Trends in Parasitology

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Review

Innate Lymphocytes and Malaria – Players or Spectators?

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Malaria remains an important global disease. Despite significant advances over the past decade in reducing disease morbidity and mortality, new measures are needed if malaria is to be eliminated. Significant advances in our understanding about host immune responses during malaria have been made, opening up opportunities to generate long-lasting antiparasitic immunity through vaccination or immune therapy. However, there is still much debate over which immune cell populations contribute to immunity to malaria, including innate lymphocytes that comprise recently identified innate lymphoid cells (ILCs) and better known innate-like T cell subsets. Here, we review research on these immune cell subsets and discuss whether they have any important roles in immunity to malaria or if they are redundant.

Highlights

There is an increasing number of innate lymphocyte subsets being discovered.

Innate lymphocytes play diverse roles in health and disease.

The roles of innate lymphocytes in malaria are now being unraveled.

Whether innate lymphocytes can influence the outcome of malaria is not known.

Innate Immunity Following *Plasmodium* Infection

Plasmodium infection triggers multiple immune mechanisms by the host that attempt to limit the spread of infection and prevent disease. The immune system is divided into two defense arms: the **innate immune system** (see Glossary) and the **adaptive immune system**. The innate immune system serves as the first line of defense, delivering a rapid response against invading pathogens [1]. This is mediated by the release of cytokines and other proinflammatory molecules by phagocytic cells, **innate-like T cells** and the more recently described **innate lymphoid cells** (ILCs) that promote pathogen capture and killing by antigen-presenting cells (APCs) and/or induction of apoptosis of infected cells [1–3].

The key difference between the two defense arms lies in the specificity of antigen receptors. Members of the innate immune system, such as myeloid cells and ILCs, express invariant antigen receptors that do not undergo recombination activating gene (RAG)-dependent rearrangement [3,4]. Instead, these cells possess **pattern-recognition receptors** (PRRs) that recognize conserved molecular structures expressed by pathogens [5–8]. The innate-like T cells, including natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells express semi-invariant **T cell receptors** (TCRs) that recognize restricted types of pathogen molecules, often without the requirement of antigen processing and expression by **major histocompatibility complex** (MHC) molecules. Additionally, innate immune cells can also sense invading pathogens indirectly through cytokines produced by infected cells or activated APCs [7,9].

Following infection, the innate immune system can recruit adaptive immune cells through their ability to sense pathogen-associated molecular patterns (PAMPs) and produce proinflammatory cytokines and chemokines, thereby establishing a more targeted and long-lasting response to infection [5,10,11]. Contrary to their innate counterparts, adaptive T and B cells express RAG-dependent rearranged TCRs and **B cell receptors** (BCRs) on their

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cell surface, respectively [12,13]. For B cells, the recognition of amino acid sequences on foreign molecules activates humoral immunity, which involves the production of pathogen-specific antibodies [14]. In the case of T cells, antigen-specific receptors engage with antigenic peptides presented by MHC molecules on APCs, thus activating cell-mediated immunity [15]. APCs, specifically dendritic cells (DCs) and macrophages, have been shown to be essential in the sustained presentation of *Plasmodium* antigens in the liver, which in turn, promote the proliferation of $CD8^+$ cytotoxic T (Tc) cells [16]. The role of CD40:CD40L interactions in mediating APC-driven expansion and differentiation of effector $CD8^+$ Tc cells is critical, and production of chemokines involved in lymphocyte recruitment was impaired in the absence of CD40 on APCs [17]. Additionally, CD40-deficient DCs in the liver expressed reduced levels of MHC class II and the costimulatory molecule CD86, resulting in impaired activation of $IFN\gamma^+$ $Tbet^+$ $CD4^+$ (Th1) cells, which subsequently compromised the production of the proinflammatory cytokine IFNγ and control of parasite growth [17]. Thus, there is clear evidence of protective roles for adaptive immune cells in malaria, and consequently, they have been the main targets for malaria vaccines to date.

