



To B or Not to B: Understanding B Cell Responses in the Development of Malaria Infection

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Malaria is a widespread disease caused mainly by the *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv) protozoan parasites. Depending on the parasite responsible for the infection, high morbidity and mortality can be triggered. To escape the host immune responses, *Plasmodium* parasites disturb the functionality of B cell subsets among other cell types. However, some antibodies elicited during a malaria infection have the potential to block pathogen invasion and dissemination into the host. Thus, the question remains, why is protection not developed and maintained after the primary parasite exposure? In this review, we discuss different aspects of B cell responses against *Plasmodium* antigens during malaria infection. Since most studies have focused on the quantification of serum antibody titers, those B cell responses have not been fully characterized. However, to secrete antibodies, a complex cellular response is set up, including not only the activation and differentiation of B cells into antibody-secreting cells, but also the participation of other cell subsets in the germinal center reactions. Therefore, a better understanding of how B cell subsets are stimulated during malaria infection will provide essential insights toward the design of potent interventions.

Keywords: B cell biology, malaria, antibodies, effective mechanism, protective immunity

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MALARIA INFECTION AND IMMUNITY

Malaria is a widespread disease mainly caused by the *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv) parasites in tropical countries. Currently, half of the world population lives in areas at risk of a malaria infection. In 2016, a global estimative enumerated 216 million clinical cases and 445,000 deaths associated with this disease (1), portraying the real magnitude of this public health problem. Most cases of malaria morbidity and mortality have been attributed to Pf infections, prevalent in sub-Saharan Africa and characterized by high parasitemias and severe complications, especially in children (2). Contrarily, Pv infections are more disseminated in American and Asian countries and induce lower parasitemia levels and milder symptoms. Rarely, Pv infections can elicit severe symptoms and kill like Pf infections (2–4).

Plasmodium parasites have a complex life cycle, with sporozoites transmitted from the *Anopheles* mosquito salivary glands to the human skin dermis during mosquito blood meals. These motile parasites cross layers of the skin and enter the bloodstream, reaching the liver within hours upon infection. Then, they invade the hepatocytes, replicating and differentiating into schizonts. In the case of a Pv infection, part of the sporozoites are transformed into dormant forms called hypnozoites, which can be activated even after a long term of parasite infection. As a result of

the hepatocyte burst, the merozoites are released in the bloodstream and invade the erythrocytes (Pf parasites) or the reticulocytes (Pv parasites), initiating the asexual blood stage of the cycle. These parasitic forms undergo several rounds of multiplication and differentiation, increasing the parasitemia levels in the host. Those forms found in infected red blood cells (iRBCs) have been identified as rings, trophozoites, schizonts, and gametocytes. Whereas the newly-released merozoites can keep re-invading the erythrocytes, a small fraction of them differentiate directly into gametocytes, giving rise to the sexual blood stage. Gametocytes are ingested during the mosquito blood meal and fuse to each other within the digestive tract, forming a zygote. The zygote differentiates into an ookinete, followed by oocyst forms, previously to the generation of infectious sporozoites that can be found in a mosquito's salivary glands (5, 6). Interestingly, the bone marrow has been described as the major parasite reservoir for early blood stage (asexual and sexual) and gametocytes in Pv infections (7, 8).

Regarding the mechanisms of immunity naturally induced by malaria, the humoral response has been described as the most important for the establishment of protection. This concept has been solidified after the finding that a passive transfer of serum samples from malaria-immune adults controlled the Pf parasitemia levels and ameliorated symptoms in acutely infected children (9). Although the elicitation of the humoral response is critical to reduce malaria morbidity and mortality, antibody-dependent protective immunity usually takes multiple parasitic exposures and may take even years to be established. The extensive genetic diversity of clinical Pf and Pv malaria episodes (10, 11) and the low frequency of malaria-specific memory B cells (MBCs) detected in residents of high endemic areas (12, 13) corroborate this statement. Considering that antibodies represent a snapshot of B cell responses at a single cell level (14), it is fundamental to understand how this cellular component is stimulated upon *Plasmodium* infection to improve vaccine formulations and consequently generate more effective antibodies against human malaria. In this review, we present the distinct aspects of B cell immunity derived from a malaria infection, ranging from the activation of naive B cells to the generation of antibody-secreting cells and the mechanisms of action by protective antibodies.

