T cell-mediated immunity to malaria

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Abstract

Immunity to malaria has been linked to the availability and function of helper CD4$^+$ T cells, cytotoxic CD8$^+$ T cells and $\gamma\delta$ T cells that can respond to both the asymptomatic liver-stage and the symptomatic blood-stage of *Plasmodium* sp. infection. These T cell responses are also thought to be modulated by regulatory T cells. However, the precise mechanisms governing the development and function of *Plasmodium*-specific T cells and their capacity to form tissue-resident and long-lived memory populations are less well understood. The field has arrived at a point where the push for vaccines that exploit T cell-mediated immunity to malaria has made it imperative to define and reconcile the mechanisms that regulate the development and functions of *Plasmodium*-specific T cells. Here, we review our current understanding of the mechanisms by which T cell subsets orchestrate host resistance to *Plasmodium* infection, based on observational and mechanistic studies in humans, non-human primates and rodent models. We also examine the potential of new experimental strategies and human infection systems to inform a new generation of approaches to harness T cell responses against malaria.

Introduction

Plasmodium species are the causative agents of malaria, a devastating disease responsible for more than 200 million infections and approximately 450,000 deaths annually[1]. Malaria is transmitted when *Plasmodium* sporozoite **[G]** forms are deposited into the dermis during *Anopheles* mosquito blood-meals (Fig. 1). Parasites exit the dermis and transit through the circulation to infect hepatocytes in the liver. Over the next several days of asymptomatic liver-stage infection, parasites undergo amplification and differentiation into merozoites **[G]**. Merozoites emerge from infected hepatocytes either singly or as part of a merosome **[G]** and represent an antigenically distinct form of the parasite that targets host erythrocytes to establish blood-stage infection, the phase responsible for all clinical signs and symptoms associated with malaria.

Both cellular and humoral adaptive immune responses are essential for limiting *Plasmodium* parasite replication and the severity of malaria (Fig. 2). As detailed below, in immune animals and partially immune humans, parasite-specific, cytotoxic CD8$^+$ T cells likely eliminate infected hepatocytes following recognition of parasite antigens presented on MHC class I molecules, whereas CD4$^+$ T cell-dependent antibody responses can prevent

sporozoite invasion of hepatocytes. Both of these immune mechanisms effectively prevent the progression from asymptomatic to clinical disease. During the blood-stage of *Plasmodium* infection in naïve or partially immune hosts, parasitized erythrocytes (which lack functional MHC expression) are indirectly targeted by CD4^{+} helper T cells and possibly γδ T cells that may orchestrate secreted antibody responses or the anti-parasitic activity of phagocytes.

Experimental malaria models in rodents and non-human primates have expanded our understanding of T cell-mediated protection against malaria and provided mechanistic insights that have guided the development of multiple experimental anti-malarial vaccine platforms. However, major challenges to immune-mediated elimination of malaria remain. The lead subunit vaccine candidate, RTS,S (Mosquirix™) **[G]** provides only short-lived, partial protection against malaria[2, 3, 4]. Thus, despite the current conceptual frameworks for αβ and γδ T cell-mediated protection against *Plasmodium*, we still lack sufficient mechanistic understanding of their formation and function, which has hampered the design of efficacious vaccines that can be deployed in malaria-endemic regions. The development of innovative anti-malarial vaccine platforms and the potential application of immunotherapies that stimulate or enhance resistance to malaria will require deeper insights into the cellular and molecular mechanisms that govern anti-*Plasmodium* T cell responses.

In this Review, we examine recent advances in our understanding of *Plasmodium*-specific αβ and γδ T cell subsets with specific emphasis on the mechanisms that these populations use to facilitate or hamper immune control of malaria. We discuss the secreted mediators that αβ and γδ T cell subsets employ to orchestrate resistance to malaria, including cytokines and pro-apoptotic factors. Although much of the mechanistic data describing both infection- and vaccine-induced T cells were generated in mouse studies, we also highlight key associations identified in human field studies and controlled human malaria infection (CHMI) models. However, missing critical information and technical limitations continue to impede progress in the field (Box 1). Despite this, we also highlight technical innovations and recent experimental advances that have facilitated critical insights into the biology of *Plasmodium*-specific T cells (Box 2) and illustrate the major gaps that remain to be addressed through future clinical and experimental studies.

CD4^{+} T cells in malaria

CD4^{+} helper T (Th) cells are activated following engagement of pathogen-specific peptides presented on MHC class II molecules and are central to orchestrating key aspects of both innate and adaptive immunity during *Plasmodium* infection. The presence of *Plasmodium*-specific CD4^{+} T cells has been identified as a correlate of protective immunity following either natural exposure or anti-malarial vaccination[5, 6, 7, 8]. When activated in the presence of specific polarizing cytokines, CD4^{+} T cells have the capacity to differentiate into one of several functionally distinct subsets. Given the complexity of the *Plasmodium* parasite lifecycle (Fig. 1), it is not surprising that a number of functionally diverse CD4^{+} T cell subsets have been identified in both experimental and clinical malaria studies and that their mechanism(s) of protection are distinctly linked to specific *Plasmodium* developmental stages (Fig. 2). Notably, accumulating evidence supports the idea that long-lived, sterilizing

immunity does not develop in humans following repeated exposure to *Plasmodium* parasites and there is also evidence of reduced efficacy of childhood vaccination in malaria-exposed individuals[2, 8, 9, 10]. These studies highlight the complexity of the regulatory circuits and immune checkpoints that become engaged during malaria. Rather than examine in detail the differentiation and regulation of CD4^{+} T cells, topics that have been recently reviewed[11, 12, 13], we focus our discussion here on the mechanisms by which functionally distinct CD4^{+} T cell subsets shape resistance to liver- and blood-stage malaria.

Th1 cells.

The presence of Th1 cells and an elevated IFNγ response are signatures of both human[14] and rodent[15, 16, 17, 18] malaria. In addition to IL-12, the formation of *Plasmodium*-specific Th1 cells has been linked to CD4^{+} T cell-intrinsic sensing of extracellular ATP by P2X7[19], and *P. falciparum*-activated human DCs may be uniquely programmed to promote Th1 cell differentiation[20]. The activity of T-bet upregulates IFNγ and approximately half of all Th1 cell-associated genes, in addition to repressing the transcriptional programmes of other CD4^{+} T cell subsets (reviewed in detail elsewhere[21]), including Th2 cell, Th17 cell and T follicular helper (Tfh) cell subsets that are described below. During experimental blood-stage infection, T-bet (*Tbx21*)-deficient mice exhibited the loss of IFNγ-producing splenic CD4^{+} T cells, which directly correlated with elevated parasite burdens[22]. Similarly, *Plasmodium* infection of *Il12^{-/-}* mice reduced T-bet and IFNγ expression in CD4^{+} T cells and abrogated control of *Plasmodium* replication[15].

The exact mechanisms by which IFNγ and effector Th1 cells contribute to host protection during blood-stage *Plasmodium* infection remain largely speculative[23], although experimental data suggests that IFNγ is critical for activating macrophages[24, 25, 26] and may tune class-switch recombination in *Plasmodium*-specific B cells[15]. IL-2, another CD4^{+} T cell-derived, Th1-associated cytokine is important for activating natural killer (NK) cells, which may participate in protective immune responses by direct cytolysis of *Plasmodium*-infected erythrocytes[27]. IFNγ-producing, TBET^{+} Th1 cells can also express macrophage colony stimulating factor (M-CSF, also known as CSF1), and CD4^{+} T cell-specific deficiency of M-CSF exacerbates the loss of CD169^{+} macrophages and abrogates the control of blood-stage infection[28]. Notably, deletion of CD169^{+} macrophages phenocopied the reduced parasite control seen in mice with a T cell-restricted deficiency in M-CSF, suggesting a key mechanistic role for M-CSF-expressing Th1-like cells in promoting the function or antigen presentation capacity of protective myeloid cells[29]. Regarding memory responses, Th1-like CD4^{+} T cells are maintained following resolution of acute experimental malaria and exhibit protective capacity upon recall in rodent models[30,31, 32, 33]. Deciphering the precise mechanisms by which memory Th1 cells orchestrate protective recall responses to blood-stage malaria remain important lines of investigation.