The traditional separation of the immune system into innate and adaptive arms has recently become less clear with the discovery of ILCs and recognition of the roles played by ILCs and innate-like T cell subsets, that include NKT cells, MAIT cells, and γδ T cells [1]. Although these cells lack the expression of specific antigen receptors (in the case of ILCs) or express a restricted diversity of antigen receptors (in the case of innate-like T cells), giving them an innate-like phenotype, their ability to facilitate immunological effector functions akin to conventional T cells, in addition to memory-like functions, make them similar to adaptive immune cells [1,2,4,10,18]. For example, NK cell activity is regulated by the expression of MHC I molecules in surrounding tissue that controls their ability to produce proinflammatory molecules such as IFNγ, as well as epigenetic and phenotypic changes that allow them to respond rapidly to the same stimulation at later times [19]. While the role of adaptive immune cells has been widely studied in *Plasmodium* infection, far less is known about the role of ILCs and innate-like T cell subsets in these infections.

ILCs and Their Potential Roles in *Plasmodium* Infection

ILCs have been implicated in the protection against, as well as the pathogenesis of numerous bacterial, viral, parasitic, and inflammatory diseases [20–24]. In a seminal review, it was suggested that ILCs be categorized under three overarching groups according to their transcription factor expression and cytokine secretion profiles, similar to the grouping of helper $CD4^+$ T (Th) cells [3]. According to this system, group 1 ILCs express the transcription factor T-bet and produce the classical Th1 cell cytokine IFNy. Group 2 ILCs express GATA3 and produce Th2 cell cytokines, such as IL-4, IL-5, and IL-13, while group 3 ILCs express the transcription factor RORγt and produce the Th17-associated cytokines IL-17A and IL-22. Recently however, it has been proposed that ILCs, like T cells, be further classed as either killer ILCs or helper-like ILCs [2] (Figure 1).

Killer ILCs include NK cells, which are made up of different subsets. Of particular interest, some NK cells can develop into liver tissue-resident natural killer (trNK) cells in addition to conventional NK (cNK) cells [2,25]. These cells can be distinguished by expression of CD49a and TNF-related apoptosis-inducing ligand (TRAIL) in trNK cells, and CD49b (DX5) on cNK cells [25,26]. In addition, while cNK cell development is dependent on the transcription factors eomesodermin (Eomes) and T-bet, trNK cell development is dependent only on T-bet [27,28].

It has been argued that trNK cells may be a subset of NK cells [25,28], but others have proposed that these $CD49a^+$ trNK cells are ILC1s [2,29]. In support of the former suggestion,

Glossary

Adaptive immune system: a part of the immune system comprising T and B cells that express antigen-specific receptors.
B cell receptor: immunoglobulin molecules that form an antigen-specific transmembrane receptor on B cells.
Controlled human malaria infection: the deliberate infection of human volunteers with malaria parasites by mosquito bite or direct injection of sporozoites (to establish liver-stage infection) or parasitized red blood cells (to establish blood-stage infection).
Humanized mice: an inbred mouse engineered to carry human genes, cells, tissues, and/or organs.
Innate immune system: a part of the immune system comprising leukocytes that do not express antigen-specific receptors, but instead express pattern-recognition receptors. It is often the first line of defence against infection, and includes innate lymphocytes, eosinophils, basophils, and mast cells, as well as phagocytes, including monocytes, macrophages, neutrophils, and dendritic cells.
Innate-like T cells: a heterogeneous group of αβ and γδ T cells with a restricted repertoire of TCRs that recognize peptides, lipids, and metabolites. They include NKT cells MAIT cells, and γδ T cells. They are often located in tissue barriers so they can rapidly respond to invading pathogens.
Innate lymphoid cells: a group of innate immune cells derived from common lymphoid precursor cells that do not express antigen-specific BCR or TCR. They include group 1 ILCs (NK cells and ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s and lymphoid tissue inducer cells) (see also Figure 1).
Major histocompatibility complex: a family of surface proteins that present peptide antigen to the TCR on $CD4^+$ and $CD8^+$ T cells to initiate their activation and expansion.
Pattern-recognition receptors: germline-encoded receptors expressed by innate immune cells that enable recognition of distinct pathogen-associated molecular patterns from invading organisms or damage-associated molecular

