

## SOLICITED REVIEW

# The spleen: “epicenter” in malaria infection and immunity

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### Abstract

The spleen is a complex secondary lymphoid organ that plays a crucial role in controlling blood-stage infection with *Plasmodium* parasites. It is tasked with sensing and removing parasitized RBCs, erythropoiesis, the activation and differentiation of adaptive immune cells, and the development of protective immunity, all in the face of an intense inflammatory environment. This paper describes how these processes are regulated following infection and recognizes the gaps in our current knowledge, highlighting recent insights from human infections and mouse models.

### KEYWORDS

T cells, B cells, Ab, cytokines, inflammation

## 1 | INTRODUCTION

Positioned on the left side of the body underneath the rib cage and stomach lies an unusual organ known as the spleen. It is roughly the size of a fist or an orange and shaped like an orange slice. Once thought by doctors as being disposable, like the appendix or wisdom teeth, the spleen was often removed from patients during surgery when it was found to be damaged and hemorrhaging. But our opinion of this organ has changed in response to studies conducted in the 1930s, as we now recognize its role in hematopoiesis and removal of old and damaged RBCs from circulation.<sup>1</sup> Most importantly, the spleen functions as a lymphoid organ serving to activate innate and adaptive immune responses and protecting the host against blood-borne pathogens, including the malaria parasite, *Plasmodium* (Fig. 1).

### Abbreviations

ASCs	Ab-secreting cells
DCs	dendritic cells
GCs	germinal centers
pRBCs	parasitized red blood cells
PRRs	pattern recognition receptors
PS	phosphatidylserine
Tfh	T follicular helper cells
Tfr	T follicular regulatory cell
Tr1	T regulatory type 1 cell
Treg	T regulatory cell

The spleen is segregated into 3 distinct architectural zones: red pulp, white pulp, and marginal zone—each capable of performing a multitude of different functions. The red pulp serves as an iron recycling site, and macrophages remove senescent RBCs. These same macrophages are instrumental in the removal of parasitized RBCs (pRBCs) during *Plasmodium* infection. The white pulp, consisting of T and B cell zones, contains the majority of the immune cells within this organ and represents a site for activation of adaptive responses. The marginal zone surrounds the white pulp separating it from the red pulp. It contains a diverse array of immune cells and stromal cells that function to screen the blood for foreign molecules and organisms (Fig. 1, inset 1).

Infection with *Plasmodium* parasites induces dramatic changes in the spleen's size (splenomegaly), architecture, and cellular composition. While this organ serves as the front line for the clearance of pRBCs, it also serves as a site of immune cell activation, hematopoiesis, and the production of protective memory lymphocytes—all of which are occurring in the face of a potentially hostile inflammatory environment. How this organ expands in size and balances these various roles while returning to a quiescent state in the matter of a few weeks is a remarkable feat. Here, this paper will focus on the spleen's position as the epicenter of the immune response to *Plasmodium* infection. Discussion will center on findings associated with the immune response to rodent species of *Plasmodium*. While it is difficult to extrapolate what features of the murine immune response to *Plasmodium* directly correlate to the events that occur in the human spleen, the findings in the rodent models will be related to what is known to occur in the human spleen.

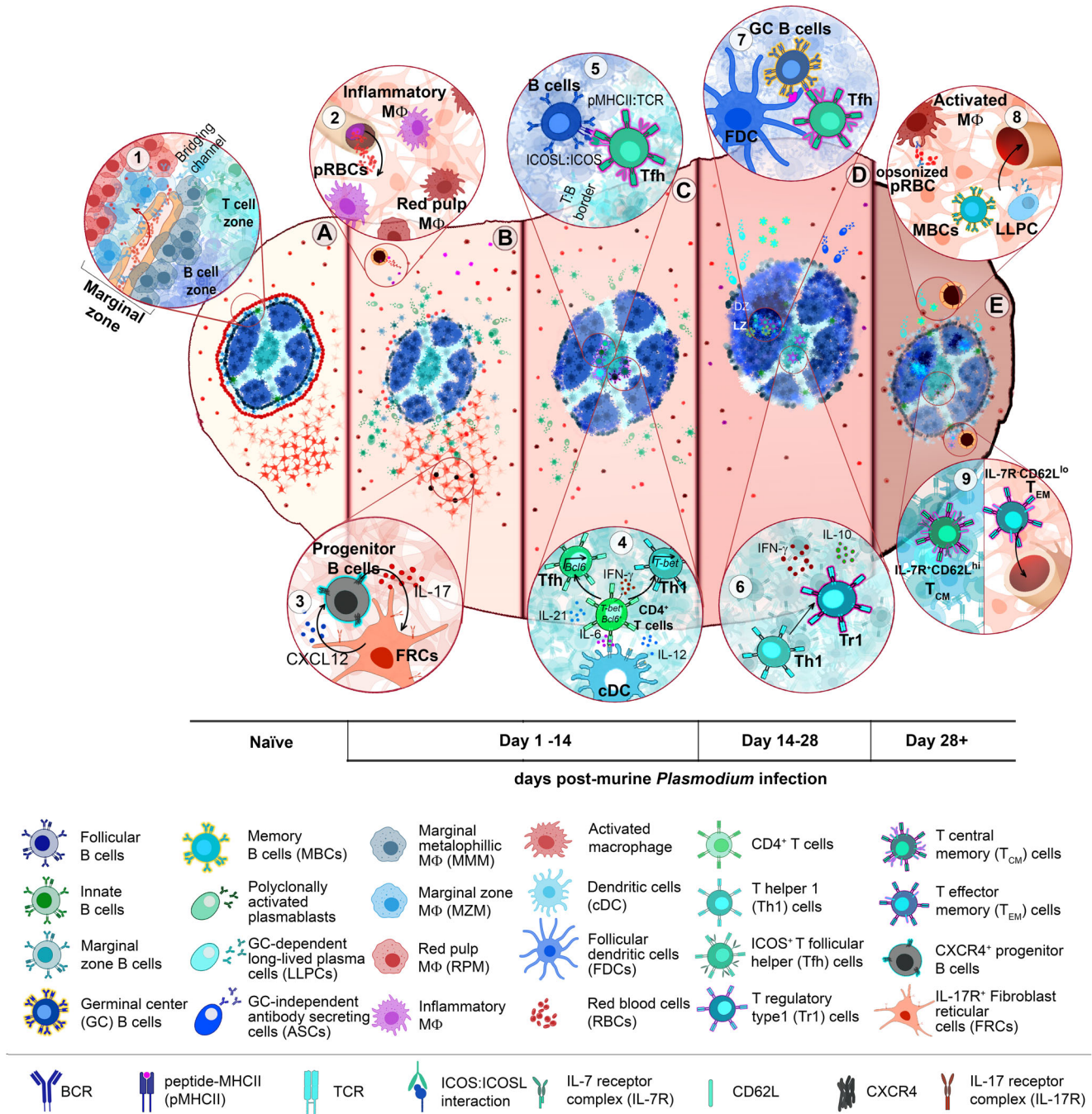
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**FIGURE 1** The spleen is a hub of activity during *Plasmodium* infection. The individual panels display the different structural features of the murine spleen and how they change over the course of a blood-stage infection with *Plasmodium* as denoted by the timeline under the spleen. (A) The white pulp is organized into distinct zones: T cell, B cell, and marginal, in the naïve spleen. Inset 1 denotes the diverse array and organization of macrophage populations in the marginal zone and red pulp and the marginal zone B cells. The red arrow denotes the flow of blood out of an arteriole in this region. Also highlighted is the marginal zone bridging channel where the T cell zone abuts the red pulp. These channels serve as an exit site for lymphocytes to move from the white into the red pulp. (B) During the early stages of the infection, polyclonal activation of B cells leads to their expansion and differentiation into Ab-secreting plasmablasts within the red pulp. Inset 2 highlights the differentiation of inflammatory monocytes recruited from the bone marrow or derived from atypical myeloid precursors in the spleen into macrophages that will aid red pulp macrophages in the phagocytosis and elimination of pRBCs. Inset 3 highlights the atypical progenitor B cells that develop into mature B cells in the spleen during infection with the assistance of CXCL12-producing FRCs. Production of IL-17 by the progenitor B cells stimulates CXCL12 production by the FRCs surrounding the white pulp. (C) Activation of CD4<sup>+</sup> T cells in the white pulp leads to their differentiation into effector phenotypes. Inset 4 displays these early activation events and the emergence of an intermediate T cell with a mixed Th1/Tfh-like phenotype before commitment of the cell toward a Th1 or Tfh cell phenotype. Inset 5 displays migration of CD4<sup>+</sup> T cells to the border of the T:B cell zones where their interaction with Ag-specific B cells, specifically ICOS:ICOSL interactions, facilitates their entry into the B cell follicle and promotes their commitment to a GC Tfh (continued on the next page)