MALARIA-SPECIFIC B CELL RESPONSES

During malaria infection, thousands of parasitic antigens are expressed in each stage of the parasite life cycle (15). However, the anti-malarial humoral responses are preferentially headed to blood stage antigens rather than the liver counterparts. Besides the differences on the antigen density, a malaria murine model has shown that the blood stage of infection weakens the humoral immunity against the liver stage antigens through the modification of lymphoid structures and the expression of cytokines and chemokines (16). Overall, these responses are mainly characterized by the generation of the antibody-secreting cells (ASCs), memory B cells (MBCs), and antibody titers. Whereas, the MBCs and antibody titers have been

found with steady levels for years in individuals living in low Pf and Pv malaria-endemic areas without the evidence of reinfection (17), these parameters are not sustained for longer periods, especially in younger individuals from high Pf-endemic areas (12, 13).

The question arises, disregarding the timing that antibodies can be detected in serum samples, how is their secretion triggered upon malaria infection? Usually, the antigen-specific antibodies are expected to be detected in the serum in <2 weeks upon any pathogen exposure. During this period, the naive B cells are activated upon B cell receptor (BCR) interaction with a parasitic antigen in the periphery, eliciting cell proliferation and differentiation into multiple subsets such as the MBCs, follicular B cells (FoBs), major players of the germinal center (GC) reactions, or marginal zone B cells (MZBs). Although all these B cell subsets express immunoglobulin (Ig) genes, only the ASCs secrete antibodies. Regarding FoBs, they form and maintain structures called the germinal centers (GCs) together with the follicular T helper cells (TFh), dendritic cells (FDCs), cytokines [IL-21, IL-6, and B cell activating factor (BAFF)], and the critical participation of co-stimulatory molecules (CD40L and ICOS). During the germinal center reactions, the GC B cells are activated and undergo several rounds of antigen selection, acquiring a mature status through the somatic hypermutations and class-switch in Ig genes. Thus, it prompts the production of high-affinity, class-switched antibodies. Models of human and murine malaria infections point to higher numbers of GC B cells and lower for MZB-like cells (18, 19). Terminal signaling triggered by activation guides the FoB cells to exit the follicles and differentiate into high-affinity, atypical, or classical MBCs (20) or short-lived, class-switched ASCs. However, there is evidence from a malaria murine model that high affinity, somatic hypermutated IgM+ MBCs dominate a recall response, being differentiated either into IgM+ or IgG+ ASCs and MBCs (21). The higher the frequency of the antigen-specific MBCs during that second encounter with the antigen, the higher will be the frequency of the antigen-specific ASCs generated. It is still controversial whether the unswitched or switched MBCs enter the GCs or form the ASCs: [reviewed by (22)]. Once generated, the ASCs migrate through circulation to the bone marrow or secondary lymphoid organs. More specifically, their physical contact with bone marrow stroma cells and the recognition of cytokines described above lead to modifications in their transcriptome profile, upregulating preferentially the expression of anti-apoptotic genes. This process culminates in their transformation from short-lived to long-lived ASCs, whose function results in the increased titers of serum antibodies (23).

On the other hand, malaria infection affects the generation of some critical cell subsets for humoral responses. Repeated parasitic exposures drive the expansion and accumulation of atypical MBCs in individuals from Pf malaria-endemic areas (24, 25). Although these cells have been mostly associated with the impaired B cell responses in this infection context, some groups have stated that atypical MBCs present a similar function as classical MBCs (26). Furthermore, this was demonstrated in an impaired GC response in a murine model of severe malaria due to the inhibition of TFh cell differentiation (27).

In addition, the murine conventional DCs presented lower BAFF expression, culminating in a reduced ASC number in the spleen (28). Noteworthy, the bone marrow ASCs have serious restrictions for sampling due to their location, being avoided in malaria clinical trials. This issue has impaired our complete understanding around the immune response triggered by malaria infection in humans.

