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Young Lives Lost as B Cells Falter: What We're Learning about Antibody Responses in Malaria

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Abstract

Plasmodium falciparum malaria remains a major public health threat for which there is no licensed vaccine. Abs play a key role in malaria immunity, but Ab-mediated protection is only acquired after years of repeated infections, leaving children in endemic areas vulnerable to severe malaria and death. Many *P. falciparum* Ags are extraordinarily diverse and clonally variant which likely contribute to the inefficient acquisition of protective Abs. However, mounting evidence suggests that there is more to the story and that infection-induced dysregulation of B cell function also plays a role. Here we review progress toward understanding the B cell biology of *P. falciparum* infection, focusing on what has been learned from population-based studies in malaria-endemic areas. We suggest ways in which advances in immunology and genomics-based technology can further improve our understanding of the B cell response in malaria and perhaps illuminate new pathways to the development of effective vaccines.

Introduction

Malaria is caused by mosquito-borne parasites of the genus *Plasmodium*. Of the five *Plasmodium* species that infect humans, *P. falciparum* is the deadliest, each year causing approximately 225 million cases of malaria and nearly one million deaths, the majority among African children and pregnant women (http://www.who.int/malaria/world_malaria_report_2011/en/) (1). Optimism that a first-generation malaria vaccine, RTS,S, may soon be licensed has been tempered by the interim results of an ongoing phase 3 clinical trial in Africa which indicated that the vaccine confers short-lived protection from malaria in only ~30% of infants (2). Clearly, the ongoing effort to develop a highly effective vaccine would benefit from a more detailed understanding of malaria immunity.

P. falciparum has a complex life cycle (Fig 1) in which only the blood stage of infection is associated with disease, typically an undifferentiated febrile illness which in a minority of cases progresses to severe disease and death (3). Epidemiologic studies in areas where individuals are repeatedly infected with *P. falciparum* show that immunity to severe, life-threatening malaria is usually acquired early in childhood, whereas immunity to uncomplicated febrile malaria is not reliably acquired until early adulthood (4), and once acquired, appears to wane (5), at least partially (6), in the absence of ongoing *P. falciparum* exposure. Despite decades of exposure, sterile immunity to infection develops rarely, if at all (4), as adults often carry blood stage parasites without symptoms. In this review we focus our attention on naturally-acquired immunity to the blood stage of *P. falciparum* infection where B cells are known to play a critical role in protection.

The central role of Abs in naturally-acquired malaria immunity was shown by early experiments in which the transfer of purified IgG from malaria-immune West African adults to children with malaria in West Africa (7), East Africa (8) or Thailand (9) led to rapid and profound reductions in parasite numbers in the blood and resolution of fever. Over 50 years after understanding that Abs mediate malaria immunity, it remains a major challenge to determine which of the ~ 5,400 predicted *P. falciparum* proteins (10) elicit protective Abs and the mechanisms by which these Abs protect (described in Fig 1) (11). Indeed, absent this information there are currently no unambiguous immune correlates of protection from malaria. It also remains incompletely understood why the acquisition of Ab-mediated immunity is so slow to develop (12, 13). The gradual acquisition of protective Abs has been proposed to be due in large part to the extensive genetic diversity of many *P. falciparum* Ags (14) as well as the parasite's ability to clonally vary the Ags it expresses on the surface of infected RBCs (iRBCs) (15). The reasoning goes that it may take years living in an endemic area for an individual to be exposed to a sufficient number of parasite clones to generate a protective Abs. However, the data supporting this view are not ironclad. Here we review studies that point toward an alternative but non-mutually exclusive explanation for the gradual acquisition of Ab immunity to malaria—that *P. falciparum* subverts B cell responses making the acquisition of protective Abs inefficient, particularly as compared to responses to other pathogens in which long-lived Ab-mediated protection is acquired after a single or few exposures.

The Ab Response to Malaria

How clone-specific are Abs that protect against malaria?

