in *P. falciparum*-infected children. The antiparasite phenotype of naturally acquired immunity is clearly attributable to the inhibitory effect of antibodies on the progressive development

of asexual stages. To date, vaccine studies with asexual stages have focused mostly on single antigens with a likely functional role in parasite replication. Although this may be a sensible approach, there is no evidence that protective immunity is restricted to the recognition of only a few antigens. In fact, it is possible that vaccines inducing broad, multiantigen immune responses may be more effective and long lasting. Also, although the prospect is daunting, we need to pursue efforts to identify conserved targets on the surface of the infected red cell. It is therefore urgent to develop extensive programs to design vaccines consisting of multiple antigens from different parasite stages. This is a major challenge as multispecific immune responses may not be easy to induce, as it is not uncommon to find antigen competition/interference when using combination vaccines. Moreover, it is not clear that there exist sensitive methodologies that can show the occurrence of additive or synergistic effects of the induced immune responses.

The extensive antigenic diversity in antigens from PE and asexual stages, which are currently studied for vaccine development is a significant hurdle. However, this should not be considered an insurmountable obstacle, as new biotechnological advances should make possible the development of multiallelic vaccine constructs. Finally, recent evidence indicating that individuals exposed to parasite infection undergo immune exhaustion and a possible inability to develop an efficient immunological memory is a matter of concern. It is unclear the extent to which these immune dysfunctions may impair the development of immune responses induced by vaccines or if it may decrease vaccine efficacy.

Resurgent interest in the sexual stages of parasites and in malaria transmission in the field has generated interest in the possibility of a transmission-blocking component of a multistage vaccine. Whether the current antigens identified or other parasite or mosquito antigens can elicit sufficiently high titers of antibodies in humans to affect transmission to the mosquito will be an important question to answer in the next 5 years. Also, we must seek vaccine formulations and expression platforms

that generate longer lasting humoral immune responses, because responses to some transmission reducing parasite proteins may not be boosted by natural infection. Finally, issues of what level of responses in a population are required to reduce transmission in a given area and how to determine that level must be approached. Nevertheless, a component of a vaccine that could aid in reducing transmission below a critical threshold would be an important contribution to malaria vaccine development.

CONCLUDING REMARKS

Characterization of immune responses induced by natural exposure to parasites has facilitated the identification of mechanisms of immunity that provide partial protection, particularly for immune responses to asexual stages. These studies show that naturally acquired immune responses against asexual stages are effective against parasites and that these responses reduce morbidity, even though they do not cause full parasite clearance. In contrast, naturally acquired immune responses against PE and sexual stages are of low magnitude and there is no clear evidence that they have a protective effect or mediate clinical immunity. The interest in immune responses to PE and sexual stages and the antigens they recognize derives from experimental vaccine studies in animal models or in human trials in the case of attenuated sporozoites, which showed the development of sterilizing immunity. The challenge now is to transform these findings into a highly effective vaccine for malaria.

Considerable advances have been made in reducing malaria in many parts of the world over the past decade using existing tools. However, the logistics and financing required to retain and to expand these advances in the face of antimalarial drug resistance and declining efficacy of insecticides will be challenging. As we consider moving beyond the current situation into an era of malaria eradication, the development and deployment of a highly effective malaria vaccine is still a critically important but unrealized component in the portfolio of responses to this global challenge. Pursuing a

more complete and detailed view of the immunologic interface between host and the various parasite stages should enhance the opportunities for developing such a vaccine.

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