NK cells possess cytotoxic abilities through the release of granules, making them more similar to $CD8^+$ T cells, while ILC1s facilitate effector functions through TRAIL-mediated cytolysis [2]. Furthermore, a transcriptome analysis of ILCs [29] showed that liver trNK cells ($CD3^-$ $CD19^-$ $CD49a^+$) possessed a core ILC signature, while cNK cells did not. Studies have also shown that these cells are capable of producing the proinflammatory cytokines IFNγ and TNF, similar to Th1 cells [2,25,30]. Given the residency of trNK cells in the liver and their ability to produce proinflammatory molecules, a role in combating liver-stage infection cannot be excluded.

NK cells were the first ILC to be associated with immune responses against *Plasmodium falciparum* in the early 1980s [31]. NK cell-mediated cytotoxicity against *P. falciparum*-infected erythrocytes was initially described [32], with NK cell production of granzyme B shown to be mediated by the expression of host-derived heat shock protein (Hsp) 70 [33]. However, by the 1990s, conflicting reports about the importance of NK cell activation and cytotoxicity in *Plasmodium*-infected individuals, as well as their roles in experimental models of malaria, were published [34–37]. NK cell-mediated protection against *Plasmodium* infection was attributed to IFNγ and TNF production [34,38], requiring interactions with APCs [39–41]. This was initially shown in *in vitro* coculture assays using peripheral blood mononuclear cells (PBMCs) from malaria-naïve donors, in direct contact with *P. falciparum*-infected erythrocytes [42,43].

patterns from the host damage or dying cells. For example, the pattern-recognition receptor Toll-like receptor 4 can recognize lipopolysaccharide expressed by Gram-negative bacteria.

T cell receptors: molecules expressed on the surface of T cells that recognize antigen peptides presented by MHC molecules on antigen-presenting cells or infected or transformed cells.

