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"Using Two Phases of the CD4 T cell Response to Blood-Stage Murine Malaria to understand Regulation of Systemic Immunity and Placental Pathology in *P. falciparum* infection"

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SUMMARY

P. falciparum infection and malaria remain a risk for millions of children and pregnant women. Here, we seek to integrate knowledge of mouse and human T helper cell (Th) responses to blood-stage *Plasmodium* infection to understand their contribution to protection and pathology. Although there is no complete Th subset differentiation, the adaptive response occurs in two phases in non-lethal rodent *Plasmodium* infection, coordinated by Th cells. In short, cellular immune responses limit the peak of parasitemia during the first phase; in the second phase, humoral immunity from T cell dependent germinal centers is critical for complete clearance of rapidly changing parasite. A strong IFN-γ response kills parasite, but an excess of TNF compared to regulatory cytokines (IL-10, TGF-β) can cause immunopathology. This common pathway for pathology is associated with anemia, cerebral malaria and placental malaria. These two phases can be used to both understand how the host responds to rapidly growing parasite, and how it attempts to control immunopathology and variation. This dual nature of T cell immunity to *Plasmodium* is discussed, with particular reference to the protective nature of the continuous generation of effector T cells, and the unique contribution of effector memory T cells.

Keywords

CD4 T cells; T follicular; *Plasmodium*; Cytokines

1. INTRODUCTION

CD4 T helper (Th) cells are required to control both parasitemia and pathology in *Plasmodium chabaudi, yoelii*, and *berghei* infection of mice, and CD4 T cell expansion correlates with protection in human *P. falciparum* infection. Several mouse studies have shown the effect of CD4 T cells in combination with B cells and phagocytes in protection from malaria in mouse models [1–3]. Upon infection with *Plasmodium spp.*, many of the

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known innate immune sensors are triggered, and multiple adaptive immune mechanisms are activated. Many of these mechanisms are coordinated by CD4 Th cells, a regulatory cell type. Their predominant effector mechanism is production of secreted cytokines, which have functions both locally and systemically. Therefore, understanding the mechanisms by which CD4 Th direct the immune response can suggest points of potential intervention upstream of many other cell types. Th cytokines are important in response to *Plasmodium* for both helping B cells to make antibody, and activating innate defense mechanisms of parasite killing. Systemic cytokine production, such as that seen when parasitemia reaches high levels, is responsible for the induction of host defense mechanisms that can also cause disease [4]. Despite this tremendous response, without treatment, infection with *Plasmodium spp.* becomes persistent. *P. falciparum* can be persistent for at least a year in humans [5,6]. Persistence is also evident in other settings of co-evolution, such as in the thicket rats (*Thamnomys*) where the parasites now used in mouse models were originally discovered [7]. In response to the urgent need to control the parasite to prevent lethal parasite-driven damage, the mammalian host has evolved a two-phase immune response to *Plasmodium* infection, which we will describe in detail in this review. The first phase following innate responses that fail to eliminate parasite is a strong cellular response, followed by a prolonged humoral response that can completely clear the parasite.

In response to infection with *Plasmodium spp.*, acute phase protein production includes complement [8], platelet activation [9], and coagulation cascade proteins induced in the liver [10,11]. In addition, endothelia are activated to make cytokines and coagulation regulators by local immune cell and parasite adhesion, as well as circulating cytokines. Inflammatory cytokines increase tissue surveillance by upregulation of adhesion molecules and chemokines. The early CD4 T cell response to blood-stage *Plasmodium* infection promotes increased phagocytosis in the spleen and liver [12]. This increase is due to the combination of recruitment of innate cells, such as CD8 DCs, to sites of parasite killing, and activation of phagocytosis and parasiticidal effector molecules within immune and structural cells [13]. Unfortunately, while this coordinated effort activates a diverse set of effector mechanisms, these early T cell responses also result in significant damage to host tissues. The spectrum of malaria disease ranges from mild to severe, where multiple organs can be affected at once, however, Th cells also regulate inflammation. In this review, we will describe studies performed in mice and humans to understand the role of CD4 T cells in coordinating this balanced response to infection with *Plasmodium spp.*, and how these cells contribute to pathology and/or protection from infection and disease.

Most findings in *P. chabaudi* infection of mice regarding adaptive immunity and pathology have been verified in *P. falciparum* infection [14]. *P. yoelii* has increasingly also been used to study basic immunology of *Plasmodium* infection, and while many features of the immune responses to these two rodent pathogens are similar, we also highlight responses that appear to be different. In this review, we will progress from what is known about the earliest activation of T cells, through interactions with B cell biology, to generation of memory T cells. Through the lens of cytokines produced by CD4 T cells, we will attempt to integrate the known mechanisms of pathology caused by T cells with what is known about how malaria disease pathology occurs and is regulated. Along the way, we will identify some of

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the many unknowns and the prognosis for this basic knowledge to be applied to successful vaccination.

2. SPECIFICITY OF THE RESPONSE TO *PLASMODIUM*

2.1 T cell detection in *Plasmodium*-immune subjects

Since the early 1990's it has been possible to detect T cell responses to *P. falciparum* in humans. Peptides have been identified from parasite antigens to which there are antibodies in the serum of exposed people, and various assays used to detect them. While proliferation in response to a peptide indicates the presence of specific T cells, many studies relied instead on cytokine production. Different cytokines in response to these peptides were found to correlate with different aspects of the response and clinical symptoms of malaria, and not necessarily to *in vitro* proliferation [15]. For example, in 1990, IL-4 in response to peptides from surface antigen RESA, was found to be correlated to the serum antibody [16], in agreement with the Th1/Th2 paradigm, first described in 1986. *Plasmodium*-specific CD4 T-cell responses was further demonstrated later by several studies both in human and murine model using intracellular staining by multi-parameter flow cytometry [17–19]. Studies in mouse models have made strides towards understanding the role of T cell specificity in the response to *Plasmodia* since generation of the first T cell receptor transgenic mice for CD4 and CD8 T cells specific for malaria [2,20–22], and some mouse MHC tetramer reagents [23]. However, the protective CD4 T cell epitopes, if there are specific protective ones, have not been identified in either mouse or human.

Overall, 596 epitopes have been identified for $CD4^+$ T cells and 147 $CD8^+$ epitopes from the *Plasmodium*-specific response in humans and mice [24]. However, the nature of specificity of the natural T cell response in terms of contribution of each epitope response to protection is less well understood. It would be useful to identify potentially protective T cell epitopes that could stimulate IFN-γ, and also help B cells make protective antibodies. This would have to be done by determining all peptides generated by eluting peptides from human antigen presenting cells MHCII from immune individuals and testing them for protection. However, thus far, it has not been feasible to test overlapping peptides from all 5,369 predicted proteins of *P. falciparum* for stimulation and protection. Due to the requirement for both B and T cell responses, it is challenging to prove that a given CD4 T cell specificity is particularly protective. Due to the mechanisms of phagocytosis and antigen presentation, T cells can recognize peptides from proteins even inside the parasite, leading to a likelihood of their recognizing a broader repertoire of proteins than B cells from the first infection. In addition, internal epitopes are likely to have less variation due to the reduction in selection by antibody-mediated neutralization and other clearance mechanisms. It should be noted that more conserved T cell epitopes could be used to enhance protective B cell epitopes. In addition, epitopes that are conserved are generally selected by an essential function, and immune responses that can target such a critical function are much more likely to be successful.

There are two primary mechanism employed by the parasite in addition to the sheer number of proteins. As an example, merozoite surface protein-1 (MSP-1) has been very well studied. Very few T cell epitopes come from the protective B cell conformational epitope in the C-

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terminus of MSP-1. While tight conserved essential structures ~~is~~ are challenging for antigen presenting cells to digest, the looser external structure is easier [25,26]. Similar camouflaged invasion mechanisms in other parasites and viruses suggest that hiding essential epitopes is a great evasion strategy. In contrast, the exposed and, hence, more variant regions are more likely to induce antibodies. Importantly, there is more latitude than commonly considered for finding T cell helper epitopes. Even though the B5 transgenic T-cell receptor (TCR) recognizes MSP-1, B5 CD4 T cell epitope is on an exposed fragment of MSP1 that is shed into the serum each time merozoites invade, while the protective 19-kDa C-terminal region of MSP-1 (p19) remains attached within the infected red blood cell, B5-specific T cells are able to help MSP-1 p19-specific B cells [2]. Unfortunately, this looser conformation also imparts more latitude for variation in the sequence of the T cell epitopes. Variation can affect the T cell response, with some alternate versions of epitopes even inhibiting other variants [27]. It would be interesting to understand the mechanisms involved in B cell help from T cells when the epitopes are not physically connected. While soluble antigen is presented to B cells by follicular dendritic cells and subcapsular macrophages, the mechanism of large antigen capture by B cells remains obscure [28]. A recent paper has shed some light on B cells capturing multiple antigens from infected cells that are nearby in space on the membrane, rather than part of the same amino acid chain [29]. This suggests that a B cell with a B cell receptor (BCR) that recognizes merozoite surface proteins can be helped by T cells recognizing any other merozoite surface proteins. In addition, DCs may be able to present antigen in immune complexes to B cells in the marginal zone by a newly described pathway using FcγRIIB that seems to bypass T cell tolerance mechanisms [30]. As T cell epitopes are less subject to selection than exposed B cell epitopes, adding T cell epitopes from less exposed sites could be a powerful method of improving vaccination for broadly neutralizing antibodies. Further study using functional assays to determine the protective contribution of particular epitopes will be essential in building up an armory of protective and immunogenic epitopes for the next generation of multi-stage, multi-antigen malaria vaccines.

2.2 Polyclonal adaptive immune activation in *Plasmodium* infection

The co-evolution of *P. falciparum* with *H. sapiens* is evident in the modern immune response to infection [31]. This process has resulted in some helpful alleles, including germline T cell receptors and natural IgM responsive to infected red blood cells [32–35]. Upon infection with *Plasmodium chabaudi* or *yoelii*, about half of the immune repertoire gets activated [36,37]. This has been observed for both CD4 T and B cells. This massive activation may be due to the huge number of proteins expressed by *Plasmodium* (from an estimated 5,369 genes [38,39]), compared to a bacterial or viral pathogen. In addition, the immunodominant repeat regions, and the switching of surface proteins with variant domains add a huge variety of unhelpful specificities. There are also polyclonal B and T cell stimulatory molecules in *P. falciparum*, which could account for this large non-specific response [40–43]. Antibodies that recognize non-*Plasmodium* antigens have even been measured in serum from *Plasmodium*-infected animals [44]. The B cell polyclonal stimulator has not been specifically identified yet. However, PfEMP1 can induce IL-12 and IL-18 from antigen presenting cells, which stimulate IFN-γ, even in the absence of MHCII stimulation [42].

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2.3 The three signals of T cell activation in response to infection with *Plasmodium spp.*

Activation of CD4 T cells during *Plasmodium* infection promotes parasite-specific B cell activation [1,45], and phagocytosis of the parasite by secretion of Th1 cytokines by cognate interaction of the T cell receptor (TCR) with the major histocompatibility complex class II (MHCII) [3]. However, these interactions are likely to be obscured by the large polyclonal response described above. Interestingly in this regard, it was found that a vast number of CD8 T cells would be required to be generated to find all infected hepatocytes in *P. yoelii* infection and affect parasitemia [46]. Given the less direct action of CD4 T cells compared to CD8, through cytokines rather than cytotoxicity, it is not known if the same barrier exists for CD4 T cell numbers, which generally proliferate less [47].

Three signals are required for Th cell stimulation in general: MHCII/TCR, co-stimulation, and cytokines. However, the sequence of events leading to T cell expansion and generation of effector and memory T cells in response to *Plasmodium* infection are under investigation. CD28 is the primary co-stimulatory molecule in the dendritic cell T cell interaction, binding to CD80 and CD86. CD28 is known to be responsible for recruitment of many of the transcription factors required for IL-2 production [48,49]. IL-2 promotes T cell expansion; however, though CD28 is a costimulatory signal thought to be required for IL-2 production, $CD28^{-/-}$ mice infected with *P. chabaudi* have similar numbers of CD4 T cells on days 7 or 11-13, suggesting that costimulatory pathways other than CD28 may contribute to T cell activation [50,51]. In infected $CD28^{-/-}$ mice, IFN-γ is significantly reduced compared to wildtype, as is switching to IgG2a/c, a critical immunoglobulin isotype in parasite clearance [52]. In addition to CD28, T cells in mouse *Plasmodium* infections upregulate several co-stimulatory and inhibitory surface molecules [22,51]. Expression of co-stimulatory molecule OX40 is beneficial for co-stimulation of T cells, regulating the size of the effector T cells (Teff) response to *P. yoelii* [53,54]. ICOS expression, while known for its enhancement of Tfh function, inhibits control of peak *P. chabaudi* parasitemia, while anti-ICOS prolongs *P. yoelii* infection suggesting an important immunoregulatory role for this molecule that differs in the two infections [55].