The proposal that the slow acquisition of protective Abs in individuals living in malaria endemic areas is due to the time required to be exposed to a large number of *P. falciparum* clones predicts that Abs to *P. falciparum* variant antigens should be highly clone specific. A major target of protective Abs in malaria appears to be *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the surface of iRBCs (16). PfEMP1s are encoded by the highly polymorphic *var* multigene family (~60 genes/genome) and their expression is clonally variant (15). PfEMP1s mediate adhesion of iRBCs to microvascular endothelium, allowing the parasite to sequester in capillaries of various organs and avoid destruction in the spleen (Fig 1) (3). In addition, sequestration of iRBCs in organs such as the brain and placenta has been linked to the pathogenesis of clinical syndromes such as cerebral malaria and pregnancy-associated malaria (Fig 1) (17). Several studies support the hypothesis that repeated infections are required to elicit a protective repertoire of PfEMP1-specific Abs (16, 18–23). However, other studies suggest that there is a limited subset of common PfEMP1s Ags that are important targets of immunity (24–27) or that protective Abs target cross-reactive or conserved epitopes on PfEMP1s (28–33). The observation that purified IgG from immune West African adults was therapeutic in children not only in West Africa but in East Africa (8) and Thailand as well (9) suggests a significant degree of Ab cross-reactivity to genetically diverse *P. falciparum* parasites (34). Together these observations raise the possibility that the gradual acquisition of protective Abs may not simply be a reflection of the time required for an individual to be exposed to all the parasite clones circulating in a given endemic area.

How long-lived is the Ab response to *P. falciparum*?

It is well established that long-lived protective Ab responses, that are induced by many pathogens and vaccines after one or a few exposures (35), depend on the generation of high affinity memory B cells (MBCs) and long-lived plasma cells (LLPCs) (36–38). LLPCs reside in the bone marrow where they constitutively secrete Abs and provide a critical first

line of defense against re-infection, whereas MBCs mediate recall Ab responses after re-exposure to their Ag by rapidly proliferating and differentiating into PCs.

Although there is significant heterogeneity in the magnitude, quality and longevity of Ab responses following infection or vaccination, in general, Ab responses are long-lived. For instance, estimates of half-lives of IgG responses range from 11 years for tetanus vaccines to >300 years for measles vaccines (35, 38). In sharp contrast, the half-lives of *P. falciparum*-specific Ab responses generally appear to be much shorter, particularly in young children. For example, in a 12-week study of Kenyan children following acute malaria, the half-lives of IgG responses specific for 5 merozoite surface Ags (39) were estimated to be 9.8 days for IgG1 and 6.1 days for IgG3. In additional longitudinal studies, *P. falciparum*-specific Ab responses have been reported to decline to undetectable or nearly undetectable levels within 3–9 months of documented malaria episodes in children (40–48). It is of interest that Ab responses to conserved (or semi-conserved) *P. falciparum* Ags (42) or conserved regions of polymorphic Ags (45), to which individuals presumably were repeatedly exposed, were similarly short lived. Together these studies in children indicate that *P. falciparum* infection does not reliably induce a stable pool of LLPCs. Short-lived *P. falciparum*-specific IgG responses have also been observed in older children and adults (42, 45, 49). On the other hand, there are studies that point toward more stable IgG responses with increasing age in areas of stable *P. falciparum* transmission (40, 44, 46, 50, 51), however, it is important to note that in such areas it is difficult to separate the effects of age and cumulative *P. falciparum* exposure on malaria immunity. Data from transmigrant studies in Indonesia suggest that adults may acquire clinical immunity to malaria more rapidly than children (52, 53), but this bears further investigation as the risk of clinical malaria was similar among previously unexposed children and adults during a malaria epidemic in Madagascar (6).

How might these differences in the estimates of the duration of Ab responses be reconciled? It is possible that studies that reported short-lived *P. falciparum*-specific Ab responses were biased by the small number of Ags examined. Indeed, the studies cited above measured IgG responses specific for one or a few Ags, often blood-stage merozoite surface Ags being developed as vaccine candidates (54) that together represent <0.5% of the predicted *P. falciparum* proteome (10). However, a recent study which employed protein microarray technology (55) to assess the IgG response to ~25% of the *P. falciparum* proteome, including proteins expressed at all stages of the parasite life cycle, showed that children exposed to intense seasonal *P. falciparum* transmission generate IgG responses to hundreds of *P. falciparum* Ags each transmission season. However, these declined rapidly during the 6 month dry season, a period of little to no *P. falciparum* transmission (Fig 2). These data suggest a preferential induction of short-lived plasma cells (SLPCs) during *P. falciparum* infection that is not restricted to a subset of intrinsically less immunogenic proteins.

