Human unconventional T cells in *Plasmodium falciparum* infection

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Abstract

Malaria is an old scourge of humankind and has a large negative impact on the economic development of affected communities. Recent success in malaria control and reduction of mortality seems to have stalled emphasizing that our current intervention tools need to be complemented by malaria vaccines. Different populations of unconventional T cells such as mucosal-associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells and γδ T cells are gaining attention in the field of malaria immunology. Significant advances in our basic understanding of unconventional T cell biology in rodent malaria models have been made, however, their roles in humans during malaria are less clear. Unconventional T cells are abundant in skin, gut and liver tissues, and long-lasting expansions and functional alterations were observed upon malaria infection in malaria naïve and malaria pre-exposed volunteers. Here, we review the current understanding of involvement of unconventional T cells in anti-*Plasmodium falciparum* immunity and highlight potential future research avenues.

Keywords Unconventional T cells · γδ T cells · MAIT cells · Vaccination · CHMI · Malaria · *Plasmodium falciparum*

Malaria biology, disease burden and pathogenesis

Malaria remains one of the most devastating infectious diseases with 228 million malaria cases globally (95% confidence interval 206–258 million) resulting in 405,000 deaths in 2018 [172]. *P. falciparum* is the most prevalent malaria parasite in the WHO African Region, responsible for 99.7% of the malaria cases in 2018 [172]. Scale-up and improvements of diagnostics and access to treatment combined with vector control measures based on insecticide treated bed-nets, indoor residual spraying and larviciding have resulted in a significant reduction of malaria prevalence and deaths between 2000 and 2015 [172]. The incidence rate of malaria declined globally between 2010 and 2018; however, this progress seems to have slowed down with 251 million cases reported in 2010 and 231 million cases in 2017 [172]. Sub-Saharan Africa is especially strongly affected by malaria – about 90% of both cases and deaths occur in this region. The most vulnerable population are children under the age of 5 years, accounting for 70% of all malaria deaths [172].

Malaria is caused by parasites of the *Plasmodium* genus and is transmitted to humans through bites of infected *Anopheles* mosquitoes [4]. The majority of malaria cases and deaths in humans are caused by *P. falciparum*, a species that is particularly prevalent in sub-Saharan Africa. During a blood meal, infected mosquitoes inject a small number of sporozoites into the skin of the human host [16, 111]. Most sporozoites will leave the skin through blood or lymph, but recent evidence from mouse models suggests that a fraction of the sporozoites can remain in the skin for several days [62]. Sporozoites can move by gliding motility, and a fraction of the injected sporozoites will invade a blood vessel, enter the blood stream and reach the liver. Interaction between sporozoites and human hepatocytes involves circumsporozoite protein, the main surface protein of sporozoites [22]. The parasites use cell traversal to escape from the liver sinusoids into the liver parenchyma, where they can traverse several hepatocytes before setting up residence inside a parasitophorous vacuole (PV) inside a hepatocyte [109]. Within the PV, the sporozoite develops into a spherical liver stage that grows extensively and ultimately gives rise to thousands of merozoites that will then enter the blood stream. For this purpose, the parasite...
induces cell death of the hepatocyte and manipulates the hepatocyte membrane, leading to formation of parasite-filled vesicles (merosomes) containing hundreds of merozoites [150]. The duration of liver-stage development is variable between few days to several weeks in different Plasmodium species, but for \textit{P. falciparum}, it takes about 6 days to complete [110]. The erythrocytic stage of development is characterized by repeated cycles of erythrocyte invasion, parasite replication, egress and invasion of other erythrocytes. Invasion of erythrocytes by merozoites is facilitated through specific interaction between parasite proteins and receptors on the erythrocyte surface [120]. Inside an erythrocyte, the parasite sets up residence inside a PV and develops into the ring-stage, while it starts to actively remodel the host cell. It feeds on haemoglobin as well as nutrients from blood plasma and develops into a trophozoite [11]. The trophozoite stage is characterized by extensive parasite growth, sustained by ingestion of large amounts of haemoglobin, leading to accumulation of toxic haem groups, which the parasite converts into the crystalline haemozoin form and stores in its food vacuole [151]. Then, the parasite undergoes a few rounds of nuclear divisions, leading to generation of 16–32 merozoites [11]. Finally, the merozoites escape through lysis of the erythrocyte and PV membranes in a protease-dependent process [138]. The free merozoites then go on to infect other erythrocytes, completing the cyclic erythrocyte development. Successful transmission of the parasite from the human host to a mosquito involves formation of a sexual parasite stage [89]. This involves the formation of male and female gametocytes, a process that only a small fraction of blood-stage parasites undergoes. During gametogenesis, the parasite develops through five morphological stages, of which only the last one is detectable in peripheral blood. The other stages occur within erythroid precursor cells in the bone marrow, enabling early gametocytes to avoid splenic clearance [3, 85]. When gametocytes are taken up by a mosquito during a blood meal, they sense a change in temperature, pH and chemical environment, inducing their development into gametes within the mosquito midgut [18]. There, male and female gametes fuse to form a short diploid stage, the zygote, which then develops into an ookinete. In the ookinete stage, meiotic recombination occurs, and the parasite traverses the epithelial cell layer in the mosquito midgut and transforms into an oocyst [12]. Ultimately, the oocyst ruptures, releasing sporozoites into the haemocoel of the mosquito from where they migrate to the salivary glands and are ready to be injected into the human host [30].