As in other infections, the co-inhibitory molecules programmed cell death-1 (PD-1), B and T lymphocyte attenuator (BTLA), and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) are upregulated on T cells upon *Plasmodium* infection. Inhibition of these molecules demonstrates the extensive network and successive waves of regulation involved in regulating the balance of parasiticidal and immuno-pathological outcomes of *Plasmodium* infections. While there is PD-1 expression on T cells in all *Plasmodium* infections studied to date, it may represent a baseline level that is present on CD4 Teff [56,57]. The most striking finding about T cell co-receptors in infection is that while blocking the inhibitory co-receptors PD-1 and LAG3 inhibited T cell mediated control of the peak parasitemia in *P. yoelii* [58]. PD-1 signaling on the other hand allows persistence later on in *P. chabaudi*, and this is modulated by PD-L2 [59]. CTLA4 on regulatory T cells (Treg) cells during priming was shown to play a critical role in regulation of the T cell response in the second week of *P. yoelii* infection [60]. BTLA, highly expressed on B and T cells, also has a regulatory effect that should continue to be studied, as it is similar to PD-1 in that it directly affects TCR signaling [61] and parasite clearance. BTLA deficient mice controlled *P. yoelii* strikingly well

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due to intrinsic effects on both B and T cells affecting both antibody and IFN-γ, suggesting a strong inhibitory effect by BTLA throughout infection [62]. In summary, co-stimulatory and inhibitory receptors are acting in concert and competition to regulate the very strong multi-modal activation initiated by *Plasmodium* infection. However, there may or may not be enough overlap between mouse models to inform human malaria therapeutics.

The third required signal in T cell stimulation is cytokines. The innate cytokine response is critical for regulation of the size and effectiveness of the T cell response. Early host factors like IFN-γ from NK cells and Type I IFN-γ from plasmacytoid cells are critical mediators of adaptive immunity [63–66]. Each parasite strain and species appears to induce a variety of early responses from different cell types [67–69], and research urgently needs to be done about the parasite alleles that can regulate innate responses changing the adaptive response. IL-2 production by CD4 T cells regulates the Treg/Teff ratio, and is required for optimal T cell expansion. However, IL-2 may be produced at low levels in *Plasmodium* infection, likely reducing levels of IFN-γ produced [70,71]. IL-12 made early on by innate cells like macrophages and dendritic cells is the predominant determinant of Th1 differentiation, and also promotes IL-10 production in Th1 cells, as does IL-27 [72,73]. Unexpectedly, IL-27 is made by a subset of T cells in *Plasmodium* infection and inhibits T cell expansion. It has been suggested that IL-27 expression reduces IL-2 which also affects later IFN-γ production, thereby inhibiting the ability of the immune response to control the peak of parasitemia [70,71]. IL-27 also regulates IL-10 production by T cells later in infection [74]. Many aspects of the T cell cytokine response have been recently reviewed [58]. Both host and parasite factors regulate the size of the T cell response. For example, parasite-encoded Macrophage Migration Inhibitory Factor (pMIF) interferes with T cell activation dramatically, including driving upregulation of inhibitory co-receptor PD-1 [75]. There should be a concerted effort to understand other mechanisms of parasite virulence using immune-evasion, for example, screening a library of parasites mutated one gene at a time to discover parasite genes that drive the compromise with the host and thereby determine host morbidity and mortality.

2.4 The cellular activation pathway of effector CD4$^+$ T cells in response to *Plasmodium* infection

In order to begin to understand the process of CD4 T cell activation in *P. chabaudi* infection, we studied surface phenotypes and identified a progression of T cell phenotypes, from more to less pluripotent, and from less to more terminally differentiated [76]. We hope that this cellular activation pathway will promote further study of both CD4 T cell effector and memory development in the context of *Plasmodium* and other infections. Using this new set of markers, one can separate terminally differentiated effector T cells, from those that are still expressing higher levels of anti-apoptotic genes. More work is needed to understand mechanisms driving the transitions from quiescent Early Teff ($CD127^{lo}CD127^{-}CD62L^{hi}$) to either proliferating Intermediate and Late Teff ($CD62L^{lo}$), or pre-memory T cells ($CD127^{+}$). It will be interesting to use this progression of distinct subsets to investigate integration of signaling for proliferation, metabolic shifts, and survival involved in T cell expansion and contraction. For example, we have recently identified the fatty acid synthesis pathway intrinsic to T cells as specifically important in generation of pre-memory T cells in

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prolonged *P. chabaudi* infection [77]. Research into early T cell decisions have converged on IL-2 as a critical cytokine driving memory CD4 T cell differentiation [78–81]. Another interesting application of this system of markers is to understand maintenance of T cell responsiveness or quiescence. For example, we and others have found that while CD27 is reported to be required for memory T cell survival, $CD4^{+}CD27^{-}$ Teff and effector memory T cells (Tem) can re-express CD27 in some conditions, presumably prolonging their survival [76,82,83]. As $CD27^{+}$ Teff produce the most cytokines, it will be important to understand additional factors involved in their survival. Understanding the basic mechanisms of T cell activation in *Plasmodium* infection will help us find pathways that could be manipulated to improve the response in vaccination.

3. TWO PHASES OF THE IMMUNE RESPONSE TO *PLASMODIUM SPP.*

To understand immunity to *P. falciparum*, which has a variety of outcomes due the immense variation and number of strains, it would seem instructive to compare the two most common mouse models used to understand anti-parasite immunity. While *P. yoelii* XNL infection of wildtype mice lasts for about twenty days [84], *P. chabaudi* AS infected mice can have relapsing remitting parasitemia for between two and three months [85]. In fact, there are reports of sub-patent *P. yoelii* infections up to 7 weeks, though that may be the result of uncloned lines being used [86]. Furthermore, the peak of *P. yoelii* does not occur until around day 15 post-infection, while *P. chabaudi* peaks between days 8-10. Two differences between these two infections that affect the immune response are the cell types they infect, and the timing of their emergence from red blood cells. Firstly, *P. yoelii* parasites prefer immature erythrocytes, called reticulocytes, while *P. chabaudi* (Figure 1A), like *P. falciparum*, are less particular. The low initial frequency of reticulocytes probably explains the slower growth of the parasite (Figure 1B). Secondly, *P. chabaudi*, like *P. falciparum*, emerges synchronously from infected erythrocytes while *P. yoelii* does not, a disease-causing process that controls release of cytokines into the serum. While kinetics of parasitemia differ, both infections require both B and T cells to fully clear infection [87]. We will discuss below the role of T cells in the early control of parasite, and the role of antibody in full clearance starting with these two rodent infections.

As originally proposed by Jean Langhorne in 1998 [88], fast-growing *P. chabaudi* elicits a notable IFN-γ response in the first week of infection. This response is followed by a change. T cells recovered after two weeks, when the parasite is well controlled, produce cytokines that help B cells make antibody (Figure 1C). This intriguing shift in the immune response over time is also seen in infection with *M. tuberculosis*, suggesting it is driven by persistent infection [89]. The mechanisms of this switch have been elucidated in that there is a change in the available antigen presenting cells from $CD8a^{+}$ to $CD8\alpha^{-}$ dendritic cells after day 10, which induce activation of less inflammatory T cells [90]. It was recently shown that B cells are also critical antigen presenting cells for generating GC Tfh cells in the first three days of *P. chabaudi* [91]. The peak of the Th1 response in both *P. chabaudi* and *P. yoelii* (which is mixed with Tfh features) occurs during the first week of infection, after which levels of the Th1 cytokine IFN-γ and T cell expression of the Th1-master regulatory transcription factor T-bet decrease [36,92]. The contraction of IFN-γ^{+} effector T cells corresponds with the increase in GC B cells seen over the course of infection (Figure 1C)

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Control of early parasitemia is due to T cells in both *P. chabaudi* and *P. yoelii*. For example, when CD4 T cells were depleted in *P. yoelii* or *P. chabaudi* infection, the parasitemia increased dramatically in the first week to ten days [93,94]. CD4 T cells responding to *P. yoelii* generate a larger IFN-γ response than that made by T cells responding to *P. chabaudi*. These highly activated T cells become exhausted by the time *P. yoelii* parasite growth peaks at the time of reticulocytosis. Inhibition of nitric oxide affects parasitemia in the second week of *P. chabaudi* infection, but not *P. yoelii* [12,95]. The strong Th1 response to fast growing parasite leads to pathology downstream of the cytokine response in *P. chabaudi*. However, *P. yoelii* is initially well controlled by a stronger Th1 response that induces anemia, but is not associated with fever or hypothermia [96–98]

Strikingly, control of T cell exhaustion by anti-PD-L1 plus anti LAG3 antibodies induced excellent control of the peak of parasitemia in *P. yoelii*, but not *P. chabaudi* [58]. It is possible that this early T cell exhaustion in *P. yoelii* is caused by the continuous presence of parasite due to the unsynchronized release of *P. yoelii*, which is atypical in malaria-causing parasites. While PD-1 has been detected on T cells in all infections [56], that is likely to represent T cell activation rather than exhaustion [57], since anti-PD-1 does not improve T cell responsiveness in *P. chabaudi*. However a variety of co-inhibitory pathways may be involved in regulating the potentially pathogenic response. In *P. vivax*, simultaneous blockade of CLTA-4, PD-1, and T-cell immunoglobulin and mucin-3 (Tim3) *ex vivo* increases cytokine production by antigen-specific CD4 T cells [99].

T cells may be helped in their control of the initial parasitemia in *P. yoelii* due to this parasite's preference for the smaller population of immature erythrocytes, compared to the large population of normocytes targeted by *P. chabaudi*, leading to almost unhindered parasite growth. Or, perhaps the stronger immune response is due to the preference for immature ($MHCI^+$) reticulocytes, leading to better control, perhaps with CD8 participation. Regardless, it has been known for many years that the peak of *P. chabaudi* infection can be reduced best, and even delayed, by a decent IFN-γ or IL-12 biased response, while the peak of *P. yoelii* is best reduced by antibody [2,12,100]. Although parasitemia of some strains of *P. yoelii* do not seem to be affected at all by inhibition of IFN-γ [68], other T cell cytokines such as LT or TNF may be at play because deleting CD4 T cells does change parasitemia from *P. yoelii* XNL both early and late [101].

While we acknowledge differences in the success of the adaptive response against the two parasite species *P. yoelii* and *P. chabaudi*, we hypothesize that the apparent difference between required immune responses in control of the peak of parasitemia is primarily due to the timing of the appearance of susceptible erythrocytes. This idea is based on the similar kinetics of the adaptive immune response with an increase in hybrid Th1/Tfh cells in both, as in *P. falciparum*, and a similar gradual increase in germinal center B cells [36,102]. This is a hypothesis that can be tested, for example, using various parasite strains to induce different levels of innate responses by NK cells and pDCs and, thereby, contributing more or less to early control. Some published work does test the hypothesis. For example, while ICOS deficient mice have very reduced peak *P. chabaudi* parasitemia, anti-ICOS actually prolongs *P. yoelii* infection. ICOS deficient T cells become more Th1-like to promote control of early parasitemia, but inhibits the clearance of parasite by GC-dependent antibody [55]. Therefore,

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ICOS deficiency may have differential effects in each infection due to its effect on IFN-γ, which may control peak *P. chabaudi*, but inhibit the germinal center for longer supporting the hypothesis we are proposing here. The differential effect of acute gamma herpesvirus reduction of humoral responses on *P. chabaudi* and *P. yoelii* also supports this idea [103].

The nature of the adaptive response is regulated very early in infection, as shown in studies cited above, where the innate response regulates the degree of the adaptive response [104]. We also show that allowing prolonged infection beyond day 3 post-infection leads to a complete change in the Th phenotypes generated compared to extended infection [36,76]. Therefore, it is critical to note that there are no known pathogenic *Plasmodium* species that are controlled completely in the first week of infection by the innate response, as are many viruses. One interpretation is that many of the features of the T cell response, including the hybrid Th1/Tfh phenotype, and the predominance of Tem compared to central memory T cell (Tcm), are due to successful immune evasion by the parasite using unknown early mechanisms.