Even though Ab responses to *P. falciparum* Ags in children are short-lived, ultimately, by adulthood stable Ab levels are acquired. The study cited above showed that with each year of *P. falciparum* exposure, the level of IgG persisting through the dry season gradually increased until young adulthood when IgG levels were maintained at high levels (Fig 2). One simple explanation for the gradual buildup of *P. falciparum*-specific IgGs with age is the incremental accumulation of LLPCs with each year of exposure. How important for protection are long-lived Ab responses? An age-adjusted analysis in the same study showed that the overall level of *P. falciparum*-specific IgG present before but not after the malaria season predicted protection from malaria (55).

Taken together, these data suggest that the gradual acquisition of clinical immunity to malaria may reflect the need for repeated infections in order to ‘fill’ the *P. falciparum*-specific LLPC compartment to the point where steady-state Ab levels exceed a protective

threshold. Conversely, MBC-mediated Ab boosting in response to acute infection, a process that generally peaks 6–10 days after Ag re-exposure, may not be rapid enough to prevent the onset of symptoms which can occur as early as 3 days after blood stage infection begins (56). Similar models have been postulated for other pathogens with short incubation periods such as *Haemophilus influenzae* type b (57), in which the infection progresses to a symptomatic threshold more rapidly than MBC-mediated Ab boosting, such that protection depends on already circulating Abs. In this light, it is likely that an effective blood-stage malaria vaccine will need to establish a critical level of circulating IgG produced by a stable pool of LLPCs, rather than depend on MBC-mediated Ab boosting. This may be especially important in areas of sporadic *P. falciparum* transmission where boosting of Ab responses does not occur through regular natural exposure. The same principle is likely applies to vaccines targeting sporozoites which are in the bloodstream and exposed to Abs only transiently before invading hepatocytes.

The continued use of high-throughput Ab profiling methods (58) in various epidemiological settings in conjunction with systems biology approaches (59) to describe the contribution of T cells and innate immunity will improve our understanding of the factors which influence the specificity, longevity and quality of Ab responses to malaria. A more detailed understanding of the mechanisms which ultimately confer protection against malaria and the reasons for their inefficient acquisition could help advance malaria vaccine development.

The MBC Response to Malaria

It was initially speculated that *P. falciparum* infection might not generate MBCs based on anecdotal evidence of waning malaria immunity in the absence of *P. falciparum* exposure, and inconsistent Ab boosting upon re-infection (39), even to conserved Ags (42). However, several recent studies have now shown that *P. falciparum*-specific MBCs can indeed be generated in response to natural infection, albeit inefficiently. For example, a longitudinal study in Mali, where there is an annual 6 month malaria season, found that the frequency of MBCs specific for two blood stage Ags, AMA1 and MSP1, increased incrementally with each malaria season, from childhood to adulthood (43). Moreover, MBCs once acquired appear to be long lived. For example, a recent study in Kenya reported that *P. falciparum*-specific MBCs were found to persist longer than Abs in the absence of ongoing *P. falciparum* exposure (60). However, the Mali study showed that despite exposure to ~60 infective mosquito bites/month at the peak of the malaria season, only ~50% of adults had detectable *P. falciparum*-specific MBCs (43). A low prevalence (~30–50%) of *P. falciparum*-specific MBCs has also been reported in adults in studies conducted in The Gambia (61), Kenya (62) and Thailand (51), even in individuals with documented *Plasmodium* infection within 6 years (51). The findings for *P. falciparum*-induced MBCs are in sharp contrast to studies that show, for example, that smallpox vaccine-specific MBCs are generated and persist in nearly all vaccinees for 50+ years in the absence of Ag re-exposure (63). Thus, it appears that *P. falciparum* infection can generate long-lived MBCs but less efficiently, at least compared to vaccines for other pathogens. Although it might be predicted that the prevalence and frequencies of *P. falciparum*-specific MBCs would be higher in areas of intense versus sporadic *P. falciparum* transmission, studies to date suggest that this is not the case (43, 51, 61, 64), raising the possibility that chronic or persistent *P. falciparum* exposure may be detrimental to MBC generation.