Th1 cell responses and IFNγ secretion have also been linked to host resistance during liver-stage *Plasmodium* infection. In *Plasmodium* exposed[34] and experimentally vaccinated[34] individuals, the presence of IFNγ-expressing, circumsporozoite **[G]** (CSP)-specific Th1 cells was linked to reduced parasite burdens and disease severity. Although the precise mechanisms by which these liver-stage-specific CD4^{+} T cells orchestrated immunity was not

explored in detail, Th1 cell-associated IFNγ[35, 36] may either directly activate inducible nitric oxide synthase in infected hepatocytes[37] or potentiate the cytotoxic activity of *Plasmodium*-specific CD8^{+} T cells via upregulation of MHC class I molecules on infected liver cells[38]. In controlled human malaria infection models[39] (CHMI, see also Box 3) and following chemoprophylaxis and sporozoite (CPS) immunization **[G]** of malaria-naïve volunteers[40], reduced blood-stage parasite burdens were associated with the presence of CD4^{+} T cells exhibiting characteristics of cytotoxic Th1-like cells. These cells exhibited elevated expression of CD38, IFNγ, CD107a, and granzyme B. The exact mechanisms by which cytotoxic CD4^{+} T cells participate in anti-*Plasmodium* immunity are not defined, but are potentially linked to the recognition and elimination of infected targets cells during either blood- or liver-stage infection (Fig. 2). In rodent models, *Plasmodium*-specific cytotoxic CD4^{+} T cells have also been identified[35, 36, 41, 42] that may recognize and kill infected hepatocytes. The extent to which these cytotoxic Th1-like CD4^{+} T cells form in malaria exposed individuals remains a question and their relative contribution to controlling either blood- or liver-stage *Plasmodium* requires further study.

Studies have also identified pathological roles for Th1 cell responses and IFNγ secretion during *Plasmodium* infection. IFNγ has been linked to atypical memory B cell formation in *Plasmodium*-exposed humans[43] and rodent models show that elevated Th1 cell and IFNγ responses[44, 45] and B cell-intrinsic IFNγ signalling[46] can either impair humoral immunity or expand phosphatidylserine-specific, self-reactive B cells that can exacerbate anemia[47]. Notably, multiple CD4^{+} T cell subsets, including Tfh cells express IFNγ during experimental malaria. Thus, fundamental questions remain regarding the relative contribution of key cellular sources of IFNγ during *Plasmodium* infection, including γδ and CD8^{+} αβ T cells. Future experimental studies that exploit conditional allelic deletions of IFNγ or its receptor should help resolve long-standing questions regarding the cell type-specific sources and targets of IFNγ and help determine whether the protective versus pathological roles of this key Th1 cell-associated cytokine evolve as the infection progresses.

Th2 cells.

Th2 cells are primarily characterized by expression of the GATA3 transcription factor and by the production of IL-4 and IL-5[48]. The role of Th2 cells in malaria is relatively unknown, as GATA3^{+} CD4^{+} T cells are rare or absent during *Plasmodium* infections[49] and strong Th1-type polarization of CD4^{+} T cells may limit Th2 cell differentiation[50]. However, IL-4, the major cytokine expressed by Th2 cells, can promote B cell class switching[51, 52] and modulate macrophage responses[53] during *Plasmodium* infection. Moreover, IL-4 has been identified as a correlate of enhanced humoral immunity in *Plasmodium* exposed humans[54]. By contrast, experimental infections showed that WT and *Il4*^{−/−} mice clear *P. chabaudi*-infected erythrocytes at identical rates[52], suggesting that IL-4 may be dispensable for protection against blood-stage malaria. Regarding liver-stage infection, as discussed in detail below, CD4^{+} T cell-derived IL-4 is important for promoting potent effector CD8^{+} T cell responses against infected hepatocytes[55], as well as maintaining functional *Plasmodium*-specific memory CD8^{+} T cell populations[56]. Thus, there may be potentially important context-dependent roles for IL-4 and Th2 cells during malaria and further work is required to define the precise role of Th2 cells, independent of their secretion of IL-4.

T follicular helper cells.

Both vaccination and infection-induced Tfh cells are broadly characterized by expression of the transcriptional repressor BCL-6, the chemokine receptor CXCR5, and the inhibitory receptor programmed cell death protein 1 (PD1)[57]. Multiple experimental systems have shown that expression of CXCR5 licenses pathogen-specific CD4^{+} T cells to undergo step-wise Tfh cell differentiation within lymph nodes; Tfh cells first localize to the 'T-B border' of the follicle, engage in cognate interactions with B cells that reinforce their differentiation, and then migrate into the light zone of the germinal centre (GC) where they provide selection, survival, and maturation signals to differentiating GC B cells[58]. CXCR5^{+}PD-1^{+} Tfh cells expand during both human and rodent blood-stage *Plasmodium* infections and are essential for promoting protective antibody responses that aid in the resolution of malaria[44, 45, 59, 60, 61, 62]. *Plasmodium*-specific Tfh cells express IL-21[63] and inducible T cell costimulator (ICOS)[64, 65], which promote maturation of *Plasmodium*-specific GC B cells and the generation of long-lived plasma cells and memory B cells (Fig. 2). As described for virus-specific Tfh cells[66], *Plasmodium*-specific Tfh cells downregulate BCL-6 and PD1 as they transition from effector to memory populations[33], leaving CXCR5 as the most reliable marker for *Plasmodium*-specific memory Tfh-like cells. Reports also suggest that virus-specific Tfh cells retain greater plasticity and secondary proliferative potential compared to their terminally differentiated Th1-like counterparts[66, 67]. However, we and others showed that *Plasmodium*-specific Tfh-like memory cells are less protective than Th1-like memory cells on a per-cell basis following adoptive transfer[32, 33]. The mechanisms by which either Tfh- or Th1-like memory CD4^{+} T cells orchestrate secondary immune reactions in malaria remain unknown.

The differentiation of *Plasmodium*-specific Tfh cells was examined in a recent single cell RNA sequencing (scRNA-seq) study that revealed early bifurcation of Th1 and Tfh cell differentiation[68]. Recent studies also showed that *Plasmodium*-specific Tfh cell development and function are promoted by IL-6[69] and countered by the activity of IRF3[70]. Notably, effector CD4^{+} Tfh cells exhibiting mixed characteristics of either Th1 or T regulatory 1 (Tr1) cells (described below) have also been reported during experimental malaria[45, 31, 33, 71]. These cells often co-express NK1.1, CXCR5, IFNγ, IL-21, and IL-10; and in one study, their formation and function did not depend on the function of BCL-6[31]. This mixed 'helper' phenotype is perhaps not surprising because like many other CD4^{+} T cell subsets, Tfh cells are also highly plastic and can adopt characteristics of either Th1, Th2, Th17, or Treg cells. In *Plasmodium*-infected humans[59] and rodents[45], BCL-6-expressing Tfh cells adopt a Th1-like phenotype[59, 72], secrete IFNγ and provide relatively inferior help to B cells[46]. Additionally, Tfh-like cells can express FOXP3 and exert regulatory function; such cells are referred to as T follicular regulatory (Tfr) cells and localize to the GC, where they suppress humoral immunity via expression of either CTLA-4[73] or PD1[74]. Notably, the role of Tfr cells during *Plasmodium* infection may be temporally distinct or context-dependent, as Tfr cells are reported to be a critical source of IL-10 that supports GC B cell responses during viral infections[75]. Although the key source of IL-10 that supports humoral immunity during malaria[46] has not been reported, it will be of interest to determine whether either Tfh, Tfr or T regulatory type 1 (Tr1) cells regulate humoral immunity via provision of IL-10. The importance of Tfh cells during acute and

chronic malaria is now well documented. Although our understanding of Tfr cells continues to expand[74, 76, 77], we still know very little about Tfr cell differentiation and function during either human or experimental *Plasmodium* infection. Aberrant *Plasmodium*-specific effector or memory Tfh or Tfr cell responses represent one possible explanation for the delayed acquisition or deficient maintenance of antibody-mediated anti-malarial immunity. Thus, future studies aimed at deciphering the phenotype, function, and role of Tfh and Tfr cells following either single or repeated *Plasmodium* exposures will be of interest.

Th17 cells.