3.1 Early roles of B cells and T cells

Transfer of serum from immune individuals can protect less immune individuals [105,106], and antibody levels can correspond to protection after vaccination [107]. Another clue pertaining to the importance of antibody is that hyperimmune serum, antiserum generated by immunization, or purified IgG or IgM significantly reduces parasitemia of a first infection in mice better than serum from a single infection [108]. Repeated infection with *P. chabaudi* or *P. yoelii* makes the immune serum more effective in parasite control, though CD4 T cells are required for this effect. These historic data support the interpretation that affinity maturation of antibody does eventually occur and that both T cells and antibody are crucial for continued immunity [109,110]. Serum from protected mice opsonizes parasite for phagocytosis [111], and B cell deficient mice never clear parasitemia [1]. Although we have not yet been able to deplete plasma cells at specific timepoints, overall there is clearly a role for antibody in protection from malaria and parasitemia. However, there is significant debate about the timing that specific antibody begins to play an effective role in elimination of parasite.

The early response includes significant serum antibody, and there is a large activation of $CD19^+$ B cells in the first week of infection. Various studies have shown a significant population of Syndecan-1 ($CD138^+$) B cells, likely to be plasmablasts, and measurable *P. chabaudi* MSP1-specific serum IgM, though little IgG [85,112,113]. The early $CD138^+$ B cells are CD4 and ICOS dependent [55]. These B cells are sometimes challenging to measure in ELISpot as antibody secreting cells either *ex vivo*, or stimulated, likely because this response is transient, with a peak of short-lived $CD138^+$ $B220^{int}$ cells lasting only one day [114–116]. However, they were detected beautifully as MSP-1 p19 tetramer-binding T cells in a new flow cytometry assay [113]. Strikingly, the T cell co-stimulatory molecule CD28 is not required for the generation of a large polyclonal and potentially non-specific IgM that is seen early in the response and provides some protection from fast growing parasitemia [50,117].

The localization and kinetics of B cell activation leading to early IgM and their dependence on T cells are just starting to become clear. However, the B cell and T cell interactions

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leading to the later antibody response dominated by IgG2a-c are more likely to be canonical. Generation of the antibodies that clear infection of mice with *P. chabaudi* is clearly T cell dependent, and likely to require the germinal center (GC) [1,45]. IgM is produced throughout the infection and IgM deficient mice show significantly increased early parasitemia [112,117]. In support of an early antibody production, there is early activation of AID, an enzyme critical for both isotype switching and affinity maturation [118], though the cell types represented and effects of this are not yet clear. Further investigation is needed into potential early isotype switching or affinity maturation, as GC are not required for isotype switching [119,120]. Intriguingly, unswitched IgM memory T cells have been recently shown to be important in reinfection, despite polyclonal antigen recognition [50,117,121]. However, while ICOS is clearly required for early CD138^{+} B cells, unswitched antibodies are often T cell-independent, and these IgM^{+} memory B cells (Bmem) have been shown to require Bcl6 intrinsically, while Bcl6 and CXCR5 expression are not required in T cells for generation of IgM^{+} Bmem [122]. The mechanisms and roles for antibody in this early response are being investigated in multiple laboratories.

IL-21, made by Teff, promotes B cell responses, including isotype switching. IL-21 is a critical actor in both early and late immune responses. This is evident from the parasitemia curve of IL-21 and IL-21R deficient mice infected with *P. chabaudi* [123]. The peak of parasitemia is significantly higher in IL-21 KO compared to wildtype controls, despite equivalent amounts of IFN-γ. High levels of IL-21 from T cells produced in this infection may also have other functions on T cells including, intriguingly, an ability to cooperate with transforming growth factor beta (TGF-β) to inhibit Treg expansion [124,125]. This is particularly relevant given that an excess of Tregs in *P. chabaudi* was shown to lead to death of the mice with greatly reduced IFN-γ and IL-10 [126]. IL-21 has also been reported to inhibit IL-2 production by Teff [127,128], which could also affect the Treg response. However, anti-IL-2 did not affect parasitemia [129]. The potential roles of Tregs in human parasitemia are under intense study; however, more work needs to be done on TGF-β and IL-21. Next, we will discuss what is known about regulation of T cell phenotype, T cells in the antibody response, and return to the role of the early cytokines in pathology and in subsequent sections.

3.2 Regulation of the Th phenotype in *Plasmodium* infection

A significant amount is known about Th differentiation *in vitro* and in acute infection. However, using these definitions we have established that IFN-γ^{+} T cells in *Plasmodium* infections do not match any of the classical Th subsets (Figure 2). This has been reported for chronic LCMV as well, with the state of CD4 T cells in that infection being called dysregulated [130]. *In vitro* and in acute *Plasmodium* infections, fully differentiated Th1 cells express high levels of T-bet transcription factor, which determines long-term commitment of a T cell to production of IFN-γ [131]. CXCR3 is the primary Th1-specific chemokine receptor, and is induced by IFN-γ [132]. Bcl6 and cMaf are the primary determining transcription factors in Tfh differentiation, though this is still an area of active research [133,134]. CXCR5 is the defining chemokine receptor of Tfh cells because it allows them to traffic to the B cell area. cMaf has recently been shown to regulate IL-10 production, and also contributes to IL-21 production [135]. In *P. chabaudi* infection, all of these markers and transcription factors

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are expressed, but not at high levels as in terminally differentiated Th1 cells [92]. Most of the effector T cells activated by *Plasmodium* express many markers of both Th1 (specifically, IFN-γ, T-bet, CXCR3, and IL-12Rβ2) and Tfh (specifically, CXCR5, IL-21, Bcl6, ICOS, and BTLA) studied to date [45,92]. The first demonstration of this was in humans. Teff in *P. falciparum* infected people express the chemokine receptors of both Th1 and Tfh, $CXCR5^+CXCR3^+$, as well as T-bet [102]. In mice, as described above, the early response has classically been described as IFN-γ^+ Th1. Many IFN-γ^+ T cells generated in response to *Plasmodium* turned out to be the now well accepted T-bet^+ IFN-γ^+IL-10^+ Th1, also known as Tr1 cells [18,136], in both mouse and human *Plasmodium* infection, and in other infections that are not immediately controlled by the innate immune system [18,130,137,138]. As in some other infections, the IL-10 produced by these cells is under the control of IL-27, cMaf, and Blimp-1 [74,135,139]. More recently, the IFN-γ^+IL-10^+ T cells were also shown to express IL-21 [123]. This IL-21 and the IL-10 also appear to be regulated by cMaf [135], which also represses IL-2, corresponding with the interpretation that autocrine or paracrine IL-2 regulates the Th1/Tfh phenotype gradient [140]. More specifically, in the first 7-10 days of infection in mouse malaria (*P. chabaudi* and *P. yoelii*) almost all of the IFN-γ producers also make IL-21, and about half of these make IL-10, complicating our understanding of Th subset differentiation. While IFN-γ^+ T cells are commonly assumed to be Th1 cells and promote phagocytosis and parasite killing, both IL-21 and IL-10 promote B cell responses [123,141], even though they have been shown to be able to be produced by either classical Th1 or Tfh cells [136,142,143]. The localization and full functionality of these cells in protection from malaria is not yet known.

While Teff in *P. chabaudi* infection express T-bet, they also express cMaf, Bcl6, and Blimp-1. We have shown that the T-bet/Bcl6 protein ratio in IFN-γ^+ Teff correlates positively with IFN-γ transcription [92], and deficiency in T-bet significantly reduces, but does not completely abolish IFN-γ. Deficiency in Bcl6 also does not dramatically increase IFN-γ. In addition, Bcl6 is not the only transcription factor regulating T-bet. Blimp-1 also inhibits T-bet as does STAT3 [36], as illustrated in Figure 2. While mechanisms of generation of these cells is under intense study in *Plasmodium* infection and other persistent infections, some driving cytokines have been identified. IL-27 and Type I Interferon (IFN-I) are definitely involved in inducing the hybrid T cells [66,74], while IL-12, cMaf and Blimp-1 have also been implicated in induction of IL-10 in IFN-γ^+ T cells [144,145].

There is an appreciable literature about cytokines and transcription factors involved in regulating the spectrum from Th1 to Tfh [140]. However, the combination of cytokines in each infection and timepoint is likely to generate a large variety of possible conformations. We found that deficiency of the Tfh defining transcription factor Bcl6 affects CXCR5 expression much more than the Tfh effector molecule, IL-21. The $CXCR5^{int}$ T cell phenotype in response to *P. chabaudi* was additionally shown to be determined by ICOS and/or CD40L ligation in the first three days of infection [91]. On the other hand, deficiency of the Th1 master regulator, T-bet, significantly affects the generation of $CXCR5^+$ IFN-γ^+IL-21^+ T cells. Classical Th1 culture also generates transient IFN-γ^+IL-21^+ Th1 cells *in vitro* [142]. This phenotype includes transient expression of Bcl6, which can bind and inhibit T-bet. [36] The early Th1-like response seen in these studies, which is highly related to mixed but transient phenotypes seen in classical Th1 culture, led us to propose the nomenclature

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"Th1/Tfh hybrid" for cells expressing markers of both Th1 and Tfh cells [92], rather than the previously used "Th1-like Tfh" [102]. T-bet regulates IFN-γ expression, though possibly to varying levels when using different parasites [146,147]. Therefore, we propose a Th1 lineage for these hybrid cells despite their expression of intermediate levels of CXCR5 [36]. In addition to this dominant Th1/Tfh hybrid population, there is a small, steady, and important $Bcl6^{hi}$ $CXCR5^{hi}$ $PD\text{-}1^{hi}$ population of GC-T follicular helper (Tfh) cells (in both *P. chabaudi* and *P. yoelii*), as shown in Figure 1 [36]. This population of genuine GC Tfh cannot be generated without B cell interaction in the first three days of *P. chabaudi* infection [91]. T-bet expression in B cells has been demonstrated to have strong effects on GC formation as well [141,148].

Despite the seeming overlap of functional markers by flow cytometry in Th1/Tfh, single cell transcriptional analysis of individual effector T cells suggests that there are likely to be populations discernable at each end of the Th1-Tfh spectrum [149]. Indeed, these are evident in one of the antibody combinations we have used to evaluate immune phenotype by flow cytometry (CXCR5, T-bet) [36] and using CXCR6, which is not expressed on $CXCR5^+$ cells. [150]. We were able to explore the functional importance of shifts along this spectrum. Upon infection of $STAT3^{fl/fl}$-CD4-Cre mice, which have a specific deletion of STAT3 in cells that have expressed *Cd4*, the Th1/Tfh hybrid cell type was shifted towards Th1, with more $IFN\text{-}\gamma^+IL\text{-}21^-$ cells being generated, and fewer $CXCR5^{int}IFN\text{-}\gamma^+IL\text{-}21^+$ cells [36].

One interpretation is that the clear separation of Th1 and Tfh lineages seen in unbiased bioinformatics analysis represents two separable terminally differentiated Teff [149], though it is not clear if Tfh can be a terminally differentiated phenotype [143]. It is also possible that the separation of two lineages within the single cell transcriptomics data is due to the high level of analogy between Tfh and Tcm expression patterns [151], which is not yet fully understood. In this reading, the early divergence of Tcm from Teff could explain the separation of Tcm/Tfh from Th1/Teff starting n day 4 post-infection in the single cell transcriptomics data. In the future, these two interpretations might be discernible using downregulation of CD62L or CCR7 on Tfh effector cells, but not on Tcm [152–154], if that is apparent in the transcriptome. The authors considered this possibility and studied memory differentiation in a separate work, which suggests that quiescence is acquired slowly over time in the effector population. While they consider a third phenotype during peak parasitemia unlikely, they allow for the possibility that the Tfh phenotype defined within the effector phase overlaps significantly with a mitochondrially fueled memory precursor that is protected from death in the contraction phase, which is challenging to detect with this method.[150] In culture, $IFN\text{-}\gamma^+IL\text{-}21^+$ Th1 cells can be driven towards a Tfh-like phenotype by varying levels of IL-2 in the culture [78,140]. This is interesting, as IL-21 seems to counter-regulate responsiveness to IL-2 [135,155]. The balance is at least partially regulated by IL-2 driven STAT5 activation, which increases transcription of Blimp-1, that can inhibit both Bcl6 and T-bet transcription. However, STAT3 has not been previously studied in this regard [140,156,157].