What might underlie the inefficient acquisition of *P. falciparum*-specific LLPCs and MBCs?

Several mechanisms have been proposed to explain the relatively inefficient acquisition of *P. falciparum*-specific LLPCs and MBCs (Table 1). Because of limited space we comment on only a few possibilities for which there is recent data.

The relative inefficiency in acquiring *P. falciparum*-specific LLPC and MBCs may be related to dysregulated B cell Ag-driven differentiation, particularly in the setting of chronic *P. falciparum* exposure. Alterations in B cell function are well described in the context of chronic infections such as visceral leishmaniasis (65), HIV (66) and HCV (67). For example, HIV (68) and HCV (69) viremia is associated with an expansion of a subset of B cells identified by the cell surface markers CD19⁺CD20⁺CD21⁻CD27⁻CD10⁻ and Fc-receptor-like-4 (FCRL4) (70). In HIV-infected individuals Moir et al (68) showed that these cells were hyporesponsive and suggested that these hyporesponsive, or 'exhausted' MBCs contribute to the humoral deficiencies observed in HIV-infected individuals.

Remarkably, an increase in a phenotypically similar subset of B cells has been observed in *P. falciparum*-exposed children and adults (71). In the context of malaria, this B cell subset has been referred to as 'atypical' rather than 'exhausted' because the function of these B cells and whether they are beneficial or detrimental in malaria remains unclear. An expansion of atypical MBCs has also been observed in malaria endemic Peru (72), The Gambia (61), Mali (73), Kenya (74) and Gabon (75), suggesting that *P. falciparum*-associated atypical MBC expansion is generalizable to genetically and geographically disparate populations; and that the degree of atypical MBC expansion correlates with *P. falciparum* transmission intensity (72). A direct causal relationship between *P. falciparum* infection and atypical MBC expansion remains to be established; however, differential expansion of atypical MBCs has recently been observed in age-matched children living under similar conditions in rural Kenya with the exception of *P. falciparum* exposure (74), suggesting that *P. falciparum* infection *per se* may drive atypical MBC expansion. Intriguingly, recent work by Muellenback et al (75) showed at the single cell level that *P. falciparum* exposure is associated with the acquisition of atypical MBCs that produce broadly neutralizing Abs specific for *P. falciparum* blood stage antigens; and furthermore, that atypical MBCs, unlike classical MBCs, show signs of active Ab secretion, although spontaneous Ab secretion was not noted in other studies (68, 71). Compared to classical MBCs, atypical MBCs also differed in their IgG gene repertoire, suggesting that they develop from different precursors (75).

P. falciparum infections could also dysregulate helper T cell responses. Interestingly, a recent study showed that PD-1, a marker of T cell exhaustion, is upregulated on CD4⁺ T cells in children following *P. falciparum* infection (76). Although the functional significance of this observation in human remains to be determined, Butler *et al.* reported that *P. yoelii* infection in mice induces phenotypic and functional CD4⁺ T cell exhaustion, and that *in vivo* blockade of the PD-1 ligand PD-L1 and the inhibitory receptor LAG-3 restored CD4⁺ T cell function, amplified the number of T_{FH} and GC B cells and plasmablasts, increased Ab levels and accelerated the clearance of blood-stage parasites (76). To understand the functional link between putative *P. falciparum*-specific CD4⁺ T cell exhaustion and Ab responses in humans will require tools that allow for the detection of *P. falciparum*-specific T cells, including MHC tetramer technology that has proved to be effective in identifying pathogen-specific T cells by flow cytometry.

Whether malaria-associated B cell dysregulation is driven by direct interactions between *P. falciparum* products and B cells or indirectly through systemic immune activation is unclear.