## 2 | ERYTHROCYTIC STAGE OF THE PLASMODIUM LIFE CYCLE AND CYTOADHERENCE

Blood-stage infection with *Plasmodium* consists of defined stages of development of the parasite within the host RBC. Upon entry, merozoites released from the liver actively invade target RBCs after binding and reorienting their apical end toward the RBC membrane.<sup>2</sup> Entry results in the establishment of the parasite inside of a parasitophorous vacuole within the RBC cytoplasm. Here, the merozoite rounds up as the apical organelles break down, leading to the emergence of the early trophozoite or ring stage. Trophozoite enlargement is associated with activated metabolism, including ingestion of host cell cytoplasm and hemoglobin's proteolysis into amino acids to be used in protein synthesis. Hemoglobin degradation occurs within a food vacuole, leading to the polymerization of free heme into hemozoin that will remain stored within the vacuole.<sup>3-5</sup> As the parasite progresses to schizogony, nuclear division and formation of the cytoplasmic organelles occur. New merozoites eventually form with nuclei and other developing organelles migrating into the developing merozoites.<sup>6</sup> The asexual replication cycle takes place over an 18–72 h period, resulting in up to 32 merozoites (both vary between species).<sup>7</sup> Through repeated rounds of invasion and replication, the parasite can establish acute infections that are often associated with high parasite burdens (e.g., *P. falciparum*) and eventually chronic infections in some cases. A subpopulation of merozoites can commit to the production of male and female gametocytes in RBCs. The formation of gametocytes is triggered by factors, including genetic, epigenetic, and environmental.<sup>7</sup> Once ingested by a mosquito, the male and female gametocytes undergo sexual reproduction in the midgut.

RBCs containing ring-stage parasites display minor modifications on their surface in the form of adhesion molecules, thereby allowing them to retain their biconcave shape and permitting them to navigate the splenic cords before returning to circulation. As the parasite shifts to the late trophozoite stage and then on to the mature schizont stage, the number of parasite-derived proteins expressed on the RBC surface increases, and the deformability of the RBC diminishes, increasing the likelihood of the infected RBC being retained and destroyed in the spleen by macrophages.<sup>8</sup> Instead, these forms of the parasite favor

sequestration in small vessels, particularly *P. falciparum*, through a process known as cytoadherence. Expression of Ags encoded by the *var*, *rifin*, and *stevor* multigene families on the surface of RBCs by mature trophozoites and schizonts promote adherence to host receptors (e.g., ICAM-1, CD36) expressed by multiple cell types including endothelial cells, in the peripheral vasculature. Thus, sequestration allows the parasite to complete asexual replication by avoiding removal in the spleen.<sup>9</sup>

Although rings are the most prevalent stage of *P. falciparum* that can be found in circulation, evidence indicates that rings can be retained within the spleen,<sup>10</sup> suggesting that they indeed undergo a degree of modification by the parasite at this early development stage. Furthermore, the deformability of uninfected RBCs is also modified by the parasite, as a proportion of uninfected RBCs are coated in parasite molecules released during invasion.<sup>11-13</sup> Thus, these decorated uninfected RBCs are subjected to retention and destruction in the spleen, which may accelerate the loss of RBCs during infection.

In the case of the sexual stages, mature gametocytes are found circulating in *P. falciparum*-infected patients, whereas immature gametocytes are only seen in circulation in splenectomized individuals.<sup>14,15</sup> Thus, the spleen's presence may influence the ability of immature gametocytes to cytoadhere in the periphery, allowing the spleen only to retain those that do not cytoadhere. In contrast, mature gametocytes may lack cytoadherence and are more deformable compared with immature forms. These traits would allow them to circulate freely and promote their transmission back to the mosquito.

## 3 | THE REQUIREMENT FOR THE SPLEEN IN PROTECTION AGAINST MALARIA

One of the main clinical signs of malaria in humans and mice is splenomegaly (Fig. 1). Adult patients with severe malaria have larger spleens than those seen in patients who died from sepsis.<sup>16</sup> The spleen turns a dark reddish-brown color during infection due to the accumulation of hemozoin in the spleen that is confined mainly in macrophages and intact mature forms of pRBCs.<sup>17,18</sup> The increase in size and weight is associated with cellular expansion in the red and white pulp<sup>16</sup> due to their prominent role during infection. The red pulp is congested with

cell phenotype. (D) During the later stages of the infection, the GC structure takes shape highlighted by distinct dark (DZ) and light (LZ) zones. While Ab-secreting cells derived from outside the GC are present at this time, those derived from the GC begin to emerge. Different functional subsets of CD4<sup>+</sup> T cells become more prevalent at this time. Inset 6 indicates the movement of Th1 effector cells toward a T regulatory type 1 (Tr1) phenotype that serves to limit the Th1 and Tfh effector response through coproduction of IFN- $\gamma$  and IL-10. Inset 7 shows the role that Tfh cells play in the GC response, serving to promote affinity maturation of GC B cells in the LZ that have undergone somatic mutation of their B cell receptors. (E) Following resolution of the infection, the spleen significantly contracts in size. The spleen color changes from a red to a darker reddish-brown due to the hemozoin that accumulates in this tissue, primarily found inside macrophages. The GCs contract in size, and the marginal zone becomes reorganized again. Inset 8 shows the emergence of long-lived plasma cells and memory B cells from the GC. Memory B cells exit this organ via the blood to circulate through lymphoid tissue surveying for Ag. In comparison, plasma cells travel to the bone marrow via the blood to seek a niche for survival and long-term Ab production. Abs produced by the plasma cells that emerge from the GC will help facilitate the clearance of the pRBCs by macrophages following opsonization. Inset 9 displays the distribution of key memory T cell populations. IL-7R<sup>+</sup>CD62L<sup>hi</sup> T<sub>CM</sub> cells are found in circulation and lymphoid tissues and express markers that allow them to traffic through lymphoid tissue. IL-7R<sup>-</sup>CD62L<sup>lo</sup> T<sub>EM</sub> cells will circulate in the blood and possess the ability to enter and exit peripheral tissues to survey for Ag. Individual cell types and key proteins expressed by these cells are highlighted in the key.

RBCs, uninfected and parasitized. Macrophage numbers are enhanced in this region<sup>16</sup> as they are primarily responsible for the removal and destruction of damaged and pRBCs from circulation.<sup>8,19</sup> Foci of Ab-secreting plasmablasts and plasma cells are present in the red pulp during primary and secondary infection,<sup>16,20–23</sup> streaming out of the bridging channels, where the T cell zone abuts the red pulp.<sup>24</sup> Also, the red pulp serves as a major site of erythropoiesis during infection in mice.<sup>25,26</sup> However, defined regions of the white pulp serve as the site of activation, expansion, and differentiation of Ag-specific B and T cell populations that are crucial for controlling blood-stage infection.<sup>27</sup>

Insight into the spleen's importance in malaria control stems from studies conducted with splenectomized mice and monkeys and findings from splenectomized humans. Infection of asplenic or splenectomized mice with *P. yoelii* 17XNL (nonlethal in intact mice) resulted in lower parasite burdens. Still, they could not control their infections within the first month, resulting in a high mortality rate.<sup>28</sup> Similarly, infection of asplenic and splenectomized mice with another nonlethal species, *P. chabaudi adami*, led to an acute patent infection, and in contrast to infection with *P. yoelii* 17XNL, resulted in higher peak parasitemia.<sup>28</sup> Although these mice were incapable of controlling an acute malaria infection, mice devoid of their spleens were resistant to rechallenge with homologous species of parasites after drug clearance of the primary infection. Thus, the spleen is absolutely critical for resolving acute infection but is not necessary for resistance to reinfection. Though the spleen may be essential for protection against acute infection with these nonlethal species of parasites, it can also exacerbate an infection with lethal species as the survival of splenectomized mice is significantly extended after infection with *P. berghei* K173 and *P. yoelii* 17XL. Although, the splenectomized mice do eventually succumb to infection.<sup>29,30</sup> Furthermore, the mouse's genetic background also influences the susceptibility of mice following splenectomy, as DBA/2 mice are resistant to *P. yoelii* 17XNL infection. In contrast, Balb/c and C57BL/6 mice are susceptible,<sup>31</sup> indicating that DBA/2 mice have additional, spleen-independent resistance mechanisms not present in the other mice. Furthermore, the interplay between host and parasite genes may influence susceptibility or resistance of these inbred strains of mice to infection with *P. yoelii* 17XNL.<sup>32</sup>