Regulation of Th1 and Tfh cytokines via this complex system of regulation throughout a long battle with the parasite clearly has evolutionary and pathological implications. The increase in $IFN\text{-}\gamma^+IL\text{-}21^-$ T cells in infected $STAT3^{fl/fl}$-CD4-Cre mice did lead to some increase in observed pathology, though no mice died. However, in a second infection of

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these mice, there was an immediate control of parasitemia, suggesting the importance of studying the cost/benefit ratio to plasticity in Th cells in responses to *Plasmodium spp*. It will be important to test if parameters of Th1 and Tfh hybrid versus IFN-γ^{+}IL-10^{-}IL-21^{-} or classical GC-Tfh cells correlate with various tests of protection in humans or not.

3.3 T cell dependent antibody in the two stages of *Plasmodium* infection

The second half of the infection, where parasite is completely cleared, absolutely depends on the generation of antibody [1,2,45]. Antibody definitely reduces peak *P. yoelii* parasitemia, while this effect is harder to measure in *P. chabaudi*. The earliest role of antibody is easiest to measure in *P. yoelii*, as the peak of parasitemia happens in the second week when germinal center B cells can be detected [87]. The effect of T cell produced IL-21 on the later *P. chabaudi* parasitemia is much more pronounced than that of the early effect described above. IL-21 deficient mice do not clear infection at all, and over weeks 2-4 post-infection, the parasitemia climbs back to peak levels [123]. This model would predict that both peak and subsequent parasitemia would be affected in *P. yoelii* infection of IL-21 deficient mice, which might even die of hyper-parasitemia, while *P. chabaudi* infected IL-21 deficient mice do not die. However, it is less likely that parasitemia would be affected days 8-10 in *P. yoelii*, as seen in *P. chabaudi*, since T cells are quite effective at this timepoint in this infection. The late action of IL-21 on *P. chabaudi* persistence was shown to be due to the presence of IL-21R on B cells and not T cells [123]. Therefore, the role of IL-21 on the persistent responsiveness of CD8 T cells shown in chronic LCMV does not seem to play a role in CD4 T cell responsiveness here [123]. Protective MSP-1 p19-specific IgG antibody is not detectable until the third week of infection in *P. chabaudi* infection [85,112]. IgG responses to the other fragments of MSP-1 have similar kinetics [2]. Therefore, it is striking that generation of species-specific isotype-switched IgG2 antibodies in both infections is around day 11p.i. [158]. IgG2 antibody has been shown to be able to inhibit parasite growth better than IgM [52,111,159].

The interactions between B and T cells leading to clearance of parasitemia in the later phase are gradually becoming clearer. In the first two weeks of a *P. chabaudi* infection, parasitemia in mice with Bcl6-deficient T cells is equivalent to wildtype [45,92]. However, Bcl6 in T cells is essential in controlling full clearance of the infection in the second two weeks [85]. As there are no GC present in mice with either T cells or B cells that are missing Bcl6, and no GC Tfh, this data suggest that GC are required for full clearance of parasite. It is important to note that most pathology occurs during the first two weeks, suggesting that in the first infection, it may not be antibody that allows survival. This is a critical point in determining what germinal centers means for patients with *Plasmodium* infection. Persistent parasitemia, particularly in immune people, does not result in clinical malaria. However, germinal centers must be important for generating high affinity neutralizing antibodies, and possibly for control of variation in parasite strains.

All subsets of cytokine-producing CD4 T cells are known to be capable of promoting B cell antibody production *in vitro* [160]. However, a highly-specialized subset of activated T cells is now known to specifically inhabit the GC and promote the affinity and diversification of the antibody response [161]. GC Tfh are defined by their location, and in flow cytometry usually

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as PD-1^{hi}CXCR5^{hi}, with both markers having critical functions for these cells. The critical cytokines for GC function are IL-10, IL-4, and IL-21[162–164]. All three of these cytokines have overlapping and unique functions in promoting B cell antibody secretion and plasma cell generation. IL-21 and IL-4 are B cell growth factors. While IL-4 also promotes IgG1 specifically, IL-21 promotes isotype switching in general. While Tfh expressing CXCR5 can be generated in cell culture with IL-21 [165], IL-21 is not sufficient for the formation of GC Tfh *in vivo* [166]. It is important to note that even though Bcl6 regulates the master regulators of other Th subsets, Tfh and other programs co-exist [160].

The potential of Th1/Tfh hybrid Teff T cells to contribute to antibody production has been shown in both human and mouse (CXCR3^{+}CXCR5^{+}, Ly6c^{+}T-bet^{+}, or NK1.1^{+}ICOS^{+}) *Plasmodium* infection [102,141,167]. However, they do not help as much as fully differentiated GC Tfh [102]. Part of the difficulty in determining the function and relevance of CXCR5-expressing Teff in *Plasmodium* infection lies in the emerging details of their differentiation pathway. For example, some CXCR5^{int}ICOS^{+} T cells are pre-Tfh and are known to require further interaction with B cells (CD40L), and further upregulation of Bcl6 and CXCR5 to enter the GC [168,169]. Indeed, the large population of CXCR5^{int} effector T cells generated in *P. berghei* ANKA infection had the potential to transition into GC-Tfh in IRF4 deficient animals [170], indicating their potential as pre-Tfh. From our perspective, the important question about hybrid Th1/Tfh cells in malaria is if they are desirable for either phase of the infection, or if they primarily contribute to prolonging the infection and/or worsening the pathology. If they are unprotective, then understanding their generation could help to avoid their promotion by vaccine strategies.

3.4 Using basic biology of B and T cells to understand weapons available against persistent and repeated *Plasmodium* infection

Variation of parasite surface proteins is a serious challenge for control of any infection by the immune response. Antigenic variation within *Plasmodium* strains can lead to low-grade chronic infection [110,171–174], and is likely a major contributor to the slow acquisition of immunity to malaria disease [175]. The most striking evidence for this is that patients whose parasites can be recognized by their own serum antibody are less likely to be severely ill [176–178]. While GCs generate high affinity antibodies, and are therefore the center for improving neutralizing specificity, the contribution of GCs to the control of persistent and repeated infection in the field is unknown, and should be studied in connection with parasite variation. However, reagents are just being developed to allow detection of variant antigens and variant-specific B cells in parallel [179–181].

On the other hand, the large repertoire of parasite antigens, the highly immunogenic but rarely protective surface antigen specificities contribute to a smokescreen of non-protective epitopes that probably protect the parasite from destruction. Not many protective B or T cell epitopes are well-defined for *Plasmodium spp* that are relevant to naturally acquired immunity [182–184], however, making the smokescreen hypothesis challenging to test. Here, we will attempt to use existing evidence to build a model of how germinal center biology could help the host recognize conserved protective antigens and compete with antigenic variation. While natural immunity may not be the best guide to possible vaccine targets,

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understanding the potential of the germinal center reaction will also be necessary to design synthetic vaccine antigens. We will also review the evidence that antibody specificity improves over time. Hyperimmune mice are refractory to further challenge with homologous *P. chabaudi* parasites after six or seven infections, but remain susceptible to heterologous challenge [110], similar to that seen in protective immunity in humans. Supporting a role for the evolution of antibodies in *Plasmodium* infection, hyperimmune serum is significantly better at controlling *P. yoelii* infection than serum collected after one infection [111]. Indeed, we observed continuous increases in antibody affinity to parasite lysate over 6 weeks post *P. chabaudi* infection [85]. The dramatic reduction of parasitemia from 30% infected erythrocytes in the first *P. chabaudi* infection at the peak, to less than 1% in the second infection is also likely due to plasma cells in the bone marrow which will continue to generate antibody over the long-term. GC have also been shown to expand much faster in the second infection than in the first [2,116,151].

GC have been studied for over 40 years, and significant detail about mechanisms of affinity maturation have emerged [185]. The GC contains a limited number of helper T cells, and also follicular dendritic cells holding antigen on Fc receptors, that serve to select B cells with ever higher affinity antibody on their surface. Higher affinity B cells take up more antigen via their surface antibody, leading to more MHC-peptide to stimulate T cells, and therefore, more B cell growth factors from T cells and stronger clonal expansion. Since selection in the GC depends on antigens captured by follicular dendritic cells, the GC would seem to be the ideal mechanism for keeping up with evolving parasite. However, protease expression is limited in the GC, and antigen can remain on FcR of follicular dendritic cells for up to three months [186], potentially allowing variants to escape.

It seems plausible that the evolution of the immune response to one *P. chabaudi* infection, which varies expression of variant antigens weekly, may be similar to repeated exposure, even within one day to infection with multiple strains and/or even species of parasite (in endemic areas). On the other hand, competing parasite strains, and even species, may result in significantly more variation in humans than in the mouse model. The kinetics of B cell division (~12 hours per division [187], parasite division (~24-72 hours per 8-32 new parasites depending on parasite species, and immune success), and the mechanisms and kinetics of variation (e.g. pre-programmed) will determine the success of one against the other. Some modelling has been done based on current knowledge of *P. falciparum* var switching and antibody responses, supporting the role of variant antigen switching in the slow acquisition of immunity, and the selection of variants by the antibody response [188–190]. While we do not know how the kinetics of antibody generation compares to that of the parasite variation, based on the evidence that variation leads to persistence, the kinetics of the germinal center reaction in *Plasmodium* infection may represent a weakness that the parasite is able to circumvent to persist in the host. GC B cells can be detected as early as day 8 post infection, and GCs are detectable by immunohistochemistry staining with peanut agglutinin on day 10 post infection with *P. chabaudi* [113,114,191]. While IgG takes some time to appear, IgM concentration stays constant throughout, and affinity also increases [113]. The kinetics of the GC, as also seen for T cell expansion, are slightly slower in the response to *Plasmodium* than after immunization with simple antigens. In a response to model antigen, GC are detectable within the first week and higher affinity antibody is evident in the second week

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[192], suggesting there are factors at play to delay their formation in response to *Plasmodium*. In agreement with this interpretation, immunohistochemistry of GCs ($GL7^+$) in *P. berghei* indicates a paucity in the time before death on day 8. Early simultaneous inhibition of IFN-γ and TNF increased the early appearance of the GC in *P. berghei* infection, and T cell intrinsic T-bet was critical for the delay of splenic GC [170]. In *Salmonella* infection, it is IL-12Rβ2 expression on T cells that inhbits accumulation of GC-Tfh [193]. It is possible that this is a broad mechanism delaying T cell dependent antibody, however, there are a few caveats worth considering. Firstly, *P. berghei* may inhibit GCs more than observed for other infections [194]. In addition, in contrast to this data suggesting that the early Th1-like response to *Plasmodium* inhibits GC formation, recent data indicates that STAT4, IFN-γR, and T-bet are all required in both B and T cells for generation of the protective isotypes IgG2a-c in other systems [87,195,196]. Indeed, we found a strong reduction in GC-Tfh cells in STAT4 deficient mice infected with *P. chabaudi* [36]. As GC-Tfh numbers did not vary significantly over the time course of *P. chabaudi* or *P. yoelii* infections, it may be that variation in the expression of T-bet during infection, such as the decrease seen from day 7 to 9 post-infection with *P. chabaudi*, allows the emergence of the GC structure. It is likely that the degree of the cytokine response determines the result of counter-regulation, allowing for a balanced and regulated Th1-biased GC response.

In addition to the evidence that GC size may be regulated early in the response, there is some evidence that GC structure is changed in the response to *Plasmodium*. It has been reported that the *Plasmodium* GC is unusually heavy in centroblasts with a loss of definition between the dark and light zones in *P. berghei* ANKA, and in *P. falciparum*-infected *Saimiri sciureus* monkeys [197,198]. This lack of definition in the GC structure in the first 7-10 days of infection was confirmed in *P. chabaudi*, and affinity maturation to the model antigen, chicken gamma globulin, during infection was shown to be reduced [191]. This result could indicate that proliferating centroblasts are not being properly selected in the light zone where follicular dendritic cells are located. It is not possible to predict if the parasite switches faster than the GC antibody can mature for effective neutralization. However, the evidence suggests that variation is a successful evasion mechanism in mice and NHP to prolong the infection [199], supporting the interpretation that the parasite surface probably changes too quickly for the B cell response to keep up, or that the parasite population as a whole becomes too diverse. More research is required to understand how the process of affinity maturation is used against variants, heterologous infection, and persistent infection in order to identify a potential for intervention.