To our knowledge the only published example of a direct *P. falciparum*-B cell interaction comes from *in vitro* studies which implicate the cysteine-rich interdomain regions 1 α (CIDR1 α) of PfEMP1 as a T cell-independent polyclonal B cell activator and Ig binding protein (77). The discovery of direct molecular interactions between *P. falciparum* and the human immune system has been accelerated by systematic protein interaction screening approaches (78). For example, a yeast two-hybrid approach identified an interaction between *P. falciparum* merozoite surface protein 1 (MSP1) and the pro-inflammatory protein S100P (79); and an avidity-based extracellular interaction screen identified basigin (CD147) as a host receptor essential for RBC invasion via the merozoite protein PfRh5 (80). Intriguingly, basigin is also expressed on B and T cells, as well as DCs, monocytes and macrophage, raising the possibility that basigin-PfRh5 interactions may modulate host responses during *P. falciparum* infection.

It is also possible that systemic mediators of B cell differentiation and survival (36), such as B-cell activating factor (BAFF), APRIL, IL-4, and IL-21 are modulated during *P. falciparum* infection. A recent study of Kenyan children (81) reported that acute malaria is associated with elevated plasma levels of BAFF and transient down-regulation of BAFF receptor expression on B cells. But interestingly, higher levels of BAFF-receptor expression on B cells were associated with more durable *P. falciparum*-specific IgG responses, suggesting that dysregulated BAFF-R expression may contribute to inefficient *P. falciparum*-specific LLPC responses. It has also been suggested (39) that binding of iRBCs to bone marrow stromal cells (82) may disrupt PC survival signals in bone marrow niches.

Chronic exposure to pathogen-associated molecular patterns (PAMPs) could possibly result in tolerance of pattern recognition receptors (PRRs) expressed on B cells and DCs which play a critical role in enhancing B cell responses (83) (84). Although few *P. falciparum*-derived PAMPs have been identified to date (85–88), recent clinical trials demonstrate that the TLR9 agonist CPG markedly enhances the IgG and MBC response to *P. falciparum* blood-stage vaccine candidates in malaria-naïve adults (89), but not in adults chronically exposed to *P. falciparum* in endemic areas (90). Further studies are needed to define how *P. falciparum* exposure modulates the function of PRRs and how this might influence homologous and heterologous LLPC and MBC responses.

It is unclear whether Ab responses in the course of repeated *P. falciparum* infections are predominantly derived from newly recruited naïve B cells or cross-reactive MBC clones generated during prior infections (46) (i.e. clonal imprinting or original antigenic sin [OAS]). It is useful to consider the case of influenza, which like *P. falciparum*, repeatedly infects much of the world's population and exhibits extensive strain diversity. Work by Wrammert et al (91) suggests that plasmablasts producing broadly neutralizing Abs induced by pandemic H1N1 influenza are predominantly derived from activated MBCs specific for epitopes conserved in several influenza strains, consistent with OAS. Similar analyses of variable gene sequences from plasmablasts induced by human experimental (56) and natural *P. falciparum* infection, particularly serial infections with genetically distinct *P. falciparum* parasites, could define the degree to which OAS operates in malaria and how it relates to malaria susceptibility.

Conclusions

Although there appears to be more questions than answers as to how malaria immunity is acquired, it is nonetheless an exciting time in malaria research. Malaria is one of the few infectious diseases in which relatively small longitudinal cohort studies of natural infection are feasible. For example, in areas of intense seasonal *P. falciparum* transmission, where nearly all individuals are predictably exposed to *P. falciparum* each year, it is possible to

analyze the human immune response before, during, and after *P. falciparum* infection and to prospectively study immune correlates of protection as well as the immunomodulatory effects of infection. Malaria is also one of the only infectious diseases for which experimental human infections are approved. These unique study populations coupled with the powerful tools made available through extraordinary advances in immunology and systems biology ought to provide a wealth of information on the impact of *P. falciparum* infections on the human immune system and on the acquisition of malaria immunity.