As mentioned, the spleen serves as the primary location for the removal of pRBCs. But the parasites, particularly the human parasite *P. falciparum*, counter this defense mechanism by cytoadhering to the vasculature through the expression of Ags such as PfEMP1, RIFIN, and STEVOR on the RBC surface, thereby allowing them to sequester in the periphery. Studies conducted with squirrel monkeys highlight that the spleen's presence or absence dictates which Ags are expressed on the surface of *P. falciparum*-infected RBCs.<sup>33</sup> In particular, *P. falciparum* strains passaged in splenectomized monkeys switched off their expression of Ags that promoted their cytoadherence to melanoma cells in vitro.<sup>34</sup> Similarly, Ag expression was also shown to be influenced by the spleen in mice, as the passage of a cloned line of *P. chabaudi chabaudi* AS in splenectomized mice resulted in a loss of sequestration in organs by the parasite.<sup>35</sup> How the parasites first sense the absence of the threat

of clearance by the spleen and then secondly alter their Ag expression in response is unclear. One possibility is that pressure exerted by the immune response triggers changes in gene expression in the parasite as is the case for the *pir* superfamily of proteins,<sup>36</sup> which are expressed by *Plasmodium* species that infect humans, monkeys, and rodents (*P. vivax*, *P. knowlesi*, *P. chabaudi*, *P. berghei*, and *P. yoelii*).<sup>37–39</sup> For instance, differences in the expression pattern of *yir* genes were noted between primary and secondary infection of immunocompetent mice with *P. yoelii*. No change in *yir* gene expression occurred between primary and secondary infection of immunodeficient mice (*Rag2*<sup>-/-</sup>).<sup>38</sup> Alternatively, vector transmission can regulate parasite gene expression during the erythrocytic cycle as in the case of the *P. chabaudi cir* genes<sup>37</sup>; hence, the Ags expressed by blood-stage parasites are predetermined before entry into the mammalian host rather than changing during the erythrocytic cycle.

In support of the latter argument, strong evidence that *P. falciparum* alters Ag expression in response to the absence of a spleen in humans is lacking. During primary infection with *P. falciparum*, the severity of disease (i.e., increased cerebral malaria), death, and parasite burden are enhanced in splenectomized patients. Also, mature forms of the parasite are more frequently found in the blood, suggesting a lack of sequestration or an inability to clear the parasites in the spleen's absence.<sup>15,40–43</sup> In support of the former, a splenectomized patient from Cameroon displayed high parasitemia and the presence of late trophozoites, schizonts, and gametocytes in circulation. RT-PCR analysis on the blood indicated that pRBCs from the patient failed to express members of the *var*, *rifin*, and *stevor* multigene families, and pRBCs failed to bind to host receptors such as CD36 and ICAM-1 in vitro.<sup>15</sup>

In contrast, the expression of surface Ags is not altered on pRBCs from naïve splenectomized patients, and their ability to bind endothelial cells in vitro is unchanged.<sup>40,42</sup> Also, the frequency of cerebral malaria is high amongst these individuals. Thus, this evidence suggests that a lack of clearance rather than a lack of sequestration causes an increase in mature forms seen in splenectomized patients' blood. Moreover, patients living in endemic areas that have undergone splenectomy (i.e., malaria immune) display higher parasite burdens and more frequent fever than spleen-intact individuals. Although these individuals have Abs specific for the parasite, the opsonized parasites are not cleared as quickly from the blood by macrophage populations in the liver and other organs, as can be accomplished by macrophages in the red pulp of the spleen. Thus, the spleen plays an essential role in parasite clearance, even in the presence of preexisting acquired protection. Although they are less efficient, the liver and other organs' contribution to removing pRBCs is essential for reducing the risk of severe disease in the absence of the spleen in malaria immune individuals.<sup>31,44,45</sup>

#### 4 | CHANGES IN SPLENIC ARCHITECTURE

In humans and mice, the spleen undergoes structural changes in response to infection with *Plasmodium*.<sup>16,46–48</sup> Pioneering microscopy

conducted by Leon Weiss and colleagues<sup>19,49,50</sup> provided visual evidence of these structural changes in the spleen of Balb/c mice in response to *Plasmodium* infection. A proportion of the blood traveling through the spleen empties into the red pulp's filtration beds (cords). Here, the vasculature is defined as "open" in that endothelial continuity between the arteries and the veins lacks.<sup>51,52</sup> The filtration beds are composed of a loose network of fibroblastic reticular cells, reticular fibers, and macrophages that serve to shunt blood from the arterioles into the venous sinuses, all the while filtering out and removing damaged and old RBCs and infectious particles from circulation (Fig. 1, inset 2). Upon infection with *P. yoelii* 17XNL, this open circulation turns into a temporarily closed system in mice due to the formation of a physical barrier made up of fibroblasts known as barrier cells.<sup>49</sup> These barrier cells are associated with fibronectin and collagen and were suggested to arise from proliferating cells in the spleen and progenitor cells brought in from the blood, possibly from the bone marrow.<sup>19,49</sup> Barrier cells are also present in the spleen of Balb/c mice infected with *P. c. adami*.<sup>53</sup>

Weiss hypothesized that the formation of the blood-spleen barrier is a defense mechanism of the spleen invoked to protect developing erythroblasts and reticulocytes in the red pulp from parasitization. Consequently, macrophages have limited access to the circulating pRBCs and cannot clear them during this period of the infection. Furthermore, Weiss and others<sup>49,54</sup> noted that pRBCs slow down and adhere in a rolling fashion to the barrier cells in the spleen, but the proteins involved in the cytoadherence remain undefined. Moreover, it is unclear how adherence of the pRBCs to the barrier cells in the spleen modulates parasite load and clearance.

Interestingly, the lifespan of the barrier is brief. Just prior to the onset of crisis (appearance of abnormal parasites characterized by a reduced number of merozoites per segmenter, stunted development to maturation, and intraerythrocytic deterioration<sup>55</sup>), maturation of erythroblasts into reticulocytes is completed, and the barrier dissolves, releasing the reticulocytes into circulation to relieve the anemia. Macrophages are again allowed access to the blood, and the pRBCs are quickly phagocytosed. There is widespread evidence of barrier cell death at this time, but some of these cells persist following resolution of the infection and contain the malarial pigment hemozoin.<sup>56</sup>

Infection of Balb/c mice with the lethal *P. yoelii* 17XL does not invoke the formation of a blood-spleen barrier as RBCs and polystyrene beads are always detectable in the filtration beds after infection.<sup>19</sup> Although activated barrier cells are visible in the spleen after infection with 17XL, they are incapable of erecting a physical barrier. Perhaps the severity of this infection and the subsequent inflammation it induces create conditions unfavorable for barrier formation. Instead, this infection is characterized by a high number of macrophages and many damaged cells in the filtration beds of the marginal zone and red pulp.<sup>19</sup>

In humans, the reticular cells/fibers that make up the filtration bed are highly ordered and close-meshed. The human spleen is an example of a sinusal spleen in which the endothelial cells of the venous sinuses

lie tight against one another. RBCs must squeeze themselves through interendothelial slits, which becomes more difficult with age due to increased rigidity, to enter the venous sinuses. Mice, on the other hand, have a nonsinusal spleen in which the interendothelial slits are more widely spaced, allowing unimpeded blood flow; hence, RBCs are not subjected to the same test imposed by the venous sinus in the human spleen.<sup>56</sup> Whether barrier cells form in the spleen of humans after *Plasmodium* infection is controversial; however, there is evidence of their existence in other pathologies.<sup>50</sup>

What is apparent after *Plasmodium* infection in humans is that the spleen significantly increases in size, and the white pulp becomes disorganized,<sup>16</sup> similar to what is seen during rodent malaria infection.<sup>20,47</sup> Marginal zones are devoid of B cells with no correlation in an increase in B-cell foci in the red pulp, suggesting that the MZ B cells are not migrating to the red pulp and differentiating into Ab-secreting cells (ASCs). As seen in the rodent malaria models, the T cell zone in the white pulp becomes disorganized, with T cells scattered throughout the B cell follicle, white, and red pulp. Unlike rodent models of malaria, no germinal centers (GCs) were apparent in the spleens of these individuals that died of *Plasmodium* infection, which may indicate that the processes of B cell activation, migration, and maturation are perturbed in cases of severe disease. However, there is much to learn about the impact of *Plasmodium* infection on splenic architecture and function in man, as the spleens analyzed in the Urban et al.<sup>16</sup> paper represent possible extremes in which the host response failed to prevent fatal infection. It is likely that splenic changes in patients capable of resolving the infection or remaining asymptomatic for disease are quite different. For instance, GCs are likely to be visible in the spleen of individuals with mild or asymptomatic infections.