Clinical malaria is characterized by cyclic episodes of fever that are caused by synchronized rupture of infected erythrocytes, releasing large amounts of parasites and parasite-derived molecules that induce a strong pro-inflammatory response. Most symptoms are relatively unspecific and include chills, headache, nausea, diarrhoea and anaemia [121]. First symptoms of \textit{P. falciparum} malaria appear 7–10 days after infection, indicating that pre-erythrocytic stages are clinically silent, while most clinical symptoms and complications occur only upon blood-stage parasitaemia [121]. A certain degree of anaemia is induced by rupture and destruction of infected erythrocytes by blood-stage parasites. However, it has become clear that the majority of cleared erythrocytes are uninfected [83, 171]. \textit{Plasmodium} parasites extensively remodel the erythrocyte and its plasma membrane by expressing a range of parasite-encoded proteins on the erythrocyte surface [178]. This leads to increased rigidity of the membrane, to binding of infected erythrocytes to endothelial cells as well as to formation of aggregates of infected and uninfected erythrocytes (rosetting) and helps the parasite to avoid splenic clearance [54]. Adherence of erythrocytes to the microvasculature leads to obstruction of blood flow, endothelial injury and increased inflammation [26].

\textit{P. falciparum} has been estimated to be older than 100,000 years resulting in an exquisite coadaptation of both, the parasite and the human host [68, 117]. Older children and adults residing in malaria-endemic countries usually develop over time naturally acquired immunity induced by repeated exposure, leading to decreasing disease severity with age [121]. Rodent and non-human primate animal models for malaria have provided essential insights into the biology of this parasite [177]. To date, no good immunological correlates of protection have been identified for malaria infection outcome or vaccination in humans [13]. It is generally accepted that studying malaria immunity in different human populations and age groups is essential for detailed understanding of this intricate host-pathogen interaction.

**Controlled human malaria infections**

By using controlled human malaria infections (CHMI), it is hoped to identify effector mechanisms and correlates of protection that could guide next-generation malaria vaccine development [27, 153]. Human challenge models for malaria are defined as the intentional infection of adult volunteers with \textit{Plasmodium} parasites under controlled conditions within a well-defined and restricted ethical framework (https://www.who.int/biologicals/expert_committee/Human_challenge_Trials_IK_final.pdf). CHMI based on \textit{P. falciparum} and \textit{P. vivax} inoculations were used as early as in the beginning of the twentieth century to treat neurosyphilis known as malariotherapy, which was rewarded with the Noble Price in Physiology and Medicine in 1927 to Julius Wagner-Jauregg (https://www.nobelprize.org/prizes/medicine/1927/wagner-jauregg/lecture/). Since the 1980s, volunteers can be reproducibly infected by the bite of reared malaria-infected mosquitoes in several centres in the USA and Europe [25, 131]. With the advent of the development of sterile, purified,
metabolically active, cryopreserved P. falciparum sporozoites by Sanaria Inc. that can be injected intradermally [25, 130, 145], intramuscularly [74, 144] and intravenously [57, 113], the number of clinical trial centres able to perform malaria CHMI studies globally has expanded rapidly. This novel approach has been particularly essential for conducting clinical studies in malaria pre-exposed populations in sub-Saharan Africa [60, 73, 87, 88]. Intravenous inoculation of parasitized erythrocytes infected with P. falciparum and P. vivax has added to the variety of CHMI approaches available for the scientific community [61, 122]. CHMI models have played major roles in clinical vaccine and drug development [39, 140], testing and validation of diagnostic markers and tools [34, 142] and research in parasite biology [8, 9, 75]. CHMI studies allowed the description of impact of malaria pre-exposure [1, 91, 118], HIV, co-infections (https://clinicaltrials.gov/ct2/show/NCT03420053) and haemoglobinopathies [101] on elicited immune responses. The main strength of CHMI relates to the defined infection timing, P. falciparum strain [23, 97] and inoculum dose and route chosen. In summary, CHMI enable the study of malaria immunity in different human populations representing an essential “human model” for this disease.