3.5 Broadly neutralizing antibodies take several years to develop

While Th1-isotypes are important, neutralization, or invasion-blocking specificities are also proven to be able to reduce parasitemia in *Plasmodium* infection. Neutralizing antibody can inhibit invasion of erythrocytes by blocking surface binding, or inhibiting proteolytic reactions that expose invasion ligands [200]. However, it is clear that of all the antibodies made to MSP-1 by exposed people, only a small fraction is neutralizing. HIV only has two antigens on its surface for the immune system to focus on, and yet, neutralizing antibodies are still rare. Mechanisms of generation of broadly neutralizing antibodies (bnAbs), as opposed to highly strain-specific antibodies, to HIV gp120 have started to become clear.

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However, the process of evolving bnAbs can take several years *in vivo* [201], and the role of T cell helper specificity is very poorly understood. Models of B cell activation show multiple critical variables, namely precursor frequency, antigen affinity, and avidity, to inform B cell vaccination strategies to promote bnAb selection in GCs [202]. Importantly, this work also suggests that if, perhaps stochastically, a suitable precursor arrives to the GC reaction, it can survive selection and the reaction is complete within a few weeks. Therefore, it is likely that the rarity of these precursor B cells is the rate-limiting step, and that their rarity may also be a result of the evolution of a successful pathogen's antigen structure and variety. Therefore, it is likely that vaccination with synthetic antigens that bypass the rarity of natural protective epitopes and promote rapid refinement in the Germinal center will be necessary to increase immunity more quickly than natural infection.

Much work is currently going into defining protective antibodies by cloning human BCRs from immune patients. For example, cloning *P. falciparum*-specific B cells led to the discovery of a host mechanism of resistance where a germline sequence, leukocyte-associated immunoglobulin-like receptor-1 (LAIR) is inserted to Immunoglobulin and adds specificity to variant antigen family epitopes [203]. This work is leading to the identification of new and promising epitopes [204,205] and will improve vaccine constructs aimed at increasing their representation in the repertoire [206]. However, work on T cell epitopes is very far behind this effort. The most likely way to identify T cell epitopes is to elute peptides from immune patients' antigen presenting cells [207–211]. In order to determine their role in protection, most likely this would require testing T cells specific to that peptide by adoptive transfer in mice. Since T cells tend to use more conserved epitopes, epitope discovery for suitable Tfh vaccine antigens could lead to more neutralizing B cell repertoires. Understanding the role of T cells in promoting the evolution of neutralizing antibody to conserved and variable protective epitopes will allow us to design ever more effective vaccine strategies.

4. THE BALANCE OF INFLAMMATORY CYTOKINES AND REGULATORY CYTOKINES PLAYS A MAJOR ROLE IN ALL MALARIA PATHOLOGY

The protective role of CD4 T cell cytokines in malaria infection has been demonstrated in murine and human *Plasmodium* infection. Most research on protection and pathology thus far has focused on the role of several highly-expressed cytokines: interferon (IFN-γ), tumor necrosis factor (TNF), and interleukin (IL)-10. Most studies analysing T cell cytokines in human malaria have generally used either serum ELISA cytokine measurements, or ELISA of culture supernatants after stimulation of PBMC with malaria peptide antigens. However, several studies lately have been studying cytokine production by intracellular cytokine staining, and experimental infections [17,18,212]. Below we discuss the roles of these cytokines in both control of parasitemia and regulation of pathology.

4.1 CD4 T cell cytokine responses to *Plasmodium* infection: Distinguishing the roles of IFN-γ and TNF in malaria immunity

The primary role of IFN-γ in cellular immunity is to activate phagocytosis and parasite killing mechanisms within macrophages and other phagocytic cells, which promotes parasite killing. Indeed, IFN-γ enhances phagocytosis of *P. chabaudi* iRBCs and frees merozoites *in*

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vitro by primary mouse macrophages [213]. Importantly, this critical parasiticidal mechanism can also be inhibited by IL-10 [214]. In *P. chabaudi* infection of mice, the IFN-γ-induced antibody isotypes, IgG2a-c, have also been shown to play an important role in parasite clearance [52]. This suggests that antibody-mediated phagocytosis via the Fc receptors, bound best by (mouse) IgG2a-c (or human IgG1, 3) rather than other isotypes, is a critical contributor to parasite control in *Plasmodium* infection. However, this is challenging to test in humans where only correlation is possible, and specificity is confounding. IgG2a-c, In mice, antibody-coated parasites are phagocytosed better than un-opsonized parasite *in vitro* and *in vivo* [159,215], and transfer of IgG2a-c definitively promotes parasite clearance in mice [40]. It would be very important to screen various parasite lines for mechanisms used to evade killing by macrophages and neutrophils [215].

Antigen-specific IFN-γ in response to both erythrocyte and liver stage malaria antigens in exposed humans has been associated with protection [216,217]. For example, over the course of multiple experimental human challenges with blood-stage *P. falciparum*, parasitemia after a challenge infection negatively correlated with numbers of circulating IFN-γ-producing $CD4^{+}$ T cells [218], and IFN-γ^{+}CD62L^{lo} T cells responding to MSP-1 show a similar trend in naturally exposed people [219]. Inhibition of major histocompatibility complex class II in these experiments leads to loss of IFN-γ production *in vitro*, identifying CD4 T cells as the main source of IFN-γ production upon *Plasmodium* infected red blood stimulation in humans [214,217]. The superior resistance inherited by members of the Fulani ethnic group to *Plasmodium* infection in Mali has also been positively correlated with serum IFN-γ [220]. In addition, there are vaccine strategies where IFN-γ producing T cells correlate best with protection. In infection-and-drug-cure immunization of people and mice, the IFN-γ-secreting CD4 T cell response correlates better with malaria protection than serum concentration of antibody [218,221]. The response to the recombinant circumsporozoite protein-viral particle-based malaria vaccine RTS,S that correlates best is the combination of anti-circumsporozoite protein antibodies and IFN-γ production [222,223].

Mouse studies primarily support the importance of IFN-γ in reducing parasitemia, in that deficiency in IFN-γ and IL-12, a strong inducer of IFN-γ-producing T cells, controls the peak of *P. chabaudi* parasitemia, representing the first phase of parasite control [52]. Early production of IFN-γ by NK and γ/δ T cells can also play a critical role in promoting very early control of *P. yoelii* parasitemia [69,224]. The early response by early sensors of infection, STING, cGAS, IL-18, and IFN-I, also critically regulate the adaptive immune response [65,104,225–228]. However, as phagocytosis and oxidative radicals do not uniformly eliminate parasites *in vivo* or *in vitro*, the precise mechanism of parasite killing is less clear [229]. Data suggests, for example, that although nitric oxide, a primary macrophage lysosome product, can kill parasites in macrophages, it is not a major effector mechanism in all models. Therefore, more work needs to be done to understand the mechanisms at work in order to determine if interventions could be developed.

An important role of TNF in the immune response to malaria has also been demonstrated in many studies, though it is even more challenging to define. Serum TNF can correlate with the synchronized rupture of infected red blood cells, and also with the severity of malaria disease in some studies on *P. falciparum* [230,231]. TNF has several measurable effects during

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Plasmodium infection, for example, on parasite killing by neutrophils [232]. However, the roles of TNF *in vivo*, either for parasite control or pathology in malaria, are not yet defined in a fully mechanistic manner [233]. In malaria endemic areas, higher frequencies of *P. falciparum*–specific CD4 T cells co-producing IFN-γ and TNF were observed in immune adults [17]. However, comparing the immune response in the first week of infection in mice using two sets of more and less lethal parasites revealed that differences in serum IFN-γ correlate better than TNF with parasite control [199]. While TNF may drive a fraction of total IFN-γ production in *P. chabaudi* infection [234], but not in *P. vinkei* [235], IFN-γ seems to be more beneficial in parasite control. Based on evidence from both mice and humans, the balance between TNF (but not IFN-γ) and IL-10 regulates some modes of pathology associated with malaria disease, as discussed further below [236].

Mouse data suggest a key role of TNF family members in malaria associated pathology such as cerebral malaria and anemia, rather than in immune-mediated parasite control, however TNF family members clearly can induce parasiticidal mechanisms. TNF does not appear to be primarily made by T cells, at least early in mouse *Plasmodium* infections [69]; however, TNF and T cell-produced IFN-γ promote each other's production. We do not know which cell types in addition to T cells make TNF during the later stages, when excess production of a TNF family member can cause pathology [234]. One study showed a lethal early increase in parasitemia in TNF deficient mice using *P. chabaudi adami* [237]. This interpretation is supported by a study showing that recombinant human TNF reduced parasitemia from *P. berghei* K173 enough to diminish death from cerebral complications [238]. In addition, the results of many studies of TNF itself are in question due to the use of antibodies that cross-react with lymphotoxin alpha (LTα), and TNF deficient mice that also express lower levels of LTα [234,239]. Critically, LTα kills parasites in neutrophils ten times better than TNF, and also causes hypoglycemia [232]. TNF family members have a critical role in driving the microstructure of the spleen, including the marginal zone, and separation of B and T cell areas, further clouding these results. Finally, TNF family members LTα2β1 and lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), also known as TNF superfamily (TNFSF)14/CD258, also play roles in *Plasmodium* infection of mice [240]. Studies of *P. berghei* ANKA infection-induced experimental cerebral malaria demonstrate that lymphotoxin beta receptor (LTα2β1), rather than the TNFRI, contributes most to pathology in the brain [241]. Infected LTβR deficient animals show similar parasitemia, but no death from *P. berghei* ANKA infection. In addition, they show strong reductions in ICAM expression and parasite binding in the cerebral vascular endothelium. However, the effects of the absence of both of these receptors on splenic microarchitecture makes it very challenging to interpret these results [242]. LIGHT is made by activated T cells, constitutively expressed on DCs, and can co-stimulate or inhibit immune responses depending on the collective activity of TNF family receptors [139]. Deficiency of LIGHT leads to increased parasite killing in *P. berghei ANKA* infection, suggesting that LIGHT plays an inhibitory role of T cells early in infection [243]. This effect is so strong that it inhibits development of experimental CM. Therefore, the role of TNF family members in lethal malaria is critical, and also very much open for further study, particularly for development of therapeutics.

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Tregs are also sometimes associated with inhibition of the response [60,244,245]. The role of Treg in immunity to mouse or human malaria is unclear, as conflicting results have been observed [244–247]. The strongest evidence so far is that upon experimental *P. falciparum* infection, increased levels of the Treg determining transcription factor, FoxP3, and one Treg mechanism, TGF-β, were increased upon infection and their levels correlated with higher rates of parasite growth and lower antigen-specific immune responses [247]. However, subsequently conflicting findings make it unclear if Tregs continue to correlate with parasite load over subsequent infections in *P. falciparum* or *P. vivax* endemic areas [245]. Interestingly, Treg activity during acute infection in children in Gambia was found to be inversely correlated with malaria-specific memory responses, suggesting a mechanism for generating quiescent memory T cells despite a prolonged inflammatory response [245]. There are also recently reported $CXCR3^+$T-$bet^+$$FoxP3^+$ Treg cells found in *P. chabaudi* [126]. In mouse models, even though there is a relatively stable number of Tregs throughout infection, increasing Tregs using a FoxP3 transgenic mouse, or IL-2 immune complex, does inhibit control of the peak of parasitemia [248,249]. Overall data support the conclusion that Tregs are highly regulated in *Plasmodium* infection and have the potential to inhibit parasite control, but are not otherwise central regulators of malaria pathology. Tregs may be more important in infants, as discussed below. In summary, the IFN-γ and TNF family member responses are the cytokines most implicated in increasing phagocytic uptake of parasites, and mechanisms of macrophage killing of these parasites. However, as we describe below, these same inflammatory cytokines are involved in pathology and in regulation of the humoral immune response.

4.2 Role of IL-10 and TGF-β and Tregs in regulation of malaria disease

Much of the evidence of the role of TNF in malarial pathology comes from studies that also demonstrate how IL-10 regulates this damaging potential. Without regulation, an excessive pro-inflammatory response controlling the parasitemia peak or parasite growth can cause tissue damage. Therefore, a balance of cytokines with the potential to regulate the effects of hyper-inflammation is important in *Plasmodium* infection to control the outcomes of the infection. IL-10 was first described in *Plasmodium* infected people in 1994 [250]. The earliest study describing the impact of the balance of anti- and pro-inflammatory response and malaria complication showed an association between a significantly higher TNF:IL-10 ratio and severe anemia in children in Ghana [251]. Further study in western Kenya confirmed this observation, showing a higher TNF:IL-10 ratio in children with severe malarial anemia compared to children with uncomplicated malaria [252]. Following this, May *et al.* observed that children with severe malarial anemia in Gabon had higher TNF:IL-10 ratios compared to non-anemic children [253]. In fact, a higher IL-10:TNF ratio in the plasma was shown to be predictive of severe anemia in this study. Those studies confirm that the balance between anti- and pro-inflammatory responses is critical for protection from complications and severe disease in malaria. Elevated levels of TNF have also been documented in cerebral malaria [254,255]. While anti-TNF antibodies, which have revolutionized treatment for some inflammatory conditions, did not ameliorate cerebral malaria [230,231,255–257], it will be important to try next generation, more specific TNF family reagents, some of which can enter the brain [258].