Furthermore, the factors contributing to these splenic architecture changes remain undefined, although several candidates were evaluated. For instance, treatment of mice with a lymphotoxin  $\beta$  receptor agonist Ab restored lymphoid architecture in the spleen after *T. gondii* infection.<sup>57</sup> However, a similar effect was not seen after *P. yoelii* 17XNL infection.<sup>47</sup> Alternatively, the absence of IFN- $\gamma$  signaling restored some of the humoral response after *P. yoelii* 17XNL infection.<sup>47</sup> It is clear there are other players at work. Simultaneous blocking of IFN- $\gamma$  and TNF with neutralizing Abs improved splenic architecture, with increased T cell retention in T cell zones and improved GC structures within B cell follicles.<sup>58</sup> A potential consequence of this splenic disorganization is that interactions between cells may be disrupted, including T:B cell interactions, which require sustained interactions to promote humoral immunity, and it is clear that acute malaria infection delays the kinetics of the humoral response after *Plasmodium* infection.<sup>47,58,59</sup>

## 5 | EXTRAMEDULLARY HEMATOPOIESIS

A common consequence of acute infection with *Plasmodium* is anemia.<sup>60</sup> Many factors contribute to the onset of this condition,

including phagocytosis of infected or deformed RBCs, retention of infected RBCs in the spleen, and a shift in hematopoiesis in the bone marrow.<sup>61</sup> The inflammatory response associated with this infection causes a change in the demand for myeloid cells, which are necessary for the clearance of infected RBCs. Consequently, the capacity of the bone marrow to produce erythrocytes and lymphocytes is reduced.<sup>62,63</sup> Instead, the spleen takes on a larger role in erythropoiesis during active *Plasmodium* infection, at least in mice,<sup>25,26,61,64</sup> as a higher total number of CFU-E and BFU-E are seen in the spleen with infection.<sup>25,26</sup> Within the mouse, splenic erythroid progenitors that differ from those that maintain steady-state erythropoiesis in the bone marrow are responsible for de novo erythrocyte production in response to anemia.<sup>65</sup> It is unclear if these atypical splenic erythroid progenitors are involved in erythropoiesis in the spleen during *Plasmodium* infection.

The mouse spleen compensates for the loss in bone marrow erythropoiesis during *Plasmodium* infection. However, it is unclear if a similar mechanism occurs in the human spleen, as no studies to date have investigated extramedullary erythropoiesis in this setting. The human spleen is implicated as a site for extramedullary hematopoiesis in cases of stress erythropoiesis that occur in response to inflammation,<sup>65</sup> suggesting it could perform a similar role in response to *Plasmodium* infection.

In addition to supporting erythropoiesis, the mouse spleen can promote other forms of extramedullary hematopoiesis, including myelopoiesis during active infection with *Plasmodium*. Infection with *P. c. chabaudi* causes a depletion in myeloid-erythroid precursors and common lymphoid precursors within the bone marrow. In their place, an atypical progenitor cell characterized by high expression of IL-7R $\alpha$  and c-Kit appears. These atypical myeloid progenitors are recruited to the spleen from the bone marrow in an IFN- $\gamma$ -dependent fashion where they predominantly give rise to myeloid cells that yield inflammatory monocytes (Fig. 1, inset 2).<sup>62,66</sup> Moreover, these inflammatory monocytes derived from atypical progenitors actively phagocytose infected RBCs and promote parasite clearance.<sup>62</sup>

On top of a loss in common lymphoid progenitors, B cell progenitor populations are depleted in the bone marrow of mice during peak parasitemia.<sup>67</sup> The loss of B cell progenitors in the bone marrow at this time correlates with an increase in a population of developing B cells in the spleen that quickly declines as the infection resolves and homeostasis returns to the bone marrow. Furthermore, mobilization of developing B cells to the spleen is seen in other inflammatory responses,<sup>63,68,69</sup> suggesting that this lymphoid organ possesses the capacity to support development outside of the bone marrow. If B cells can develop in this environment, it is unclear how negative selection would be impacted. If more self-reactive B cells reach maturation in the spleen, this offers a potential link between inflammation and autoimmune disease induction. Moreover, immature/T1 B cells can respond to infection through stimulation of TLRs.<sup>70</sup> The result is the production of low-affinity poly-reactive Abs (some of which are class-switched) during the early stages of the immune response in a similar fashion as marginal zone and B-1 B cells.<sup>71</sup> Whether this happens during *Plasmodium* infection is unknown.

As was the case for myeloid cells, an atypical lymphoid progenitor population emerges within the spleen of mice in response to infection with *Plasmodium* and several other pathogens defined by their expression of Sca-1 and a lack of c-Kit expression.<sup>72</sup> This atypical lymphoid population gives rise to B lymphocytes in the spleen capable of responding to the active infection by differentiating into GC B cells, memory B cells, and ASCs. However, their role in and necessity for parasite control remains unresolved. Similar to other progenitor cell populations,<sup>73,74</sup> these atypical lymphoid progenitors express TLRs, indicating they can detect and respond to pathogen stimuli. Although, no defect in the expansion of these progenitor cells in the spleen occurs in response to *Plasmodium* infection in the absence of MyD88,<sup>72</sup> indicating that other pattern recognition receptors (PRRs) can contribute to the activation and expansion of these cells.

Furthermore, the factors and cells that contribute to the development of B cells in the spleen remain unclear. Although the studies of Ghosh and colleagues<sup>72</sup> ruled out a role for IL-7, Flt3L, and BAFF, they implicated a role for CXCL12 in promoting the development of B cells from atypical lymphoid progenitors.<sup>75</sup> B cell development in the bone marrow is primarily regulated by stromal cell populations that secrete growth factors and chemokines. They also express adhesion markers that facilitate interactions between progenitor and stromal cells. The spleen is enriched for several stromal cell populations that could potentially support extramedullary hematopoiesis. Endothelial and stromal cells can express stem cell factor, and subsets of stromal cells produce CXCL12,<sup>76</sup> 2 factors involved in B cell development in the bone marrow. A key modulator of CXCL12 production, particularly in tertiary lymphoid tissue, is the proinflammatory cytokine IL-17.<sup>77-81</sup> In the study by Ghosh et al.,<sup>75</sup> the authors indicated that atypical lymphoid progenitor cells are the primary source of IL-17 in the spleen during *Plasmodium* infection. Moreover, stromal cells deficient in IL-17R signaling produce less CXCL12 in vitro and are less able to support the development of B cells from the atypical lymphoid progenitor cells.

Hence, they proposed a model in which IL-17 production by lymphoid progenitor cells promotes the secretion of CXCL12 by splenic stromal cells to aid in the tethering of the progenitor cells to the stromal cells, thus establishing a niche in the spleen to promote their development into B cells (Fig. 1, inset 3). Although this study implicated follicular reticular cells as the source of CXCL12, further studies are needed to determine if this is indeed the population of stromal cells that supports B cell development in the spleen during malaria infection and to determine what other factors are involved in this process. The effects of CXCL12 may not be limited to progenitor cells as the delivery of exogenous CXCL12 to *P. berghei*-infected mice increased dendritic cell (DC) recruitment in the spleen.<sup>82</sup>

Although the mouse spleen can support the production and development of these atypical progenitor populations, the ability of similar populations to develop and participate in the control of a blood-stage *Plasmodium* infection in the adult human spleen is currently unknown. However, many studies are investigating extramedullary hematopoiesis under various conditions in humans.<sup>83</sup>

## 6 | GENERATION OF PROTECTIVE IMMUNE RESPONSES

### 6.1 | Activation of T cell responses

Not only is the spleen a generative organ for hematopoietic cells, but it is also the primary site for the initiation of the adaptive immune response against blood-stage infection. Although the parasite resides in RBCs, which are devoid of MHC class I and II Ag processing machinery, CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses are mounted against blood-stage infection.<sup>84</sup> DCs typically play a crucial role in priming T cell responses in the spleen, which is the case for blood-stage *Plasmodium* infection in mice (Fig. 1, inset 4).<sup>84–88</sup> Although multiple DC subsets are implicated in activating T cells at different points of the blood-stage infection, including CD8<sup>+</sup> DCs<sup>84–86,88</sup> and CD8<sup>−</sup> classical DCs<sup>85–87,89</sup> but not plasmacytoid DCs.<sup>85</sup> In the steady-state or early on in infection, CD8<sup>+</sup> DCs are superior to CD8<sup>−</sup> DCs in their ability to process, present, and activate CD8<sup>+</sup> and CD4<sup>+</sup> T cells.<sup>86,88</sup> Furthermore, the depletion of CD8<sup>+</sup> DCs decreased CD8<sup>+</sup> and CD4<sup>+</sup> T cell activation in response to *P. c. chabaudi* and *P. berghei* ANKA infection.<sup>90,91</sup> However, by day 7 post-*P. c. chabaudi* infection, CD8<sup>−</sup> DCs are more efficient at MHC II presentation than CD8<sup>+</sup> DCs, mainly because the latter population shows increased cell death at this time after infection.<sup>86</sup> The early superiority of the CD8<sup>+</sup> DCs in T cell activation may be attributed to their ability to acquire Ag, particularly cell-associated Ag, as CD8<sup>+</sup> DCs only outperformed other DC subsets when Ag was provided in a RBC-associated form, but not when the Ag was soluble.<sup>88</sup>