In the presence of IL-6, IL-23, and TGFβ, naïve CD4^{+} T cells can adopt a functional programme governed by RORγt, an essential transcription factor regulating IL-17 expression. Th17 cells have been linked to orchestrating neutrophil recruitment and function during multiple scenarios of microbial infection (reviewed in ref.[78]). Although pathological neutrophil responses have been associated with severe malaria[79, 80], only a limited number of studies describe the presence of *Plasmodium* infection-induced Th17 cells. The first study described CD4^{+} T cells in malaria exposed individuals from Mali with the capacity to express IL-17A following *in vitro* stimulation[81]. In experimental models, only a very limited number of Th17 cells are detected in the spleens of *Plasmodium*-infected mice[82, 83, 84, 85]. Mechanistic studies failed to identify any role for Th17 cells and the genetic deficiency in *Il17a* did not influence either disease severity[82] or protective immunity[83]. However, Th17 cells can also secrete IL-21[86], suggesting that Th17 cells, when present, may play a modest role in supporting GC reactions. IL-21 may also support CD8^{+} T cell responses, as has been described in *Toxoplasma gondii* infection[87, 88]. Moreover, rodent models have shown that the Th17 cell-associated cytokine IL-22 was essential for protecting against inflammatory pathology in the lung and liver in mice with malaria[83]. CD8^{+} T cells were found to express IL-22, although whether Th17 cells also served as a source of IL-22 was not determined. Further work is necessary to define the role of Th17 cells during *Plasmodium* infections. Whether Th17 cell populations can also be expanded in anti-malarial vaccinations and meaningfully contribute to host protection also remains to be addressed.

IL-27-producing CD4^{+} T cells.

IL-27 is a cytokine that has been linked to both pro- and anti-inflammatory immune functions. Initial analyses of the role of IL-27 during experimental malaria revealed its importance in limiting immunopathology in either primary or secondary blood-stage malaria[89, 90, 91]. First described as a myeloid-derived immunoregulatory factor, recent work using experimental *P. berghei* infection in mice revealed that IL-27 can also be produced by parasite-specific CD4^{+} T cells[92]. This study showed that IL-27 is produced by a subset of IL-10^{−}IFNγ^{−} CD4^{+} T cells during acute *Plasmodium* infection and that the primary effect of IL-27 is to suppress IL-2 production by other CD4^{+} T cells[92]. Given their immunosuppressive function, determining whether the expansion of IL-27-expressing CD4^{+} T cells is a potential signature of either acute or chronic *Plasmodium* infection warrants further investigation.

IL-10-producing CD4$^+$ T cells.

T regulatory type 1 (Tr1) cells express high levels of both BLIMP1 and T-BET, are FOXP3-negative, and co-express IFNγ and IL-10[93, 94, 95]. These cells are widely regarded as a subset of terminally differentiated Th1 cells that have been reprogrammed to express IL-10. The precise mechanisms by which this transition occurs remain incompletely understood. However, experimental data support the view that CD4$^+$ T cell-intrinsic IL-27[90], IL-10[96], and type I IFN signaling[94] can promote Tr1 cell formation in *Plasmodium*-infected mice, whereas unspecified signals from CD8a$^+$ cDC1s restrain the development of Tr1 cells[97],. Type I IFNs also appear to promote rapid Tr1 cell formation following CHMI[98]. Tr1 cells have been established as the primary source of IL-10[93, 94, 95] that both prevent immunopathology and facilitate parasite persistence during protozoan infections[93, 99, 100]. Malaria is no exception and Tr1 cells appear to limit protective immunity and parasite control[93, 94], by either suppressing humoral immune responses[94] or possibly by reducing the capacity of antigen presenting cells (APCs) to sustain T cell activity. An early cross-sectional study in Gambia identified that children with mildly symptomatic malaria had an increased proportion of IL-10-producing CD4$^+$ T cells compared to children with severe clinical malaria[101]. More recently, the presence of CD4$^+$ Tr1 cells was associated with increased parasitemia during human *Plasmodium* infections[95], yet was also associated with a decreased risk of severe clinical disease[102]. Therefore, while Tr1 cells can down-regulate pro-inflammatory responses during malaria, IL-10-production by Tr1 cells may impair parasite control[93]. Thus, both FOXP3$^+$ Treg cells (discussed below) and FOXP3$^-$ Tr1 cells are critical regulators of host anti-malarial immunity, with FOXP3$^-$ Tr1 cells primarily serving to limit malarial disease severity. The precise mechanisms by which Tr1-derived IFNγ and IL-10 modulate protective versus pathological responses remain to be deciphered. Understanding of the factors that regulate Tr1 cell activity during *Plasmodium* infection will have important implications for controlling malaria-associated immunopathology.

Treg cells.

Treg cells are a class of CD4$^+$ T cells delineated by their expression of the transcription factor FOXP3. Although Treg cells were originally identified as expressing high levels of CD25 and low levels of CD45RB on their cell surface, we now know that there is no single surface marker that demarcates all Treg cells. Along with FOXP3, the current definition of Treg cells encompasses the epigenetic 'Treg signature', of CpG demethylation in *Foxp3* conserved non-coding region 2, *Tnfrsf18*, *Ctla4*, *Ikzf4* and *Il2ra* genes[103, 104, 105]. Treg cells are known to define host immune responses to human and mouse malaria, yet their impact and mode of action have remained controversial and contentious[106].

Most of the inconsistencies observed in the mouse models of malaria (elaborated in various reviews[106, 107, 108]) can be traced back to the incomplete characterizations of various T cell subsets, limitations in the methodologies used to deplete Treg cells, or variations in the infection models used. For example, CD25 is expressed by activated conventional CD4$^+$ and CD8$^+$ T cells and is not expressed by a significant proportion of Treg cells[109], yet anti-CD25 (PC61) has been commonly used to 'deplete' Treg cells[106, 107, 108] in mice. When mice were treated with an anti-CD25 antibody at the onset of infection, better control of *P. yoelii* infection was seen in BALB/c mice[110], but not in C57BL/6 mice[93]. Furthermore, treatment

with anti-CD25 exacerbated *P. chabaudi* infection in BALB/c mice[111]. In addition, the timing of depletion of Treg cells in mouse studies has been inconsistent, with these studies often failing to consider the Treg cell kinetics associated with blood-stage malaria[73]. When Treg cells were depleted using anti-CD25 treatment at the peak of their population expansion (as opposed to at the onset of infection[93]) in *P. yoelii*-infected C57BL/6 mice[73], parasitemia was better controlled. Given that Treg cells rapidly repopulate the host in 1–2 days after their complete depletion[112, 113], informed Treg cell-depletion strategies may need to be tailored to the kinetics of Treg cell expansion in malaria. For instance, C57BL/6 mice treated with anti-CD25 antibody before the onset of *P. berghei* (ANKA) infection were better protected from experimental cerebral malaria (ECM)[114, 115]. But ECM symptoms remained unchanged with precise depletion of Treg cells in FOXP3-Diphtheria toxin receptor (DTR) transgenic (DEREG) C57BL/6 mice[116], suggesting a minimal role for Treg cells in the ECM model. Malaria is a complex disease in which Treg cell numbers and function are kinetically modulated during its course. A better understanding of the Treg cell kinetics and employment of precise Treg cell-depletion strategies accordingly might help understand the underlying mechanisms of immunoregulation in malaria, using the mouse model.

Longitudinal or cross-sectional studies in human subjects from endemic areas naturally or experimentally infected with *Plasmodium* consistently show that Treg cell populations expand in blood-stage malaria; and lower Treg cell frequencies are associated with lower parasite loads along with more favorable disease outcomes[73, 108, 117, 118, 119]. Our inability to manipulate the immune system in humans has made it difficult to know if the expansion of Treg cell populations is a cause or consequence of enhanced parasite loads. Lower frequencies of functionally deficient Treg cells are associated with lower parasitemia levels in the African Fulani people, who are naturally more resistant to malaria, compared to the sympatric Mossi people[118]. This suggested that Treg cell expansion in humans may be a consequence, rather than the cause, of increasing parasite loads in malaria. In addition, longitudinal studies showed that clinical malaria was associated with higher Treg cell frequencies compared to pre-infection or convalescent levels[73]. Also, higher pre-infection Treg cell frequencies correlated with increased risk of febrile malaria[120]. Further longitudinal, controlled infection studies with human malaria will help draw better parallels on the nature of Treg cell responses and functions in the human and mouse model of malaria; and might advance our mechanistic understanding of immunoregulation by Treg cells in blood-stage malaria.