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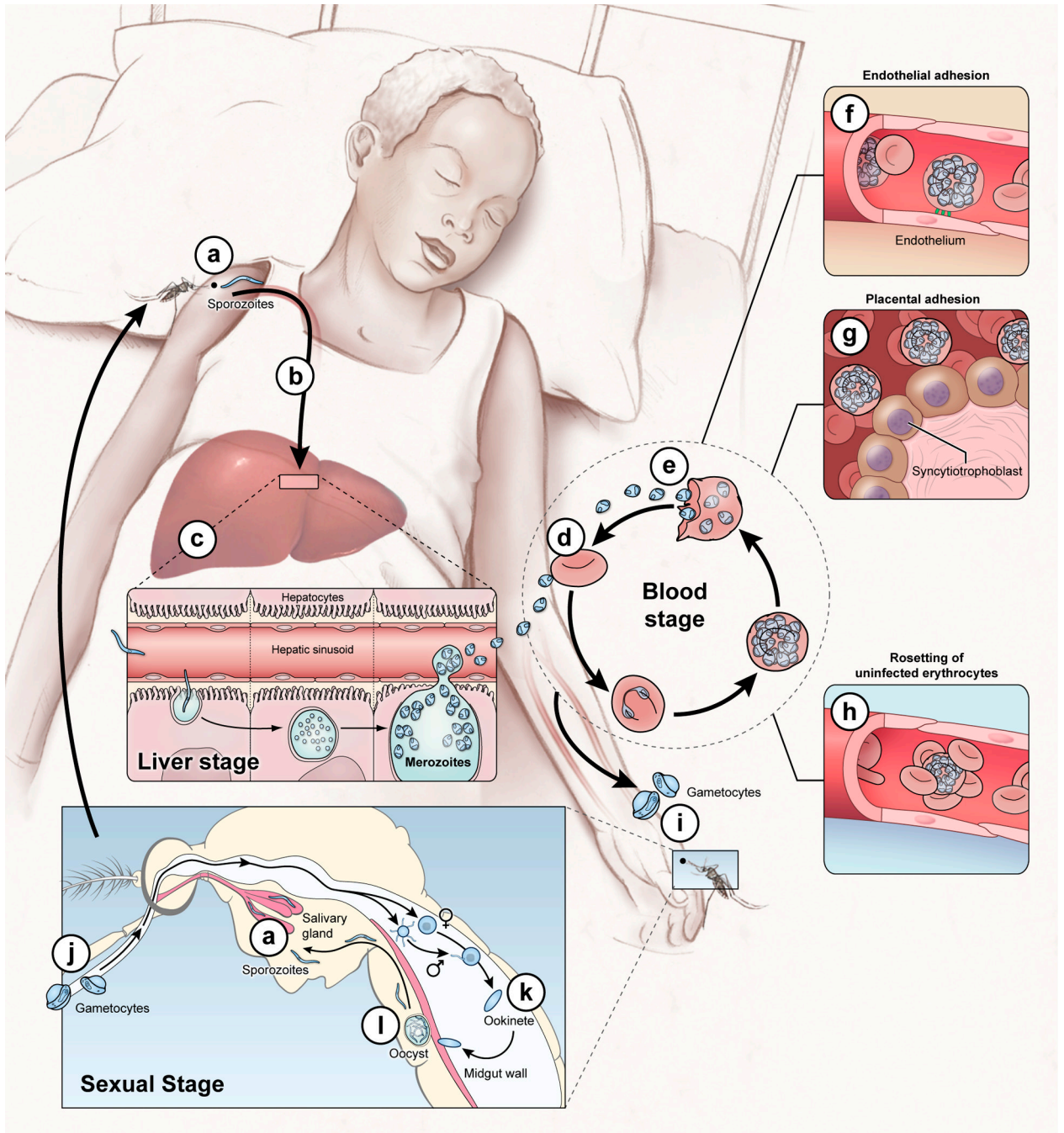