T cells in human malaria immune responses

T lymphocytes are key components of the immune system contributing to the anti-P. falciparum immunity and are of major interest in malaria research. The vast majority of studies dedicated to T cell-mediated immunity have focused on “conventional” adaptive CD4+ and CD8+ T cells [31, 95, 96]. Those cells express αβ T cell receptors (TCR) that recognize peptide antigens presented by highly polymorphic major histocompatibility complex (MHC) class I and II molecules. Once activated, CD4+ and CD8+ T cells undergo clonal expansion and differentiation into special subsets with unique functions to orchestrate key aspects of innate and adaptive immunity during P. falciparum infections (reviewed in [96]). CD8+ T cells develop into cytotoxic T cells with the ability to target and kill mainly the Plasmodium-infected hepatocytes but not infected erythrocytes as they lack MHC class I expression. CD4+ T cell subsets provide help to CD8+ T cells, B cells and activation of antigen-presenting cells (APC) such as dendritic cells and macrophages [95, 96]. Clinical trials to induce strong and durable anti-P. falciparum T cell responses by subunit and whole parasite vaccination approaches have yielded so far limited success, thought to be based on diversity of antigen expression during different development stages, heterogeneity in the P. falciparum strains and suboptimal vaccine formulations and delivery [27, 108]. One of the barriers that needs to be overcome rests with the donor-specific, highly polymorphic MHC (human leucocyte antigen (HLA)) molecules presenting distinct peptide repertoires to diverse T cell populations [42, 43]. In addition, DCs and macrophages were shown to fail in upregulating HLA-DR expression upon P. falciparum infection [162, 163] limiting their capacities to activate conventional antigen-specific T cells.

In contrast, unconventional T cells target highly conserved, monomorphic MHC class Ib and MHC-I like molecules and other ligands. These include unconventional αβ T cells such as MAIT and NKT cells that are restricted to metabolite-presenting MHC class I-related proteins (MR1) and lipid-presenting cluster of differentiation 1 (CD1) molecules, respectively [53, 165]. In addition, some γδ T cells can recognize MR1 and CD1 molecules, while the vast majority recognizes butyrophilin (BTN) and BTN-like (BTN-L) proteins or stress-induced ligands [36, 53, 80] allowing them to participate in health and diseases.

Mucosal-associated invariant T cells

MAIT cells are a special subset of MR1-restricted T cells carrying features of the innate immunity. They are present mainly in mucosal tissues, the liver and lymphoid tissues but also recirculate in peripheral blood [114, 158]. The highest frequencies are found in liver and peripheral blood in which they represent up to 45% and 10% of T cells, respectively [44, 51, 98, 154], while they are less frequent in lymphoid tissues and comprise around only 1% of splenic αβ T cells [158]. MAIT cells are characterized by the expression of the semi-invariant TCR composed of the Vα7.2 chain rearranged with Jα33, Jα12 and Jα20, paired with a constrained TCRβ repertoire (enriched for TRBV6 and TRBV20-1) and high levels of surface expression of CD161 [102, 119, 125, 157]. They are restricted to MR1 [159] and depend on the presence of commensal microbiota and their metabolites for development and activation [100, 159]. The canonical, activating MAIT cell antigens presented by MR1 were identified as ligands generated from precursors to riboflavin in the riboflavin synthesis pathway of bacteria and fungi [93]. Further characterization revealed the diverse nature of these antigens—they are derived from 5-amino-6-d-ribitylaminouracil (5-A-RU), an intermediate of the riboflavin biosynthesis pathway. 5-A-RU reacting with glyoxal or methylglyoxal leads to generation of potent MR1-ligands 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil, respectively [28]. These antigens are unstable in solution but get stabilized in the antigen-binding pocket of MR1 by formation of a Schiff base with lysine amino acid residue 43 [28]. This interaction possibly influences their potency, which is high compared with non-stabilized 6,7-dimethyl-8-D-ribityllumazine [107, 141]. Blood-derived MAIT cells respond to different antigens by distinct changes in the surface marker expression [107, 141]. Further studies
showed the existence of a broader MR1 ligand repertoire including ligands derived from riboflavin-auxotroph bacteria [56, 67], mammalian cells [103] and drugs [92]. Some metabolites of folic acids such as 6-formylpterin (6-FP) and acetyl-6-FP as well as bacterial ligands do bind to MR1 but act as antagonists [45, 67, 93] suggesting that the function is modulated depending on the specific antigen-MR1 interaction. Therefore, MAIT cells are predominantly activated by cells infected with riboflavin-synthesizing bacteria and fungi, but not by uninfected or virus-infected cells [166]. In the absence of TCR engagement, MAIT cells can be activated by IL-12 and IL-18 resulting in IFNγ-and TNFα production [105, 166], an effect that is conserved across many CD161-expressing T cells [47, 164]. In summary, MAIT cells are rapidly responding innate-like T cells with a restricted TCR repertoire that recognize metabolite-derived antigens and show high responsiveness to pro-inflammatory cytokines.