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TGF-β is the other major regulatory cytokine known to play a role in *Plasmodium* immunity and pathology . Severe *P. falciparum* infection in children was associated with elevated levels of TGF-β in plasma whereas IL-2 or IL-12 were lower in uncomplicated malaria [259]. Some mechanisms of IL-10 and TGF-β in regulation of the pro-inflammatory cytokine response in *Plasmodium* infection have been investigated in mouse studies. IL-10 knock-out mice infected with *P. chabaudi* revealed worse pathology (hypothermia, hyperglycemia, weight loss, and anemia) and 60% mortality compared t wildtype [234,260]. Some signs of cerebral malaria were detected downstream of hyper-inflammation including cerebral edema, hemorrhages, behavioral changes, and vascular congestion including leukocytes [261,262]. Neutralization of TGF-β in IL-10 KO mice leads to uncontrolled pathology and mortality increased to 100% of the mice [234]. Although many cell types can secrete IL-10 (Breg, T cells subsets, NK cells and γδ T cells), it has been shown that the IL-10 that protects animals from *P. chabaudi*-induced mortality does come from T cells, as IL-$10^{fl/fl}$CD4^{Cre} mice do not die [74]. IL-10 induced by *P. chabaudi* and *P. yoelii* is downstream of IL-27 [73,74]. The potential effect of IL-10 driven by *Plasmodium* infection on clinical illness has been highlighted by the recent finding that the fraction of *P. falciparum*-specific IL-10-positive T cells (IFN-γ^{-}TNF^{-}) correlates well with decreased odds of symptoms in children from an area of high exposure [18]. It will be interesting to determine if these serve a more regulatory or Tfh function (*e.g.* IL-21 or CXCR5+), as IL-10 also promotes antibody secreting B cells. In human *Plasmodium* infection, it is not yet known if the majority of the specific IL-10 is made by CD4 T cells.

4.3 Autoregulation of T cell responses affects development of malaria immunity

In addition to the roles of IL-10 in regulating pathology, IL-10 and TGF-β can also regulate the innate response by antigen presenting cells and phagocytes. It is well known that IL-10 inhibits IL-12 production and antigen presentation, thereby reducing the proliferation and Th1 differentiation of T cells at the time of priming [263]. In the field, detectable plasma IL-10 and T cells that can make IL-10 in the intracellular cytokine assay were correlated with recent *P. falciparum* positive smears, or high parasite burden during a recent infection [18,264–266], suggesting that a possible reduction in immunity to parasite growth correlated with IL-10 production. In mouse studies, IL-10^{+}IFN-γ^{+} T cells also directly correlate with the fraction of infected erythrocytes, suggesting a potential cofounding factor in that the same T cells make both paraciticial and anti-inflammatory cytokines [36]. Indeed, a high IL-10:TNF ratio in the plasma was predictive of hyperparasitemia (>250,000 parasites/μL) in exposed Gabonese children [253].

IL-10 and Tregs have also been found to correlate with permissive parasite growth in neonates. Lower proportions of Teff and higher IL-10 response in cord blood were associated with an increased risk of *P. falciparum* infection during the first year of life [267,268]. *In utero* exposure to *Plasmodium* also increased of Treg cells in cord blood [268–270]. Though much more needs to be done to understand the mechanisms involved in susceptibility to infection of newborns, this data supports the interpretation that IL-10 and Tregs may play an inhibitory role on the immune response most conducive to parasite elimination in some settings.

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These opposing functions of IL-10 can also be detected in the mouse *Plasmodium* literature. For example, IL-10 deficient animals show decreasing parasitemia in some laboratories, but primarily increasing malarial pathology in others [139,145,234,262]. Similarly, although infection of Blimp-1 deficient mice results in quite a substantial reduction in IL-10 production, this results in the death of animals from *P. chabaudi* AS in some laboratories, but not others [36,145]. Perhaps a difference in the strain of parasite or mouse strains, or microbiomes evolving over time in different laboratories causes these differential outcomes. In fact, both TNF and IL-10 are differentially induced by different clones of *P. chabaudi* and different species, supporting the interpretation that IL-10 plays a double-edged role in immunity to *Plasmodium* infection [139,271].

TGF-β has similar dual functions [272]. TGF-β can suppress macrophage activation and inhibit antigen presentation, antagonizing upregulation of IL-12 in synergy with IL-10. In mice, increasing TGF-β led to reduced inflammatory cytokine production and decreased severity of malaria disease, while neutralization of TGF-β also led to more rapid parasite growth [97,234]. In human infection, upregulation of TGF-β, FoxP3, and $CD25^+$ $CD4^+$ T cells was also shown to correlate with more rapid parasite growth in *P. falciparum* sporozoite challenge [247]. However, though TGF-β levels correlated with Treg numbers, about half of the TGF-β detectable as intracellular in PBMCs was found in monocytes. Interestingly, although TGF-β needs to be cleaved to be active, several *Plasmodium* species have a metalloprotease that actives TGF-β [273]. The critical variables shifting the importance of IL-10 and TGF-β in inhibiting pathogenic outcomes versus inhibiting control of parasitemia for the two major outcomes of *P. falciparum* infection are not yet known.

An interesting insight, however, is emerging from immunological studies in a highly endemic area of Uganda. Frequency of exposure is an important variable for determining the amount of IL-10, with more IL-10 being found co-produced by effector T cells from children in a higher transmission setting compared to lower transmission [17]. The authors report that IL-10 decayed over time since last exposure, but also correlated inversely with the ability of T cells to proliferate *ex vivo* to parasite antigens. Jagannathan *et al.*, therefore, tested the potential of anti-IL-10Rα to enhance T cell proliferation and found a small but significant role for IL-10 in inhibition of T cell proliferation to parasite lysate that was confirmed in 8/9 subjects [265]. Therefore, heavily exposed children maintain a high enough level of IL-10 to actually inhibit T cell proliferation. This has also been seen in cord blood, as IL-10 inhibited IFN-γ production [274]. The mechanism is suspected to be either by a T cell autocrine loop, or via inhibition of MHCII and antigen presentation [275]. This work suggests that the T cell specificities are still present despite frequent parasitemia, which could suggest an absence of *Plasmodium*-specific T cells. Overall the data indicates that the downside of inhibition of the immune response by IL-10 is balanced by the upside of reduction of pathology only in a highly exposed host.

4.4 Role of CD4 cytokines in regulating malaria pathologies in newborns and children

While acknowledging that T cell regulation can have a cost in parasite growth, an excessive inflammatory T cell response to *P. falciparum* can have a devastating effect in newborns or toddlers, and therefore does require regulation. CD4 T cells clearly play a critical role in

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both the protective immune response to malaria in the first years of life, and the immunoregulation of the dangerous side-effects. Several studies have shown the important role of CD4 T cells in the regulation and protective immune response to malaria in the first years of life with increasing Treg or Teff frequencies [268,270,276,277]. Inflammatory mediators such as IL-1β, IL-6, and IL-8, which have been implicated in fever, are made in response to *P. falciparum*-infected erythrocytes, even by uninfected children's immune cells. However, during fever-inducing infection, these children's PBMCs, produced lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines (IL-10, TGF-β) [277]. This regulatory effect has been attributed to specific $CD4^+$ Teff that co-produce IL-10, IFN-γ, and TNF. Therefore, T cells regulate the exposure-dependent *P. falciparum*–specific reduction in pathologic fever response [277]. Additional mechanisms are likely to be at play in regulation of the fever response including changes in antigen presenting cell responsiveness known as TLR tolerance [278,279]. Similar tolerance mechanisms may be at play in anemia, which is transient, even in the presence of prolonged infection [234,280]. As reviewed in detail elsewhere some kind of tolerance also regulates the newborn immune response after exposure of the fetus to malaria during pregnancy [281]. Essentially, the tolerance to fever induced by repeated *Plasmodium* infections malaria may lead to the side-effect of increased susceptibility to infections in general.

Some mechanistic insights into disease tolerance suggest roles for TLR tolerance and IL-10. *P. falciparum* infection during pregnancy is associated with increased frequencies of Tregs and alterations in the response to TLR ligands in newborns exposed to *P. falciparum* during first months of life [267,268]. We also saw evidence of higher IL-10 upon stimulation of whole blood with TLR stimulation in newborns born to infected mothers. Therefore, elevated IL-10 activity in exposed newborns may inhibit T cell responsiveness via inadequately activated antigen presenting cells. Supporting this idea, increased proportions of Tregs, have been associated with high concentrations of IL-10 in cord blood in children exposed *in utero* to *Plasmodium* infection [269,270,282,283].

4.5 Role of CD4 T cells in tissue pathology of malaria infection

Cerebral malaria is the most common and most lethal of the outcomes of *Plasmodium* infection, and still kills more than half a million people worldwide every year. Severe malarial anemia is also very common, as are malaria-associated poor outcomes in pregnancy and birth. The role of cytokines is appreciated as a cause of placental malaria, and there is growing appreciation for the role of inflammatory cytokines in human cerebral malaria. As CD4 T cells regulate cytokine production by innate and adaptive cell types thus controlling both parasite killing and inflammatory pathology, they are at the center of these lethal pathologies.

There is an ongoing debate about the causes of cerebral malaria, which can be resolved by allowing that it is a multi-factorial syndrome. The most striking histopathological observation ever made in autopsy studies from cerebral malaria patients is the heavy parasitization of the blood vessels of the brain. Historically, the prevalence of parasites found adherent to vascular endothelium and within vessels, and the relative paucity of leukocytes found in the brain upon autopsy, led to the common interpretation that

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sequestration, not inflammation (interpreted as accumulation of leukocytes rather than cytokine production), drives cerebral malaria [284]. However, the concept of inflammation includes systemic and local cytokine and chemokine levels, and not just infiltration of a target organ by leukocytes. In addition, there is a growing appreciation for the effect of leukocytes within the vasculature, including T cells on the integrity of the endothelium and tissue function [285–288]. The role of T cell IL-10 in *P. chabaudi* infection is the perfect example. IL-10 produced by T cells is required to protect the host from dying of a syndrome that includes severe cerebral symptoms, and yet, T cells do not leave the vasculature in the brain [74,262]. T cell cytokines also have the potential to cause endothelial activation, an important pathological feature of cerebral malaria, including upregulation of the adhesion molecules used by the parasite to adhere for sequestration [240]. T cells can also recognize and kill endothelial cells with captured *Plasmodium* antigens [289–295]. Therefore, there is strong significance to the study of inflammation in mouse models that recapitulate some of the features of cerebral malaria, such as *P. berghei* ANKA infection of C57/BL6 mice. In this infection, a deficiency in either CD4 or CD8 T cells, as well as several T cell cytokines, effector molecules, chemokines, and several adhesion molecules can eliminate the behavioural symptoms associated with cerebral dysfunction [13]. CD8 T cells were recently identified in the cerebral vasculature of people who died from cerebral malaria [296]. As the immune response to *P. berghei* ANKA, and its relation to cerebral malaria has been extensively reviewed elsewhere [295,297], we will not dwell on it here, except where mechanisms seem to overlap with anemia and placental malaria.

Severe malarial anemia is also at least partially driven by and regulated by T cells. Anemia is not only the result of the death of parasite-infected erythrocytes. While this contributes to the reduction of functional erythrocytes, additional mechanisms are being discovered. In addition to inappropriate killing of uninfected erythrocytes, dyserythropoesis is evident in mouse and human. The serum cytokine levels in patients with severe malarial anemia have a measurably different ratio of IL-10 to IL-12 than serum from malaria patients with less severe disease, suggesting a role for T cell regulation, as shown for fever [298,299]. TNF inhibits erythroid expansion *in vitro*, as does addition of infected red blood cells or infected cell lysates [300,301]. In this review, we have chosen to use placental malaria as an example of what is known about how regulation of inflammatory cytokines by CD4 T cells contribute to the pathological consequences of this blood-borne infection.