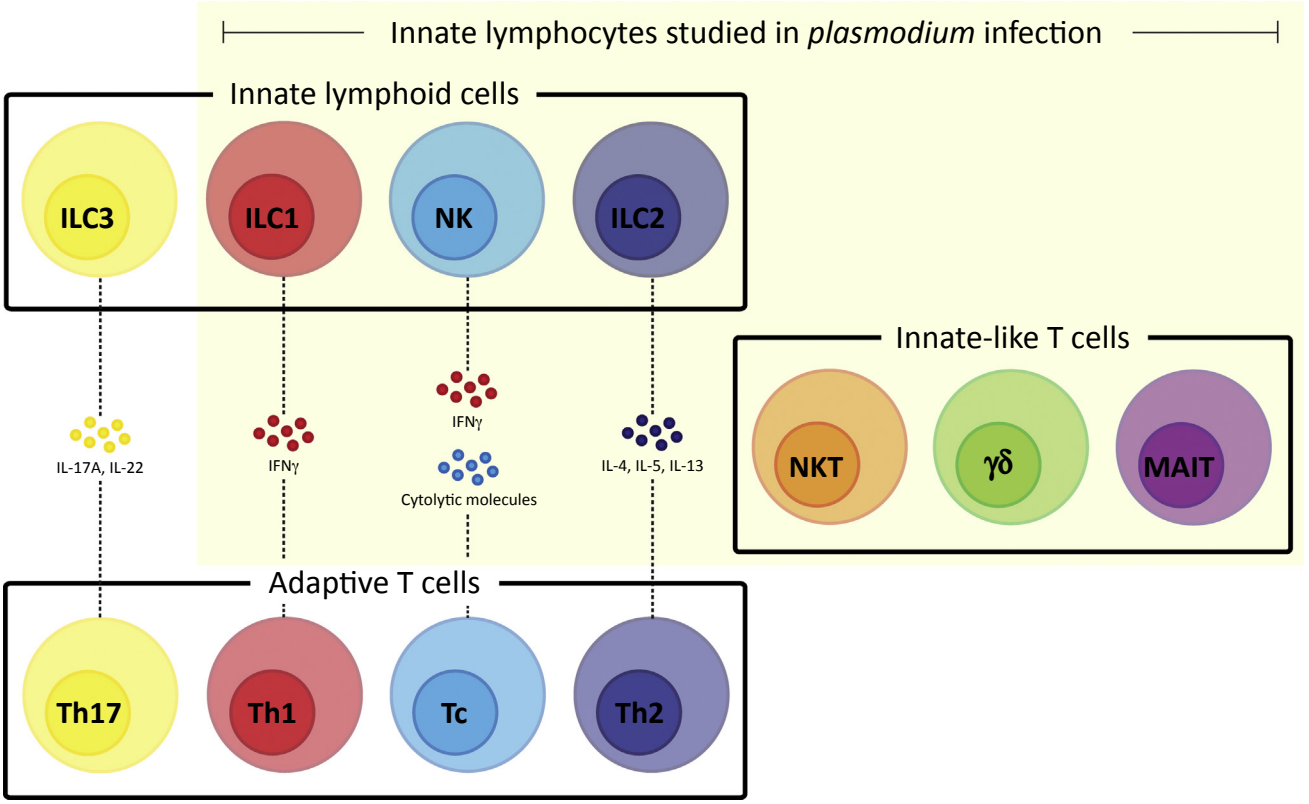

Figure 1. Innate Lymphocytes Studied in *Plasmodium* Infection. The illustration shows the innate lymphoid cells (ILCs) and innate-like T cell subsets that have been studied in *Plasmodium* infection. ILCs are innate counterparts of T helper (Th) cells and mirror their cytokine profiles. Amongst these, the roles of natural killer (NK) cells, ILC1s, and ILC2s during *Plasmodium* infection have been investigated. Additionally, the responses of natural killer T (NKT) cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells have been described. The role of ILC3s remains to be elucidated in the context of *Plasmodium* infection.

Support for this antiparasitic role for NK cells comes from the finding that human NK cells can clear *P. falciparum* in a humanized mouse model [44]. Several studies in experimental mouse models of malaria have shown that IFNγ production by NK cells early during *Plasmodium* infection is dependent on T cell-derived IL-2, and IL-12 production by monocytes and DCs [34,45–47]. This observation is consistent with results from *in vitro* and *in vivo* human studies [11,48,49], although heterogeneity in the ability of NK cells to produce cytokines response has been reported [50]. In addition to cytokine-dependent effector mechanisms, NK cells have been reported to exert cytotoxic effects through the activation of Fc receptors (FcR) upon binding by the Fc portion of antibodies [51]. This mechanism, termed antibody-dependent cell-mediated cytotoxicity (ADCC), was recently reported to promote the activation of NK cells, and consequently, lysis of *P. falciparum*-infected red blood cells (RBCs) [52]. Thus, while it is difficult to envision a way to target and expand these FcR-expressing NK cells via vaccination, we may be able to better promote the production of specific immunoglobulin isotypes able to mediate NK cell ADCC.

A recent study comparing transcriptomes of splenic NK cells in humans and mice with blood NK cells from the same species showed that the tissue NK cells had a more active and diverse transcriptional profile [53]. Given the limited opportunities to study tissue NK cells from malaria patients, future studies on NK cells in malaria are more likely to be conducted with these cells isolated from PBMCs. Therefore, tissue-dependent differences must be considered when interpreting experimental data, and alternative approaches to studying tissue NK cells considered, such as from autopsy material or nonhuman primates.