Few reports have described a potential involvement of MAIT cells in malaria immunity. Tanzanian volunteers undergoing intradermal CHMI by purified *P. falciparum* sporozoites developed asexual blood-stage parasitaemia [116]. We examined the frequency of MAIT cells (CD3+Vα7.2+CD161β) on study days 0 and 9 post-infection and then after the malaria treatment on days 28, 56 and 168. Using a single-cell RNA-seq approach, we found that the MAIT cell population displayed a distinct RNA expression profile suggesting that they responded to the malaria challenge by day 9 after sporozoite inoculation and did not return to baseline transcriptional status by day 28. Durable expansion of circulating CD8+ MAIT cells for several months after parasite infection might be possibly driven by homeostatic expansion [116]. In a follow-up study conducted in Tanzanian volunteers, ex vivo MAIT cell activation (upregulation of CD38) and expansion after asexual blood-stage parasitaemia were confirmed [137]. This data suggest that MAIT cells might contribute to immune responses, but the underlying molecular and cellular mechanism remains unknown. There are currently no defined antigens in *P. falciparum* known to be presented by MR1; MAIT expansion and activation could be indirectly driven by cytokines produced by activated APC.

Dendritic cells and monocytes/macrophages are known to produce IL-12 and IL-18 in response to danger- and pathogen-associated molecular patterns. Elevated pro-inflammatory cytokines including IL-12 and IL-18 have been detected in severe cases of malaria-infected individuals [94, 106], and IL-12 has been shown to be involved in cellular immune responses to blood-stage infections [32]. Indeed, in vitro MAIT cell proliferation and activation were induced by co-culture of infected red blood cells (iRBCs) with autologous DCs and PBMCs collected from these malaria pre-exposed volunteers [137]. MAIT cells from *P. falciparum*-infected humans were still responsive to IL-12, IL-15 and IL-18 activation and produced IFNγ, IL-17A and Granzyme B (grzB) [116] and did not succumb to malaria-induced immunosuppression [116]. Alternatively, activation of MAIT cells might be a consequence of impaired gut barrier integrity and function during malaria. Histopathological sections in severe malaria cases indicated intense adherence and sequestration of iRBC into the gut [123, 143] possibly resulting into gut tissue damage and microbial translocation, thus activating gut- and liver-homing MAIT cells.

Hence, MAIT cells are emerging as an unconventional T cell population involved and possibly contributing to anti-*P. falciparum* T cell immunity.

**CD1-restricted T cells**

Humans express four CD1 antigen-presenting molecules, divided into two groups: CD1a, CD1b, CD1c (group 1) and CD1d (group 2) [53, 165]. CD1 molecules are MHC class I-like molecules expressed on APCs, and they present distinct lipid-based antigens of a diverse origin [53, 165]. Group 1 CD1 molecules are differentially expressed in APCs with high expression of CD1a in skin-resident DCs and Langerhans cells, CD1b in myeloid DCs in lymphoid tissue and CD1c in DCs and B cells. In contrast, CD1d is widely expressed on many cells types including monocytes, B cells and epithelial cells [53, 165]. CD1d-presented lipid antigens are recognized by the iNKT cell TCR comprised of an invariant Vα24Jα18 chain and a TCRβ chain of a limited repertoire [53, 165]. iNKT cells recognize the model antigen αGalCer, and although many foreign lipids are recognized by these T cells, it is not clear whether NKT cells encounter them under non-experimental conditions [17].