ACTIVATION OF B CELLS

Similar to pathogenic infections such as *Trypanosoma cruzi* (the etiologic agent of Chagas disease) or HIV, malaria infection elicits the polyclonal activation of B cells. Among the major factors contributing to this condition are the parasite-specific antigens and cytokines. Consequently, malaria patients present hypergammaglobulinemia, i.e., the increased serologic IgG titers. Furthermore, asymptomatic individuals with high parasitemia usually display broader antibody responses than the asymptomatic individuals with low parasitemia or symptomatic individuals (29). Thus, the malarial parasite load derived from the acute phase of infection seems to drive the ASC response as described for HIV and SIV (30) and Silveira et al., manuscript in preparation. A primary parasite exposure elicits the activation and differentiation of naive B cells into *Plasmodium*-specific MBCs and ASCs. These antigen-specific MBCs, activated through engagement of BCR or Toll-like receptors (31), can also be differentiated into ASCs, enhancing the antibody secretion upon Pf infection (32–35). Alternatively, MBCs induced by *S. mansoni* worms may cross-react with Pf antigens and become activated in an malarial-specific manner (36, 37). However, this humoral response does not reach enough concentration in the serum to provide protection. As already mentioned, the individuals from malaria-endemic areas develop an antibody-derived natural immunity only after multiple parasite exposures.

Among the several potential parasite antigens that could induce hypergammaglobulinemia under infection, the domain C1DR1a of EMP1 from a cloned strain (FCR3S1.2), a blood-stage antigen, has been identified in Pf (38). It remains elusive whether the same antigen derived from Pf isolates can also trigger hypergammaglobulinaemia. Interestingly, the domain C1DR1a of PfEMP1 preferentially promotes the MBC activation and proliferation (39). Another *Plasmodium* variant antigen (MSP-1) has been described to activate antigen-specific IgM+ MBCs as the earlier responders upon a malaria re-challenge in mice. Both IgM- and IgG-secreting cells can be generated from the differentiation of those IgM+ MBCs (21). It is still obscure whether the domain C1DR1a is capable of eliciting a similar response during malaria infection in humans. Considering that autoimmunity has been commonly detected during malaria infection as a result of hypergammaglobulinemia, molecular mimicking cannot be ruled out for parasite antigens. In fact, the serum samples of systemic lupus erythematosus (SLE) patients displayed the ability to recognize the Pf malarial antigens (40). Cardiolipin, histones, and DNA are among the auto-antigens usually targeted by the anti-*Plasmodium* antibodies (41).

Another important piece of the polyclonal B cell activation puzzle is related to the modifications in the cytokine profile during malaria infection. BAFF is known to support B cell differentiation into ASCs and potentially elicit hypergammaglobulinemia. Increased levels of this cytokine have been found in the plasma of volunteers upon the Pf challenge (42), in acute Pf-infected children (43), in pregnant women (44), and in Pv infection (45). Moreover, the IL-10 has shown increased levels detected upon the Pf or Pv infections (46) and can influence the serological BAFF levels (47).

B Cell Subsets

Strikingly, human and murine malaria infections strongly alter the composition of B cell subsets. A murine malaria model showed severe reductions in the bone marrow-derived B cell progenitor numbers (48). Alternately, the infection enhances the numbers of atypical hematopoietic stem and progenitor cells (HSPCs) in the murine spleen. Considering their potential to differentiate into B cells and generate the GC B cells, MBCs, and antigen-specific ASCs *in vivo* (49), the HSPCs could repopulate the immune cell repertoire by counteracting the deficiency in B cell progenitors. Besides progenitor B cells, the Pf and Pv infections also affect the frequency of the peripheral B cells. Whereas the kinetics of classical MBCs and ASCs seem paradoxical in infected individuals living in endemic or non-endemic areas, the transitional B cells (TBCs) and atypical MBCs consistently have shown increased numbers in the blood of all individuals (42, 50).