In general, ILC2s help to maintain gut homeostasis, mediate protection during micronutrient deficiency, and help to promote fat metabolism, but they also cause inflammation-related diseases in the skin and lungs [10,22,54–58]. Recently, lineage (CD4, CD11b, CD11c, NK1.1, CD3ε, Ter119, FcεRI, Siglec F, Gr1, CD49b, CD5, F4/80)-negative $CD45^+$ $ST2^+$ $ICOS^+$ ILC2s were reported to promote protection against experimental cerebral malaria caused by *Plasmodium berghei* ANKA (*Pb*A) [20]. In this study, the administration of recombinant IL-33 expanded ILC2s which produced IL-4, IL-5, and IL-13 that stimulated polarization of anti-inflammatory macrophages, which, in turn, expanded $Foxp3^+$ regulatory T cells that mediated protection against disease. Although the physiological relevance of this study is questionable, it does identify a potential protective mechanism that could involve ILC2s in local tissue sites. Whether this can be exploited through practical immune modulation in humans should be explored.

Although there are limited data on group 3 ILC subsets, including $Nkp46^+$ $ROR\gamma t^+$ ILC3s, $CD90^{hi}$ $ROR\gamma t^+$ ILC3s and human $CD3\varepsilon^-$ $IL\text{-}7R^+$ ILC3s in malaria, they have been reported to mediate gut immunity and have been implicated in gut-associated infectious and inflammatory diseases such as caused by *Citrobacter rodentium* infection, as well as inflammatory bowel disease (IBD) [59–61]. These observations suggest a potential role in pathogenesis, but this has not been reported as yet. Similarly, little is known about any roles for ILC1s in malaria, but $NKp46^+$ $NK1.1^+$ $T\text{-}bet^+$ $Eomes^-$ ILC1s have been shown to protect against the protozoan parasite *Toxoplasma gondii* through the production of IFNγ and TNF [24]. Again, if similar mechanisms of activation occur during *Plasmodium* infection, these cells may help to promote removal of infected RBCs by activating phagocytic cells. However, given that ILCs generally comprise less than 0.1% of peripheral blood leukocytes, it is unlikely that their impact on enhancing phagocytic activity will be great.

The Roles of Innate-like T Cells in *Plasmodium* Infection

The innate-like T cell subsets have also been reported to influence protection and/or pathology during *Plasmodium* infection. Innate-like T cells consist of T cells that express a restricted

repertoire of TCRs that recognize peptides, lipids, and metabolites [9,62–64]. These include NKT cells and MAIT cells that express semi-invariant TCR chains [65], as well as γδ T cells, that are postulated to bridge the gap between the innate and adaptive immune systems [63,66,67].

An antiparasitic role for NKT cells during liver-stage, but not blood-stage, infection, was suggested by studies that administered the NKT cell activating agent α-galactosylceramide (α-GalCer) following *P. yoelii* (strain: 17XNL) and *P. berghei* (strain: NK65) sporozoite and blood-stage challenge in mice [68]. These cell populations have also been reported to expand in blood relatively early during human blood-stage *P. falciparum* and *P. vivax* infection, then contract during recovery from infection [69]. A similar early expansion of NKT cells was also reported in C57BL/6 mice infected with *P. chabaudi* AS, and these splenic NKT cells were capable of producing IFNγ, suggesting a potential protective role [70]. NKT cells in the liver and spleen of *P. yoelii*-infected mice were also capable of coproducing IFNγ and TNF [71,72]. Additionally, it has been reported that NKT cell IFNγ production during *P. berghei* ANKA infection caused increased splenic B cell numbers, resulting in splenomegaly, thereby indicating pathogenic roles for these cells during infection [64].