CD1c expression was increased on monocytes and inflammatory CD16+ DC during blood-stage infection in malaria naïve volunteers after CHMI [156] suggesting that CD1 molecules have the capacities to present *P. falciparum* or self-lipid antigens to CD1-restricted T cells. A comprehensive lipid analysis of asexual blood-stage *P. falciparum* profiled 300 lipids [64], which might – if presented by group 1 and/or 2 CD1 molecules – activate unconventional T cells in the liver or other organs. Moreover, as endogenous lipids presented by CD1 group 1 molecules can activate T cells [38], *P. falciparum* infection might have an influence on this subset of unconventional T cells.

Very few studies have addressed the role of CD1d-restricted iNKT cells in human malaria infections, natural or experimental during CHMI. One study demonstrated that the proportion of blood-derived unconventional T cells with NK phenotype (CD3+CD56+ or CD3+CD57+) is increased in malaria patients, especially in adults [169]. In contrast, a more recent study demonstrated that the frequency of invariant CD3+ Vα24Jα18+ NKT cells did not change significantly following controlled malaria infection in peripheral blood
[116]. This might be due to the lack of stimulating CD1d antigens and/or lipid presentation during malaria in blood-stage infections but does not exclude iNKT cells from having an antiparasitic role and/or participating in anti- \textit{P. falciparum} immunity in other organs such as the liver. In order to determine their anti-malaria potential, further studies should investigate CD1-restricted T cells in individuals experimentally challenged by CHMI or naturally infected in endemic areas.

\textbf{γδ T cells}

T cells, defined by their expression of the γδ TCR, are a heterogeneous population of lymphocytes and are not limited to antigen presentation by MHC proteins, CD1 and MR1. They represent up to 5% of T cells in the lymphoid and non-lymphoid organs in humans [168]. Furthermore, the rapid activation of γδ T cells [69] may critically regulate tissue immunogenicity by modulating the microenvironment so as to accommodate time-delayed adaptive responses of B cells and αβ T cells [70] in response to infections and vaccines.

Human γδ T cells have a reduced number of V and J gene segments to select from during rearrangement, but γδ TCR diversity is largely achieved through extensive junctional diversity. The Vγ chains mainly pair with Vδ1, Vδ2 and Vδ3 chains although more δ chains exist [2]. Moreover, some Vγ pseudogenes have been described in the γ locus without “functional” rearrangement found so far in humans [2]. Interestingly, the length of the complementarity determining region 3 (CDR3) of the γδ TCR is much less constrained compared to the αβ TCR repertoire, especially the CDR3 of the TCR δ chain can be significantly longer. This is reminiscent of the CDR3 length distribution of antibodies, and it has been implied that γδ T cells recognize antigen in an antibody-like manner [129]. The CDR3-driven ligand recognition allows the γδ T cell to interact with target cells in an MHC-unrestricted manner [115] and therefore does not require expression of CD4 and CD8 coreceptor [90]. Knowledge of molecular mechanisms leading to γδ TCR recognition of antigens and functional consequences for γδ T cell physiology remain scarce. γδ TCRs might recognize and directly interact with several host-derived ligands expressed on the surface of human cells [69, 173]. Various γδ T cell subsets are modulated in their activation upon interactions with B7-like BTN and BTN1 proteins [6]. Innate-like Vγ9Vδ2 T cells, highly present in peripheral blood, are activated by human cells expressing the BTN3A1 molecule, which is essential for binding phosphorylated antigens [139, 167]. A recent study further demonstrated that BTN2A1 is a direct ligand for the Vγ9Vδ2 TCR acting in unison with BTN3A1 to licence γδ T cell responses to phosphorylated antigens [128]. Another study showed direct binding of human Vγ4+ TCR to BTN3L resulting in T cells activation in a superantigen-like binding mode, while endothelial protein C receptor (EPCR) binds to Vγ4Vδ5 TCR in a CDR3-dependent and antibody-like binding mode that mediates adaptive immunity [175]. The non-Vδ2 T cells responsive to the autoantigen EPCR respond to CMV-infected fibroblast and endothelial cells by direct interactions of the TCR to the ligand and other costimulatory ligands [174]. Thus, full activation, Vδ1 T cells might rely on integration of several signals, both TCR-mediated and TCR-extrinsic. Other Vδ1 T cells have been shown to recognize phycoerythrin [179], monomorphic CD1 molecules presented on APCs [10, 135, 148, 161] and MR1 overexpressed in cell lines [99]. Because of low abundance of CD1-, MR1- and PE-recognizing γδ T cells in humans, it seems unlikely that these Vδ1 T cells represent expanded effector Vδ1 clonotypes in diseases such as malaria. There is evidence that γδ T cells can get activated by cytokines in a TCR-extrinsic fashion [49, 50] increasing the possibilities of γδ T cells to participate in bridging innate and adaptive immune responses.