In terms of TBCs, their frequency has been directly correlated to high parasitemias and an impaired immunity to Pf infection (50). Interestingly, the BAFF receptor signaling has been connected to the control of the immature B cell differentiation to TBCs (51). Considering that BAFF levels are significantly higher during the acute phase of Pf and Pv malaria (43, 45), this cytokine could indeed stimulate a stronger TBC proliferation. Regarding the atypical MBCs in malaria, these cells have been mainly characterized as exhausted cells with a decreased capability to differentiate into ASCs and secrete antibodies (24, 25), as for HIV infection (52). However, the monoclonal antibodies cloned out from those cells majorly recognized the Pf-infected RBCs and neutralized Pf parasites. Furthermore, it has been speculated that their contribution for the neutralizing IgG titers would be similar to the classical MBCs (26). Although total atypical MBCs significantly expand upon Pf or Pv malaria infections, parasitic-specific atypical MBCs still present similar frequencies to classical counterparts (26). In terms of longevity, it remains undetermined whether atypical MBCs represent the majority of the malarial-specific MBCs detected years after parasite exposure in primed individuals without reinfection (17). In infected mice, malarial-specific atypical MBCs were recently associated to short-lived responses that were dependent on the presence of the parasite (53). It would be plausible that the atypical MBCs are high-affinity, somatic hypermutated MBCs in human malaria. Indeed, these cells have been described as the first cells to expand during a recall malaria challenge in mice (21) and both cell subsets

can differentiate into IgG⁺ ASCs, strongly related to malaria immunity.

Follicular T Helper Cells

To clear microbial infections, the immune system simultaneously triggers cellular and humoral responses that converge to the onset of a long-lasting, protective response. Although it correlates with the titers of high-affinity, class-switched antibodies, B cells are not the unique players in this scenario. In fact, the generation of these antibodies relies on assistance from a particular CD4⁺ T cell subset called the follicular T helper (TFh) cells. More specifically, the germinal center (GC) B cells physically interact with the activated CD4⁺ T cells in structures of lymphoid organs called follicles. Within the GCs, B-T cell talk leads to B cell activation, which mature through somatic hypermutations in V(D)J Ig genes as well as the Ig isotype switch, and differentiation into ASCs. Importantly, some TFh cytokines have shown to be critical in this process, such as the IL-6 and IL-21 that regulate B cell survival and cell differentiation.

It has been demonstrated that Pv malaria stimulates the expansion of the TFh cells and secretion of the TFh cytokines (54). Interestingly, Pf infection stimulates a less-functional Th1-like Tfh cell subset, whose function does not correlate to ASC differentiation and antibody secretion. Due to the increased secretion of IFN γ and TNF α during the Pf acute malaria, TFh cell precursors increase the T-bet expression and hinder their differentiation into mature TFh cells (55). Moreover, B cells are also affected by that IFN γ secretion, expressing high levels of T-bet and mainly expanding IgG3 class-switched cells that have the phenotype of atypical MBCs (20). Similarly, this issue is also seen in murine malaria models, implicating lower frequencies of the GC B cells and ASCs as well as the decreased antibody titers. Noteworthy, the murine bone marrow reconstituted with T-bet KO CD4⁺ T cells had the TFh cell functionality restored, followed by the elicitation of GC formation and higher antibody titers (27). Alternately, IL-10 signaling restricts the T-bet expression and rescues the GC formation and antibody responses upon malaria infection in mice (56). Hence, the IL-21 has also demonstrated an important role during this process since the IL-21 KO mice showed decreased numbers of splenic GC B cells and ASCs in the bone marrow, lower antibody titers, and, consequently, a failure to control the parasitemia levels upon challenge (57).

Moreover, other factors can disturb the TFh cell differentiation and influence the effectiveness of humoral responses, such as the expression of particular MHC class II molecules by B cells or co-infections. Regarding the MHC class II expression, humanized mice expressing only HLA-DR0401 as the only MHC class II molecules had impaired antibody responses and could not clear parasitemia after a challenge with a strain causing murine malaria. An expanded subset of the regulatory T cells (Tregs) was found to interact with B cells in those mice, rather than the TFh cells. However, the Treg depletion or HLA-DR0401 co-expression with murine MHC class II molecules boosted the antibody titers and, consequently, the parasitemia dropped to undetectable levels in these mice (58). The expression of T-bet and the influence of Th1 cytokines,

such as IFN γ , have been associated with that Treg expansion which impairs the Tfh cell differentiation and survival as well as wanes the secretion of malaria-specific antibodies (59, 60). In terms of co-infections, an acute gammaherpesvirus (MHV68) infection decreased the resistance against a non-lethal malaria in mice. This co-infection diminished the frequencies of the Tfh cells, GC B cells, and ASCs, suppressing the humoral response to malaria (61).