4.6 Role of CD4 T cells in placental malaria immune cell infiltration and pathology

Plasmodium-infected erythrocytes adhere to endothelial cells and placental trophoblasts using host cell receptors contributing to severe complications, including cerebral or placental malaria [302]. Among the parasite surface proteins that contribute to sequestration, PfEMP1 (*P. falciparum*-Erythrocyte Membrane Protein-1) locus encodes about 60 members of the VAR gene family. The VAR2CSA-type has unique affinity to placental-expressed chondroitin sulfate A (CSA) [302,303] leading to binding of infected erythrocytes in the placenta [304–306]. Essentially, binding to VAR2CSA selects parasite variants that can expand in immune adults by infecting the new tissue formed only during pregnancy. In addition to maternal malaria, there are adverse effects of systemic inflammation induced by infection on outcomes of pregnancy, including pre-eclampsia [307]. High levels of infected red blood cells at the

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placental:fetal interface leads to a pro-inflammatory response in the placenta to kill the parasite, which also leads to pathological damage to the placenta. This localized pathology limits the function of the intervillious interface, which is to nourish the fetus and provide oxygen and therefore can induce intrauterine fetal death, maternal morbidity, premature birth, and low birth-weight infants, all of which is also seen in mouse models [308–311]. Additional effects on newborns as they grow up are just now becoming clear.

Placental pathology showed syncytiotrophoblast disruption in Gambian women [312]. Chronic intervillositis, or cellular infiltration of the open circulation in the placental intervilious space, has been reported, that ultimately affects maternal-fetal exchange [313]. Several studies have shown the expansion of circulating $CD4^+$ Teff during malaria infection during pregnancy [266,314]. Furthermore, the presence of immune T cells and monocyte/macrophages has been observed in *Plasmodium*-infected placentas. Increased frequencies of $CD4^+$ T cells secreting-IFN-γ and TNF were demonstrated in *P. falciparum* infected placenta compared to uninfected [315]. This study also found $IL\text{-}12^+$ monocyte/macrophages within placental tissue by flow cytometry. Elevated cytokine-secreting T cells and pro-inflammatory cytokine levels in placenta indicate that T cells actively participate to inflammatory responses to *Plasmodium* infection in the intervillous space. Several studies have demonstrated the impact of placental cytokine levels due to malaria infection with poor pregnancy outcomes. Specifically, increased concentrations of TNF, IFN-γ, IL-2, and IL-10 have been measured [316,317]. IFN-γ and IL-2 indicates the presence of CD4 T cells and suggests local proliferation, which can also regulate the other cell types identified in the tissue [318]. Investigators showed that higher TNF and IFN-γ in infected placentas from Kenyan women with *P. falciparum* were associated with both low birth weight and maternal anemia [319].

Mouse and human placentas share the hemotrichorial type and some, but not all features; therefore, we include relevant lessons learned from mouse models here [320]. Placentas of *P. berghei*-infected mice and rats show necrosis as well as hyperplasia of trophoblasts, peri-vascular edema, and sinusoid constriction [311,321–323]. Fibrin-type fibrinoid deposition was also seen associated with placental necrosis [312]. Pathology is also evident in the placenta of *P. chabaudi*-infected dams including placental disruption and fibrin thrombi [324]. Fibrin accumulation is also seen in mouse and human cerebral malaria and anti-coagulants have shown promise as adjuvant therapy in both pathologies in mice [324,325]. While sequestration contributes to congestion in vessels in the brain, lungs, kidney, and placenta, there are other important factors such as coagulation, leukocyte binding and activation, and endothelial and immune cell activation contributing to dysfunction of parenchymal tissue. It is also important to note that while there is definitely vascular congestion, there is little to no evidence that brain or placental pathology is downstream of hypoxia or necrosis caused by vessel blockage. Therefore, it is critical to continue searching for all of the contributing mechanisms of malaria pathology.

In *P. chabaudi* infected pregnant mice, high systemic production of the proinflammatory cytokines IFN-γ and IL-1β was seen while sustained TNF was measured in the placenta itself more than systemically [326]. Systemic TNF has also been shown to be able to induce the loss of the fetus, with infected dams more sensitive to its effects [327]. Monocyte/ macrophages have been identified in the placentas of *P. falciparum*, *P. chabaudi*, and *P.*

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berghei [328,329]. Regulatory IL-10 and inflammatory chemokines (IP-10, MIP-1, MCP and I-309), and cytokines (IFN-γ and TNF) [118,319,330,331], are produced in response to infected erythrocytes by placental mononuclear cells and lymphocytes from patients infected during pregnancy. Systemic neutralization of TNF (and LT) preserved placental architecture, which was severely affected in infected pregnant animals.

The data so far suggest that as in experimental cerebral malaria, T cells both make inflammatory cytokines in placental circulation that promote macrophage activation, and that must be regulated by T cell cytokines. In many immunopathologies, T cell cytokines amplify and regulate inflammatory monocyte function, which do not perform prolonged inflammatory functions in tissues without this positive and negative regulation. Both cytokine producing T cells and monocytes have been increased in infected placentas compared to uninfected placenta [315], and are likely to be the leukocytes seen in damaged areas of the placenta [332]. Damaged areas can also be marked by parasite hemozoin indicating prior parasite binding in these same locations. Potentially as a result of damage to the maternal trophoblast barrier to direct blood exchange, parasite antigens are thought to gain access to fetal blood, leading to *P. falciparum*-specific T and B cell responses by fetal umbilical cord mononuclear cells [282].

Overall these studies suggest a cellular inflammatory etiology to the damage in the placenta that may be done by hyper-activated inflammatory monocytes that leads to poor maternal fetal exchange that is both exacerbated and regulated by T cells. While it is not obvious that this same cellular mechanism can occur in the brain without significant cellular infiltration, these interactions may occur in the vasculature. Another possibility is that the brain is exquisitely sensitive to systemic cytokines, and that local amplification by tissue sentinel cells such as microglia drives neurological dysfunction. Better understanding of the interactions in the brain vasculature and intervillous space could also apply in the open circulation of the bone marrow where cytokines can affect erythropoiesis. Cytoadhesion of the parasite in various organs is also common to tissue pathologies, suggesting another potential contributor to induction of local inflammation. A new understanding of the specific pathologic mechanisms downstream of the contributions of TNF family members and coagulation to various pathways of pathology could lead to new clinical trials.

5. CD4 T cell memory to *Plasmodium spp.*

As CD4 T cells are so critical both in regulating effector functions important to parasite control and elimination, as well as effector functions that contribute to pathology, we must endeavour to understand both the quantity and quality of durable T cells that remain after an initial infection. Detecting *Plasmodium*-specific T cells in humans has been challenging, as described above. However in the last 20 years, development of mice with defined T cell receptors has allowed study of *Plasmodium*-specific T cells as they develop into effector and memory T cells [2,20,22]. Both in humans and mice, the majority of CD4 memory phenotype T cells specific to MSP-1 have an effector memory phenotype [219,333]. However, the CD62L or $CCR7^{lo}$ phenotype generally used includes both short-lived Teff and longer-lived Tem. We have recently taken advantage of the complete but transient downregulation of the IL-7Rα (CD127) on effector T cells to distinguish them from the effector memory phenotype T cells

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generated by *P. chabaudi* infection. The mixture of cells present two months after infection includes a mixture of effector, effector memory, and central memory T cells (in a ratio of about 10:80:10, [333]). Persistent infection, which maintains protection in humans and mice, also maintains *Ifng*+ T cells [333], which are a mixture of effector and effector memory T cells [334]. While the mixture of T cells divide in response to re-infection, they do not accumulate in number, or "boost," and their production of cytokines may be their most significant response [335,336]. When we tested all of the various T cell phenotypes found in the memory phase for protection, we found that the most differentiated, late effector memory phenotype T cells (Tem Late) contributed more to protection of immunocompromised mice than memory T cells with a central memory phenotype [337]. In addition, the Tem Late subset maintains a proportion of IFN-γ^+ T cells while they are alive [334]. We have recently shown that depletion of IFN-γ prior to re-infection inhibits the improved immunity to reinfection imparted by persistent infection [334]. This is in agreement with the finding that long-term immunity is boosted by recombinant IFN-γ [338]. While IFN-γ is likely to boost phagocytosis and maintain a state of heightened basal innate immunity, the mechanisms at play in persistent or recurrent *Plasmodium* infection have not yet been defined. Decay of immunity in the absence of exposure has been shown to correlate better with decay of T cells rather than antibody [221], and we recently showed that effector T cells actually protect better than any subset of memory T cells [337]. Intriguingly, this suggests that long-lived memory T cells generated to natural infection are not effective. That does not mean that there is not a potentially protective type of memory T cell that could be generated by vaccination. Further studies of the function [335] and metabolism [77] of these cells may provide some insight into differences with Tmem that protect in other infections. For example, there is a significantly different mitochondrial response to inhibition of fatty acid synthesis in persistently *Plasmodium* exposed T cells compared to Tmem generated in a shortened infection [77]. This could suggest that central memory T cells generated by acute stimulii [339], which can be promoted with drugs that stimulate fatty acid oxidation, rather than fatty acid synthesis alone, would be a better match for the parasite.

As suggested above, the surface phenotype can be one indication of protectiveness, however balanced cytokine production and effector function, including help for B cells, must be a critical component. Our work and that of others suggests that improving the production of IFN-γ in the memory phase, or continuous generation of this powerful cytokine may be as critical as maintaining protective levels of neutralizing antibodies in maintaining protection. In addition, stimulation of regulatory cytokines that promote less pathogenic responses to infection are critical. IL-10 increases over the course of one infection in *P. chabaudi* [74], and is made earlier in a second infection than a first [335,336]. While regulation of IL-10 transcription involves Blimp-1, and cMaf, it is not well understood, and mouse T cells *in vitro* do not show maintenance of IL-10 over time, like a fully differentiated subset. Similarly, most IL-10 producers in *P. yoelii* were short-lived [72]. Nevertheless, patients last exposed to *P. vivax* up to six years in the past still had T cells that could make IL-10 (in a six-day assay), but not IFN-γ (24-hour assay). While children make more IL-10 in highly exposed settings, adults do not, supporting the apparent self-limiting production of this cytokine [265]. This is particularly important now that IL-10 has been correlated not only with immunity to severe disease, but also to tolerance to fever downstream of the inhibition of

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multiple inflammatory responses from innate cells [277]. In this context, it is important to understand which cytokine responses are the best for reinfection, particularly with heterologous parasite, as in the field.

Both *P. falciparum* and *P. vivax* can be quite persistent, with *P. vivax* persisting in the liver and *P. falciparum* being able to evade immunity for up to two years, even in the absence of re-exposure [5,6]. As in other parasite infections, and other persistent pathogens, persistent infection can be the best prevention of recurrent pathology. For example, the Leishmanization strategy with active parasites still employed in Iran to avoid Leishmania [340]. In *P. falciparum*, IFN-γ^{+} MSP-1-specific T cells with a CD62L^{lo} Teff/Tem phenotype accumulate over time with exposure [219]. While Tem with a CD27^{-} phenotype are somewhat efficacious in mice, the efficacy of central versus effector memory T cells in humans against *Plasmodium spp.* is unknown [337]. Recent studies in highly exposed people in Uganda suggest that cytokines associated with the central memory T cell phenotype (TNF, IL-2) correlate with better T cell proliferation and lower parasitemia [341]. Re-expansion is often used as a ruler to measure efficacy of memory T cell responses; however, only central memory T cells appear to have this capacity. Much more needs to be understood about effector memory T cells before we can conclude whether they are useful for protection. In our transcriptome analysis comparing Tcm to Tem, we have found much more similarity between all memory T cell subsets than difference. For example, despite the expression of several “activation markers,” Tem do not show a significantly different metabolic transcriptome, or a broadly different balance of pro- and anti-survival or cell cycle genes than Teff (our unpublished observations). This phenotypic information can be used to reverse engineer vaccine strategies that will promote long-lived *Plasmodium*-specific T cell responses for long-term protection. Therefore, the take home message is likely to be that maintenance of effector T cells rather than memory T cells is a dominant mechanism protecting most adults from clinical malaria. This suggests that new mechanisms of chronic vaccination may be successful at prolonged protection, whereas all current methods of protection by vaccination are short-lived.