Treg cells can be broadly classified into two subsets based on their origins: thymically derived Treg (tTreg) cells, which differentiate in the thymus from immature CD4$^+$CD8$^+$ precursors, and peripheral Treg (pTreg) cells, which originate from conventional CD4$^+$ T cells in peripheral tissues that have upregulated FOXP3 in response to chronic or sub-optimal T cell receptor (TCR) stimulation, homeostatic cues or commensal bacteria[121, 122, 123, 124, 125]. The presence of a diverse array of non-overlapping TCRs in tTreg and pTreg cells, indicates a distinct, non-redundant role for either Treg cell subsets in the recognition of antigens[105, 126]. Yet, the relative contributions of pTreg or tTreg cells in most infections, including blood-stage malaria, remain unresolved. This is partly due to the absence of precise markers that can distinguish pTreg cells from tTreg cells. Although the expression of Neuropilin-1 in mice and Helios in humans are sometimes used to separate

tTreg cells from pTreg cells[105], their lack of accuracy has confounded our abilities to segregate their functional roles in various infection models[122, 127]. Some earlier studies with experimental human malaria have suggested that pTreg cells may be induced in response to blood-stage malaria, through TGFβ activation[107, 119, 128]. However, the role of TGFβ is now considered to be mostly limited to inducing Treg cells *in vitro*[122, 129, 130]. Other studies have suggested that tTreg cell populations may expand in response to blood-stage malaria in both humans and mice[93, 111, 114, 131]. Therefore, the relative contributions of, one, antigen-driven proliferation, two, cytokine-mediated differentiation, and three, recruitment to peripheral tissues, to the generation and function of tTreg or pTreg cells in blood-stage malaria remain unsettled. More research needs to be done to help understand the relative contributions of tTreg or pTreg cells to the overall impact of Treg cells in combating blood-stage malaria.

The mechanism by which Treg cells function in malaria is also a relatively understudied area. Recent work from our own laboratory using a mouse model of malaria infection showed that Treg cells use CTLA-4 expression to hinder productive interactions between Tfh cells and B cells in GC reactions in lymphoid tissues; this resulted in compromised humoral and cell-mediated immune responses to blood-stage malaria[73]. Populations of Treg cells that expand in febrile malaria in humans and mice express higher levels of CTLA4; and therapeutic blockade of CTLA4 tailored to the kinetics of CTLA4 expression or Treg cell expansion in blood-stage malaria resulted in better control of the infection in the mouse model. In addition to expressing CTLA4, Treg cell populations that expand in *P. yoelii*-infected mice transcriptionally upregulate genes that are associated with Treg function, including *Gpr83*, *Penk1*, *GzmB*, *Socs2* and *Il10*[132]. More extensive investigations in the future will help determine the roles of these genes, as well as the molecular mechanisms of immunoregulation in blood-stage malaria.

The immuno-regulatory responses to malaria, spearheaded by Treg cells help rein in the immune system from damaging the host. Malaria is a protracted disease with multiple phases of complex host-pathogen interactions leading to discrete waves of cellular and humoral immune responses. Only mechanistic studies using the mouse model, with concurrent observational studies in humans may help decipher the mechanistic underpinnings of immunoregulation by Treg cells in primary or subsequent blood-stage malaria infections.

CD8+ T cells in malaria

CD8+ T cells recognize pathogen-derived peptides bound to surface MHC class I molecules on APCs or infected cells and contribute to the clearance and immune memory against many intracellular pathogens. Malaria parasite-specific CD8+ T cells have been described in the blood of humans living in endemic areas[133, 134] and after vaccination[135, 136, 137]. In experimental malaria, CD8+ T cells specific for sporozoite antigens, liver-stage antigens (the pre-erythrocytic stages) and blood-stage antigens (erythrocytic stage) (Fig. 2) have been described after infection or vaccination[138, 139, 140]. Although CD8+ T cells are primed against the various pre-erythrocytic stages of malaria in the vertebrate host, their relevance to protection in a primary infection is contentious[140, 141]. This is largely because an infected mosquito delivers only a few hundred sporozoites into the host dermis[142], leading to a very

low proportion of infected hepatocytes prior to release of blood-stage merozoites[140, 143]. Further, liver-stage malaria offers a short window of opportunity (~7 days in humans and ~2 days in mice)[144, 145] to mount optimally functional[146] effector CD8^{+} T cell responses[106, 144, 147]. Additionally, repeated exposure to *Plasmodium* infections does not generate sufficient immunity against the liver-stages in humans despite eliciting disease-limiting humoral immunity against the pathogenic blood stage[148, 149, 150]; the precise reasons for this remain a major knowledge gap. However, if sufficient antigen-specific CD8^{+} T cell responses are generated against the pre-erythrocytic stages of *Plasmodium* by immunization, progression to blood-stage malaria can be prevented in humans, non-human primates and the mouse models[5, 151, 152, 153]. Hence the underlying mechanisms of priming, dynamics and function of CD8^{+} T cell responses to malaria are areas of intense research in the quest for an anti-malarial vaccine.

For a long time it was assumed that CD8^{+} T cell responses against pre-erythrocytic stages of *Plasmodium* were primed by infected hepatocytes[154]. Yet, the unlikelihood of rare naïve CD8^{+} T cells, encountering infrequently infected hepatocytes in the liver made this event improbable based on the existing paradigms of T cell priming mechanisms[155]. Early studies revealed that CD11c^{+} dendritic cells (DCs) played a vital role in priming CD8^{+} T cell responses to pre-erythrocytic developmental stages of *Plasmodium*[156]. Sporozoite antigen-specific CD8^{+} T cell responses are generated in the skin-draining lymph nodes at the site of inoculation, primarily by the uptake and cross-presentation of sprorozoites by CD8^{+} CD11c^{+} DCs[157, 158]. However, the observation that late liver-stage arresting genetically attenuated *Plasmodium* parasites (GAPs) elicited better protective CD8^{+} T cell responses compared to early liver-stage arresting GAPs or radiation attenuated *Plasmodium* sporozoites (RAS) suggested that the developmental progression of *Plasmodium* in infected hepatocytes had a decisive role in generating better, perhaps antigenically broader CD8^{+} T cell responses[159]. Recent findings from our lab identified a class of monocyte-derived CD11b^{+}CSF1R^{+}CD207^{+}F4/80^{+}CD11c^{+} APCs in the liver that acquired *Plasmodium* following hepatocyte infection, to prime CD8^{+} T cell responses against liver-stage specific antigens, in the liver-draining lymph nodes (Kurup et al submitted). This finding uncovers how a broad spectrum of CD8^{+} T cell responses are primed against the *bona fide* liver stages of development in malaria. The precise mechanisms by which APCs acquire *Plasmodium* liver-stage antigens from infected hepatocytes remain to be elucidated, and may involve extrinsic or cell-intrinsic innate immune pathways. In contrast to natural infections, live-attenuated vaccines using RAS generate sterilizing CD8^{+} T cell mediated immune responses only after intravenous inoculation of the parasites[160, 161]. Here, CD8^{+} T cell responses may be primed in the spleen, in addition to the liver-draining lymph nodes[162]. Furthermore, other immune cells including NK cells, helper T cells and regulatory T cells influence the generation and maintenance of productive CD8^{+} T cell responses[55, 163, 164, 165]. A deeper understanding of the dynamics of CD8^{+} T cell priming in natural *Plasmodium* infections or live-attenuated malaria vaccines will help us devise strategies that can help engender stronger and long-lasting protective immunity to malaria.

Intravital and *in vivo* imaging studies showed that adoptively transferred sporozoite-specific effector CD8^{+} T cells clustered around infected hepatocytes, resulting in loss of *Plasmodium*-GFP signal indicating the likely destruction of the parasite and infected host

cell[166, 167]; and it is assumed that memory CD8^{+} T cells may function similarly. Activated CD8^{+} T cells are capable of elaborating a number of anti-microbial effector mechanisms, broadly divided into cytolytic and cytokine pathways[168, 169]. Among the various effector pathways and molecules associated with CD8^{+} T cell function, while granzymes and Fas/FasL mediated pathways appeared dispensable for the effector functions of CD8^{+} T cells against liver-stage malaria in mice, IFNγ, TNF and perforin contributed to protective immunity, depending on the *Plasmodium* species and genetic background of the host[170, 171, 172, 173, 174, 175]. IFNγ is known to directly impair *Plasmodium* development in human hepatocytes in culture[176]. Mechanistic studies with the mouse models of malaria indicated that IFN-γ produced by CD8^{+} T cells induced nitric oxide synthase (and hence nitric oxide) in the infected hepatocytes to help eliminate them[177]. However, adoptively transferred *Plasmodium* circumsporozoite protein (CSP)-specific CD8^{+} T cells deficient in IFNγ were able to confer protection from *P. yoelii* liver-stage malaria[178]. The variation in the functional roles of CD8^{+} T cells depending on the parasite species and the genetic background of the host[179, 180] indicates that effective CD8^{+} T cell responses in malaria may be influenced by cell-extrinsic and natural host defence mechanisms. The precise mechanisms by which CD8^{+} T cells function to limit liver-stage infection in human malaria remain unknown, and a better understanding of those will help further our ability to tailor sterilizing immunity to malaria.