An important feature of γδ T cells is their capability to home to epithelial and mucosal surfaces [19]. In the liver, preferentially Vδ1 and Vδ3 T cells are present with high diversity of the TCR repertoire after durable and subject-specific expansion [36, 37, 77]. Their physiological roles might include production of pro-inflammatory cytokines and growth factors to recruit immune cells, activate phagocytic cells and promote DC maturation [19] as well as tissue repair and regeneration [84]. γδ T cells are able to provide B cell help and present antigens to other T cell subsets further contributing to protection against infectious diseases, wound healing and epithelial cell maintenance [19]. A number of studies support the idea that γδ T cells are involved in immunity during \textit{P. falciparum} infections. First, γδ T cells home to organs including skin, blood and body sites which are preferentially exposed to \textit{P. falciparum} parasites. Second, γδ T cells expand upon \textit{P. falciparum} infections in children and adults from malaria-endemic and non-endemic regions [71, 72, 79, 132, 133, 176]. Third, γδ T cells express a broad range of effector cytokines including IFNγ and TNFα secretion and display cytotoxic activities in response to the parasite [29, 49, 82].

The most abundant γδ TCR expressed in humans is Vδ2, preferentially paired with Vγ9 [146], and these T cells proliferate extensively in malaria infections [71, 72, 79, 132, 133, 176]. During CHMI, but not repeated immunizations with live, purified, attenuated \textit{P. falciparum} sporozoites delivered intravenously, expansion of Vδ2 T cells in individuals developing asexual blood-stage infections has been observed [71, 137] supporting previous observations that asexual blood-stage infections drive Vγ9Vδ2 T cells expansion. Vγ9Vδ2 T cells likely respond to different stimuli expressed by infected RBC and merozoites [14, 41, 59, 81, 86, 104, 149, 170]. Further characterization of ligands revealed that stressed cells
and *P. falciparum* produce phosphoantigens including isopentenyl pyrophosphate and E-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) with the capacity to activate Vγ9Vδ2 T cells [14, 15, 55]. Recognition of phosphoantigens requires the expression of BTN3A and BTN2A molecules on target cells critical for Vγ9Vδ2 T cell activation [66, 127, 167]. *P. falciparum*-infected RBC do not express butyrophilin 3, and Vγ9Vδ2 T cell activation does not require direct cell-to-cell contact involving TCR engagement [63].

Similarly, the mechanisms by which free merozoites are recognized by Vγ9Vδ2 T cells remain unknown [29]. One possible mechanism might be antibody-dependent cellular cytotoxicity, as Vγ9Vδ2 T cells can express the Fc receptor CD16 [5]. Dendritic cells and possibly other cell types might recognize iRBC and merozoites by presentation of self- or pathogen-derived phosphoantigens in context of by BTN3A/BTN2A.

Despite their effector functions in malaria, Vγ9Vδ2 T cells are also known to express surface molecules with the potential to mediate immune regulation [24, 126]. After CHMI in malaria pre-exposed volunteers, Vγ9Vδ2 T cells did not upregulate the programmed cell death 1 (PD1) and lymphocyte-activation gene 3 [137]. This data suggested either the lack of sufficient TCR stimulation or differences in the kinetic of γδ T cells in peripheral blood after CHMI, as zolodronate induced activation of Vγ9Vδ2 T cells induced PD-1 expression in vitro [76]. In addition to the production of pro-inflammatory cytokines such as TNFα, IFNγ and cytotoxic mediators including granulysin [33, 82], Vγ9Vδ2 T cells can produce GM-CSF [81] and might thereby influence monocyte differentiation into DCs and recruitment of other immune cells including T cells [147]. Overall, the data suggests that innate-like Vγ9Vδ2 T cells might participate in anti-malaria immunity in various ways.