As for humans, the role of DCs in priming T cell responses is murkier. The majority of the data on DCs from humans come from peripheral blood DCs circulating in patients currently or previously infected with *Plasmodium* or in vitro studies looking at DC responses to parasite-derived material using DCs derived from the peripheral blood.<sup>89</sup> Therefore, the data must be viewed cautiously as the behavior, populations, and phenotype of the DCs present in lymphoid tissue such as the spleen may differ from those present in the blood. Moreover, there is evidence in mice and humans of impaired DC function with blood-stage infection,<sup>92–99</sup> particularly in high transmission settings associated with frequent reinfection and higher parasite burdens.<sup>89</sup>

Lastly, an additional layer to T cell activation is that B cells are sufficient for the activation and differentiation of CD4<sup>+</sup> T cells into T follicular helper (Tfh) cells in response to blood-stage *P. yoelii* 17XNL infection in mice (Fig. 1, inset 5).<sup>100</sup> This result stands in stark contrast to the previously proposed model in which DCs prime the initial activation and differentiation of Tfh cells followed by later maintenance by B cells.<sup>101</sup> Moreover, the depletion of conventional CD11c<sup>+</sup> DCs before *P. yoelii* 17XNL infection impaired T cell activation,<sup>102</sup> suggesting they are the dominant Ag-presenting cell. Although CD11c is expressed by several cell types other than DCs, including B cells,<sup>103</sup> help explain these findings. The Arroyo study indicated that the loss of B cells did not eliminate Tfh cell differentiation, suggesting other cells like DCs work in their place. However, the absence of B cells did significantly reduce the capacity of CD4<sup>+</sup> T cells to up-regulate CXCR5 and

PD-1 at early stages after infection.<sup>100</sup> Thus, B:T cell interactions serve to bias the Th cell response toward the Tfh cell phenotype that typically dominates the CD4<sup>+</sup> T cell response to *P. yoelii* 17XNL. Moreover, when MHC class II expression was limited to B cells, the frequency and number of Tfh cells were not reduced after *P. yoelii* 17XNL infection, unlike infection with LCMV. This result suggests that early Tfh cell differentiation can occur independently of DCs in response to *Plasmodium* infection. Whether the same holds true in humans is still unknown. The prominent role of B cells in promoting T cell activation and differentiation toward Tfh cells may be the immune system's way of dealing with the DC dysfunction that results from this parasitic infection.

### 6.2 | CD8 T cells

Although there is strong evidence indicating that CD8<sup>+</sup> T cells are not required to control blood-stage infection in mice,<sup>104–106</sup> they are activated and differentiated into effector cells capable of producing IFN- $\gamma$  and displaying cytotoxic activity in the spleen after *Plasmodium* infection of mice.<sup>84,107</sup> Furthermore, CD8<sup>+</sup> T cells are implicated as significant contributors to the immune pathology seen in the experimental cerebral malaria model in mice.<sup>108</sup> However, some reports suggest that CD8<sup>+</sup> T cells contribute to the control of blood-stage infection. For instance, in the *P. yoelii* 17XNL model, CD8<sup>+</sup> T cells recognize parasite-infected erythroblasts that express MHC class I,<sup>109</sup> causing externalization of phosphatidylserine (PS) on the erythroblast membrane in a Fas-FasL-dependent manner, leading to their clearance by macrophages.<sup>110</sup> In the case of *P. c. chabaudi*, which causes a persistent relapsing infection after the resolution of acute parasitemia, CD8<sup>+</sup> T cells contribute to the control of the acute and persistent infection in mice.<sup>111–113</sup> Although, similar to findings during chronic viral infections and cancer,<sup>114</sup> these parasite-specific CD8<sup>+</sup> T cells undergo significant PD-1-dependent exhaustion.<sup>112</sup> As *P. falciparum* and *P. vivax* can also infect erythroblasts,<sup>115,116</sup> the involvement of CD8<sup>+</sup> T cells in the protective immune response in humans is feasible.

### 6.3 | CD4 T cells

Although the role of CD8 T cells in protective immunity against blood-stage *Plasmodium* infection is debatable, there is more concrete evidence pointing toward an essential role for CD4<sup>+</sup> T cells in mediating protection against this stage of the infection.<sup>97,105,117–119</sup> After activation, CD4<sup>+</sup> T cells take on several fates, including Th1 or Tfh cells, with the majority taking on the latter fate (Fig. 1, inset 4).<sup>100</sup> To complicate matters, CD4<sup>+</sup> T cells that emerge during the early stages of the infection in mice show a mixed Th1/Tfh cell phenotype. These T cells are characterized by the coproduction of IL-21 and IFN- $\gamma$ , coexpression of the Th1 and Tfh canonical transcription factors T-bet and Bcl6, and the expression of CXCR5 and ICOS markers associated with Tfh cells.<sup>59,120,121</sup> This mixed Th1/Tfh cell phenotype is not unique to the rodent model as they are present in the blood of children from malaria-endemic regions.<sup>122</sup> Further insight into the pathway of CD4<sup>+</sup> T cell differentiation during blood-stage *Plasmodium* infection comes from a transcriptomic analysis. This analysis revealed that Th1 and Tfh cells

emerge from a common intermediate developmental stage that more closely resembles a Tfh signature than a Th1 signature,<sup>123</sup> matching other *in vitro* findings.<sup>124,125</sup> Also, this transcriptomic analysis indicated that T regulatory type 1 (Tr1) cells defined by their ability to coproduce IL-10 and IFN- $\gamma$ <sup>126</sup> are derived directly from Th1 cells (Fig. 1, inset 6).<sup>123</sup>

Interestingly, these T cells with a mixed Th1/Tfh cell phenotype are not necessarily a transient state in development but may also reflect a defined outcome. For instance, circulating CXCR5<sup>+</sup>PD-1<sup>+</sup> Tfh cells present in Malian children without an active *Plasmodium* infection can be subdivided based on their expression of CXCR3, a chemokine receptor expressed by Th1 cells in a T-bet-dependent manner.<sup>127</sup> The CXCR5<sup>+</sup>PD-1<sup>+</sup>CXCR3<sup>+</sup> Tfh cells showed a preference for providing B cell help after *in vitro* reactivation compared with the CXCR5<sup>+</sup>PD-1<sup>+</sup>CXCR3<sup>-</sup> Tfh cells, although the latter population was preferentially activated during acute malaria.<sup>122</sup> Similar populations of Tfh cells were found in the blood of adults infected with *P. vivax*, but differences in functional activity were not assessed.<sup>128</sup> In mice, Th1 and Tfh-like effector and memory CD4<sup>+</sup> T cells also express CXCR3,<sup>129</sup> indicating this chemokine receptor may be a common signature of *Plasmodium*-specific CD4<sup>+</sup> T cells.

Overall, these findings suggest that the model of CD4<sup>+</sup> T cell differentiation is not necessarily strictly linear during *Plasmodium* infection. Instead, it fans out from a common precursor, as indicated by Soon and Haque,<sup>130</sup> and continues to be fine-tuned based on cell-to-cell interactions and the cytokines produced within the local milieu throughout and after clearance of the infection. However, it is not explicitly clear whether the formation of this intermediate T cell population with a mixed Th1/Tfh phenotype is advantageous to the host or a pathway supported by the parasite to suppress protective immunity and promote its persistence.

Importantly, plasticity within the splenic memory T cell pool remains after reactivation, as memory T cells with either a Th1- or a Tfh-like phenotype display overlapping functions during secondary recall responses in mice.<sup>129</sup> Unlike the studies in humans, the Th1-like memory T cells found in this study expressed proteins associated with Tfh cells and provide help to B cells *in vitro* and *in vivo*. They also supported B cell responses more effectively than the Tfh-like memory T cells, suggesting that these Th1-like memory T cells could be harnessed to promote humoral immunity upon reactivation under the right circumstances. For instance, by fostering conditions that favor the expression of genes associated with Tfh cells (e.g., ICOS, CXCR5, CD40L) while limiting the expression of genes related to Th1 responses such as IFN- $\gamma$  these Th1-like memory T cells could favor a humoral rather than a cell-mediated response.

## 6.4 | Ab responses

One of the primary functions of CD4<sup>+</sup> T cells within the spleen is to promote a T-dependent humoral response. The product of this response is Abs, which are critical for the control and clearance of blood-stage parasites in mice and man.<sup>27,131-134</sup> Abs are produced within the mouse model in 2 waves with contributions from the

extrafollicular and GC responses (Fig. 1). The extrafollicular response dominates the early stages of the infection. It is characterized by polyclonal activation of B cells and their differentiation into short-lived plasmablasts that reside within the spleen's red pulp. In contrast, in the latter stages of the infection and after resolution, the GC reaction is the primary source of ASCs and memory B cells (Fig. 1, inset 7-8). Some of these ASCs go on to become long-lived plasma cells that reside in the bone marrow of the host, whereas others are short-lived, turning over in the spleen with the clearance of the infection. In comparison, memory B cells circulate in and out of secondary lymphoid tissues, surveying for Ag and providing an immediate source of Abs upon reexposure to the parasite.