In contrast to NKT cells, MAIT cells were shown to decrease early in the blood of African volunteers infected with *P. falciparum* in a **controlled human malaria infection** (CHMI) study, with little evidence of activation [65]. However, the frequency of MAIT cells increased above baseline levels at day 168 postinfection, although the cause of this late cell expansion is not yet clear and the consequences, if any, are unknown. Given that these cell recognize metabolites, they may play a role in early detection of parasite infection in tissue, but this remains speculation and more research will be needed to better understand what these cells do during malaria.

The role of γδ T cells during *Plasmodium* infection has also been investigated since the mid-1990s [73]. Early studies suggested that γδ T cells contribute significantly to the early production of IFNγ, in response to *P. falciparum*-infected erythrocytes from malaria-naïve donors [74]. This observation was verified *in vivo* where γδ T cells and αβ T cells emerged as the dominant IFNγ-producing subsets during experimental *P. falciparum* infection [75]. Intriguingly, this study also suggested that γδ T cells were capable of immunological memory. In line with these observations in *P. falciparum* infection, γδ T cells were shown to produce IFNγ during *P. chabaudi adami* 556KA infection in mice, suggesting that these cells were the key early mediators of protection, rather than NK or NKT cells [76]. Another study reported that the $V\delta2^+$ subset of γδ T cells that have intrinsic reactivity to malaria antigens, proliferated and conferred antiparasitic immunity in children, but their frequencies in circulation were reduced upon repeated infections with *P. falciparum* [77]. This decline in circulation correlated with reduced proinflammatory cytokine production by these cells, and consequently a reduction in symptoms associated with malaria. Additionally, proinflammatory cytokine production by γδ T cells was significantly higher in Papua New Guinea children with severe malaria, compared to healthy children and those with uncomplicated malaria, suggesting a role in pathogenesis [78]. Hence, γδ T cells are emerging as one of the most influential innate-like T cell subsets during malaria with the potential to contribute to both antiparasitic immunity and susceptibility to severe disease.

The Proposed Redundancy of Human ILCs in Immunosufficient Settings

A recent study reported evidence for ILC redundancy in humans [79]. In most cases where severe combined immunodeficiency (SCID) patients underwent hematopoietic stem cell transplantation, they had successful reconstitution of adaptive immune cells but not ILCs. Nonetheless, these patients were not significantly more susceptible to infections in the long term.

While limitations in this report have been suggested [80], subsequent studies have shown a decrease in the frequency and numbers of ILCs and innate-like T cell subsets following *Plasmodium* infection [65,81]. Additionally, depletion of ILC3s alone during *C. rodentium* infection failed to significantly impact the outcome of disease, in the presence of T cells [82,83]. Hence, these studies in experimental models of disease have reported the complete or partial redundancy of ILCs in the presence of T cells, including in mouse models of malaria [81,84,85].

This raises the question about the importance of ILCs in diseases like malaria. A simple conclusion could be that they have a limited role, if any, in disease outcome. However, ILCs have been proposed to help establish immunological niches in tissues during early development, prior to the existence of a functional adaptive immune system [86]. This hypothesis is supported by mouse data showing the emergence of ILCs around the time when hematopoiesis occurs from the fetal liver prior to birth [87]. In humans, ILC progenitors are found in the fetal liver and cord blood [88], and eventually develop into the tissue-resident ILCs that are more abundant than circulatory ILCs [80]. Additionally, current evidence suggests that the expansion of ILCs within tissue sites are derived locally [89], although circulatory precursors of ILCs that arise from a hematopoietic precursor may also contribute to replenishing effector ILCs within tissue sites [88]. Thus, although a direct antiparasitic role for ILCs may be unlikely, especially given their low numbers relative to their T helper cell counterparts (less than 0.1% of peripheral blood leukocytes), their role in conditioning tissues to be able to generate and maintain effective, long-lasting immune responses may be important. As such, their roles in conditioning tissues to respond positively to malaria vaccination may be one way that they can be manipulated for clinical advantage. For example, if they could be stimulated to make the liver more amenable to recruitment of antiparasitic T cells and enhance their retention in this organ, they could help to improve vaccine responses. However, a much greater understanding about their behavior and functions in specific tissue sites will first be required if we wish to use these approaches.