As indicated above, the protective role of CD8^{+} T cell responses generated against pre-erythrocytic developmental stages of a primary *Plasmodium* infection are possibly relevant in subsequent infections or in vaccinations. Although the higher frequencies of peripheral CD8^{+} T cell responses elicited by various immunization strategies against *Plasmodium* may reflect the higher chances of protection from subsequent challenges with live sporozoites[9, 153, 180, 181], if and how they functionally and numerically contribute directly to protection against liver-stage malaria is currently unknown. Nevertheless, a tangible pool of liver-resident CD8^{+} T cell populations specific for the liver-stage antigens of malaria, that might directly target infected hepatocytes, are generated and maintained after immunizations with RAS[182] or through 'prime and trap/target' subunit vaccinations that target the liver[182, 183, 184]. These LFA1^{hi} CXCR6^{+} CD69^{+} CXCR3^{+} CD8^{+} T cells that establish a niche within the hepatic sinusoids can be primed outside of the liver and offer protective immunity to subsequent sporozoite challenges[182, 184, 185, 186]. Although liver-resident memory CD8^{+} T cells appeared essential for immunity after RAS immunization of mice[182], whether these cells alone are sufficient for sterilizing immunity, or provide a 'sensing and alarm' function[187] to recruit circulating *Plasmodium*-specific CD8^{+} T cells remains an important knowledge gap. A thorough understanding of the origin, dynamics and functional mechanics of *Plasmodium* specific liver-resident CD8^{+} T cells may help tailor this population to better protect from future liver-stage *Plasmodium* infections.

Although CD8^{+} T cells are vital for protection from liver-stage malaria, they are thought to contribute little to control of the blood-stage of malaria infection, mostly owing to the lack of MHC class I on erythrocytes[188, 189, 190]. Yet, robust CD8^{+} T cell responses directed at various *Plasmodium* erythrocytic-stage antigens are primed, primarily in the spleen, in blood-stage malaria[191, 192]. Of note, human CD8^{+} T cells generated in infection with *P. vivax* that preferentially colonizes reticulocytes, specifically eliminated parasitized

reticulocytes though an HLA-I mediated, granulysin driven mechanism, possibly contributing to protection[193]. Although the breadth of their role in protection to blood-stage malaria with other *Plasmodium* species may be limited, CD8+ T cells are speculated to drive the highly pathogenic cerebral malaria in humans, based on parallel studies in the mouse model[194, 195, 196, 197]. The pathogenic role of CD8+ T cells in murine experimental cerebral malaria is mediated directly by perforin and Granzyme B, and indirectly by IFN-γ-driven accumulation of parasitized RBC in the brain[196, 198, 199]. Cerebrovascular endothelial cells are possibly targeted by CD8+ T cells in an antigen-specific manner, to drive blood-brain barrier dysfunction, subsequent vascular leakage and neuronal death[194]. Of note, cerebral malaria is limited to certain mouse strains, *Plasmodium* species or isolates[200, 201, 202, 203, 204]. Recent experiments from our laboratory suggest that the magnitude of certain blood-stage specific CD8+ T cell responses, which is largely predicated on the size of the precursor pool in specific mouse strains, may contribute to disease specificity[205]. Although the functional role of CD8+ T cells in orchestrating cerebral malaria in humans remains unresolved[206, 207], mostly owing to the ethical implications of invasive studies, only further research can enhance our understanding in this relatively understudied yet clinically critical area of malaria pathogenesis.

γδ T cells in malaria

γδ T cells are a subgroup of T cells that express distinct TCRγ and TCRδ chains and account for around 4% of all T cells in healthy adult humans[208, 209, 210, 211]. The precise contributions of γδ T cells to host immunity remain unresolved, primarily owing to the wide spectrum of effector functions they possess, that may be governed by the immediate tissue microenvironments[212]. Predictably, the contributions of γδ T cells to anti-malarial immunity also remain poorly understood and disputed[213]. Although γδ T cell populations, specifically those expressing the Vγ9+Vδ2+ chains (which constitute ~75% of all γδ T cells in humans), expand in primary *P. falciparum* or *P. vivax* infections[214, 215, 216, 217] and correlate with protection[218], how they function during *Plasmodium* infection remains to be determined. It is remarkable that in human malaria, Vγ9+Vδ2+ γδ T cell populations appear to expand during acute, primary infections, but possibly contract with each subsequent exposure to malaria, despite reactivation each time[216, 217, 219]. Although the progressive improvement in tolerance to clinical malaria with multiple exposures in endemic regions has been attributed to the decline of Vγ9+Vδ2+ γδ T cells[220], it is hard to ascertain if the γδ T cell kinetics is also a cause or an effect in this relationship. Considering that the frequencies of Vγ9+Vδ2+ γδ T cells naturally increase with age[221], and most humans are exposed to malaria from childhood in endemic regions, recurrent *Plasmodium* challenges that hinder γδ T cell expansion[220] may indeed help control clinical malaria with age in endemic regions. It is noteworthy that severity of symptomatic malaria (excluding cerebral malaria) and mortality in primary infections increase with age[222, 223] and could be a direct function of naturally increasing frequencies of γδ T cells. However, based on studies using the mouse model of malaria, γδ T cells were observed to undergo clonal expansion, albeit disproportionately, in various tissues as a consequence of blood-stage malaria. γδ T cells are reported to serve as a source of IL-21 that may support Tfh cell responses[224], and γδ T cells helped control *Plasmodium* recrudescence in a TCR-dependent fashion, possibly via by their production of

colony stimulating factor-1 (M-CSF)[225]. Of note, γδ T cells are also a correlate of protective efficacy in experimental RAS immunizations[5, 8, 226]. Depletion of γδ T cells at the time of RAS vaccination in mice hindered the influx of CD11c^{+} DCs into the liver, prevented optimal effector CD8^{+} T cell responses and sterilizing immunity to future sporozoite challenges. In contrast, γδ T cell ablation immediately before challenge of RAS immunized mice did not diminish protection[226]. These data suggest that γδ T cells may help facilitate effective CD8^{+} T cell responses that provide immunity to liver-stage malaria, although the underlying mechanisms remain a major knowledge gap. Future mechanistic studies that segregate the pre-erythrocytic and erythrocytic developmental stages of malaria will help improve our understanding of γδ T cell function in *Plasmodium* infections and anti-malarial vaccines in humans.

The fundamental mechanisms of γδ T cells function in the context of infections are also poorly understood. γδ T cells are thought to be among the first lines of defence against infections, with abilities to contextually stimulate or repress immune responses through distinct natural or induced cell subsets[212, 227, 228, 229, 230]. γδ T cells can also be recalled in reinfections, target pathogens directly in a TCR dependent or independent manner, or indirectly by enlisting other cell subsets[208, 212, 231]. These properties that bridge the distinctions of innate and adaptive responses make γδ T cells functionally unique. Vγ9[+]Vδ2[+] γδ T cells may help control primary *Plasmodium* infections in humans through the production of various immune mediators, such as IFNγ, TNF or granzyme B, in addition to possibly killing the merozoites directly in blood-stage malaria[72, 214, 232, 233]. In human malaria, Vγ9[+]Vδ2[+] γδ T cell populations undergo polyclonal expansion upon sensing of phosphoantigens derived from *P. falciparum* or *P. vivax* apicoplast **[G]**, is independent of classical antigen presentation, but requires the presence of monocytes, CD4^{+} T cells or exogenous cytokines[214, 215, 220, 225, 234, 235, 236, 237]. By contrast, in *P. chabaudi* blood-stage malaria in mice, Vδ6.3^{+} (also known as TRAV15N-1[+]) γδ T cells specifically undergo clonal expansion and exhibit a unique transcriptional and functional profile that contributes to protection[225]. In summary, there is emerging evidence for key roles of γδ T cells in the control and pathogenesis of malaria and these should prompt further studies to decipher the mechanisms involved.