In contrast to the T cell zone, the B cell follicle structure remains intact throughout infection with *Plasmodium*. However, infection with *Plasmodium* results in a perturbation in the B cell compartment in the spleen. For starters, the marginal zone in the spleen disperses after infection in mice and humans.<sup>16,20</sup> Although marginal zone B cell numbers initially expand after infection in mice, their numbers quickly drop off after this wave of expansion and do not return to baseline numbers until after the infection resolves.<sup>20</sup> Whether these cells migrate to other areas of the spleen, differentiate into ASCs, or phenotypically change the markers used to distinguish them from follicular B cells by flow cytometry, is unknown. As these B cells are positioned within a region of the spleen with direct contact with the blood as it filters through this organ, it is plausible that these cells will bind to and detect parasite-derived material. Hence, after their initial expansion, marginal zone B cells could quickly differentiate into ASCs, contributing to early Ab production after infection. Whether these B cells specifically respond in an Ag-specific manner is unclear. Furthermore, their contribution to the GC response is unknown. However, in other models, marginal zone B cells are active participants in the GC response.<sup>135</sup>

As mentioned, infection with *Plasmodium* elicits the polyclonal activation of B cells, resulting in hypergammaglobulinemia, that is, increased serum IgG titers.<sup>136</sup> This occurs in a T-dependent manner, as the loss of T cells diminishes Ab production after infection in mice.<sup>27</sup> It is unclear if a specific Ag induces polyclonal activation of B cells, but activation is not limited to mature B cells as transitional B cells also respond.<sup>137</sup> One potential candidate for this mitogenic response is the C1DR1 $\alpha$  domain of the EMP1 protein from *P. falciparum*.<sup>138</sup> Although the C1DR1 $\alpha$  domain preferentially activates memory B cells<sup>139</sup> in a TLR-dependent fashion,<sup>140</sup> TLR activation is also implicated in the polyclonal activation of B cells in response to *Plasmodium* infection in mice.<sup>141</sup> In particular, activation of TLR9, a DNA sensor, leads to the activation of an atypical population of autoreactive B cells that secrete anti-PS Abs.<sup>141</sup> These auto-Ab-producing B cells express CD11c and T-bet, and they are not unique to *Plasmodium* infection, as they are also associated with age-related and chronic autoimmune disorders.<sup>142,143</sup> Whether this type of auto-reactive response is erroneous or induced by design as a mechanism to recognize stressed RBCs that are infected with parasites is unclear. A consequence of the production of anti-PS Abs is the removal of uninfected RBCs that express PS on their surface in the spleen, thereby contributing to the anemia associated with this infection.<sup>141</sup> Anti-PS Abs may contribute to the anemia seen with



*P. falciparum* and *P. vivax* infection, as IgG auto-Abs against normal RBCs are found at an increased quantity in patients with anemia and are inversely correlated to hemoglobin levels.<sup>144-147</sup> Abs against PS are not the only auto-Abs induced by *Plasmodium* infection, as Abs against nuclear and brain Ags and ssDNA are often commonly found in patients in endemic regions.<sup>148-150</sup> However, the increase in auto-Ab production seen with malaria infection does not appear to be a generalized effect. Increases in auto-Abs against thyroglobulin, smooth muscle, gastric parietal cells, or dsDNA are not found above normal frequency, suggesting that some degree of Ag specificity along with a nonspecific mitogenic signal is responsible for the activation and expansion of these auto-Ab producing B cells.<sup>148,149,151</sup>

The auto-Abs produced during acute *Plasmodium* infection are derived mainly from plasmablasts generated through an extrafollicular response in the spleen, as seen in mice.<sup>21,152</sup> Although it is widely believed that these early plasmablasts play an essential role in providing an initial source of protective Abs against malaria and other types of infections, they may also impede Tfh cell activation and function.<sup>153</sup> Indeed, although no direct evidence of plasmablasts restricting Tfh cell responses was demonstrated, Vijay et al.<sup>21</sup> indicated that abrogation of plasmablast differentiation or deletion of plasmablasts during an established *P. yoelii* 17XNL infection increased the magnitude of the GC response. Furthermore, these authors went on to show that the plasmablasts consume a large quantity of glutamine. Thus, they may serve as a metabolic sink during infection, depriving other B cell populations of valuable nutrients needed for their growth and expansion. Whether the activation and differentiation of B cells into plasmablasts, which are mostly nonspecific for *Plasmodium* Ags, serve a greater benefit to the host or the parasite is still debatable. However, these findings open up the possibility of utilizing interventions to alter host metabolism to suppress plasmablast expansion and support the GC response to promote protective humoral immunity in the host.

## 7 | FACTORS THAT PROMOTE AND RESTRICT THE HUMORAL IMMUNE RESPONSE

### 7.1 | Costimulatory molecules

Expansion and differentiation of B cells into non-Ag-specific plasmablasts is just one of many recently described mechanisms, including costimulatory molecules, cytokines, and transcription factors that restrict the host's humoral response during *Plasmodium* infection. For instance, simultaneous blockade of LAG-3 and PD-L1 in mice increased Tfh cell, GC B cell, and plasmablast numbers and T cell production of cytokines such as IFN- $\gamma$  and TNF, leading to enhanced production of protective Abs and clearance of infection with *P. yoelii* 17XNL.<sup>154</sup> As expression of PD-1 and LAG-3 are associated with T cell exhaustion, blockade of signaling through these receptors restores T cell function in this model and suggests that prolonged *Plasmodium* infection in mice leads to exhaustion of CD4<sup>+</sup> T cell functionality.

CTLA-4 expression on T regulatory (Tregs) cells is one of the primary mechanisms used by these cells to suppress the immune response, as the binding of CTLA-4 to CD80 or CD86 on Ag-presenting cells limits costimulation of CD4<sup>+</sup> T cells. Pathogen-specific CD4<sup>+</sup> T cell numbers plateau or fall off after their initial activation, then rise again before *P. yoelii* 17XNL clearance. Kurup et al.<sup>155</sup> showed that this stall in effector T cell expansion correlated with an expansion in Treg numbers. As a proportion of these Treg cells express markers associated with Tfh cells (Tfr cells) and localize to the B cell follicle, the authors speculated that these Tfr cells limit humoral responses through binding of CTLA-4 to B7 molecules expressed by B cells. Indeed, treatment of mice with an anti-CTLA-4 Ab during infection with *P. yoelii* 17XNL (starting at the point in which CD4<sup>+</sup> T cell expansion halts) enhanced the total numbers of CD4<sup>+</sup> T cells, Tfh cells, and Tfr cells. Additionally, a corresponding increase in absolute numbers of splenic B cells, GC B cells, plasmablasts, and parasite-specific Ab titers was observed. The end result was accelerated control of the infection with anti-CTLA-4 treatment. Even though increases in T and B lymphocyte populations were seen with CTLA-4 blockade, splenomegaly was considerably reduced in these mice. As a result of the spleen's reduced swelling, the white pulp's architecture was not disrupted, as is typically seen with this infection. Hence, blocking Treg cell function during active blood-stage infection results in the immune response's ability to mount a more efficient response to control infection with *P. yoelii* 17XNL.

Furthermore, stimulation of the costimulatory molecule OX40 during active infection with *P. yoelii* 17XNL<sup>156</sup> or the loss of the coinhibitory molecule BTLA<sup>157</sup> can also improve immunity against blood-stage infection. A key costimulatory molecule essential for promoting the GC response is ICOS (Fig. 1, inset 5). This costimulatory molecule is up-regulated on CD4<sup>+</sup> T cells after activation. Although T cells with a Tfh-like phenotype are generated in the absence of ICOS during *P. c. chabaudi* and *P. yoelii* infection, they are not maintained.<sup>59,158</sup> Specifically, these Tfh-like cells do not progress in their development toward a GC Tfh cell phenotype, and GC structures fail to form in the spleen. However, the loss of ICOS does not entirely impair the humoral response, as class-switched parasite-specific Abs are produced. Still, the titers are not maintained long term, resulting in an inability of the mice to resolve their infection with *P. c. chabaudi*.<sup>59</sup> Although, resolution of infection with *P. yoelii* 17XNL does occur when ICOS signaling is blocked in mice.<sup>158</sup> Thus, these results suggest that parasite-specific Abs do not have to undergo extensive affinity maturation in the GC to control infection with *P. yoelii* 17XNL. Yet high-affinity Abs are required to resolve an infection with *P. c. chabaudi*, highlighting the differences in the immune response to these 2 genetically distinct rodent species of *Plasmodium*.