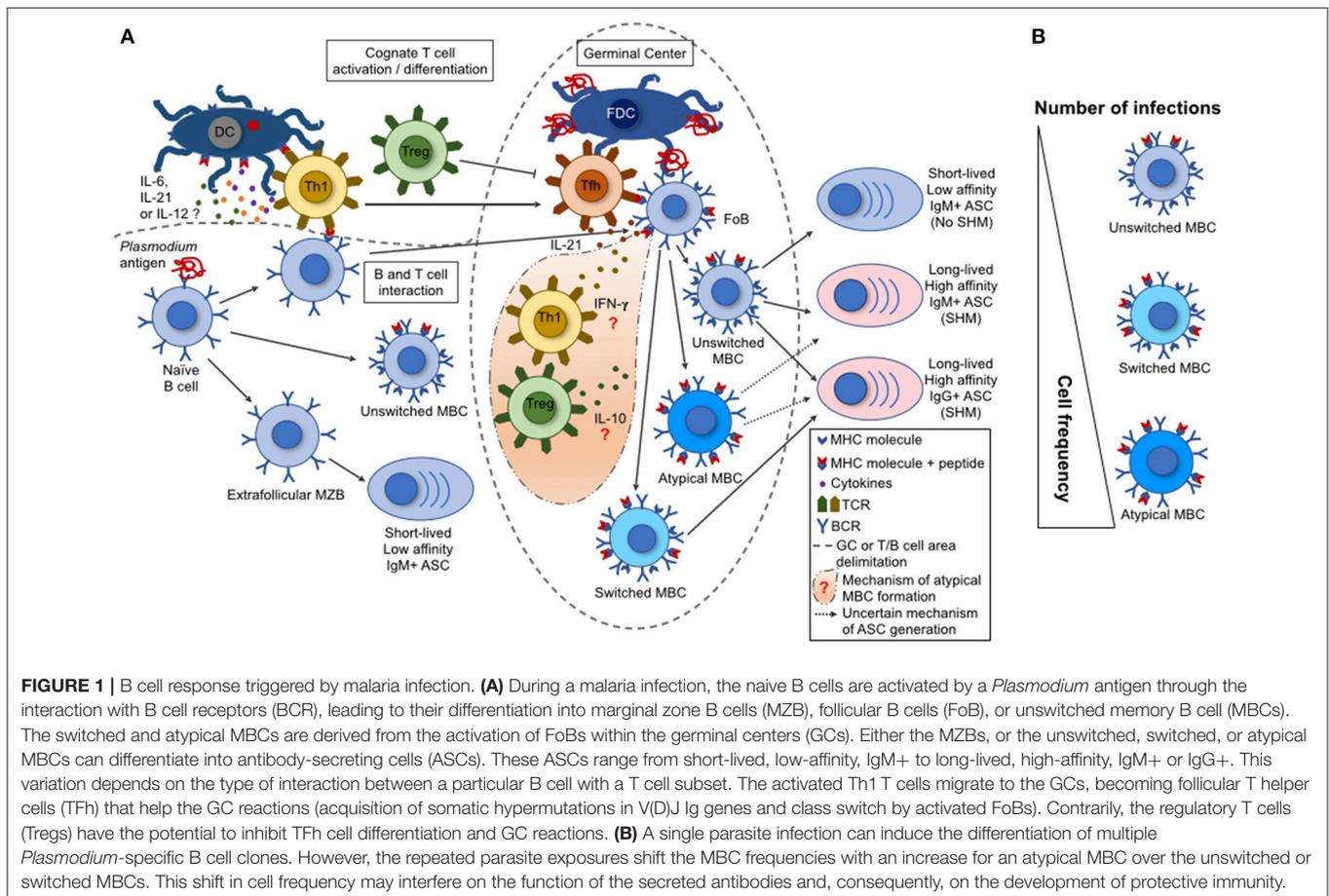
Antibody-dependent Protective Immunity

The malarial-specific antibody responses derived from Pf exposure are usually transient since their titers decrease by the next parasitic transmission season. After their contraction, the antibody levels are still maintained in a higher magnitude than the respective titers detected in the previous parasite exposure (62). For the Pv infection, it follows an opposite pattern (17, 54). However, once secreted at a certain level in the serum, the antibodies can provide protection against human malaria. Serology data against blood stage antigens have determined an inverse correlation between the antibody titers specific for Pf MSP-2-, MSP-3-, and AMA-1 and Pf morbidity in the infected individuals. Thus, an increased breadth of antibody specificity would be associated with a lower chance to experience a clinical episode or be admitted to hospital with severe Pf malaria (63).

To provide antibody-dependent protection, the immune system launches different mechanisms of action upon malaria infection. Considering that the blood stage of infection breaks humoral immunity against the liver stage antigens in murine malaria models (16), malaria-specific antibodies would preferentially opsonize the merozoites. Subsequently, they could trigger effector functions, such as inhibition of cell invasion, phagocytosis, activation of respiratory burst, or complement-derived parasite death [reviewed by (64)]. In the context of the inhibition of cell invasion, it has been challenging to study the breadth of antibody responses which could block sporozoite or merozoite invasion into the hepatocytes or erythrocytes (Pf) / reticulocytes (Pv), respectively. To assess the antigens linked to protective humoral responses against Pf malaria, the high-throughput technologies have been utilized. Among the identified antigens were liver and blood-stage antigens (65). Regarding the reactivity against the *Plasmodium* liver stage antigens, most of such response is driven to the circumsporozoite protein (CSP). The most immunogenic region of Pf CSP for antibodies consists of the central repeat-region. Recently, a