Figure 1. The *P. falciparum* life cycle: no shortage of Ab targets

The *P. falciparum* life cycle in humans includes the asymptomatic liver stage; the blood stage which causes disease; and the sexual gametocyte blood stage which infects mosquitoes that transmit the parasite. Infection begins when *Anopheles* mosquitoes inject sporozoites into the skin and blood (a) which migrate to the liver and invade a small number of hepatocytes (b). Each sporozoite gives rise to thousands of asexual parasites called merozoites (c). ~1 week after hepatocyte invasion merozoites exit the liver into the bloodstream and begin a 48 hr cycle (d) of RBC invasion, replication, RBC rupture, and merozoite release (e). Once inside RBCs the parasite exports variant surface antigens

(VSAs) such as PfEMP1 to the RBC surface. VSAs mediate binding of iRBCs to the microvascular endothelium of various organs (**f**) and placental tissue (**g**) allowing parasites to avoid splenic clearance and promoting the inflammation and circulatory obstruction associated with clinical syndromes such as cerebral malaria (coma) and pregnancy-associated malaria. VSA-mediated rosetting (binding of iRBCs to RBCs) may also contribute to disease (**h**). A small number of blood-stage parasites differentiate into sexual gametocytes (**i**) which are taken up by mosquitos (**j**) where they differentiate into gametes that fuse to form a motile zygote, the ookinete (**k**), where meiosis occurs. The ookinete crosses the midgut wall and forms an oocyst (**l**) that develops into sporozoites that enter the mosquito salivary gland to complete the life cycle (**a**).

Abs that sterily protect by neutralizing sporozoites (**a**) and/or blocking hepatocyte invasion (**b**) are rarely if ever acquired through natural infection (4). Abs induced by the RTS,S vaccine target the circumsporozoite (CS) protein on the sporozoite surface (98) and correlate with sterile protection in malaria-naïve adults (99); but in African children RTS,S generally protects against disease not infection and the correlation between Abs and protection is less clear (100, 101). Abs are a key component of naturally-acquired blood stage immunity (7) but the Ag targets and mechanisms of protection are incompletely understood and likely multifaceted. Abs may contribute to protection by clearing merozoites (102) and iRBCs (**e**) through opsonization (103) or complement-mediated lysis (104); inhibiting merozoite invasion of RBCs (**d**) (105); and/or blocking adhesion of iRBCs to vascular endothelium (**f**) (106). Non-neutralizing Abs may contribute to protection through Ab-dependent monocyte- (107) or NK cell-mediated cytotoxicity. Blood stage vaccines have focused primarily on generating Abs to merozoite proteins but with limited success (54, 108), probably due to Ag polymorphism (14, 108) and redundant RBC invasion pathways (109). Of note, the conserved merozoite protein PfRh5 appears to be essential for RBC invasion (80) and may be susceptible to vaccine-inducible cross-strain neutralizing Ab (110). Protection from pregnancy-associated malaria correlates with IgG specific for VAR2CSA, a conserved protein which mediates adherence to placental tissue (**g**) (111). Vaccine-induced Abs targeting Ags on gametocytes and gametes that are ingested with the blood meal (**i-I**) could prevent parasite development in the mosquito and block or reduce transmission (112).