### 7.2 | Cytokines

Cytokines are a key element in regulating the immune response to blood-stage infection, and they also restrain the humoral immune response. In particular, blockade or loss of type I IFN signaling leads

to enhanced B-cell and Ab-responses against *P. yoelii* 17XNL, resulting in the accelerated control of this infection. This finding is due to the ability of type I IFN signaling to limit the accumulation of Tfh and Th1 cells,<sup>158,159</sup> while promoting the accumulation of Tr1 cells that coproduce IFN- $\gamma$  and IL-10 (Fig. 1, inset 6).<sup>159</sup> Coproduction of these cytokines by Tr1 cells is key to their suppressive activity, as only when IFN- $\gamma$  and IL-10 are simultaneously blocked do mice mimic the phenotype observed with anti-IFNAR treatment. However, these 2 studies provided different conclusions regarding the cell type in which IFNAR-signaling occurs to produce the observed phenotype. The Sebina et al.<sup>158</sup> study concluded that IFNAR-signaling via classical DCs is required to limit Tfh cell and GC B-cell responses. In contrast, the Zander et al.<sup>159</sup> study indicated that T cell-intrinsic type I IFN signaling directly induced the Tr1 phenotype, which subsequently limits humoral immunity. Moreover, as neither study ruled out the other option, it is quite possible that type I IFN signaling through multiple cell types can suppress the immune response to blood-stage infection.

Interestingly, although IL-10 showed suppressive activity on the humoral response in the presence of IFN- $\gamma$ , it also can promote humoral immunity to *Plasmodium* infection in mice.<sup>160</sup> Treatment of mice with an anti-IL-10R Ab led to a reduction in GC B cells and parasite-specific Ab production. Intrinsic IL-10R signaling in B cells is key to controlling the parasite and promoting the humoral response through the down-regulation of T-bet expression in B cells. As the expression of T-bet leads to down-regulation of another transcription factor, Bcl-6, and impaired GC B cell development. In contrast, IFN- $\gamma$  suppresses the GC response by promoting T-bet expression in B cells and favoring the production of B cells with an atypical phenotype (CD11c<sup>+</sup>T-bet<sup>+</sup>) and short-lived plasmablasts that do not play an appreciable role in controlling the infection and may instead promote pathology.<sup>21,161</sup> Furthermore, IFN- $\gamma$  and TNF can reduce the activation of Tfh cells skewing the response toward a Th1 response during infection with *P. berghei* ANKA. However, it is vital to keep in mind that IFN- $\gamma$  does promote Ig class-switching to IgG isotypes that promote opsonization and killing of parasites by macrophages.<sup>162-164</sup>

What is unclear at this time is how the activity of IL-10 can modulate between promoting and limiting the immune response during *Plasmodium* infection. As the majority of IL-10 producing CD4<sup>+</sup> T cells coexpress IL-10 and IFN- $\gamma$ , a phenotype that favors suppression of humoral immunity and T cell responses, this suggests that other IL-10 producing cells could be the source of IL-10 that promotes humoral immunity after *Plasmodium* infection. It is also possible that the CD4<sup>+</sup> T cells that secrete IL-10, but not IFN- $\gamma$ , possess a different role in the immune response compared to the Tr1 cells. Alternatively, the timing of IL-10 production after infection could influence its functionality, with IL-10 favoring humoral immunity support early after infection and suppressing T cell responses during the resolution phase of the infection.

Another cytokine produced by CD4<sup>+</sup> T cells critical for humoral immunity to blood-stage infection is IL-21, as mice deficient in IL-21 or IL-21R fail to resolve their infection with *P. c. chabaudi* or *P. yoelii* due to inadequate parasite-specific IgG responses.<sup>165</sup> Mixed bone marrow

chimera experiments indicated that IL-21 production by CD4<sup>+</sup> T cells and IL-21R signaling in B cells was necessary for controlling a persistent infection with *P. c. chabaudi*. Similarly, loss of IL-6 negatively affected parasite control following infection with *P. yoelii* 17XNL due to impaired Ab production, GC B cell development, and ICOS expression on Tfh cells.<sup>166</sup> However, IL-6-deficient mice do not display a defect in parasite-specific IgG production only IgM after *P. c. chabaudi* infection, nor was Tfh cell accumulation impaired. Furthermore, splenic plasmablast formation rather than GC B cell accumulation was more dependent on IL-6.<sup>166</sup> It appears that IL-6 influences early events associated with humoral immunity, including extrafollicular plasmablast development, whereas later production of IL-21 in the GC acts to promote affinity maturation of IgG Abs.

### 7.3 | Transcription factors and signaling molecules

As alluded to above, the transcription factor T-bet strongly influences the events associated with *Plasmodium* infection in mice and men. Although T-bet expression is essential for promoting a Th1 response and promoting class-switching to specific IgG subclasses, it has become scrutinized lately due to its modulation of the humoral response.<sup>160,167</sup> In particular, its ability to promote the production of atypical B cells.<sup>141,161</sup> As highlighted above, IFN- $\gamma$  signaling in B cells stimulates T-bet expression, limiting GC B cell differentiation and GC derived Ab responses.<sup>160</sup> Although this result suggests that T-bet restricts a potentially beneficial response, the expression of T-bet by GC B cells may be advantageous to the outcome of the GC reaction and long-term protection. Approximately 60% of splenic B cells express CXCR3, a well-defined transcriptional target of T-bet, early after infection with *P. berghei* ANKA,<sup>167</sup> indicating that T-bet has a considerable influence over gene expression in B cells in response to *Plasmodium* infection. Furthermore, the T-bet<sup>+</sup> GC B cells that form during *Plasmodium* infection favor localization and a gene expression profile associated with residence in the dark zone. Moreover, T-bet expression reduces the number of low-affinity memory B cell precursors in the GC. It also prevents the premature cessation of GC cycling and exit of B cells from the GC, thereby promoting Ab affinity maturation.

What remains unknown regarding the function of T-bet is how it influences the development and function of atypical B cells, as T-bet expression is not necessarily required for their generation.<sup>168</sup> In addition to IFN- $\gamma$  signaling, *Plasmodium* DNA enhances T-bet expression in B cells in a TLR9 intrinsic manner.<sup>141</sup> As mentioned, many of these B cells are not specific for parasite-derived Ag but instead are autoreactive.<sup>141,145</sup> Importantly, the absence of T-bet expression affects the production of anti-PS Abs but not the quality or quantity of parasite-specific Abs in response to *P. yoelii* 17XNL infection.<sup>141,160</sup> Thus, contrasting with the results observed in the *P. berghei* ANKA model described above.<sup>167</sup> Although these B cells are classified as atypical memory B cells in mice and humans<sup>145,161,169-172</sup> in the setting of a *Plasmodium* infection, it is unclear if they are memory B cells or a subset of activated B cells. Specifically, these atypical B cells are not maintained in mice with the resolution of persistent infection with

*P. c. chabaudi*,<sup>161</sup> contrasting with a finding indicating that T-bet<sup>hi</sup> memory B cells persist in the spleen up to at least 100 days after influenza infection.<sup>173</sup> Thus, calling into question whether this T-bet<sup>+</sup> population of B cells generated after *Plasmodium* infection is indeed a memory B cell population or if they play any subsequent role upon reinfection of the host. Nor is it clear if these cells contributed to the production of protective Abs at some point during active infection.

In contrast, T-bet expression in CD4<sup>+</sup> T cells affects the outcome of infection differently, as the loss of T-bet expression in T cells leads to increased Tfh cell accumulation and an enhanced B cell response in its absence following infection with *P. berghei* ANKA.<sup>58,167</sup> In particular, loss of T-bet expression by T cells leads to increased expression of Bcl-6, which is not surprising given the ability of T-bet to repress the expression of this Tfh cell defining transcription factor and other markers of Tfh cells.<sup>124</sup> However, in response to infection, CD4<sup>+</sup> T cells appear to progress through a transitional stage during their development where they express proteins associated with Th1 and Tfh cells, including T-bet and Bcl6.<sup>124,130</sup> From this transitional stage, it is unclear what factors push the T cell toward terminal differentiation into a Th1 or Tfh cell. Contributing factors may include co-stimulatory signaling, cytokine milieu, and TCR affinity for binding peptide:MHC complexes. Together this data indicates that whereas T-bet expression in CD4<sup>+</sup> T cells limits the magnitude of the GC response, its expression in B cells serves to control the quality of the B cell response.