Pf CSP-specific mAb (CIS43) displayed a high capacity of parasite neutralization, with its binding region identified in the junction between the N-terminal and central repeat regions of CSP (66). Another anti-Pf CSP repeat-region mAb (2A10) has been shown to elicit protection in mice challenged with chimeric Pb-Pf sporozoites. After being cloned into a adeno-associated virus vector, 2A10 was expressed for long-term and reduced parasite burden, providing protection in mice either by sporozoite injection or mosquito bites (67). The N-terminal region flanking those Pf CSP repeat-regions also possess a linear protective B cell epitope recognized by the mAb 5D5. The antibody-binding inhibits the CSP proteolytic cleavage,

neutralizing the hepatocyte invasion. Whenever administrated in combination with mAb 2A10, these mAbs enhanced the sporozoite neutralization *in vivo* (68). Contrarily, the antibody reactivity had multiple targets against the blood stage antigens. Anti-EBA-175 mAbs (R217 and R218) have been described as inhibitory for Pf invasion in RBCs. Whereas R217, the more inhibitory mAb, engages fundamental antigen residues for RBC binding, R218 interacts with F1 region residues, irrelevant for RBC binding (69). The subdomains I and II of Duffy binding protein (DBP) have also been targeted by the neutralizing antibodies detected in high concentration in the serum of individuals from Pv malaria-endemic areas. Mutations in those antibody sequences accumulate over parasitic exposures, enhancing their breadth and potency (70). Furthermore, although barely recognized during infection even in residents of Pf malaria-endemic areas (71), the anti-RH5 antibodies have demonstrated a great capacity of inhibiting invasion of the Pf merozoites in erythrocytes (72–76). Due to a subcellular location, the RH5 antigen has been detected around the moving junction that is assembled just before the erythrocyte invasion. Thus, RH5 would be accessible to antibodies only during the short contact between Pf merozoites and erythrocytes. Crystallography data showed that anti-RH5 bNabs bind at or close to the basigin-binding site, blocking the interaction