In contrast, adaptive-like Vδ1 T cells are highly abundant in peripheral epithelial tissues including skin, gut and liver [20, 40, 65, 77]. In the liver, Vδ1 T cells represent the largest population of γδ T cells with unique characteristics indicative for resident and circulating T cells [77]. Early work published in the 1990s indicated an increased frequency of Vδ1 T cells circulating in peripheral blood of healthy individuals living in a malaria-endemic region in Ghana as well as a rapid but transient expansion of Vδ1 T cells upon malaria chemotherapy in children [78, 79]. Further studies showed that Vδ1 T cells and other non-Vδ1 T cell subsets were expanded in malaria children and/or adults in Thailand, Ethiopia and Laos [72, 155, 176]. Vγ2,3,4 were the most common chains among expanded Vδ1 T cells, and the diverse usage of TCRγ chains indicated a polyclonal response of Vδ1 and Vδx T cells from malaria-exposed individuals [79, 155]. Length spectra-typing of the CDR3 of these Vδ1 T cells did not reveal a dominant public clonotype to be expanded across all patients in a cross-sectional analysis [79]. Recent data suggested that non-Vδ2 and Vγ9-Vδ2+ T cell subsets seem to have an unfocused, highly diverse and donor-specific TCR repertoire as in naïve Vδ1 T cells [35–37, 77, 124]. Clonal selection of naïve Vδ1 T cells that might respond to microbial/nonmicrobial stress upon infection might be the main driver of T cell expansion. Indeed, a proportion of Vδ1 T cells expanded oligoclonally and displayed a unique signature of surface molecules in individuals from malaria-endemic regions [137]. Expansion and phenotypic differentiation of Vδ1 T cells were only present in individuals with asexual blood-stage infections [136]. Clonal expansion of Vδ1 T cells was shown to be associated with differentiation from naïve CD27+ T cells into long-lived effector CD27+ T cells with phenotypical and functional changes. These T cells displayed an effector phenotype based on low expression of CD27, CCR7, CD28, IL-7R and CD62L as well as rapid activation and proliferation in response to TCR signalling [35]. In line with these observations, activated Vδ1 T cells had low expression of CD27, lacked CD57 indicating activation and differentiation from naïve CCR7+ Vδ1 T cells into early effector CCR7+ Vδ1 T cells [137]. Further phenotypic characterization of these expanding Vδ1 T cells revealed a distinct pattern by co-expressing CD38 and PD1 which is distinct from other unconventional T cells. These molecules are associated with an exhausted phenotype in CD4+ T cells during malaria infection [21] and might influence Vδ1 T cell function by modulating inflammatory cytokines production and cytotoxicity during liver- and blood-stage infections. The diversity in expression of a range of surface markers points towards a so far unappreciated heterogeneity in Vδ1 T cell responses [20, 40, 65, 77, 137]. Vδ1 T cells produced selectively ex vivo IFNγ in response to hepatocyte-derived cell lines [7, 46, 48, 124]. Whether circulating asexual blood-stage parasites represent an immune stimulus for liver resident Vδ1 T cells resulting in clonal expansion remain to be investigated. It has been shown that established clonotypes are long-lived and persist for several months to years in human peripheral blood [35, 124]. A population of Vδ1 T cells, characterized by the expression of CD38 and PD1, displayed durable expansion of clonally distinct T cells after CHMI [137]. Studying the TCR repertoire of Vδ1 T cells and characterization of expanded clonotypes are essential steps to determine how Vδ1 T cells actually respond to malaria.

So far, few γδ TCR ligands have been identified, and there is no evidence that Vδ1 T cells recognize directly asexual blood-stage parasites [58, 72], which is in line with our observation that Vδ1 T cell expansion depended on the presence of iRBC and autologous DCs [137]. Expansion of Vδ1 T cell clones might have been facilitated by cytokines produced by monocyte-derived DC, monocytes or activated non-Vδ1 cells in PBMCs [137]. Indeed, IL-15 produced by activated monocytes, fibroblasts and epithelial cells [152] and hepatocyte cell lines [134] might promote activation and proliferation of γδ T cells. In addition, *P. falciparum*-activated non-Vδ1 T cells
might produce IL-2 contributing to the observed response ex vivo.