Other intracellular proteins that shape the humoral response during malaria infection include the SLAM-associated protein (SAP) and Bcl6. Loss of Bcl6 expression in CD4<sup>+</sup> T cells leads to impaired Tfh cell development and GC formation, resulting in the inability of these mice to resolve their persistent infection with *P. c. chabaudi*. Intriguingly, *Bcl6<sup>fl/fl</sup>Cd4-cre* mice failed to produce any *P. c. chabaudi*-specific IgG subtypes, indicating a need for Bcl6 expression by T cells to promote class-switching by B cells,<sup>174</sup> although the mechanism behind this finding requires further clarification. In the case of the SAP-deficient mice, approximately 50% of the mice showed sub-patent parasitemia beyond 100 days after infection with *P. c. chabaudi*, indicating a defect in the complete clearance of parasites.<sup>174</sup> Like ICOS, SAP signaling is not required for the initial development of T cells with a Tfh-like phenotype. Instead, it is necessary to maintain and localize Tfh cells to the GC.<sup>59,174</sup> Unlike *Icos<sup>-/-</sup>* mice, GC center structures were apparent within the spleen of SAP-deficient mice, albeit at significantly reduced numbers.

One other signaling protein found to modulate the balance between Th1 and Tfh cell development during malaria infection is STAT3. As its absence in CD4<sup>+</sup> T cells leads to outgrowth in Th1 polarized cells that express IFN- $\gamma$  and T-bet at the expense of T cells that coproduce IFN- $\gamma$  and IL-21 in response to infection with *P. chabaudi*.<sup>120</sup> Although no defect in GC Tfh cell accumulation was seen, parasite-specific IgG titers and GC B cells were reduced in *Stat3<sup>fl/fl</sup>Cd4-cre* mice. However, the *Stat3<sup>fl/fl</sup>Cd4-cre* mice could control their persistent infection, though some pathology differences were noted. Strikingly, *Stat3<sup>fl/fl</sup>Cd4-cre* mice show enhanced control of parasite burden upon reinfection. A higher frequency of memory T cells in these mice expressed T-bet and IFN- $\gamma$ , suggesting the Th1-biasing of the

memory T cells promoted better control of secondary parasite burden. In comparison, STAT3 signaling in T cells can promote Tfh cell differentiation.<sup>175</sup> However, that does not appear to be the case here, as it primarily served to limit the Th1 response during *Plasmodium* infection.

Overall, these findings indicate that factors that promote a Th1 response while beneficial to promoting cell-mediated immunity also limit and restrict the development of the humoral immune response, particularly the GC response. However, it is essential to understand that the Th1 response, particularly IFN- $\gamma$ , does not entirely suppress humoral immunity but instead delays it. An unfortunate side effect of this delay is that the infection takes longer to resolve itself, and inflammation dominates during the transition from the cell-mediated to the GC response in mice. In the end, parasite-specific high-affinity IgG Abs are produced that help eliminate primary infections and serve as a frontline defense against reinfection. Moreover, memory T cells that express markers associated with a Th1 phenotype (Th1-like) can support Ab production after rechallenge through up-regulation of the cell markers related to Tfh cells.<sup>129</sup>

## 8 | THE ROLE OF INNATE CELLS IN RESOLVING THE INFECTION

In addition to its role in activating the adaptive immune response and promoting the production of high-affinity class-switched Abs, the spleen also serves as the primary site for eliminating parasitized RBCs. Key to this process are neutrophils and macrophage populations, including red pulp macrophages and monocytic populations recruited to and capable of differentiating into macrophages in the spleen (Fig. 1, inset 2). These cells utilize complement and Fc receptors to bind opsonized merozoites and pRBCs, leading to phagocytosis or the release of soluble factors that inhibit parasite growth (Fig. 1, inset 8). A process known as Ab-dependent cellular inhibition.<sup>176</sup> Abs that facilitate opsonic phagocytosis are associated with protection from malaria in children in Kenya and Papua New Guinea.<sup>177,178</sup> Opsonization of merozoites by Abs can promote killing by neutrophils via respiratory burst,<sup>179</sup> although microbial components of neutrophil granules may also be involved in parasite killing.<sup>180</sup> Though there is evidence that neutrophils can kill parasites. Their contribution to clearance of pRBCs in vivo is questionable given the findings that circulating neutrophils of *P. falciparum*-infected children display reductions in their oxidative burst capacity due to expression of heme oxygenase-1.<sup>181</sup> One unique activity of macrophages within the red pulp is their ability to carry out pitting, which is the removal of parasites from inside of RBCs without destroying the RBC.<sup>182</sup> Although this process can occur naturally in the spleen during infection, the rate of pitting increases after antimalarial treatment.<sup>183,184</sup> The importance of this process in clearing infected RBCs is highlighted by findings in asplenic patients in which RBCs containing dead parasites continue to circulate for months after antimalarial treatment.<sup>183</sup>

## 9 | CONCLUDING REMARKS

The spleen is critical for removing damaged and infected RBCs from circulation, activation of malaria-specific T and B cell responses, and serves as a hub for hematopoiesis, all of which contribute to malaria control. Additionally, this organ plays a crucial role in forming the components of the memory compartment that will provide protective immunity upon subsequent reexposure (Fig. 1, inset 8–9). Although significant inroads have been made in understanding these events, numerous questions regarding the role this organ plays in malaria remain unanswered. For instance, the influx of hematopoietic cells from the blood and their subsequent expansion in the spleen contributes to the splenomegaly associated with this infection. Remarkably, at least in mice, this organ's swelling diminishes quickly upon resolution of the infection. Yet, it is unclear how this tissue can swell and shrink over just a few weeks. Does the inflammation associated with this infection trigger a wound-healing program in the spleen that mirrors what is seen in other nonlymphoid tissues? Furthermore, the factors that contribute to the disorganization and reorganization of the white pulp remain unknown. However, the mediators associated with the Th1 response are likely to play a central role in guiding these changes given the recent evidence linking these factors (e.g., IFN- $\gamma$ , T-bet) to the restriction of the humoral response during malaria. Alterations in the composition and number of stromal cells within this organ and their interplay with immune cells may contribute to the spleen's structural changes. Moreover, it is unclear if splenomegaly and the resultant disorganization of the white pulp impact cell migration. Further advances in microscopy techniques will be key to shedding light on these events.

Hemozoin, a byproduct associated with *Plasmodium* infection, remains in the host well after clearance of infection. In mice, free hemozoin can be found in the blood and deposited in the spleen, liver, bone marrow, brain, and lungs, often associated with macrophages that have phagocytosed it.<sup>185,186</sup> In vitro studies indicate that the presence of hemozoin in human monocytes leads to impaired expression of MHC class II, costimulatory molecules, and several adhesion molecules. It also inhibited their ability to differentiate into DCs.<sup>187–189</sup> Therefore, this raises questions about whether this remnant of *Plasmodium* infection negatively impacts the immune response to other infections. Indeed, there is evidence that hemozoin and the molecules bound to it can impair innate immunity against systemic bacterial infections.<sup>190</sup> As hemozoin and the DNA bound to it can serve as a potent activator of TLR9 and other PRRs,<sup>191</sup> it is possible that the hemozoin that lingers after clearance of the infection serves to tolerize or train innate cells to respond differently to other infections or stimuli. Indeed, hemozoin can train monocytes to hyper-respond to secondary stimulation with a TLR agonist through increased production of proinflammatory cytokines, a response that differs from other infectious stimuli.<sup>192</sup> Although this finding hints at the potential of hemozoin to alter gene expression in monocytes in vitro, whether it can do the same in vivo remains unanswered.

Lastly, although studies in mice have aided our understanding of the immune response to malaria in the spleen, it is still unclear if these

findings will translate to humans. Given the differences in the blood flow and organization of the white pulp in the human spleen compared with the rodent spleen, it would not be surprising if the events and the cells that orchestrate the immune response to blood-stage infection differ between the 2 hosts. Although the events that lead to the activation of T and B cells and the formation of GCs, and the disruption of the splenic architecture, are most likely similar between both host species, the influence of environmental factors differs. For instance, it is unclear how exposure to multiple infectious bites from *Anopheles* mosquitoes over the course of a transmission season shapes the spleen's immune response. Does reinfection interrupt ongoing GC reactions, thereby affecting the generation of protective affinity matured Abs in favor of a robust proinflammatory response driven by IFN- $\gamma$ ? Does the trained immunity reported in hemozoin primed monocytes<sup>192</sup> lead to more robust or weakened inflammatory responses to reinfection or infection with other organisms? Children under the age of 5 are at the highest risk for developing severe disease and dying from *Plasmodium* infection.<sup>193</sup> Since the immune system, particularly the adaptive immune system, is still in its infancy and developing at birth, it is unclear how the immune system's immaturity affects the subsequent response to *Plasmodium* infection in the spleen during this stage of life. Whether studies in newborn mice will inform us of the events that occur in the spleen early in humans' lives needs to be addressed. Until we address these shortcomings in our understanding of this disease's pathogenesis, our pathway to developing rational vaccines or therapeutics to limit pathology will continue to be blocked.

## AUTHORSHIP

J.S.S. conceptualized the paper and wrote the original draft of the manuscript. J.S.S. and D.G. wrote, reviewed, edited, and revised the paper.

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## DISCLOSURES

The authors declare no conflicts of interest.

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