between RH5 and basigin (77) that is critical for Pf merozoite invasion. However, this protein interaction does not seem to be the unique spot for the anti-RH5 mAb neutralizing activity. Considering that distinct RH5-derived B cell epitopes have been described with those bNabs, it suggests that the RH5 sequences may suffer some immunological pressure (78). Other Pf antigens have been described as stimulators of malaria bNabs, such as EXP1, MSP-3, GLURP, RAMA, SEA, and EBA-181. Those antigens were discovered after an investigation of serum samples from the cured Pf malaria patients and individuals with subsequent recrudescence infection. The cured patient samples had higher antibody titers against all those antigens and consequently, a higher capacity to inhibit the erythrocytes invasion by Pf merozoites (79). Recently, a mechanism based on interchromosomal DNA transposition was described as the contributor to the antibody diversity in the context of Pf malaria infection. A DNA insertion from a sequence of a collagen-binding inhibitory receptor (LAIR1) into V(D)J Ig genes was described to generate broad reactive antibodies against the Pf-infected erythrocytes (80). Although these LAIR1-containing antibodies were found in 5-10% of residents of Pf malaria-endemic areas and recognized distinct members of the RIFIN family, they did not confer protection against the disease (80, 81).



Similar to the importance of immunoglobulin variable regions for effective humoral responses, Fc regions also have a fundamental role in mediating protection against infectious diseases. Receptors for immunoglobulin Fc regions have been described to be involved in cellular processes such as phagocytosis, antibody-dependent cellular cytotoxicity, and inflammation, among others (82). In the context of Pf malaria infection, the inoculations of human anti-MSP1₁₉ IgG protected transgenic mice for human Fc gamma receptor after challenge with chimeric Pb-Pf sporozoites. Contrarily, protection was not obtained when the same mAb was tested in non-transgenic mice, suggesting that the antibody interaction with MSP1₁₉ is not sufficient, while the presence of the Fc region is critical for parasite clearance (83). Few studies about human single nucleotide polymorphisms (SNPs) of the Fc gamma receptor have already validated the relevance of this opsonizing antibody-dependent phagocytosis for Pf malaria immunity [reviewed by (64)]. Noteworthy, the merozoite opsonisation has been associated with several conserved antigens and induces immunity against multiple Pf parasite strains (84).

Among the cells that can phagocyte and eliminate an opsonised Pf merozoite are neutrophils through the activation of respiratory burst [reviewed by (64)]. A SNP study has associated the increased levels of nitric oxide (NO) to Pf malaria protection (85). Furthermore, ROS levels have been correlated to natural acquired Pf malaria immunity (86). Once secreted to the extracellular medium, NO and ROS can both dampen the growth of Pf parasites *in vitro* (64, 87).

In terms of antibody-dependent complement activation, complement C1q proteins have shown to be deposited on the merozoite surface after the antibody-antigen interaction, allowing the further formation of the membrane attack complex (MAC) for parasite destruction. MSP-1 and MSP-2 have been identified as the main Pf merozoite targets involved in this protective mechanism. The higher is the C1q deposition, the higher is the protection. Interestingly, older children from Pf endemic areas presented higher C1q deposition than the younger children (88).

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CONCLUDING REMARKS

After decades of suffering with malaria morbidity and mortality in several tropical areas, the at risk-populations are long overdue for effective strategies to contain this epidemic. The development of *Plasmodium*-specific vaccines already tested in clinical trials has not presented a considerable degree of protection yet. Hence, it has been strongly demonstrated that *Plasmodium* parasites have an enhanced resistance against the anti-malarial drugs on a daily basis. Thus, other alternatives of preventative or therapeutic treatments for malaria should be considered. Following all the characteristics presented in this manuscript about malaria-derived B cell immunity (Figure 1), those formulations must prioritize the best conditions for optimal B cell activation and development of the GC, TFh, and ASC responses. In terms of antibodies which are the final and effector products of the potential formulations, there are several reports of neutralizing antibodies identified for human malaria. However, their mechanisms of action have not been elucidated yet. Overall, it has been widely seen that antibody-based prophylaxis or therapeutical approaches possess a great efficacy against multiple pathogens. Therefore, strategies that properly stimulate malarial B cell responses may be beneficial not only in inhibiting infection, but also in reducing the morbidity and mortality numbers and disease transmission.

AUTHOR CONTRIBUTIONS

ELVS and MRD wrote the draft of this manuscript. ISS reviewed the manuscript and contributed significantly to the final draft. All authors read and approved the final manuscript.

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