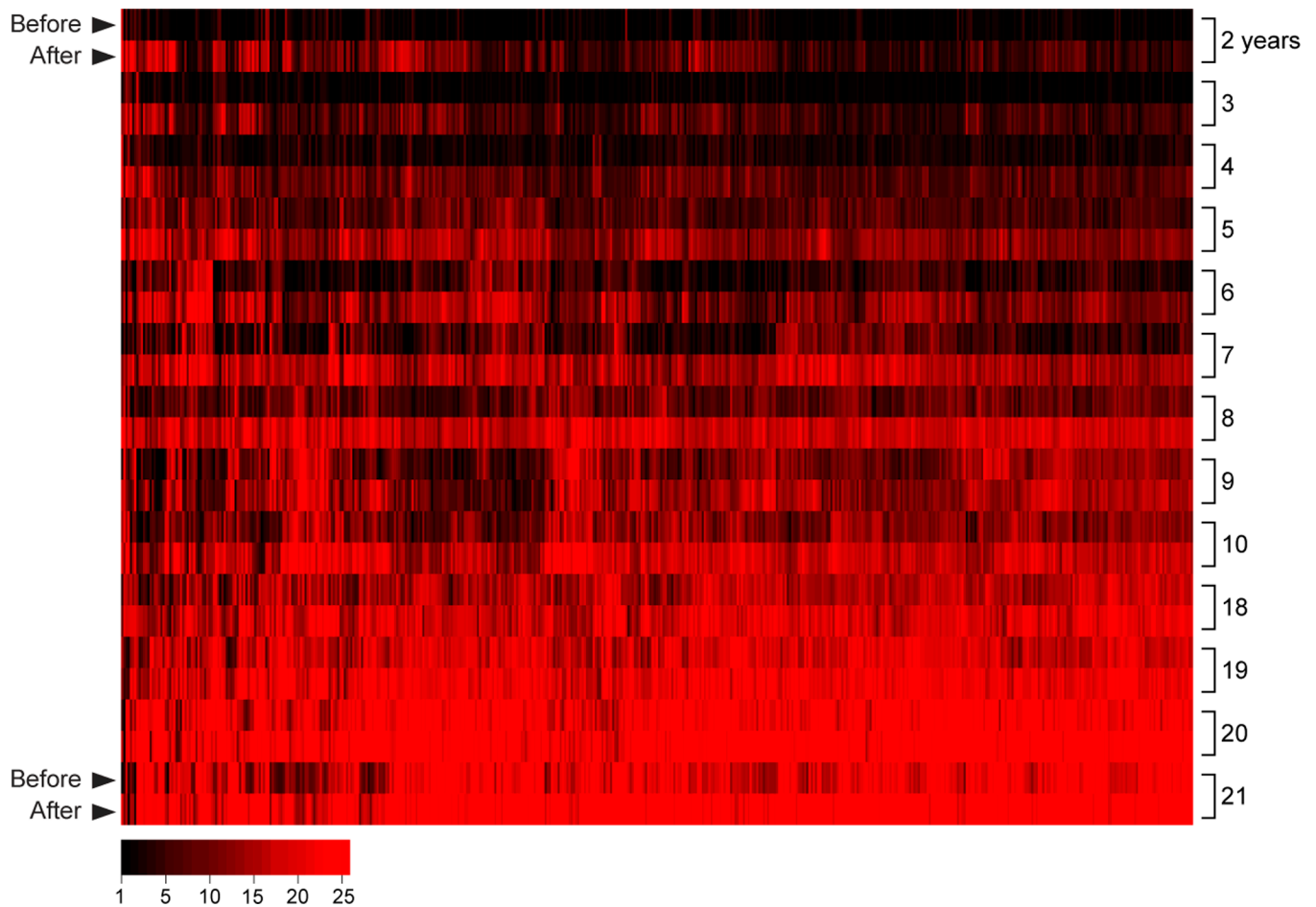


Figure 2. The *P. falciparum*-specific IgG response is broad but short-lived in children
 Shown is IgG reactivity (red) specific for 491 *P. falciparum* proteins (columns) in plasma collected from children (n=157) and adults (n=37) before and after an intense 6 month malaria season [(55) for detailed Methods and Results]. The average IgG level for each protein is stratified by 1-yr age groups from 2–21 yrs, and within each age group from before to after the malaria season (rows). A broad *P. falciparum*-specific IgG response is present after the 6-month malaria season in children as young as 2 yrs, however, the IgG response in young children is short-lived based on IgG levels at the end of the 6 month dry season, a period of little to no *P. falciparum* transmission. With increasing age, IgG levels at the end of the dry season gradually increase until plateauing in adulthood.

Table 1

Potential factors contributing to the inefficient acquisition of protective Abs.

Factor	References
Clonal Ag variation	(15)
Ag genetic diversity	(14)
GC disruption	(92)
Lack of Ab avidity maturation	(93)
Marginal zone B cell induction	(94)
B cell 'exhaustion'	(71)
Increased immature transitional B cells	(95)
CD4+ T cell exhaustion	(76)
Polyclonal B cell activation	(77, 96)
BAFF/BAFF-R dysregulation	(81)
TLR tolerance	(90)
Self-reactive antibodies/anergy	(97)
Clonal imprinting/OAS	(46)