T cells and vaccine design

Multiple anti-malarial vaccine platforms are being evaluated in humans for their capacity to effectively target and block either sporozoite- or liver-stage progression, or trigger humoral responses that reduce the severity or delay the onset of blood-stage malaria and clinical disease (recently reviewed in[238, 239]). RTS,S, the most clinically advanced anti-malaria subunit vaccine, is believed to stimulate the production of antibodies that may either target sporozoites for destruction or prevent their ability to reach the liver. The humoral immune response to RTS,S likely involves T cell help and there is some evidence of CD4 T cell responses in vaccinees, although much work remains to characterize these responses and determine why immunity after RTS,S vaccination is of short duration[238, 239]. While whole parasite vaccines, including RAS, GAP, CPS, and chemically attenuated blood-stage platforms, are likely to trigger the expansion and function of *Plasmodium*-specific CD8^{+} and CD4^{+} T cells and antibodies responses, similar to rodent models discussed above,

information on which of these platforms provides the best T cell responses in humans remains limited. Despite the many correlates observed in experimentally vaccinated humans, the true requirements for either Tfh, Th1, or cytotoxic CD4^{+} and CD8^{+} T cells and the precise effector mechanism by which these T cell subsets might mediate protection in humans are not understood. Advances in basic mechanistic insights into the role of T cells in malaria should provide a roadmap to address these issues in human vaccines.

Due to the low parasite burden and lack of disease association, the liver-stage of *Plasmodium* infection remains a particularly attractive target for T cell-based vaccines. Attenuated whole parasite vaccines such as RAS elicit potent protective liver-stage immunity in animals and humans[240, 241]. The protective mechanisms of these vaccines are likely a function of their ability to generate T resident memory in the liver targeting multiple parasite antigens[162]. However, such whole parasite vaccines are complicated by the cumbersome production process, the need to deliver large numbers of sporozoites multiple times via intravenous immunizations to achieve potent immunity and reduced efficacy in the field[242]. Prime-boost approaches with viral vectored antigens designed to elicit T cells have shown some promise in vaccine trials in malaria naïve subjects, although the number of antigens investigated to date is small and it is unclear how potent the current viral vectored immunization approaches are at eliciting liver T resident memory populations[243].

An intriguing possible solution to the latter issue, that has had success in animal models, is to initially prime circulating T cell responses and then pull or trap these cells in the liver during the boosting phase, with liver-tropic viral antigen delivery systems such as adeno-associated virus[182] or liver-targeted antigen and adjuvant containing nanoparticles[244]. Another potential solution is to combine subunit and RAS vaccinations, to take advantage of the ability to RAS to generate liver T resident memory but limit the number of cumbersome RAS immunizations[184]. Further work to optimize these systems and evaluate them in clinical trials should be a priority.

Additionally, it is unclear from human vaccine trials whether targeting a single antigen will permit generation of sufficient T cells to provide sterilizing liver-stage immunity to *Plasmodium*.

The field of malaria subunit vaccines is desperately in need of new target antigens to evaluate and the capacity to carry out whole parasite immunization studies in human volunteers could be a fertile basis for such antigen discovery. Importantly, recent work in animal models shows that detection of a T cell response to a *Plasmodium* antigen does not ensure that antigen will elicit T cells that can protect against infection[245]. Thus, additional screening approaches, perhaps based on demonstrated antigen-specific recognition of infected hepatocytes, must be developed to identify protective antigens and determine if protective antigens share characteristics such as surface or secreted localization or differentiation stage specificity, that will facilitate their identification and potential incorporation into a successful malaria vaccine.

In summary, recent mechanistic studies of the T cell response to malaria from the combination of animal models and human vaccine/challenge studies have provided new

insights and identified critical knowledge gaps to overcome in developing potent, T cell-based vaccines for malaria. Emphasis should be placed on identifying of the mechanisms that determine the formation and persistence of liver-resident memory CD8^{+} T cells, as well as circulating memory CD4^{+} Th1 and Tfh cells with the capacity to orchestrate phagocytic and humoral responses. New mechanistic insight should facilitate refinements in the formulation, delivery, and induction of protection by next-generation anti-malarial vaccines.

Conclusions and perspectives

Cell-mediated immune responses are critical for immunity against malaria. Here, we have summarized our current understanding of the roles of various T cell subsets that contribute to protection against both pre-erythrocytic and erythrocytic stages of malaria, as understood from experimental or natural infections in humans or animal models. However, there are many fundamental questions that remain unanswered (Box 4). Addressing these gaps should help advance our understanding of the mechanical underpinnings of the immunology of malaria and move the field closer to developing more practical, feasible, and reliable immunization and therapeutic interventions that would help control or eliminate malaria.

Acknowlegements

Glossary terms:

Sporozoite
Plasmodium parasite life form transmitted by mosquito bite capable of initiating the asexual cycle of growth and differentiation in the vertebrate host

Merozoite
Plasmodium parasite life form that first develops in infected hepatocytes and is capable of initiating either sexual or asexual cycles of development in host red blood cells

Merosome
Host cell-derived, membrane-bound structures containing multiple merozoites that bud from infected hepatocytes during *Plasmodium* egress from the liver. Merosomes release merozoites into circulation after rupture

RTS,S (Mosquirix™)
Candidate anti-malarial vaccine furthest along in global development. RTS,S is comprised of two subdomains of the *Plasmodium falciparum* circumsporozoite protein that are associated with units of the hepatitis B surface antigen and formulated with the adjuvant AS01 (3-*O*-desacyl-4'-monophosphoryl lipid A and the saponin QS-21). Infection is prevented by inducing antibodies that either immobilize sporozoites in the skin or prevent sporozoites from infecting the liver

Circumsporozoite protein (CSP)

Immunodominant, high-density surface antigen expressed by *Plasmodium* sporozoites that is the target of humoral and cellular immune responses that either block sporozoite infection of the liver or eliminate infected hepatocytes, respectively

Chemoprophylaxis and sporozoite (CPS) immunization

Vaccination strategy whereby virulent sporozoites are delivered by either mosquito bites or needle injection with prophylactic delivery of a drug targeting *Plasmodium* blood-stages. Parasites initiate and complete liver-stage development, release merozoites, and initiate the first wave of blood-stage infection before being eliminated by the drug. Vaccinated individuals are thereby exposed to antigens that derive from multiple parasite lifecycle stages while remaining protected against clinical disease by the anti-blood-stage drug

Apicoplasts

Non-photosynthetic organelles that characterize protists within the Phylum Apicomplexa that are likely derived from an algal endosymbiont. The composite of apicoplast functions are not fully known but defined activities primarily relate to essential metabolic pathways necessary for the viability of *Plasmodium* and other Apicomplexans

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Box 1:

Unravelling T cell responses in human malaria

Below, we summarize some of the main challenges to determining the mechanisms of T cell-mediated protection in natural and controlled human *Plasmodium* infections.

Specificity:

High-resolution studies of *Plasmodium*-specific T cell responses generally requires knowledge of the identity of the peptide epitopes and MHC restriction elements. Identifying immunodominant subsets of CD4^{+} and CD8^{+} T cells will necessitate a greater emphasis on deciphering immunodominant T cell epitopes that are targeted by liver- or blood-stage-specific T cells. Notably, some dominant T cell epitopes targeted by substantial populations of T cells do not represent protective epitopes[205]; this is likely due to large numbers of precursor T cells bearing T cell receptors (TCRs) that can recognize the epitope presented by professional antigen-presenting cells (APCs), but a lack of this epitope on infected cells. Determining which of the detectable epitope-specific T cell responses are the most protective represents a major goal.

Location:

With regard to liver-stage *Plasmodium* T cell-mediated protection, experimental data suggests that liver-resident T cells are key to eliminating *Plasmodium*-infected hepatocytes before merozoites emerge to establish clinical malaria. This presents a substantial hurdle for sampling and studying these cells in patients with malaria who have been either naturally exposed or exposed through controlled human malaria infections (see Box 3) or experimental vaccination platforms. Identifying circulating T cell populations that serve as a surrogate for protective tissue-resident T cells represents a major limitation for understanding the mechanisms by which liver-stage, and potentially blood-stage, T cells mediate protective immunity.