The massive polyclonal B cell activation during malaria infections might lead to subsequent reactivation of CMV and Epstein-Barr virus (EBV) and further B cells proliferation, which might in turn induce Vδ1 T cell proliferation [80]. It might be of interest to screen volunteers involved in malaria immunity studies for pre-existing viral co-infections like CMV and EBV which might impact indirectly on type and size of ensuing γδ T cell activation after malaria infection.

Interestingly, a Vδx T cell clone expressing a Vγ4Vδ5 TCR can recognize EPCR [174]. EPCR is present on many different cells in various organs such as liver and blood vessels [52] and can bind lipids such as phosphatidylcholine or lysophosphatidylcholine and platelet-activating factor [112]. This γδ TCR engages with EPCR when the intercellular adhesion molecule 1 (ICAM-1) is co-expressed, but this interaction is independent of displayed lipids. In malaria, *P. falciparum* erythrocyte membrane protein 1 expressed on iRBC binds to EPCR with increased binding of parasites isolated from patients suffering from severe malaria [160]. Therefore, EPCR might represent a molecule displaying stress such as viral infections and other stress conditions. Whether asexual blood-stage infections represent such stress conditions and modulate EPCR expression and subsequent TCR-mediated T cell activation remains open. Clearly, identification of ligands recognized by Vδ1 and Vδx T cells during malaria will provide a more comprehensive understanding of γδ TCR ligand activation.

### Summary

Cell-mediated immune responses are critical for anti-*P. falciparum* immunity. It has long been recognized that unconventional T cells, especially γδ T cells, respond to asexual blood-stage infections. The fact that MAIT and γδ T cell frequencies and selective clonotypes expand during asexual blood-stage infections suggests that these cells have a role in malaria immunity. While innate-like T cells including Vγ9Vδ2, NKT and MAIT cells have the ability to rapidly respond and elicit effector functions without clonal expansion and differentiation, Vδ1 and Vδx T cells display more features of adaptive-like T cells. Unconventional T cells home to non-lymphoid tissue sites such as skin, liver, spleen and gut, which are key tissues in the context of malaria infections. The involvement of other unconventional T cells restricted to non-polymorphic antigen presenting molecules like HLA-E, HLA-G and CD1 in malaria immunity remains to be defined. The incorporation of innate-like and adaptive-like unconventional T cells into research studies has the potential to provide novel approaches for vaccine development and immunotherapy in malaria.

### Concluding remarks and outlook

In malaria research, unconventional T cells gain growing attention due to their diverse and specific functions in bridging innate and adaptive immunity. Unconventional innate-like T cells respond rapidly to infections and stress-induced signals and have the capacity to become T cell populations with tissue-resident and/or circulating features. Therefore, targeting these cell populations might provide an opportunity to increase efficacy of currently pursued experimental vaccine approaches. To unravel mechanisms of protection or participation in pre-erythrocytic anti-*P. falciparum* immunity, getting access to liver biopsies for characterization of liver residing T cells at the single-cell level is essential. Technological advances for improved gene coverages and simultaneous characterization of the TCR by scRNA-seq have become available. These approaches will help to understand the intrinsic heterogeneity among antigen-specific T cells and their tissue-specific roles in malaria. Vδ1 T cells recognize stress-induced molecules and respond to cytokines induced by viruses, and therefore investigating viral co-infections in volunteers undergoing CHMI might provide new insights into the well-known heterogeneity of anti-malaria immunity. Furthermore, the microbiome has been shown to impact communicable and non-communicable diseases, and certain intestinal communities might even promote protection against *P. falciparum* infections. Inclusion of microbiome studies in future field trials might unravel important new mechanisms driven by the interaction with unconventional T cells. CHMI studies can now be conducted safely in more clinical research centres than ever before in the field of malaria. This opens exciting new possibilities to address critical knowledge gaps in different human populations with several degrees of malaria pre-exposure, genetic background and co-infection status.

What are unappreciated ligands and stimuli of non-Vγ9Vδ2 T cells, MAIT cells and NKT cell subsets in human malaria?

Are unconventional T cells stage specific in malaria immunity and do they develop memory?

Does malaria induce tissue-specific metabolic changes that can be sensed by unconventional T cells?

Which micro-environmental changes are induced by the long-lasting clonal expansion of unconventional T cells after asexual blood-stage infections?

Do chronic malaria infections promote dysregulation of unconventional T cells leading to variations of clinical outcomes?

How do unconventional T cells communicate with innate immune cells and conventional lymphocytes?
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Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

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