Outstanding Questions

Are innate lymphocytes needed to condition tissues early in life to allow development of effective immune responses?

Do innate lymphocyte subsets contribute to the outcome of malaria?

Can innate lymphocytes be targeted to make vaccines, or immune therapies work better?

Concluding Remarks and Future Perspectives

As advancements in immunotherapy are being made for diseases such as cancer, it is becoming apparent that harnessing the power of the human immune system can be equally beneficial in the fight against parasitic infectious diseases [90,91]. This is especially important given the rise of parasite resistance towards existing chemotherapy options [92].

However, the potential of boosting the immune system to fight diseases either through immunotherapy or vaccination requires an extensive knowledge about the roles that different immune cells play, and the synergy and relationships between these cells in the context of diseases. This includes cells such as ILCs and innate-like T cell subsets that have been shown to produce both pro- and anti-inflammatory cytokines during *Plasmodium* infection, but whose contributions to disease outcome are still unclear [20,44,68,78]. Nevertheless, increasing our understanding about the mechanisms regulating the functions of these cells, and distinguishing them from related Th cells subsets, may allow selective targeting of cells to limit pathology and tissue damage associated with diseases, while promoting antiparasitic immunity.

Current questions related to the role of ILCs and innate-like T cell subsets in humans have been addressed using *in vitro* or *ex vivo* studies. Analysis of innate lymphocyte frequencies and numbers allows researchers to postulate the importance of these cells through correlations and association [65,77,81]. However, better methods to address the functions of these cells need to be developed to move our understanding of these cell populations forward. This could

include using *Plasmodium*-infected non-human primates [93,94] or humanized mouse models [95]. The latter has already identified unique roles for human ILCs, whereby mouse studies showed that the absence of group 1 ILCs via genetic, chemical, or antibody-mediated techniques resulted in minimal impact on parasitemia during *P. chabaudi* AS infection in mice [81], while a study employing antihuman CD56 to deplete NK cells in **humanized mice** infected with *P. falciparum* reported exacerbated parasitemia following depletion of NK cells, albeit with relatively low blood parasitemia [44].

Although a redundant role for ILCs has been proposed in humans with a functional adaptive immune system, malaria is a disease frequently afflicting patients that are coinfected with other pathogens, including HIV [96]. In such cases, $CD4^+$ T cells, which serve as hosts for HIV, are rapidly lost, resulting in Th cell deficiency leading to AIDS [97]. A previous report has suggested the loss of ILCs during HIV infection, which can be prevented with administration of antiretroviral treatment (ART) in the early stages of acute HIV infection [98]. This was shown to occur by Fas-mediated apoptosis [98,99], which suggests the possibility that further understanding of ILCs may allow the use of methods to sustain these cells in the absence of $CD4^+$ T cells. This could allow the maintenance ILC subsets with similar functions to $CD4^+$ Th cell subsets, potentially allowing compensatory mechanisms of immunity to be maintained. A similar role for NKT cells has been previously proposed [100]. Regardless, there is still a great deal for us to learn about the behavior of ILCs and innate-like T cell subsets during infections such as malaria.

There are still many questions in regard to the roles of innate lymphocytes in malaria (see Outstanding Questions). It has been suggested that these cells play an important role in establishing tissue niches for adaptive immune cells. Given the increasing recognition of the important role for tissue-resident memory T cells for maintaining long-lasting protective immunity, it will be important to establish whether innate lymphocytes contribute to their development and maintenance. Critically for malaria, we need better evidence for the roles of innate lymphocytes in determining disease outcome, as this will determine whether they should be targeted through prophylactic or therapeutic strategies.

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