Regulation:

Malaria is well known to engage various co-stimulatory, co-inhibitory, and immunoregulatory circuits that alter the function of parasite-specific effector T cells. The number, complexity, and temporally overlapping expression patterns of these pathways represent significant challenges to understanding how such circuits govern the development and function of *Plasmodium*-specific memory T cells.

Polyfunctionality:

Plasmodium-specific effector and memory CD4 T cells commonly exhibit mixed characteristics of both Th1, Tfh, and Tr1 cells, including the simultaneous expression of T-bet, Bcl-6, IFNγ, IL-10 and IL-21. The extent to which these multifunctional cells either promote or hinder the development and function of long-lived adaptive immune responses remains a question.

Box 2:

Tools, systems, and approaches to study *Plasmodium*-specific T cell responses

In recent years, multiple new tools have been developed to enhance resolution and focus studies on mechanisms of T cell function during experimental malaria. Technologies now exists to interrogate the development, persistence, and precise effector functions of *bona fide Plasmodium*-specific T cells. Novel T cell receptor (TCR) transgenic mouse models have stimulated new insight into the biology and regulation of *Plasmodium*-specific T cells. Investigators can also generate fluorescently labeled multimeric peptide–MHC complexes to directly identify, quantify, and phenotype *Plasmodium*-specific, effector and memory CD4$^+$ and CD8$^+$ T cells using flow cytometric methods. Already such tools have been used to describe the single-cell transcriptomic profile of *Plasmodium*-specific, effector CD4$^+$ T cells[68] and critical factors that regulate the formation and protective mechanisms of effector and memory blood- and liver stage T cell responses[33, 70, 185, 246, 247]. Combining these methods with functional assays has greatly expanded our understanding of the quantitative, qualitative, and functional features of *Plasmodium*-specific CD4$^+$ T cells and CD8$^+$ T cells.

Box 3:

Controlled human malaria infection models

Major advances in our understanding *Plasmodium* pathogenesis, host immunity, and correlates of protection are projected to come from controlled human malaria infection (CHMI) models. CHMI involves either the delivery of live sporozoites by mosquito bites or by direct injection, or via delivery of *Plasmodium*-infected erythrocytes by needle injection. The development of fulminant blood-stage infection and clinical disease are blocked by the use of defined parasite strains with well-established drug sensitivity profiles in combination with the timely delivery of effective and appropriate antimalarial drugs. Studies typically cure infections at either a pre-determined parasite density or when the infection becomes detectable by microscopic examination of blood smears or PCR. The utility of such seemingly risky studies is apparent when we consider that candidate vaccines may be rapidly evaluated for efficacy before costly clinical trials are initiated in malaria endemic areas. CHMI models also facilitate examination of immune correlates of resistance and susceptibility. In malaria-naïve adults from non-endemic regions, CHMI has made it possible to examine dynamic changes in myeloid and lymphoid populations that arise in response to a first exposure to *Plasmodium*. Such studies have already revealed unexpected and potentially critical expansion of clonal populations of γδ T cells and cytotoxic CD4 T cells. In malaria-exposed individuals, CHMI should also reveal whether specific populations of effector or regulatory immune cell subsets are "boosted" following subsequent parasite exposures. Several such CHMI trials have taken place in malaria endemic settings[248, 249, 250], and future CHMI studies will continue to expand our understanding of how the host responds and coordinates immune responses against *Plasmodium* parasites. These studies will also better define the precise mechanisms by which T cells contribute to controlling liver- and blood-stage *Plasmodium* infections.

Box 4

Key outstanding questions on T cell mediated immunity to malaria

- To what extent do phagocytes mediate clearance of circulating parasites and does IFNγ directly regulate these processes?

- What factors govern the generation, maintenance, and function of Tfh cells during malaria? Does repeated *Plasmodium* parasite exposure distinctly program effector and memory Tfh cell responses?

- Which sets of signals regulate the differentiation and function of IL-27- and IL-10-expressing effector CD4$^+$ T cells during malaria? Do these functionally distinct subsets persist as memory cells and do they also regulate recall responses? Are IL-27-expressing cells a signature of malaria? What are the primary cellular targets of IL-27 and IL-10 during malaria? Do the effects of IL-27 and IL-10 evolve as infection progresses?

- What are the contributions of distinct populations Th1 and Tfh memory CD4$^+$ T cells in protection against repeated exposure to malaria? How do inflammation, antigen persistence, and the presence of specific immunoregulatory circuits impact the formation and function of *Plasmodium*-specific memory CD4 T cells?

- What are the relative roles of either self- or antigen-specific peripheral and thymic Tregs in primary and repeated *Plasmodium* infections?

- What signals regulate liver resident CD8$^+$ T formation, dynamics, mechanisms of maintenance and protection in malaria?

- What are the key functional dynamics of CD8$^+$ T cells in human cerebral malaria?

- What antigens stimulate oligoclonal γδ T cell expansions and do memory γδ T cell populations persist? By what mechanisms do γδ T cells protect against liver-stage and blood-stage malaria? Do the γδ T cells elicited by vaccination meaningfully contribute to host resistance to malaria?
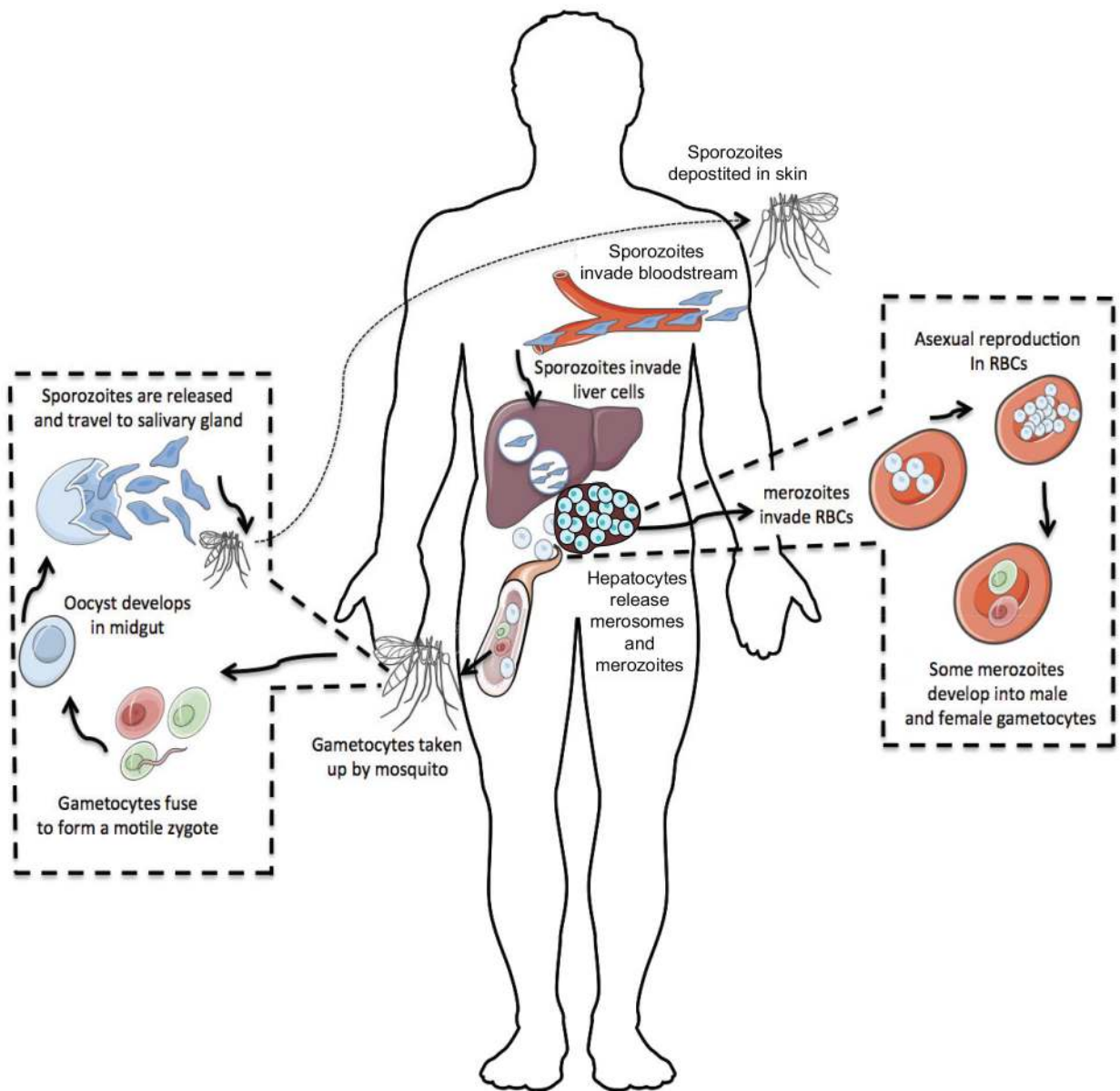
FIGURE 1: *Plasmodium* **life cycle.**