As we have seen above, information gleaned from the surface phenotype needs to be combined with information about protective functionality, including cytokine expression for antibody help, phagocyte activation, and also regulatory capacity. The last is likely the most critical aspect for understanding human immunity to disease. This is highlighted by the clear absence of sterilizing immunity in many people, especially if they leave an endemic area [175]. Cytokine studies are often performed separately from surface phenotype analysis due to technical limitations. However, we can understand cytokine production in immune people by the relationship with the age of the persistently exposed person. As this field is developing, variation in the frequency of exposure and parasite species still confound integration of the data collected on this point so far.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

In summary, the two primary functions of T cells in protection from *Plasmodium* infection itself are help for phagocytosis and antibody. While these two facets of the response are largely separated in time, they are carried out by the same T cells interfering with the

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exquisite immune architechture that separates these functions and thereby drives affinity maturation. As we have already seen, increased understanding of the molecular mechanisms of Th phenotype regulation will contribute to better therapeutics for many immune-mediated and chronic diseases. The symptoms caused by the inflammatory response are self-regulated by T cell cytokines as well. The overlapping functions within effector T cells are likely to lead to vulnerabilities that the parasite is able to use to evade immunity using its large genome, and capacity for antigenic variation. While regulatory cytokines are necessary to ensure the survival of the host, they also inhibit the parasiticidal immune response enough to allow persistent infection. Although much is understood about this response, there are still many outstanding questions, and we highlight some below. A major limitation to studying the *Plasmodium*-specific CD4 T cell response in humans is the lack of known protective T cell epitopes or MHCII tetramers. Without these reagents, we will not be able to compare mechanistic observations from mouse infection with *Plasmodium*-specific human T cell responses, or understand the role of the polyclonal T cell response in control of parasitemia or pathology in humans. However, the search to define proteins and epitopes that can drive protective responses by vaccination is well under way. To prevent disease caused by blood-stage infection, it is likely to be important to add blood-stage epitopes to the current liver-stage targeted vaccines, unless their effectiveness can be increased to 100%.

Another critical area of study is to determine the major determinants of protection provided by CD4 T cells. While effector T cells can protect in the presence of continuous or repeated infection, central memory T cells cannot be effectively generated under these conditions. Further work on new methods to generate memory and effector T cells with new vaccine platforms, particularly to drive particularly protective effector function combinations will be essential. It will be important to determine if strain variation is the primary reason that we continue to be susceptible to malaria disease long after the first infection, unlike in chicken pox. We now have the tools to detect PfEMP-1 variant gene expression and it will be important to understand the correlation of switching, which seems to be programmed, with selection by antibodies to exposed parts of the molecule. T cell epitopes, which can be derived from unexposed portions of proteins, and thus may be less variable, could provide a reliable vaccine component. While it is known that chronic infection is possible in part due to expression of variant antigens by the parasite, it is harder to determine the role of the immune response in the strain variation present in each population center. However, tools are being developed to answer these questions, though they will require close collaboration between immunologists and those studying genetic diversity of the parasite.

Another urgent need is to understand the role of TNF family members in pathology caused mostly, but not solely, by *P. falciparum*. As the balance of TNF and IL-10 has been clearly linked to each of the most severe pathologies, we should search for vulnerabilities in this system to utilize in therapeutics. Further understanding of the cell types involved in production and the various actions of TNF on the diverse tissue types targeted by immunopathology in malaria are required to identify druggable targets among the broad and cross-reactive TNF and TNFR families. In addition to these pressing, clinically relevant questions, there are many ways that understanding of the response to *Plasmodium* infection can inform our understanding of inflammation, immunopathology, and basic immunology.

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Recent work from many new and established laboratories are using *Plasmodium spp.* as a tool to define basic mechanisms of B and T cell biology, and we have highlighted a few here. A major conundrum will be deciphering mechanisms of affinity maturation that contribute to the clearance of the persistent parasite, and determining the role of this process in repeated and heterologous infection in humans. Some interesting areas are how the persistent infection or the host metabolic response to infection affect the T cell response, or how the parasite or T cell response regulates exhaustion. Now that parasite genetics are tractable, there should be a concerted effort to understand mechanisms of parasite virulence and immune-evasion, for example, screening a library of parasites mutated one gene at a time to discover parasite genes that drive the compromise with the host. This complex and important pathogen provides an amazing opportunity to delve into the weapons of war used by the host and pathogen and better understand their strengths and limitations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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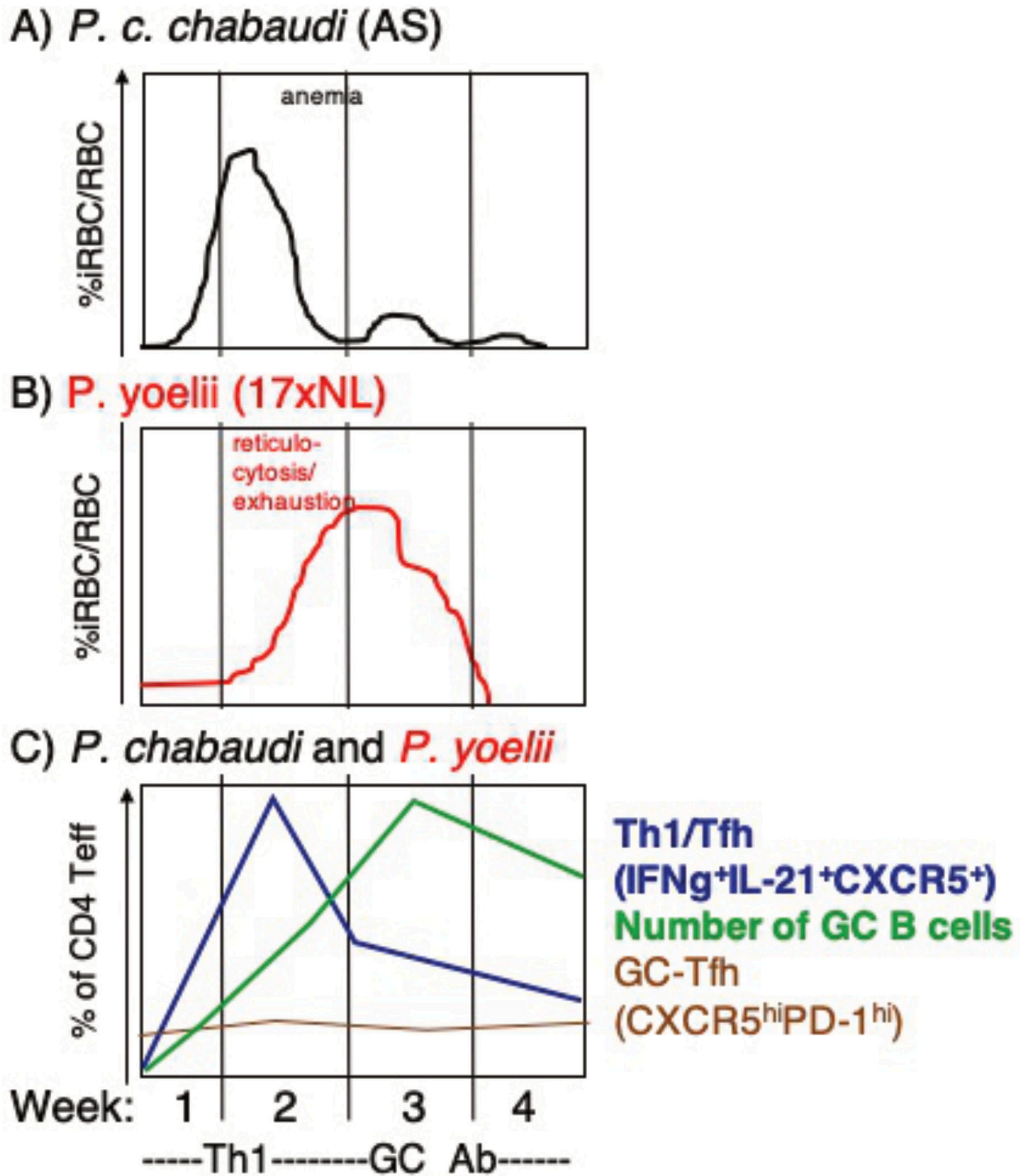

Figure 1.

Two phase adaptive immune response to *Plasmodium spp.*. Alignment of parasitemia of **A)** *P. chabaudi chabaudi* (AS) with **B)** *P. yoelii* (17XNL) and **C)** kinetics of T cell phenotypes in mice each week of infection. Th1-like response peaks preceeding strong germinal center response, which does not coincide with increase in GC-Tfh cells. Based on data from [36]

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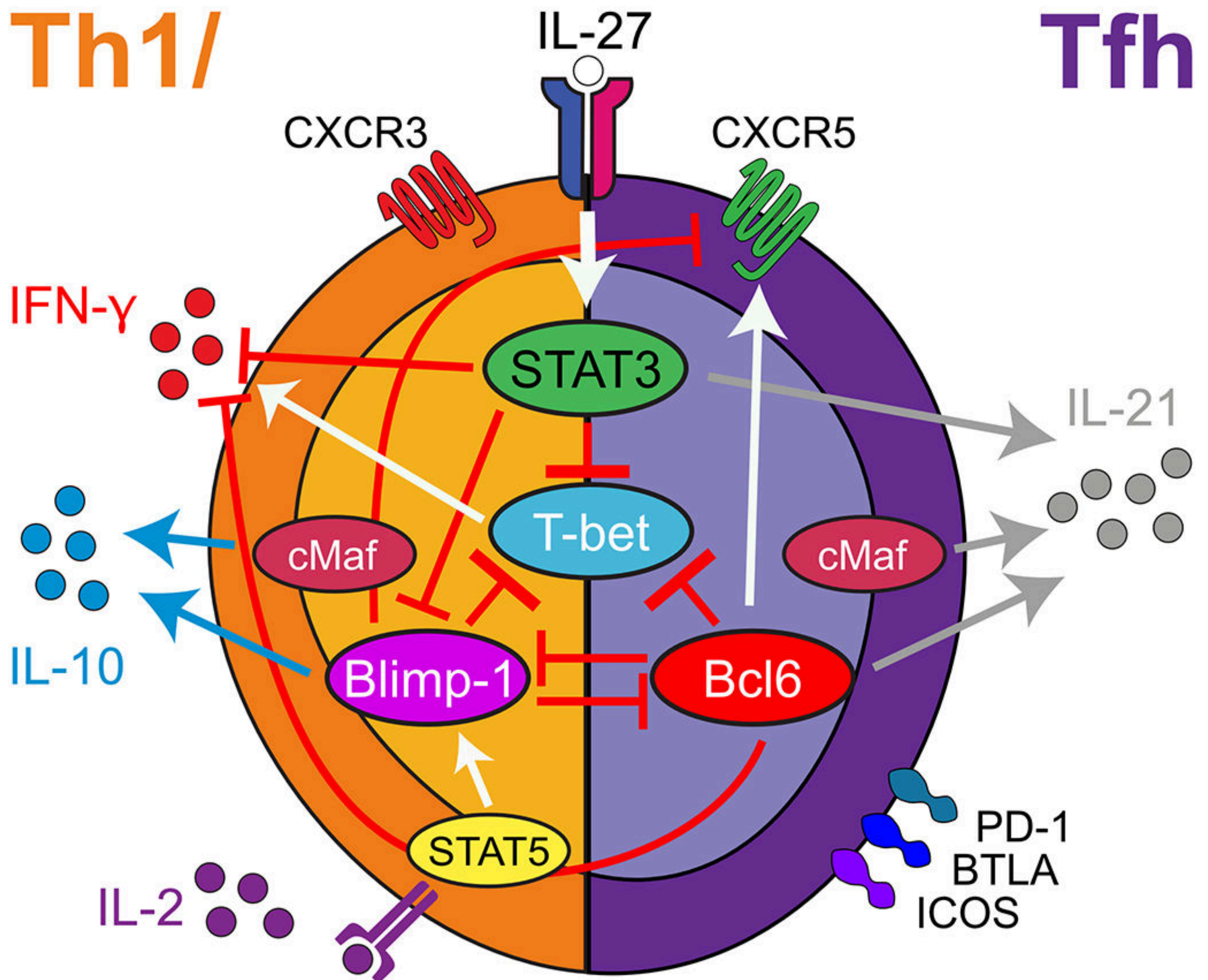

Figure 2.

Several transcription factors regulate T-bet in *Plasmodium*-induced hybrid Th1/Tfh cells. Each marker and cytokine is regulated by a unique set of overlapping signals. Based on data from [36]