The life cycle begins when a *Plasmodium*-infected female *Anopheles* mosquito takes a blood meal from a human host and deposits *Plasmodium* sporozoites into the skin. Motile sporozoites exit the dermis and travel through the blood to access hepatocytes. Sporozoites invade liver cells via interactions between *Plasmodium* circumsporozoite protein (CSP) and heparin sulfate molecules expressed on hepatocytes. One *P. falciparum* sporozoite will undergo differentiation over 6–7 days and amplify into ~10,000 merozoites. Infected hepatocytes release merozoites and merosomes, which are membrane bound packets of merozoites, into the blood stream where they proceed to invade erythrocytes. Merozoites undergo repeated rounds of asexual replication. A minor proportion of merozoites will differentiate into either male or female gametocytes that can be ingested by other female *Anopheles* mosquitos. In the mosquito midgut, male and female gametocytes fuse and

develop into a motile ookinete. Ookinetes embed within the mosquito midgut wall and develop further into oocysts. Each oocyst produces thousands of sporozoites over a period of two weeks. Sporozoites eventually migrate to the salivary glands and poise the mosquito to transmit malaria to a new host.
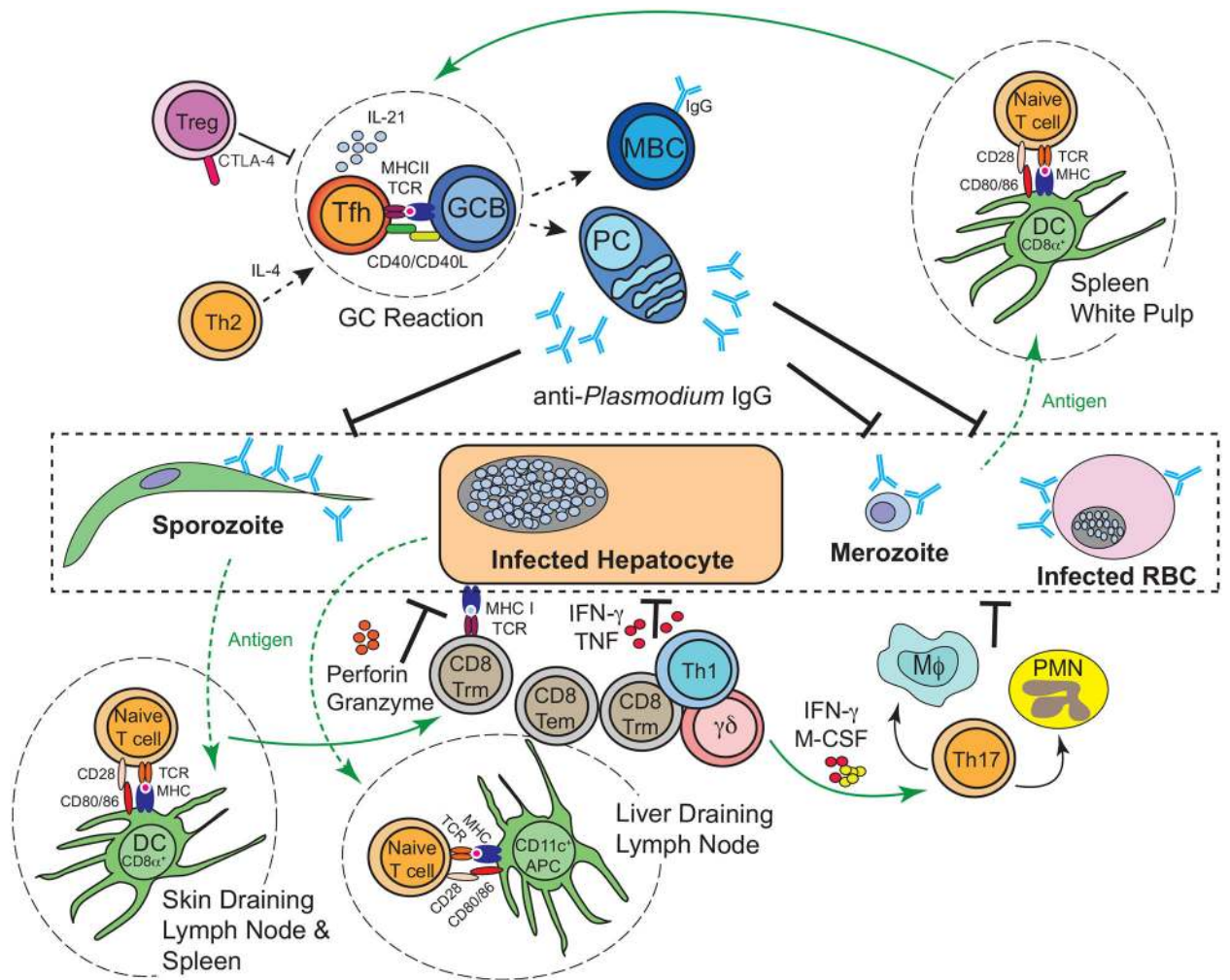
FIGURE 2: Overview of tissue-specific, T cell-mediated immune resistance networks during *Plasmodium* infection.

CD8α^{+} dendritic cells (DC) in the skin-draining lymph nodes and spleen, as well as CFS1R^{+} CD11c^{+} cells in the liver-draining lymph nodes, serve as antigen presenting cells and play an important role in bridging innate and adaptive immune responses during malaria. Upon phagocytosis of merozoites, parasitized RBC (pRBC), sporozoites, debris from infected hepatocytes, or circumsporozoite protein formulated as part of the RTS,S vaccine, DCs will process and present *Plasmodium* antigens to activate naïve CD4^{+} and CD8^{+} T cells. DC production of specific cytokines, such as IL-12 and IL-6, skew CD4^{+} T cell differentiation toward T helper 1 (Th1) and T follicular helper (Tfh) lineages. Th1 cells produce the cytokine IFN-γ that activates macrophages to enhance their phagocytic function and stimulates production of reactive oxygen species that are toxic to the parasite. Tfh cells engage parasite-specific B cells and orchestrate the germinal centre (GC) reaction, where they express co-stimulatory factors (CD40L) and secreted soluble factors (IL-4 and IL-21) that promote GC B cell (GCB) antibody isotype switching, affinity maturation, and somatic hypermutation, as well as the generation of memory B cells (MBC) and long-lived antibody-secreting plasma cells (PC). Parasite-specific antibodies potentially function to immobilize or target sporozoites for antibody dependent cellular cytotoxicity (ADCC), block merozoite

invasion of RBCs, opsonize pRBC to enhance their phagocytosis, target merozoites and pRBC for ADCC, and activate the classical complement-pathway. Sporozoite- or liver-stage-specific, tissue-resident (Trm) CD8^{+} T cells elaborate the cytokines IFN-γ and TNF and trigger extrinsic cell death pathways via expression of perforin and granzyme to kill infected hepatocytes. Cytotoxic CD4^{+} T cells may function similarly to kill infected target cells expressing MHC class II. Cytotoxic CD8^{+} T cells also have the potential to kill infected reticulocytes that transiently retain expression of MHC. B cells and CD4^{+} and CD8^{+} T cells are subject to regulation by other αβ T cells, including Tregs, IL-27-secreting CD4^{+} T cells, and Tr1 cells (the latter two subsets are not depicted). γδ T cells are activated in response to liver and blood-stage infection in response to unknown cues. These cells express cytokines that may include IFN-γ and myeloid activating factors like M-CSF. γδ T cells likely promote blood and liver stage parasite clearance by orchestrating and amplifying the activity of phagocytes. The contributions of Th17 and Th2 cells are less clear, but may be related to either recruiting and activating phagocytes or promoting *Plasmodium*-specific GC B cell reactions.